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Biomarkers of immunotherapy in glioblastoma

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Abstract

Glioblastoma (GBM) is the most common primary brain cancer, comprising half of all malignant brain tumors. Patients with GBM have a poor prognosis, with a median survival of 14–15 months. Current therapies for GBM, including chemotherapy, radiotherapy, and surgical resection, remain inadequate. Novel therapies are required to extend patient survival. Although immunotherapy has shown promise in other cancers, including melanoma and non-small lung cancer, its efficacy in GBM has been limited to subsets of patients. Identifying biomarkers of immunotherapy response in GBM could help stratify patients, identify new therapeutic targets, and develop more effective treatments. This article reviews existing and emerging biomarkers of clinical response to immunotherapy in GBM. The scope of this review includes immune checkpoint inhibitor and antitumoral vaccination approaches, summarizing the variety of molecular, cellular, and computational methodologies that have been explored in the setting of anti-GBM immunotherapies.

Keywords

biomarkers | cancer vaccine | cancer immunotherapy | glioblastoma | immune checkpoint inhibitor

Glioblastoma (GBM) is the most common and aggressive primary brain malignancy in adults, accounting for approximately 50% of all malignant brain tumors.[1] The disease has a devastating impact on patients, with a median overall survival of only 14–15 months and a 5-year survival rate of approximately 5%.[1,2] The current standard of care for newly diagnosed patients involves a multimodal treatment approach, including maximal surgical resection followed by adjuvant radiotherapy and temozolomide chemotherapy.[3] Despite this aggressive treatment regimen, GBM remains nearly universally fatal.[2] There is a pressing need for novel therapeutic options that can improve patient outcomes and extend survival.

Immunotherapy has emerged as a promising treatment option for various aggressive cancers, including melanoma[4] and non-small cell lung cancer (NSCLC).[5] Immunotherapies harness the patient's immune system to target cancer cells specifically, leading to prolonged survival in many cases.[6] Inspired by these successes, researchers have begun investigating potential immunotherapeutic approaches in GBM.[7] Various modalities, such as immune checkpoint inhibitors,[8] cancer vaccines,[9] and chimeric antigen receptor T-cell therapy,[10] have been explored as potential therapeutic options for GBM patients. Although preclinical studies have demonstrated encouraging results, successful translation into human studies remains fleeting.

There are multiple factors limiting the effectiveness of immunotherapy in GBM.[11] First, the highly heterogeneous nature of the tumor limits the ability of immunotherapies to target all cancerous cells.[12] Second, the uniquely immunosuppressive tumor microenvironment of GBM is hostile to antitumor immunologic activity, with an increased presence of myeloid-derived suppressor cells and a variety of suppressive T cells and their associated proteins, such as programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4).[13,14] This relative abundance of immunosuppressive cellular and molecular factors is compounded by the relative paucity of infiltrating T cells in GBM. Recent studies have demonstrated that GBM is often associated with T-cell sequestration in the bone marrow, leading to clinically significant lymphopenia[15]; relatedly, the

quantity of infiltrating effector T cells is significantly correlated with patient survival.[16] While quantitative[17] and qualitative[18] T-cell dysfunction in GBM has been documented since the 1970s, recent work has more specifically categorized the many contributors to reduced T-cell surveillance in this setting. These factors include senescence due to telomere shortening, tolerance via regulatory T cells and T-cell elimination, anergy due to impaired IL-2 production and T-cell proliferation, exhaustion resulting from both transcriptional and metabolic alterations following repeated antigenic exposure, and ignorance due to tumor-related antigens that are insufficiently concentrated or anatomically inaccessible, preventing robust T-cell activation and activity.[19] The blood-brain barrier presents one such anatomical obstacle, limiting the delivery and penetration of many immunotherapeutic agents, in addition to contributing to T-cell ignorance as described by *Woroniecka* et al.[20]

To overcome these challenges, it is crucial to identify long-term survivors and explore these patients' tumoral and immunological characteristics. Studying the immune landscape of these tumors may help uncover novel immune targets or mechanisms that can be exploited to improve the efficacy of immunotherapies, such as the classical immune checkpoints PD-L1 and CTLA-4. For instance, recent studies have elucidated the role of additional cell-surface markers CD39 and LAG-3 in inducing T-cell exhaustion; CD39 enhances the immunosuppressive activity of regulatory T cells, while LAG-3 competes with CD4 for binding to MHC-II, hindering T-cell activation.[14] These molecular discoveries suggest novel avenues to modulate the immunosuppressive intra- and peri-tumoral milieu characteristic of GBM. Additionally, further investigation into the role of specific biomarkers in the context of GBM immunotherapy may help guide patient stratification and inform the design of future clinical studies.[21]

Recent trials demonstrating the efficacy of immunotherapies in a subset of GBM patients[22,23] have accelerated the need to identify and validate novel immune biomarkers that can better inform patient stratification and guide clinical studies.[21,24] These emerging biomarkers can help predict responsiveness to specific immunotherapeutic approaches, facilitating the development of personalized treatment regimens.[25,26] In this review, we focus exclusively on biomarkers that are clearly linked to relevant clinical outcomes (Table 1); given the complexity of the tumor microenvironment and the litany of failed immunotherapy trials in GBM, we limit the scope to only the most promising markers.[27]

Results

Checkpoint Inhibitors

Cancer cells commonly hijack or evade intrinsic cellular regulatory mechanisms. One such example is the elaboration by tumor cells of programmed cell death-ligand 1 (PD-L1), which binds to programmed cell death protein-1 (PD-1) on the surface of T cells. This PD-L1/PD-1 interaction inhibits T-cell activation, promoting immunosuppression and enabling continued tumor proliferation.[28] Immune checkpoint blockade immunotherapies, such as anti-PD-1 monoclonal antibodies, have shown remarkable efficacy in treating NSCLC,[29] advanced melanoma,[30] Hodgkin's lymphoma,[31] locally advanced rectal adenocarcinoma,[32] and liver cancer,[33] among other advanced cancers.[34] In GBM, however, immune checkpoint blockade therapies have generally been ineffective; in clinical trials, only a small subset of GBM patients have responded to immune checkpoint inhibitors. For instance, in the first large-scale randomized controlled trial of PD-1 signaling pathway inhibition in GBM, CheckMate 143, just 8% of patients receiving anti-PD-1 therapy demonstrated a treatment response, defined as an investigator-assessed complete response or partial response.[11,35] Despite results suggesting that the use of neoadjuvant PD-1 blockade enhances the antitumor-immune response,[36] there is now a relative paucity of ongoing or future clinical trials exploring immune checkpoint blockades in GBM. Attention has been redirected towards retrospectively identifying potential biomarkers among subsets of patients who exhibited durable treatment responses, with the goal of prospectively identifying such patients to inform future studies and clinical management.

For instance, recent analysis by *Reardon* et al. retrospectively explored potential biomarkers among participants in the phase 1b KEYNOTE-028 study, which enrolled 26 patients with histologically confirmed recurrent GBM and PD-L1 positive tumors.[37] These patients were given the anti-PD-1 monoclonal antibody, pembrolizumab, every 2 weeks for up to 24 months, depending on dose-limiting toxicities, adverse events, disease progression, or other events that necessitated termination. Akin to the results of the CheckMate 143 trial, anti-PD-1 therapy induced a durable response in only a subset of patients; in this trial, this subset consisted of only 2 patients. *Reardon* et al. sought to explore potential biomarkers in these archival samples. Yet, the baseline tumor PD-L1 expression in the 2 responders was 1% and 100%. Furthermore, assessment of microsatellite instability (MSI), an 18-gene gene expression profile (GEP), O^6-methylguanine-DNA-methyltransferase (MGMT) methylation status, dexamethasone use, and baseline absolute lymphocyte count yielded no significant differences between responders and non-responders, although these analyses were limited by the small sample size.

Similarly, a post hoc analysis performed by *George* et al. evaluated the ability of magnetic resonance imaging (MRI) radiomics-based machine learning to predict overall survival (OS) and progression-free survival (PFS) in GBM patients treated with durvalumab, another monoclonal PD-L1 inhibition therapy. This Phase II study enrolled 162 patients at 8 sites in the United States and Australia; 113 patients had complete imaging data which allowed inclusion in the post hoc, radiology-based study.[38] The analysis assessed the predictive strength of radiomics data derived from pretreatment and first on-treatment MRI scans, and found that first on-treatment MRI scans yielded radiomics features with a high predictive value for OS and PFS in patients treated with durvalumab. This work highlights the potential role of machine learning, which can detect patterns imperceptible to the human eye, to prognosticate treatment efficacy on the basis of early, noninvasive data,

Table 1. Summary of Biomarkers of Immunotherapy in Glioblastoma

Authors	Clinical trial identifier	Immunotherapy	Tumor	# of patients	Biomarker	Reference #
Immune checkpoint inhibitors						
George et al., 2022	NCT02336165	Durvalumab	GBM	113	First on-treatment MR imaging features (shape, intensity, and texture) were predictive of OS and PFS	38
Zhao et al., 2019	-	Nivolumab or pembrolizumab	GBM	66	*PTEN* mutations are decreased and MAPK pathway alterations are increased in responders	39
Xiao et al., 2022	NCT02852655	anti-PD-1	rGBM	15	mRNA levels of CD44 are increased in non-responders	42
Arrieta et al., 2021	—	anti-PD-1	rGBM	62	ERK1/2 phosphorylation is predictive of response	46
Chen et al., 2022	—	anti-PD-1	GBM	17	High antigen processing and presenting machinery (APM) signature-based risk score is predictive of reduced OS	47
Yang et al., 2022	—	anti-PD-1	GBM	159	C3 immune subtype andTC-6 cell subset are predictive of reduced treatment efficacy and reduced OS	50
Fan et al., 2022	—	anti-PD-1	Glioma	1,024	Chemokine-based gene signature negatively correlated with survival and treatment response	51
Wang et al, 2020	—	anti-PD-1, anti-CTLA-4	GBM	501	Patient classification based on GBM cell differentiation-related genes (GDRGs) predicts OS and immunotherapy response	53
Wang et al., 2021	-	anti-PD-1, anti-CTLA-4	GBM	518	Stemness subtype based on mRNA stemness index (mRNAsi) calculated from normal PSCs predicts immunotherapy response	54
Cancer vaccines						
Weller et al., 2017	NCT01480479	EGFRvIII vaccination	GBM	745	Significant residual disease positively correlated with long-term survival	23
Wen et al., 2019	NCT01280552	ICT-107 vaccination	GBM	124	HLA-A2 + patients had longer FPS and OS than controls	59
Izumoto et al., 2010	—	WT1 vaccination	GBM	21	WT1 expression positively correlated with PFS	62
Erhart et al., 2018	NCT01213407	DC vaccination	GBM	43	ELISPOT IFNγ and blood CD8 +T cells positively correlated with OS	65
Erhart et al., 2020	NCT01213407	DC vaccination	GBM	74	Protein levels of HIP1, RBP1, FTH1, and FAK2 were negatively correlated with survival; microRNA levels miR-216b, miR-216a, miR-708 and let-7i were positively correlated with survival	66
Narita et al., 2019	-	Personalized peptide vaccination	rGBM	88	OS was positively correlated with a higher proportion of CD3 + CD4 + CD45RA-T cells and lower proportion of immunosuppressive monocytes (CD11b + CD14 + HLA-DR^{low} and CD11b + CD14 + HLA-DR-) in pre-vaccination peripheral blood mononuclear cells (PBMCs)	70
Wang et al., 2022	NCT02122822	HSPPC-96 vaccination	GBM	19	Low MxA expression correlated with longer OS	71
Takashima et al., 2016	—	WT1 vaccination	GBM	53	Low SDC-4 mRNA levels correlated with longer OS	72
Fong et al., 2012	NCT00068515, NCT00612001	DC vaccination	GBM	24	Post-vaccination decrease in peripheral bloodT cell expression of CTLA-4 and regulatoryT-cells correlated with longer OS	73
Everson et al., 2014	NCT00068510, NCT00612001	DC vaccination	GBM	21	Increased post-vaccination functional responsiveness in cytotoxicT cells was associated with increased OS	74
Jan et al., 2018	—	Autologous dendritic cell therapy	GBM	47	Low PD-1+/CD8 + ratio in tumor infiltration lymphocytes correlated with longer OS and PFS	76

GBM, glioblastoma; rGBM, recurrent GBM; OS, overall survival; PFS, progression-free survival.

thereby aiding in the selection of patients for immunotherapy and informing clinical care. This work can be advanced by incorporating advanced MRI sequences, such as perfusion and DWI/ADC, and by assessing the generalizability of these predictions in a larger, more diverse patient population.

A multi-center coalition involving neuro-oncology researchers at Columbia University and Northwestern University pooled longitudinal data from patients who received anti-PD-1 therapy to explore molecular predictors of anti-PD-1 treatment efficacy. This study included 66 patients who had received anti-PD-1 inhibitors, nivolumab or pembrolizumab, following standard therapy. *Zhao* et al. did not find more non-synonymous single nucleotide variants (nsSNVs) nor increased neoepitope loads in responsive tumors versus non-responsive tumors,[39] which was contrary to findings in other tumor types such as metastatic melanoma[40] and NSCLC.[41] Furthermore, there was no statistically significant difference in human leukocyte antigen (HLA) zygosity or tumor purity. However, there was a statistically significant enrichment of *PTEN* mutations in non-responders relative to responders, and RNA sequencing analysis suggested that these *PTEN* mutations may induce an immunosuppressive tumor microenvironment, with a reduction in T-cell infiltration in tumors with *PTEN* mutations. Single-cell RNA sequencing indicated that these immunosuppressive features seen in *PTEN*-mutant tumors may be due to an overexpression of CD44, an M2 tumor-associated macrophage marker.

With regard to CD44 expression, *Xiao* et al. analyzed 1,395 glioma samples from The Cancer Genome Atlas (TCGA) and Chinese Glioma Genome Atlas (CGGA) using bulk, spatial, and single-cell RNA sequencing to demonstrate that CD44 promotes an immunosuppressive milieu and tumor progression; according to RNA sequencing and immunohistochemical analysis, the degree of CD44 transcription positively correlated with M2 macrophage infiltration.[42] *Xiao* et al. reported that high levels of CD44 expression were nonsignificantly associated with ineffective anti-PD-1 therapy.

In addition to their findings regarding *PTEN*, *Zhao* et al. noted that among the 17 long-term responders to anti-PD-1 therapy, there were significantly more mutations in mitogen-activated protein kinase (MAPK) signaling pathway genes.[39] The concept that alterations in the MAPK signaling pathway can increase the efficacy of anti-PD-1 therapy is supported by prior preclinical[43] and clinical studies involving other tumor types, including melanoma[44] and breast cancer.[45]

The same group expanded upon these findings in a subsequent study involving 29 patients with recurrent GBM treated with anti-PD-1 therapy. *Arrieta* et al. used immunohistochemical staining to classify tumor samples according to the extent of ERK1/2 phosphorylation, which is downstream of MAPK activation.[46] In patients treated with anti-PD-1 therapy, high ERK1/2 phosphorylation (p-ERK) was predictive of significantly better overall survival (OS) relative to low-p-ERK; this trend was not seen in patients who did not receive anti-PD-1 therapy, however, suggesting that p-ERK is a useful biomarker in identifying potential anti-PD-1 responders. *Arrieta* et al.[46] posit that computer-based p-ERK quantification could lead to a rigorous, consistent method for predicting immunotherapy treatment efficacy. In particular, tumor samples—and subsequent p-ERK classification—acquired prior to anti-PD-1 therapy were most valuable in predicting response to PD-1 blockade. To explore possible mechanisms, multiplex immunofluorescence and single-cell RNA sequencing (scRNA-seq) were utilized to assess alterations in the tumor microenvironment. This analysis demonstrated that high p-ERK staining is associated with increased infiltrating microglia, and furthermore, microglia from high p-ERK tumors have increased MHC class II expression relative to low p-ERK tumors.

Other groups have utilized computational approaches to identify changes in the tumor microenvironment and, relatedly, to predict treatment response. *Chen* et al.[47] expanded on the notion that variations in antigen presentation—for instance, alterations in MHC expression, as reported by *Arrieta* et al.[46]—can affect the efficacy of immune checkpoint blockade therapies. Rather than analyzing data from a small clinical trial, *Chen* et al. pursued a large-scale bioinformatics approach, investigating antigen presentation in 1,013 glioma samples from the CGGA and 672 glioma samples from TCGA.[47] Using single sample gene set enrichment analysis (ssGSEA), the authors determined the expression level of genes related to antigen presentation and used these results to create an antigen processing and presenting machinery signature and risk score. Using the Tumor-Immune Dysfunction and Exclusion (TIDE) algorithm, a computational model of tumor evasiveness,[48] *Chen* et al. found that glioma patients with high-risk scores were more likely to respond to anti-PD-1 therapy.

Other groups have used similar approaches to group GBM patients on the basis of their GEPs. While *Chen* et al. created prognostic signatures and risk scores based on genes related to antigen presentation, *Huang* et al. used RNA sequencing and survival data from the CGGA dataset to identify immune-related genes with prognostic implications.[49] Using the expression of 6 immune-related genes, the authors generated risk scores, akin to those generated by *Chen* et al., and a model which predicted that patients in the high-risk category were more likely to exclude lymphocytes from infiltrating tumor tissue, and thus, these patients were less likely to be responsive to immune checkpoint blockade therapy. Similarly, using RNA-sequencing data from 159 GBM patients in the TGGA dataset, *Yang* et al. used single-cell RNA sequencing (scRNA-seq) and bulk RNA sequencing to identify immune signatures and molecular subtypes among GBM patients.[50] The authors identified an immunosuppressive GBM subtype, TC-6, which was rich in immunosuppressive tumor-associated macrophages (TAMs) and was associated with reduced efficacy of anti-PD-1 therapies, including a reduced OS.

Fan et al. explored immune characteristics in the tumor microenvironment of patients treated with immune checkpoint blockade therapy by evaluating the clinical and RNA-sequencing data of 1,024 patients with glioma.[51] In this report, the authors generated a risk stratification by creating a "4-chemokine signature," composed of CCL2, CCL5, CCL18, and CXCL16. When evaluated by both mRNA expression and immunohistochemistry, the resultant high-risk group had a significantly higher level of immune checkpoint gene expression. Most significantly,

the authors' 4-chemokine signature-derived risk score outperformed both the T-cell inflamed signature (TIS) and the aforementioned TIDE score in predicting OS in patients receiving anti-PD-1 therapy. Rather than creating a risk score and model, *Xu* et al. assessed the level of FCER1G expression as a potential biomarker for responsiveness to immunotherapy.[52] FCER1G is an innate immunity gene involved in numerous pathologies, including clear cell renal cell carcinoma, meningioma, childhood leukemia, and eczema. The FCER1G level was determined using gene set enrichment analysis, and the correlation of these data with clinical outcomes was determined using the CGGA, TCGA, and gene expression omnibus datasets. Using the TIDE, TIS, Subclass Mapping (SubMap), and ImmuneCell AI models, the authors predicted that high FCER1G levels would be associated with increased responsiveness to immunotherapy in patients with glioma. In sum, the authors suggest that FCER1G levels may provide valuable prognostic data when considering immunotherapeutic interventions such as anti-PD-1 therapies. However, the merits of this assertion must be validated in large, prospective trials.

Rather than delineating GBM subtypes based on their immune profiles, *Wang* et al. sought to predict responsiveness to immunotherapies according to GBM cell differentiation states. First, using single-cell and bulk RNA-sequencing data from the Gene Expression Omnibus (GEO), TCGA, and CGGA datasets, *Wang* et al. identified 498 GBM cell differentiation-related genes (GDRGs) and classified GBM patients based on their expression of these GDRGs.[53] The authors report that, within a given GBM cell, the expression of immunological molecules, such as PD-1, PD-L1, CTLA-4, CD80, and CD86, was related to that cell's differentiation pattern. Cells that belonged to the group labeled molecular cluster 1 expressed more PD-1 and PD-L1, while cells belonging to molecular cluster 2 expressed more CTLA-4; unsurprisingly, cells within the molecular cluster 1 cluster were predicted to be more sensitive to anti-PD-1 therapy, while molecular cluster 2 cells were predicted to be more sensitive to anti-CTLA-4 therapies. The following year, the same group furthered this analysis by utilizing transcriptome analysis to divide 518 GBM patients from the TCGA dataset into 2 subtypes according to their mRNA stemness index (mRNAsi) or GEPs, termed Stemness Subtype I and Stemness Subtype II.[54] Stemness Subtype I was comprised of patients who were generally younger, with more *IDH* mutations, *TP53* mutations, somatic mutations, and copy number alterations. According to the TIDE algorithm, these patients were more likely to respond to immunotherapy (44.6%) than patients in Stemness Subtype II (21.8%). As a result, the authors propose that classifying GBMs based on their tumor stemness may help select appropriate patients for immunotherapy.

In summary, efforts to utilize immune checkpoint clinical trial data to identify potential biomarkers have been limited by small sample sizes. Alternative approaches—including radiomic, RNA sequencing, immunohistochemical, and bioinformatic techniques—have contributed to a complex landscape wherein various RNA sequencing and IHC-derived signatures are promising yet largely untested. For instance, numerous groups have suggested that CD44 overexpression may induce an immunosuppressive tumor microenvironment, and thus, resistance to anti-PD-1 therapy. Alternatively, various independent investigational modalities have found that mutations in the MAPK signaling pathway may confer sensitivity to anti-PD-1 therapy. These molecular stratification schemes are likely closest to clinical utility; however, large-scale computational approaches to cluster GBM patients according to their GEPs and susceptibilities may ultimately provide the most fruitful means to select appropriate patients for immune checkpoint blockade immunotherapies. In this way, there is growing hope for a future in which clinical trials prospectively stratify patients according to their biological profiles and their predicted responsiveness to immune checkpoint therapeutics.

Cancer Vaccine

It is well-established that, in GBM, merely inducing cell death and exposing the immune system to tumor-associated or tumor-specific antigens (TSAs) is insufficient to stimulate an antitumor T-cell mediated immune response.[55] Cancer vaccines, which introduce antigens exogenously, could potentially address this failure to initiate a cellular immune response, leading to greater activation, infiltration, and T-cell-mediated cell death. Cancer vaccine targets fall into 2 categories: Tumor-associated antigens (TAAs) or TSAs. TAAs, such as survivin and Wilms tumor 1 (WT1), are highly expressed in tumor cells but also exist elsewhere in the body. TSAs, including EGFRvIII and heat-shock proteins, are unique to malignant cells and thus are considered superior targets.[56] Numerous trials testing specific TSAs or TAAs have demonstrated survival benefits in preliminary trials. Though these results have not been replicated in larger cohorts, some patients exhibit prolonged survival.[9] Within these cohorts, investigators seek to identify defining biological characteristics of these patients via assessment of tumoral and immunological features.

Tumor phenotype.—Biomarkers can be divided into tumor intrinsic and immune-related factors. The former group focuses on understanding which baseline tumor characteristics are shared across treatment responders. Hypermethylation of the MGMT gene is predictive of response to temozolomide[57] and radiotherapy[58] in GBM. Two recent phase II trials of vaccine therapy in GBM have suggested that MGMT methylation may correlate with improved survival; however, these trials were limited by small sample size[59] and the lack of a control arm,[60] respectively. Conversely, a larger phase II trial of an autologous tumor-lysate DC vaccine in newly diagnosed GBM found no significant association between MGMT-methylation status and survival in the treated arm across 76 patients.[61] Thus, at present MGMT remains an unreliable biomarker for responsiveness to vaccine therapy.

In pursuit of an alternative biomarker, researchers have evaluated markers explicitly related to the vaccine, including levels of expression of the target antigen in the tumor cells. A phase II trial of a WT1 peptide vaccine in patients with HLA-A*2402–positive recurrent GBM demonstrated that patients with higher WT1-immunostaining scores had significantly longer PFS than patients with lower WT1-immunostaining scores.[62] However, patients

with a moderately elevated WT1-immunostaining score had a longer PFS than patients with the highest WT1-immunostaining score. The authors speculate that the highest WT1-immunostaining score may reflect insurmountable proliferative activity, suggesting that tumors with moderately elevated WT1-immunostaining may be most amenable to WT1 vaccination therapy. Additionally, this investigation documented higher WT1-specific cytotoxic T lymphocytes (CTLs) in WT1-positive GBM patients than in healthy controls, suggesting a potential biomarker; however, among these WT1-positive GBM patients, there was no significant correlation between the quantity of WT1-specific CTLs in the peripheral blood and WT1 vaccination treatment efficacy. A subsequent analysis confirmed that across 37 patients both the median PFS and OS were significantly longer in the group with high WT1 expression compared to those with low expression, validating the idea that higher target antigen expression may correspond with improved survival.[63] Importantly, when stratifying patients according to MIB-1 staining, a marker of cellular proliferation, there was no statistically significant difference in PFS or OS, suggesting that it is WT1 antigen expression, rather than proliferative activity, that is most predictive of treatment response.

However, the usefulness of target antigen expression on tumor cells is undercut by the outcome of one of the few phase III vaccine trials in GBM, ACT IV, which evaluated treatment of EGFRvIII+ GBM with EGFRvIII peptide vaccination plus granulocyte-macrophage colony-stimulating factor (GM-CSF).[23] Despite prior phase I and II trials suggesting significant survival benefits, the trial was terminated due to futility. There was no consistent correlation between humoral and clinical responsiveness. Nevertheless, subsequent exploratory analysis identified a potential long-term survival benefit in patients with significant residual disease following initial tumor resection and chemoradiation, leading the authors to postulate that residual disease (and its associated increased EGFRvIII expression) may be necessary to make anti-EGFRvIII therapy efficacious. Notably, even in those not treated by anti-EGFRvIII therapy, it has been reported that approximately half of the tumors expressing the EGFRvIII mutation will lose EGFRvIII expression at recurrence[64]; the ACT IV trial corroborated these findings, as, among patients with tissue samples available at recurrence, approximately 60% had lost EGFRvIII expression. These data highlight the difficulty of targeting EGFRvIII due to its fickle nature and, more broadly, the limitations of targeting a single antigen with vaccine therapy.

More recent vaccine trials have sought to target multiple antigens. For instance, a 2019 phase II trial investigated a vaccine (ICT-107) containing 6 TAAs in newly diagnosed GBM patients.[59] As 2 of the TAAs were A1 specific and the other 4 were A2 specific, inclusion criteria limited enrollment to HLA-A1+ or HLA-A2+ patients. Analysis of the tumor specimens during the trial revealed that > 90% of HLA-A2+ patients expressed all of the A2 antigens while only 38% of HLA-A1+ patients expressed the A1 antigens. Unsurprisingly, HLA-A2+ patients, especially those with MGMT methylation, had a longer median PFS and OS than the control group. In this way, the results of this ICT-107 trial underscore the dual importance of target antigen expression and presentation in devising successful anti-GBM vaccine strategies.

Spurred by the inadequacy of factors such as single tumor antigens, HLA-expression, and MGMT-methylation as reliable biomarkers, more comprehensive, unbiased approaches have emerged in the quest for prognostic utility. For instance, *Erhart* et al. used an integrative method, melding quantitative proteomics with microRNA sequencing, to examine results from a phase II clinical trial with a tumor-lysate-charged DC vaccination,[65] identifying expression of huntingtin interacting protein (HIP1) and retinol-binding protein 1, among others, as significant correlates of reduced survival.[66] Conversely, microRNAs miR-216b, miR-216a, miR-708, and let-7i were associated with prolonged survival. While further studies are needed to delineate the precise roles that these mRNA sequences and proteins have in tumorigenesis and tumor-immune interactions, this unbiased, systems approach paves the way for uncovering robust biomarkers, and perhaps, unveiling new adjuvants or therapeutic targets for vaccine development.

These large-scale proteomic and transcriptomic analyses have highlighted the theoretical utility of tumor-lysate-loaded vaccines, which target a much more diverse set of antigens and thereby reduce the tumor's evasiveness relative to standardized antigen vaccines.[21] This theoretical advantage has been validated by the recently reported trial involving a dendritic cell (DC) tumor-lysate-loaded vaccine (DCVax-L); this study garnered much enthusiasm, as it was the first phase III trial since 2005 to demonstrate a survival benefit in recurrent GBM.[22] While the use of external controls due to the trial's unconventional crossover design warrants caution in interpreting these results,[67] the authors note that future analyses will investigate the predictive capacity of potential immune biomarkers. The field awaits these data to determine if the most promising vaccine therapy in recurrent GBM can become more efficacious with tailored, biomarker-derived patient stratification and targeting.

Immunophenotyping.—Immunophenotyping, which involves the analysis of immune cell populations via techniques such as flow cytometry and immunohistochemistry, has been explored in the context of vaccination as a potential biomarker. While this review is focused on the utility of immunophenotypic biomarkers related to immunotherapies, it is important to note that these approaches have also been explored in the setting of standard-of-care treatment. For instance, *Alban* et al. utilized flow cytometry and mass cytometry time-of-flight analyses to determine that increased levels of myeloid-derived suppressor cells were associated with poor prognosis in GBM, suggesting a potential role for MDSC blockade to supplement traditional chemotherapeutics in GBM.[68] Furthermore, among GBM patients receiving anti-angiogenic combination therapy in the form of bevacizumab and lomustine, increased CD8+ T-cell infiltration was associated with an increase in OS and PFS.[69] In this way, the study of the immune cell populations involved in combating GBM progression has utility in the setting of varied antitumoral pharmacologic approaches.

Yet, immunophenotyping has particular appeal with respect to identifying biomarkers in the setting of vaccine therapy, given that the immune system itself is the therapeutic agent. Efforts to identify immune-related biomarkers have largely concentrated on the pretreatment presence and shifts in immune subsets such as CD4+ or CD8+T cells, found either in the peripheral blood or the tumor microenvironment. For example, while a recent phase III trial of a personalized peptide vaccine involving 88 patients with (HLA)-A24+ GBM yielded no significant difference between experimental and placebo groups in OS or PFS, subsequent analysis focused on the T-cell populations of patients of varying outcomes.[70] The authors noted that patients with longer OS had a higher proportion of CD3+CD4+CD45RA- T cells and a lesser proportion of immunosuppressive monocytes (CD11b+CD14+HLA-DR^{low} and CD11b+CD14+HLA-DR-) in pre-vaccination peripheral blood mononuclear cells (PBMCs). No other cellular subsets served as significant correlates of clinical response. Similarly, in the phase II trial for an autologous tumor-lysate DC vaccine discussed previously, there was a significant correlation between pre-vaccination peripheral levels of CD8+T cells in peripheral blood mononuclear cells and OS.[65] While these data suggest promise for simply quantifying peripheral T-cell populations prior to vaccination, the results of other studies are less encouraging: for instance, a phase I trial involving heat-shock protein complex 96 (HSPPC-96) vaccination did not identify any correlation between T-cell infiltration levels and clinical response.[71] In this way, metrics involving T-cell prevalence are hindered by inconsistent results and thus, are currently limited in their predictive capacity, warranting the exploration of other potential immunophenotypic biomarkers.

In addition to investigating cellular characteristics, *Wang* et al. investigated the potential of pre-vaccination levels of MxA protein, which is involved in interferon signaling, to predict response to heat-shock protein peptide complex-96 (HSPPC-96) immunotherapy, finding that low MxA expression was associated with favorable prognosis and long-term survival.[71] Interestingly, further analysis revealed the association of high MxA expression with the loss of a TCR clone, CDR3-2, that predicted durable survival in glioma patients receiving therapeutic peptide vaccination. Others have used a computational approach to identify potential immune-related factors that could predict treatment response. *Takashima* et al. analyzed PMBCs from 53 patients in the phase II WT1 peptide vaccine trial[62] and found, among an initial set of 25,000 genes, that low SDC-4 mRNA expression levels were associated with significantly prolonged survival.[72] However, these markers must be evaluated in larger, more diverse, and prospective studies to assess their utility in clinical practice. In summary, while there are encouraging data regarding the use of various immunophenotyping approaches, standalone cellular or molecular metrics are currently insufficient to prognosticate regarding vaccine therapy efficacy.

Numerous groups have explored alternative approaches to assess changes in the tumor microenvironment during or after vaccine therapy. While these would be immediately useful only for monitoring, rather than predicting, treatment efficacy, a more nuanced understanding of the immunophenotypic changes induced by vaccine therapy may help identify future biomarkers with predictive capabilities.

Specifically, recent trials have sought to characterize the relevant immune populations functionally, whether via changes in cell-surface markers or secretory testing. Using data and tissue samples collected during 2 phase I DC vaccine trials, *Fong* et al. found that a decrease in both peripheral blood regulatory T-cell levels and CTLA-4 expression on CD4+ and CD8+T cells after vaccination significantly correlated with overall survival.[73] Using the same dataset, the group assessed the functional responsiveness of pre- and post-vaccination peripheral blood lymphocytes using immunostimulatory cytokines. Increased functional responsiveness, quantified by determining the downstream phosphorylation of STAT-5 within cytotoxic T cells, was associated with extended survival.[74]

Similarly, a phase I trial investigating HSPPC-96 demonstrated that patients with high tumor-specific immune response following IFN-γ release ELISPOT assay had increased PFS and OS,[75] while the ICT-107 trial noted that prolonged patient survival was associated with increased DC functioning, as determined by the in vitro magnitude of IL-12 secretion in response to CD40L stimulation.[59] Lastly, a trial of 47 de novo GBM patients randomized to receive postsurgical adjuvant autologous DC vaccine or conventional chemoradiation found that, among the 27 patients who received the autologous DC vaccine, longer OS and PFS were associated with tumor-infiltrating lymphocytes with a low PD-1+/CD8 + ratio.[76] Specifically, patients in this subset with a PD-1+/CD8 + ratio ≤ 0.21 had a longer PFS (11.2 vs. 4.4 months, $P < .008$) and OS (61.0 vs. 20.1 months, $P < .001$) than patients with a PD-1+/CD8 + ratio > 0.21. Yet, other markers of immune activation such as CD45+, CD4+, and CD8+ lymphocyte counts were not significant prognostic factors for OS or PFS, highlighting the inconsistent nature of potential immunophenotype biomarkers for vaccine therapies.

In summary, there is a relative paucity of validated prognostic biomarkers for vaccine therapies. The recent development of a variety of approaches to evaluate the immunological milieu, however, will hopefully provide reliable methods to monitor treatment efficacy and permits optimism for future prognostic biomarkers.

Macrophage Targets

Macrophages, given their role in mediating both innate and adaptive immune responses, represent another promising target for immunotherapy. TAMs have historically been classified into more tumor-suppressive (M1) phenotype and tumor-supportive (M2) phenotype, with the TAMs generally becoming more pro-tumorigenic as the tumor progresses.[77,78] Recent research has focused on discovering and targeting markers of pro-tumor macrophage activity. A 2020 study by *Sa* et al. identified 30 genes upregulated in mesenchymal-associated pro-tumor TAMs compared to non-mesenchymal-associated TAMs.[79] Of these, macrophage receptor with collagenous structure (MARCO) emerged as the most highly differentially expressed gene[79] and was reported to be a "master regulator" of pro-tumor macrophage activity, inducing a shift in glioma stem cells

toward a mesenchymal state. MARCO expression in GBM patients has subsequently been correlated with worse overall and disease-free survival.[80] Anti-MARCO antibodies have demonstrated efficacy in mouse melanoma models and may offer synergistic effects when strategically coupled with immunotherapies such as anti-PD-1.[80–82]

Another potential immunotherapy target associated with macrophages is found in the CD47-SIRPα axis. Expressed by normal cells, CD47 functions as a "do not eat me signal," protecting cells from phagocytosis by macrophages expressing SIRPα. Tumor cells exploit CD47 to evade phagocytosis by such macrophages.[78] Anti-CD47 therapy aims to disrupt the interaction between CD47 and SIRPα, thereby increasing phagocytosis of tumor cells and enhancing antigen presentation to T cells. Preclinical models have demonstrated the efficacy of anti-CD47 in augmenting antitumor phagocytic activity, and clinical trials for lymphoma and blood cancers have shown promising results.[83]

Numerous preclinical and clinical trials have attempted to induce immunosuppressive, M2 GBM-associated macrophages and microglial cells towards an antitumor, M1 phenotype via blockade of the colony-stimulating factor-1 receptor (CSF1R) pathway. While CSF1R-targeted drugs, such as PLX3397,[84] have failed in clinical trials, recent in vitro work suggests that newer agents, such as GW2580, may be more efficacious than drugs previously selected for human studies.[85] These preclinical results suggest that GW2580 induces phenotype-defining transcriptional changes, including the downregulation of immunosuppressive cytokines such as CCL13 and CD38; monitoring of these transcriptional alterations may have utility in determining treatment efficacy, but prospective biomarkers for CSF1R blockade therapy are not yet well elucidated.

Along the same lines, recent studies have explored the role of triggering receptor expressed on myeloid cell 2 (TREM2) modulation in anti-GBM therapy. TREM2 is integral to microglial function in various neurodegenerative pathologies; in GBM, it has been shown that TREM2 expression is associated with poor prognosis via its induction of an M2-macrophage phenotype. Furthermore, loss of TREM2 function and expression leads to increased macrophage phagocytic and cytotoxic activity, contributing to reduced tumor growth and increased survival in a mouse model.[86] While this preclinical work has not yet allowed for the validation of potential biomarkers to predict responsiveness to TREM2-related therapy, *Sun* et al. noted that mice with low TREM2 expression had increased PD-1+/CD8+T cell infiltration into the tumor microenvironment, suggesting that reduced TREM2 expression may confer increased cytotoxic T cell activity and increased sensitivity to anti-PD-1 therapy. Further studies are needed to explore targets such as pro-tumor macrophage markers in human subjects with GBM.

Alternatively, future efforts to induce myeloid-derived cells toward an antitumoral phenotype may leverage genetic engineering. *Canella* et al. recently demonstrated that bone-marrow-derived myeloid cells (BMDMs), when genetically altered to release IL-2, can reprogram the tumor microenvironment to promote cytotoxic T cell and NK cell recruitment and antitumor activity.[87] The authors had previously suggested that the malignant progression of glioma from low to high grade is fundamentally associated with the progression of myeloid cells toward an immunosuppressive phenotype.[88] Thus, while the *Canella* et al. study was performed in a mouse model of low-grade glioma, the survival advantage conferred by these genetically altered myeloid cells warrants further exploration. Specifically, the totality of the data from these recent studies suggests that these genetic alterations, when delivered at the appropriate moment in myeloid cell phenotypic plasticity, may markedly alter the tumor microenvironment and delay progression from low-grade to high-grade glioma, thereby extending survival.

Conclusion

While a variety of immunotherapeutic approaches have shown remarkable efficacy in several advanced cancers, the translation of these advances to clinical practice in GBM remains limited. It is important to consider how the factors that hinder neuro-oncologic progress broadly have affected the translation of immunotherapies in GBM. First, the field remains limited by a relative dearth of longitudinal data, in no small part due to the limited survival of patients with GBM. This makes assessing treatment efficacy and potential biomarkers particularly challenging. Furthermore, radiographic evaluation of treatment efficacy is complicated by the phenomenon of pseudo-progression, wherein off-target chemoradiation effects induce contrast enhancement that mimics true tumor progression.[89] This post-treatment radiographic ambiguity necessitates biopsy to distinguish true tumor progression from these reactive, treatment-related inflammatory processes; yet, most post-treatment recurrent GBM biopsies are mixed specimens, with histopathological features of both recurrent tumor and treatment effect.[90] This contributes to marked inter-pathologist inconsistency in establishing a diagnosis.[91] In this way, the characteristic heterogeneity of GBM,[92] together with these radiographic and histopathological limitations, hinders the evaluation of treatment efficacy of all anti-GBM therapeutics, including immunotherapies.

Importantly, despite the aforementioned molecular and diagnostic challenges, these immunotherapies have demonstrated sustained treatment response in a subset of GBM patients, especially among patients receiving treatment via convection-enhanced delivery directly into the tumor cavity.[93] These encouraging results, in conjunction with recent technological advancements, which suggest that chronic convection-enhanced delivery is safe, feasible, and effective,[94] accentuate the urgent need to identify potential biomarkers that can monitor immunological changes begetting antitumor activity and, ultimately, predict patient responsiveness. In this review, we summarize existing and emerging biomarkers predicting response to immunotherapy in patients with GBM, covering immune checkpoint blockade, cancer vaccination modalities, and cellular targets (Figure 1).

In immune checkpoint inhibitor therapy, retrospective analyses have attempted to identify biomarkers that can predict treatment response, but small sample sizes and conflicting results hinder definitive conclusions. Nevertheless,

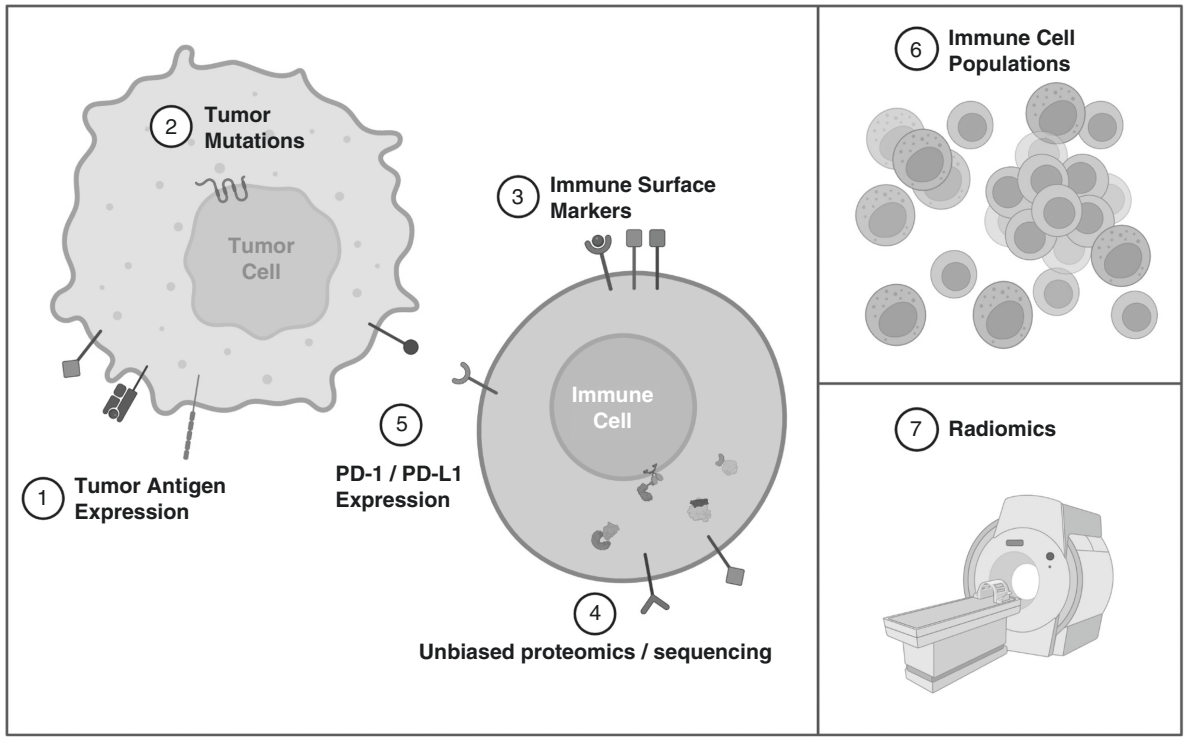

Figure 1. Categories of clinical biomarkers.

there are encouraging data regarding a variety of potential molecular markers. For example, there is converging evidence that alterations in the MAPK pathway may predict treatment efficacy in patients receiving anti-PD-1 therapy, while *PTEN* mutations may induce an immunosuppressive tumor microenvironment that is not conducive to immune checkpoint blockade.[39] Furthermore, ERK1/2 phosphorylation has been shown to be correlated with response to anti-PD-1 therapy.[46] Research investigating novel markers is ongoing, using chemokine signatures and GBM cell differentiation genes to stratify patients receiving immune checkpoint inhibitor therapy.[51,53]

Among anti-GBM vaccination methods, while targeting single tumor antigens remains futile in most instances, targeting a more diverse repertoire of antigens via tumor-lysate-derived vaccination approaches may minimize a tumor's immune evasion.[21,22,67] Studies by *Erhart* et al. represent an effort to apply unsupervised, comprehensive analyses to explore potential biomarkers to vaccination response,[65,66] and there is growing hope that combining findings from tumor phenotype and immunophenotype investigations may help stratify patients according to predicted treatment efficacy.

Importantly, there is a need for larger, well-powered clinical trials that systematically and prospectively aim to validate the efficacy of potential immunotherapy-related biomarkers. Considering the complicated landscape of these potential biomarkers, it is essential to design clinical trials that consider all facets of immunogenicity, including, for instance, both target antigen expression and HLA class. A coordinated approach involving multiple centers and collaborations will be crucial to collect and share sufficient data to yield meaningful results.[95] Some authors have argued that there is an ethical and scientific imperative to develop novel, streamlined clinical trial designs, accelerating both patient access to exploratory therapeutics and scientific progress.[22] Emerging computational tools such as machine learning and scRNA-seq are already providing new insight regarding a variety of novel radiographic and molecular biomarkers. For instance, *Lupo* et al. used susceptibility-weighted imaging, an MRI modality adept at detecting vascular alterations, in 25 GBM patients to demonstrate that radiographic evidence of increased vascularity predicted response to anti-angiogenic therapy.[96] The parallel advancement of radiographic and targeted molecular approaches, including those involving immunotherapy, may allow for the emergence of additional radiographic biomarkers of this nature. Furthermore, exploration of novel immunotherapy targets such as those involved in pro-tumor macrophage activity will continue to enhance our understanding of the tumor-immune microenvironment and uncover potential avenues to modulate it toward an anti-tumor state. Integrating these approaches into future studies will provide deeper insights into the mechanisms underlying immunotherapy response and resistance in GBM. While current biomarkers of immunotherapy response in GBM may be insufficient in isolation, the summation of prognostic tools in molecular and cellular biology, antigen targeting and presentation, and machine learning is most likely to identify robust and reliable biomarkers. Continued investigations regarding biomarkers, including the implementation of larger clinical

trials, the integration of computational approaches, and the exploration of novel targets, are essential in providing targeted immunotherapeutic care for patients with GBM.

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Conflict of interest statement

JNB has a consulting agreement with Theracle, Inc. No other author has any declarations of interest.

Authorship statement

Conceptualization and design: W.M.S., M.D.Y., A.J.T., C.P.S., and M.G.A.. Literature review and data interpretation: W.M.S., M.D.Y., and A.J.T.. Writing of the initial draft: W.M.S., M.D.Y., A.J.T.. Manuscript revision and approval of the final version: W.M.S., M.D.Y., A.J.T., C.P.S., M.G.A., A.R.A., N.Y., P.C., and J.N.B..

Data availability

No new data were generated or analyzed in support of this research.

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