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Increased Immune Gene Expression and Immune Cell Infiltration in High-Grade Astrocytoma Distinguish Long-Term from Short-Term Survivors

Andrew M. Donson,*† Diane K. Birks,†‡ Stephanie A. Schittone,*† Bette K. Kleinschmidt-DeMasters,‡*§ Derrick Y. Sun,‡ Molly F. Hemenway,*† Michael H. Handler,‡*§ Allen E. Waziri,‡ Michael Wang,*† and Nicholas K. Foreman,*†‡

Survival in the majority of high-grade astrocytoma (HGA) patients is very poor, with only a rare population of long-term survivors. A better understanding of the biological factors associated with long-term survival in HGA would aid development of more effective therapy and survival prediction. Factors associated with long-term survival have not been extensively studied using unbiased genome-wide expression analyses. In the current study, gene expression microarray profiles of HGA from long-term survivors were interrogated for discovery of survival-associated biological factors. Ontology analyses revealed that increased expression of immune function-related genes was the predominant biological factor that positively correlated with longer survival. A notable T cell signature was present within this prognostic immune gene set. Using immune cell-specific gene classifiers, both T cell-associated and myeloid lineage-associated genes were shown to be enriched in HGA from long-term versus short-term survivors. Association of immune function and cell-specific genes with survival was confirmed independently in a larger publicly available glioblastoma gene expression microarray data set. Histology was used to validate the results of microarray analyses in a larger cohort of long-term survivors of HGA. Multivariate analyses demonstrated that increased immune cell infiltration was a significant independent variable contributing to longer survival, as was Karnofsky/Lansky performance score. These data provide evidence of a prognostic anti-tumor adaptive immune response and rationale for future development of immunotherapy in HGA.


Median survival in high-grade astrocytoma (HGA), consisting predominantly of glioblastoma (GBM) and anaplastic astrocytoma (AA), is 15 mo and 3 y, respectively (1, 2). Few robust prognostic factors have been identified in HGA, hindering patient care and stratification in clinical trials. Currently established clinical risk factors include Karnofsky/Lansky performance score, a measure of patient well-being that is widely used in oncology, and age (3). O-6-Methylguanine-DNA methyltransferase promoter methylation status and isocitrate dehydrogenase 1 mutational status have been established as molecular prognostic factors in adult HGA (4, 5).

Gene expression microarray analyses have been used to identify novel prognostic biomarkers in HGA. This unbiased genome-wide approach has the additional benefit of providing insight into the biological mechanisms of tumorigenesis that can be exploited for the development of more effective therapies. Despite numerous studies that have identified prognostic gene signatures in HGA using microarray technology, there remains no predictor of survival that has proved robustly reproducible from study to study (6–10). This may be due to the effects of biological heterogeneity inherent in HGA combined with the typically limited duration and range of survival.

Although prognosis is poor for the majority of HGA, a small but discrete subgroup of long-term survivors exists, with 3–5% of GBM patients surviving longer than 3 y. The driving hypothesis for this study is that a more pronounced and biologically informative prognostic gene signature could be obtained by gene expression microarray analysis of long-term survivors of HGA. Using this approach, the current study identified immune function as the predominant ontology associated with long-term survival in pediatric and adult HGA. Histological validation in a larger cohort of long-term survivors demonstrated that increased infiltration of immune cells was prognostically favorable.

Materials and Methods

Patient cohort and sample collection

Both adult and pediatric HGA samples, including GBM and AA, were included in this study cohort. Using broad age and diagnostic categories substantially increased the number of long-term survivors available to the study. This inclusive approach also has the potential to identify broad
prognostic factors in HGA. Multivariate analyses were used to address the confounding effect of age, tumor grade, and a number of other potentially prognostic factors in this cohort.

For the initial discovery cohort (gene expression microarray analysis), surgical tumor samples were obtained from 26 patients who presented between 1990 and 2008 for treatment of HGA at the University Hospital or The Children’s Hospital (Aurora, CO) who were diagnosed with GBM or AA according to World Health Organization guidelines (11). Tumor was either snap frozen in liquid nitrogen or placed in RNALater storage solution (Qiagen, Valencia, CA) at the time of resection. Survival data were available for all patients in this study, which was conducted in compliance with institutional review board regulations (COMIRB 95-500 and 05-0149). Included in this study were three long-term HGA survivors (two adult GBM and one pediatric GBM), with a median survival of 7.0 y (range, 1–28 y). Details are provided in Supplemental Table I. The microarray contains 54,675 probe sets including previously identified survival-associated factors of age at diagnosis, Karnofsky/Lansky performance score, diagnosis, tumor location, extent of surgery, and therapy received. The microarray analysis control cohort consisted of 23 standard survival HGA samples (21 GBM and 2 AA) of which 18 were from pediatric patients. The median survival for this cohort was 12 mo (range, 1–80 mo).

Validation of the results of gene expression microarray analyses was performed in a publicly available Affymetrix genechips data set used by Phillips et al. (10) to identify molecular subclasses of high-grade glioma (GSE4271). Gene expression profiles were available for 50 primary samples of GBM from deceased patients. The median survival of this cohort was 14.7 mo (range, 0.7–74 mo), of which 8 survived longer than 3 y.

In the secondary validation cohort, formalin-fixed paraffin-embedded (FFPE) tissue from 53 patients was obtained from archival diagnostic specimen banks of the pathology departments of the University Hospital or The Children’s Hospital. Included in this study were 14 long-term HGA survivors (12 GBM and 2 AA) of which 4 were pediatric. For the purposes of this study, long-term survivors were defined as anyone who survived longer than 5 y with a diagnosis of GBM and 15 y with a diagnosis of AA. This long-term survival cohort included the same three long-term survivors used in the microarray study. The median survival for the HGA long-term survivor cohort was 8.2 y (range, 5.5–16.6 y). The control cohort consisted of 19 standard survival HGA samples (13 GBM, 6 AA) of which 10 were pediatric. The median survival for this cohort was 10 mo (range, 1–31 mo).

Gene expression microarray analysis

Five micrograms of RNA that had been extracted from tumor was amplified, biotin-labeled (ENZO, Farmingdale, NY), and hybridized to Affymetrix HG-U133 Plus 2 microarray chips (Affymetrix, Santa Clara, CA). Analysis of gene expression microarray data was performed using Bioconductor functions written in the R programming language (http://www.bioconductor.org). Microarray data CEL files were background corrected and normalized using the guanine cytosine Robust Multiblend Average (gcRMA) algorithm (12), resulting in log2 expression values. The Affymetrix HG-U133 Plus 2 microarray contains 54,675 probe sets including multiple probe sets for the same gene. Three HGA survival-associated gene lists were thus created for each immune cell lineages was strengthened by using replicate gene symbols (Supplemental Table II). The ability of classifier gene lists to distinguish discrete immune cell lineages was strengthened by using only genes that were 15-fold higher than controls.

Enrichment of HGA survival-associated genes in gene expression microarray validation data set

HGA gene expression microarray profiles were filtered to generate gene lists that were either positively or negatively correlated with survival (p < 0.05). Genes that were positively correlated with survival were then filtered to contain only genes that were associated with “immune response” GO term (6955) (Supplemental Table II). The three HGA survival-associated gene sets were combined with the specific immune cell lineage classifier gene sets described earlier for GSEA analysis of the Phillips validation data set.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5-μm FFPE tumor tissue sections. Slides were deparaffinized and then subjected to optimal Ag retrieval protocols. Subsequent steps were performed using the EnVision-HRP kit (Dako, Glostrup, Denmark) on a Dako autostainer according to standard protocol. Incubation with primary Ab was performed for 2 h. The following dilutions of primary Ab were used and applied to the sections for 1 h: 1:250 allograft inhibitory factor-1 (AIF1) (cat. no.01-1974) from Waco Pure Chemicals (Richmond, VA); 1:2 PreDiluted CD4 (SP5; cat. no. 104R-18) from Cell Marque (Rocklin, CA); and 1:100 CD6 (C8/144B; cat. no. M7103) from Dako. Each of these Abs stained a discrete subpopulation of cells that were distributed throughout the parenchyma of the tumor. Sections were counterstained with hematoxylin. Slides were analyzed with the Olympus BX40 microscope, ×40 objective lens and ×10 eyepiece (Olympus, Center Valley, PA). Images were captured using an Optronics MicroFire 1600 × 1200 camera and PictureFrame 2.3 imaging software (Optronics, Goleta, CA). Slides were scored in a blinded fashion, with infiltrating cell abundances measured as the mean number of positive staining cells per multiple fields of view (number dependent on sample size) at ×400 magnification.

Statistical analysis

The Kaplan–Meier method was used to estimate the probability of survival as a function of time. Survival was calculated from the date of initial di-
agrosis to the date of death from any cause; patients alive at time of analysis were censored. Differences between survival curves were analyzed for significance using the log-rank test. Multivariate analysis of the relative importance of factors to survival was performed using the Cox proportional hazards method.

Results

Genes positively correlated with long-term survival in HGA are predominantly immune-related

In the initial gene ontology analysis, DAVID was used to identify enriched biological functions in genes associated with survival as a continuous variable. As input for DAVID ontology analysis, a list of 1106 genes that positively correlated ($p < 0.05$ estimated by Pearson correlation test) with survival in HGA ($n = 26$) was created from all 20,722 genes. Using the same approach, a list of 469 genes that were negatively correlated with survival was also created. DAVID showed that the predominant ontology in genes positively correlated with survival were immune-related (Table I). Cell cycle and M-phase–related ontologies were found to be enriched in genes that were negatively correlated with survival. These cell cycle ontologies reached greater statistical significance than the positive survival correlate enriched ontologies. GSEA was used as a secondary analysis of functional enrichment in survival-associated gene lists. Similar to the DAVID results, GSEA identified three of the highest 10 ontologies in genes that positively correlated with survival as immune-related (Table I). In the reverse analysis, among those genes that were negatively correlated with survival, the highest enriched GO terms were cell cycle-related.

Ontological analysis using GO is dependent on curator-driven annotation of gene functions based on traceable author statements and/or inferences from electronic annotations. As a consequence, genes that have not yet been annotated are ignored by the computer-based ontological analyses described above. Therefore, the 1106 genes positively correlated with survival genes used above were

<table>
<thead>
<tr>
<th>Rank</th>
<th>GO Term Annotation</th>
<th>GO Term ID</th>
<th>Fold</th>
<th>$p$ Value</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Immune system process</td>
<td>2376</td>
<td>2.69</td>
<td>2.16 x 10^{-32}</td>
</tr>
<tr>
<td>2</td>
<td>Immune response</td>
<td>6955</td>
<td>3.02</td>
<td>1.61 x 10^{-29}</td>
</tr>
<tr>
<td>3</td>
<td>Regulation of immune system process</td>
<td>2682</td>
<td>2.84</td>
<td>4.27 x 10^{-14}</td>
</tr>
<tr>
<td>4</td>
<td>Defense response</td>
<td>6952</td>
<td>2.35</td>
<td>1.50 x 10^{-13}</td>
</tr>
<tr>
<td>5</td>
<td>Regulation of immune response</td>
<td>50776</td>
<td>3.42</td>
<td>4.76 x 10^{-13}</td>
</tr>
<tr>
<td>6</td>
<td>Positive regulation of immune system process</td>
<td>2684</td>
<td>3.32</td>
<td>8.00 x 10^{-12}</td>
</tr>
<tr>
<td>7</td>
<td>Cell activation</td>
<td>1775</td>
<td>3.04</td>
<td>1.34 x 10^{-12}</td>
</tr>
<tr>
<td>8</td>
<td>Response to external stimulus</td>
<td>9605</td>
<td>1.99</td>
<td>5.26 x 10^{-12}</td>
</tr>
<tr>
<td>9</td>
<td>Response to wounding</td>
<td>9611</td>
<td>2.35</td>
<td>1.04 x 10^{-11}</td>
</tr>
<tr>
<td>10</td>
<td>Inflammatory response</td>
<td>6954</td>
<td>2.79</td>
<td>1.51 x 10^{-11}</td>
</tr>
</tbody>
</table>

The top 10 enriched ontologies for positive and negative correlates of survival using DAVID and GSEA ranked according to $p$ value and normalized enrichment score, respectively.
manually reviewed to identify those with a documented predominant role in specific immune mechanisms. This process identified 19% (205 of 1106) genes that were related to immune function. The results of this analysis, with genes listed and categorized into subgroups according to their documented role in specific immune mechanisms, are provided in Supplemental Table IB. Notably, 99 immune-related genes (48%) were found that did not have an annotation of “immune response” (GO term 6955), including a number of genes of key CD immune markers such as CD2, CD3, CD33, and CD40, underscoring the importance of manual review of gene lists as performed here.

A number of notable genes associated with the adaptive immune system were identified in those genes positively correlated with longer survival in HGA. In particular a large number of genes known to be expressed by T cells (CD3D, CD3E, CD3G, CD8B, TRAC, TRAT1, VAV1, and ZAP70) were shown to be associated with long-term survival. Additionally, multiple components of the innate immune system, including genes associated with microglia/macrophages (AIF1, CD68, CD86, CIITA, HLA-DOA, HLA-DQB2, HLA-DRB1, HLA-DRB6, NOD2), were present in this prognostic immune gene set. Several TLRs were also associated with long-term survival (TLR 2, 3, 5, 6, 7, and 8).

Known negative regulators of immune function in gliomas were examined to identify any association with shorter survival in HGA. IL-10 was found to be associated with longer survival (p = 0.0127), and no significant survival association was observed for IL-13, FOXP3, STAT3, TGFβ1, or TGFβ2 gene expression.

Lymphoid lineage-specific genes are enriched in HGA long-term survivors

To clarify which immune cell types might account for the immune function enrichment identified earlier, genes uniquely specific to each immune cell lineage were identified using existing publicly available gene expression data. Gene expression microarray data obtained from purified immune cell lineages were used to create classifier gene sets. These classifier gene sets were then used in combination with GSEA to indicate the presence of specific types of infiltrating immune cells in HGA from long-term survivors. This analysis revealed that lymphoid cell lineage classifiers Th cell (CD4), combined T cell (CD4 and CD8), and NK cell (CD56) were statistically significantly enriched (p < 0.05) in the long-term survivor-associated genes (Table II). Cytotoxic T cells (CD8) and myeloid lineages (combined myeloid lineage [CD14 and CD33], myeloid cell [CD33], and monocyte [CD14]) approached significance (p < 0.1). B cells (CD19) and dendritic cells (BDCA4) were the least enriched cell types. Conversely, none of these immune cell lineage classifiers were enriched in the genes associated with shorter survival in HGA.

Validation of survival-associated immune response gene expression signature

After primary analysis of the in-house HGA data set, a publicly available gene expression microarray data set (Phillips; GSE4271) was used to confirm our findings (10). The patient cohort represented by this data set contained 50 primary GBM that were from deceased patients of which 8 survived longer than 3 y (range, 3.5–6.2 y).

This data set was subjected to the same ontology and immune cell lineage analyses as described earlier. Expression of all 19,110 HG-U133A and U133B best-expressed probe sets in the Phillips data set were correlated (Pearson) with survival as a continual variable. This approach identified 1391 genes that were positively correlated with survival (p < 0.05). Ontology analysis of these genes using DAVID identified immune response as the most highly enriched biological process associated with long-term survival GBM samples (Table III). Similar to the DAVID results, GSEA identified two of the highest 10 ontologies in genes that positively correlated with survival as inflammation and immune-related (Table III). These results match the results of the in-house analysis thus suggesting the hypothesis that host immunity contributes significantly to long-term survival in HGA. In the converse analysis, 1181 genes were significantly correlated with shorter survival. DAVID and GSEA analyses demonstrated that the predominant ontologies associated with short-term survival were mitosis-related, again matching the results of the in-house data set analysis (Table III). Enrichment of genes associated with specific immune cell lineages in the Phillips data set was measured using GSEA as described earlier, but with the addition of HGA positive and negative correlate gene sets generated from our in-house data set (n = 1106 and n = 469, respectively). A third gene set was created by using only “immune response” (GO term 6955) annotated genes that were significantly positively correlated with survival (n = 117). Seven of the nine immune cell lineages were shown to be associated with long-term survival (Supplemental Table IC). In contrast to the HGA in-house data set, monocyte and myeloid lineage-associated genes were significantly enriched in long-term survivors in the Phillips data set. HGA positive and negative survival-correlate genes were significantly enriched in the corresponding Phillips data set survival-correlated genes. Of note, a higher enrichment was seen in “immune response” annotated HGA positive correlates than in the full HGA positive correlate gene set.

Immune-related genes positively correlated with survival in the Phillips were compared with those identified in the HGA data set. Fifteen “immune response” GO term annotated genes were shared between the two data sets. These included myeloid-lineage expressed genes GPR183, IRF8, IL6R, KYNU, NCF4, STXB2, TNFAIP8L2, and TNFSF13 (APRIL) based on BioGPS, suggesting a common myeloid-lineage enrichment in long-term survivors from the two data sets. Those immune-related genes that distinguished the Phillips from the HGA data set included a number of Fc-receptors (CD16A and B, CD32, CD64) and MHC class I HLA-A, MHC class II and T cell-associated genes that had been identified in the HGA gene set were absent from the Phillips data set. Negative regulators of immune function were studied to identify any association with shorter survival in the Phillips data sets. IL-4 was found to be associated with shorter survival (p = 0.0175) supporting a link between TH2 immune response polarization and a shorter survival. Conversely, FOXP3 and TGFβ1 were significantly associated with long-term survival (p = 0.0203 and p =

Table II. Immune cell lineage enrichment analysis of genes correlated with survival in HGA

<table>
<thead>
<tr>
<th>Rank</th>
<th>Immune Cell Lineage</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD4 Th cell</td>
<td>2.02</td>
</tr>
<tr>
<td>2</td>
<td>T cell lineage (CD4 and CD8 combined)</td>
<td>1.96</td>
</tr>
<tr>
<td>3</td>
<td>CD56 NK cell</td>
<td>1.95</td>
</tr>
<tr>
<td>4</td>
<td>Myeloid lineage (CD14 and CD33 combined)</td>
<td>1.74</td>
</tr>
<tr>
<td>5</td>
<td>CD8 cytotoxic T cell</td>
<td>1.65</td>
</tr>
<tr>
<td>6</td>
<td>CD33 myeloid cell</td>
<td>1.65</td>
</tr>
<tr>
<td>7</td>
<td>CD14 monocye</td>
<td>1.61</td>
</tr>
<tr>
<td>8</td>
<td>CD19 B cell</td>
<td>1.41</td>
</tr>
<tr>
<td>9</td>
<td>BDCA4 dendritic cell</td>
<td>1.32</td>
</tr>
</tbody>
</table>

NES, Normalized enrichment score.
Positively correlated with survival

1. Immune response
2. Defense response
3. Regulation of transcription from RNA polymerase II promoter
4. Enzyme linked receptor protein signaling pathway
5. Vascular development
6. Cellular defense response
7. Taxis
8. Chemotaxis
9. Blood vessel development
10. Positive regulation of protein kinase activity

Negatively correlated with survival

1. DNA metabolic process
2. Cell cycle
3. DNA replication
4. Cell cycle process
5. Response to DNA damage stimulus
6. Cell cycle phase
7. DNA repair
8. M phase
9. Mitotic cell cycle
10. Cell division

In a histological validation cohort of HGA, immune cell infiltration and Karnofsky/Lansky performance score contribute to long-term survival.

On the basis of results of microarray analysis of long-term survivors of HGA, it was hypothesized that increased tumor infiltration of microglia and/or T cells is associated with longer survival. To validate this hypothesis, IHC was used to measure immune cell infiltration in a larger cohort of long-term survivors of HGA. The patient cohort for this study included 14 long-term survivors (median survival, 8.5 y; range, 5.5–17 y) and 19 patients whose survival was in the typical range (median survival, 9 mo; range, 1–31 mo). Immunostaining of FFPE diagnostic samples was performed for CD8 and CD4, specific markers for cytotoxic T cells and Th cells, respectively (Fig. 1A). The association of a significant number of putative myeloid/monocyte function and lineage-specific genes with long-term survival in HGA also implied the presence of infiltrating microglia/macrophages in these tumors. To confirm the presence of microglia/macrophages, the most abundant myeloid-lineage cells in the CNS, immunostaining for AIF1 was performed (Fig. 1B). AIF1, also known as Iba1, is a specific marker for the microglia/macrophage population in the CNS (18). Frequency of infiltration of specific immune cells was scored as number of stained cells per 400 field of vision. Immunostaining representing high and low location, extent of surgery, and therapy received—were included in the survival analyses to address any potential confounding factors.

Univariate Kaplan–Meier analysis was first used to identify factors that were significantly associated with survival. Factors addressed in this study consisted of noncontinuous variables (tumor grade,
adult/pediatric, radiation treatment, temozolomide treatment, supratentorial location, thalamic location, and gross total resection) and continuous variables that were divided at the mean value into high/low [Th cell (CD4) infiltration, cytotoxic T cell (CD8) infiltration, microglia/macrophage (AIF1) infiltration, age at diagnosis, and Karnofsky/Lansky performance score]. Factors significantly associated with survival were performance score greater than the mean of 80 ($p = 0.00387$), cytotoxic T cell infiltration greater than the mean ($p = 0.0154$), and Th cell infiltration greater than mean ($p = 0.0272$). Mean cytotoxic and Th cell infiltration was 1.26 (range, 8.2–0) and 1.98 (range, 37–0) cells per 3400 field of view, respectively. Association of survival with greater than mean microglia/macrophage infiltration showed a trend toward significance ($p = 0.089$). To investigate further the influence of microglia/macrophage infiltration on survival, the cutoff for high infiltration in this cell population was made more stringent by using the 75th percentile, which showed a significant association with survival ($p = 0.0146$). The 75th percentile score for microglia/macrophage infiltration was 100 (range, 220–0) cells per 3400 field of view. None of the remaining factors showed a significant association with survival.

Significant variables were next subjected to multivariate analysis. Each of the three immune cell population infiltrations showed a significant correlation with each other by either Pearson or Spearman approaches. Because these factors were not therefore independent, it was considered appropriate also to combine them (microglia/macrophage >75th percentile or cytotoxic T cell greater than mean or Th cell greater than mean) into one measure as a single variable. By Kaplan–Meier analysis, the combined immune cell variable was significantly associated with survival ($p = 0.0066$) (Fig. 1C).

Combined immune cell and individual immune cell variables were modeled with Karnofsky/Lansky performance score and analyzed using Cox proportional hazard (Table IV). This analysis showed that when combined with performance score, each of the immune variables apart from Th cell infiltration were independent variables for survival. The best model appeared to consist of performance and combined immune cell infiltration, with both factors contributing significantly to longer survival ($p = 0.00268$ and 0.00772, respectively) (Table IV).

**Discussion**

HGAs are heterogeneous but almost uniformly fatal with only a small percentage of long-term survivors. An unbiased genome-wide microarray approach, with validation in an independent data

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**Table IV. Multivariate survival model of Karnofsky/Lansky performance score and immune cell infiltration using Cox’s proportional hazards regression analysis**

<table>
<thead>
<tr>
<th>Model</th>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% Confidence Interval</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karnofsky/Lansky performance and microglia/macrophage infiltration (AIF1)</td>
<td>Performance $&gt;$ mean</td>
<td>0.216</td>
<td>0.086–0.538</td>
<td>0.00101</td>
</tr>
<tr>
<td></td>
<td>AIF1 $&gt;$ 75th percentile</td>
<td>0.138</td>
<td>0.031–0.618</td>
<td>0.00963</td>
</tr>
<tr>
<td>Karnofsky/Lansky performance and Th cell infiltration (CD4)</td>
<td>Performance $&gt;$ mean</td>
<td>0.402</td>
<td>0.169–0.957</td>
<td>0.0394</td>
</tr>
<tr>
<td></td>
<td>CD4 $&gt;$ mean</td>
<td>0.202</td>
<td>0.026–1.59</td>
<td>0.1282</td>
</tr>
<tr>
<td>Karnofsky/Lansky performance and cytotoxic T cell infiltration (CD8)</td>
<td>Performance $&gt;$ mean</td>
<td>0.317</td>
<td>0.132–0.762</td>
<td>0.0102</td>
</tr>
<tr>
<td></td>
<td>CD8 $&gt;$ mean</td>
<td>0.206</td>
<td>0.047–0.913</td>
<td>0.0375</td>
</tr>
<tr>
<td>Karnofsky/Lansky performance and combined immune (AIF1 $&gt;$ 75th percentile or CD4 $&gt;$ mean or CD8 $&gt;$ mean)</td>
<td>Performance $&gt;$ mean</td>
<td>0.253</td>
<td>0.103–0.620</td>
<td>0.00268</td>
</tr>
<tr>
<td></td>
<td>Combined immune</td>
<td>0.183</td>
<td>0.0523–0.638</td>
<td>0.00772</td>
</tr>
</tbody>
</table>

---

**FIGURE 1.** Representative histology of (A) greater than median tumor infiltration of cytotoxic T cells (CD8; red) and Th cells (CD4; brown) in long-term survivor HGA11, and (B) $>$75th percentile tumor infiltration of microglia/macrophages (AIF1; brown) in long-term survivor HGA12. IHC was performed using FFPE tumor sections with hematoxylin counterstaining (original magnification $\times400$). (C) Kaplan–Meier survival analysis of combined immune cell infiltration. High combined immune cell infiltration was defined as greater than median cytotoxic T cell or Th cell or $>$75th percentile microglia/macrophage infiltration. Thirty-three HGA samples were used.
set, was used to analyze this broad diagnostic class to determine whether increased enrichment of immune genes and cells was associated with better patient survival as had been seen in a similar study of another brain tumor, ependymoma (19). The results of this study showed that this was indeed the case, with immune function ontologies, immune cell lineage-specific genes, and infiltrating immune cell frequency being significantly associated with long-term survival in HGA. These data provide additional support to the theory that host immunity can control tumor growth in a subset of HGA and provide a potential explanation for instances of long-term survival in this otherwise uniformly and rapidly fatal disease.

A large number of large-scale gene expression clustering studies have identified sets of genes reportedly predictive of prognosis in HGA. Rich et al. (6) identified a migration/invasion molecular signature that was associated with a worse survival in GBM. Using an agglomerative approach, Phillips et al. (10) defined three molecular subgroups of HGA (proliferative, proneural, and mesenchymal), one of which conveyed a better prognosis. However, the gene sets identified in these studies share few genes in common. To address this problem, Zhang et al. (9) combined a number of independent data sets and identified a 23-gene classifier that outperformed previously reported classifiers from the independent cohorts. Of note, 2 of 23 genes were immune-related, the remaining 21 being related to cellular proliferation.

The majority of studies aimed at identifying prognostic factors in HGA have used standard survival patient cohorts that contain few long-term survivors. The narrow survival range associated with these cohorts presents difficulty in assigning good and bad outcomes. The inclusion of a large proportion of long-term survivors in the current study, although not representative of natural survival distribution in HGA, provides a wider survival range and thus more clearly delineated good and bad outcomes. These more clearly defined survival cohorts allow for stronger inferences to be made from associated gene expression analyses. To explore this hypothesis, one other study, in addition to the current study, compared primary GBM from seven long-term survivors (>2 y) with primary GBM from 13 short-term survivors (<9 mo) (20). Using a genomic approach, they identified a prognostic fingerprint of 43 genes. Consistent with the current study, six survival-associated immune genes were identified (CD34, IGHG1, IL13RA1, IL17, IL22, and SERPING1) of which two were also associated with longer survival in the current analysis (IL17 and SERPING1).

Data from the current study, which identified apparently reciprocal prognostic cell cycle and immune functions, support the theory that an equilibrium may exist between immune system and tumor, known as immunoeediting (21). A shift in this equilibrium can result in either complete elimination of tumor by the immune system or alternatively escape from immune control leading to tumor progression. In long-term survivors of HGA, therapeutic intervention (surgery, radiation, and/or chemotherapy) may have skewed this equilibrium in favor of the host immune system, resulting in clinically significant tumor control. Opposing prognostic cell cycle and immune function gene sets have previously been observed in GBM and ependymoma (9, 19).

That genes related to immune functions are associated with long-term survivors of HGA implies that the host immune system may be involved with control of tumor growth in these cases. Further details of the specific immunological mechanism through which this occurs is of significant interest, potentially providing a basis for the design of more effective immunotherapeutic strategies. A rational approach to improvement of immunotherapeutic approaches would be to design strategies based on data taken from direct clinical studies of human host anti-CNS tumor immune responses.

This report potentially illustrates just such a response, underscoring the value and potential impact of these findings.

The data described in the current study can provide some mechanistic detail of a putative antitumor immune response in the human CNS. Biological inferences can be gained by examining the specific immune function genes and cell types that are associated with HGA long-term survivors and by comparison with the good-outcome-associated immune genes found in other CNS tumors. An earlier study by our group identified a similar association of immune gene and cell enrichment in ependymoma (EPN), a glial tumor of childhood (19). EPN commonly has a less aggressive phenotype than HGA, with a recurrence rate of ∼50%. A number of T cell-specific genes are seen in the HGA survival-associated genes that were not identified in EPN outcome-associated immune genes. This includes multiple components of the T cell immune synapse (CD2, CD3D, CD3E, CD3G, CD8B, TCRGC2, TRBC1, TARP, and TRAT1), cytotoxic mediators (granzyme B, H, K, and M), and key signaling molecules restricted to activated T cells (CD69, ZAP70, CARD11, and VAV1). The presence of CD8B and cytotoxic mediators, in addition to the above-listed T cell-specific genes, implies that cytotoxic T cells are an important cellular correlate of survival in HGA. This theory is supported by multivariate analysis of a larger cohort of HGA, which showed that frequency of tumor-infiltrating cytotoxic T cells (CD8) was significantly associated with longer survival.

Some of the immune genes upregulated in long-term survival HGA are associated with specific T cell functions. Prior studies have demonstrated that the majority of T cells within most human GBM are Th2-biased; however, these data were not generated from long-term survivors (22). Polarization of infiltrating T cells in long-term survivors to the Th1 phenotype is implied by the presence of GIMAP4, HAVCR2 (TIM3), and STAT4 (23–25). Together, these data provide preliminary evidence that, beyond the simple presence of an immune infiltrate, the phenotype and function of that infiltrate may influence survival in HGA. This conclusion is consistent with the report by Galon et al. (26) demonstrating that the type (specifically Th1), density, and location of immune cells within human colorectal tumors predict clinical outcome better than current staging criteria. A number of histological studies have been performed to assess the role of the host immune system in control of HGA growth and patient outcome. Three studies identified a positive correlation of lymphocyte infiltration with outcome (27–29). The results of three other studies, however, showed no correlation (30, 31) or a negative correlation (32). It should be noted that these studies relied on morphometry to identify immune infiltrates and unlike the current study were not able distinguish subtypes of T cells nor identify microglia/macrophages.

Much attention in the study of the interaction of HGA with the host immune system has been devoted to immunosuppressive factors that have shown to contribute to a poor prognosis. A number of soluble factors released by HGA, most notably TGF-β, IL-4, IL-10, and IL-13, have been shown to be immunosuppressive (33–35). The current study demonstrated that IL-4 expression correlated with shorter survival, but no other immunosuppressive factors were shown to be associated with a worse outcome. More recently, attention has been focused on a number of specific immune cells shown to contribute to an immunosuppressive milieu in the tumor. These include regulatory T cells, myeloid-derived suppressive cells, and, most recently, neutrophils (36–40). STAT3-induced signaling in gliomas has been implicated in induction of inflammation and mesenchymal transformation resulting in a poor prognosis (41, 42). No correlation of these immunosuppressive cells and signaling pathways with a poor prognosis was observed in either data set in the current analysis.
The findings of the current study provide evidence that despite the immunosuppressive nature of HGA, immunologically mediated control of tumor growth may arise in some instances, resulting in prolonged survival. Further characterization of the mechanism of immune control of HGA in long-term survivors is warranted and may aid in the design of more effective immunotherapies.

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References


