Characterization of the Clinical Significance of PD-1/PD-Ls Expression and Methylation in Patients With Low-Grade Glioma

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Abstract

Background: Immune checkpoints play crucial roles in the immune escape of cancer cells. However, the exact prognostic values of expression and methylation of programmed-death 1 (PD-1), programmed-death-ligand 1 (PD-L1) and PD-L2 in low-grade glioma (LGG) have not been well-defined yet. **Methods:** A total 514 LGG samples from the Cancer Genome Atlas (TCGA) dataset containing gene expression, DNA methylation, and survival data were enrolled in our study. Besides, a total of 137 primary LGG samples from the Chinese Glioma Genome Atlas (CGGA) database were also extracted for the survival analysis of the prognostic values of PD-1/PD-Ls expression. **Results:** PD-1/PD-Ls had distinct co-expression patterns in LGG tissues. The expression and methylation level of PD-1/PD-Ls seemed to be various in different LGG subtypes. Besides, overexpression and hypo-methylation of PD-1/PD-Ls were associated with worse prognosis. In addition, PD-1/PD-Ls expression was positively associated with TIICs infiltration, while their methylation was negatively associated with TIICs infiltration. Moreover, PD-1/PD-Ls and their positively correlated gene mainly participated in immune response related biological processes. **Conclusion:** To conclude, overexpression and hypo-methylation of PD-1/PD-Ls predicted unfavorable prognosis in LGG patients, suggesting those patients may benefit from PD1/PD-Ls checkpoint inhibitors treatment.

Keywords

PD-1, PD-L1, PD-L2, prognosis, low-grade glioma

Abbreviations

WHO, the World Health Organization; LGG, low-grade glioma; GBM, glioblastoma; PD-1, programmed-death 1; CD279, cluster of differentiation 279; PD-L1, programmed-death-ligand 1; PD-Ls, PD-L1 and PD-L2; TCGA, the Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas; TIICs, tumor-infiltrating immune cells; GO, gene ontology; GSEA, gene set enrichment analysis; OS, overall survival; CNV, copy number variations

Introduction

Glioma is a common neuroepithelial-derived primary brain tumor, which is one of most fetal malignancies worldwide. As a heterogeneous disease, classification of glioma is essential for therapeutic guidance and prognostic assessment, which largely relied on tumor histopathologic features.[1] World Health Organization (WHO) grade system, the most authoritative classification, divides glioma into two main classes, which contains low-grade glioma (LGG) and glioblastoma (GBM). LGG is slower growing than their high-grade counterparts, accounting for 10%-20% of all primary intracranial tumors.[2] Although

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surgical resection is the preferred therapeutic strategy for glioma, substantial efforts have also been made to recognize the critical interplay between glioma and immunity.[3,4]

Immunotherapy is one of the most encouraging strategies for tumor treatment, and the most common therapy is to interrupt the interaction between immune checkpoints expressed on tumor and immune cells, which blocks the immune escape of tumor cells to some extent.[5] Programmed-death 1 (PD-1), also termed as cluster of differentiation 279 (CD279), is an important immunosuppressive molecule expressed on T cells and other immune cells membrane, which has been widely reported across multiple malignant tumors.[6] Programmed-death-ligand 1 (PD-L1) and PD-L2 are transmembrane proteins that are accounted to play critical roles in triggering the cancer immunity escape by binding to their receptor PD-1.[7,8] Previously, PD-L1 and PD-L2 (PD-Ls) expression have been revealed to be correlated with poor prognosis of glioma.[9,10] As we all know, two main glioma subtypes, LGG and GBM, exhibit different biological patterns and PD-Ls expression. However, we failed to obtain an integrated study on PD-1/PD-Ls expression and methylation in LGG. Only several researches observed the promising prognostic impact of PD-L1 in GBM.[11,12] Thus, the relationship between expression, methylation, and prognostic values of PD-1/PD-Ls in LGG needs to be further explored.

In this research, to define the PD-1/PD-Ls expression and regulatory factors in LGG, we took advantage of the Cancer Genome Atlas (TCGA), including RNA-sequencing mRNA expression, DNA methylation, and copy number data. Besides, we further checked the prognostic values of PD-1/PD-Ls expression and methylation status in subpopulations. Moreover, LGG samples from the Chinese Glioma Genome Atlas (CGGA) database were used to validate the prognostic values of PD-1/PD-Ls expression. This is the first integrative research that systematically characterizes PD-1/PD-Ls expression and methylation in LGG molecularly and clinically, providing a comprehensive insight into the values of PD-1/PD-Ls in predicting prognosis in patients with LGG.

Materials and Methods

Acquisition of TCGA and CGGA Data

The data of RNA-sequencing (IlluminaHiSeq), DNA methylation (Methylation450 k), and copy number (gistic2 thresholded) as well as clinical information in TCGA-LGG dataset were downloaded from UCSC Xena (https://xenabrow ser.net/datapages/). The gene expression level was assessed as in $\log_2(x+1)$ transformed RSEM normalized count. Main clinical data contained the histological type, IDH mutation status, WHO grade, and survival information. For further analysis, a total of 514 samples containing both gene expression, DNA methylation, and survival data were extracted. The basic clinico-pathological features of reserved samples were shown in Supplementary Tab. S1.

Besides, the RNA-sequencing data and survival information in the CGGA database (mRNAseq_325) were also obtained from the official website (http://www.cgga.org.cn/index.jsp). For survival analysis, a total of 137 primary LGG samples from the CGGA database, which contained both gene expression and survival data were extracted.

Tumor-Infiltrating Immune Cells Analysis

TIMER (https://cistrome.shinyapps.io/timer/) is an integrated web platform for systematic analysis of immune infiltration across various cancer types from TCGA datasets, including 10,897 samples across 32 cancer types.[13] TIMER applies a deconvolution method to speculate the abundance of tumor-infiltrating immune cells (TIICs) according to gene expression profiles.[14] We assessed the correlation between PD-1/PD-Ls expression and methylation with the infiltration levels of TIICs according to infiltrating data obtained from TIMER, including B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells.

Gene Co-Expression and Enrichment Analysis

To explore genes that shared co-expressed pattern with PD-1/PD-Ls, the online database Linked Omics (http://www.linkedo mics.org/login.php) was applied,[15] which containing gene expression data with the RSEM normalized count. PD-1/PD-Ls co-expressed genes were analyzed statistically using Pearson's correlation coefficient. Function module of Linked Omics performs analysis of Gene Ontology (GO) by gene set enrichment analysis (GSEA). The criterion for GO analysis as follows: minimum number of genes was 3, simulations were 500, and analysis method was affinity propagation.

Statistical Analysis

R 3.6.3 and GraphPad Prism 8 were applied as main tools for the statistical analysis and figures exhibition. Most of the data between the two groups were analyzed by Student's t-test. All data are presented in violin plots. Correlation analysis were assessed by Pearson correlation analysis. Survival analysis were conducted in R 3.6.3 by batch analysis methods using self-compiled program, and the groups were divided based on the median level PD-1/PD-Ls gene expression or methylation. Kaplan-Meier survival plots were generated with survival curves compared by log-rank test. For all analyses, differences were considered statistically significant when P values were less than 0.05.

Results

PD-1/PD-Ls Expression Levels in LGG

We first checked the expression levels of PD-1/PD-Ls in LGG tissues. As exhibited in Figure 1A, the expression of these three immune checkpoints in LGG showed obvious clustering. Expression of PD-1/PD-Ls was generally determined in LGG samples. PD-L2 exhibited the highest expression while the expression of PD-1 was lowest among these three immune
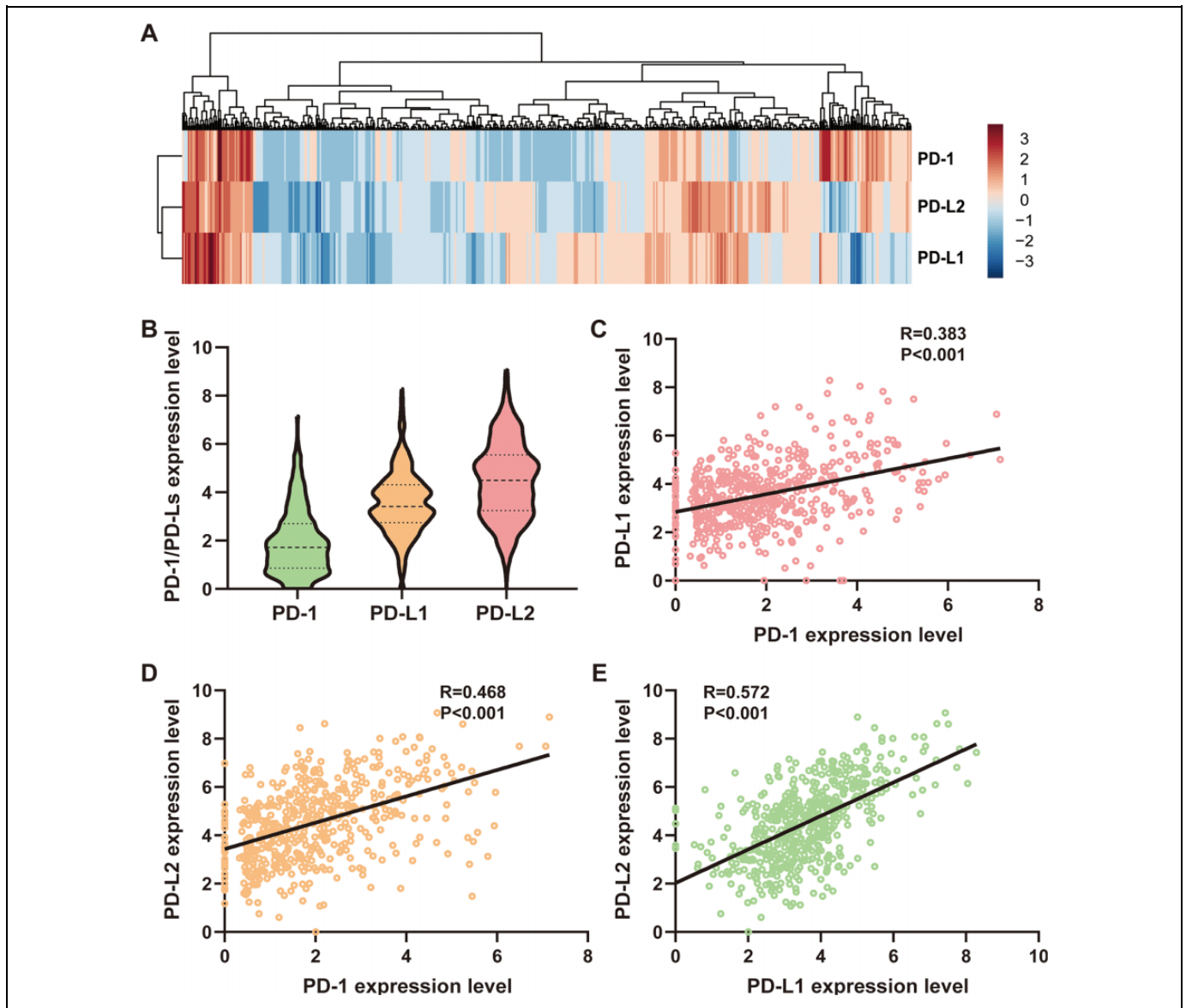
Figure 1. Expression of PD-1/PD-Ls in LGG. (A) A clustering heat map of gene expression of PD-1/PD-Ls in LGG; (B) expression of PD-1/PD-Ls were generally determined; (C) PD-1 expression was positively correlated with PD-L1 expression; (D) PD-1 expression was positively correlated with PD-L2 expression; (E) PD-L1 expression was positively correlated with PD-L2 expression.

checkpoints (Figure 1B). To further identify the correlation between PD-1/PD-Ls expression, we conducted correlation analysis using Pearson test. The results suggested that PD-1/PD-Ls had distinct co-expression patterns. PD-1 expression was positively associated with PD-L1 (Figure 1C) and PD-L2 expression (Figure 1D), the expression levels between PD-L1 and PD-L2 were also significantly correlated (Figure 1E). These results uncovered that PD-1, PD-L1, and PD-L2 might collaborate on specific molecular and biological functions in LGG.

Clinical Significance of PD-1/PD-Ls Expression

Due to notable heterogeneity of molecular nature across different LGG subtypes, PD-1/PD-Ls expression levels were evaluated according to the histological type, WHO grade system, as well as IDH mutation status. The results showed that astrocytoma exhibited the highest PD-1/PD-Ls expression when compared with oligoastrocytoma and oligodendroglioma (Figure 2A-C). Moreover, when WHO grade system was applied as a sub-classifier, we found that in grade 3 LGG showed significantly upregulated PD-1/PD-Ls expression (Figure 2D-F). Furthermore, IDH mutant-type showed universally lower expression of PD-1/PD-Ls than that of IDH-wt LGG (Figure 2G-I). These findings suggested that PD-1/PD-Ls related immune response were obviously various, which may further reflect different biological patterns across LGG subtypes.

To acquire the novel insight into the influence of on survival, we checked the prognostic values of PD-1/PDLs
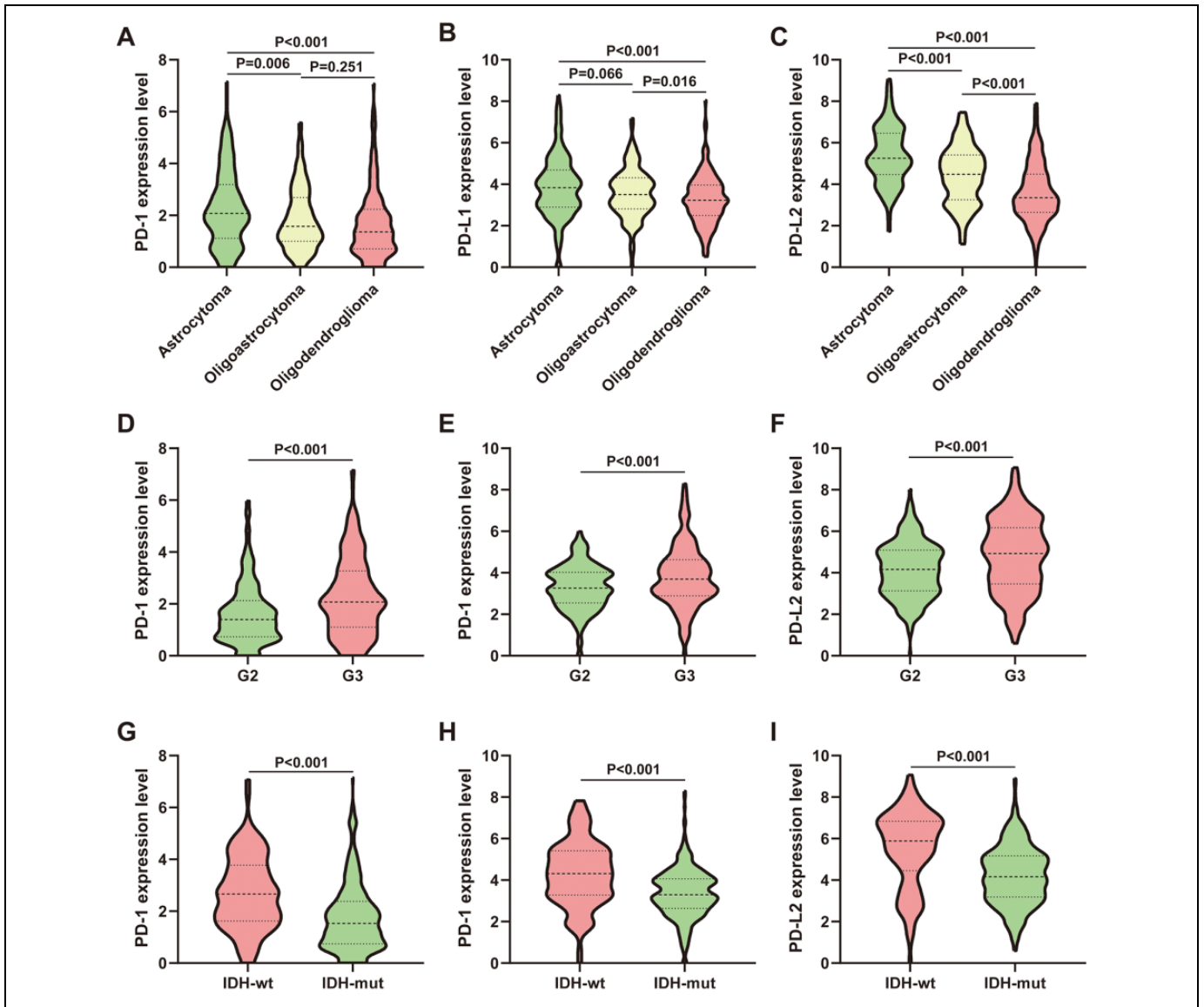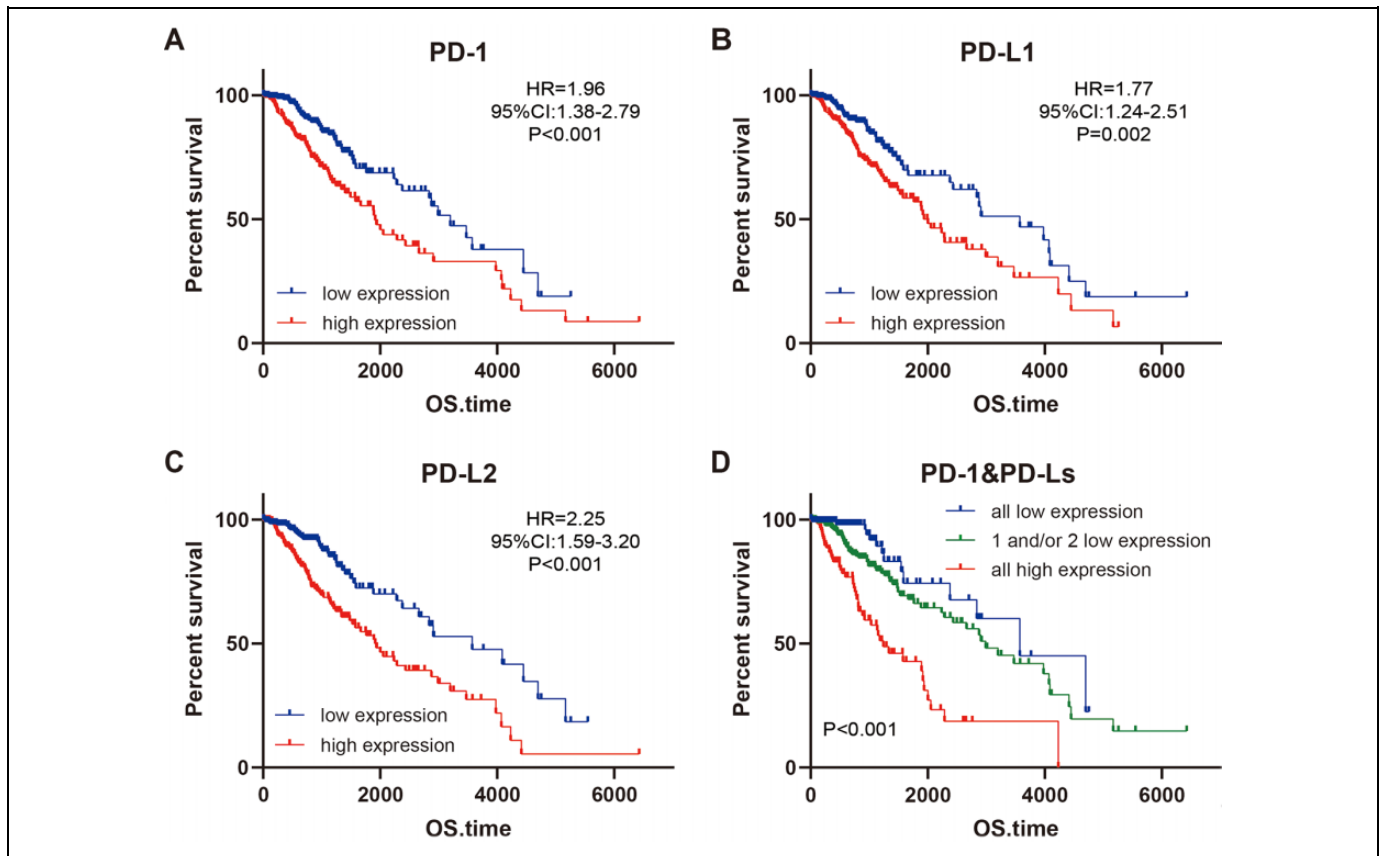
Figure 2. Expression of PD-1/PD-Ls in different LGG subtypes. (A) PD-1, (B) PD-L1, and (C) PD-L2 were highly enriched in astrocytoma subtype; (D) PD-1, (E) PD-L1, and (F) PD-L2 were highly enriched in grade 2 LGG; (G) PD-1, (H) PD-L1, and (I) PD-L2 were highly enriched in IDH wild type LGG.

expression in LGG. As shown in Figure 3, patients who expressed higher PD-1 in tumor tissues exhibited a significantly shorter overall survival (OS) than the counterparts (Figure 3A). Besides, overexpression of PD-L1 (Figure 3B) and PD-L2 (Figure 3C) also predicted poor OS in LGG patients. Moreover, we combined these three immune checkpoints expression to evaluate the prognosis of LGG patients. The result showed patients with PD-1/PD-Ls high expression had significantly poor OS than other cohorts, and the prognosis of patients with PD-1/PD-Ls low expression was best (Figure 3D). In addition, these results were also validated in the CGGA database, namely, higher PD-1/PD-Ls expression were significantly associated with poor prognosis (Figure S1A-C). Overall, these findings suggested that PD-1/PD-Ls were both negative prognostic indicators in LGG.

Regulatory Factors for PD-1/PD-Ls Expression

In view of the fact that PD-1/PD-Ls had various expression patterns in LGG and significant prognostic values were observed, we next try to the regulatory factors that responsible for dys-regulation of PD-1/PD-Ls at gene level according to available data. DNA copy number variations (CNV) are most common genetic alterations that affect tumorigenesis of cancers *via* mediating tumor-related gene expression.[16-18] However, the expression levels of PD-1 and PD-L1 showed no significant difference across different CNV status (Figures S1A, S1B), while different, CNV status significantly influence PD-L2 expression. Copy gain was associated with notably upregulated PD-L2 levels compared with the copy-neutral (diploid) and copy-loss (shallow deletion and deep deletion) samples (Figure S1C).

Figure 3. Survival analysis for PD-1/PD-Ls expression. Kaplan–Meier survival analysis showed that high expression of (A) PD-1, (B) PD-L1, and (C) PD-L2 were associated with significantly worse prognosis in LGG patients; (D) combined PD-1, PD-L1, and PD-L2 expression defined various prognosis in LGG patients.
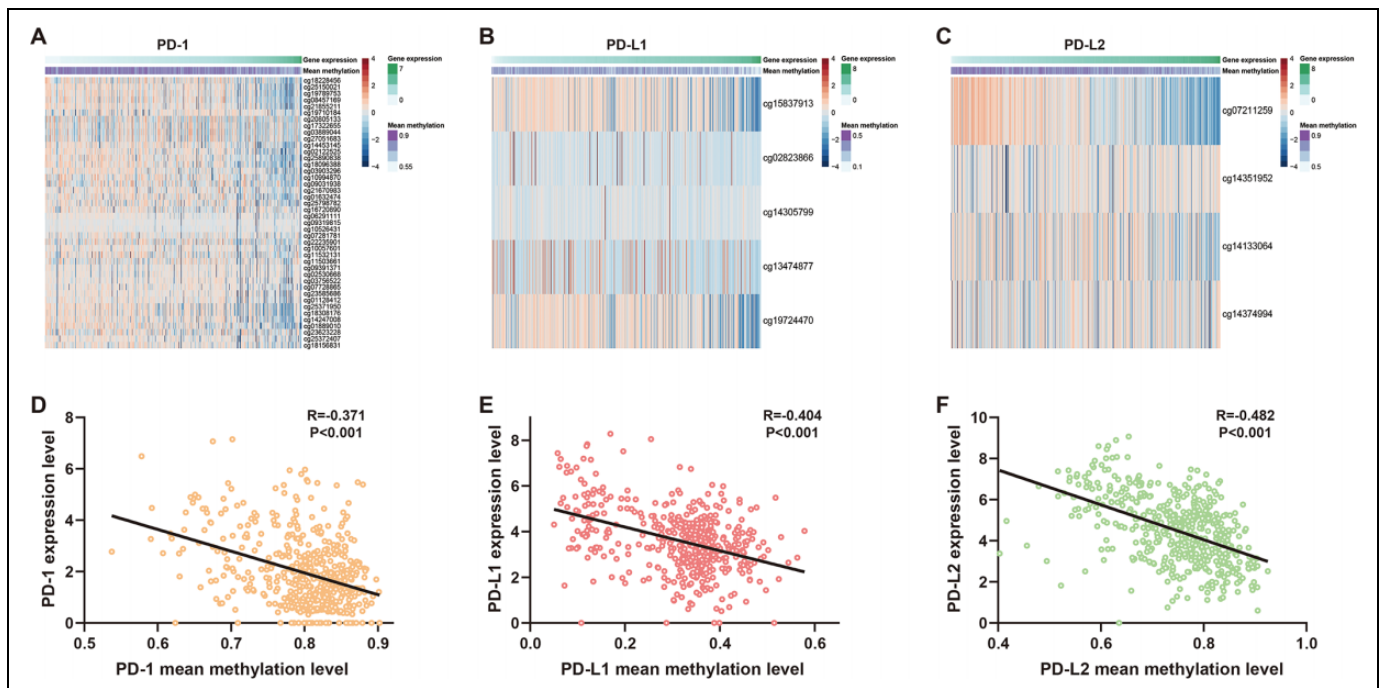
Figure 4. DNA methylation of PD-1/PD-Ls and correlation with expression. The expression clustered methylation of (A) PD-1, (B) PD-L1, and (C) PD-L2; methylation levels of (D) PD-1, (E) PD-L1, and (F) PD-L2 were negatively associated with their mRNA expression.
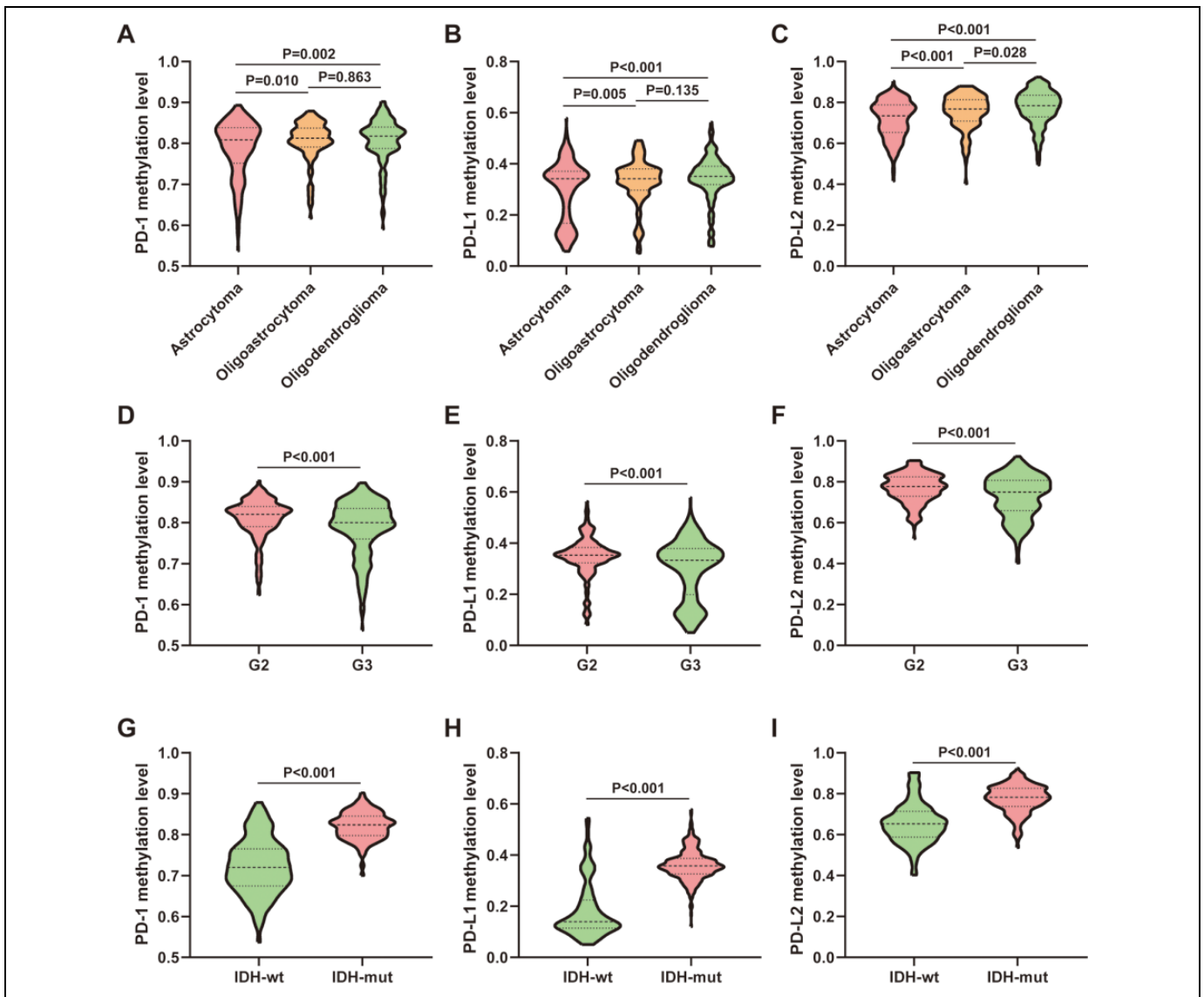
Figure 5. Methylation of PD-1/PD-Ls in different LGG subtypes. (A) PD-1, (B) PD-L1, and (C) PD-L2 were hypo-methylated in astrocytoma subtype; (D) PD-1, (E) PD-L1, and (F) PD-L2 were hypo-methylated in grade 2 LGG; (G) PD-1, (H) PD-L1, and (I) PD-L2 were hypo-methylated in IDH wild type LGG.

DNA methylation is another common regulatory factor at gene level, which causes low gene expression. We performed DNA methylation clustered based on the expression of PD-1/ PD-Ls (Figure 4A-C), which showed that most CpG sites had obviously negative correlations with PD-1/PD-Ls expression. Next, we calculated the mean levels of PD-1/PD-Ls methylations and conducted Pearson correlation analysis to confirm the associations between DNA methylation and mRNA expression. The results exhibited that PD-1 methylation level was negatively associated with PD-1 expression (Figure 4D). Besides, expression levels of PD-L1 and PD-L2 were regulated by methylation as well (Figure 4E, 4F). Taken together, DNA methylation was a crucial factor for dysregulated PD-1/PD-Ls expression, which might be used as an indicator for immune checkpoints expression prediction.

Clinical Significance of PD-1/PD-Ls Methylation

Considering the DNA methylation was a crucial factor in regulating PD-1/PD-Ls expression, we further analyzed the methylation levels according to different clinical subtypes. Contrary to gene expression, astrocytoma exhibited the lowest PD-1/PD-Ls methylation levels when compared with oligoastrocytoma and oligodendroglioma (Figure 5A-C). Besides, we found that in grade 3 LGG showed significantly hypomethylation levels than grade 2 (Figure 5D-F). Moreover, IDH mutant-type showed commonly hyper-methylation of PD-1/ PD-Ls than that of IDH-wt LGG (Figure 5G-I). These results suggested that PD-1/PD-Ls methylation levels were potential markers for different LGG subtypes.

DNA methylation has been identified as potential prognostic markers in tumors.[19] Thus, we also checked the prognostic

values of PD-1/PDLs methylation in LGG. We first evaluated the prognostic impacts of a single CpG site in LGG, the results were exhibited in (Table 1), and most CpG sites were positive prognostic indicators in LGG. When it came to mean methylation levels, as shown in Figure 6, patients with low PD-1 methylation in tumor tissues showed a significantly worse OS than the counterparts (Figure 6A). Besides, hypermethylation of PD-L1 (Figure 6B) and PD-L2 (Figure 6C) also predicted favorable OS in LGG patients. When we combined PD-1/PD-Ls methylations to assess the prognosis of LGG patients, the result showed patients with PD-1/PD-Ls hypomethylations had notably poor OS than other cohorts (Figure 6D). To sum up, PD-1/PD-Ls methylations might be other effective prognostic indicators prognostic indicators in addition to mRNA expression in LGG.

Prognostic Values of PD-1/PD-Ls in LGG Patients With Various Subtypes

Given the promising prognostic values of PD-1/PD-Ls expression and methylation in LGG, we next try to check the prognostic effects of PD-1/PD-Ls in LGG patients with various subtypes. As shown in (Table 2), in astrocytoma, both PD-1/PD-Ls expression and methylation levels were the effective indicators in predicting prognosis. In oligoastrocytoma, only PD-1/PD-Ls methylation levels were prognostic indicators. In oligodendroglioma, in addition to PD-L1 methylation, the other 5 factors had prognostic values. For LGG with IDH wild status, both PD-1/PD-Ls expression and methylation levels had prognostic impacts. While in IDH mutant subtype, only PD-1 methylation was the prognostic biomarker. Besides, In WHO grade 2 LGG, low PD-1 methylation and PD-L2 methylation levels were associated poor prognosis, while in WHO grade 3 LGG, in addition to PD-L1 expression, the other 5 factors had prognostic values. Taken together, these findings suggested the promising roles of PD-1/PD-Ls as potential prognostic indicators in LGG patients with specific subgroups.

Association Between PD-1/PD-Ls Expression as well as Methylation and Immune Infiltration

The survival times of patients in multiple tumors is affected by the quantity and activity status of TIICs.[20-22] At first, we determined the prognostic values of different immune cells infiltration, including B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells, the results identified that infiltration of these six TIICs all possessed excellent biomarker potential for assessing prognosis (Table 3). Next, we explored the relationship between PD-1/PD-Ls expression as well as methylation and the infiltrating immune cells in LGG tissues. The heatmap showed the general correlation between PD-1/PD-Ls expression as well as methylation and infiltration levels of six TIICs (Figure 7A). Specifically, PD-1, PD-L1, and PD-L2 expression were positively associated with six immune cells infiltrations (Figure 7B), while PD-1, PD-L1, and PD-L2 methylation were negatively associated with those TIICs

Table 1. Prognostic Values of Specific CpG Sites in PD-1/PD-Ls in LGG.

Genes	CpG sites	HR	Low 95%CI	Up 95%CI	P value
PD-1	cg18228456	0.69	0.49	0.98	0.038
PD-1	cg25150021	0.54	0.38	0.77	<0.001
PD-1	cg19789753	0.48	0.34	0.69	<0.001
PD-1	cg08457169	0.59	0.41	0.84	0.002
PD-1	cg21855211	0.52	0.37	0.74	<0.001
PD-1	cg19710184	0.61	0.43	0.86	0.005
PD-1	cg20805133	0.36	0.25	0.51	<0.001
PD-1	cg17322655	0.44	0.31	0.64	<0.001
PD-1	cg03889044	0.51	0.35	0.72	<0.001
PD-1	cg27051683	0.46	0.32	0.65	<0.001
PD-1	cg14453145	0.41	0.29	0.58	<0.001
PD-1	cg02122525	0.39	0.27	0.56	<0.001
PD-1	cg25890838	0.66	0.47	0.94	0.021
PD-1	cg18096388	0.42	0.30	0.61	<0.001
PD-1	cg03903296	0.64	0.45	0.91	0.012
PD-1	cg10994870	0.67	0.47	0.96	0.022
PD-1	cg09031938	0.73	0.51	1.04	0.073
PD-1	cg21670983	0.67	0.47	0.95	0.023
PD-1	cg01632474	0.55	0.39	0.79	0.001
PD-1	cg25798782	0.41	0.29	0.59	<0.001
PD-1	cg16720890	0.62	0.43	0.88	0.007
PD-1	cg06291111	0.61	0.43	0.87	0.006
PD-1	cg09319815	0.89	0.63	1.26	0.498
PD-1	cg10526431	0.75	0.53	1.07	0.111
PD-1	cg07281781	0.77	0.55	1.10	0.152
PD-1	cg22235901	0.81	0.57	1.16	0.232
PD-1	cg10057601	0.60	0.42	0.86	0.003
PD-1	cg11532131	0.51	0.36	0.73	<0.001
PD-1	cg11503661	0.65	0.46	0.93	0.015
PD-1	cg09391371	0.52	0.36	0.73	<0.001
PD-1	cg02530668	0.58	0.40	0.82	0.002
PD-1	cg03756522	0.59	0.42	0.84	0.003
PD-1	cg07728865	0.68	0.48	0.96	0.029
PD-1	cg23585686	0.47	0.33	0.66	<0.001
PD-1	cg01128412	0.60	0.42	0.85	0.004
PD-1	cg25371950	0.44	0.31	0.62	<0.001
PD-1	cg18308176	0.45	0.32	0.65	<0.001
PD-1	cg14247008	0.54	0.38	0.77	0.001
PD-1	cg01889010	0.65	0.46	0.92	0.015
PD-1	cg23623228	0.71	0.50	1.02	0.055
PD-1	cg25372407	0.54	0.38	0.77	0.001
PD-1	cg18156831	0.66	0.47	0.95	0.021
PD-L1	cg15837913	0.31	0.22	0.45	<0.001
PD-L1	cg02823866	0.65	0.45	0.92	0.014
PD-L1	cg14305799	1.08	0.76	1.54	0.669
PD-L1	cg13474877	0.40	0.28	0.57	<0.001
PD-L1	cg19724470	0.38	0.27	0.54	<0.001
PD-L2	cg07211259	0.38	0.27	0.55	<0.001
PD-L2	cg14351952	0.73	0.51	1.04	0.076
PD-L2	cg14133064	0.54	0.38	0.77	<0.001
PD-L2	cg14374994	0.70	0.49	1.00	0.045

infiltrations (Figure 7B). Therefore, these results further confirmed that PD-1/PD-Ls were specifically correlated with infiltrating immune cells in LGG, revealing that PD-1/PD-Ls functioned as critical roles in the regulation of tumor and immune cell interaction in the LGG microenvironment.
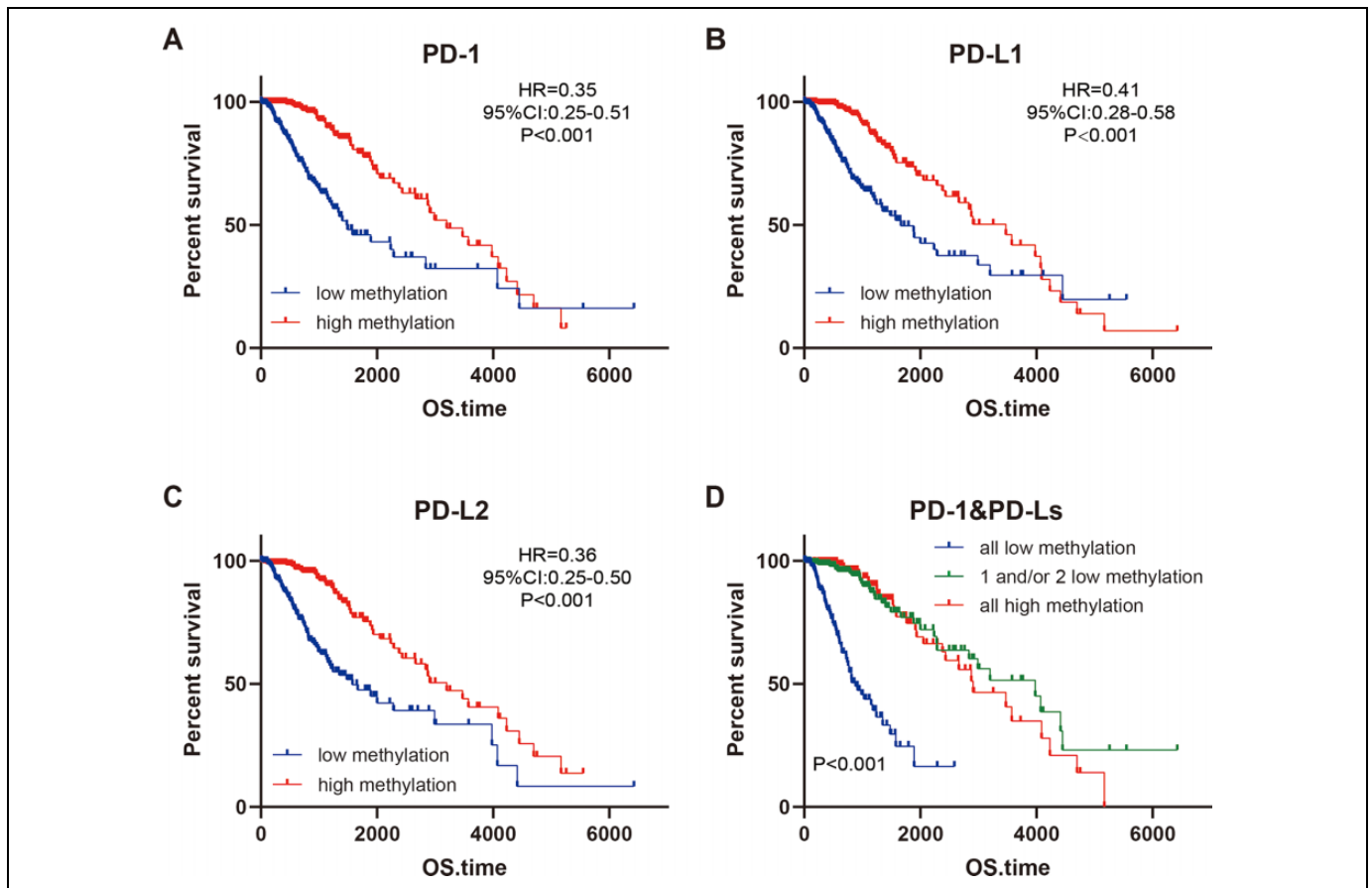
Figure 6. Survival analysis for PD-1/PD-Ls methylation. Kaplan-Meier survival analysis showed that hyper-methylation of (A) PD-1, (B) PD-L1, and (C) PD-L2 were associated with significantly worse prognosis in LGG patients; (D) combined PD-1, PD-L1, and PD-L2 methylation defined various prognosis in LGG patients.

PD-1/PD-Ls Associated Biological Process

To explore the biological features of LGG with different PD-1/PD-Ls expression, we screened the genes that strongly correlated with PD-1/PD-Ls expression, respectively (Figure 8A-C). To obtain an exact result, notably related genes were submitted for GO analysis. The results showed that PD-1/PD-Ls co-expressed genes positively regulated immune response related biological processes, such as cellular defense response, adaptive immune response, cellular defense response, and so on (Figure 8D-F), while those correlated genes were more related to negatively regulate physiological biological processes, including glutamate receptor signaling pathway, mitochondrial gene expression, etc. (Figure 8D-F). Overall, these results revealed that PD-1, as well as PD-L1, PD-L2 were induced as immune inhibitors in the tumor microenvironment where inflammatory and immune response were relatively active, suggesting PD-1/PD-Ls had similar molecular functions in LGG compared with other common solid tumors.

Discussion

LGG is the main subtype of gliomas, which is characterized with lower aggressiveness and well differentiation than GBM

counterpart. A growing number of studies focus on the interplay between glioma and immunity, but most research attaches great attentions to the GBM.[23-25] LGG seems to be ignored from the field of cancer immunotherapy due to its better prognosis than GBM. Several studies have uncovered that GBM tends to express higher PD-L1 than LGG.[26,27] However, whether PD-1/PD-Ls play key roles in LGG and whether patients could benefit from immunotherapy should be explored to further improve curative effect and prognosis.

Immune checkpoints play crucial roles in tumor immune escape. PD-1/PD-Ls axis is the most important immune checkpoints in cancer immunity, but whether PD-1/PD-Ls have notable influence on LGG biology is largely unknown. We have noticed two studies presented by Prof. Jiang's group reported that PD-L1 and PD-L2 were correlated with WHO grade system and poor prognosis in gliomas and GBM, as well as PD-L2 also had promising prognostic value in LGG.[9,10] In this research, we systematically evaluated PD-1/PD-Ls expression and their prognostic values in LGG. PD-1/PD-Ls had distinct co-expression patterns and were upregulated in astrocytoma subtype and higher grade LGG. Moreover, expression levels of PD-1/PD-Ls were companied by IDH mutation, indicating that IDH wild-type LGG exhibited more tumor-derived

Table 2. Prognostic Values of PD-1/PD-Ls Expression and Methylation in LGG Patients With Different Subtypes.

Subtypes	Indicators	HR	Low 95%CI	Up 95%CI	*P* value
Astrocytoma	PD-1 expression	2.05	1.22	3.46	0.007
	PD-1 methylation	0.25	0.15	0.43	<0.001
	PD-L1 expression	2.00	1.19	3.36	0.009
	PD-L1 methylation	0.23	0.13	0.40	<0.001
	PD-L2 expression	2.18	1.29	3.69	0.003
	PD-L2 methylation	0.37	0.22	0.63	<0.001
Oligoastrocytoma	PD-1 expression	1.13	0.52	2.49	0.744
	PD-1 methylation	0.37	0.16	0.84	0.008
	PD-L1 expression	1.25	0.56	2.77	0.579
	PD-L1 methylation	0.33	0.15	0.74	0.006
	PD-L2 expression	2.25	1.03	4.94	0.058
	PD-L2 methylation	0.33	0.15	0.75	0.004
Oligodendroglioma	PD-1 expression	2.36	1.28	4.35	0.007
	PD-1 methylation	0.36	0.19	0.67	0.001
	PD-L1 expression	2.23	1.20	4.13	0.011
	PD-L1 methylation	0.65	0.35	1.20	0.162
	PD-L2 expression	2.05	1.11	3.78	0.023
	PD-L2 methylation	0.51	0.27	0.96	0.029
IDH wild	PD-1 expression	1.79	1.03	3.11	0.037
	PD-1 methylation	0.52	0.30	0.92	0.015
	PD-L1 expression	2.55	1.44	4.52	<0.001
	PD-L1 methylation	0.36	0.20	0.62	<0.001
	PD-L2 expression	3.05	1.72	5.41	<0.001
	PD-L2 methylation	0.43	0.25	0.75	0.002
IDH mutant	PD-1 expression	1.22	0.77	1.94	0.389
	PD-1 methylation	0.56	0.35	0.90	0.010
	PD-L1 expression	1.07	0.67	1.71	0.769
	PD-L1 methylation	0.76	0.47	1.20	0.225
	PD-L2 expression	1.06	0.67	1.68	0.809
	PD-L2 methylation	0.68	0.43	1.09	0.092
G2	PD-1 expression	1.75	0.91	3.36	0.089
	PD-1 methylation	0.40	0.20	0.79	0.003
	PD-L1 expression	1.67	0.87	3.22	0.132
	PD-L1 methylation	0.75	0.39	1.44	0.370
	PD-L2 expression	1.37	0.71	2.64	0.351
	PD-L2 methylation	0.50	0.26	0.99	0.030
G3	PD-1 expression	1.53	1.00	2.33	0.047
	PD-1 methylation	0.33	0.21	0.50	<0.001
	PD-L1 expression	1.47	0.97	2.24	0.075
	PD-L1 methylation	0.25	0.16	0.38	<0.001
	PD-L2 expression	2.19	1.44	3.33	<0.001
	PD-L2 methylation	0.33	0.22	0.51	<0.001

Table 3. Survival Analysis of TIICs Infiltrating Levels in LGG.

| TIICs	HR	Low 95%CI	Up 95%CI	*P* value
B cell	2.11	1.49	3.00	<0.001
CD4+ T cell	1.83	1.29	2.61	0.001
CD8+ T cell	1.66	1.17	2.36	0.006
Neutrophil	2.33	1.64	3.31	<0.001
Macrophage	2.28	1.60	3.24	<0.001
Dendritic cell	1.94	1.37	2.76	<0.001

immune response than IDH mutant LGG. Additionally, PD-1/PD-Ls high expression were both correlated with poor prognosis in LGG patients, which could be served as promising prognostic indicators. All results suggested that PD-1/PD-Ls

expression was associated with more aggressive biological process in LGG.

We further explored the regulatory factors responsible for the dys-regulated PD-1/PD-Ls expression. In this research, DNA methylation levels were found to be negatively correlated with PD-1/PD-Ls expression, and PD-L2 expression also was regulated by CNVs. In Berghoff *et al*'s report, PD-L1 expression was negatively mediated by methylation level of CpG site cg15837913,[28] revealing DNA methylation was a significant regulatory factor for PD-L1 expression in LGG. However, RNA-sequencing could not reflect the cellular resources of expression data, which might not explain the precise mechanism of PD-1/PD-Ls expression regulation. Moreover, we also noticed that PD-1/PD-Ls methylations were more effective
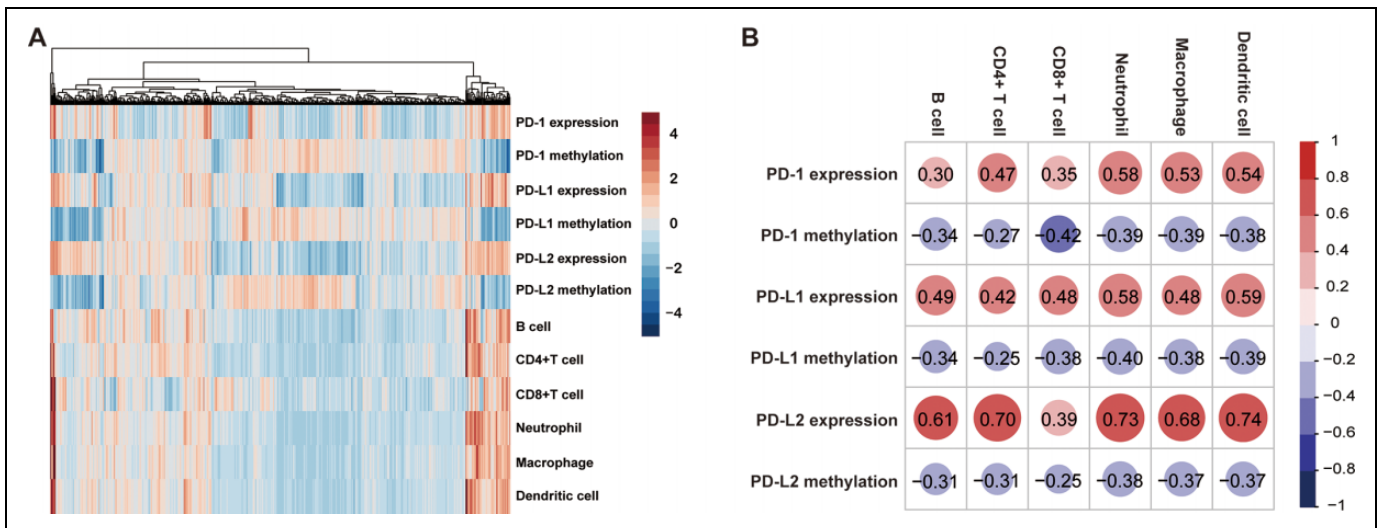
Figure 7. Correlation analysis of PD-1/PD-Ls expression as well as methylation and infiltration levels of immune cells in LGG. (A) Correlation analysis between PD-1/PD-Ls expression as well as methylation and infiltration levels of six TIICs was summarized in the heatmap; (B) PD-1/PD-Ls expression positively correlated with infiltration levels of TIICs, while PD-1/PD-Ls methylation negatively correlated with infiltration levels of TIICs.
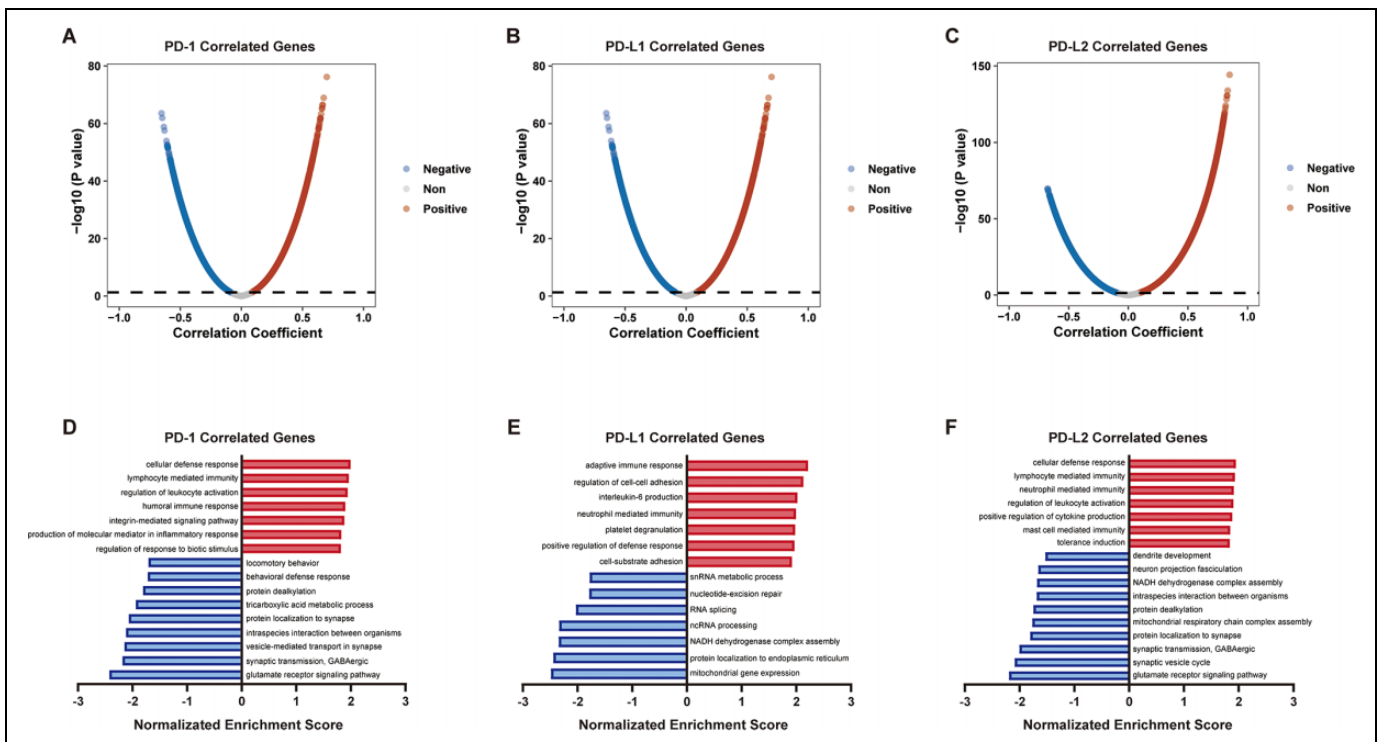
Figure 8. GO analysis of PD-1/PD-Ls co-expressed genes in LGG. The global (A) PD-1, (B) PD-L1, and (C) PD-L2 highly correlated genes identified by Pearson test in LGG. (A) significantly enriched BP annotations of (D) PD-1, (E) PD-L1, and (F) PD-L2 in LGG.

indicators for LGG subtypes and prognosis. According to previous studies, PD-L1 methylation mediated PD-L1 expression and functioned as a promising prognostic marker in melanoma.[29] Besides, PD-L1 promoter methylation was also associated with negative PD-L1 expression, and the development of advanced gastric cancer.[30] Therapeutic subtype analysis revealed that the methylation of PD-1/PD-Ls had more

broad-spectrum prognostic values in LGG patients receiving various therapeutic strategies. Collectively, PD-1/PD-Ls methylation could be innovative biomarkers for assessing LGG patients' prognosis in addition to PD-1/PD-Ls expression.

Immune checkpoints commonly play significant roles in triggering the cancer immunity escape by mediating the interaction between tumor cells and TIICs, so their expressions

were commonly positively correlated with TIICs abundance.[31,32] Deng *et al* established an immune-related prognostic signature and found that LGG patients with high-risk had higher levels of infiltrating B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells,[33] indicating TIICs infiltration was an unfavorable prognostic biomarker in LGG. Hao *et al* identified that infiltration of TIICs was associated with poor prognosis in specific types of LGG.[34] In our research, we found that the high infiltration of TIICs predicted worse outcomes in total LGG patients. Besides, PD-1/PD-Ls expression and methylation were both correlated TIICs infiltration. Although PD-1/PD-Ls methylation exhibited more obviously promising prognostic values, their expression had tighter associations with TIICs infiltration, suggesting the various roles of expression and methylation in assessing prognosis and immune infiltration, respectively.

We subsequently identified co-expressed genes of PD-1/PD-Ls to summarize their potential biological functions in LGG and the GO enrichment analysis suggested PD-1/PD-Ls co-expressed genes enriched in immune response related biological processes, which conformed to the defined roles of PD-1/PD-Ls in multiple solid cancers. Given the similar immunosuppressive roles of PD-1/PD-Ls and adverse prognostic values of TIICs in LGG, we speculated that patients who expressed higher PD-1/PD-Ls might notably suffer from the blocking of anti-tumor effect of specific TIICs, in other words, those patients might benefit more from immunotherapy.

Conclusion

In this study, we reported the expression and methylation status of PD-1/PD-Ls in different subtypes of LGG. High PD-1/PD-Ls expression and hypo-methylation of PD-1/PD-Ls were associated with poor survival of LGG patients. PD-1/PD-Ls expression were demonstrated to be related with immune cells infiltration. Besides, the PD-1/PDLs correlated gene profiles were screened, the GO enrichment analysis of which focus on immune response related biological process. To sum up, LGG patients with PD-1/PD-Ls high expression, whose prognosis was poorer, might benefit from PD1/PD-Ls checkpoint inhibitors treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding author.

Declaration of Conflicting Interests

Funding

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Supplemental Material

Supplemental material for this article is available online.

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