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Original Article

Expression and Prognostic Value of CD80 and CD86 in the Tumor Microenvironment of Newly Diagnosed Glioblastoma

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ABSTRACT: ***Background:*** Strategies to modulate the tumor microenvironment (TME) have opened new therapeutic avenues with dramatic yet heterogeneous intertumoral efficacy in multiple cancers, including glioblastomas (GBMs). Therefore, investigating molecular actors of TME may help understand the interactions between tumor cells and TME. Immune checkpoint proteins such as a Cluster of Differentiation 80 (CD80) and CD86 are expressed on the surface of tumor cells and infiltrative tumor lymphocytes. However, their expression and prognostic value in GBM microenvironment are still unclear. ***Methods:*** In this study, we investigated, in a retrospective local discovery cohort and a validation TCGA dataset, expression of CD80 and CD86 at mRNA level and their prognostic significance in response to standard of care. Furthermore, CD80 and CD86 at the protein level were investigated in the discovery cohort. ***Results:*** Both CD80 and CD86 are expressed heterogeneously in the TME at mRNA and protein levels. In a univariate analysis, the mRNA expression of CD80 and CD86 was not significantly correlated with OS in both local OncoNeuroTek dataset and TCGA datasets. CD80 and CD86 mRNA high expression was significantly associated with shorter progression free survival (PFS) ($p < 0.05$). These findings were validated using the TCGA cohort; higher CD80 and CD86 expressions were correlated with shorter PFS ($p < 0.05$). In multivariate analysis, CD86 mRNA expression was an independent prognostic factor for PFS in the TCGA dataset only ($p < 0.05$). ***Conclusion:*** CD86 could be used as a potential biomarker for the prognosis of GBM patients treated with immunotherapy; however, additional studies are needed to validate these findings.

RÉSUMÉ : L'expression et la valeur pronostique des molécules CD80 et CD86 dans le micro-environnement tumoral du glioblastome nouvellement diagnostiqué. ***Contexte*** **:** Les stratégies visant à moduler le micro-environnement tumoral (MET) ont permis d'offrir de nouvelles voies thérapeutiques qui ont démontré une efficacité intertumorale étonnante mais hétérogène dans de nombreux cancers, dont les glioblastomes. Aussi la recherche sur les acteurs moléculaires du MET pourrait-elle aider les chercheurs à mieux comprendre l'interaction entre les cellules tumorales et le MET. Les protéines du point de contrôle immunitaire comme les Cluster of Differentiation 80 (CD80) et 86 (CD86) s'expriment à la surface des cellules tumorales et des lymphocytes infiltrant les tumeurs. Toutefois, nous ne connaissons pas très bien leur expression et leur valeur pronostique dans le micro-environnement du glioblastome (GB). ***Méthode*** **:** L'étude, fondée sur une cohorte d'exploration locale, rétrospective et sur une base de données TCGA de validation, visait à examiner l'expression des molécules CD80 et CD86 au niveau de l'ARNm et à leur valeur pronostique chez les patients traités par le standard de soins. Les CD80 et CD86 ont aussi fait l'objet de recherche pour ce qui est des protéines dans la cohorte d'exploration. ***Résultats*** **:** Les molécules CD80 et CD86 s'expriment toutes les deux de manière hétérogène dans le MET pour ce qui est de l'ARNm et des protéines. D'après une analyse univariée, l'expression de l'ARNm des molécules CD80 et CD86 n'était pas associée de manière significative avec la survie globale tant dans la base de données locale OncoNeuroTek (ONT) que dans la base de données TCGA. Toutefois, une expression marquée de l'ARNm des CD80 et CD86 était significativement associée à une diminution de la survie sans progression (SSP) ($p < 0{,}05$). D'ailleurs, les résultats ont été validés dans la cohorte TCGA; en effet, une expression marquée des molécules CD80 et CD86 était en corrélation avec une SSP plus courte ($p < 0{,}05$). D'un autre côté, l'expression de l'ARNm de la CD86 s'est révélée, dans une analyse multivariée, un facteur pronostique indépendant de la SSP dans la base de données TCGA seulement ($p < 0{,}05$). ***Conclusion*** **:** La molécule CD86 pourrait servir de biomarqueur pronostique du GB chez les patients traités par immunothérapie; il faudrait toutefois réaliser d'autres études afin de valider les résultats obtenus.

Keywords: Glioblastoma; Microenvironment; Immune checkpoint proteins; Prognosis

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Introduction

Glioblastoma (GBM) is the most common and aggressive glioma in adults. The latest World Health Organization guideline classifies GBM as grade IV glioma.[1] Over the last years, massive efforts have led to a better understanding of the pathology and the genetic of GBM.[2] To date, the most effective and approved standard therapeutic regimen is maximum surgical resection of the tumor followed by concurrent chemoradiation and adjuvant chemotherapy with temozolomide (TMZ).[1] Despite this very intensive therapeutic regimen, newly diagnosed GBM patients have a dismal outcome with a median overall survival (OS) below 18 months.[3] The main known prognostic factors are (i) age, (ii) Karnofsky performance status (KPS), (iii) *MGMT* promoter methylation status, and (iv) IDH mutational status.[4]

Immunotherapies have dramatically improved melanoma prognosis[5] and other nonneurological solid tumors.[5] In the setting of primary brain cancer, results from clinical trials are still disappointing.[6] Nonetheless, specific GBM patients responded, supporting the identification of biomarkers to stratify patients in the prescription of immunotherapies. Immune checkpoint proteins such as Cluster of Differentiation 80 (CD80; known as B7-1) and CD86 (known as B7-2) are expressed on the surface of tumor[7] and immune cells[8] but not glial cells.[9] CD80 protein expression was observed in infiltrative tumor lymphocytes in melanoma.[10]

Cytotoxic T-lymphocyte-associated antigen–4 (CTLA-4) and Cluster of Differentiation 28 (CD28) are located on T-lymphocytes. Both CD28 and CTLA-4 proteins bind to their ligands on the antigen-presenting cells and major histocompatibility complex.[11] The interaction between immune checkpoint proteins and their coreceptor at the surface of T-lymphocytes delivers the signal to activate or inhibit T cells function, that is, CTLA-4 has a higher affinity to CD80 and CD86, and when bound to its ligands, T cells remain exhausted.[12]

In preclinical studies, antibodies targeting CTLA-4 were used in to block CTLA-4 from binding to its ligands.[13] Ipilimumab – anti-CTLA4 – has also shown responses in patients with brain metastases, highlighting efficacy within the central nervous system.[14] Expression of the most studied immune checkpoint proteins, programmed death-ligand (PD-L1), was inversely correlated with OS in GBM patients.[15] However, the expression of CD80 and CD86 in GBM tissues and their prognostic significance in the tumor microenvironment (TME) of newly diagnosed GBM patients has not been reported yet. This study investigated the mRNA and protein expression of CD80 and CD86 in the TME of newly diagnosed GBM patients, aged below 70 years old and with KPS above 70% treated with the standard of care. In addition, this study highlighted a possible correlation between CD80 and CD86 expression and the immune cell populations in the TME of newly diagnosed GBM patients.

Materials and Methods

Patient Samples

OncoNeuroTek (ONT) is a local brain tumor tissue bank collecting samples from patients operated at the University Hospital La Pitié-Salpêtrière. All samples were collected with informed consent from patients. The inclusion criteria of the discovery local cohort (47 patients) were as follows: (i) newly diagnosed and histologically verified GBM, (ii) age at diagnosis is below 70 years, (iii) KPS above 70%, (v) known *MGMT* promoter methylation status, (vi) known IDH status, (vii) treated with the standard first-line therapeutic regimen including maximal safe surgery, chemoradiation and adjuvant TMZ, and (viii) a documented clinical follow-up. The validation cohort (121 patients, TCGA cohort) clinical information and RNA-sequencing data (read counts) were downloaded from the National Cancer Institute's Genomic Data commons Data portal and from the NCBI GEO GSE62944, respectively. Similar inclusion criteria were used for both cohorts.

Immunohistochemistry Staining

Paraffin-embedded tissue blocks (5–7 µm) from biopsies of newly diagnosed GBM patients were received from the ONT biobank. The slides were obtained from diagnostic blocks and were selected to get a homogeneous group of patients for prognostic studies. Indeed, we have selected the patients aged below 70 years old, with a KPS > 70% and treated with the standard of care to be in line with inclusion criteria of the clinical trial that has established the standard of care.[4] Tissue sections (two sections per patients) were deparaffinized using xylene and rehydrated. For antigen retrieval, each slide was embedded in citrate buffer at pH 4.0 and heated for 15 min in the microwave at 800 W. 10% goat serum with 5% fetal bovine serum in 0.2% triton phosphate buffer saline was used as a blocking buffer. 3% hydrogen peroxide was used to block tissue peroxidation. Antihuman CD80 antibody (A16039; Abclonal) and antihuman CD86 antibody (A2353; Abclonal) were used at 1:500 dilution in blocking solution and incubated on the tissue slides overnight at room temperature. Avidin–Biotin Complex kit was used as a signal enhancer before the incubation in 3,3′-Diaminobenzidine (DAB). Slides were embedded in hematoxylin dye and rinsed with tap water for nuclear staining; gradual alcohol and xylene baths were used for dehydration and mounted with a hydrophobic mounting medium (Sigma, 24845633). All stained tissues were scanned via ZEISS Axio Scan 40× for bright field imaging.

Quantification of IHC Staining

Following all slides' imaging, three regions of interest with known dimensions (528 * 528 µm) were randomly selected for each tissue section and quantified using an in-house quantification Fiji code. Shortly, each image was imported to the Fiji program.[16] Using the color deconvolution tool, the area positive for DAB staining was isolated and quantified using a semiautomated in-house generated code. The percentage of DAB positive areas was calculated, and the mean value from the three images was calculated and used in the survival analysis.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA samples were obtained from ONT bank and used to synthesize cDNA. Reverse transcription of RNA samples was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, K1442) according to the manufacturer's recommendation with 100–250 ng of RNA. Quantitative reverse transcriptase polymerase chain reaction was used to quantify the expression levels of CD80 and CD86 in patients. PPIA gene was used as a house reference gene for normalization as previously described.[17] Primers were designed using Universal Probe Library (UPL) for Human. Primer's sequences were as follows: PPIA (left: atgctggacccaacacaaat; right: tctttcactttgccaaacacc;

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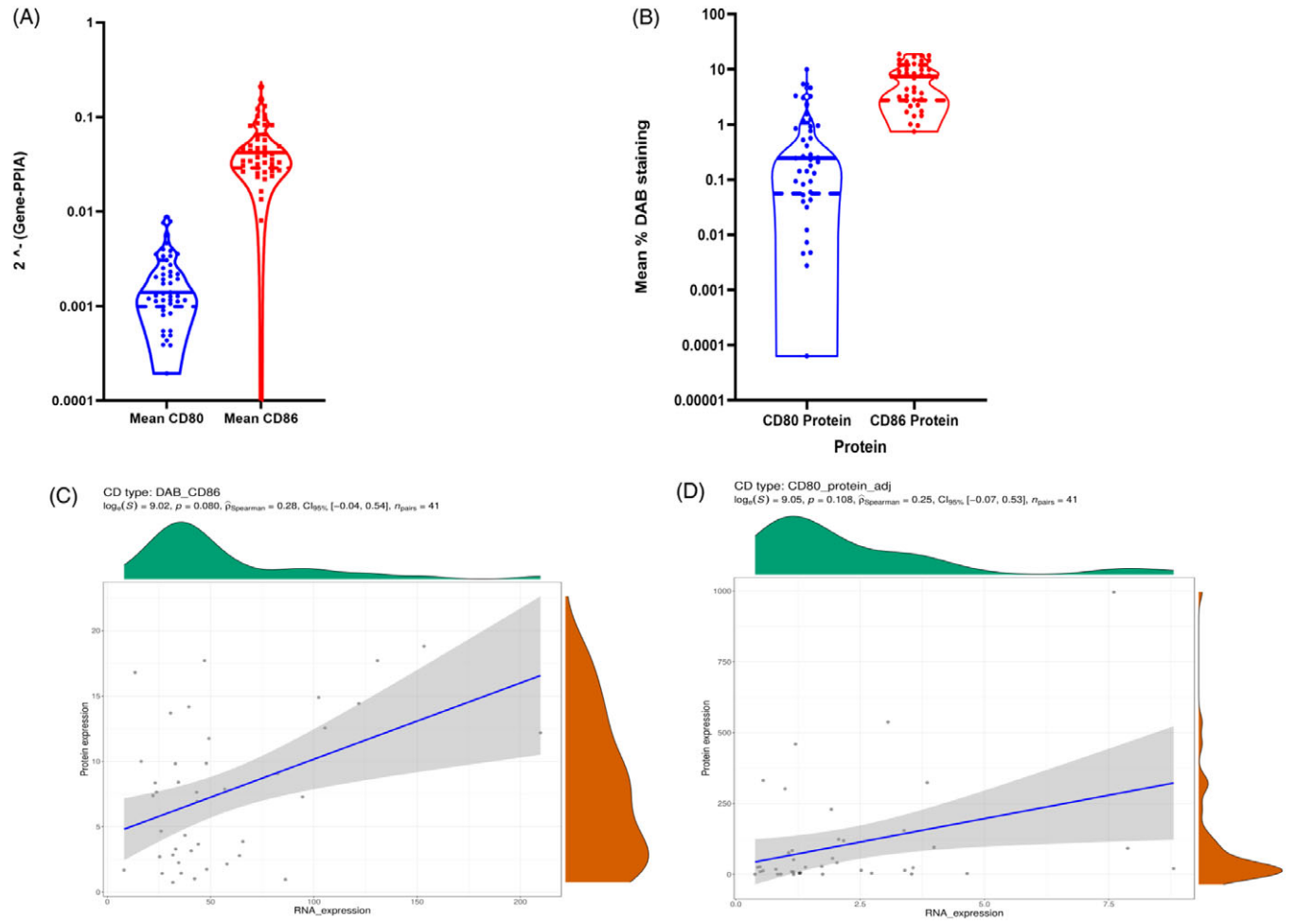

Figure 1: (A) Violin plot to visualize the data distribution of CD80 and CD86 mRNA expression in ONT database; (B) shows CD80 and CD86 protein expression in ONT database. (C–D) Spearman correlations between CD86 protein values and CD86 RNA values. (C) represents CD86 protein quantification based on the mean percentage of positive DAB signals correlation with mRNA values. (D) shows CD80 protein values quantified based on the mean percentage of positive DAB signals correlation with mRNA values.

UPL probe 48) CD80 (left: gaagcaaggggctgaaaag; right: ggaa gttcccagaagaggtca; UPL probe 10) and CD86 (left: cagaagcagc-caaaatggat; right: gaatcttcagaggagcagcac; UPL probe 15). cDNA samples were analyzed using the Light Cycler Probe Master mix 2× (Roche, 04887301001) and the UPL detection system (Roche, 04483433001) in a Light Cycler 96 (Roche). For each qPCR, two independent experiments were completed with duplicate samples in each experiment. The mean of $2^{-(CT^{\text{gene of interest}}-CT^{PPIA})}$ from the two different experiments was used in all analyses.

Statistical Analysis

A violin plot was used to visualize our data's full distribution (GraphPad Prism).[14] Spearman correlation between the expression values (RNA or protein) and age was evaluated to discard age bias. Survival analysis was performed by an open-source validated approach[18,19] by finding a supervised cutoff value for the CD80 or CD86 expression independently using the "survminer::surv_cutpoint" function, which determines the cut point based on the highest/lowest value of the log-rank statistics (low or high expression values), and then using these categories for Kaplan–Meier analysis or Cox proportional hazard regression modeling testing at each variable independently or to adjust for multiple variables including CD80/CD86 expressions and *MGMT* promoter methylation status *p*-values lower than 0.05 were considered significant.[20,21] Furthermore, we have used TCGA database to evaluate and profile tumor infiltrating immune populations and whether it differ among the highly expressed C86 tumor cells. TCGA immune data (i.e., CIBERSORT calculated immune populations) was retrieved from https://cavei.github.io/example-datasets/panCancerAnnotation.RData. Comparisons were performed by two-side Wilcoxon-test and *p*-values were corrected for multiple comparisons using FDR method.

Results

Patients and Tumors Characteristics

Forty-seven patients with a confirmed GBM diagnosis fulfilled the inclusion criteria: 14 men and 33 women (percentage 29.8%–70.2%). The patients' median age at diagnosis was 55.9 years (range: 24.3–69.5 years). KPS was 70% and above in all patients. The median OS is 559 days (range 31–2539), and the median PFS is 266 days

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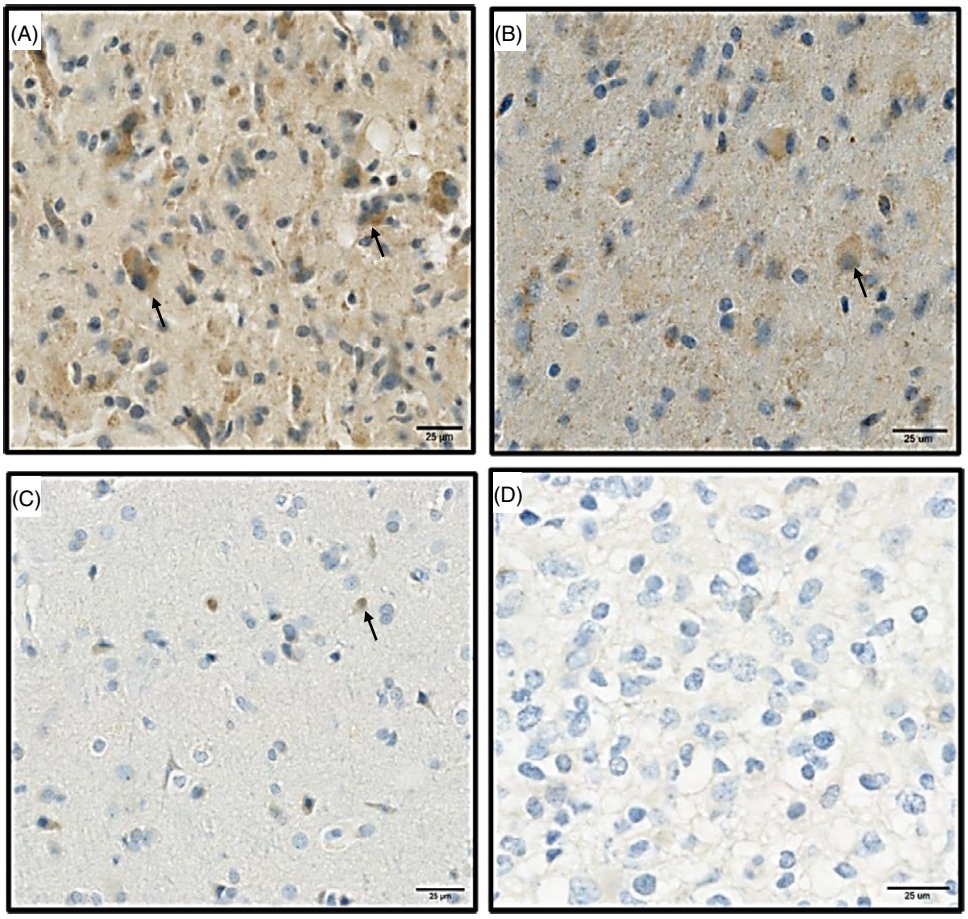

Figure 2: Represents the protein expression of CD86 and CD80 proteins in paraffin sectioned GBM samples. (A) High expression of CD86 protein. (B) Low expression of CD86. (C) High expression of CD80. (D) Low expression of CD80. Black arrows (brown signals) highlight a positive staining for CD80 and CD86 proteins and represent the signals that were used for quantifications, blue staining correspond to hematoxylin dye which was used as counterstaining.

(range 26–1355). The IDH status was evaluated as mutant for two patients (4.3%) while wildtype for 45 (95.7%). Furthermore, *the MGMT* promoter was methylated in 16 patients (34%) and unmethylated in 31 (66%). All patients were treated with the standard of care first-line treatment including maximal safe surgery, radio chemotherapy, and adjuvant chemotherapy with TMZ.

CD80 and CD86 Expression at mRNA and Protein Level

At the mRNA level, CD86 expression was quantitatively higher than CD80 expression in the TME (Figure 1A). In agreement with mRNA expression, immunohistochemistry (IHC) analysis showed that the expression of CD86 is higher than CD80 in our discovery cohort (Figure 1B). Based on the IHC staining, CD80 and CD86 are observed in the cell membrane and/or the cytoplasm (Figure 2). Following protein quantification, we observed a positive correlation between RNA and protein expression for CD86 (Spearman coefficient of correlation Rho = 0.28; $p = 0.08$; Figure 1C). However, we observed a weaker correlation between mRNA and protein expression for CD80 ($p = 0.108$; Rho = 0.25; Figure 1D).

Prognostic Value of CD80 and CD86 Expression

Our patient's cohort was used as a discovery cohort, while the TCGA dataset was used as a validation cohort. In a univariate analysis, mRNA expression of CD80 and CD86 was not significantly correlated with OS in both the ONT cohort and TCGA dataset (Table 1). On the other hand, CD80 and CD86 mRNA high expression was significantly associated with shorter PFS ($p = 0.04$ and $p = 0.005$, respectively; Figure 3A,B). Moreover, these findings were validated using the TCGA cohort; higher CD80 and CD86 expressions were correlated with shorter PFS (p-value; 0.0428, 0.00283; Figure 3C,D). Interestingly, higher CD86 protein expression was associated with shorter PFS in the ONT cohort ($p < 0.005$; Table 2). CD80 and CD86 protein expression were not available in the TCGA dataset for validation purposes. However, we have used TCGA database to profile tumor-infiltrating immune cells in the selected cohort.

As expected, *MGMT* promoter methylation was associated with longer PFS and longer OS in the ONT cohort ($p < 0.05$ and $p < 0.05$, respectively) and TCGA dataset ($p < 0.05$ and $p < 0.05$, respectively) (Tables 1 and 2). Furthermore, *IDH* mutations were also associated with better OS and PFS in the TCGA database ($p < 0.05$ and $p < 0.05$, respectively); however, in the ONT cohort, the limited number of IDH-mutant GBM did not allow a robust analysis (n = 2). In multivariate analysis, CD80 mRNA expression did not provide additional prognostic information to *MGMT* promoter methylation in the ONT cohort. On the other hand, multivariate analysis of CD86 mRNA expression was an independent prognostic factor for PFS in the TCGA dataset only ($p < 0.05$; Figure 4). We have observed a similar trend ($p = 0.27$; Figure 4) in the ONT cohort, yet the trend was not significant, which could be related to the lower number of patients (n = 47) in the ONT cohort compared to (n = 121) in the TCGA database.

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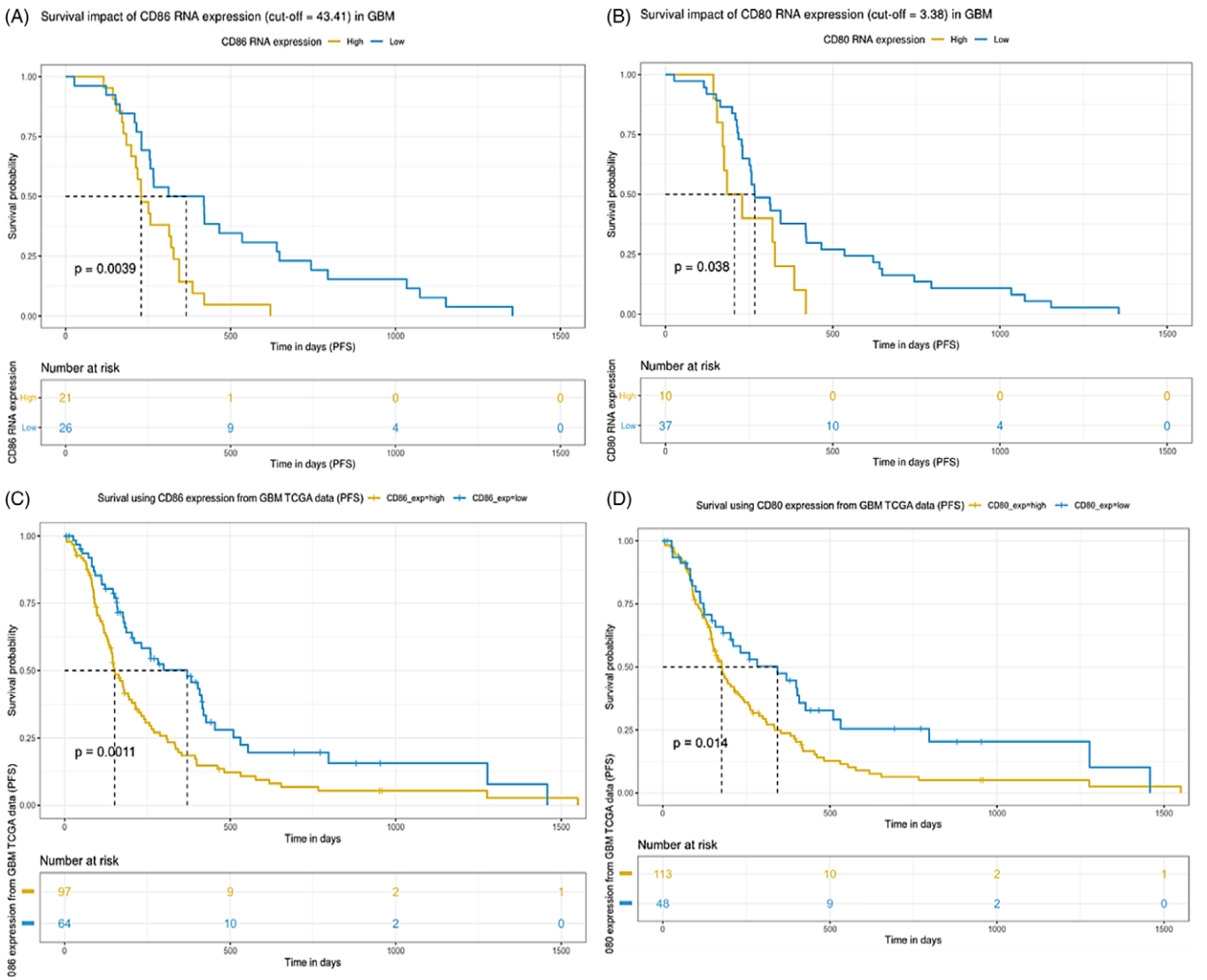

Figure 3: CD80 and CD86 mRNA expression and outcome in GBM in both ONT and TCCA database. (A) Kaplan–Meier PFS estimates in GBM patients in relation to CD86 (ONT database) (B) Kaplan–Meier PFS estimates in GBM patients in relation to CD80 (ONT database). (C) Kaplan–Meier PFS estimates in GBM patients in relation to CD86 (TCGA database). (D) Kaplan–Meier PFS estimates in GBM patients in relation to CD80 (TCGA database).

The Relationship Between CD86 Expression and Immune Cell Populations

Immune cell populations were evaluated using CIBERSORT, and we compared the immune cell populations between patients expressing both CD80 and CD86 as high and low expression. CD80 and CD86 are expressed on the surface of tumor-associated macrophages' surface suggesting a role in immunosuppressive TME. Immune cell population analysis showed low fraction of classically activated macrophages (M1) and higher fraction of immunosuppressive macrophages (M2). High CD86 expression group contained more patients with high M2 macrophages fraction ($p = 0.00013$; Figure 5). On the other hand, high CD86 expression group contained more patients with low tumor-infiltration lymphocytes fraction ($p = 0.005$; Figure 5). This effect was not observed in CD80 expression patients. Additionally, high CD86 expression group contained more patients with low $CD8^+$ cell fraction ($p = 0.039$; Figure 5) whereas, the low CD86 expression group contained more patients with high $CD8^+$ fraction. Although further studies are warranted, these data suggest association between high CD86 expression, immunosuppressive TME, and low activity of $CD8^+$ cytotoxic T lymphocytes.

Discussion

CD80 and CD86 molecules play an essential role in influencing the immune recognition of GBM cells. They bind to the CD28 molecule with a costimulatory signal for T-lymphocytes activation. On the other hand, they bind to CTLA-4, resulting in an immunosuppressive effect. CTLA-4 has a higher affinity to CD80 and CD86, making these molecules' role in immunosuppressive effect higher than their costimulatory effect.[21] The current study has linked CD80 and CD86 expression on GBM TME to PFS. We observed a low correlation between mRNA and protein expression of CD80. However, a better correlation was observed between CD86 protein and mRNA expression. Low correlation between the mRNA and protein expression might be due to posttranscriptional mechanisms involved in turning mRNA into protein. Not to

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Table 1: Univariate analysis (Cox-P regression) for OS in both ONT and TCGA database

Characteristics		ONT					TCGA				
		N = 47	Percentage %	median OS (days)	*P*-value	HR [95% CI]	N = 121	Percentage %	median OS (days)	*P*-value	HR [95% CI]
MGMT	Methylated	16	34.04	986.5	**0.00032**	0.266 [0.129–0.547]	50	41.32	457	**0.0066**	0.544 [0.350–0.844]
	Unmethylated	31	65.95	441			71	58.67	273		
IDH	Wildtype	45	95.74	502	0.321	2.062 [0.493–8.623]	113	93.38	333	**0.0045**	5.39 [1.69–17.22]
	Mutant	2	4.25	1220			8	6.61	845		
CD80 mRNA	High	5	10.63	488	0.192	0.525 [0.200–1.382]	104	85.95	306	0.07	0.573 [0.314–1.046]
	Low	42	89.36	585			17	14.04	485		
CD86 mRNA	High	31	65.95	568	0.09	0.55 [0.27–1.11]	36	29.75	421	0.376	1.223 [0.783–1.911]
	Low	16	34.04	500			85	70.24	333		
		N = 41	Percentage %	median OS (days)	*P*-value	HR [95% CI]					
CD80 protein	High	8	19.51	950	**0.011**	3.53 [1.34–9.33]					
	Low	33	80.48	470							
CD86 protein	High	24	58.53	486	0.202	1.537 [0.794–2.972]					
	Low	17	41.46	568							

Boldface values are considered significant.

Table 2: Univariate analysis (Cox-P regression) for PFS in both ONT and TCGA database

Characteristics		ONT					TCGA				
		N = 47	Percentage %	Median PFS (Days)	*P*-value	HR [95% CI]	N = 121	%	Median PFS (Days)	*P*-value	HR [95% CI]
MGMT	Methylated	16	34	587.5	**0.00013**	5.12 [2.22–11.8]	50	41.32	194	**0.0095**	1.788 [1.15–2.77]
	Unmethylated	31	66	251			71	58.67	157		
IDH	Wildtype	45	95.7	266	0.407	0.54 [0.128–2.30]	113	93.38	158	**0.0117**	4.467 [1.40–14.3]
	Mutant	2	4.3	242.5			8	6.61	488		
CD80 mRNA	High	10	21.27	206.5	**0.0426**	0.464 [0.221–0.975]	80	66.11	156	**0.0428**	0.621 [0.392–0.985]
	Low	37	78.72	267			41	33.88	203		
CD86 mRNA	High	21	44.68	229	**0.0049**	0.38 [0.199–0.75]	72	59.50	145	**0.00283**	0.509 [0.327–0.793]
	Low	26	55.31	365.5			49	49	210		
		N = 41	Percentage %	Median PFS (Days)	*P*-value	HR [95% CI]					
CD80 Protein	High	25	60.97	229	0.0841	0.565 [0.296—1.08]					
	Low	16	39.02	402							
CD86 Protein	High	13	31.70	218	**0.0429**	0.48 [0.244—0.977]					
	Low	28	68.29	329							

Boldface values are considered significant.

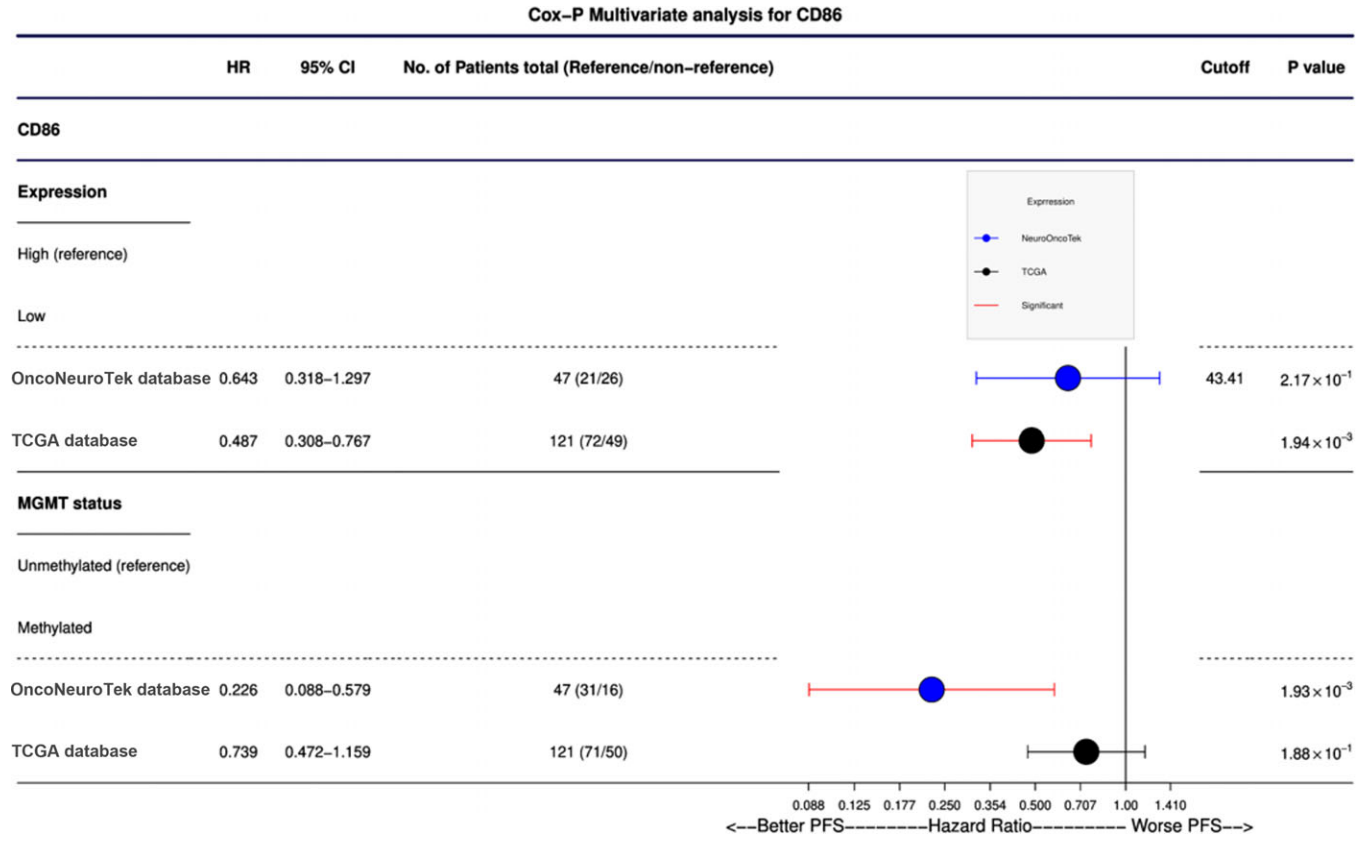

Figure 4: Cox-P (proportional hazards) multivariate analysis of CD86 protein expression and mRNA expression. CD86 was found to be an independent prognostic factor in TCGA database ($p = 0.0019$); mRNA expression of CD86 is a more predictive prognostic factor than MGMT methylation. A nonsignificant trend was observed in our ONT cohort.

mention, there is a possible error and noise in protein quantification and mRNA extraction that could influence mRNA stability and protein expression.[20] In addition to DAB staining intensity used in our study, quantification of protein using the number of positive cells should also be evaluated in future IHC analyses to better understand expression of proteins and mRNA of interest.

Number of patients ($n = 47$) in the ONT cohort is lower than the number of patients in the TCGA dataset ($n = 121$). The higher number of TCGA GBM samples could be one reason that affected the statistical analysis and provided a better prognostic value than the ONT cohort. Indeed, GBM samples' availability with comprehensive clinical and biological annotations and fulfilling the inclusion criteria is a limitation for a larger cohort. Larger patient cohort is needed to evaluate the prognostic value of CD86 expression in the TME of GBM patients. Using TCGA data to profile immune cell populations interestingly revealed that CD86 expression is associated with an immunosuppressive TME with low activity of cytotoxic T cells however protein analysis of immune cell populations is needed to validate our findings from TCGA immune cell population profiling. Indeed, high CD86 expression is associated with a cold immune microenvironment with a limited antitumor immune response promoting tumor growth and poor prognosis.

The expression of 50 immune checkpoint molecules was investigated in breast cancer. The study showed that high expression of costimulatory immune checkpoint molecules was associated with better PFS. However, no significant effect on prognosis was associated with CD80 and CD86 expression in the selected cohort.[22] Feng et al.[23] reported that low expression of CD80 is a predictive biomarker for poor prognosis in gastric adenocarcinoma. Furthermore, CD80 and CD86 were found to be potential biomarkers for better prognosis survival in nasopharyngeal carcinoma.[24] Additionally, the molecular characterization of PD-L1 expression was correlated with other checkpoint proteins, that is, CD80, highlighting that higher levels of immunosuppression are associated with GBM than lower-grade gliomas (LGG).[25] In myeloma cell lines, silencing the CD28–CD86 pathway resulted in myeloma cells' significant cell death.[26] A recent study constructed a more robust model, using GBM and LGG data from the TCGA and Chinese Glioma Genomic Atlas, and identified that low expression of CD86 molecules is a good prognostic indicator for OS. PFS analysis was not applied in this study.[27]

In 2017, Berghoff et al. described a specific signature to predict the success of TMZ in *MGMT*-methylated patients. They showed that the TME signature could be used to indicate an individual's TMZ sensitivity. The TME was identified to be different between *IDH*-mutant and *IDH*-wildtype. A richer tumor infiltrative lymphocyte and a higher expression of PD-L1 were observed in *IDH*-wildtype tumors.[28] However, to date, no studies have linked *MGMT* promoter methylation with the TME. A recent research article has studied the expression of immune checkpoint inhibitor Tim3 and *MGMT* methylated status. They identified that a high expression of Tim3 in *MGMT*-unmethylated patients is linked to poor prognosis.[29] Pratt et al.[30] have reported that the expression of PD-L1 is a negative prognostic biomarker in recurrent *IDH*-wildtype GBM. In line with these findings, our study supports that

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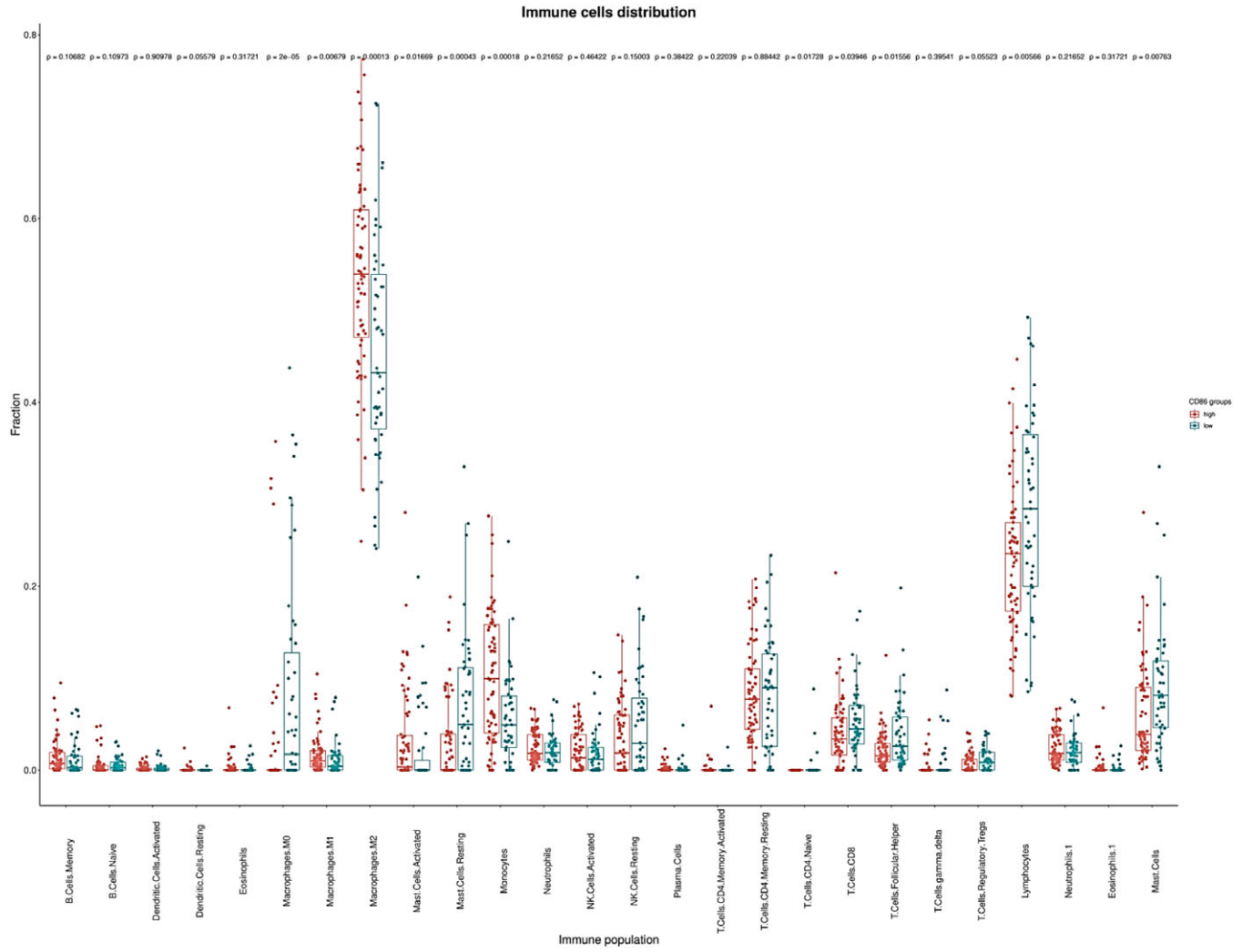

Figure 5: CIBERSORT calculated tumor infiltrating immune populations in TCGA database. Immune cell populations represented fraction of the X-axis immune cells to the whole gene expression mixture. Box plots depicting the estimated relative fractions of immune cell types by GBM category according to CD86 expression. The Y-axis here show the relative proportion which can range from 0 to 1. Relative fraction estimates the percentage of a given cell population in the total tumor infiltrate. In our analyses immunosuppressive M2 macrophages and lymphocytes were the most frequently observed immune phenotypes.

the expression of immune checkpoint inhibitors may inhibit T-lymphocyte and antitumor reaction. A recent integrated analysis of the prognostic value of CD86 reveals that CD86 is heterogeneously expressed in gliomas and is an independent unfavorable prognostic value in LGG.[31]

CD86 molecular status could be explored as a predictor of response to immunotherapies in the setting of future clinical trials dedicated to GBM patients. Our study suffers from the limitation of retrospective studies with a limited number of patients. Nonetheless, our results were validated in an independent dataset and support investigations of immune checkpoint molecules as potential prognostic biomarkers and potential predictive biomarkers of response to immunotherapies in GBM.

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Author Contributions. MA and AI designed the experiments, wrote the manuscript, and approved the manuscript's final version. MA performed the experiments. IHV performed the statistical analysis and revised the manuscript. FB, JL, and MV provided a technical support for IHC optimization and protein quantification. All authors reviewed the manuscript.

Ethics Approval. All samples were collected with informed consent from patients.

Data Statement. All annotated data will be available upon request from the authors.

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