Tumour Mutational Burden is Associated with Poor Outcomes that Cannot Be Predicted by Pan-cancer Targeted Sequencing in Diffused Glioma

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Abstract

Aims Tumor mutational burden (TMB) is a biomarker for immune checkpoint therapy. The impact of TMB on clinical outcomes and the correlation coefficient between exome sequencing and targeted sequencing in glioma have not yet been explored. Methods Somatic mutations in the coding regions of 897 primary gliomas, clinical and RNA-seq information for 654 patients In TCGA dataset were analyzed. Descriptive and correlational analyses were conducted with TMB. Enrichment Map and GSEA was also performed. Results TMB was higher for the group of mutant genes that are frequently mutated in glioblastomas (GBMs) and lower for the group of mutant genes that are frequently mutated in lower-grade gliomas (LGGs). Patients with a higher TMB exhibited shorter overall survival. TMB was associated with grade, age, subtype and mutations affecting genomic structure. The signaling pathways of the cell cycle and immune effector processes were enriched in the TMBHigh group. TMB was higher in the mismatch repair (MMR) gene mutant group compared to the wildtype group, but the MMR pathway was enriched in the TMBHigh group of gliomas without mutations in classical MMR genes. The correlation between TMBs calculated through exome sequencing and targeted sequencing was moderate. Conclusions TMB is associated with poor outcomes in diffuse glioma. High proliferative activity in the TMBHigh group could account for the shorter survival of these patients. This association was not reflected by a pan-cancer targeted sequencing panel.

Introduction

Glioma is the most common malignant primary brain tumor in adults. Molecular classification via genomics, transcriptomics and methylomics has revealed the potential value of diagnosis based on molecules[1, 2, 3]. With the publication of the 2016 WHO classification, integrated diagnosis including mutational and histological phenotypes is broadly applied in pathological typing. In some cases, the genotype even trumps the histological phenotype [4].

Tumour mutational burden (or tumour mutational load) is a biomarker of immune checkpoint inhibitors in many cancer types, as neoantigens are generated by somatic tumour mutations [5]. T cell-inflamed GEPs (gene expression profiles) are used to predict the response to PD–1 blockade and combined TMB to predict the effects of anti-PD–1 treatment [6]. TMB is also a poor prognostic marker for neuroblastoma but a good prognositc marker for non-small-cell lung cancer [7, 8]. Furthermore, deficiency of the MMR complex leads to the accumulation of mutations [9, 10]. Exome sequencing or targeted sequencing is used to measure TMB. As it is widely agreed that panel-based TMB is highly correlated with TMB calculated by exome sequencing [9, 11], panel-based TMB is commonly used in cancer patients to predict survival after immunotherapy [5].

In glioma, the reports of TMB seem to be controversial. TMB is higher in LGG compared to that in GBM [12]; on the other hand, the TMB of LGG is lower than that of GBM [13]. It has also been reported that the correlation of TMB and grade is not significant [14]. The prognostic value and related signaling pathways of TMB in glioma are still not known. In this study, using multiomics data from TCGA, we systematically

analyzed the correlations between TMB and mutational distribution, clinical features and transcriptomic data, revealing potential value for predicting the prognosis and related enrichment pathways of TMB in glioma. Furthermore, we found that MMR pathways were activated in high-TMB glioma patients without mutations in MMR genes. Finally, we evaluated the correlation between exome sequencing-based TMB and targeted sequencing-based TMB, which indicated that it was inappropriate to predict TMB with pan-cancer panels in glioma.

Materials And Methods

Data Source

Our data included exome sequencing data (level 2, n = 897), RNA-seq data (n = 669) and clinical data (n = 1105) from patients with LGG and GBM from TCGA. Mutational data including variant allele frequencies of mutations were obtained from cBioPortal (http://www.cbioportal.org)[15, 16]. RNA-seq data were obtained from Gliovis (http://gliovis.bioinfo.cnio.es/)[17]. Clinical data were collected from Gliovis and cBioPortal. Integrated diagnoses were performed according to the World Health Organization (WHO) classification (2016).

TMB (tumour mutational burden)

The size of the targeted (coding) genomic region has been defined as 36 Mb. For the estimation of TMB, we used the same approach as was outlined in a recent study [9], i.e., counting all coding somatic base substitutions and indels in the targeted regions, including “stop_/start_lost/frameshift_/missense_/inframe_” alterations. The software used to estimate TMB was Personal Cancer Genome Reporter software [18].

Statistical Analysis

The Mann-Whitney test was performed to compare the TMBs of two different groups. The Kruskal-Wallis test was used to compare the TMBs of more than two different groups. Spearman’s rank correlation test was used to examine the association between TMB and age/gene expression. Patient survival was analyzed by the Kaplan-Meier method. $P<0.05$ was considered statistically significant. (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

GSEA and Enrichment Map

GSEA (gene set enrichment analysis) was performed with GSEA software (http://software.broadinstitute.org/gsea/downloads.jsp)[19], and GO biological process analysis including 4436 gene sets was performed (http://software.broadinstitute.org/gsea/msigdb/genesets.jsp?

collection = BP). Enrichment Map was used to visualize the results of GSEA according to previously reported methods [20].

Results

Mutational distribution according to the elevation of TMB

To understand the value of TMB in glioma, we first studied the types and distribution of nonsynonymous mutations (Supplementary table 1). As we suspected, in the majority of genes, such as PTEN and EGFR, TMB was higher in the mutant group compared to the wildtype group (median TMB: PTEN, 1.05 VS 0.67 mutations/Mb; EGFR, 1.08 VS 0.69 mutations/Mb; Figure 1). However, for genes such as IDH1, TP53 and ATRX, TMB was lower in the mutant group (median TMB: IDH1, 0.53 VS 1.03 mutations/Mb; TP53, 0.67 VS 0.81 mutations/Mb; ATRX, 0.61 VS 0.81 mutations/Mb; Figure 1). As previously reported, the mutation frequencies of IDH1, TP53 and ATRX were higher in LGG, and the mutation rates of PTEN and EGFR were higher in GBM. We confirmed the conclusion through the analysis of mutational frequencies in LGG and GBM (IDH1, 77% VS 7%; TP53, 43% VS 25%; ATRX, 33% VS 9%; PTEN, 5% VS 29%; EGFR, 5% VS 17%; Supplementary Figure 1, 2). The data indicated that TMB might be elevated in GBM compared to LGG.

TMB is associated with worse outcomes in glioma patients.

To test this hypothesis, we analyzed the relationship between TMB and clinical features (Supplementary table 2, n = 654). Patients without clinical or mutational information were excluded (Figure 2A). As we expected, TMB increased according to grade (median TMB, 0.47 VS 0.64 VS 0.99 mutations/Mb; Supplementary Figure 3A). Through ROC analysis, we determined the cut-off value (0.655 mutations/Mb) of TMB, and the patients were divided into TMB^{High} and TMB^{Low} groups (Figure 2B). Overall survival was decreased in patients with a high TMB compared to those with a low TMB (hazard ratio 3.91, 95% confidence interval 3.33–5.70; P<0.001, log-rank; Figure 2C). Patients in the TMB^{High} group exhibited median overall survival of 23.0 months, whereas those in the TMB^{Low} group exhibited median overall survival of 105.2 months. We confirmed the prognostic effect of TMB in the LGG (hazard ratio 3.04, 95% confidence interval 2.57–5.73; P<0.001, log-rank) and high-grade glioma (HGG, hazard ratio 2.67, 95% confidence interval 2.00–3.58; P<0.001, log-rank) subgroups (Figure 2D, E). Overall survival was also worse for patients harboring more than one mutation per Mb compared to those with less than one mutation per Mb (Figure 2F). Furthermore, we analyzed the distribution of clinical features accompanied by an elevated TMB (Figure 2G). It seemed that TMB might be associated with age, grade, methylation of the MGMT promoter, codeletion of 1p/19q and Chr.7.gain/Chr.10.loss. The calculation of statistical significance showed that TMB was increased in old patients but was not associated with sex

(Supplementary Figure 3B, C). In the subgroup analysis of integrated diagnosis, TMB was found to be elevated in the anaplastic astrocytoma IDH-wildtype group compared to the other astrocytoma group (Supplementary Figure 3D). TMB was also increased for classic-like and mesenchymal-like subtypes compared to other IDH-wildtype subtypes and for G-CIMP-low subtype compared to other IDH-mutant subtypes (Supplementary Figure 3E) [5]. Mutational analysis revealed that the patients exhibiting an unmethylated MGMT promoter, non-codeletion of 1p/19q and Chr.7.gain/Chr.10.loss exhibited a higher TMB (Supplementary Figure 3F). Taken together, these data indicated that TMB could be an effective prognostic biomarker of glioma.

TMB^{High} gliomas exhibit elevated proliferative activity and immune responses.

To clarify the mechanism of the association between the TMB and poor outcomes of glioma patients, we analyzed the data from patients with TMB information and RNA-seq data (n = 654). Gene set enrichment analysis (GSEA) coupled with Enrichment Map analysis was performed to visualize the enriched biological processes. The TMB^{High} group was enriched in transcriptional programs related to the cell cycle, DNA replication and immune effector processes. By contrast, the transcriptional programs of adenylate cyclase activity and synaptic transmission were enriched in the TMB^{Low} group (Figure 3A, B). These transcriptomics data indicated that high-TMB gliomas exhibit intensive proliferative activity, which might result in a worse prognosis. Furthermore, TMB exhibited a modest correlation with inflammatory biomarkers of checkpoint inhibitor-based immunotherapy (Figure 3C), which was consistent with previous reports based on the pan-cancer dataset [6].

A high TMB is associated with the mismatch repair pathway in gliomas without mutations in classical MMR genes.

It is reported that deficiency of MMR (mismatch repair) is associated with a higher TMB in gliomas [14], and we confirmed this finding in the TCGA dataset. Only 3.6% of glioma patients harbored MMR gene mutations (32 in 897 glioma patients). TMB was elevated in patients exhibiting MLH1, MSH2, MSH6, PMS2, PODL1 or POLE gene mutations (Figure 4A). We further performed GSEA in patients without mutations in MMR genes to confirm whether high TMB was associated with low mismatch repair function. Interestingly, mismatch repair-associated transcriptional programs were also enriched in the TMB^{High} group but not in the TMB^{Low} group (Figure 4B, C). The correlation analysis of TMB and the expression of MMR genes further demonstrated that a high TMB was associated with the expression of MLH1, MSH2, MSH6, PODL1 and POLE in gliomas without MMR mutations (Figure 4D). These data

indicated that when the MMR genes are not mutated, TMB exhibits a positive correlation with MMR function.

Pan-cancer targeted sequencing cannot predict prognosis in glioma.

Considering the cost of exome sequencing, targeted sequencing is widely used to predict TMB in pan-cancer analyses. We calculated TMB using 468 genes (Supplementary table 3) from MSK-IMPACT [11] and analyzed the Spearman correlation with TMB calculated on the basis of exome sequencing (Figure 5A, Supplementary table 4). Interestingly, unlike other cancer types, the correlation between panel-based TMB and exome sequencing-based TMB was moderate in glioma (r = 0.3105). This result was confirmed with two other panels (Supplementary table 3) that are used in China (Supplementary Figure 5, r = 0.2753/0.3461). We further performed AUC analysis for panel-based TMB (Figure 5B), which was significantly different from the AUC of exome sequencing-based TMB (Figure 2B). Unlike exome sequencing-based TMB, TMB calculated by targeted sequencing was decreased in GBM (Figure 5C). These data indicated that, at least in glioma, pan-cancer panel-based TMB cannot represent exome sequencing-based TMB and is not suitable for prognosis.

Discussion

In this study, to understand the value of TMB in glioma, we analyzed the relationship between TMB and the mutation distribution. For most genes, TMB was elevated in the mutant group compared to the wildtype group, which is reasonable, as TMB is an aggregate of mutations that result in protein alterations. However, for genes that were mutated in lower-grade glioma, TMB was lower in the mutant group. Through the analysis of clinicopathological parameters, we found that TMB was significantly elevated with an increasing grade and was associated with poor survival of patients. According to enrichment MAP analysis with GSEA, the TMB^{High} group exhibited activation of cell proliferation and immune reactions. As reported in pan-cancer studies, the correlation between TMB and T cell immunity was moderate, and TMB was elevated in the MMR gene mutant group. Interestingly, in the MMR gene wildtype group, the transcriptional programs of MMR enriched in the TMB^{High} group and TMB were positively correlated with the expression of MMR genes. Finally, by analyzing pan-cancer targeted sequencing, it was found that TMB based on panel analysis was not highly correlated with TMB calculated by exome sequencing in glioma. No prognostic value existed for targeted sequencing-based TMB.

With the development of large-scale sequencing, thousands of somatic mutations have been revealed in cancer samples. Different cancer types exhibit distinct mutational signatures [12]. The mutational landscape is reported to illustrate driver mutations and develop individualized cancer treatments [21]. In glioma, the reported TMB is paradoxical. The mutational load calculated by exome sequencing is

associated with the tumor grade and age of patients [22]. However, in another cohort, targeted sequencing-based TMB was not correlated with grade [14]. To study the application of targeted sequencing in the prediction of TMB in glioma, we analyzed the correlation between exome sequencing-based TMB and targeted sequencing-based TMB in a TCGA cohort. Although the correlation was significant, the coefficient was modest. For most gliomas with a TMB <2, targeted sequencing-based calculation could not exactly predict TMB. Although targeted sequencing is more economical than exome sequencing, pan-cancer targeted sequencing-based TMB may be inappropriate for predicting the prognosis of glioma patients. Glioma-customized panels should be designed to precisely predict the mutational load.

Emerging data imply that neoantigens resulting from nonsynonymous mutations could serve as biomarkers for checkpoint blockade therapy. The mutation that results in tumour initiation could also be targeted by the immune system [15]. In a mouse model, radiation plus anti-PD–1 treatments improved survival compared to radiation alone [23]. Through immune checkpoint inhibitor treatment, the tumor size of glioblastomas with hypermutation is significantly reduced [24]. However, the failure of CheckMate–143 indicates that nivolumab does not improve the OS of patients with recurrent glioblastoma compared to bevacizumab treatment [25]. These data imply that the mutational state should be analyzed before immune checkpoint inhibitor treatment. We analyzed the relationship between TMB and other biomarkers of immune checkpoint inhibitors and illustrated the possible mechanism by which TMB was associated with poor survival. Our work revealed potential biomarkers for improving survival in glioma patients. However, our data were mainly obtained from patients treated with routine chemoradiotherapy. TMB should be tested in patients treated with immune checkpoint inhibitors. Recently, one clinical study concluded that there was no association between a higher TMB and improved survival in glioma patients treated with an immune checkpoint inhibitor, and the trend was toward worse survival [5]. However, as we noted above, TMB was calculated by targeted sequencing, which may not be concordant with the real TMB in glioma. Exome sequencing should be performed to verify the conclusions, and the survival bias induced by the mutational load must be considered.

Unlike non-small-cell lung cancer and melanoma, treatment with an immune checkpoint inhibitor in glioma presents many challenges, as TMB, T-cell infiltration, and mutations in MMR genes are lacking in these cancers, and they are protected by the blood brain barrier. Although the results of the application of nivolumab in recurrent glioma were disappointing, presurgery treatment via PD–1 monoclonal antibody blockade is reported to significantly extend overall survival, especially for newly diagnosed patients [26, 27]. This neoadjuvant treatment is associated with higher immune cell infiltration but downregulation of cell cycle-related gene expression, which is also related to TMB. It is inspiring that neoadjuvant treatment significantly improved the outcomes of glioma patients in such a small cohort. As tumor tissue from patients treated with pre- and postimmune checkpoint therapy and patients not receiving immune checkpoint therapy treatment are available,the calculation of TMB through exome sequencing to illustrate the relationship with the response to neoadjuvant PD–1 blockade treatment is promising.

Take home messages

1. TMB is associated with shorter overall survival in glioma patients.
2. proliferative activity and immune response are activated in TMBHigh gliomas.
3. TMB is higher in MMR gene mutant group, but the MMR pathway is enriched in the TMBHigh group.
4. Pan-cancer targeted sequencing-based TMB cannot predict prognosis in glioma.

Declarations

Authors' contributions:

TL conceived this work, performed the statistical analysis, and wrote the paper. LW collected and preprocessed the data from TCGA. LW generated the panel for subtype analyses and panel-based TMB. YL, JG, YT, ML and SD helped to interpret the results. TL revised the manuscript. TL supervised the whole study.

Conflict of Interest Statement:

The authors declare that they have no competing interests.

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Figures

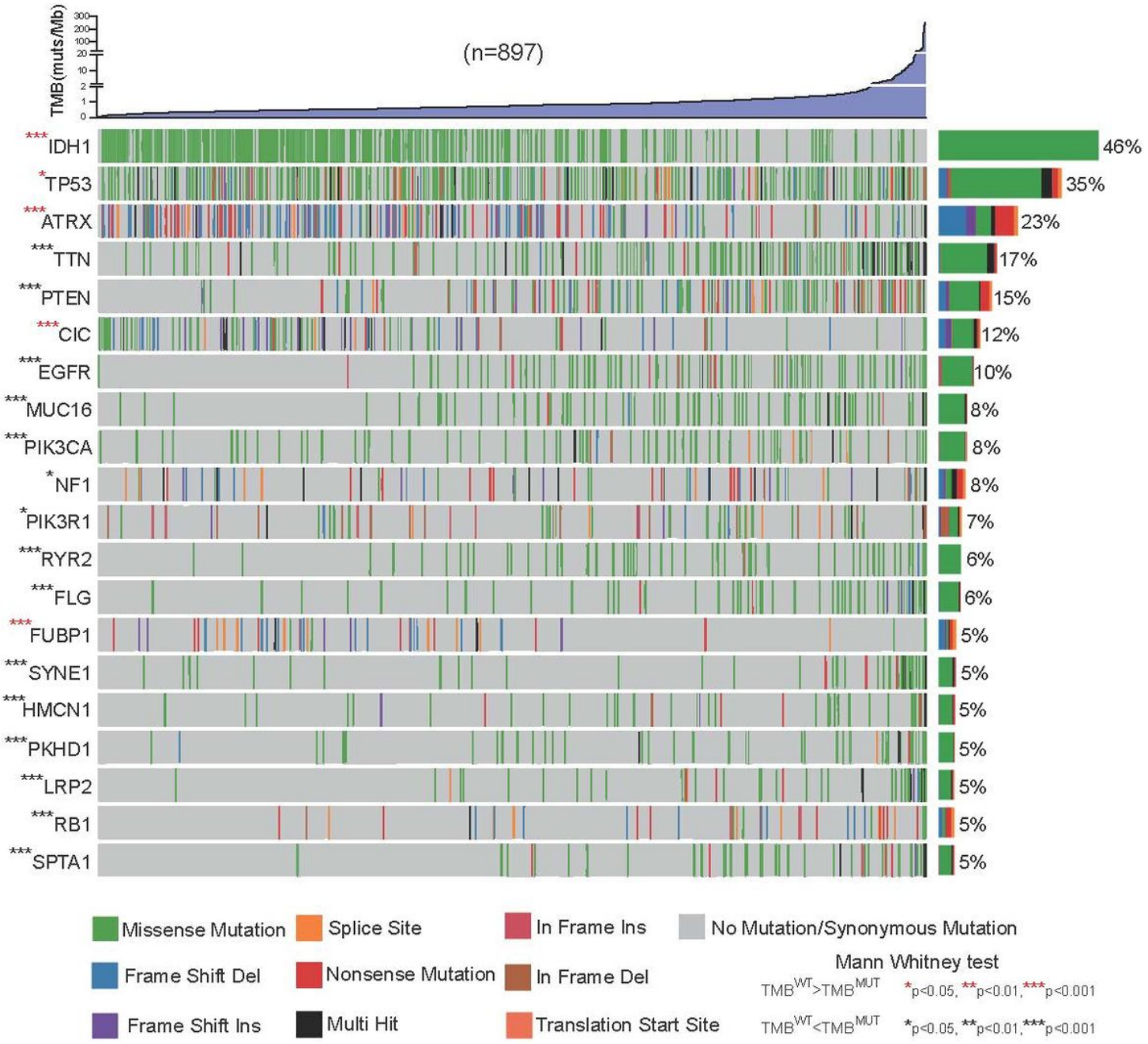

Figure 1

Heatmap showing the top 20 genes' mutational frequencies and their types in glioma (n=897). The statistical significance of the indicated genes was as described above.

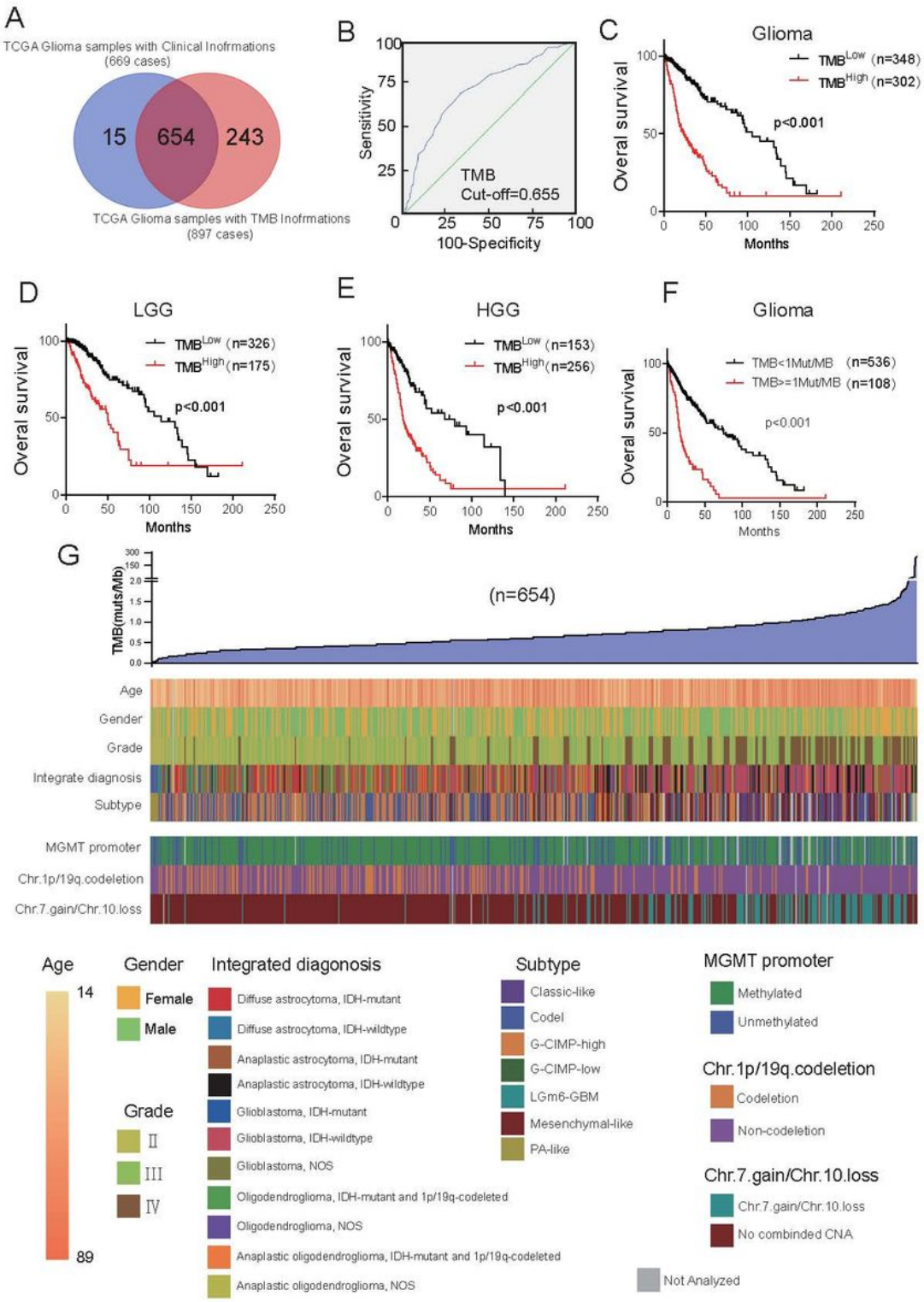

Figure 2

TMB is associated with worse outcomes in glioma patients. (A) Venn map showing the patients included in further analysis. (B) The cut-off value was determined via ROC analysis, and the patients with a higher TMB (>=0.655 mutations/Mb) were referred to as the TMB^{High} and those with a lower TMB (<0.655 mutations/Mb) as the TMB^{Low} group. (C-E) Kaplan–Meier curves of the overall survival of glioma patients (C, n=650, 4 patients lacked survival information)/lower-grade glioma (LGG, D,

n=501)/high-grade glioma (HGG, E, n=409) with high TMB (TMB^{High}) versus low TMB (TMB^{Low}). (F) Survival analysis was performed in glioma patients (n=650) with the indicated TMBs. (G) Heatmap showing the distribution of clinical features and genetic characteristics in glioma specimens (n=654).

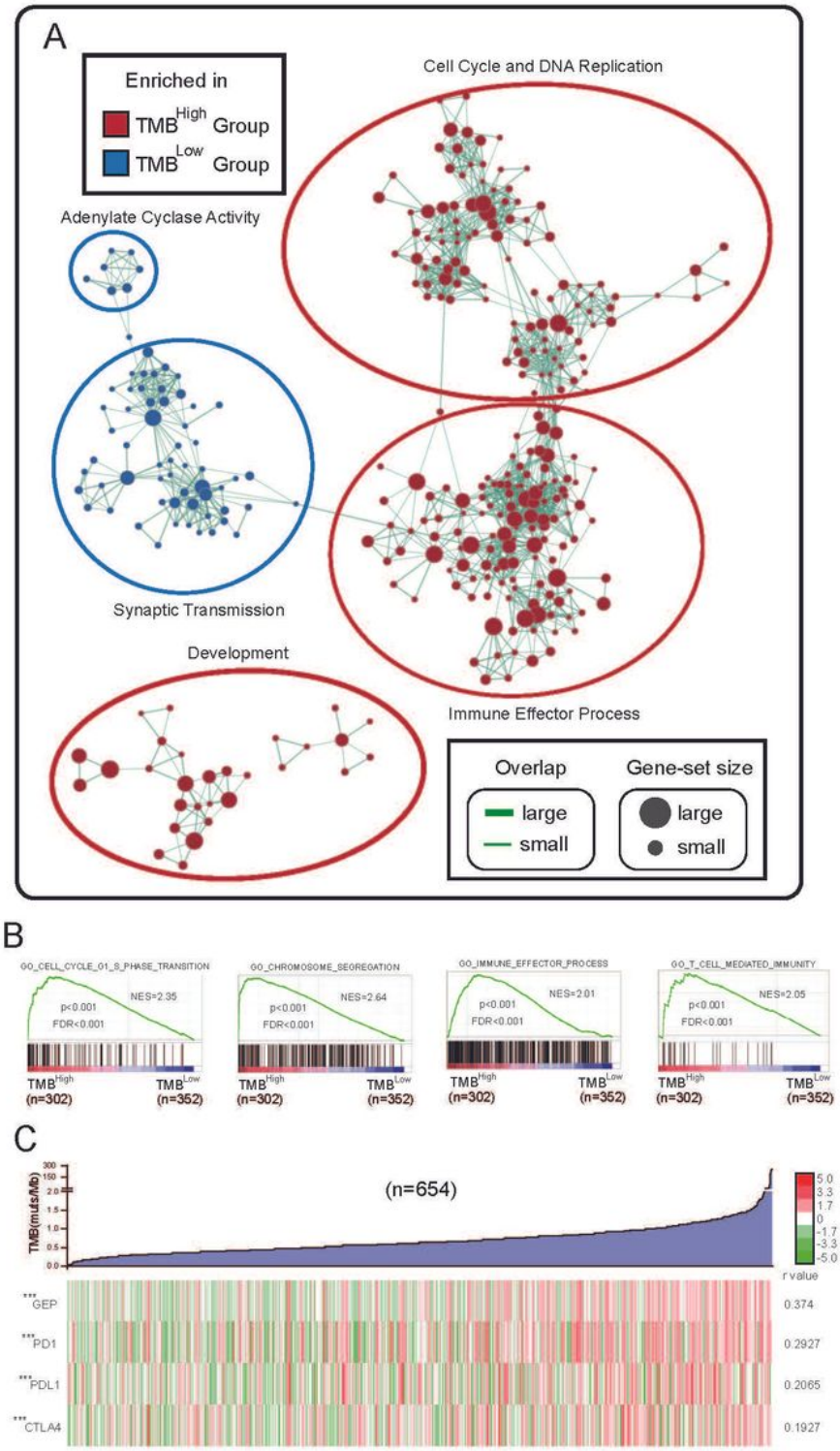

Figure 3

TMB^{High} gliomas exhibit an increased proliferative activity and immune response. (A) GO biological progress enriched by GSEA in the TMB^{High} group (n=302) versus the TMB^{Low} group (n=352) using an enrichment map. Node size represents the number of genes in gene sets. Line width represents the number of overlappinggenes. (B) Representative GSEA enrichment plots in (A). The NES (normalized enrichment score), p value and FDR (false discovery rate) were calculated with GSEA software. (C) Heatmap showing the distribution and correlation of the indicated gene set/genes was visualized using Java Treeview in glioma specimens. Spearman's r value and significance were calculated.

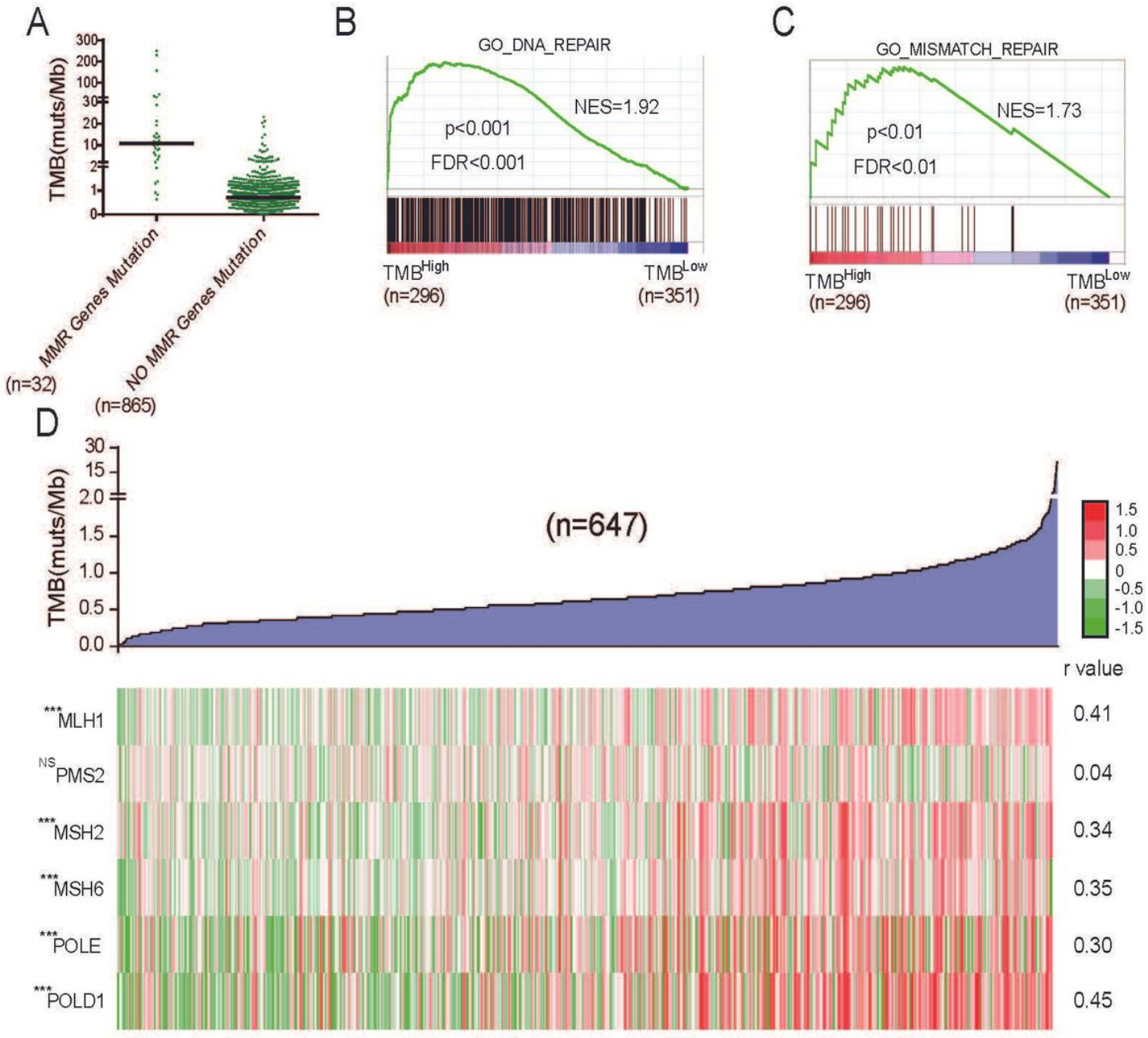

Figure 4

The mismatch repair pathway is activated in the TMB^{High} group in gliomas without mutations in classical MMR genes. (A) TMB of gliomas with/without mutations in 6 classical MMR genes. (B) DNA repair and mismatch repair functions were analyzed by GSEA in the TMB^{High} group versus the TMB^{Low} group. (C) Heatmap analysis of the distribution and correlation of the indicated genes was performed. Spearman's r value and significance were calculated.

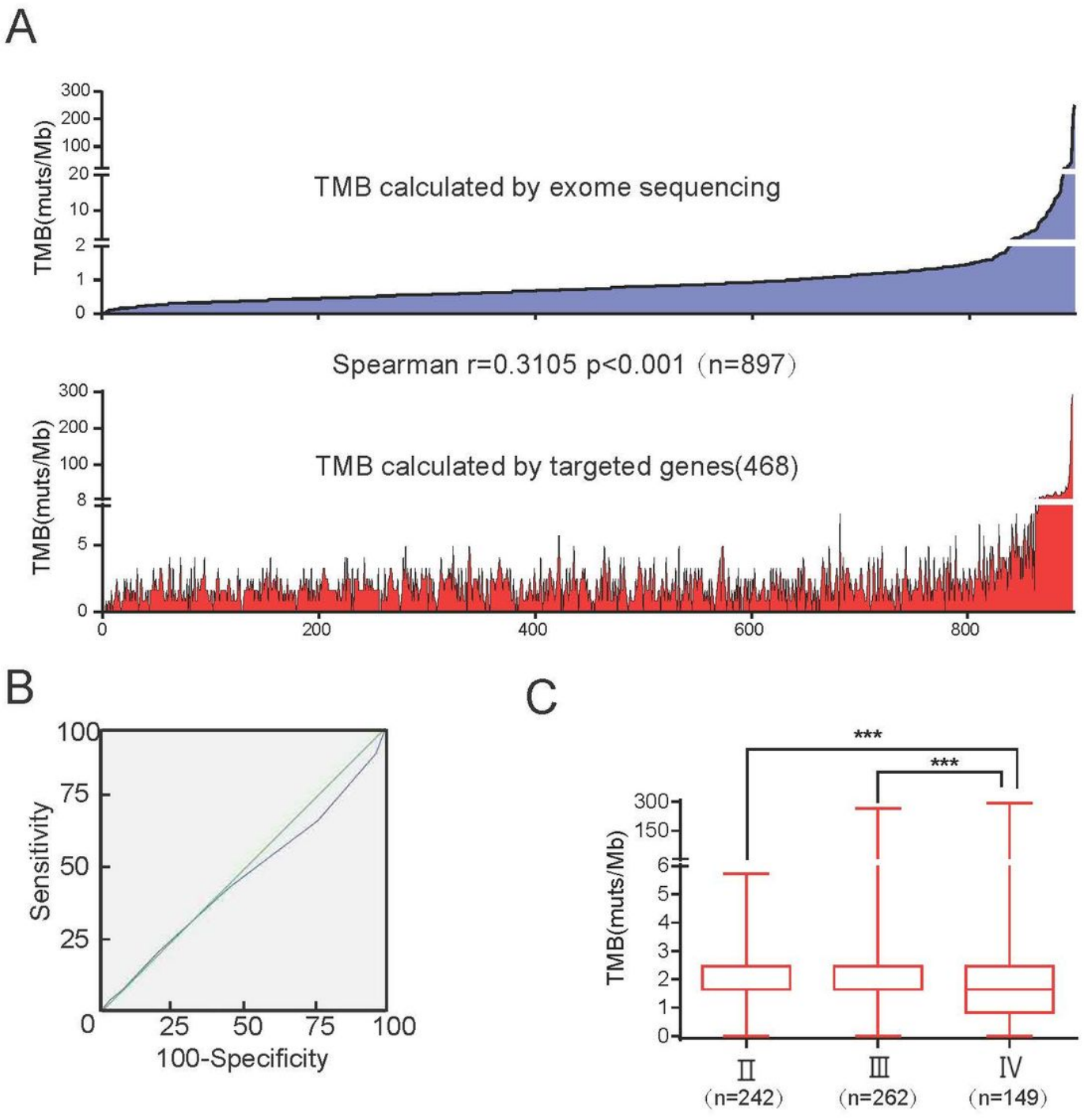

Figure 5

Pan-cancer targeted sequencing-based TMB cannot predict prognosis in glioma. (A) Correlation of TMB calculated on the basis of exome sequencing and targeted genes. The list of genes is from the FDA-approved targeted next generation sequencing panel (MSK-IMPACT™). Spearman's r value and significance were calculated. (B) AUC analysis with TMB calculated using the MSK-IMPACT panel. (C) TMB calculated using the MSK-IMPACT panel for different grades. Statistical significance was calculated with the Kruskal-Wallis test for more than two groups.

Supplementary Files

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- supplement1.xlsx
- supplement1.xlsx
- supplement3.xlsx
- supplement4.xlsx