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Correlation of immune phenotype with *IDH* mutation in diffuse glioma

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ABSTRACT

Background: Tumor infiltrating lymphocytes (TILs) and programmed death ligand 1 (PD-L1) are targets of immune checkpoint inhibitors.

Methods: 43 WHO grade II/III gliomas (39 *IDH*-mutant, 4 *IDH*-wt) and 14 *IDH*-mutant glioblastomas were analyzed for TIL (CD3+; PD1+) infiltration and PD-L1 expression. Results were compared with the data of a previously published series of 117 *IDH*-wild type glioblastomas. *PD-L1* gene expression levels were evaluated in 677 diffuse gliomas grade II-IV from The Cancer Genome Atlas (TCGA) database.

Results: TILs and PD-L1 expression were observed in approximately half of WHO grade II/III gliomas. *IDH*-wt status was associated with significantly higher TIL infiltration and PD-L1 expression among all (grades II-IV) cases (n=174, p<0.001) and within the cohort of glioblastomas (n= 131, p<0.001). In the TCGA low grade glioma (LGG) and glioblastoma cohorts, significantly higher *PD-L1* gene expression levels were evident in *IDH*-wt compared to *IDH*-mutant samples (LGG: N=516; p= 1.933e-11, GBM: N=161; p < 0.009). Lower *PD-L1* gene expression was associated with increased promoter methylation (Spearman correlation coefficient -0.36; p<0.01) in the TCGA LGG cohort. *IDH*-mutant gliomas had higher *PD-L1* gene promoter methylation levels than *IDH*-wt gliomas (p<0.01).

Conclusions: The immunological tumor microenvironment of diffuse gliomas differs in association with the *IDH* mutation status. *IDH*-wt gliomas display a more prominent TIL infiltration and higher *PD-L1* expression than *IDH*-mutant cases. Mechanistically this maybe at least in part be due to differential *PD-L1* gene promoter methylation levels. Our findings may be relevant for immune modulatory treatment strategies in glioma patients.

Keywords: low grade glioma; glioblastoma; PD-L1; immune microenvironment; promoter methylation; *IDH* mutation

IMPORTANCE OF THE STUDY

Here, we show that the immunological tumor microenvironment of diffuse gliomas differs in dependence of the molecular tumor status with *IDH*-wild type cases showing more prominent infiltration by lymphocytes and higher expression of the immune check-point molecule programmed death ligand 1 (*PD-L1*) than *IDH*-mutant cases. The difference in PD-L1 expression was evident not only at the protein level as assessed by immunohistochemistry, but also on the gene expression level, as confirmed in a large series of diffuse gliomas from the TCGA database. As potential mechanistic link between *PD-L1* gene expression and *IDH* mutations we identified increased *PD-L1* gene promoter methylation in the *IDH*-mutant subpopulation. Given the possible predictive value of TIL infiltration and PD-L1 expression as biomarkers for response to immune checkpoint inhibitors, our findings may be relevant for immune modulatory treatment strategies in glioma patients.

INTRODUCTION

Diffuse gliomas are the most common primary brain tumors of adults and comprise a heterogeneous group of neoplasms that differ with regard to their natural course and sensitivity to chemo- and radiotherapy. Traditionally, diffuse gliomas have been separated in astrocytic versus oligodendroglial neoplasms and in three tumor grades based on histological features.[1] However, in recent years, distinct molecular classes of diffuse gliomas have been identified and the revised 4th edition of the internationally accepted World Health Organisation (WHO) Classification of CNS Tumors published in 2016 has incorporated important molecular features as integral part of glioma sub-classification.[2] These include the mutational status of the isocitrate dehydrogenase (*IDH*) 1 and 2 genes, the co-deletion status of chromosome arms 1p and 19q and histone 3 mutational status that have been shown to distinguish biologically and clinically distinct diffuse glioma types.[3,4] Although neurosurgical resection and adjuvant radio- and chemotherapy may prolong patient´s survival times, most diffuse gliomas recur and limit life expectancy. So far, novel treatments based on biological insights have not been able to improve patient outcomes and new treatment modalities are needed for patients with diffuse gliomas.

Immunotherapies blocking specific immunomodulatory molecules, so called immune checkpoint inhibitors, have shown clinically relevant efficacy in a number of tumor types and have emerged as novel treatment paradigm in clinical oncology. Monoclonal antibodies targeting the immunosuppressive molecule programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) have proven particularly successful and have been approved in melanoma, lung cancer, renal cell cancer and other tumor types. Among diffuse gliomas, glioblastoma has repeatedly been described to over-express PD-L1 and contain tumor-infiltrating lymphocytes (TIL).[5-7]

Moreover, efficacy of PD-1/PD-L1 inhibitors has been observed in preclinical glioma models and in individual human cases.[8-11] Several clinical trials including large international randomized studies are enrolling glioblastoma patients and will report efficacy data in this glioma type in the near future. However, so far little data on the immune composition of the tumor microenvironment of other diffuse gliomas are available. Intriguingly, a recent paper reported some differences in the frequency of TILs and PD-L1 expression between tumor types and tumor grades as defined by histological features and the WHO 2007 classification.[12,13] Associations of molecular subtypes with tumor immunogenicity and the capacity for immune evasion have been reported for several tumor types. We hypothesized that the recently defined molecular glioma subtypes may also associate with distinct immunological tumor characteristics.[14-16] Therefore, we compiled a large series of cases across all diffuse glioma types as defined by the WHO 2016 classification and characterized them with regard to infiltration by TIL subsets and expression of PD-L1. To validate our findings, we performed an analysis of *PD-L1* gene expression levels in diffuse gliomas derived from The Cancer Genome Atlas (TCGA) database.

METHODS

Vienna WHO II/III glioma cohort

Patients treated for diffuse astrocytoma, anaplastic astrocytoma, oligodendroglioma or anaplastic oligodendroglioma at the Medical University of Vienna were identified from the Neuro-Biobank of the Institute of Neurology, Medical University of Vienna. Diagnosis was performed according to the WHO 2016 classification by a board certified Neuropathologist.[2] The ethics committee of the Medical University of Vienna approved the study (Vote 078/2004).

Vienna glioblastoma cohort

Data on TIL infiltration and PD-L1 expression in 117 *IDH-R132H* negative newly diagnosed glioblastomas were available from a previous study.[6] For the present study, we expanded this cohort by 14 newly diagnosed glioblastoma cases harboring an immunohistochemically detected *IDH-R132H* mutant. The overall glioblastoma cohort analyzed in this study therefore encompassed 131 cases.

Immunohistochemistry and molecular pathology

Tumor tissue was formalin fixed and paraffin embedded (FFPE) according to standard laboratory practice. Specimens presenting with a specific anti-IDH-R132H immunohistochemical signal were scored as *IDH* mutated (*IDH*-mut). Anti-IDH-R132H negative WHO II/III glioma cases underwent genetic sequencing of *IDH1* and *IDH2* genes to detect less common forms of *IDH* mutations.[17] Only WHO II/III glioma specimens with no evidence of *IDH1/2* mutations at immunohistochemistry and gene sequencing were classified as *IDH* wild type (*IDH*-wt).[18] Immunohistochemistry for CD3 and PD1 was performed as previously published on a Ventana Benchmark Ultra

immunostaining system.[6] Tissue of human non-malignant lymph nodes was used as positive control and omission of primary antibody was used as negative control. Immunohistochemistry for PD-L1 was performed using a Dako AutostainerPlusLink immunostaining system and the monoclonal mouse antibody Clone 5H1 (dilution 1:400; kindly provided by Dr. Lieping Chen), which was used in several studies correlating PD-L1 expression and response to immune checkpoint inhibitors as well as in our previous studies of our group investigating PD-L1 expression.[6,19-23] In brief, antigen retrieval was performed using the TRS high pH9 buffer (Dako Glostrup, Denmark) followed by antibody incubation using a 1:100 dilution and detection using the EvVision FLEX and visualization system (Dako Glostrup, Denmark. Human placenta served as positive control and omission of the primary antibody as negative control. The 1p19q status (1p19q co-deleted [codel] vs. 1p19q non-codeleted [non-codel]) was evaluated in all WHO grade II/III gliomas using fluorescence *in situ* hybridization (FISH) as described previously.[24]

Evaluation of Immunohistochemistry

Tumor infiltrating Lymphocytes

Density of CD3+ and PD1+ TILs was evaluated semiquantitatively by overall impression at low microscopic magnification (100x) and scored absent (less than 4 TILs in the entire specimen), sparse (more than 4 but no accumulation), moderate (single areas with accumulation of TILs), dense (TILs throughout the tumor section) or very dense (high frequency of TILs throughout the entire tumor section) according to previously published criteria.[6,25] Further, accumulation of TILs in predefined areas (within the viable tumor tissue, in the perivascular region and, if applicable, in the invasion zone to the surrounding brain parenchyma) was analyzed at higher magnification (200x – 400x).

PD-L1 expression

PD-L1 expression was evaluated according to a previously published algorithm: diffuse/fibrillary PD-L1 expression was semiquantitatively assessed according to the following criteria: (i) no positive tumor areas; (ii) expression in < 25% of non-necrotic tumor area; (iii) expression in > 25% and < 50% of non-necrotic tumor area; (iv) expression in > 50% and < 75% of non-necrotic tumor area; (v) expression in > 75% of non-necrotic tumor area.[6] Further, membranous PD-L1 labeling was recorded as percentage of tumor cells presenting with strong, complete, or membranous PD-L1 staining. For subsequent statistical analysis specimens with fibrillary/diffuse PD-L1 expression in >25% of viable tumor tissue, or membranous PD-L1 expression in at least 1% of tumor cells were defined as “PD-L1 positive”.

The Cancer Genome Atlas Dataset

TCGA RNA-Seq Level 3 (normalized) data for WHO grade II/III diffuse glioma (TCGA LGG dataset) and glioblastoma (WHO grade IV, TCGA GBM dataset) samples were obtained through the NCI GDC portal, and was pre-processed to produce single data frames of expression values with sample annotations (sample type, *IDH* mutation status) based on supplementary tables 2 & 3 from Ceccarelli et al. [3] For the *PD-L1* gene expression analysis, TCGA WHO grade II/III gliomas and glioblastoma samples were selected for which RNA-Seq data and annotation information were available (N=516). For the correlation analysis of *PD-L1* gene expression and promoter CGI CpG methylation levels, a smaller subset of samples was selected due to the lack of glioblastoma samples which have both RNA-Seq and Infinium450k data available (N=51 *IDH-wt* and 4 *IDH-mut*). Infinium450k probes measuring DNA methylation at the *PD-L1* promoter were selected for analysis based on the previously described

methodology.[26] Briefly, *PD-L1* promoter probes exhibiting functional methylation were determined 1) based on their annotated location, and 2) exhibiting a significant negative correlation methylation / *PD-L1* expression (Spearman correlation) in the TCGA-LGG dataset. TCGA data has been downloaded between November 2015 and January 2016, and has been pre-processed to produce single data frames of expression values for each dataset. Compiled molecular information, comprising *IDH*1/2 mutation status, 1p19q codeletion status, and CIMP status were kindly made available by Pierre Bady. [27]

Statistical analysis

All pairwise comparisons with two groups were performed using Student's T-Test or Chi Square test as appropriate. Comparisons with more than two groups were compares using ANOVA, with pairwise comparisons performed using Tukey's HSD post-hoc analysis. P-values < 0.05 were considered as statistically significant. No survival analysis was performed due to the high percentage of censored patients (>90%). Due to the exploratory and hypothesis-generating design of the present study, no adjustment for multiple testing was applied.[28] All statistical analysis was performed with a statistical package for the social sciences (SPSS) 20.0 software (SPSS Inc) or in R. [29]

RESULTS

TIL density and PD-L1 expression in the Vienna WHO grade II/III glioma cohort

Overall, tissue samples of 43 patients with WHO grade II and III diffuse gliomas were available. Tumor typing according to WHO 2016 classification and patient characteristics are given in **Table 1**. Infiltration of TILs was present in 22/43 (51.2%) specimens. The TIL density overall was only sparse to moderate, and none of the investigated specimens presented with dense or very dense infiltration of any TIL subtype (**Figure 1**). Infiltration was observed diffusely throughout the viable tumor tissue. TILs were only infrequently observed in the invasion zone to the surrounding brain parenchyma, and if present only at sparse density. PD-1+ TILs were not detected in any of the investigated WHO grade II/III glioma samples (**Supplemental Table 1**).

No correlation between histology (*IDH-mut*/1p19q codel vs. *IDH-mut*/1p19q non-codel) and CD3+ TIL density was detected (p=0.408; Chi Square test). Correlation of TIL infiltration and *IDH* status was not performed due to limited statistical power (only 4/43 *IDH*-wt specimens).

Diffuse/fibrillary PD-L1 expression in tumor tissue was observed in 22/43 (51.2%) specimens (**Figure 1E**). Membranous PD-L1 expression of individual tumor cells was evident in 3/43 (7.0%) specimens (**Supplemental figure 1**; **Supplemental Table 1)**. Only 1/43 (2.3%) cases, an anaplastic astrocytoma *IDH*-mut, displayed membranous PD-L1 expression in approximately 10% of viable glioma tumor cells. No statistical difference in frequency of PD-L1 expression according to molecular subtype (*IDH-mut*/1p19q codel vs. *IDH-mut*/1p19q non-codel) was observed (p=0.855 Chi Square

test). Correlation of PD-L1 expression and *IDH* mutation was not performed due to limited statistical power (only 4/43 *IDH-wt* specimens).

TIL density and PD-L1 expression in the Vienna glioblastoma cohort (WHO grade IV)

In the 14 cases of *IDH*-mut glioblastoma we found CD3+ TILs in 3/14 (21.4%) cases. PD1+ TILs were absent in all 14 *IDH*-mut glioblastoma cases. Diffuse/fibrillary and membranous PD-L1 expression was evident in 1/14 (7.1%) of these specimens, while none of the cases showed membranous PD-L1 expression. Comparing the results from this series of 14 *IDH*-R132H-positive glioblastomas to the data from our previously reported series of 117 *IDH*-R132H-negative glioblastomas [6] , we found a strong correlation of *IDH* status with characteristics of the inflammatory microenvironment: *IDH*-wt glioblastoma presented significantly more frequently with CD3+ TILs (66.7% vs. 21.4%; p=0.001; Chi Square test), PD1+ TILs (17.1% vs. 0.0%; p=0.002; Chi Square test), fibrillary/diffuse PD-L1 expression (84.6% vs. 7.1%; p<0.001; Chi Square test) and membranous PD-L1 expression (58.1% vs. 0%; p<0.001; Chi Square test) than *IDH*-mut specimens (**Supplemental Table 1**).

TIL density and PD-L1 expression in the overall Vienna glioma cohort (WHO grades II, III and IV)

IDH mutation status also correlated with characteristics of the inflammatory microenvironment in the entire Vienna glioma cohort containing 43 WHO grade II/III gliomas and 131 glioblastomas (total n=174). *IDH*-wt glioma presented significantly more frequently with CD3+ TILs (66.1% vs. 43.4%; p=0.005; Chi Square test; **Figure 2A; Table 2**), with PD1+ TILs (16.5% vs. 0%; p=0.002; Chi Square test; **Figure 2B; Table 2**), with fibrillary/diffuse PD-L1 expression (56.2% vs. 5.7%; p<0.001; Chi

Square test; **Figure 2C; Table 2**) and with membranous PD-L1 expression (56.2% vs. 5.7%; p<0.001; Chi Square test; **Figure 2D; Table 2**) compared to *IDH*-mut glioma.

PD-L1 **gene expression and** ***PD-L1*** **gene promoter methylation in the TCGA dataset**

PD-L1 expression differed significantly between molecular glioma subtypes. The lowest expression was observed in *IDH-mut*/1p19q codel gliomas, followed by *IDH-mut*/1p19q non-codel, *IDH-wt* and glioblastoma (p<0.001; **Figure 3A**). *PD-L1* gene expression was statistically significantly higher in *IDH-wt* WHO grade II/III gliomas compared to *IDH-mut* WHO grade II/III gliomas (p = 1.933e-11). However, there was no statistical difference in *PD-L1* gene expression levels between *IDH*-wt WHO grade II/III glioma and glioblastoma cases.

PD-L1 gene promoter methylation was studied as a possible explanation for these differing results. A negative correlation of *PD-L1* gene expression with *PD-L1* gene promoter methylation was observed (cg15837913, -0.36 (Spearman correlation coefficient), p<0.01, **Figure 3B;** cg19724470, -0.27, p<0.01 **Supplemental Figure 1**). In line, *PD-L1* gene promoter methylation levels were higher in *IDH*-mut as compared to *IDH*-wt samples, supporting the notion that *PD-L1* gene promoter methylation may be causally linked to the lower *PD-L1* expression levels in *IDH*-mut versus *IDH*-wt samples (p<0.01; **Figure 3B**). In the TCGA glioblastoma cohort the number of *IDH*-mt cases with 450K and RNA-seq data was too small for meaningful analysis (4 of 55).

DISCUSSION

In this project, we investigated TIL infiltration and PD-L1 expression in diffuse gliomas and report a significant association of these immunological parameters with their molecular tumor subtype. A previous paper by Garber et al. has already documented some difference in TIL infiltration and PD-L1 expression among histological glioma types. [12] Our study indicates that the main factor influencing the extent of TIL infiltration and presence of PD-L1 expression in diffuse gliomas is the *IDH* mutational status. *IDH*-wt cases had more TIL and PD-L1 expression and may be considered more immunologically activated than *IDH-mut* cases. The association of PD-L1 expression with *IDH* status was evident both at protein-based analysis using immunohistochemistry in our series and at the gene expression level in the TCGA dataset.

The mechanistic basis for the association of the *IDH* mutation with the immunologic make-up of the tumor microenvironment remains to be determined. However, the lower *PD-L1* gene expression was associated with increased promoter methylation in the *IDH-mut* gliomas. Based on our data, we hypothesize that the higher *PD-L1* promoter methylation is associated with the characteristic hypermethylator phenotype of *IDH-mut* gliomas that has been shown to be induced by the oncometabolite 2-hydroxglutarate. [30-32] Further factors influencing the different immune phenotypes of *IDH-wt* and *IDH-mut* gliomas may include epigenetic alterations in other immune-relevant signaling pathways, and reprogramming of the metabolism. [33,34] In addition, the effects of 2-hydroxglutarate on the tumor microenvironment including TIL will need to be considered. [31,35]

Clinical trials are currently evaluating the role of PD-1/PD-L1 inhibitors in newly diagnosed and recurrent primary glioblastoma. Should these trial efforts show a positive therapeutic effect of these drugs in this tumor type, expansion of the subset of non-glioblastoma diffuse gliomas without *IDH* mutation should be considered. Such cases, i.e. diffuse and anaplastic astrocytoma with *IDH-wt* status have poor clinical outcome and limited treatment options and are in need of new therapies. [2] Our data and the TCGA data may suggest that these cases may be amenable to immune checkpoint inhibition.

Preclinical studies suggest that *IDH* mutations may serve as a specific target for vaccination approaches in glioma and a clinical trial evaluating this approach is currently recruiting patients with *IDH-mut* diffuse gliomas (NCT02454634, NOA-16).[35] Our findings show a low base-line infiltration by TILs in *IDH-mut* gliomas and combination of an IDH1R132H-specific vaccine with other immune-stimulatory agents boosting immune cell migration into the tumor microenvironment may be useful to facilitate an efficient anti-tumor response.[10] Given the low *PD-L1* expression found in *IDH-mut* cases, likely silenced through methylation of the *PD-L1* gene promoter, PD1/PD-L1 immune checkpoint inhibitors are not indicated, and other strategies need to be employed e.g. agonists of co-stimulatory checkpoint molecules. Further, high mutational load is considered as an emerging biomarker for response to immune checkpoint inhibitors and pediatric hypermutant glioblastoma resulting from a germline mismatch repair deficiency presented with response to immune checkpoint inhibitors. [36,37] However, LGG present with a rather low mutational load (0.77 mutations/ Mb) in comparison to glioblastoma (2.2 mutations/ Mb) and especially other tumor entities with high response rates to immune checkpoint inhibitors like melanoma (12.9 mutations/ Mb) and lung cancer (9.9 mutations/ Mb).

[38,39] A subset of *IDH-mut*/1p19q non-codel LGG and glioblastoma have been reported to present with a hypermutator phenotype after temozolomide based therapy. [40,41] The hypermutator phenotype has been found in *MGMT* methylated glioma at recurrence, usually associated with the acquisition of a mutation in *MSH6*, or another gene of the mismatch repair (MMR) pathway that provides resistance to temozolomide treatment.[41,42] The plethora of acquired mutations may yield neo-epitopes and render the tumors sensitive to immunotherapies. However, the frequency of the hypermutator phenotype is unknown, as only small series have been published so far.[40-42] It has been proposed that *MSH6* mutations and mutations in other MMR genes may serve as a biomarkers to detect the hypermethylation phenotype in temozolomide treated patients with *MGMT* methylated glioma. The latter is common in *IDH-mut* LGG or GBM (>90%), and close to 50% in *IDH-wt* glioma (WHO grade II to IV), and most importantly these patients are usually treated with an alkylating agent.[27]

Therefore, combinational strategies taking into account the specific characteristics of the immune microenvironment like TIL density and PD-L1 expression, as well as mutational characteristics and previously applied therapies might in the future define the immune modulatory therapy approach.

Several different antibodies and protocol have been used for the detection on PD-L1 by immunohistochemistry. Importantly, the resulting signal can vary according to the main targeted domain as antibodies targeting the extracellular domain produce a rather cytoplasmatic signal and antibodies targeting the cytoplasmic domain a rather membranous signal. [43,44] In the current study, we used the antibody clone 5H1, which has been used by previous studies to study PD-L1 expression in glioma and other tumor types by our group and others to correlate PD-L1 expression likelihood of

response to immune checkpoint inhibitors. [6,23,20,21] Here, we observed a diffuse/fibrillary as well as a membranous staining pattern, both of which have been described previously.[45] We believe that these staining patterns resemble the heterogeneous microarchitecture of glioblastoma, with the membranous labeling being only visible on epitheloid cancer cells and the diffuse/fibrillary staining reflecting membrane-staining on the delicate tumor cell process forming the pathognomic “neurofibrillary matrix” of glial tumors. Most likely only studies on ultrastructure level e.g. using electron microscopy can answer to with component the PD-L1 is bound in case of the fibrillary staining patterns. Importantly, so far no standard protocol has been published for the detection of PD-L1 expression in glioma and the optimal method for routine clinical use as well as the cut-off values need to be defined. [13] A further interesting parameter could be PD1 expression on tumor infiltrating lymphocytes besides the proposed predictive value of PD-L1 expression on tumor cells, macrophages or TILs.[23,46,47] Indeed, a retrospective study suggests an increased likelihood of response to PD1 axis targeting immune checkpoint inhibitors in patients with dense infiltration of PD1+ TILs.[48] The currently on-going clinical trials on immune checkpoint inhibitors in glioma patients will provide deeper insights on which characteristics of the inflammatory microenvironment might be of predictive value.

In conclusion, our data show that the immunological tumor microenvironment of diffuse gliomas differs in association with their *IDH* mutation or CIMP status, respectively, although the mechanistic basis of the observed relationship remains to be elucidated. Our findings suggest that the characteristics of the inflammatory microenvironment may differ according to the genetic glioma subtype and may be

relevant for the further conduction and planning of clinical trials investigating the therapeutic value of immune modulatory treatment strategies in glioma patients.

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Figure legend

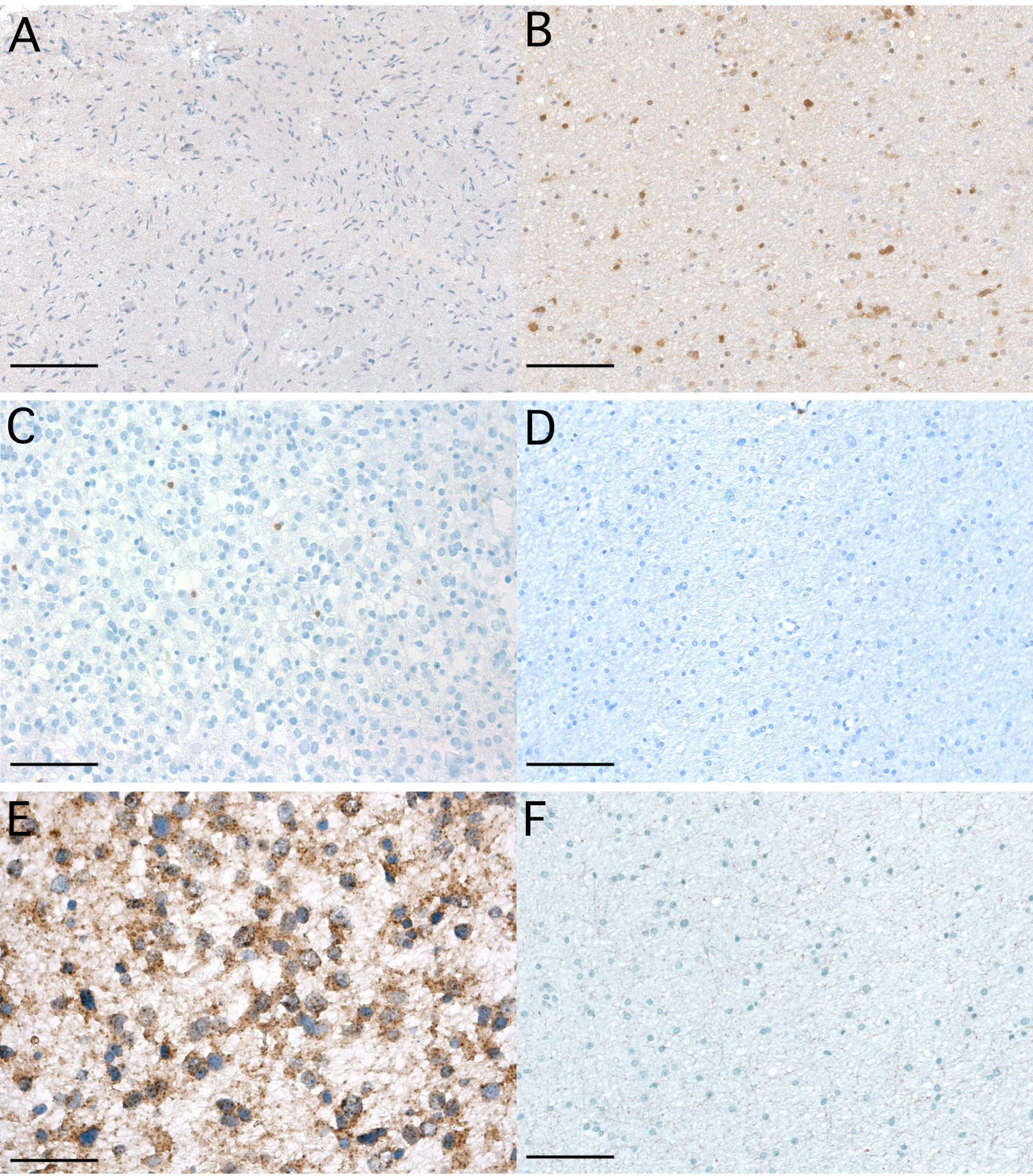
Figure 1: Difference in TIL density and PD-L1 expression in *IDH-wt* and *IDH-mut* glioma **A/C/E** *IDH*-wt glioma WHO grade III without immunohistochemical staining for *IDH*-R132H mutant and lack of *IDH* gene 1 or 2 mutations based on gene sequencing (magnification x 100; A; scale bar 250µm), scattered infiltration with CD3+ Tumor infiltrating lymphocytes (magnification x 200; C; scale bar 100µm), fibrillary expression of PD-L1 (magnification x 400; E); **B/D/F** *IDH*-mut WHO grade II glioma presenting with anti-*IDH*-R132H immunostaining (magnification x 100; B), absence of CD3+ Tumor infiltrating lymphocytes (magnification x 200; D; scale bar 100µm), lack of PD-L1 expression (magnification x 200; F; scale bar 100µm)

Figure 2: Bar graphs illustrating the correlation of TIL infiltration and PD-L1 expression with *IDH* status in the overall Vienna cohort of WHO grade II-V diffuse glioma (n=174). A CD3+ TILs in *IDH*-mut and *IDH*-wt glioma. **B** Fibrillary/diffuse PD-L1 expression in *IDH*-mut and *IDH*-wt glioma. C Membranous PD-L1 expression in *IDH*-mut and *IDH*-wt glioma.

Figure 3: *PD-L1* gene expression and gene promoter methylation in diffuse glioma. A *PD-L1* gene expression is significantly lower in *IDH*-mut WHO II/III glioma than in *IDH*-wt WHO II/III glioma of the TCGA WHO LGG cohort, while *IDH*-wt WHO II/III glioma are not different from the TCGA GBM cohort (for which RNA seq data is available). **B** *PD-L1* gene expression levels show a significant negative correlation with *PD-L1* gene promoter methylation, illustrated for a representative functional CpG (probe cg15837913, −0.36 [Spearman], $p<0.01$) in WHO II/III glioma of the TCGA LGG dataset. Black lines in the plot show the fit of linear regression, in grey local

regression using lowess smoothing. Methylation of the *PD-L1* promoter is significantly higher in *IDH*-mut WHO II/III glioma than *IDH*-wt WHO II/III glioma (beta-values, p<0.01, two-sided Student's T-test).

Supplemental Figure 1 (linked to Figure 3B)**:** *PD- L1* gene expression levels show a significant negative correlation with *PD-L1* gene promoter methylation, illustrated for a functional CpG interrogated by the probe cg19724470 (−0.27 [Spearman], p<0.01) in WHO II/III glioma. Black lines in the plot show the fit of linear regression, in grey local regression using lowess smoothing. Methylation of the *PD-L1* promoter is significantly higher in *IDH*-mut WHO II/III glioma than *IDH*-wt WHO II/III glioma (b-values, p<0.01, two-sided Student's T-test).


A
B
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D
E
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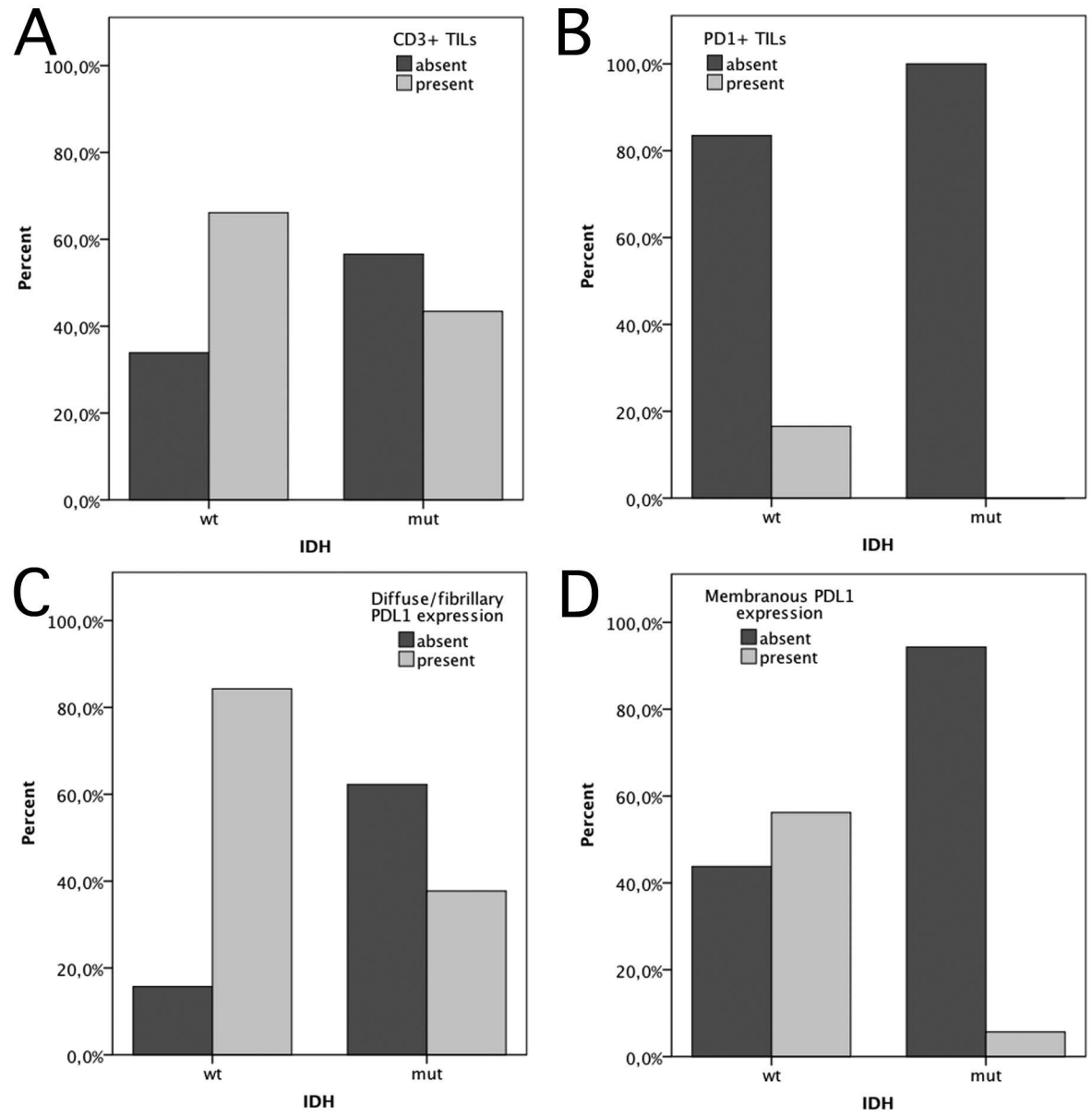
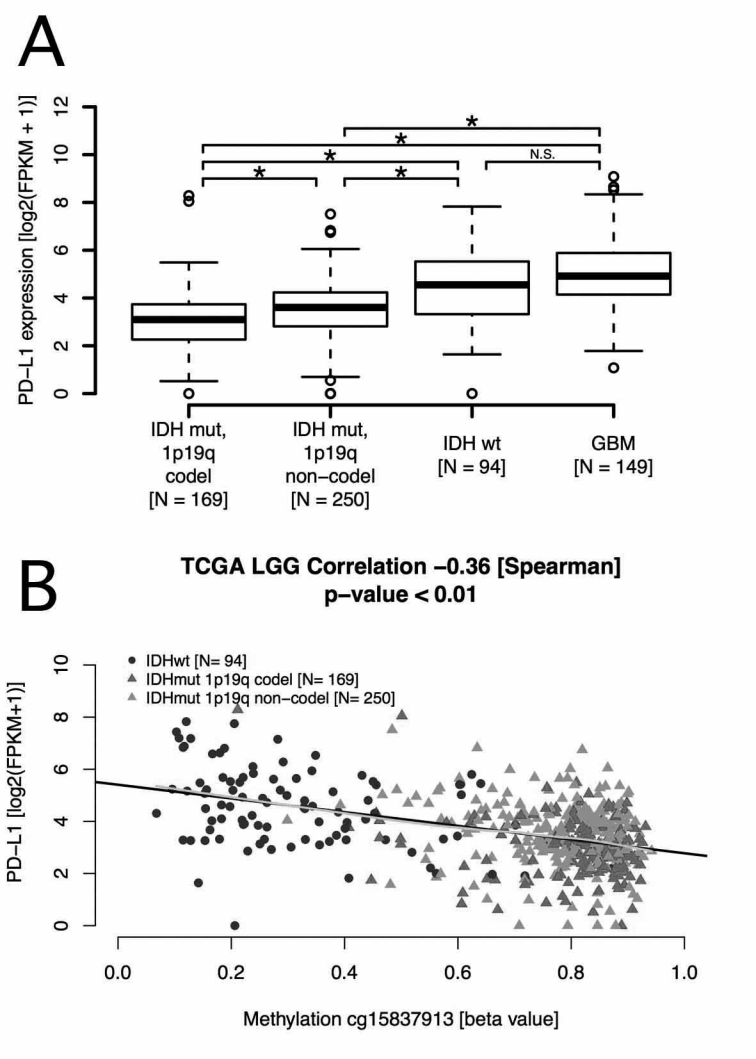

A
CD3+ TILs
absent
present
100,0%
80,0%
60,0%
40,0%
20,0%
0,0%
Percent
wt
mut
IDH
B
PD1+ TILs
absent
present
100,0%
80,0%
60,0%
40,0%
20,0%
0,0%
Percent
wt
mut
IDH
C
Diffuse/fibrillary
PDL1 expression
absent
present
100,0%
80,0%
60,0%
40,0%
20,0%
0,0%
Percent
wt
mut
IDH
D
Membranous PDL1
expression
absent
present
100,0%
80,0%
60,0%
40,0%
20,0%
0,0%
Percent
wt
mut
IDH


A
PD–L1 expression [log2(FPKM + 1)]
0
2
4
6
8
10
12
*
*
*
*
*
N.S.
IDH mut, 1p19q codel [N = 169]
IDH mut, 1p19q non–codel [N = 250]
IDH wt [N = 94]
GBM [N = 149]
B
TCGA LGG Correlation –0.36 [Spearman]
p–value < 0.01
IDHwt [N= 94]
IDHmut 1p19q codel [N= 169]
IDHmut 1p19q non–codel [N= 250]
PD–L1 [log2(FPKM+1)]
0
2
4
6
8
10
0.0
0.2
0.4
0.6
0.8
1.0
Methylation cg15837913 [beta value]