

Siglecs, Novel Immunotherapy Targets, Potentially Enhance The Effectiveness of Existing Immune Checkpoint Inhibitors in Glioma Immunotherapy

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Guan-Zhang Li^{1,*}
Ke-Nan Zhang^{1,*}
Zheng Wang²
Hui-Min Hu¹
Zhi-Liang Wang¹
Ruo-Yu Huang¹
Hao-Yu Jiang²
You Zhai¹
Yue-Mei Feng¹
Yuan-Hao Chang¹
Ren-Peng Li²
Fan Wu¹
Fan Zeng¹
Tao Jiang¹⁻⁴
Wei Zhang²

¹Department of Molecular Neuropathology, Beijing Neurosurgical Institute, Capital Medical University, Beijing 100070, People's Republic of China; ²Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing 100070, People's Republic of China; ³Center of Brain Tumor, Beijing Institute for Brain Disorders, Beijing 100070, People's Republic of China; ⁴China National Clinical Research Center for Neurological Diseases, Beijing 100070, People's Republic of China

*These authors contributed equally to this work

Correspondence: Wei Zhang; Tao Jiang
Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, No. 119 South Fourth Ring Road West, Fengtai District, Beijing, People's Republic of China
Tel +86 180 1021 2611
Email zhangwei_vincent@126.com; taojiang1964@163.com

Background: Inhibitors of immune checkpoints have shown little effect in clinical trials involving glioma patients. Here, we explored novel targets for use in future treatments. Previous studies showed the sialic acid-binding Ig-like lectin (Siglec) family to have a specific role in immunosuppression. We aimed to study the characteristics and immune function of Siglec family members.

Methods: Transcriptome data from 1024 glioma samples and 1551 glioma single cells were used in our study. Clinical and molecular pathology information was also included. Statistical, bioinformatical methods, and single-cell sequencing analysis were applied to investigate the role of Siglec family members.

Results: Siglecs-5, -7, -9, and -16 showed a significant correlation with immunosuppression in glioma. They are typically expressed in higher grade, IDH-wildtype, and mesenchymal subtype gliomas. Siglec-5, -7, and -9 had a similar immune function to TIM-3, while Siglec-16 was similar to PD-L1, suppressing tumor immunity via different mechanisms. Joint use of Siglec-inhibitors and immune checkpoint inhibitors could prolong the survival of glioma patients.

Conclusion: Siglec-5, -7, -9, and -16 suppressed tumor immunity in different ways. Joint usage of inhibitors may be an effective means to improve the efficacy of glioma immunotherapy.

Keywords: glioma, Siglec family, immunotherapy, single-cell sequencing analysis, prognosis

Introduction

Gliomas, the most common and most malignant brain tumors in adults, are highly resistant to traditional treatments.¹ Despite aggressive treatment approaches, consisting of neurosurgical resection followed by adjuvant chemo- and radiotherapy, prognosis remains poor.² Newly developed immunotherapy approaches may improve outcomes in glioma patients.^{3,4}

The 2018 Nobel Prize for Medicine Prize was awarded for work showing that the inhibition of negative immune regulation can be used to treat cancer. Various inhibitors of immune checkpoints have been investigated in research and clinical trials, demonstrating remarkable effectiveness.⁵⁻⁷ PD-1/PD-L1-targeting therapy has been shown to be effective in several tumor types.^{3,6-9} Thus, the US Food and Drug Administration (FDA) has approved the clinical application of immune checkpoint inhibitors in melanoma, breast cancer, renal cancer, and lung cancer. However, PD-1/PD-L1 inhibitors showed little therapeutic effect in glioma.¹⁰⁻¹² Phase II and phase III clinical trials of durvalumab and nivolumab found no significant improvement in median overall survival.^{13,14} Therefore, it is imperative to explore novel targets to develop effective treatments or for use in joint treatments.

The sialic acid-binding Ig-like lectins (Siglecs) are a protein family of classical immunoglobulin-like lectins. Under physiological conditions, only 15 human and nine murine Siglec molecules are expressed on the surface of myeloid cells and immune cells mediating immune suppression.^{15,16} Recent research showed that Siglec members are broadly over-expressed in human cancer cells and tumor-infiltrating myeloid cells,^{17,18} and that their distribution is mutually exclusive with that of PD-L1.¹⁹ In particular, Siglec-10 was identified as the receptor of CD24, delivering a “don’t eat me” signal;²⁰ it was also discovered on extracellular vesicles inhibiting T cell activity.²¹ Similarly, Siglec-15 was shown to function as an immune suppressor in colorectal cancer.²²

However, the relationship between Siglec family members and checkpoints in glioma remained unknown. Here, we performed an integrative investigation of Siglecs in 325 glioma samples and validated the results using The Cancer Genome Atlas (TCGA) database and single-cell RNA sequencing (RNA-seq) data from the GSE89567 dataset. This is the first integrative study describing the molecular and clinical characteristics of Siglecs in glioma and demonstrating the potential use of Siglec inhibitors in joint treatment with immune checkpoint inhibitors.

Materials and Methods

Patients and Samples

Sample collection and data analyses were approved by the Beijing Tiantan Hospital institutional review board (IRB), and written informed consent was obtained from each patient. Samples with more than 80% tumor cells were selected for transcriptome sequencing on an Illumina HiSeq platform. Each sample was diagnosed by two independent neuropathologists. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. TCGA RNA-seq data were downloaded from the official website (<https://cancergenome.nih.gov>). Single-cell sequencing data were obtained from GSE89567 on the GEO website.

Gene Set Variation Analysis (GSVA)

GSVA analysis was performed with the GSVA package.²³ The list of gene ontology terms was obtained from the GSEA Web portal (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>). The analysis was performed with default parameters. Relationships between genes and biological functions were determined using Pearson correlation analysis.

T-Distributed Stochastic Neighbor Embedding (T-SNE) Analysis

The t-SNE analysis was performed with the *Rtsne* package. Perplexity was set to 20. Identification of cell types used specific cell markers obtained from the official CellMarker website (<http://bioacc.hrbmu.edu.cn/CellMarker/>).

Statistical Analysis

Statistical analyses and visualization were performed in R 3.5.0, SPSS software 25.0, and Microsoft Office 2016. SPSS statistical software was used for the Cox regression analysis. Radar charts were created in Microsoft Office 2016. Other analyses were performed with R packages, including *ggplot2*, *pROC*,²⁴ and *pheatmap*. The log-rank test was used in Kaplan–Meier survival analysis. A p-value less than 0.05 was considered statistically significant.

Results

Siglec-5, -7, -9 and -16 are Independent Prognostic Factors Associated with Malignant Progression in Glioma

The expression landscape of Siglec family members in glioma showed that most members were differentially expressed in both the CGGA and TCGA databases, except Siglec-6 (Figure 1A and B). The multivariate Cox analysis revealed that Siglec-5, -7, -9 and -16 are independent to clinical and molecular pathological factors in both databases (Figure 1C). In addition, Siglec-5, -7, -9 and -16 showed higher expression levels in high-grade gliomas (Figure 1D and E), suggesting that these Siglecs are associated with tumor progression in glioma.

Siglec-5, -7, -9, and -16 are Enriched in IDH-Wildtype and MGMT Promotor Unmethylated Glioma

IDH mutation and MGMT promotor methylation status are the two most significant prognostic biomarkers for glioma.²⁵ Therefore, we explored the relationships between IDH mutation and MGMT promotor methylation status and the expression of Siglec-5, -7, -9 and -16. As shown in the column diagrams, all four Siglec members showed significantly higher expression in the IDH-wildtype and MGMT promotor unmethylated groups (Figure 2A and B, Figure S1A and B). Subsequent receiver operating characteristic (ROC) curve analysis showed that Siglec-5, -7, -9 and -16 were specifically enriched in IDH-wildtype gliomas (Figure S2A and B).

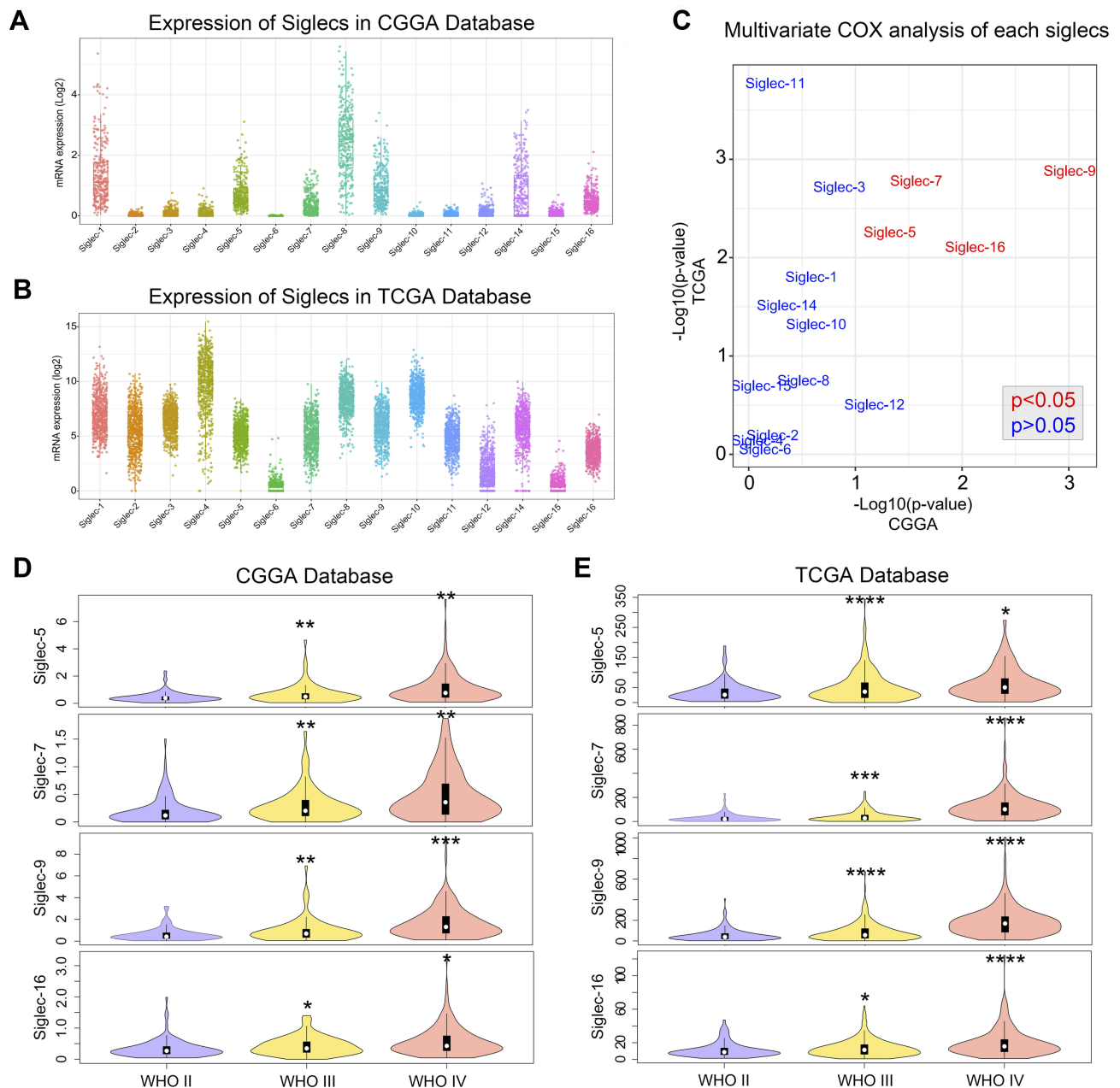


Figure 1 Expression landscape of Siglec family members in glioma. **(A, B)** Transcriptome expression map of Siglecs in the CGGA and TCGA database. **(C)** p-values for multivariate Cox analysis of each Siglec member in the CGGA and TCGA databases. Variables in the multivariate Cox analysis included Siglec member, WHO grade, age, and IDH mutation status. Red font indicates an independent prognostic factor. **(D, E)** Violin plot showing the expression of Siglecs in each WHO grade glioma according to the CGGA and TCGA databases. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

All these results were mutually verified using the CGGA and TCGA databases.

Siglec-5, -7, -9, and -16 are Potential Markers for the Mesenchymal Molecular Subtype

Specific enrichment in the mesenchymal molecular subtype is an important feature of immune checkpoints.²⁶ Thus, we explored the distribution of four Siglec members

in different molecular subtypes defined by the TCGA network.²⁷ As shown in Figure 3, four Siglec family members had higher expression in the mesenchymal subtype. Accordingly, the enrichment of Siglec-5, -7, -9, and -16 in the mesenchymal subtype were also specifically validated by ROC curve analysis (Figure S2E and F). The specific expression pattern was found in both the CGGA and TCGA databases, indicating a potential immune-related feature of Siglecs.

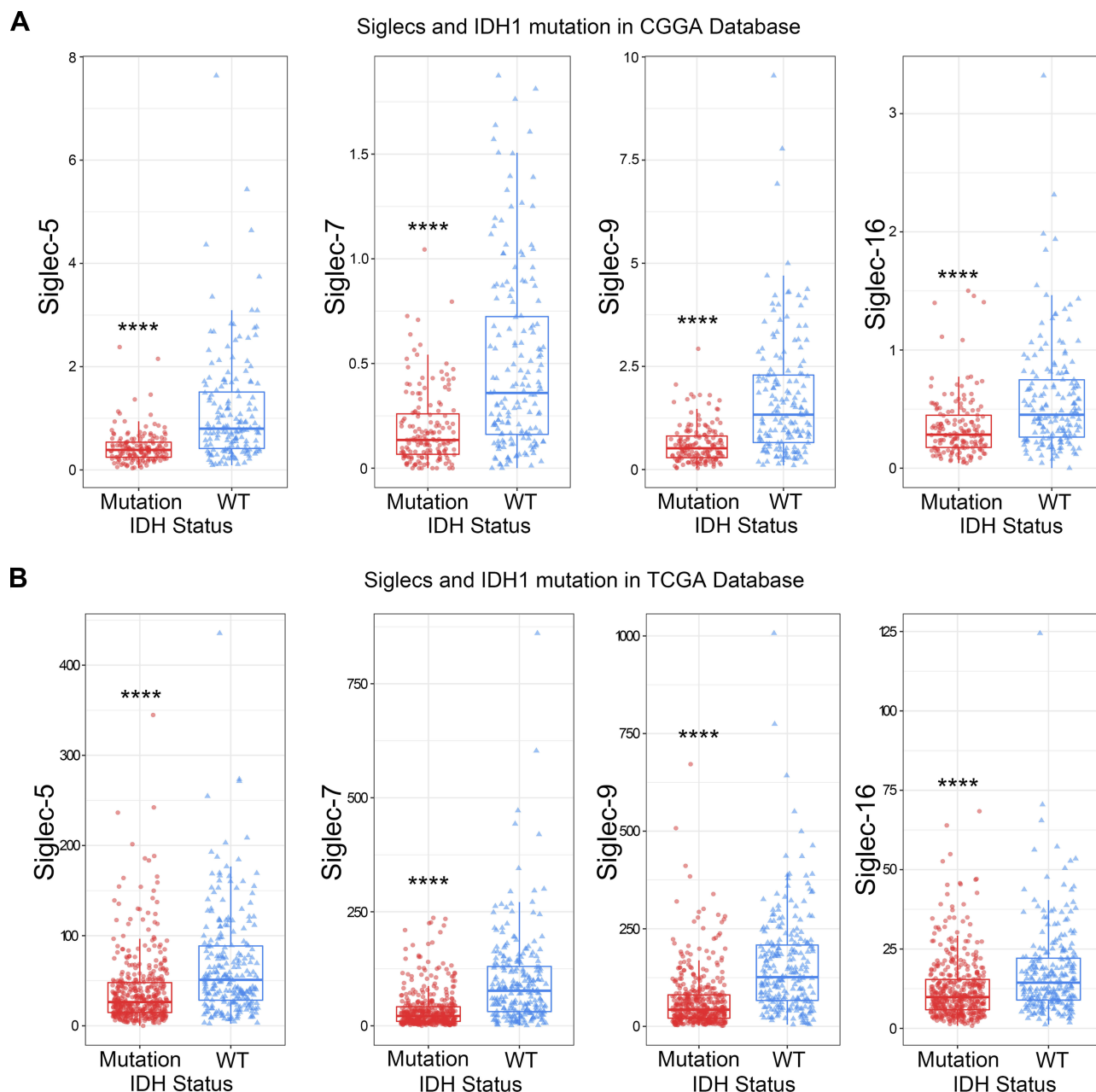


Figure 2 Siglec-5, -7, -9, and -16 are highly expressed in IDH-wild-type gliomas. The expression of Siglec-5, -7, -9, and -16 was higher in IDH-wildtype gliomas than in IDH-mutated gliomas, according to the CGGA (**A**) and TCGA (**B**) databases. **** $p < 0.0001$.

Siglec-5, -7, -9, and -16 are Closely Related to Immune Functions in Glioma

Unsupervised clustering analysis was used to determine the expression patterns of Siglecs and known immune checkpoints. Siglec-5, -7, and -9 had similar expression patterns, whereas that of Siglec-16 was quite different (Figure 4A and C). We further analyzed the correlation of the four Siglec family members and 4436 biological functions in 14 functional classes to investigate related biological processes. All four Siglecs were positively correlated with approximately 99%

of immune functions (Figure 4B and D). All related biological functions are listed in [Supplementary Table 1](#). Highly consistent results were observed with the CGGA and TCGA databases, indicating that Siglec-5, -7 and -9, and -16 are commonly immune-related, but in slightly different ways from each other.

Siglec-5, -7, -9, and -16 Synergize with Different Immune Checkpoints

To explore the distinctions between the immune-related functions of Siglec-5, -7, -9, and -16, we performed

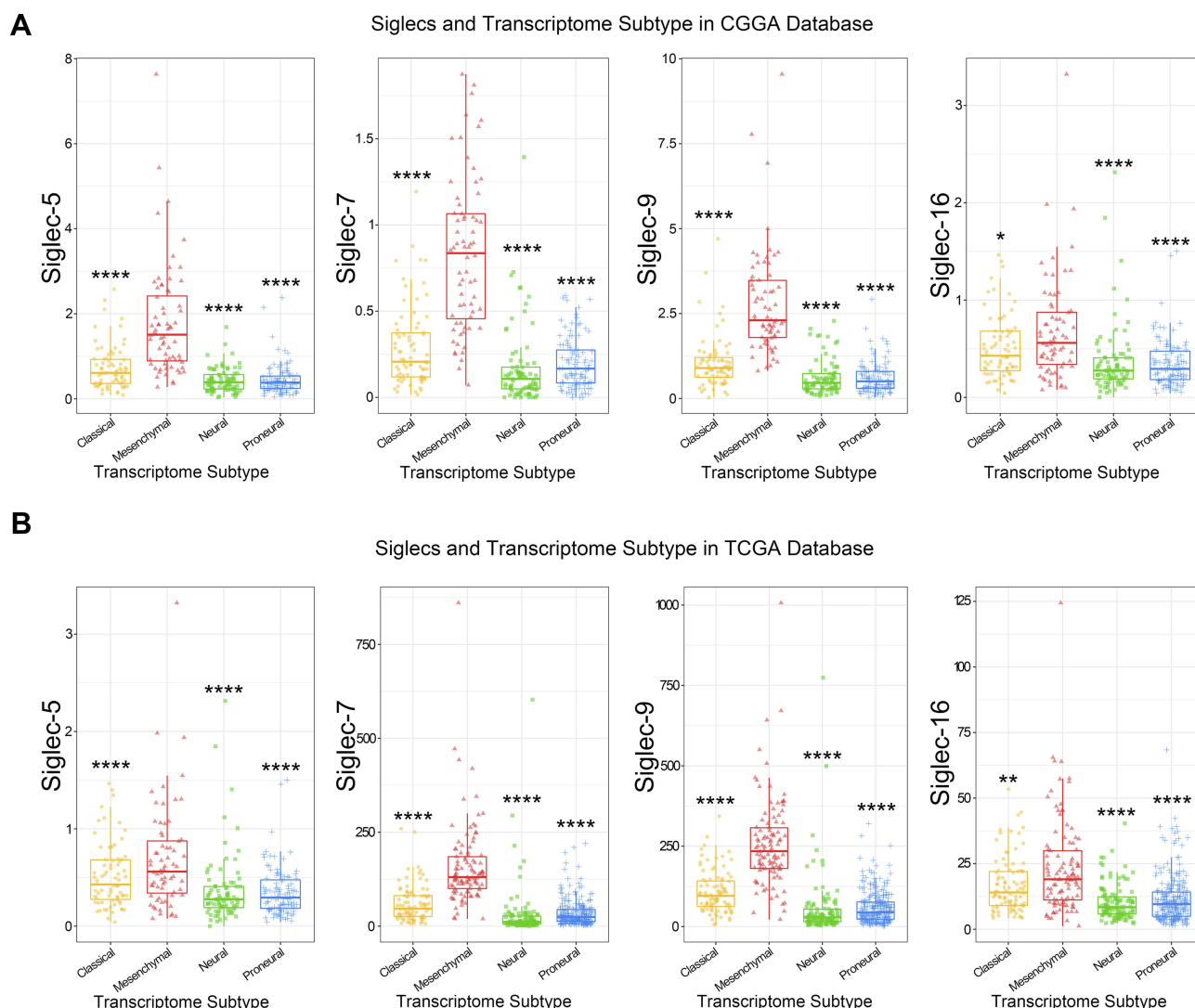


Figure 3 Siglec-5, -7, -9, and -16 are highly enriched in mesenchymal subtype. The expression of Siglec-5, -7, -9, and -16 was higher in mesenchymal subtype gliomas than others, according to the CGGA (**A**) and TCGA (**B**) databases. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

t-SNE analysis for the expression levels of Siglecs and known immune checkpoints. There were similarities among Siglec-5, -7, and -9, TIM-3, galectin-9, B7-1, and CD40; and Siglec -16 and PD-L1, PD-1, CTLA4, B7-2, CD28, and HVEM had similar roles in both the CGGA and TCGA databases (Figure 5A and C). To further validate the relationship between Siglecs and immune functions, we calculated the correlation with inflammatory activities. As shown in Figure 5B and D, Siglec-5, -7, and -9, TIM-3, and galectin-9 had almost the same inflammatory activation status. Siglec-16, B7-2, PD-1, PD-L1, and CTLA4 were also similar to each other (Figure 5B and D).

Single-Cell Sequencing Reveals the Difference Between Siglec-5, -7, and -9 and Siglec -16

The development of single-cell sequencing technology enabled a deeper understanding of the heterogeneity among cells.^{28,29} We found that Siglec-5, -7, and -9 expression was mutually exclusive in cells in WHO grade II gliomas. However, the expression pattern changed in higher-grade gliomas (WHO III and WHO IV), where different Siglecs were simultaneously expressed on the same cells (Figure 6A). However, Siglec-16 was expressed exclusively to Siglec-5, -7, and -9 in glioma cells of all grades (Figure 6A). Next, we studied the

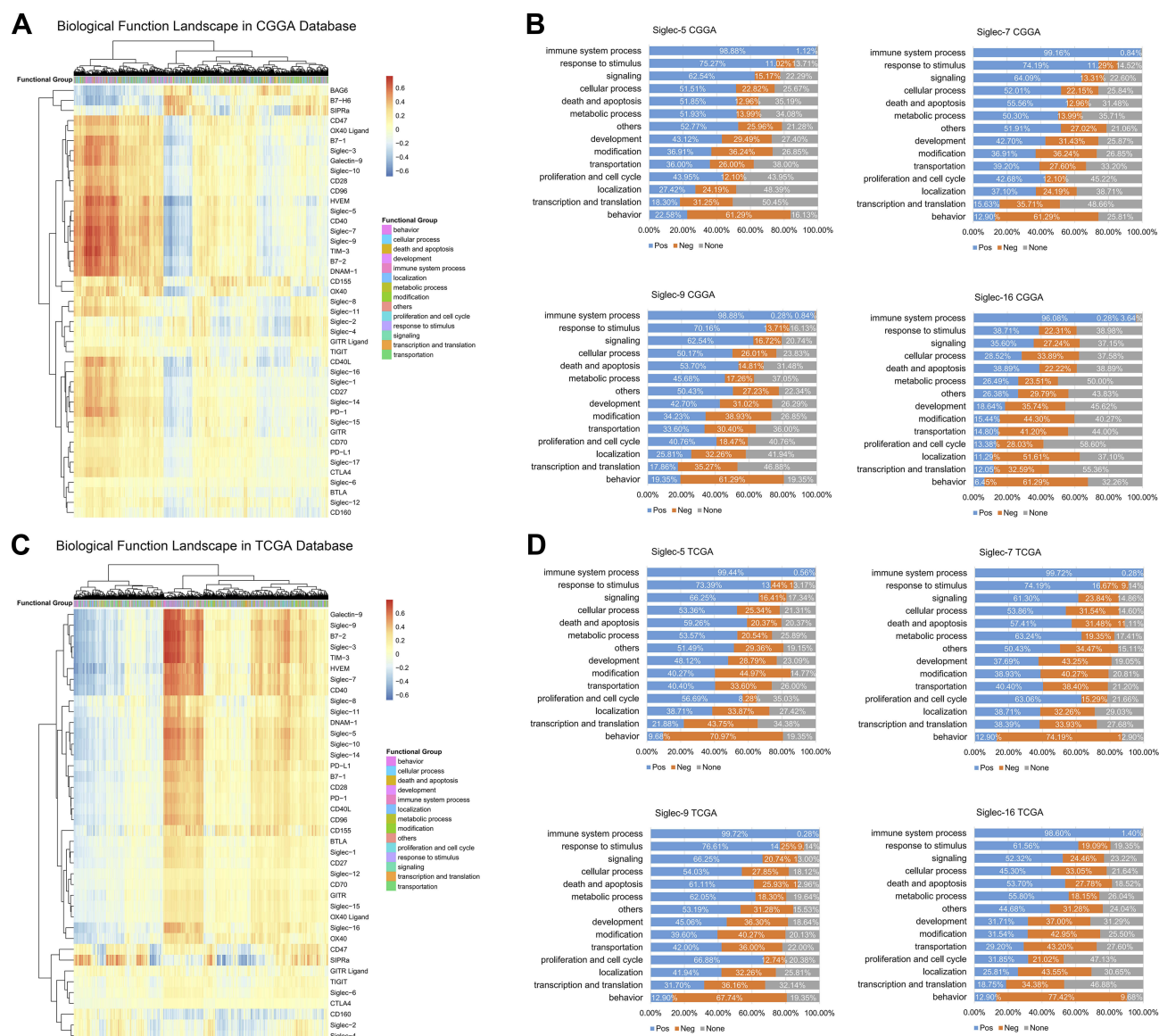


Figure 4 Siglec-5, -7, -9, and -16 are closely related to immune functions in glioma. **(A, C)** Functional heatmaps of Siglecs and immune checkpoints in the CGGA and TCGA databases. **(B, D)** Proportion of Siglec-related biological functions in each function class. Blue parts represent positive correlation; orange represents negative correlation; gray indicates no significant correlation.

relationships between Siglec-5, -7, -9, and -16 and immune checkpoints. Siglec-16 expression was exclusive to PD-1, PD-L1, CTLA4, CD28, and HVEM (Figure 6B), whereas no exclusive pattern was found for Siglec-5, -7, and -9 and TIM-3, galectin-9, B7-1, and CD40 (Figure S3). Interestingly, spatial mapping analysis revealed that Siglec-5, -7, and -9, but not Siglec-16, gradually transferred from tumor cells to immune cells with increasing malignancy (Figure 6C and D). Further subdividing the Siglec-expressing immune cells, we found that macrophages and tumor-associated macrophages (TAMs) accounted for a major

portion (Figure 6E). In an open-access single-cell sequencing database, the main distribution was also in macrophages, providing an external validation of our results (Figure S4).³⁰

Joint Usage of Siglec Inhibitor and Immune Checkpoint Inhibitor May Benefit Glioma Patients

To explore the potential effects of joint usage of a Siglec inhibitor and immune checkpoint inhibitor on survival, a combined prognostic analysis was performed. In the

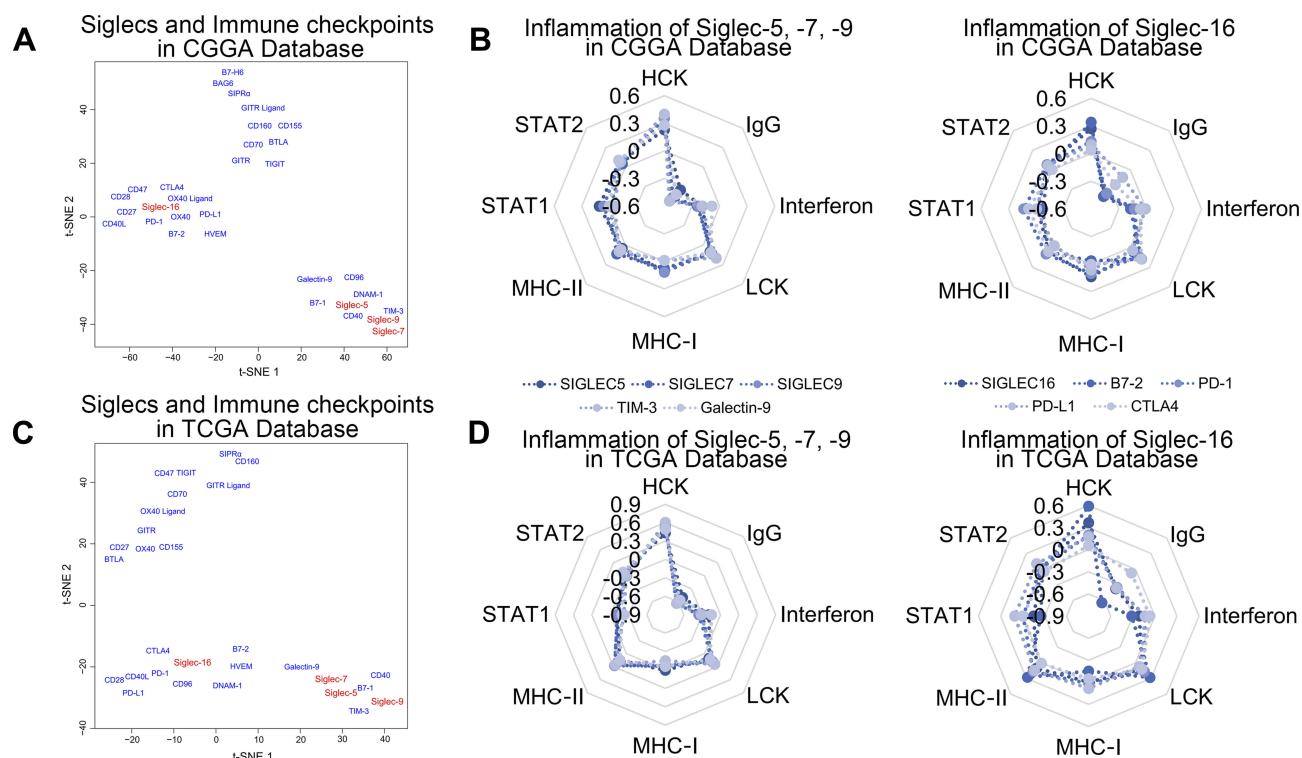


Figure 5 Siglec-5, -7, -9, and -16 and immune checkpoints share biological functions. **(A, C)** Functional distribution of Siglec-5, -7, -9, and -16 and immune checkpoints by t-SNE analysis in the CGGA and TCGA databases. **(B, D)** Radar diagrams of the correlation between Siglec-5, -7, -9, and -16 and inflammatory activity. The coordinates in the figure are the R values from Pearson correlation analysis of the Siglecs and inflammatory response scores.

former retrospective survival analysis, patients with over-expression of immune checkpoints were regarded as applicable target patients. The Kaplan–Meier curves revealed that patients with low expression of both Siglecs and synergistic immune checkpoints had better survival than patients in the other three groups, according to both the CGGA and TCGA databases (Figure 7). All these results indicated a better prognosis in patients under combined treatment compared with those receiving single immune checkpoint inhibitor therapy, indicating an enhancer role for Siglec inhibitors in the combined treatment.

Discussion

Owing to its great success in the treatment of non-small-cell lung carcinoma, acute lymphocytic leukemia, etc., tumor immunotherapy has flourished in the past few years.^{6,7,31,32} However, the immune checkpoint inhibitors often lose efficacy gradually over time, eventually leading to failure of the treatment.^{10,12,33} A series of studies confirmed that the PD-1/PD-L1 pathway was responsible for immunosuppressive status in less than 40% of solid tumors.^{31,34} Thus, it is necessary to screen out new targets for immunotherapy. The Siglec family of novel immune-

regulators has attracted research attention in this regard.¹⁶ Recently, Siglec-15 was screened out as a new therapeutic target by a high-throughput functional screening system (TCAA), expressed exclusively in PD-1/PD-L1 in tumors.¹⁹ In glioma, Santegoets et al found that the Siglecs were highly expressed in tumor cells and immune cells.³⁵ Based on the above studies, we hypothesized that Siglecs might significantly regulate tumor immunity in glioma.

In this work, we found a specific expression pattern of Siglec family members in 1024 gliomas and confirmed Siglec-5, -7, -9, and -16 as independent prognostic factors. Their correlations with IDH mutation, MGMT promotor methylation status, and mesenchymal subtype suggested their potential as biomarkers. Although the correlation with MGMT promotor methylation status was not significant, this may have been due to missing data. Further functional analysis revealed the immune-related features of these Siglecs, including leukocyte activation, natural killer cell differentiation, positive regulation of macrophage activation, and regulation of T cell chemotaxis, indicating that they deserve further future research. Siglec-5, -7, and -9 were also identified in a study by

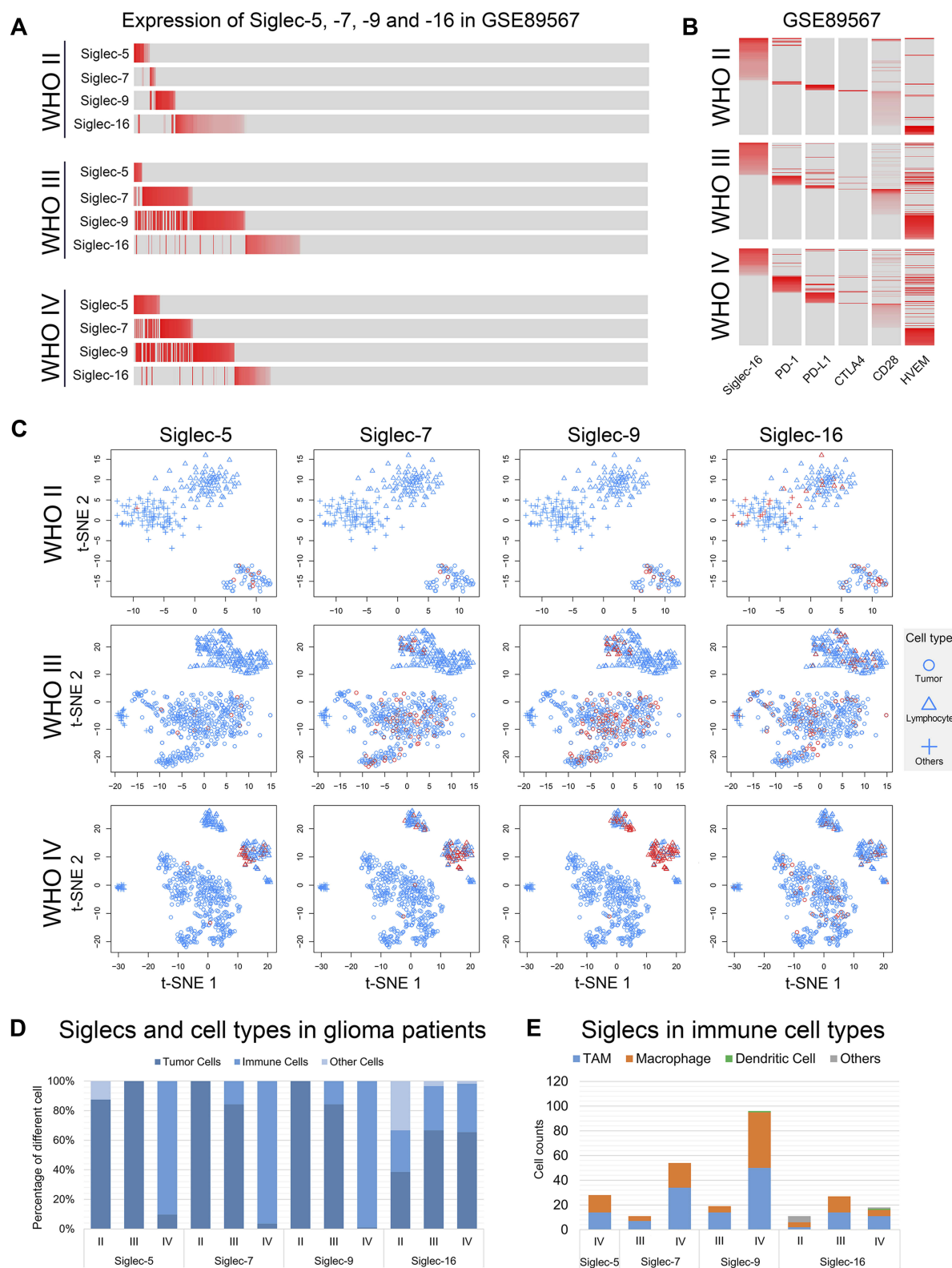


Figure 6 Immunological characteristics are different in Siglec-5, -7, -9, and -16. **(A)** Expression of Siglec-5, -7, -9, and -16 in glioma cells of each WHO grade. The expression level is represented by the shade of red. Gray indicates no expression. **(B)** Expression of Siglec-16 and immune checkpoints in glioma cells of each WHO grade. The expression level is represented by the shade of red. Gray indicates no expression. **(C)** Spatial mapping analysis of each WHO grade glioma. Red indicates that Siglec was expressed in the cell. Cells without Siglec expression are colored blue. **(D)** The distribution proportion of Siglec members in each cell type. **(E)** Number of immune cells with Siglec members expressed.

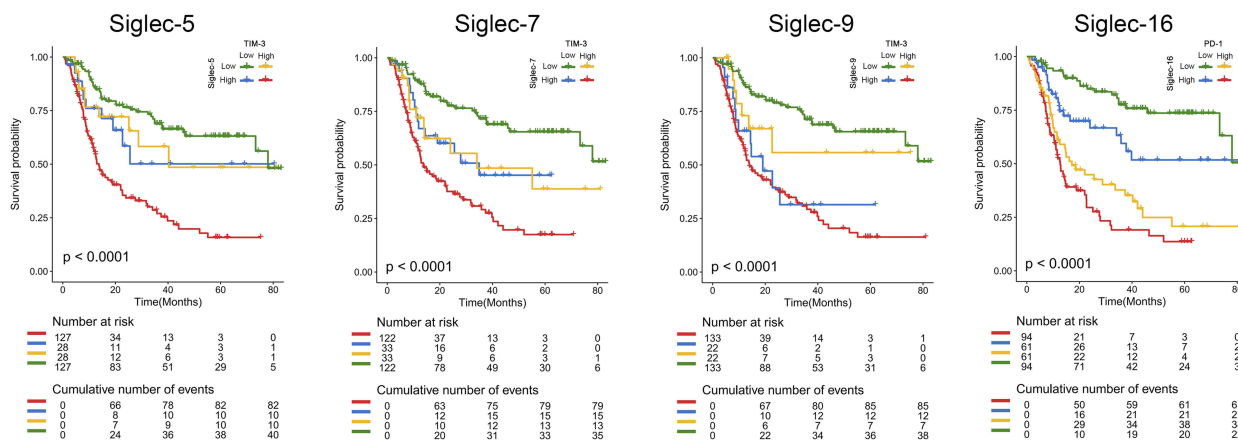
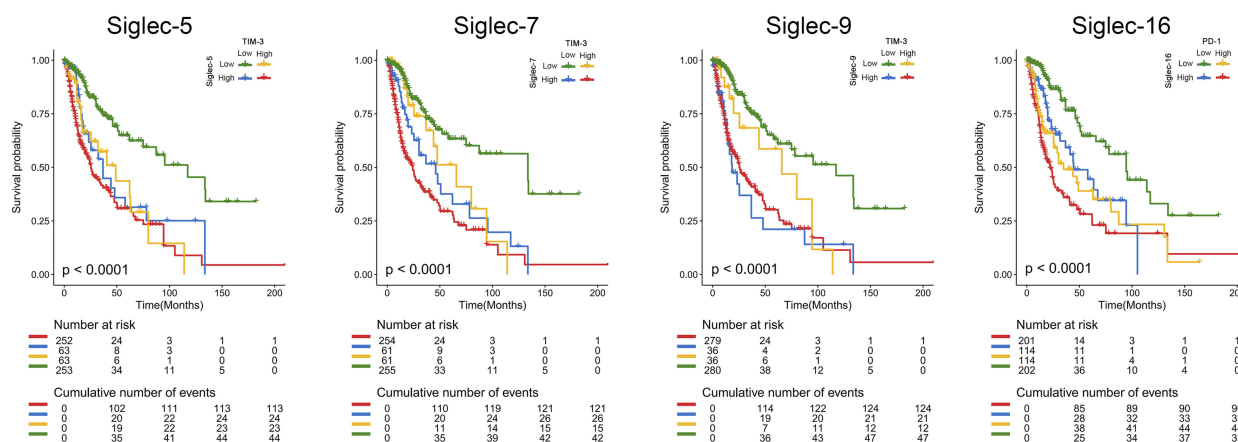
A**Survival analysis of Siglecs in CGGA Database****B****Survival analysis of Siglecs in TCGA Database**

Figure 7 Joint usage of Siglec inhibitor and corresponding immune checkpoint inhibitor may benefit glioma patients. (**A**, **B**) Kaplan–Meier curves of patients with different expression patterns of Siglec members and immune checkpoints in the CGGA and TCGA database. Patients with low expression of Siglec members and corresponding immune checkpoints had the longest overall survival time. The log-rank test was used for survival analysis.

Santegoets³⁵ and were shown to have similar immune functions to TIM-3,³⁶ whereas Siglec-16 was different, with a function complementary to that of PD-1/PD-L1.²⁶

Single-cell sequencing analysis revealed that Siglec-5, -7, -9, and -16 were expressed synergistically with TIM-3, mainly in macrophages and TAMs; this has been reported in leukemia, glioma, etc.^{17,19,35} Interestingly, we first reported the expression shift of Siglec-5, -7, and -9 from tumor cells to macrophages with increasing tumor malignancy, possibly owing to trogocytosis of macrophages.³⁷ This may induce immune escape in high-grade gliomas. However, no such expression shift was detected in Siglec-16 expressing cells, indicating the selectivity of macrophage trogocytosis. Siglec-16 was expressed exclusively with PD-1, PD-L1, and CTLA4 in gliomas, similar to the reported function of Siglec-15.¹⁹

In the survival analysis, patients were divided into four groups based on the expression of Siglec members and immune checkpoints, and their Kaplan–Meier curves were compared, as in previously published research.³⁸ The results indicated that patients with low expression of both Siglecs and the corresponding immune checkpoints had a better prognosis than others, suggesting that the joint usage of a Siglec inhibitor and corresponding immune checkpoint inhibitors may prolong the overall survival of glioma patients.

Conclusions

In conclusion, Siglec family members showed specific expression patterns in gliomas and had diverse roles in the immunosuppression process, interacting with different

immune checkpoints. This indicates the high potential of a combination of Siglec inhibitors and immune checkpoint inhibitors to solve the current problems of immunotherapy in glioma.

Ethics Approval and Informed Consent

Sample collection and data analyses were approved by Beijing Tiantan Hospital IRB. Written informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki.

Data Availability

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Acknowledgments

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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