Tumor-Infiltrating Lymphocytes in Glioblastoma Are Associated with Specific Genomic Alterations and Related to Transcriptional Class

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Abstract

Purpose: Tumor-infiltrating lymphocytes (TIL) have prognostic significance in many cancers, yet their roles in glioblastoma have not been fully defined. We hypothesized that TILs in glioblastoma are associated with molecular alterations, histologies, and survival.

Experimental Design: We used data from The Cancer Genome Atlas (TCGA) to investigate molecular, histologic, and clinical correlates of TILs in glioblastomas. Lymphocytes were categorized as absent, present, or abundant in histopathologic images from 171 TCGA glioblastomas. Associations were examined between lymphocytes and histologic features, mutations, copy number alterations, CpG island methylator phenotype, transcriptional class, and survival. We validated histologic findings using CD3G gene expression.

Results: We found a positive correlation between TILs and glioblastomas with gemistocytes, sarcomatous cells, epithelioid cells, and giant cells. Lymphocytes were enriched in the mesenchymal transcriptional class and strongly associated with mutations in NF1 and RB1. These mutations are frequent in the mesenchymal class and characteristic of gemistocytic, sarcomatous, epithelioid, and giant cell histologies. Conversely, TILs were rare in glioblastomas with small cells and oligodendroglioma components. Lymphocytes were depleted in the classical transcriptional class and in EGF receptor (EGFR)-amplified and homozygous PTEN-deleted glioblastomas. These alterations are characteristic of glioblastomas with small cells and glioblastomas of the classical transcriptional class. No association with survival was shown.

Conclusions: TILs were enriched in glioblastomas of the mesenchymal class, strongly associated with mutations in NF1 and RB1 and typical of histologies characterized by these mutations. Conversely, TILs were depleted in the classical class, EGFR-amplified, and homozygous PTEN-deleted tumors and rare in histologies characterized by these alterations. Clin Cancer Res; 19(18); 4951–60. ©2013 ACR.
Translational Relevance

Tumor-infiltrating lymphocytes (TIL) are present in a subset of glioblastomas, suggesting that some are more capable of eliciting an adaptive immune response. We hypothesized that TILs are differentially distributed among specific molecular classes of glioblastoma and used histologic data linked to multiplatform molecular analysis from The Cancer Genome Atlas (TCGA) to identify correlates. We have shown that TILs are associated with specific genomic alterations and related to transcriptional class, which may provide insight into the biologic significance of TILs in glioblastoma and predict response to immunotherapy.

present within the stroma of other cancers including melanoma, colorectal, and ovarian carcinoma, where their presence is associated with improved patient outcomes (8–17). A recent study suggested that TILs have prognostic significance in glioblastoma, yet others have shown lymphocytes may impart a less favorable prognosis (18–21).

A complex relationship exists between the development of cancer, the immunologic response to it, and the immunosuppression it causes. In glioblastoma, tumor-mediated immunosuppression is thought to explain functional deficits in cytotoxic lymphocytes (22). Regulatory lymphocytes are elevated in patients with glioblastoma and suppress antitumor activity by inhibiting secretion of cytotoxic cytokines from effector lymphocytes (23, 24). Thus, while lymphocytes and other immune cells infiltrate glioblastoma, an immunosuppressive tumor milieu likely prevents successful immune-mediated tumor eradication. Recent clinical trials have focused on augmenting the antitumor immune response with tumor vaccines or reversing tumor-mediated immunosuppression.

Molecular alterations in glioblastoma and other cancers may be immunogenic. For example, microsatellite instability and methylation aberrations are independent predictors of lymphocyte density in colorectal cancer (14). Given its molecular and histologic heterogeneity, subsets of glioblastoma may be more immunogenic and responsive to immunotherapy, yet little is known about the relation between immune cells and the tumor microenvironment or molecular classes in glioblastoma. Therefore, the purpose of this study was to identify molecular and histologic correlates of the immune response in glioblastoma. We used whole-slide digitized images of glioblastomas from The Cancer Genome Atlas (TCGA) linked to multiplatform molecular data. These may provide insight into the biologic significance of TILs in glioblastoma and impact therapy.

Materials and Methods

We conducted an integrated molecular and histologic analysis using data from TCGA, which molecularly characterized glioblastomas across multiple platforms, including single-nucleotide polymorphism (SNP) genotyping, mRNA and microRNA profiling, DNA sequencing, and methylation analysis (2). Clinical data, including treatment and survival, is also available.

Digitized images used for morphologic analysis

Permanent section histologic slides from TCGA glioblastomas were provided by contributing institutions. Slides were scanned and digitized at 20× resolution by a high-throughput digital scanner (Aperio, Inc.) at the TCGA Biospecimen Core Resource located at the International Genomics Consortium (Integen). The number of slides available for review ranged from 1 to 9 per case (median, 3).

Ratings of TILs and other histopathologic features

TCGA consortium neuropathologists annotated digitized permanent section histologic slides from 122 TCGA cases for 18 histopathologic features including inflammation, angiogenesis, necrosis, lymphocytes, common morphologic subtypes, and others (Table 1). “Inflammation” as a general category was defined by the presence of inflammatory cells, including lymphocytes, macrophages, neutrophils, or their combination. Lymphocytes were identified as small round cells with scant cytoplasm and darkly staining nuclei. Lymphocytes and all other histopathologic features were categorized as absent (0), present (1+), or abundant (2+) by 2 neuropathologists (M.L. Cohen, K. D. Aldape, R.E. McLendon, N.L. Lehman, C.R. Miller, M.J. Schniederjan, D.J. Brat) and adjudicated by a third. Cases with a complete absence of lymphocytes were labeled 0 (absent). Cases with lymphocytes in less than 50% of tumor tissue were categorized 1+ (present), whereas cases with lymphocytes in ≥50% were categorized 2+ (abundant; Fig. 1). In addition to these 122 cases, an additional 49 TCGA cases from Emory University Hospital and Henry Ford Health System were annotated for the same 18 histopathologic features using the same criteria by 2 TCGA neuropathologists (D.J. Brat, M.J. Schniederjan). All digitized histologic sections and TCGA neuropathology ratings were obtained from the TCGA portal (http://tcga-data.nci.nih.gov.proxy.library.emory.edu/tcga/tcgaHome2.jsp; last accessed November 28, 2012). Digitized images and corresponding neuropathology ratings were the primary source of data.

Mutations, copy number alterations, methylation status, and transcriptional class

We obtained TCGA mutation and copy number data from the Memorial Sloan Kettering Cancer Genomics Portal (http://www.cbioportal.org/public-portal; last accessed November 28, 2012; ref. 25). Mutation data from whole exome sequencing (NextGen) was obtained for 99 cases. Putative copy number calls determined with GISTIC 2.0 were obtained for 153 cases. CpG island methylator phenotype (G-CIMP) status was available for 124 cases. Transcriptional class labels for the Verhaak classification were obtained for 162 cases from the TCGA Advanced Working Group. The updated Verhaak labeling extends the original labeled set presented by Verhaak and colleagues by using
the originally labeled samples along with Affymetrix HT_HG-U133A data to label previously unclassified samples (3).

Validation dataset

CD3G is the gene that encodes the T-cell surface marker CD3. We used CD3G expression data from TCGA as a marker of lymphocytes to validate our histologic findings in the same set of tumors. CD3G expression data were obtained from the TCGA portal (http://tcga-data.nci.nih.gov.proxy.library.emory.edu/tcga/tcgaHome2.jsp; last accessed February 1, 2012).

Statistical analyses

Associations between lymphocytes and other histopathologic features were assessed using the Mantel–Haenzel \( \chi^2 \) and exact test and Spearman correlation for ordinal comparisons (0, 1+, 2+) and other histopathologic features (0, 1+, 2+). Mantel–Haenzel exact \( \chi^2 \) test was used when 25% of the cells had less than 5 observations. Effective sample size was 171. Bolded \( P \) values are <0.05.

Table 1. Eighteen histopathologic features were categorized as absent (0), present (1+), or abundant (2+) in 171 TCGA glioblastomas

<table>
<thead>
<tr>
<th>Histopathologic feature</th>
<th>Mantel-Haenzel ( \chi^2 ) P</th>
<th>Spearman correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>&lt;0.001</td>
<td>0.93</td>
</tr>
<tr>
<td>Pseudopalisading necrosis</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>Zonal necrosis</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.09</td>
<td>0.13</td>
</tr>
<tr>
<td>Macrophages</td>
<td>&lt;0.001</td>
<td>0.32</td>
</tr>
<tr>
<td>Microvascular hyperplasia</td>
<td>0.59</td>
<td>0.04</td>
</tr>
<tr>
<td>Endothelial hyperplasia</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>Epithelial metaplasia</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Gemistocytes</td>
<td>0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>Giant cells</td>
<td>0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>Oligodendrogial cells</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>Sarcomatous metaplasia</td>
<td>&lt;0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>Small cells</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>Mineralization</td>
<td>0.68</td>
<td>0.03</td>
</tr>
<tr>
<td>Satellitosis</td>
<td>0.20</td>
<td>0.10</td>
</tr>
<tr>
<td>White matter invasion</td>
<td>0.09</td>
<td>0.13</td>
</tr>
<tr>
<td>Cortex invasion</td>
<td>0.71</td>
<td>0.03</td>
</tr>
</tbody>
</table>

NOTE: TILs are associated with specific histopathologic features in GBMs. Mantel–Haenzel \( \chi^2 \) test and Spearman correlation were used to examine associations between lymphocytes (0, 1+, 2+) and other histopathologic features (0, 1+, 2+). Mantel–Haenzel exact \( \chi^2 \) test was used when 25% of the cells had less than 5 observations. Effective sample size was 171. Bolded \( P \) values are <0.05.
count was less than 5 in 25% or more of cells. Associations between lymphocytes and transcriptional class were examined using $\chi^2$ and Fisher’s exact tests. The Spearman correlation was used to measure the correlation between lymphocytes and CD3G expression. Wilcoxon 2-sample or Kruskal–Wallis tests were used to detect differences in CD3G expression according to lymphocytes, mutation, or copy number status and transcriptional class.

**Survival analysis**
Clinical data were obtained from the TCGA data portal. Survival was taken as "days to death" for uncensored patients and "days to last follow-up" for right-censored patients. The association between TILs and survival was examined using the log-rank test.

All $P$ values reported are 2-sided and regarded as statistically significant if $P < 0.05$. The software used for statistical analysis was SAS Version 9.3 (SAS Institute Inc.).

**Results**

**TILs are differentially distributed in glioblastoma**
Within the 171 glioblastomas reviewed for this study, TILs were absent (0) in 93 cases (54%), present (1+) in 59 cases (35%), and abundant (2+) in 19 cases (11%). There was no significant association between the number of slides analyzed for each case and the level of lymphocytes annotated.

**TILs are associated with specific histopathologic features in glioblastoma**
Glioblastomas show a tremendous degree of histologic variability and numerous morphologic subtypes have been recognized including fibrillary, gemistocytic, epithelioid, small cell, giant cell, gliosarcoma, and glioblastoma with oligodendroglioma component. To determine whether TILs correlated with specific glioblastoma morphologies or other histopathologic features, we examined their associations using the Mantel–Haenzel $\chi^2$ test and Spearman correlation (Table 1). All variables, including lymphocytes and other histopathologic features, were categorized as 0, 1+, or 2+. We detected a strong positive correlation between TILs and specific tumor cell morphologies that included gemistocytes, sarcomatous cells, epithelioid cells, and giant cells (all $P < 0.05$; Fig. 2). Conversely, TILs were depleted in glioblastomas characterized by small cells and oligodendroglial cells (both $P < 0.05$). Among other features analyzed, TILs were most tightly correlated with the findings of inflammation (as a general category) and macrophages (both $P < 0.05$). TILs were also statistically associated with both forms of necrosis annotated (pseudopalisading and zonal; both $P < 0.05$).

**TILs are associated with specific mutations in glioblastoma**
In the initial TCGA analysis of glioblastoma, 8 genes were identified as significantly mutated, including TP53, PTEN, NF1, EGFR, ERBB2, RB1, PIK3R1, and PIK3CA (genes attaining a false discovery rate $< 0.1$; ref. 2). A subsequent, unbiased genomic analysis identified recurrent mutations in isocitrate dehydrogenase 1 (IDH1; ref. 26). We examined associations between lymphocytes (0, 1+, 2+) and mutations (mutant vs. wild-type) for these genes using $\chi^2$ and Fisher’s exact test (Table 2). Our data set contained no cases with ERBB2 mutations.
We examined the association between TILs and these recur-
65% of cases with absent (0) lymphocytes (53 of 82) tests (Table 3). Lymphocytes were depleted in
tumors (absent (0) in 52, present (1+)
EGFR
global analysis publication, including amplifications of
76% of cases with absent (0) lymphocytes (53 of 82) compared with 48% of cases with present (1+) or abundant (2+) lymphocytes. Only 35% of cases with abundant (2+) lymphocytes were EGFR-amplified.

TILs were also depleted in tumors with homozygous deletions of 
PTEN (P < 0.05). Seventy-eight percent of cases with homozygous 
PTEN deletion (14 of 18) had absent (0) lymphocytes. Seventeen percent of 
PTEN-deleted cases had present (1+) lymphocytes (3 of 18), whereas only 5% of cases had abundant (2+) lymphocytes (1 of 18). There was a trend toward significance for 
PTEN deletions (P = 0.07). No other associations between TILs and CNAs were noted.

TILs are not associated with the CIMP
G-CIMP–positive tumors represent less than 10% of glioblastomas but have significantly improved survival compared with G-CIMP–negative tumors (27). IDH1 mutations have been shown to establish the G-CIMP phenotype (28). We examined the association between TILs and the CIMP using \( \chi^2 \) and Fisher’s exact tests (data not shown). TILs were not associated with G-CIMP status (P > 0.05).

TILs are related to transcriptional class
Verhaak and colleagues identified 4 transcriptional classes of glioblastoma using TCGA gene expression data: proneural, neural, classical, and mesenchymal (3). To determine whether there was an association between transcriptional class and TILs, we examined each class for its distribution of lymphocytes using \( \chi^2 \) and Fisher’s exact tests (Table 4). We found that TILs were strongly associated with transcriptional class (P < 0.05).

TILs were enriched in the mesenchymal class compared with all other classes combined (P < 0.05). Forty-two percent of cases (30 of 72) with a lymphocytic infiltrate (1+ or 2+) belonged to the mesenchymal class. However, when classical tumors were excluded, there was no statistically significant enrichment of TILs in the mesenchymal class compared with the proneural and neural classes (P > 0.05). Cases with abundant lymphocytes were heavily

### Table 2. TILs are associated with specific mutations in glioblastoma

<table>
<thead>
<tr>
<th>Gene</th>
<th>n</th>
<th>( \chi^2 ) P (0, 1+, 2+)</th>
<th>( \chi^2 ) P (0 vs. 1+, 2+ combined)</th>
<th>( \chi^2 ) P value (0, 1+ combined vs. 2+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>33</td>
<td>0.13</td>
<td>0.06</td>
<td>0.15</td>
</tr>
<tr>
<td>PTEN</td>
<td>29</td>
<td>0.15</td>
<td>0.06</td>
<td>0.44</td>
</tr>
<tr>
<td>EGFR</td>
<td>26</td>
<td>0.82</td>
<td>0.54</td>
<td>1.00</td>
</tr>
<tr>
<td>NF1</td>
<td>16</td>
<td>0.03</td>
<td>0.74</td>
<td>0.04</td>
</tr>
<tr>
<td>RB1</td>
<td>10</td>
<td>0.04</td>
<td>0.04</td>
<td>0.22</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>9</td>
<td>0.67</td>
<td>0.49</td>
<td>1.00</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>7</td>
<td>0.72</td>
<td>1.00</td>
<td>0.50</td>
</tr>
<tr>
<td>IDH1</td>
<td>4</td>
<td>0.53</td>
<td>1.00</td>
<td>0.32</td>
</tr>
<tr>
<td>ERBB2</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE: \( \chi^2 \) test was used to examine associations between lymphocytes (0, 1+, 2+) and mutations (0, 1). Fisher’s exact test was used when 25% of the cells had less than 5 observations. Effective sample size was 99. Categories were combined to identify associations driven by cases with either complete absence or abundance of lymphocytes. Bolded P values are <0.05.

Among cases with mutational data, lymphocytes were absent (0) in 52, present (1+) in 38, and abundant (2+) in 9. We found lymphocytes were strongly associated with mutations in neurofibromatosis 1 (NF1) and retinoblastoma 1 (RB1) (both P < 0.05). Nine cases with absent (0) lymphocytes were NF1-mutant (9 of 52, 17%), whereas 44% of cases with abundant (2+) lymphocytes harbored mutations in NF1 (4 of 9). Two cases with absent (0) lymphocytes harbored mutations in RB1 (2 of 52, 4%). Of cases with lymphocytes present (1+), 6 were RB1 mutants (6 of 32, 16%). Two cases with abundant (2+) lymphocytes harbored mutations in RB1 (2 of 9, 22%).

There was a trend toward significance for TP53 mutations (P = 0.12), particularly when cases with present (1+) and abundant (2+) lymphocytes were combined (P = 0.06). Twenty-five percent of cases with absent (0) lymphocytes were TP53-mutant (13 of 52), whereas 43% of cases with present (1+) or abundant (2+) lymphocytes harbored mutations in TP53 (20 of 47).

As mutations in TP53 are characteristic of both the proneural and mesenchymal transcriptional class, we investigated whether mutations were overrepresented in 1 of these 2 transcriptional classes (3). Twenty cases harbored mutations in TP53 and had present (1+) or abundant (2+) lymphocytes. Nine (45%) belonged to the mesenchymal transcriptional class and 5 (25%) belonged to the proneural class (P > 0.05).

### TILs are associated with specific CNAs in glioblastoma

Significant CNAs were also identified in the initial TCGA global analysis publication, including amplifications of EGFR, CDK4, PDGFRα, MDM2, MDM4, MET, CDK6, MYCN, CCND2, PIK3CA, and AKT3 and deletions of CDKN2A/B, PTEN, CDKN2C, RB1, PARK2, and NF1 (2). We examined the association between TILs and these recurrent amplifications and deletions using \( \chi^2 \) and Fisher’s exact tests (Table 3). Lymphocytes were depleted in EGFR-amplified tumors (P < 0.05). Amplification of EGFR was present in 65% of cases with absent (0) lymphocytes (53 of 82)
enriched in mesenchymal transcriptional class. Seventy-one percent of cases (12 of 17) with abundant lymphocytes (2+) belonged to the mesenchymal class, whereas 12% were proneural, 12% neural, and 6% were classical, representing statistically significant enrichment ($P < 0.05$). When classical cases were excluded, there was still a strong trend toward statistically significant enrichment of abundant (2+) TILs in the mesenchymal class compared with the proneural and neural classes ($P = 0.07$).

Conversely, TILs were depleted in the classical transcriptional case compared with all other classes combined ($P < 0.05$). Seventy-four percent of classical cases (31 of 42) were characterized by absent (0) lymphocytes, representing statistically significant depletion ($P < 0.05$). Only one case with abundant (2+) lymphocytes belonged to the classical transcriptional class (1 of 17). When mesenchymal tumors were excluded, there was still a trend toward statistically significant depletion of TILs (1+ and 2+) in the classical class compared with the proneural and neural classes ($P = 0.053$).

**CD3G gene expression is positively correlated with lymphocytes, mutations in TP53, RB1 and the mesenchymal transcriptional class and negatively correlated with EGFR amplification, PTEN deletion, and the classical transcriptional class**

We used CD3G expression data to validate findings uncovered by our morphologic analysis. There was a

<table>
<thead>
<tr>
<th>Table 3.</th>
<th>TILs are depleted in EGFR-amplified and homozygous PTEN-deleted tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNA</td>
<td>%</td>
</tr>
<tr>
<td>Amplifications</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>49.3</td>
</tr>
<tr>
<td>CDK4</td>
<td>14.5</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>14.1</td>
</tr>
<tr>
<td>MDM4</td>
<td>9.9</td>
</tr>
<tr>
<td>MDM2</td>
<td>9.3</td>
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<tr>
<td>MET</td>
<td>8.8</td>
</tr>
<tr>
<td>CDK6</td>
<td>7.0</td>
</tr>
<tr>
<td>CCND2</td>
<td>4.0</td>
</tr>
<tr>
<td>AKT3</td>
<td>3.2</td>
</tr>
<tr>
<td>MYCN</td>
<td>2.6</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>2.4</td>
</tr>
<tr>
<td>Deletions</td>
<td></td>
</tr>
<tr>
<td>CDKN2A</td>
<td>62.0</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>61.0</td>
</tr>
<tr>
<td>PTEN</td>
<td>10.3</td>
</tr>
<tr>
<td>RB1</td>
<td>3.6</td>
</tr>
<tr>
<td>CDKN2C</td>
<td>3.6</td>
</tr>
<tr>
<td>PARK2</td>
<td>2.0</td>
</tr>
<tr>
<td>NF1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

NOTE: $\chi^2$ test was used to examine associations between lymphocytes (0, 1+, 2+) and CNAs. Fisher’s exact test was used when 25% of the cells had less than 5 observations. Effective sample size was 153. Categories were combined to identify associations driven by cases with either complete absence or abundance of lymphocytes.

<table>
<thead>
<tr>
<th>Table 4.</th>
<th>TILs are enriched in the mesenchymal class and depleted in the classical class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>1+</td>
<td>10</td>
</tr>
<tr>
<td>2+</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
</tr>
</tbody>
</table>

NOTE: $\chi^2$ test was used to examine associations between lymphocytes (0, 1+, 2+) and transcriptional class. Fisher’s exact test was used when 25% of the cells had less than 5 observations. Effective sample size was 162.
positive correlation (0.30) between the histologic categorization of lymphocytes and CD3G expression ($P < 0.001$). Glioblastomas with higher levels of TILs had significantly higher CD3G expression ($P < 0.001$). Those with absent (0), present (1+), and abundant (2+) lymphocytes had CD3G expression of 15.34 (SD ± 7.81), 17.55 (SD ± 10.99), and 20.23 (SD ± 13.51), respectively.

We examined levels of CD3G expression in cases harboring mutations in $RBI$, $TP53$, and $NF1$. Cases with $RBI$ mutations tended to have higher levels of CD3G expression than wild-type cases (19.1 vs. 16.2). Cases with $TP53$ mutations also showed higher levels of CD3G expression than wild-type (17.4 vs. 16.0). Cases with $NF1$ mutations had lower levels of CD3G expression compared with wild-type (15.6 vs. 16.5; all $P > 0.05$).

We also investigated CD3G expression in $EGFR$-amplified and $PTEN$-deleted cases. $EGFR$-amplified cases had lower levels of CD3G expression than wild-type (15.6 vs. 16.7). Likewise, $PTEN$-deleted cases also had lower levels of CD3G expression (15.5 vs. 16.3; both $P > 0.05$).

Finally, we analyzed CD3G expression levels by transcriptional class. There was a statistically significant difference in expression according to transcriptional class ($P < 0.001$). Cases belonging to the mesenchymal transcriptional class had the highest CD3G expression (19.6), which was significantly higher than that of other classes combined (15.1; $P < 0.001$). Conversely, CD3G expression was significantly lower in the classical transcriptional class compared with all other classes (14.8 vs. 17.1; $P < 0.001$).

**TILs are not associated with prolonged survival**

TILs were not associated with prolonged survival in univariate analyses. We compared the survival of cases with absent (0) lymphocytes, present (1+), and abundant (2+) lymphocytes using a log-rank test ($P > 0.05$; Fig. 3). We found patient age was a highly significant predictor of survival ($P < 0.001$); however, lymphocytes did not vary according to age. The mean age of patients with absent (0), present (1+), and abundant (2+) lymphocytes was 56.9 (SD ± 14.6), 57.3 (SD ± 12.1), and 54.8 years (SD ± 13.1), respectively ($P > 0.05$). TILs were not a significant predictor of survival in an age-adjusted Cox proportional hazards model (data not shown; $P > 0.05$). If tumors belonging to the classical transcriptional class were excluded, there were no survival differences according to TILs among proneural, neural, and mesenchymal tumors ($P > 0.05$). Similarly, if mesenchymal tumors were excluded, there were no survival differences according to TILs among proneural, neural, and classical tumors ($P > 0.05$).

**Discussion**

Lymphocytes are present in the stroma of many human cancers. The histologic finding of TILs is often associated with prolonged survival. Tumors with TILs may have distinctive clinicopathologic features or underlying genetic alterations, which could be relevant for future tailored therapies.

Although prognostically significant in other cancers, the clinical relevance and genetic associations of TILs in glioblastoma remain unclear. The TCGA data set offers an opportunity to study the relationship between morphologic features, molecular alterations, and survival in glioblastoma (3, 4). Verhaak and colleagues used TCGA gene expression profiles to identify 4 transcriptional classes, whereas independent studies of genome methylation uncovered a hypermethylated, G-CIMP+ subset characterized by $IDH$ mutations and overproduction of the oncometabolite 2-hydroxyglutarate (2-HG; refs. 3, 26, 29). Recent evidence suggests that glioblastomas within the mesenchymal transcriptional class have a better response to immunotherapy, suggesting this class may be more immunogenic (30). We hypothesized that TILs are differentially distributed within specific morphologic and molecular classes of glioblastoma.

We found that TILs were not uniformly distributed among glioblastomas, suggesting some are more capable of eliciting an immune response. Indeed, lymphocytes were absent in more than half, as determined morphologically by a panel of TCGA neuropathologists. TILs were strongly enriched in the mesenchymal transcriptional class of glioblastoma, which suggests tumors belonging to this subtype are more immunogenic. Seventy-one percent of tumors with abundant (2+) lymphocytes belonged to the mesenchymal class. No other transcriptional class had more than 12% with abundant (2+) TILs, indicating a fundamental difference in transcriptional class with regard to TILs.

Variation of TILs could potentially be accounted for by the extent of necrosis, as the mesenchymal class signature is heavily influenced by the degree of necrosis and the presence of lymphocytes was associated with necrosis in this study (31). However, mesenchymal glioblastomas with abundant (2+) TILs did not have substantially different levels of necrosis than those with no TILs. Neither zonal or pseudopalisading necrosis were associated with transcriptional class. Moreover, molecular alterations associated with the mesenchymal class are more likely to be...
responsible for the association with TILs. Lymphocytes were strongly associated with mutations in NF1 and RB1 and a trend was noted for TP53 mutations. Mutations of NF1 and RB1 are characteristic of the mesenchymal transcriptional class, and TP53 mutations are common in both the mesenchymal and proneural subtypes. Interestingly, we found that TP53 mutant tumors with abundant (2+) TILs were more common in the mesenchymal than the proneural class, but this did not reach statistical significance.

We also found that TILs were more common in certain morphologic subtypes of glioblastomas, including those with sarcomatous, giant cell, epithelioid, and gemistocytic components. This was of interest since prior studies have shown that gliosarcoma, giant cell glioblastoma, and gemistocytic astrocytomas are all characterized by a high frequency of TP53 mutations (32–34). Our own studies using TCGA data indicated that sarcomatous components in glioblastoma were associated with NF1, RB1, and TP53 mutations; giant cells were associated with TP53 and RB1 mutations; and epithelioid cells were associated with NF1 and RB1 mutations (data not shown). We did not find an association between TP53 mutations and gemistocytic cells, likely because of inclusion of tumors with low levels of this histologic finding as compared with prior studies, which included morphologically pure gemistocytic astrocytomas of lower grade. Nonetheless, we found that the presence of TILs was strongly associated with the mesenchymal transcriptional class as well as the mutations and tumor morphologies associated with this gene signature.

We also found that TILs were rare in tumors of the classical transcriptional class, suggesting that these may exclude lymphocytes and prevent immune-mediated tumor eradication. Only one case with abundant (2+) lymphocytes belonged to the classical transcriptional class (1 of 17). We also noted that TILs were depleted in EGFR-amplified glioblastomas, which are frequent in the classical transcriptional class of glioblastomas but less common in other classes, including mesenchymal. As small cell glioblastomas have been shown to have a high frequency EGFR amplification, we were also encouraged that the histologic presence of small cells within glioblastomas from the TCGA data set was associated with TIL depletion (35). Recent evidence suggests that EGFR activation may repress the adaptive immune response, potentially through attenuation of MHCI and MHCII expression, and therefore explain the relation between EGFR amplification and lymphocyte depletion (36). We also found that TILs were depleted in PTEN-deleted tumors (homozygous). Loss of PTEN has been shown to increase expression of the immunosuppressive protein B7 homolog 1 (B7-H1) and therefore may account for the association between homozygous PTEN deletion and lymphocyte depletion (22).

We used CD3G expression data from TCGA to validate our morphologic findings. The CD3G gene encodes a protein that forms the T-cell receptor–CD3 complex and is highly specific to T lymphocytes and is present in all subsets. The correlation between lymphocytes and CD3G expression was highly statistically significant (P < 0.001), however, the strength of the correlation was moderate (0.3). Although the tissue used for molecular analysis by TCGA was from the same neoplasm as the tissue used to create slide images, the distribution of TILs in tumors can be irregular and may contribute to a weak correlation. In addition, the detection and evaluation of TILs by molecular and pathologic methods differ considerably and may also lead to a weakened correlation. However, we were encouraged that mutations associated with TILs (RB1 and TP53) tended to have higher levels of CD3G expression, whereas CNAs associated with depletion of lymphocytes (EGFR amplification and homozygous PTEN deletion) had lower CD3G expression. Tumors of the mesenchymal transcriptional class had significantly higher CD3G expression than all other subtypes, whereas those of the classical subtype had significantly lower expression, which corroborates the associations uncovered in our morphologic analysis.

Glioblastomas annotated as having zonal necrosis by TCGA neuropathologists did not have significantly higher levels of CD3G expression as those with pseudopalisading necrosis had lower levels of CD3G expression, suggesting that TILs may indeed represent an antitumor adaptive immune response rather than a response to necrosis.

In summary, our analysis of digitized, whole slide hematoxylin and eosin (H&E)-stained images from TCGA had the advantage of a large number of cases and high-quality, multiplatform molecular analysis. It illustrates the power of networks such as the TCGA to link multiplatform molecular analysis to histopathologic findings in cancer with implications for future therapy. The categorical classification of lymphocytes as 0, 1+, and 2+ was not optimal as statistical associations with molecular and clinical variables were not as strong. We have not adjusted for multiple comparisons, as the purpose of the analysis was to identify common molecular alterations in glioblastoma related to the immune response for future tissue-based analyses. However, our analysis was limited to alterations known as significant and recurrent events in glioblastoma pathogenesis. Furthermore, we are encouraged that each statistically significant association has a biologic rationale. Finally, this classification does not account for the functional activity of lymphocytes or specific lymphocyte subsets. While effector lymphocytes are thought to mediate the antitumor response, regulatory lymphocytes may suppress the cytotoxic activity of effector lymphocytes. Thus, effector and regulatory lymphocytes may have distinct molecular and histologic associations. Future studies that examine the molecular correlates of lymphocyte subsets will add substantially to the field of immunotherapy.

Disclosure of Potential Conflicts of Interest
HH. Saltz is a consultant/ advisory board member for Appistry. No potential conflicts of interest were disclosed by the other authors.

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References


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