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Pre-radiation Nivolumab plus ipilimumab in patients with newly diagnosed high-grade gliomas

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ABSTRACT

The limited success of immune checkpoint inhibitors (ICIs) in the adjuvant setting for glioblastoma highlights the need to explore administering ICIs prior to immunosuppressive radiation. To address the feasibility and safety of this approach, we conducted a phase I study in patients with newly diagnosed Grade 3 and Grade 4 gliomas. Patients received nivolumab 300 mg every 2 weeks and ipilimumab 1 mg/kg every 6 weeks until disease progression or unacceptable toxicity. Fifteen patients were treated, with four patients on dexamethasone at treatment initiation and five tumors having *MGMT* promoter methylated. Treatment began a median of 38 days post-surgery. The most common treatment-related adverse events (AEs) were rash, pruritus, fatigue, nausea, and anorexia. Grade 3 AEs were lipase increased ($n = 2$), anorexia ($n = 1$), pruritus ($n = 1$), and rash ($n = 3$), and one Grade 4 cerebral edema occurred. Median progression-free survival (mPFS) was 1.3 months and median overall survival (mOS) was 19.3 months (95% CI, 12.9-NA). Three patients deferred conventional radiochemotherapy for over seven months while ten eventually received it. Progressing tumors tended to exhibit higher LAG-3 levels at baseline compared to shrinking tumors. Analysis of paired pre-treatment and post-progression tissue ($n = 5$) showed trends of up-regulated TGF-β, ERBB2, ERBB3, and ERBB4 signaling pathways, downregulated PPAR signaling, decreased B cell proportions, and increased monocytes proportions in tumors post-treatment. We show nivolumab plus ipilimumab can be safely administered prior to standard radiotherapy for newly diagnosed gliomas and is operationally feasible. Clinicaltrials.gov NCT03425292 registered February 7, 2018.

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Introduction

Surgical resection followed by radiochemotherapy, with or without the Optune device, prolongs overall survival of patients with newly diagnosed glioblastoma (GBM), yet nearly all patients inevitably experience disease recurrence and median overall survival remains about 14 to 16 months.[1–4] Radiotherapy, although effective against neoplasms, often leads to significant and permanent neurological complications that cause cognitive decline and decreased quality of life.[5,6] Approximately two percent of patients refuse treatment with radiation for these reasons and others.[7] Additionally, standard radiotherapy decreases lymphocyte counts, immune activation status, and recruits suppressive myeloid cells, all of which contribute to deficiencies in innate and adaptive immune responses to clear tumors.[8–11] Finding alternate strategies to delay conventional radiotherapy could potentially improve patient quality of life as well as the effectiveness of immunotherapy for this intractable disease.

Evaluation of therapeutic agents prior to radiation therapy in newly diagnosed disease is not a new concept for brain cancers, though the use of immunotherapy at this juncture is novel. About fifty clinical trials have utilized a window-of-opportunity strategy to examine treatments prior to radiotherapy in patients with malignant gliomas. Several of these trials have demonstrated the feasibility and safety of administering one to four cycles of chemotherapy prior to radiation therapy, and that the delay to radiation does not appear to compromise patient outcome.[12–16] These window-of-opportunity trials set precedence to now evaluate the impact of immunotherapy prior to radiotherapy and allow multiomic analyses without confounding issues secondary to radiation.

Therapies targeting the immune inhibitory checkpoint axis of programmed cell death protein 1 (PD-1), its ligand PD-L1, and cytotoxic T lymphocyte antigen-4 (CTLA-4) have achieved breakthroughs in many cancers and are the most widely used FDA-approved immunotherapeutic agents for solid tumors. These successes, however, have not been replicated in brain

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cancer despite intense investigation. Immune checkpoint blockade has failed to improve survival for GBM patients in the adjuvant setting, as shown by trials like CheckMate-548,[17] CheckMate-498,[18] and BN007.[19] Yet, responses of brain metastases[20,21] and subgroup analyses of primary brain cancer trials suggest that specific tumor characteristics and microenvironmental factors may drive immunotherapy responsiveness.[22–24] Identifying reliable biomarkers that enable the selection of tailored treatments remains a priority. Additionally, the timing of treatment may also be critical for GBM susceptibility to immunotherapy, as antitumor responses have been observed in recurrent GBM treated with pre-surgical neoadjuvant anti-PD-1 therapy.[25,26]

We hypothesize that immunotherapy regimens in the upfront setting prior to radiation may be administered safely and facilitate early recognition of active and inactive combination regimens. Therefore, we initiated a single-center phase I trial to investigate the feasibility and safety of anti-PD-1 and anti-CTLA-4 treatment administered prior to radiotherapy in adult patients with newly diagnosed Grade 3 and 4 gliomas. Furthermore, a longitudinal assessment of tumor and microenvironmental evolutions will add to the knowledge base of this devastating disease and may reveal underlying characteristics predisposing cancers to differential immunotherapy responsiveness.

Methods

Trial design

The Longitudinal Assessment of Tumor Evolution in Patients with Brain Cancer (NCT03425292) was an investigator-initiated, single institution phase I clinical trial conducted at Providence Saint John's Health Center. The primary objective was to determine the safety and tolerability of administering pre-radiation immunotherapy in newly diagnosed high-grade glioma, as defined by the rate of dose limiting toxicities occurring during the first 28 days of treatment. The study protocol is included in online supplemental materials. Results of patients treated with nivolumab (anti-PD-1) plus ipilimumab (anti-CTLA-4) are presented here.

Patients

Eligible patients were 18 years or older being evaluated for a potential diagnosis of Grade 3 or Grade 4 glioma (World Health Organization 2016 classification) and planning to undergo surgical resection or having undergone surgery and had not received any additional treatment. Patients had Karnofsky performance status (KPS) of 60 or higher and confirmed histological diagnosis of high-grade glioma prior to initiating study treatment. Patients were excluded if they received more than 8 mg daily dexamethasone within 7 days of treatment initiation.

Treatment regimen

Treatment was targeted to start within 42 days from cytoreductive surgery and continued until disease progression or unacceptable toxicity. Nivolumab 300 mg was administered intravenously every two weeks and ipilimumab 1 mg/kg (rounded to 50 or 100 mg) was administered intravenously every six weeks. This regimen was chosen to reduce side effects from combination treatment when integrating observations of toxicity and efficacy profiles in lung cancer.[27–29]

Safety assessments

Safety evaluations were performed prior to treatment and as clinically indicated and consisted of clinical laboratory assessments, vital signs, physical exam, performance status assessment, and neurological exam. Adverse events were monitored throughout the trial and graded according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.03.

Imaging and response assessments

Patients were assessed by magnetic resonance imaging (MRI) scan obtained before the first dose of study treatment, every 4 weeks for the first three cycles and every 8 weeks thereafter, or more frequently if clinically indicated. Radiographic response and disease progression were assessed according to Immunotherapy Response Assessment for Neuro-Oncology (iRANO).

Tumor clinical molecular profiling

Formalin-fixed paraformaldehyde-embedded (FFPE) tumors from study participants were used for molecular profiling as part of medical management. FFPE blocks were cut into 5 µm sections onto positively charged slides. Of each block, one section was stained with hematoxylin and eosin (H&E) and assessed by a board-certified anatomical pathologist for tumor representation adequacy, tissue preservation quality, signs of necrosis, and fixation or handling issues. PTEN and p53 immunohistochemistry (IHC) were performed by NeoGenomics. The Caris Molecular Intelligence® comprehensive tumor profiling approach was used to assess DNA (genome sequencing), RNA (RNA sequencing), and proteins (immunohistochemistry: IHC). OmniSeq Advance® combined next generation sequencing (NGS)- based comprehensive genomic profiling, tumor mutational burden and microsatellite status, PD-L1 and CD8 proteins by IHC, and RNA-sequencing gene expression profiling of the tumor microenvironment.

Differential gene expression analysis of RNA sequencing

The output data (FASTQ files) from RNA sequencing performed by Caris Life Science were mapped to the target genome (GRCh38) to establish raw count expression using the Kallisto program.[30] Normalization of the full dataset and analysis of differential expression between sample groups were performed using the R/Bioconductor package edgeR.[31] Heatmaps were generated using the heatmap.2 function from the gplots R package. Pathway Analysis was performed on genes differentially expressed between groups with a p-value

<0.05 using Ingenuity Pathway Analysis (IPA) software (QIAGEN). Only pathways with a z-score less than or greater than 0 were included.

Immune cell infiltration estimation from RNA sequencing

Immune infiltration estimation from RNA sequencing performed by Caris Life Science was performed using TIMER 2.0 software.[32] Immune cell composition characterization and quantification of the absolute abundance of eight immune and two stromal cell populations were performed using CIBERSORT[33] and the Microenvironment Cell Populations-counter (MCP-counter)[34] methods, respectively. Graphical representation and statistical analyses for immune cell abundance and gene expression (expressed as transcript per million) were performed using Prism software (GraphPad) and significance was determined at $p < 0.05$ using two-tailed paired Student's t test.

Immune-related assessment of tumor microenvironment with targeted RNA sequencing and IHC

Targeted RNA sequencing of immune-related genes was performed by OmniSeq (Labcorp) FFPE tumor specimens with less than 5% tumor tissue content or greater than 50% necrosis were excluded from analysis. In most cases, with or without tumor microdissection, tissue from 3–5 unstained slide sections was required to meet RNA (10 ng) and DNA (20 ng) requirements of the assay. RNA was extracted from each sample, and gene expression was quantified by RNA-seq, as previously described.[35] RNA (ribogreen staining) was measured by Qubit fluorometer (Thermo Fisher Scientific). Gene expression was measured by RNA sequencing of 395 transcripts on samples meeting validated quality control (QC) thresholds. RNA and DNA libraries were sequenced to appropriate depth on the Ion Torrent S5XL sequencer (Thermo Fisher Scientific). Tumor mutation burden (TMB) was assessed by DNA sequencing the full coding regions of 409 cancer-associated genes, then calculating non-synonymous mutations per megabase (Mut/Mb) of sequenced DNA on samples with greater than 30% tumor nuclei.

Cell surface PD-L1 expression was assessed by Dako PD-L1 IHC 22C3 pharmDx (Agilent, Santa Clara, CA). Expression was scored by a board-certified anatomical pathologist according to published guidelines,[36] where a tumor proportion score (TPS) greater than 1% was declared a positive result (PD-L1+) and a TSP less than 1% declared a negative result (PD-L1-).

Statistical analysis of clinical outcomes

The study data were collected and managed using REDCap (Research Electronic data Capture), a secure, web-based software application hosted at Providence St. Joseph Health.[37] Safety was assessed for all patients who received at least one dose of study treatment. Descriptive statistics were used for evaluation of baseline patient characteristics and adverse events. The Kaplan–Meier method was used to estimate progression-free survival (PFS) and overall survival (OS) and was performed with statistical R software, version 4.1.2. Comparison of survival based on *MGMT* promoter methylation status used the two-sided long-rank test. Although no formal statistics were used to calculate sample size for this pilot study, a reasonable sample size of 15 is adequate to direct future trial development given the rarity of tumor type, similar to a common enrollment number of the first stage of a Simon's two-stage design, with one or more responses warranting further investigation.

Results

Patients and treatment

Between March 2018 and August 2019, 15 patients with glioblastoma were enrolled to the nivolumab plus ipilimumab study treatment cohort (Figure S1). Descriptive analysis of baseline patient characteristics is summarized in Table 1 and provided by individual patient (Table S1) are in line with expected medians for age (median 66, range 39–78), sex (73.3% male) and O^{6}-methylguanine DNA methyltransferase (MGMT) promoter methylation (33.3%). At the time of study entry, all 15 tumors were classified as glioblastoma, although two of the patients would be reclassified as astrocytoma IDH mutant according to the 2021 World Health Organization central nervous system tumor classification.[38] Thirteen (13) patients had undergone gross total resection and 2 patients had subtotal resection. Five tumors had *MGMT* promoter methylation, nine were unmethylated, and one was equivocal (Table S2). Four patients were taking daily dexamethasone 4

Table 1. Baseline characteristics of patients treated with nivolumab + ipilimumab (N = 15).

Characteristic	No. of Patients	%
Age, years		
Median	66	
Range	39-78	
Age, number		
<50 yr	3	20
≥50 yr	12	80
Gender		
Male	11	73·3
Female	4	26·7
Racial Origin		
White	14	93·3
Other	1	6·7
Ethnicity		
Hispanic or Latino	2	13·3
Not Hispanic or Latino	13	86·7
Karnofsky Performance Status		
100	1	6·7
90	9	60
80	3	20
70	2	13·3
60	1	6·7
Extent of Surgery		
Gross total resection	13	86·7
Subtotal resection	2	13·3
MGMT Promoter		
Unmethylated	9	60
Methylated	5	33.3
Equivocal	1	6.7
Steroid Use at Treatment Start*		
<2 mg/d	3	20
4 mg/d	1	6·7
None	11	73·3

*Converted to dexamethasone-equivalent dose. Baseline corticosteroid dose was defined as the average dose within 4 days prior to the first study treatment.

mg or lower at the time of treatment initiation and the remaining eleven patients had discontinued steroid use prior to treatment initiation (Fig. S2). Nivolumab and ipilimumab were initiated within a median of 38 days from surgery (95% confidence interval [CI]: 29 to 57). The median number of doses of nivolumab and ipilimumab was 3 (range 2–39) and 1 (range 1–13), respectively.

Safety and tolerability

Adverse events were generally mild at grades 1–2, and the most common treatment-related adverse events were fatigue, pruritus, anorexia, rash, nausea, lipase increased, diarrhea, fever, headache, constipation, arthralgia, and dysgeusia. Grade 3 treatment-related adverse events included rash ($n = 3$), lipase increased ($n = 2$), anorexia ($n = 1$), and pruritus ($n = 1$). One Grade 4 treatment-related cerebral edema considered serious was observed (patient #4). No Grade 5 adverse events dose-limiting toxicities occurred. Table 2 summarizes the number of patients with treatment-related toxicities by grade.

Four patients were on steroids at the time of treatment initiation. Patient #12 was receiving dexamethasone 1 mg once a day (QD) and remained on this dose throughout study treatment, patient #19 was receiving dexamethasone 1 mg QD and discontinued dosing after six weeks of study treatment, patient #33 received dexamethasone 5 mg on the day of treatment initiation then received 1 mg QD for two weeks, and patient #34 was receiving dexamethasone 2 mg BID, temporarily reduced dose to 2 mg/1 mg BID for two weeks, increased back to 2 mg BID for 12 days, then increased to 4 mg four times a day as disease progressed (Fig S2). Six patients (#1, 3, 4, 16, 19, and 34) received prednisone, methylprednisolone, budesonide, or hydrocortisone after treatment initiation for treatment-related adverse events.

Table 2. Number of patients with treatment-related adverse events (N = 15).

Adverse Event	Nivolumab + Ipilimumab			
Grade	1	2	3	4
Endocrine disorders				
Hypothyroidism		1 (7%)		
TSH increased		1 (7%)		
Gastrointestinal disorders				
Constipation	3 (20%)			
Diarrhea	1 (7%)	2 (13%)		
Nausea	5 (33%)			
General disorders and administration site conditions				
Edema- extremity	1 (7%)			
Fatigue	5 (33%)	4 (27%)		
Fever	2 (13%)	1 (7%)		
Investigations				
ALT increased	1 (7%)			
Amylase increased	1 (7%)			
AST increased	1 (7%)			
Lipase increased	2 (13%)		2 (13%)	
Weight loss	1 (7%)			
Metabolism and nutrition disorders				
Anorexia	3 (20%)	2 (13%)	1 (7%)	
Musculoskeletal and connective tissue disorders				
Arthralgia	1 (7%)	1 (7%)		
Generalized arthralgia	1 (7%)			
Generalized muscle weakness	1 (7%)			
Generalized weakness	1 (7%)			
Pain	1 (7%)	1 (7%)		
Stenosing tenosynovitis- digit	1 (7%)			
Nervous system disorders				
Cerebral edema				1 (7%)
Dysgeusia		2 (13%)		
Headache	2 (13%)	1 (7%)		
Lethargy		1 (7%)		
Psychiatric disorders				
Insomnia	1 (7%)			
Renal and urinary disorder				
Urinary urgency	1 (7%)			
Skin and subcutaneous tissue disorders				
Alopecia	1 (7%)			
Dry skin	1 (7%)			
Pruritus	4 (27%)	3 (20%)	1 (7%)	
Rash maculopapular	2 (13%)	1 (7%)	2 (13%)	
Rash	5 (33%)	1 (7%)	1 (7%)	

Antitumor activity

Thirteen of 15 patients had measurable disease at the pretreatment scan before starting CPI. Five patients had stable disease, one of whom had stable disease for 11 months and another for three years; ten patients had progressive disease. Tumor shrinkage was observed in five patients, though criteria for objective response were not met (Fig. S3). Median progression-free survival was 1.3 months (95% CI, 0.92 to 2.99) (Figure 1a). At the time of progression, 3 patients received standard radiochemotherapy; 6 patients underwent a second resection, 5 of whom then initiated radiochemotherapy and 1 who elected alternate therapy; 4 patients elected for additional systemic therapy rather than radiation, 2 of whom subsequently received radiochemotherapy; 1 patient passed away before subsequent treatment due to disease progression; and 1 patient withdrew consent for follow-up. Median overall survival was 19.3 months (95% CI, 12.9 to not available [NA]) (Figure 1b). There was a significant difference in overall survival between patients whose tumors had *MGMT* promoter methylation and those whose tumors did not ($p = 0.004$, methylated vs. unmethylated HR 0.08; 95% CI: 0.01 to 0.64) (Figure 1c). The median overall survival among patients with methylation was 35.7 months (95% CI: 35.7 to NA), as compared with 12.6 months (95% CI: 4.3 to NA) among those without *MGMT* promoter methylation. Representative images of patient #1 in Figure 2 show initial shrinkage of enhancing tumor until clear progression at nine months after treatment initiation.

Molecular assessments from RNAseq

Bulk RNA-sequencing was performed by Caris on tumor tissue collected prior to treatment initiation (Table S3), on tumor tissue collected from five patients who had a second surgical resection upon disease progression immediately following treatment with nivolumab plus ipilimumab (Table S4 and Fig S4), and on tumor tissue collected from one patient who received additional therapy after study treatment before undergoing a second resection (Table S4 and Fig S4). To explore the global impact of nivolumab plus ipilimumab treatment on tumor molecular profiles, we compared gene expression between pre- and post-treatment tumor samples from patients using available RNA-sequencing raw data (Table S5). No significant difference was observed with an adjusted p-value less than 0.05. We then performed pathway analysis on 450 genes

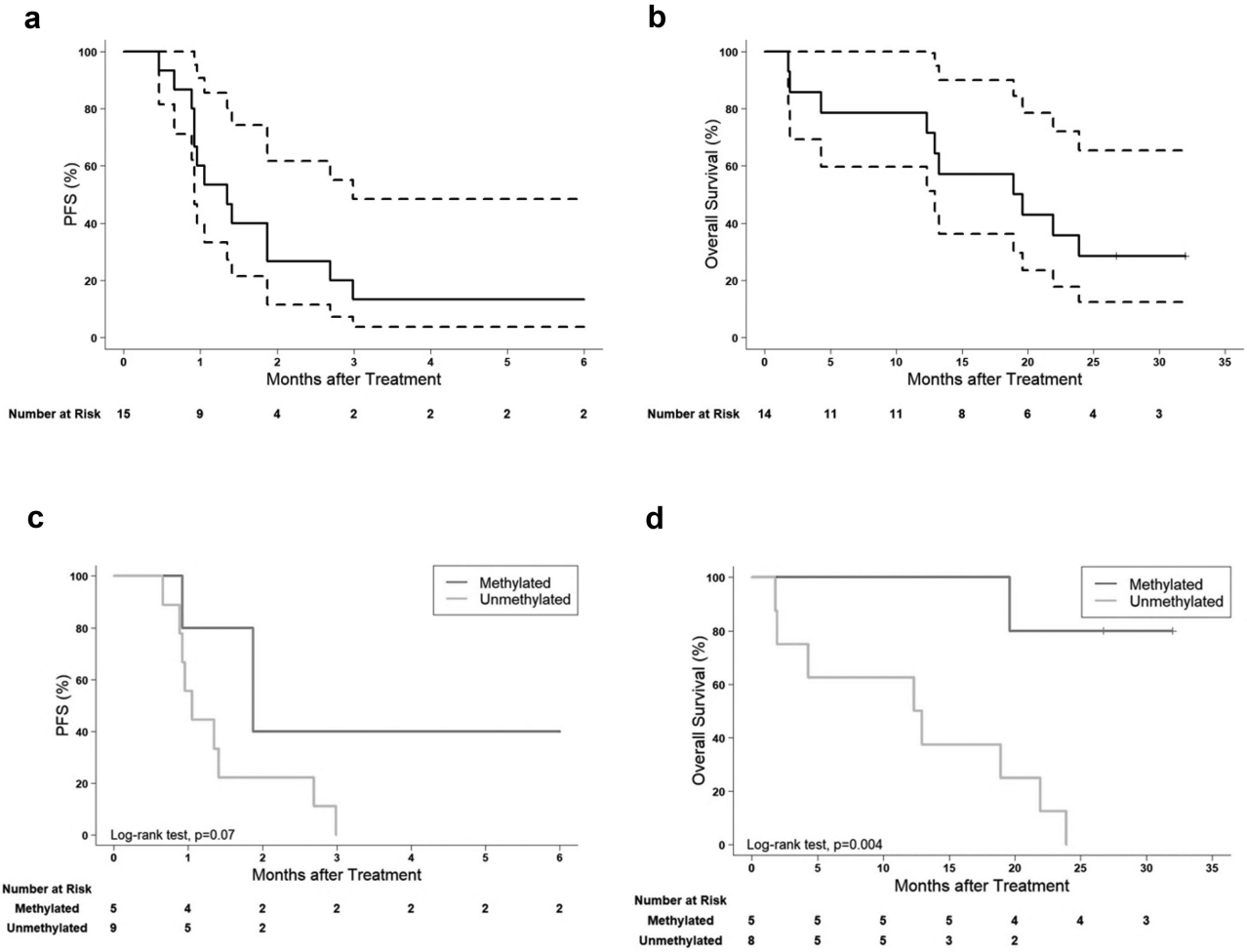

Figure 1. Kaplan–Meier curves of survival probability among patients treated with nivolumab and ipilimumab ($N = 15$). (a) progression-free survival in all patients. (b) overall survival in all patients. (c) progression free survival among patients with high-grade glioma containing a methylated *MGMT* promoter and an unmethylated *MGMT* promoter. (d) overall survival among patients with high-grade glioma containing a methylated *MGMT* promoter methylation and an unmethylated *MGMT* promoter. Censored patients are annotated by a small vertical line.

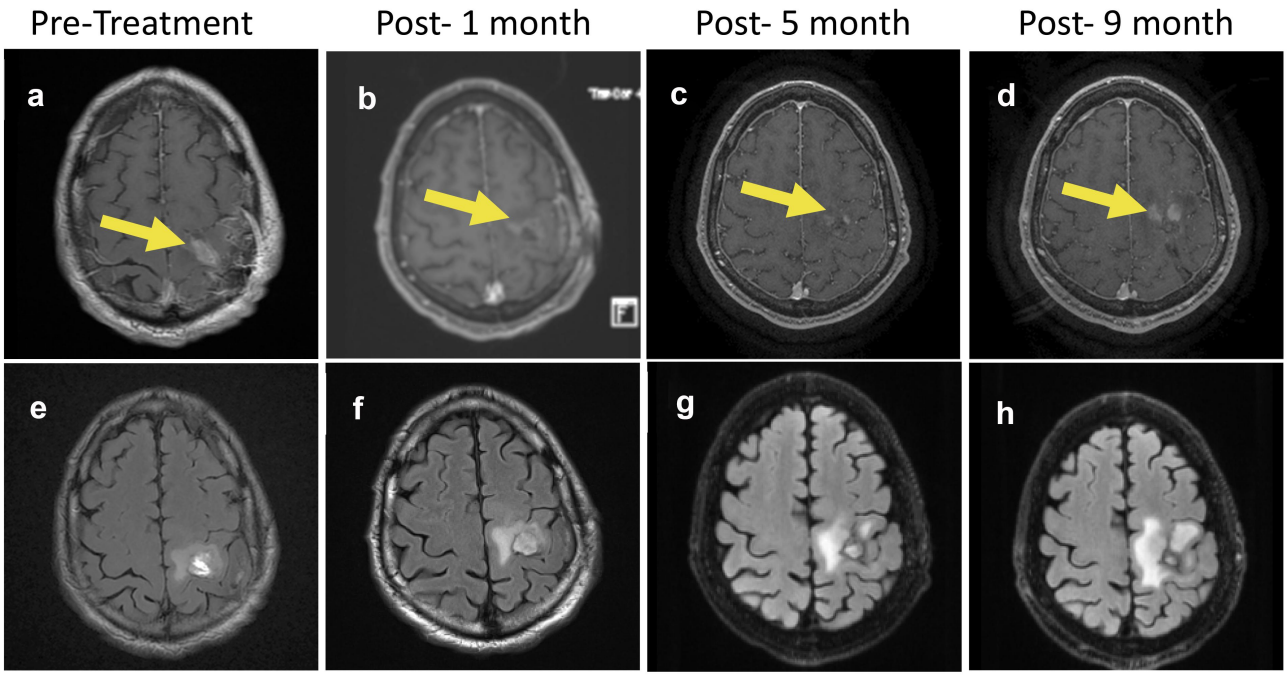

Figure 2. Magnetic resonance imaging of patient 301.Brain MRI shows axial T1 post-gadolinium images (a-d) and corresponding T2-FLAIR images (e-h) of a left frontal residual tumor (arrows) before treatment with nivolumab and ipilimumab (a, e), and 1 month (b, f), 5 months (c, g), and 9 months (d, h) after treatment initiation. The images show improving enhancing disease for over 5 month before progression at 9 months.

differentially expressed with a p-value less than 0.05 using Ingenuity Pathway Analysis (IPA) Software (Figure 3a,b). We observed up-regulation of TGF-β, ERBB2, ERBB3, and ERBB4 signaling pathways and downregulation of PPAR signaling in tumors post-treatment, consistent with pathways involved in disease progression.[39–41]

To identify changes in the tumor immune microenvironment following treatment, we performed immune cell infiltration estimation from the RNA-seq data using TIMER2.0 software. Consistent with previous studies,[42] CD4+ T cells, NK cells, and at a lower extent, CD8+ T cells, were present in all tumor samples (Figure 3c). No significant changes were observed between pre- and post-treatment samples except for a trending decrease in the proportion of B cells and an increase in the proportion of monocytes post-treatment (Figure 3c).

For further insight into whether molecular and/or cellular features at baseline could be used as biomarkers of responsiveness in the absence of objective response, we assigned the 6 patients with paired tissues into two categories based on changes in tumor size: shrinking (decrease in tumor size: patients #1, 3, and 16) and growing (increase in tumor size: patients #5, 17, and 29). From immune cell infiltration estimation, no statistically significant difference was observed in global T cells (78.52 ± 4.422 vs 80.48 ± 11.09, $p = 0.665$) or CD8+ T cells (0.8509 ± 0.5183 vs 0.357 ± 0.247, $p = 0.316$) between shrinking and growing groups at baseline (Figure 3d, e). We then asked whether the exhaustion status of immune cells could explain the different outcomes, as evidenced by the expression of PD-1, CTLA-4, TIM-3, and LAG-3 immune checkpoints.[43] Transcript Per Million (TPM) normalized data was used to analyze the expression of PDCD1 (PD-1) (693.6 ± 62.91 vs 525.9 ± 238.5), CTLA4 (1158 ± 69.56 vs 1190 ± 166.8), HAVCR2 (TIM-3) ($0.56.1 \pm 0.43.7$ vs 0.467 ± 0.26), and LAG-3 (0.62 ± 0.225 vs 0.901 ± 0.257). No statistically significant difference was observed between the two groups, though there was a trend for higher LAG-3 expression at baseline in growing tumors (Figure 3f,g). We next asked whether the presence of costimulatory molecules could explain the different outcomes. Again, no statistically significant difference was observed for CD40LG (0.312 ± 0.202 vs 0.091 ± 0.087, $p = 0.185$), CD86 (0.632 ± 0.274 vs 0.158 ± 0.058, $p = 0.129$), and CD80 (0.173 ± 0.108 vs 0.11 ± 0.077, $p = 0.534$), though there was a trend for lower expression of costimulatory markers at baseline in growing tumors (Figure 3h).

Finally, for a global overview of molecular differences between shrinking and growing tumors at baseline, we performed differential gene expression between the groups. Only two genes were significantly differentially expressed with an adjusted p-value less than 0.05: TBC1D3C and AC004980.9. We therefore performed pathway analysis on 731 genes differentially expressed with a p-value less than 0.05 (Table S6) using IPA Software (Figure 3i,j). At baseline, shrinking tumors exhibited up-regulation of the PD-1-PDL-1 pathway and NK cell signaling compared to growing tumors, suggesting a potential responsiveness to immune checkpoint inhibitors (Figure 3j). Surprisingly, pathways involved in chemo-resistance as well as tumor cell proliferation such as PI3K/AKT and ATM pathways were also up-regulated in shrinking tumors at baseline.

Targeted tumor immune microenvironment assessments

Bulk RNA-sequencing of 395 immune-related transcripts as well as cancer testis antigen burden (CTAB)[44] and tumor mutational burden (TMB) was performed by OmniSeq on tumor tissue collected prior to treatment initiation from 12 patients and tumor tissue collected from 5 patients who had

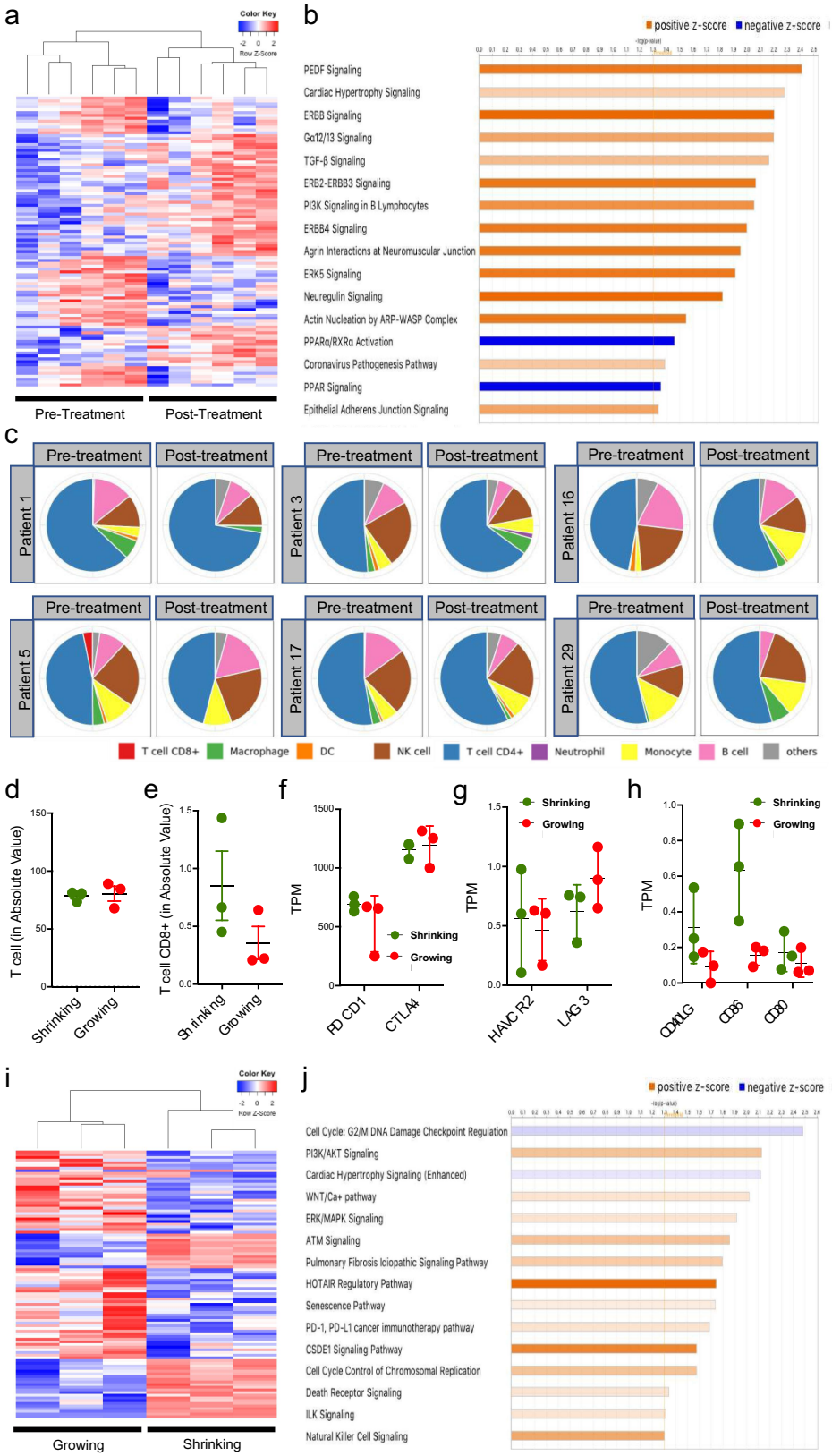

Figure 3. Gene expression profile of tumors from 6 patients pre- and post-treatment with nivolumab and ipilimumab. (a) heatmap of log counts per million (log-cpm) values for top 100 differentially expressed genes (DEGs) with a p-value <0.05 in tumor samples of 6 patients collected pre- and post- treatment with nivolumab and ipilimumab. Red coloration represents relatively high expression of a given gene and blue coloration represents relatively low expression. Lighter shades and white coloration represent genes with intermediate expression levels. Samples and genes were arranged by hierarchical clustering. A dendrogram is shown for the sample clustering. (b) graph representing the most significant canonical pathways from DEGs with a p-value <0.05 in pre- versus post-treatment samples. Orange-colored bars indicate predicted pathway activation; blue-colored bars indicate predicted inhibition. (c) pie plot showing the proportion of immune cell types in tumor samples pre- and post- treatment. Abundance of 8 immune cell types and infiltration levels in samples compared amongst, and within, patients were estimated by TIMER2.0 using CIBERSORT algorithm. (d and e) abundance, expressed as *mcp-counter score* in absolute value, of pre-treatment samples from patients with tumor shrinkage compared to patients with tumor growth of T cells (d) and CD8+ T cells (e). (f-h) gene expression (expressed as transcript per million (TPM)) in pre-treatment samples from patients

a second surgical resection upon disease progression immediately following treatment with nivolumab plus ipilimumab (Table S4). Differential gene expression analyses revealed upregulation of 12 genes with a p-value less than 0.05 following treatment with nivolumab and ipilimumab (Table S7). Upregulated transcripts included genes affecting leukocyte trafficking such as *CCL22*, *CX3CL1*, and *CXCR3*, immune effector cell activation such as *GZMA, IFNG*, and *NKG7*, and downregulated transcripts included genes implicated in myeloid cell trafficking (*S100A8/9)* and negative regulation of T cell receptor-mediated signaling (*SIT1*).

PD-L1 expression levels in tumor tissue collected prior to treatment initiation were explored by immunohistochemistry for potential responsiveness to treatment; however, no significant difference was observed between tumors classified as shrinking versus growing (Figure S5A). To describe the degree of intratumoral immune activity as a whole, including tumor and immune cells, a tumor immunogenic signature (TIGS)[45] was calculated for each baseline tumor sample from targeted RNA-sequencing. Although there was no difference in TIGS at baseline between tumors classified as shrinking versus growing, TMB and CTAB were significantly higher in tumors classified as shrinking (Fig S5A), suggesting a potential immune response to neoantigens as well as cancer testis antigens in shrinking tumors.

Gene expression analyses of baseline tumor samples revealed 35 genes significantly differentially expressed with a p-value less than 0.05 in shrinking tumors compared with growing tumors (Table S8). At baseline, shrinking tumors exhibited downregulation of interferon pathway genes such as *MX1*, *OAS-1*, *IFI6*, and *ISG15*, downregulation of SNAI2 involved in epithelial-mesenchymal transition, downregulation of pro-inflammatory chemokines *CXCL10* and *CXCL11*, and upregulation of CCL3 (FigS5B). Ingenuity Pathway Analysis revealed the *Role of Hypercytokinemia/hyperchemokinemia in the pathogenesis of influenza signaling pathway* as the most downregulated in shrinking tumors compared to growing tumors, followed by downregulation of *interferon signaling pathway* and upregulation of the *COVID pathogenesis pathway* (Figure S5C).

Discussion

High-grade gliomas remain a dismal unmet clinical need despite decades of intense research. Although important to overall therapeutic success, radiation to the brain can cause severe neurological deficits and iatrogenic suppression of immune responses as well as promote a more aggressive phenotype at recurrence.[5,6,8–11] Innovative approaches to treatment are needed to improve outcomes over conventional treatment. We therefore evaluated a novel immunotherapeutic approach of administering anti-PD1 and anti-CTLA4 treatment prior to radiotherapy in patients with newly diagnosed high-grade glioma. We report it is feasible to initiate immunotherapy within six weeks from surgery and no unexpected toxicities occurred. Delaying radiation did not appear to negatively impact survival in this limited dataset. Though the study was not randomized with standard of care or powered to test non-inferiority to historical control, the results encourage larger confirmatory studies with combinatorial treatment.

Adverse events were consistent with known side effects of nivolumab and ipilimumab and the rate of Grade 3 and 4 treatment-related adverse events was approximately 9% in this cohort. The potential risk of administering upfront immunotherapy prior to radiation is that adverse events from treatment might delay initiating standard of care radiation, or poor treatment response may increase future surgical risk and/or side effects from a larger radiation field. After disease progression on study treatment, all patients were able to receive additional treatment except for patient #9 who succumbed to disease progression and poor functional status.

Progression-free survival was short at a median of 1.3 months. It is unclear whether pseudoprogression or true disease progression was the case for several tumors and future studies could include research biopsies or blood biomarker analysis to help define this better. It is possible that additional agents should be combined with immunotherapy to increase tumor cell death and antigen generation. Furthermore, administration of immunotherapy prior to surgical debulking might better leverage antigenic burden to activate tumor-specific T cells.

At the time of progression, additional treatment was selected at the investigator's discretion (e.g., repeat surgery, chemoradiation, etc.). Interestingly, one third of patients elected to receive alternative therapy after study treatment to further defer radiotherapy. Two-thirds of patients did receive radiotherapy at some point along their disease management. Molecular profiling was performed to explore intrinsic tumor properties that might render some gliomas more responsive to PD-1 and CTLA-4 blockade. Univariate analysis identified MGMT promoter methylation as significantly correlated with improved overall survival. While not surprising that methylation status serves as a prognostic factor, this finding adds to the growing body of literature that MGMT promoter methylation could potentially identify patients who benefit more from immunotherapy, as suggested by retrospective studies[46,47] and larger phase 3 studies of dendritic cell vaccination[48] and nivolumab.[49]

Tumor tissue was collected from five patients at the time of progression on study treatment and transcriptome analyses showed a downregulation of PPAR signaling and upregulation of TGF-β and ERBB signaling pathways compared to baseline. These dysregulated pathways are associated with impaired immune cell effector functions, mesenchymal transformation, and stem-like properties in cancer cells, which drive resistance to standard therapies and ICIs.[50–53] Combining inhibitors of TGF-β and ERBB signaling may help to overcome therapeutic resistance. Finding reliable biomarkers to select combinatorial treatment for

with tumor shrinkage compared to patients with tumor growth of *PDCD1* (PD-1) and *CTLA4* (f), *HAVCR2* (TIM-3) and *LAG-3* (g), and costimulatory molecules *CD40L, CD86* and *CD80* (h). For graphs F-H, data are presented as means ± SEM and significance was determined with two-tailed Student's *t* test, *$p<.05$ **$p<.01$. i) heatmap of log-cpm values for top 100 DEGs with a p-value <0.05 in pre-treatment samples from patients with tumor shrinkage and patients with tumor growth. High- and low-expression is marked in red and blue, respectively. Samples and genes were arranged by hierarchical clustering. A dendrogram is shown for the sample clustering. j) graph representing the most significant canonical pathways from DEGs with a p-value <0.05 in pre-treatment samples from patients with tumor shrinkage and patients with tumor growth. Orange-colored bars indicate predicted pathway activation; blue-colored bars indicate predicted inhibition.

each patient remains essential given cardiac and skin toxicity observed in trials targeting TGF-β and the failure of pan-HER inhibitors to improve survival in unselected glioma patients.

No significant differences in immune cell populations were observed following treatment in this limited sample size, however the trend of increased monocytes, which share a common phenotype and morphology with monocytic MDSCs,[54] suggests a potential increase in MDSCs infiltration that could partially account for disease progression. Molecular analyses of baseline tumor tissue point toward evaluating upregulated PD-1/PD-L1 pathway and NK cell signaling for CPI responsiveness along with higher TMB and cancer testis antigen burden. Failure of adequate antigen presentation may also partially explain the lack of response, with an apparent decrease in the expression of co-stimulatory molecules CD40L and CD86 and increase of early exhaustion marker LAG-3. LAG-3 along with other checkpoints have been shown to confer resistance to PD-1 inhibitors in gliomas and a combination approach may yield more activity.[55–57]

There were several limitations of the study including a small sample size, lack of a control group, tumor heterogeneity, and bias from a multi-cohort study without randomization. However, our study served to demonstrate the feasibility of administering immunotherapy prior to radiation and the impact of PD-1 and CTLA-4 blockade without the confounding effects of radiation. Other groups have also recognized the need to reevaluate the timing of treatment strategies to stimulate the immune system in the context of standard of care and have conducted window of opportunity trials of neoadjuvant PD-1 blockade in resectable, recurrent GBM.[25,26,58] These neoadjuvant studies have begun to tease apart pharmacodynamic immune stimulation and suppression within the GBM microenvironment that can be targeted to enhance clinical benefit. Although molecular profiling analyses in our study were limited by bulk tissue sampling, estimation of infiltrating immune cell abundances and phenotypes, and variable disease courses for tissue sampling timepoints, our study leverages molecular profiling available through routine medical management to create the initial datasets from radiation-naïve tumors progressing after PD-1 and CTLA-4 blockade. This is a critical step toward understanding resistance mechanisms and identifying patient subsets most likely to derive benefit from this investigational approach. Furthermore, due to small sample size, we were unable to evaluate the changes in stem cell populations, which have also been shown to confer resistance to immune therapies.[59–61]

To our knowledge, we present the first clinical trial of immune checkpoint inhibitors administered to patients with newly diagnosed high-grade gliomas prior to radiation. Use of the pre-radiation model can serve to address whether applying immunotherapy earlier in the course of the disease could alter the overall disease trajectory. Further studies are needed to better understand the mechanistic underpinnings of therapeutic response for tailoring treatment combinations and identifying specific patient populations more likely to respond to various therapies. In future neoadjuvant studies, we suggest incorporating research tissue and liquid biopsies to enable deeper insights into tumor microenvironmental changes due to treatments. This may give more individualized insights to develop personalized drug combinations such as adding TGF-b and ERBB inhibitors to CPI to improve tumor response by turning cold tumors hot. Other approaches can involve short-course radiation therapy that is less immunosuppressive.

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Declaration of interests

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Authorship

SK and TMJ contributed equally to this manuscript and created the study concept and design. SK, TMJ, and AW drafted the manuscript. All authors were involved in the acquisition, analysis, or interpretation of the data. SK is responsible for the overall content as guarantor. All authors have reviewed and revised the manuscript and approved the submission.

Data availability statement

The de-identified patient clinical data generated in this study are available upon reasonable request from the corresponding author. The human sequence data were generated at Caris Life Sciences and OmniSeq (Labcorp) and are not publicly available due to patient privacy requirements. Derived data supporting the findings of this study are available from the corresponding author upon reasonable request.

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