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Inflammatory response and cell cycle pathways contribute to innate resistance to anti-PD-1 therapy in glioblastoma

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Abstract. Glioblastoma (GBM), as the most prevalent malignant primary brain tumor in adults, is characterized by limited treatment options and poor prognosis. Immune checkpoint inhibitors have revolutionized cancer therapy, prompting interest in their potential application in GBM treatment. This study identified potential targets for enhancing the efficacy of GBM immunotherapy by a statistical analysis of genomic and transcriptional data coupled with an exploration of the molecular mechanisms governing patient responses to immunotherapy. Our analysis revealed that the effectiveness of anti-PD-1 immunotherapy in GBM is closely associated with the mutational burden of the tumor and the age at which treatment is initiated. In addition, we found that the gene set signature of cell cycle regulation is upregulated, while the immune response regulation pathways are enriched in the tumors from non-responsive patients. These findings underscore the potential effectiveness of targeting these pathways in the context of anti-PD-1 immunotherapy, with the promise of enhancing patient outcomes in GBM.

Keywords: Glioblastoma, Immunotherapy, PD-1 Inhibitor, Precision Medicine

1. Introduction

Glioblastoma is a prevalent primary brain malignancy in adults, and the current standard of care for newly diagnosed cases has limited effectiveness, resulting in a median overall survival of approximately 16-20 months [1]. This concern is further exacerbated by the lack of effective treatments available for progressive or relapsed glioblastoma, which unfortunately occurs in most patients. In recent years, immunotherapy employing checkpoint inhibitors has emerged as a promising approach in the treatment of various types of cancers, including advanced melanoma [2], non-small-cell lung cancer [3], and Hodgkin's lymphoma [4]. The success of immunotherapy in these cancers has sparked interest in exploring its potential in glioblastoma treatment. However, a recent clinical trial investigating the use of programed cell death 1 (PD-1) immune checkpoint inhibitors in recurrent glioblastoma revealed a low response rate with only a small subset of patients (8%) demonstrating objective responses [5]. This finding raises questions about the underlying mechanism accounting for the variation in response patterns.

Understanding the factors that contribute to the limited effectiveness of immunotherapy in glioblastoma is crucial for improving treatment outcomes. One plausible explanation for the low response rate observed in the clinical trial could be the intricate tumor microenvironment in glioblastoma [6]. Glioblastoma tumors are known to exhibit a highly immunosuppressive environment, characterized by the presence of immunosuppressive cells, such as regulatory T cells and

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myeloid-derived suppressor cells, as well as immunosuppressive cytokines and chemokines. These factors may hinder the ability of checkpoint inhibitors to activate the immune system and mount an effective anti-tumor response. Furthermore, the genetic and molecular heterogeneity intrinsic to glioblastoma tumors may contribute to the unpredictable response to immunotherapy [7]. The existence of different molecular subtypes within glioblastoma tumors could impact the expression of immune checkpoint molecules and their interaction with immune cells. Additionally, the presence of genetic alterations, such as mutations in the genes encoding PD-1 or its ligands, could affect the efficacy of PD-1 immune checkpoint inhibitors. Another potential explanation for the limited response to immunotherapy in glioblastoma could be the immune escape mechanisms employed by the tumor cells [8]. Glioblastoma tumors are known to exhibit mechanisms that allow them to evade immune surveillance and destruction, including the downregulation of major histocompatibility complex (MHC) molecules, which are essential for antigen presentation to immune cells. These immune escape mechanisms may, therefore, render immunotherapy less effective in targeting and eliminating tumor cells. In addition to tumor-related factors, patient-related factors can also exert influence the response on the response to immunotherapy in glioblastoma. Patient characteristics, such as age, performance status, and comorbidities, could potentially impact the immune response and the ability to tolerate immunotherapy [9]. Moreover, the presence of pre-existing immune dysfunction or immunosuppression, either due to the disease itself or prior treatments, may also affect the response to immunotherapy.

Studies have demonstrated that a higher mutational burden in tumors [10, 11] and increased levels of T cell infiltration in the tumor microenvironment [12] are associated with an improved response to anti-PD-1 therapy across various cancer types. This suggests that tumors with higher mutational burdens may present more antigens to the immune system, leading to a stronger anti-tumor immune response. However, glioblastoma, in contrast to melanoma or non-small-cell lung cancer, exhibits a lower burden of somatic mutations [13], limiting the availability of potential antigens for recognition by the immune system. In addition, the tumor microenvironment in glioblastoma is immunosuppressive, which further hinders the immune response against the tumor. [6]. One significant contributing mechanism for immunosuppression in glioblastoma involves T cell exhaustion and apoptosis mediated by the expression of PD-1 ligands (PD-L1/2) on tumor cells [14]. The binding of PD-1 on the surface of cytotoxic T cells to these ligands impedes their ability to mount effective anti-tumor responses. Consequently, PD-1 inhibitor therapy is designed to disrupt this immune checkpoint and restore the anti-tumor immune response. However, the response to PD-1 inhibitor therapy in glioblastoma patients remains highly variable and unpredictable, highlighting the need for further investigation into the underlying factors influencing treatment outcomes.

In order to gain a comprehensive understanding of the factors that influence the response to immunotherapy in patients with glioblastoma, we conducted an extensive profiling of 66 individuals at different time points. This profiling process entailed the collection of DNA, RNA, tissue imaging, and clinical data. Our primary objective was to elucidate the genomic and stromal characteristics associated with clinical outcomes, with an ultimate goal of deciphering the underlying mechanisms underpinning the response to immunotherapy. Through this comprehensive analysis, we aspire to identify potential biomarkers or predictors of response that can serve as valuable guides for treatment decision-making and ultimately enhance the prospects of patient outcomes in the context of glioblastoma.

2. Methods

2.1. Patient and Sequencing Data Information

We obtained a cohort of 34 patients diagnosed with glioblastoma (GBM) who received with anti-PD1 treatment. Among these patients, 17 exhibited no response to the treatment, while the remaining 17 showed responses. The baseline characteristics of our patient cohort are provided in **Table 1**. The tumor samples sequenced in this study consistently achieved an average coverage of 100-fold across the exome-wide target. This level of coverage indicates that the DNA sequences of interest were thoroughly sampled multiple times, thereby increasing the accuracy and reliability of the genetic data obtained from

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these specimens. On the other hand, the matched blood normal samples attained an average coverage of 60-fold, which is slightly lower than that of the tumor samples.

2.2. Survival Analysis

We conducted a Kaplan-Meier survival analysis on the two patient cohorts (non-responders *versus* responders). This statistical method allowed us to assess the survival rates and estimate the probability of survival over a specified time period for each group. Additionally, we used the log-rank test to assess the significance of these differences.

2.3. Gene Set Enrichment Analysis (GSEA)

In order to gain a deeper understanding of the underlying molecular mechanisms that influence patient response to anti-PD1 treatment, the Gene Set Enrichment Analysis (GSEA) was conducted [15]. The GSEA approach involves a comparative assessment of gene set expression levels within samples categorized as responders and non-responders to immunotherapy. By assessing the enrichment of specific gene sets between these two groups, we aimed to pinpoint the biological pathways intricately associated to treatment response.. The goal of this analysis was to identify potential targets capable of enhancing the effectiveness of GBM immunotherapy.

3. Results and Discussion

In our initial investigation, we set out to examine the association between response to PD-1 inhibitor immunotherapy and overall survival in patients. The results indicated that the response to anti-PD-1 immunotherapy was significantly correlated with the duration of overall survival starting from the commencement of immunotherapy. Specifically, patients who exhibited a responsive pattern to immunotherapy demonstrated a median survival period of 1626 days, whereas non-responsive patients had a median survival of 951 days. This difference in survival duration was determined to be statistically significant (p=0.0358, log-rank test) (**Figure 1A**). These findings suggest that the response to PD-1 inhibitor immunotherapy plays a crucial role in determining the overall survival of patients. Patients who responded positively to the treatment experienced a prolonged median survival compared to those who did not respond. This highlights the potential efficacy of anti-PD-1 immunotherapy in improving patient outcomes.

Table 1. The information of sequenced patient treated with anti-PD1 therapy

Category	Patient Number	Correlation (p-value)
Response Status		
Response	17	
Non-response	17	
Mutation (Number)		0.0129
20-60	15	
60-100	16	
Gender		0.3147
Male	19	
Female	15	
Age Start		0.0004
30-60	18	
60-80	16	

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We conducted a comprehensive univariate survival analysis to invetigate the demographic and clinical characteristics of individuals, encompassing factors such as gender and age at the onset of treatment. Our findings revealed a significant correlation between the age at treatment initiation and the response to anti-PD-1 immunotherapy. The results of our analysis indicated that younger age at the start of treatment was associated with a more favorable response to anti-PD-1 immunotherapy (**Table 1 and Figure 1B**). These outcomes underscore the pivotal role played by the patient's age in predicting their response to therapy. In essence, individuals who initiate treatment at a younger age may experience better outcomes in terms of response to the therapy. It is important to note that while age was found to be a significant factor in predicting response, other variables such as gender did not show a significant association. These findings have important implications for the clinical management of patients undergoing anti-PD-1 immunotherapy. Healthcare providers should factor in the age of patients when making treatment initiation decisions, recognizing that younger patients may be more inclined to respond positively to the therapy. However, it is essential to acknowledge that our study represents a preliminary step, and further research is imperative. Subsequent investigations should delve into the underlying mechanisms that underpin this age-related response, and validation of these findings in larger, more diverse patient populations is essential..

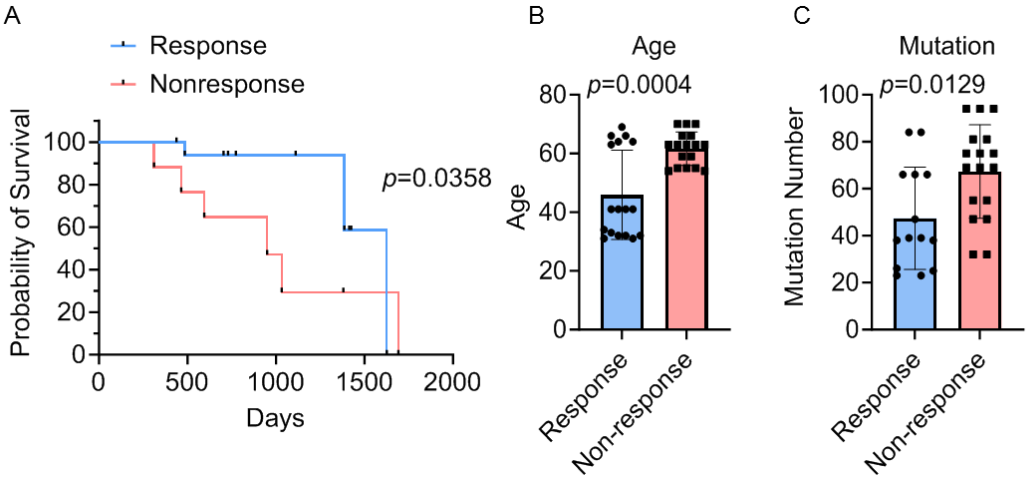

Figure 1. Anti-PD1 response correlates with age and mutation burden. A) The Kaplan–Meier survival curves of responders and non-responders of GBM patients for anti-PD1 treatments. **B)** The age of responders and non-responders of GBM patients for anti-PD1 treatments. **C)** The numbers of mutated genes in GBM tumors from responders and non-responders of GBM patients for anti-PD1 treatments. The data are shown as mean with SD. The Log-rank test was used for panel A; the unpaired student t-test was use for panel B and C. $p<0.05$ was considered as significant difference.

In our study, we analyzed a total of 31 tumors and detected a median of 58 non-synonymous somatic mutations. The range of mutations varied from 23 to 94, which is consistent with previous observations in glioblastoma multiforme (GBM) . Interestingly, we observed a higher number of non-synonymous single nucleotide variants (nsSNVs) in the responsive tumors compared to the non-responsive baseline tumors. This finding aligns with previous studies conducted in different tumor types [16]. Our analysis revealed that non-responders had a median nsSNV count of 46, whereas responders had a median count of 67 (**Figure 1C**). This difference was found to be statistically significant ($p=0.0129$, student-t test). These findings suggest that the presence of a higher number of nsSNVs may be associated with a better response to treatment in GBM patients. Overall, our study provides evidence for the correlation between the mutational profile of tumors and treatment response in GBM.

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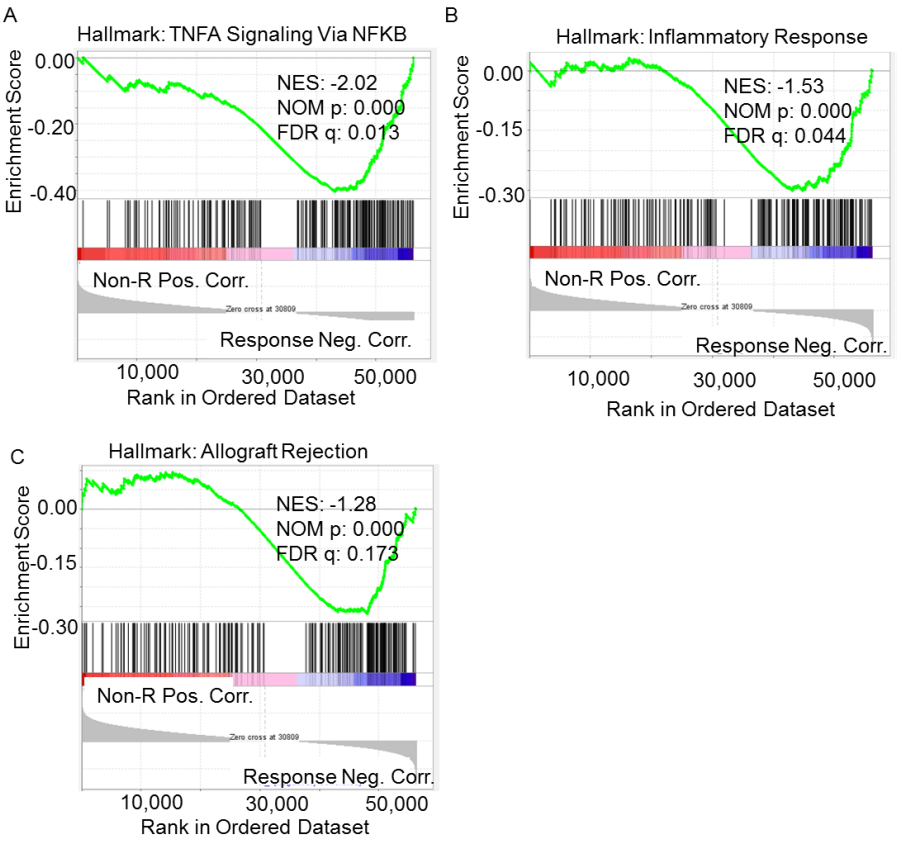

Figure 2. The immune-related gene sets are enriched in the tumors from responders compared to tumors from non-responders of GBM patients treated with anti-PD1 therapy. A)-C) The distribution of the three listed gene sets in the rank list of genes' expression changes in the tumors from non-responders compared to tumors from responders of GBM patients treated with anti-PD1 therapy (non-responders vs responders). The graphs were generated using GSEA 4.3.0 (https://www.gsea-msigdb.org/gsea/index.jsp) according to Hallmark Gene Set Database (doi: 10.1016/j.cels.2015.12.004). NES: Normalized Enrichment Score; NOM p-val: Nominal p value; FDR q-val: False Discovery Rate of q-value.

Table 2. The significantly enriched KEGG Pathway Gene Sets in tumors from response patients compared to tumors from non-response patients treated with anti-PD1 therapy.

Gene Set Name*	NES	NOM p-val	FDR q-val
Allograft Rejection	-1.86	0.006	0.016
Graft Versus Host Disease	-1.73	0.006	0.035
Hematopoietic Cell Lineage	-1.63	0	0.059
Cell Adhesion Molecules Cams	-1.62	0	0.055
Ascorbate And Aldarate Metabolism	-1.51	0.039	0.094
Intestinal Immune Network For Iga Production	-1.38	0.014	0.193
Porphyrin And Chlorophyll Metabolism	-1.35	0.06	0.204

*: The analysis was conducted using GSEA 4.3.0 (https://www.gsea-msigdb.org/gsea/index.jsp) according to KEGG Gene Set Database (KEGG). NES: Normalized Enrichment Score; NOM p-val: Nominal p value; FDR q-val: False Discovery Rate of q-value.

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We conducted a differential enrichment analysis utilizing HALLMARK and KEGG gene sets to probe the effects of PD-1 inhibitor treatment on gene sets intricately linked to the immune response. Our primary aim was to elucidate the impact of this treatment on the immune-related pathways.. The results of our analysis revealed that, prior to the initiation of the treatment, the top-ranked gene sets were significantly enriched in pathways related to the immune response. Through our analysis, a total of ten pathways were identified to be statistically significant, with a p-value of less than 0.05 and a false discovery rate q-value of less than 0.250. This statistical significance implies the likely biological relevance of these pathways and underscores their pivotal role in shaping the response to PD-1 inhibitor treatment. These findings suggest that the gene sets implicated in these pathways play a crucial role in the immune response and hold the potential for targeted therapeutic intervention. Overall, our study highlights the importance of exploring the impact of PD-1 inhibitor treatment on gene sets associated with the immune response. The identification of these pathways presents a promising avenue for the development of targeted interventions aimed at enhancing the efficacy of PD-1 inhibitor treatment while bolstering the immune response in patients. Thus, our study paves the way for further investigation in this domain, facilitating the development of novel strategies to optimize the therapeutic outcomes of PD-1 inhibitor treatment..

Interestingly, among the statistically significantly enriched pathways comprising twelve Hallmark Gene Sets and twelve KEGG pathways (**Table 3 & Table 4**) in non-response group, four of them are related to cell cycle regulation, including G2M Checkpoint, E2F Targets, Mitotic Spindle, and Cell Cycle gene sets. Cell cycle control systems, similar to the immune system, play a crucial role in regulating the different phases of the cell division cycle. This control is essential in preventing uncontrolled cell division, which is a hallmark of cancer development.

Table 3. The significantly enriched Hallmark Gene Sets in tumors from non-response patients compared to tumors from response patients treated with anti-PD1 therapy.

Gene Set Name*	NES	NOM p-val	FDR q-val
G2m Checkpoint	2.0188794	0	0
E2f Targets	1.9598504	0	4.36E-04
Myc Targets V1	1.8828586	0	8.72E-04
Oxidative Phosphorylation	1.8540591	0	6.54E-04
Dna Repair	1.7651566	0	0.001050928
Spermatogenesis	1.7344183	0	0.00116378
Mitotic Spindle	1.7088575	0	0.001493628
Peroxisome	1.6128508	0.001060445	0.005004191
Fatty Acid Metabolism	1.5694866	0	0.006611298
Adipogenesis	1.4623746	0	0.025461573
Bile Acid Metabolism	1.4323065	0.004192872	0.03359608
Pancreas Beta Cells	1.3914847	0.052009456	0.05133176

*: The analysis was conducted using GSEA 4.3.0 (KEGG) according to Hallmark Gene Set Database (doi : 10.1016/j.cels.2015.12.004). NES: Normalized Enrichment Score; NOM p-val: Nominal p value; FDR q-val: False Discovery Rate of q-value.

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Table 4. The significantly enriched KEGG Pathway Gene Sets in tumors from non-response patients compared to tumors from response patients treated with anti-PD1 therapy.

Gene Set Name*	NES	NOM p-val	FDR q-val
UBIQUITIN MEDIATED PROTEOLYSIS	1.8929709	0	0.001873718
SELENOAMINO ACID METABOLISM	1.8338487	0	0.007074765
CELL CYCLE	1.8334739	0	0.00471651
RNA DEGRADATION	1.8249701	0	0.003774562
SPLICEOSOME	1.7948121	0	0.005862449
PROTEASOME	1.7511322	0	0.010703397
RIBOSOME	1.7245507	0	0.014044377
PEROXISOME	1.6984931	0	0.017175686
PURINE METABOLISM	1.6928145	0	0.01600388
PYRIMIDINE METABOLISM	1.6905106	0	0.014792683
PROPANOATE METABOLISM	1.688515	0.003496504	0.013969905
PROGESTERONE MEDIATED OOCYTE MATURATION	1.6826808	0	0.013670911

*: The analysis was conducted using GSEA 4.3.0 (https://www.gsea-msigdb.org/gsea/index.jsp) according to KEGG Gene Set Database. NES: Normalized Enrichment Score; NOM p-val: Nominal p value; FDR q-val: False Discovery Rate of q-value.

The mechanism of action for checkpoint control is a finely orchestrated process driven by site-specific protein phosphorylation [17], which primarily relies on cyclin-dependent proline-directed protein kinases. For instance, cyclin D1 [18] and CDK4/6 [19] are downstream targets of growth-initiating signaling pathways that promote cellular proliferation. Inhibitors such as Palbociclib, which targets CDK4/CDK6, have been approved for the treatment of certain types of breast cancer, highlighting the importance of checkpoint control in cancer therapy. Another key regulatory protein in the cell cycle is E2F, encoded by the E2F proto-oncogene[20]. This protein is known to drive cell competence, allowing quiescent cells to enter the S phase, and is also involved in oncogene addiction as a molecular survival factor. In fact, clinical trials exploring tumor-targeted gene therapy blocking E2F have been conducted.

4. Conclusions

In this study, we delved into the intricate landscape of glioblastoma therapy, with a particular focus on PD-1 inhibitor immunotherapy. Our findings have illuminated critical facets of this treatment approach, underlining its pivotal role in shaping patient outcomes. Notably, our research unveiled a strong association between treatment response and overall survival, with responders experiencing significantly extended survival periods. Furthermore, age at treatment initiation emerged as a crucial factor, with younger patients exhibiting more favorable responses. Intriguingly, our mutational analysis demonstrated that a higher number of non-synonymous somatic mutations correlated with better treatment responses. Equally noteworthy, our differential enrichment analysis uncovered the profound impact of PD-1 inhibitor treatment on immune-related gene sets, reinforcing their therapeutic relevance. Particularly, the identification of cell cycle regulation pathways within the non-response group highlighted potential connections between cell cycle control and immune response modulation. Overall, our study has underscored the multifaceted interplay of factors influencing glioblastoma treatment response, offering valuable insights for the refinement of therapeutic strategies aimed at enhancing

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patient outcomes. The promising avenues unveiled in this research beckon for further exploration, heralding a brighter future in glioblastoma therapy.

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