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Tumor-infiltrating lymphocytes in glioblastoma are associated with specific genomic alterations and enriched in the mesenchymal transcriptional class

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Abstract

Tumor-infiltrating lymphocytes in glioblastoma are associated with specific genomic alterations and enriched in the mesenchymal transcriptional class

By William Caleb Rutledge

Glioblastoma (GBM) is a highly malignant astrocytoma with an extremely poor prognosis. While tumor-infiltrating lymphocytes (TILs) have prognostic significance and potential therapeutic relevance in many cancers, their roles in GBM have not been fully defined. We hypothesized that TILs in GBM are associated with specific molecular alterations and histologies and variably contribute to the host immune response.
We used publicly available data from The Cancer Genome Atlas (TCGA) to investigate clinical, molecular, and histologic correlates of TILs in GBM. Lymphocytes were categorized as absent (0), present (1+), or abundant (2+) in digitized, whole slide histopathologic images from 171 TCGA GBM cases. TILs were absent (0) in 93 cases (54%), present (1+) in 59 cases (35%), and abundant (2+) in 19 cases (11%). Associations were examined between TILs and histologic features, mutations, copy number alterations, G-CIMP status, gene expression class, and clinical outcome.
We found a positive correlation between TILs and GBMs with abundant gemistocytes, sarcomatous cells, epithelioid cells, and giant cells. Conversely, TILs were rare in GBMs characterized by small cells and oligodendroglioma components. TILs were enriched in the mesenchymal transcriptional class, with 71% of cases containing abundant (2+) TILs within this class. TILs were strongly associated with mutations in *NF1* and *RB1* and trended towards significance for *TP53*. These mutations are frequent in the mesenchymal transcriptional class and characteristic of gemistocytic, sarcomatous, epithelioid, and giant cell histologic subtypes of GBM. In contrast, TILs were depleted in *EGFR*-amplified and homozygous *PTEN*-deleted GBMs. No association with survival was demonstrated.
We found that TILs were enriched in GBMs in the mesenchymal transcriptional class, strongly associated with mutations in *NF1* and RB1, and typical of histologic subtypes characterized by these mutations. TILs were depleted in *EGFR*-amplified and *PTEN*-deleted GBMs, as well as those with small cell and oligodendroglioma components. Immunogenic mechanisms underlying these molecular associations remain to be further explored.

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INTRODUCTION AND BACKGROUND

Glioblastoma (GBM), the highest grade and most common astrocytoma (World Health Organization, grade IV), is an aggressive disease that infiltrates surrounding brain parenchyma, preventing complete surgical resection. Despite advances in neuroimaging and navigation, surgery, radiation and chemotherapy, GBM remains incurable with an average survival of only 15 months.[1] Although characterized by dramatic molecular and histologic heterogeneity, current adjuvant treatments inhibit cell division nonspecifically and are not tailored to specific subsets.[1, 2] Gene expression, genomic and epigenetic molecular classification of GBMs has revealed molecularly distinct subclasses with clinical relevance.[3, 4] With better understanding of molecular alterations and their biologic correlates, future therapies could be targeted to class-specific mechanisms.

Immunotherapy represents a treatment option that could be tailored to specific disease subsets. Already FDA-approved for prostate cancer and metastatic melanoma and in clinical trials for other cancer types, immunotherapy remains promising for patients with GBM, offering the potential advantages of high tumor-specificity and long-term tumor surveillance.[5-7]

Tumor infiltrating lymphocytes (TILs) are present in specific subsets of GBM, yet their biologic activities and clinical relevance have not been fully explored. As opposed to macrophage infiltrates or acute inflammation, which are often associated with the necrosis that characterizes GBM, the presence of TILs suggests an engaged, anti-tumor adaptive immune response. Lymphocytes are present within the stroma of many other forms of cancer, including melanoma, colorectal, and ovarian carcinoma, where their presence is associated with improved survival.[8-17] A recent study has suggested that

lymphocytes have prognostic significance in GBM, 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 that it causes. In GBM, tumor-mediated immunosuppression is thought to explain functional deficits in cytotoxic CD_8^+ lymphocytes.[22] $CD_4^+ CD_{25}^+ Foxp3^+$ regulatory lymphocytes (T-regs) are elevated in patients with GBM, both within the tumor and peripheral blood.[23, 24] T-regs suppress anti-tumor activity by inhibiting secretion of cytotoxic cytokines from effector CD_8^+ lymphocytes. Thus, while lymphocytes and other immune cells infiltrate GBM, an immunosuppressive tumor milieu likely prevents successful immune-mediated tumor eradication. Recent clinical trials have focused on augmenting the anti-tumor immune response with tumor vaccines or reversing tumor-mediated immunosuppression.

Molecular alterations in GBM 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 GBM 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 GBM. The purpose of this study was to identify molecular and morphologic correlates of the immune response in GBM. We have used whole slide digitized images of GBMs within the TCGA data set that are linked to multiplatform molecular analysis. These studies may provide insight into the biologic significance of TILs in GBM and could potentially impact therapy.

METHODS

Hypothesis

We hypothesized that specific molecular alterations or transcriptional classes of GBM are immunogenic and predict the density of the lymphocytic response and are related to patient outcome.

Specific Aims

1. Identify specific histopathologic features associated with TILs.
2. Identify specific molecular alterations associated with TILs, including recurrent mutations and copy number alterations, methylation status, and transcriptional class.
3. Determine whether TILs are associated with survival differences.

The Cancer Genome Atlas

We performed an integrated morphological and molecular analysis using data from The Cancer Genome Atlas (TCGA). The TCGA project molecularly characterized tissue from several hundred patients with GBM across multiple platforms, including single-nucleotide polymorphism (SNP) genotyping, mRNA and microRNA profiling, DNA sequencing and methylation analysis, and identified recurrent mutations, copy number alterations, methylation status, and gene expression patterns.[2] Clinical data, including treatment and survival, is also available for each case.

Exclusion criteria

GBM cases were identified based on surgical pathology reports and clinical records. Frozen tissues were reviewed for a minimum of 80% tumor nuclei and a maximum of 50% necrosis.[2]

Human Subjects

All human subjects data was publicly available de-identified data from TCGA and not designated as human subjects research. No Institutional Review Board approval was required.

Digitized Images used for Morphological Analysis

Permanent section histologic slides from GBM cases submitted to TCGA were provided by contributing institutions. Slides were scanned and digitized at 20X resolution on an Aperio scanner at the TCGA Biospecimen Core Resource located at the International Genomics Consortium (Intgen, Phoenix, AZ). The number of slides available for review ranged from 1-9 per case (median, 3).

Rating of TILs and other Histopathologic Features

GBMs show a tremendous degree of histologic variability and numerous morphologic subtypes have been recognized, including fibrillary, gemistocytic, epitheliod, small cell, giant cell, gliosarcoma, and GBM with oligodendroglioma component. TCGA consortium neuropathologists annotated 122 TCGA cases for 18 histopathologic features (Table 1), including lymphocytes and morphologic subtypes, which allowed an integrated analysis of histopathology, molecular alterations, and clinical outcomes. All histopathologic features, including lymphocytes, were categorized as absent (0), present (1+) or abundant (2+) by two neuropathologists and adjudicated by a third. In addition to 122 cases annotated by TCGA neuropathologists, an additional 49 cases submitted to TCGA from Emory University Hospital and Henry Ford Health System were annotated for the same 18 histopathologic features using the same criteria by two TCGA neuropathologists (DJB, MS). 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).

Mutations, Copy Number Alterations, Methylation Status and Transcriptional Class

We obtained mutation data and copy number alterations from the Memorial Sloan Kettering Cancer Genomics Portal (http://www.cbioportal.org/public-portal*;* last accessed November 28, 2012).[25] In the initial TCGA analysis of GBM, eight genes were identified as significantly mutated, including *TP53*, *PTEN*, *NF1*, *EGFR*, *ERBB2*, *RB1*, *PIK3R1* and *PIK3CA* (genes attaining a false discovery rate <0.1).[2] A subsequent, unbiased genomic analysis identified recurrent mutations in isocitrate dehydrogenase 1 (*IDH1*).[26] We restricted our analysis to these genes. Mutation data from whole exome sequencing (NextGen) was obtained for 311 cases. Corresponding histopathologic ratings were available for 99 of these cases. We excluded *ERBB2* from our analysis as no cases in our dataset harbored mutations.

Significant copy number alterations (CNAs) were also identified in the initial TCGA global analysis publication, including amplifications of *EGFR*, *CDK4*, *PDGFRA*, *MDM2*, *MDM4*, *MET*, *CDK6*, *MYCN*, *CCND2*, *PIK3CA*, and *AKT3* and deletions of *CDKN2A*/B, *PTEN*, *CDKN2C*, *RB1*, *PARK2,* and *NF1*.[2] Similarly, we restricted our analysis to these alterations. Putative copy-number calls determined using GISTIC 2.0 were obtained for 497 cases. Corresponding histopathologic ratings were available for 153 of these cases.

G-CIMP positive tumors represent less than 10% of GBMs, but have significantly improved survival compared to G-CIMP negative tumors.[27] *IDH1* mutations have been

shown to establish the G-CIMP phenotype.[28] CpG island methylator phenotype (G-CIMP) status was available for 234 cases. Corresponding histopathologic ratings were available for 124 of these cases.

Verhaak *et al*. used gene expression data from TCGA to identify transcriptional classes of GBM, the proneural, neural, classical, and mesenchymal classes.[3] Transcriptional class labels for the Verhaak classification were obtained from the TCGA Advanced Working Group. The updated Verhaak labeling extends the original labeled set presented in Verhaak *et al*. 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 to validate findings from the morphologic analysis. *CD3G* expression data was obtained from the TCGA portal (http://tcga-data.nci.nih.gov.proxy.library.emory.edu/tcga/tcgaHome2.jsp; last accessed February 1, 2013).

Statistical Analysis

The association between TILs (categorized as 0, 1+, 2+; absent, present or abundant) and other histopathologic features (also categorized as 0, 1+, 2+; absent, present or abundant) was assessed using the Mantel-Haenzel chi-square and exact test and the Spearman correlation. The Mantel-Haenzel exact test was used when the cell count was <5 in 25% or more of cells. Chi-square and Fisher's exact test were used for all dichotomous comparisons (0, 1+ vs 2+; absent or present vs abundant and 0 vs 1+, 2+; absent vs present and abundant combined). Categories were combined to identify

associations driven by cases with complete absence or abundance of TILs. The association of TILs with mutations, copy number alterations, and G-CIMP status were also assessed using chi-square and Fisher's exact test. Fisher's exact test was used when the cell count was <5 in 25% or more of cells. Logistic regression was used to estimate an effect size for variables with significant associations with TILs in univariate analyses. The associations between TILs and transcriptional class were examined using chi-square and Fisher's exact test. Two-sample Student's t tests were used to detect differences in mean *CD3G* expression according to TILs, specific mutations, copy number alterations, and transcriptional class.

Clinical data was obtained from the TCGA data portal (http://tcga-data.nci.nih.gov.proxy.library.emory.edu/tcga/tcgaHome2.jsp; last accessed February 1, 2013).

Survival time was calculated from date of initial pathologic diagnosis to date of death or date of last follow-up for censored patients. Associations between TILs and survival were examined using the log-rank test.

Patient age was also obtained to determine if TILs varied according to age, as age is one of the main prognostic factors in GBM, with older patients having significantly shorter survivals.[29]

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., Cary NC).

RESULTS

Tumor-infiltrating lymphocytes are present in a subset of GBMs

Tumor-infiltrating lymphocytes (TILs) were absent (0) in 93 cases (54%), present (1+) in 59 cases (35%), and abundant (2+) in 19 cases (11%).

TILs do not vary according to age in GBM

The mean age of patients with absent (0), present (1+), and abundant (2+) TILs was 56.9 (Standard deviation (SD) ± 14.6), 57.3 (SD ± 12.1), and 54.8 years (SD ± 13.1), respectively. Differences in age were not statistically significant ($p>0.05$, Figure 1).

TILs are associated with specific histopathologic features in GBM

We detected a strong positive correlation between TILs and specific tumor cell morphologies that included gemistocytes, sarcomatous cells, epithelioid cells and giant cells (all $p<0.05$) (Table 1, Figure 2). Conversely, TILs were depleted in GBMs characterized by small cells and oligodendroglial cells (both $p<0.05$) (Table 1, Figure 1). Among other features analyzed, the presence of TILs was most positively correlated with the findings of inflammation (a general category that includes acute and chronic inflammation and macrophages) and with macrophage infiltrates (Table 1). TILs were also positively correlated with both forms of necrosis annotated (pseudopalisading and zonal) (Table 1).

TILs are associated with specific mutations in GBM

We examined the association between TILs (0, 1+, 2+) and recurrent mutations (wild-type vs. mutant) in *TP53*, *PTEN*, *NF1*, *EGFR*, *RB1*, *PIK3R1*, *PIK3CA*, and *IDH1*. There were 99 cases with mutation data and corresponding histopathologic ratings. TILs were absent (0) in 53% (52/99), present (1+) in 38% (38/99), and abundant (2+) in 9%

(9/99). We found TILs were strongly associated with mutations in neurofibromatosis 1 (*NF1*) and retinoblastoma 1 (*RB1*) (both $p<0.05$) (Table 2).

Mutation in *NF1* was a significant predictor of TILs (odds ratio 5.20, 95% confidence interval 1.22 – 22.13, $p=0.026$). Nine cases with absent (0) TILs were *NF1*-mutant (9/52, 17%), while 44% of cases with abundant (2+) TILs harbored mutations in *NF1* (4/9, 44%).

Mutation in *RB1* was also a significant of predictor of TILs (odds ratio 5.13, 95% confidence interval 1.03 – 25.53, $p=0.049$). Two cases with absent (0) TILs harbored mutations in *RB1* (2/52, 4%). Of cases with TILs present (1+), six were *RB1* mutants (6/32, 16%). Two cases with abundant (2+) TILs harbored mutations in *RB1* (2/9, 22%).

There was a trend towards significance for an association between TILs and *TP53* mutations ($p=0.118$), particularly when cases with present (1+) and abundant (2+) TILs were combined ($p=0.064$). 25% of cases with absent (0) TILs were *TP53*-mutants (13/52), while 43% of cases with present (1+) or abundant (2+) TILs harbored mutations in *TP53* (20/47).

Since mutations in *TP53* are most characteristic of both the proneural and mesenchymal transcriptional class, we investigated if mutations were overrepresented in one of these two transcriptional classes.[3] In our dataset, 20 cases harbored mutations in *TP53* and had present (1+) or abundant (2+) TILs. Nine (45%) belonged to the mesenchymal transcriptional class and five (25%) belonged to the proneural class ($p>0.05$).

TILs are associated with specific copy number alterations in GBM

We examined the association between TILs and recurrent amplifications of *EGFR*, *CDK4*, *PDGFRA*, *MDM2*, *MDM4*, *MET*, *CDK6*, *MYCN*, *CCND2*, *PIK3CA*, and *AKT3* and deletions of *CDKN2A*/B, *PTEN*, *CDKN2C*, *RB1*, *PARK2,* and *NF1*. We found that TILs (1+, 2+; present or abundant) were depleted in *EGFR*-amplified tumors ($p<0.05$) (Table 3). Amplification of *EGFR* was present in 65% of cases with absent (0) TILs (53/82) compared to 48% of cases with present (1+) or abundant (2+) TILs. Only 35% of cases with abundant (2+) TILs were *EGFR*-amplified.

We also found that TILs were depleted in tumors with homozygous deletions of *PTEN* (Table 3). 78% of cases with homozygous *PTEN*-deletion (14/18) had absent (0) TILs. 17% of *PTEN*-deleted cases had present (1+) TILs (3/18), while only 6% of cases had abundant (2+) TILs (1/18). There was a trend towards significance for *NF1* deletions ($p=0.07$). No other associations between TILs and copy number alterations were noted.

TILs are not associated with the CpG island methylator phenotype (G-CIMP)

We examined the association between TILs and tumors with the CpG island methylator phenotype. TILs were not associated with G-CIMP status ($p>0.05$).

TILs are enriched in the mesenchymal transcriptional class

To determine if there was an association between transcriptional class and TILs, we examined each class for its distribution of TILs. We found that TILs were strongly associated with transcriptional class ($p=0.02$). TILs were heavily enriched in mesenchymal transcriptional class ($p<0.05$). 71% of cases (12/17) with abundant TILs (2+) belonged to the mesenchymal class (Table 4). Among the 17 GBMs with abundant (2+) TILs, 12% were proneural, 12% neural and 6% were classical. TILs were also

significantly depleted in classical transcriptional class ($p<0.05$). Of all cases with abundant (2+) TILs, only one (1/17) belonged to the classical transcriptional class.

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

We found that cases with higher levels of TILs tended to have higher *CD3G* expression. Cases with absent (0), present (1+), and abundant (2+) TILs had increasing *CD3G* expression, 15.34 (SD ± 7.81), 17.55 (SD ± 10.99), and 20.23 (SD ± 13.51), respectively ($p>0.05$).

We also examined levels of *CD3G* expression in cases harboring mutations in *NF1*, *RB1*, and *TP53*. Although cases with *NF1* mutations had lower levels of *CD3G* expression compared to wild type (15.6 versus 16.5, $p>0.05$), cases with *RB1* mutations tended to have higher levels of *CD3G* expression than wild type cases (19.1 versus 16.2, $p>0.05$). Cases with *TP53* mutations also showed higher levels of *CD3G* expression than wild type (17.4 versus 16.0, $p>0.05$).

Next we examined the level of *CD3G* expression in *EGFR*-amplified and *PTEN*-deleted cases compared to wild type. *EGFR*-amplified cases had significantly lower levels of *CD3G* expression than wild type (15.6 versus 16.7, $p=0.048$). *PTEN*-deleted also were characterized by lower levels of *CD3G* expression (15.5 versus 16.3, $p>0.05$).

Finally we analyzed the level of *CD3G* expression according to transcriptional class. Cases belonging to the mesenchymal transcriptional class had the highest level of *CD3G* expression (19.6, SD ± 11.3). *CD3G* expression was significantly higher in the mesenchymal transcriptional class compared to all other classes (19.6 versus 15.1,

p<0.05). Conversely, *CD3G* expression was significantly lower in the classical transcriptional class compared to all other classes (14.8 versus 17.1, p-value<0.05).

TILs are not associated with prolonged with survival

TILs were not associated with prolonged survival in a univariate analysis. We compared the survival of cases with absent (0), present (1+) and abundant TILs (log-rank p-value=0.92, Figure 3).

DISCUSSION/CONCLUSION

Lymphocytes are present in the stroma of many human cancers and suggest an engaged host adaptive immunologic response. In ovarian and colorectal cancer, the histologic finding of tumor infiltrating lymphocytes (TILs) is associated with prolonged survival. Cancers with TILs may have distinctive clinicopathologic features or specific genetic alterations, which could be relevant for future tailored therapies. For example, in colorectal cancer, the presence of lymphocytes is noted most frequently in those tumors with microsatellite instability and methylation aberrations[14]. While lymphocytes may reflect a robust immune response and active tumor surveillance, the immunosuppressive effects of cancer, at least in part mediated through regulatory T-cells (T-regs), serves as a counterbalance.

Although prognostically significant in other cancers, the clinical relevance of TILs in GBM remains unclear. Little is known about the interaction of lymphocytes with the tumor microenvironment or genetic subsets in GBM. The TCGA data set, which includes comprehensive genomic characterization of GBM, offers an opportunity to study the relationship between morphologic features, molecular alterations and clinical outcome.[3, 4] Verhaak *et al*. used TGCA gene expression profiles to identify four transcriptional classes (proneural, neural, classical, and mesenchymal), while independent studies of genome methylation uncovered a hypermethylated, G-CIMP+ subset that is characterized by IDH mutations and overproduction of the oncometabolite 2-hydroxyglutarate (2-HG).[3, 26, 30] Both survival and response to therapy appear to differ by class, suggesting that future therapies could be directed at class-specific mechanisms. We hypothesized that TILs are differentially distributed within specific morphologic and

molecular classes of GBM. Recent evidence suggests that GBMs within the mesenchymal transcriptional class have a better response to immunotherapy, suggesting that this class may be more immunogenic.[31]

We found that TILs were not uniformly distributed among GBMs, suggesting that some are more capable of eliciting an immune response. Indeed, TILs were absent in over half, as determined morphologically by a panel of TCGA neuropathologists. TILs were strongly enriched in the mesenchymal transcriptional class of GBM, which provides additional evidence that tumors belonging to this subtype are more immunogenic. Seventy-one percent of tumors with abundant (2+) TILs belonged to the mesenchymal class. No other transcriptional class had more than 12% with abundant TILs, indicating fundamental difference in transcriptional class with regard to TILs. Variation of TILs could potentially be accounted for by the extent of necrosis, since the mesenchymal class signature is heavily influenced by the degree of necrosis and the presence of TILs was associated with necrosis in this study.[32] However, we did not find that mesenchymal GBMs with abundant (2+) TILs had substantially different levels of necrosis than those with no TILs. Neither zonal or pseudopalisading necrosis were associated with transcriptional class in this study. Moreover, there is reason to suggest that specific molecular alterations associated with the mesenchymal class could be responsible for the association with TILs. TILs were strongly associated with mutations in *NF1* and *RB*1 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 tumors with abundant

(2+) TILs in *TP53* mutant GBMs were slightly 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 specific morphologic subsets of GBM, 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 high frequency of *TP53* mutations.[33-35] Our own studies using TCGA data indicated that sarcomatous components in GBM 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. 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 to prior studies, which included more 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 noted that TILs were depleted in *EGFR*-amplified GBMs, which are frequent in the classical transcriptional class of GBMs, but less common in other classes, including mesenchymal. Since small cell GBMs have been shown to have a high frequency *EGFR* amplification, we were also encouraged by the finding that the histologic presence of small cells within GBMs from the TCGA data set were associated with TIL depletion.[36] Recent evidence suggests that *EGFR* activation may repress the host adaptive immune response, potentially through its attenuation of MHCI and MHCII expression, and therefore explain the relation between *EGFR* amplification and

lymphocyte depletion.[37] 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] There was only a trend towards significance for deletion in *NF1*, likely because only three cases in our dataset harbored deletion in the neurofibromatosis 1 gene.

We used *CD3G* expression data to validate our findings. The *CD3G* gene encodes the protein, CD3-gamma polypeptide, which forms the T cell receptor-CD3 complex. The protein is highly specific to T lymphocytes and is present in all T lymphocyte subsets. We noted that cases categorized with present (1+) or abundant (2+) TILs had higher levels of *CD3G* expression. Moreover, mutations associated with TILs tended to have higher levels of *CD3G* expression. Copy number alterations associated with depletion of TILs had lower levels of *CD3G* expression. *EGFR*-amplified tumors had significantly lower levels of *CD3G* expression. We also found that the mesenchymal transcriptional class had significantly higher levels of *CD3G* expression than all other subtypes, while the classical subtype had significantly lower levels of expression, which corroborates the associations uncovered in our morphologic analysis.

Cases with zonal necrosis did not have significantly higher levels of *CD3G* expression and those with pseudopalisading necrosis had lower levels of *CD3G* expression, suggesting that TILs represent an engaged, anti-tumor adaptive immune response rather than a response to necrosis, and that specific molecular alterations in GBM account for variation in TILs.

Our analysis of digitized, whole slide H&E-stained images from TCGA had the advantage of a large number of cases and high quality, multi-platform molecular analysis, providing a unique opportunity for a correlative analysis of TILs in GBM. It illustrates the power of networks such as TCGA to link multiplatform molecular analysis to important histopathologic findings in cancer with implications for future therapy.

The categorical classification of lymphocytes as 0, 1+, and 2+ was not optimal since statistical associations with molecular and clinical variables were not as strong. We have not attempted to correct for the problem of multiple comparisons since the purpose of the analysis was to identify common molecular alterations in GBM that may be related to the immune response for future tissue-based analyses. We limited our analysis to alterations that had been previously identified as significant and recurrent events in GBM pathogenesis. Furthermore, we are encouraged that each statistically significant association we detected has a biologic rationale.

In addition to cases annotated by TCGA neuropathologists, 49 cases submitted to TCGA from Emory University Hospital and Henry Ford Health System were annotated by two TCGA neuropathologists (DJB, MS) for this study, introducing a potential source of bias. DJB and MS are expert neuropathologists and participated in the original TCGA consortium review. All cases were reviewed using the same criteria. 61% of these cases were identified as having present (1+) or abundant (2+) lymphocytes in the secondary review compared to 46% in the original TCGA consortium review. Although not statistically significant, cases were more likely to be categorized as having present (1+) or abundant (2+) lymphocytes in the secondary review. However, it is possible that the

original TCGA consortium review may have underestimated the presence of lymphocytes, illustrating the need for additional tissue-based analyses.

Finally, this classification does not account for the functional activity of lymphocytes or specific lymphocyte subsets. While CD_8^+ lymphocytes are thought to mediate the anti-tumor response, $Foxp3^+$ T-regs 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.

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TABLES AND FIGURES

Table 1. Tumor-infiltrating lymphocytes are associated with specific histopathologic features in GBM.

Histopathologic Feature	Mantel-Haenzel chi-square p-value	Spearman correlation
Inflammation	**<0.0001**	0.93
Pseudopalisading necrosis	**0.01**	-0.19
Zonal necrosis	**0.04**	0.16
Neutrophils	0.09	0.13
Macrophages	**<0.0001**	0.32
Microvascular hyperplasia	0.59	-0.04
Endothelial hyperplasia	0.05	-0.15
Epithelial metaplasia	**0.04**	0.16
Gemistocytes	**0.01**	0.22
Giant cells	**0.02**	0.18
Oligodendroglial cells	**0.01**	-0.19
Sarcomatous metaplasia	**<0.01**	0.22
Small cells	**<0.01**	-0.24
Mineralization	0.68	0.03
Satellitosis	0.20	-0.10
White matter invasion	0.09	-0.13
Cortex invasion	0.71	-0.03

Histopathologic features were categorized as absent (0), present (1+), or abundant (2+). Mantel-Haenzel chi-square test and Spearman correlation were used to examine associations between tumor-infiltrating lymphocytes (0, 1+, 2+) and other histopathologic features (0, 1+, 2+).

Mantel-Haenzel exact chi-square test was used when 25% of the cells had <5 observations.

Effective Sample Size=171.

Table 2. Tumor-infiltrating lymphocytes are associated with specific mutations in glioblastoma.

Gene	Number mutations	p-value (0, 1+, 2+)	p-value (0 vs. 1+, 2+)	p-value (0, 1+ vs. 2+)
TP53	33	0.130	**0.064**	0.155
PTEN	29	0.153	0.061	0.442
EGFR	26	0.824	0.539	1.000
NF1	16	**0.030**	0.744	**0.036**
RB1	10	**0.036**	**0.044**	0.225
PIK3R1	9	0.671	0.492	1.000
PIK3CA	7	0.724	1.000	0.498
IDH1	4	0.529	1.000	0.321
ERBB2	0	-	-	-

Tumor-infiltrating lymphocytes were categorized as absent (0), present (1+), or abundant (2+). Cases were defined as wild-type (0) or mutant (1).

Chi-square test was used to examine associations between tumor-infiltrating lymphocytes (0, 1+, 2+) and mutations (0, 1).

Fisher's exact test was used when 25% of the cells had <5 observations.

Effective Sample Size=99.

Table 3. Tumor-infiltrating lymphocytes are depleted in *EGFR*-amplified and homozygous *PTEN*-deleted tumors.

Copy Number Alteration	Frequency (%)	p-value (0, 1+, 2+)	p-value (0 vs 1+, 2+)	p-value (0, 1+ vs 2+)
EGFR amp	49.30	**0.05**	**0.04**	0.06
CDK4 amp	14.49	0.37	0.22	0.47
PDGFRA amp	14.08	0.61	0.73	0.47
MDM4 amp	9.86	0.23	0.24	0.64
MDM2 amp	9.26	0.81	0.82	1.00
MET amp	8.85	0.57	0.79	0.48
CDK6 amp	7.04	0.75	0.65	0.69
CCND2 amp	4.02	1.00	1.00	1.00
AKT3 amp	3.22	1.00	1.00	1.00
MYCN amp	2.62	1.00	0.62	1.00
PIK3CA amp	2.41	0.69	0.60	1.00
CDKN2A del	62.0	0.23	0.09	0.48
CDKN2B del	61.0	0.23	0.09	0.48
PTEN del	10.3	0.09	**0.03**	0.69
RB1 del	3.62	0.87	1.00	0.57
CDKN2C del	3.62	0.71	1.00	0.51
PARK2 del	2.01	1.00	1.00	1.00
NF1 del	1.21	0.07	0.10	0.30

Tumor-infiltrating lymphocytes were categorized as absent (0), present (1+), or abundant (2+). Copy number status was defined as neutral (0), amplified or deleted (1).

Amplifications and deletions were considered separately.

Chi-square test was used to examine associations between tumor-infiltrating lymphocytes (0, 1+, 2+) and recurrent copy number alterations (0, 1).

Fisher's exact test was used when 25% of the cells had <5 observations.

Effective Sample Size=153.

Table 4. Tumor-infiltrating lymphocytes are enriched in the mesenchymal class.

Lymphocytes	Transcriptional Class				
	Classical	**Mesenchymal**	**Neural**	**Proneural**	**Total**
0	31	27	13	19	90
1+	10	18	9	18	55
2+	1	12	2	2	17
Total	42	57	24	39	162

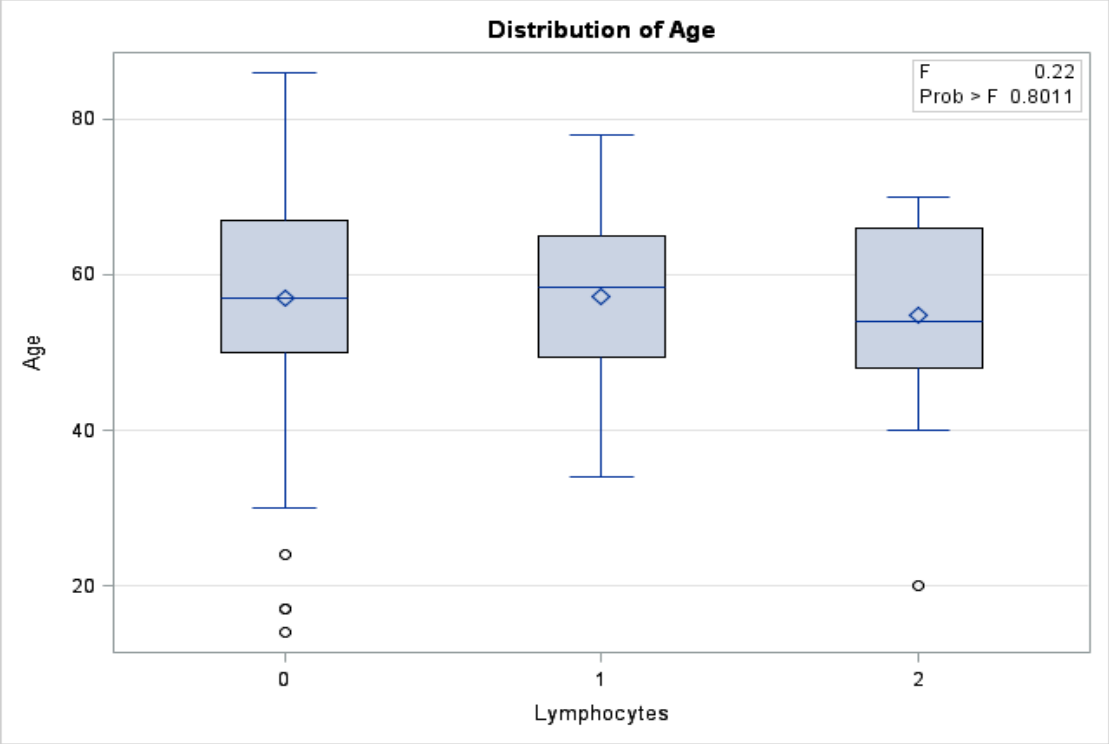
Tumor-infiltrating lymphocytes were categorized as absent (0), present (1+), or abundant (2+).

Chi-square 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 <5 observations.

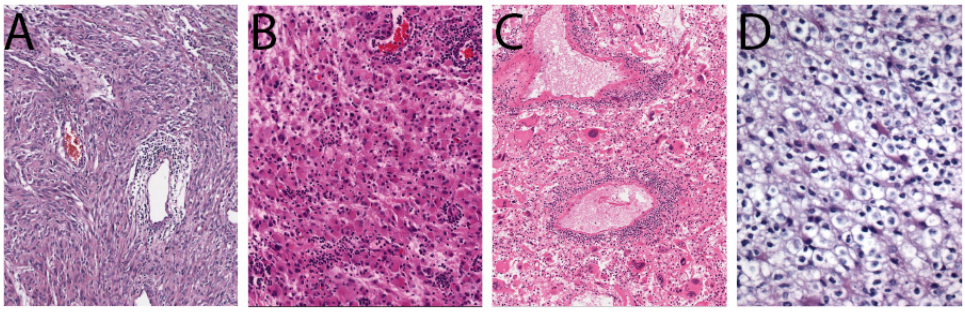
Effective sample size = 162.

Figure 1. Tumor-infiltrating lymphocytes in glioblastoma do not vary according to age.



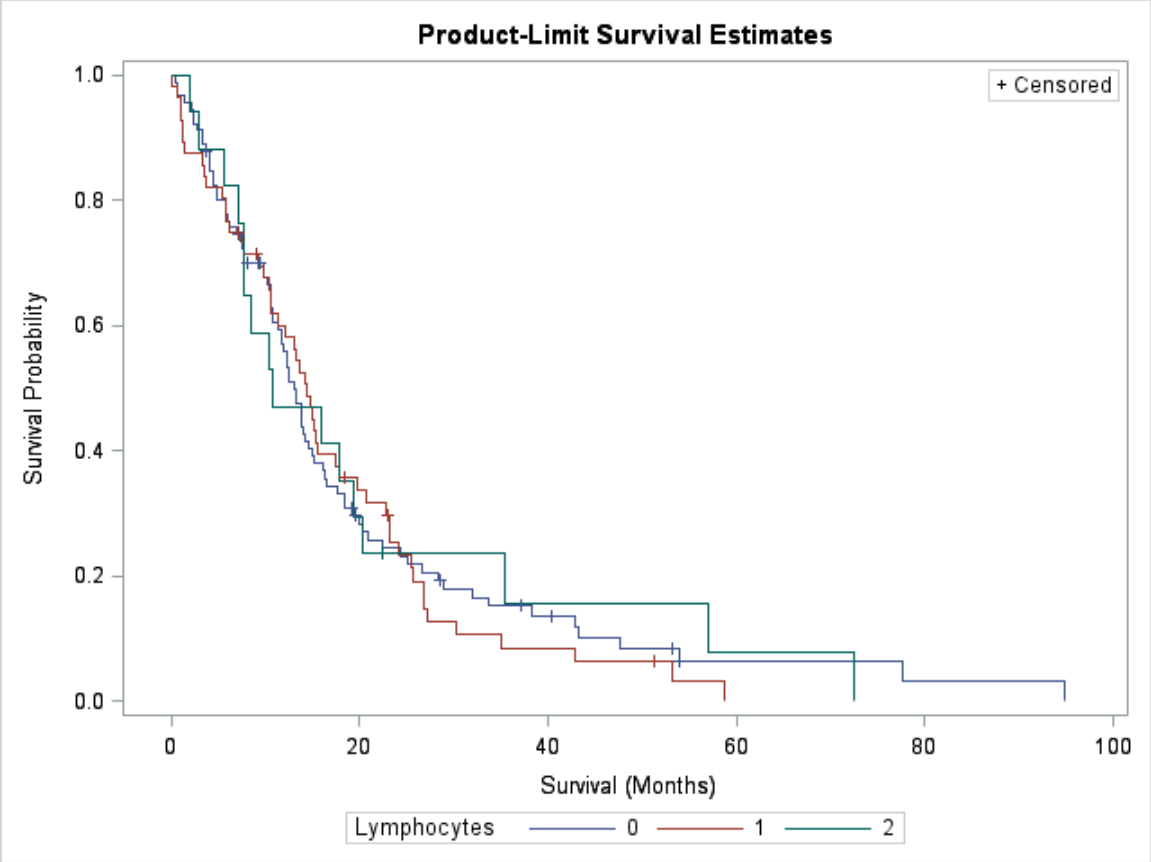
The mean age of patients with absent (0), present (1+), and abundant (2+) TILs was 56.9 (Standard deviation (SD) ± 14.6), 57.3 (SD ± 12.1), and 54.8 years (SD ± 13.1), respectively. Differences in age were not statistically significant ($p>0.05$).

Figure 2. Tumor-infiltrating lymphocytes are enriched in glioblastomas with sarcomatous cells (A), gemistocytes (B), epithelioid cells (not shown), and giant cells (C). TILs are depleted in GBMs with oligodendroglial cells (D) and small cells.



Representative examples of morphologic subtypes in TCGA GBM permanent section histologic slides

Figure 3. Tumor-infiltrating lymphocytes are not associated with improved survival.



Kaplan–Meier Estimates of Survival According to Lymphocytes

Number at Risk						
	0	**20**	**40**	**60**	**80**	**100**
0	91	21	9	2	1	0
1+	56	17	4	0	0	0
2+	17	5	2	1	0	0