

CD80 and CD86: expression and prognostic value in newly diagnosed glioblastoma

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Abstract

Purpose Strategies to modulate the tumor microenvironment (TME), including the vascular and immune components, have opened new therapeutic avenues with dramatic yet heterogeneous intertumor efficacy in multiple cancers, including brain malignancies. 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 glioblastoma (GBM) is still unclear. Methods In this study, we have 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. CD80 and CD86 at the protein level were also investigated in the discovery cohort. Results Both CD80 and CD86 are expressed heterogeneously in GBM at mRNA and protein levels. In a univariate analysis, the mRNA expression of CD80 and CD86 was not significantly correlated with OS in both ONT and TCGA datasets. On the other hand, CD80 and CD86 mRNA high expression was significantly associated with shorter 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 Additional studies are warranted to validate our findings and to explore the expression of CD80 and CD86 in GBM patients treated with immunotherapy and, more specifically, with CTLA-4 inhibitors.

Introduction

Glioblastoma (GBM) is the most common and aggressive glioma in adults. The latest World Health Organization (WHO) 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 [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 non-neurological 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 cells [7]. Furthermore, CD80 protein expression was observed in infiltrative tumor lymphocytes in melanoma [8]. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and Cluster of Differentiation 28 (CD28) are located on T cells. Both CD28 and CTLA-4 proteins bind to their ligands on the antigen-presenting cells and major histocompatibility complex (MHC) [9]. CTLA-4 has a higher affinity to CD80 and CD86, and when bound to its ligands, T cells remain inactive and exhausted [10].

Antibodies targeting CTLA-4 were used in preclinical studies in multiple solid tumors, resulting in many ongoing clinical trials [11]. Ipilimumab - anti-CTLA4- has also shown responses in patients with brain metastases, highlighting efficacy within the central nervous system [12]. Expression of the most studied immune checkpoint proteins, programmed death-ligand (PD-L1), was inversely correlated with overall survival in GBM patients [13]. However, the expression of CD80 and CD86 in GBM tissues and their prognostic significance has not been reported yet. This study investigated the mRNA and protein expression of CD80 and CD86 in GBM patients, aged below 70 and with KPS above 70 treated with the standard of care. In addition, we have investigated possible correlation with prognosis.

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 follow: (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 chemoradiation and adjuvant temozolomide 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 (GDC) Data portal and from the NCBI GEO GSE62944, respectively.

Immunohistochemistry (IHC) staining

Paraffin-embedded tissue blocks (5–7 μm) from biopsies of newly diagnosed GBM patients were received from the ONT biobank. Tissue sections 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 800w 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. Anti-human CD80 antibody (A16039; Abclonal) and anti-human 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 (ABC) 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 40x 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 [14]. Using the color deconvolution tool, the area positive for DAB staining was isolated and quantified using a semi-automated in-house generated code. The percentage of DAB positive areas were calculated, and the mean value from the three images was calculated and used in the survival analysis.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR)

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. qPCR was used to quantify the expression levels of CD80 and CD86 in patients. PPIA gene was used as a house reference gene for normalization. Primers were designed using Universal Probe Library (UPL) for Human. Primer's sequences were as follow; PPIA (left: atgctggaccacaacaaat; right: tctttcactttgccaaacacc; UPL probe 48) CD80 (left: gaagcaaggggctgaaaag; right: ggaagttcccagaagaggta; UPL probe 10) and CD86 (left: cagaagcagcaaaatggat; right: gaatcttcagaggagcagcac; UPL probe 15). cDNA samples were analysed using the Light Cycler Probe Master mix 2 \times (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 $2^{-(\text{CT}^{\text{gene of interest}} - \text{CT}^{\text{PPIA}})}$ 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 [15, 16] by finding a supervised cut-off value for the CD80 or CD86 expression independently using the *surv_min_er*: $surv_{cut}$ 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 [17, 18].

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% to 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 (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 received the standard of care first-line treatment regimen.

CD80 and CD86 expression at mRNA and protein level

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

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 2, A-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 2, C and 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.

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) (Table 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 3). We have observed a similar trend ($p=0.27$; Figure 3) in the ONT cohort, yet the trend was not significant, which could be related to the lower patient numbers ($n=47$) in the ONT cohort compared to ($n=121$) in the TCGA database.

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 [18]. The current study has linked CD80 and CD86 expression on GBM tumor microenvironment 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 post-transcriptional mechanisms involved in turning mRNA into protein. Another reason could be related to the stability of both mRNA and protein in our patient's samples. Finally, there is a possible error and noise in protein quantification and mRNA extraction that could influence mRNA stability and protein expression [17].

The 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 CD80 and CD86 expression in GBM samples. In our protein analysis, co-staining of CD80 and CD86 is needed to determine these proteins' expression in different immune cell populations. Furthermore, other immune checkpoint proteins could be evaluated in future studies.

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 [19]. Feng et al. reported that low expression of CD80 is a predictive biomarker for poor prognosis in adenocarcinoma [20]. Furthermore, CD80 and CD86 were found to be potential biomarkers for better prognosis survival in nasopharyngeal carcinoma [21]. Additionally, the molecular characterization of PDL1 expression was correlated with other checkpoint proteins, *i.e.*, CD80, highlighting that higher levels of immunosuppression are associated with GBM than lower-grade gliomas (LGG) [22]. In myeloma cell lines, silencing the CD28-CD86 pathway resulted in myeloma cells' significant cell death [23]. A recent study constructed a more robust model, using GBM and LGG data from the TCGA and CGGA (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 [24].

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 wildtype. A richer tumor infiltrative lymphocyte (TIL) and a higher expression of PDL1 were observed in IDH-wildtype tumors [25]. 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 [26]. Pratt *et al* have reported that the expression of PD-L1 is a negative prognostic biomarker in recurrent IDH-wildtype GBM Pratt, Dominah, Lobel, Obungu, Lynes, Sanchez, Adamstein, Wang, Edwards, Wu, Maric, Giles, Gilbert, Quezado and Nduom [27]. In line with these findings, our study supports that the expression of immune checkpoint inhibitors may inhibit T-lymphocyte and anti-tumor reaction.

CD86 expression could be used as potential biomarkers predicting the efficacy of ipilimumab in GBM patients. Furthermore, it could be used as a biomarker for patients' stratification for future clinical trials. 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.

Declarations

Acknowledgments

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Conflicts of interest

Ahmed Idbaih reports grants and travel funding from Carthera, research grants from Transgene, Sanofi, Air Liquide and, Nutritheragene, travel funding from Leo Pharma, advisory board from Novocure and Leo Pharma.

Author's contribution

MA, 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 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.

Availability of data

mRNA and protein expression for our ONT cohort were made publicly available using Dryad, Dataset and are accessible through this link: <https://doi.org/10.5061/dryad.7m0cfxptd>

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Tables

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

Table 2: Univariate analysis (Cox-P regression) for PFS 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							

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							

Figures

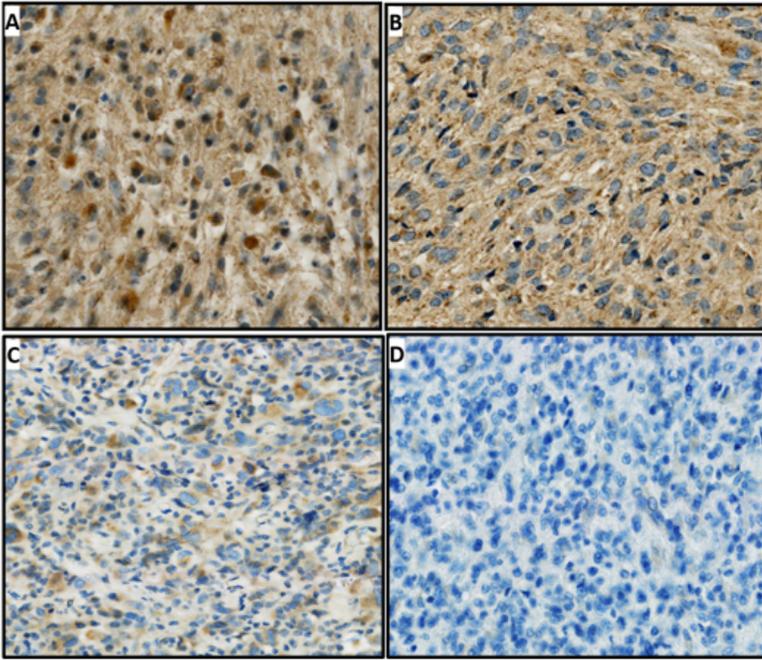


Figure 1

Represent the protein expression of CD86 and CD80 proteins in paraffin sectioned GBM samples. Panel A: high expression of CD86 protein. Panel B: low expression of CD86. Panel C: High expression of CD80. Panel D: low expression of CD80.

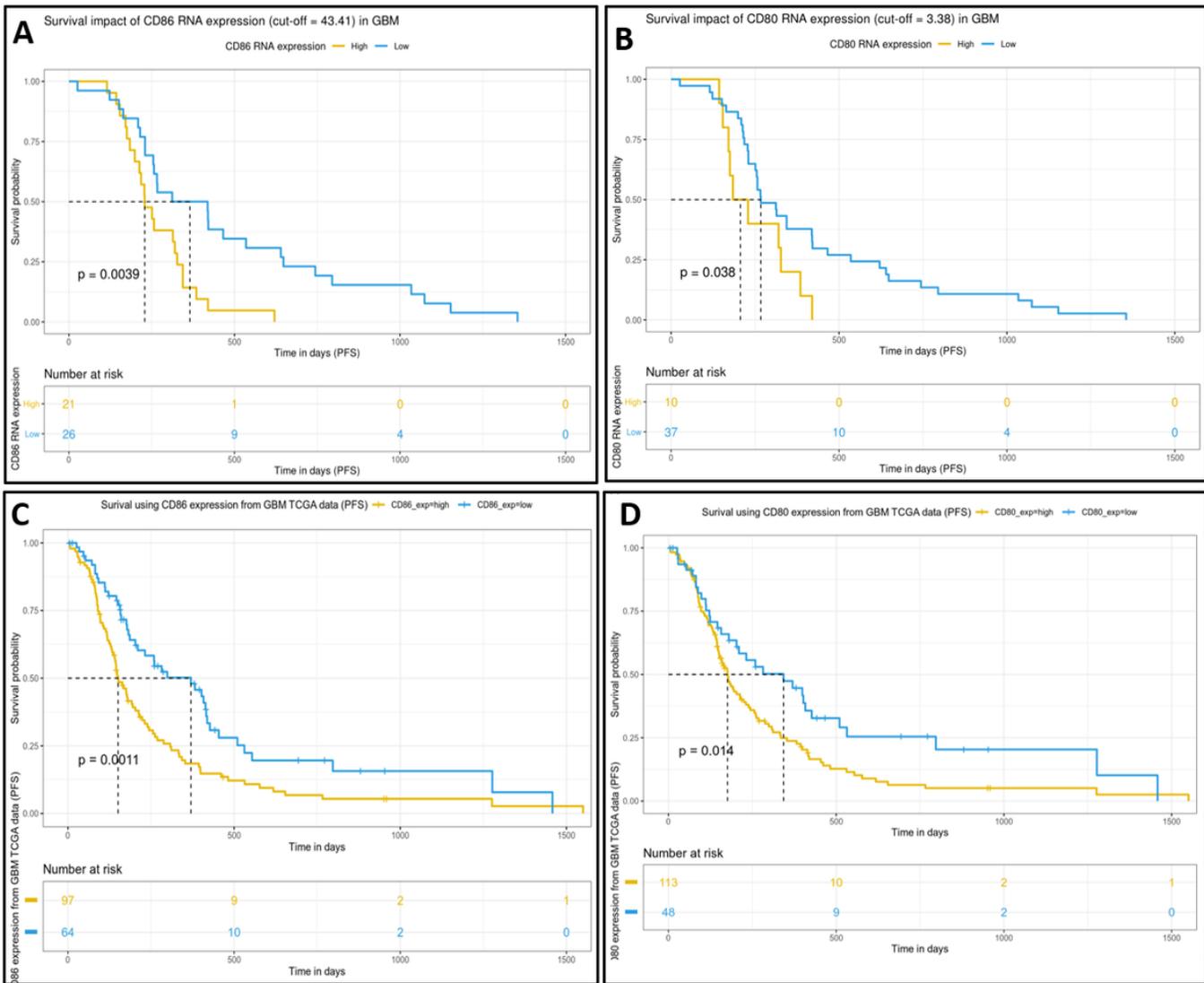


Figure 2

CD80 and CD86 RNA expression and outcome in GBM in both ONT and TCGA database. Panel A: Kaplan-Meier PFS estimates in GBM patients in relation to CD86 (ONT database) Panel 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)

Cox-P Multivariate analysis for CD86

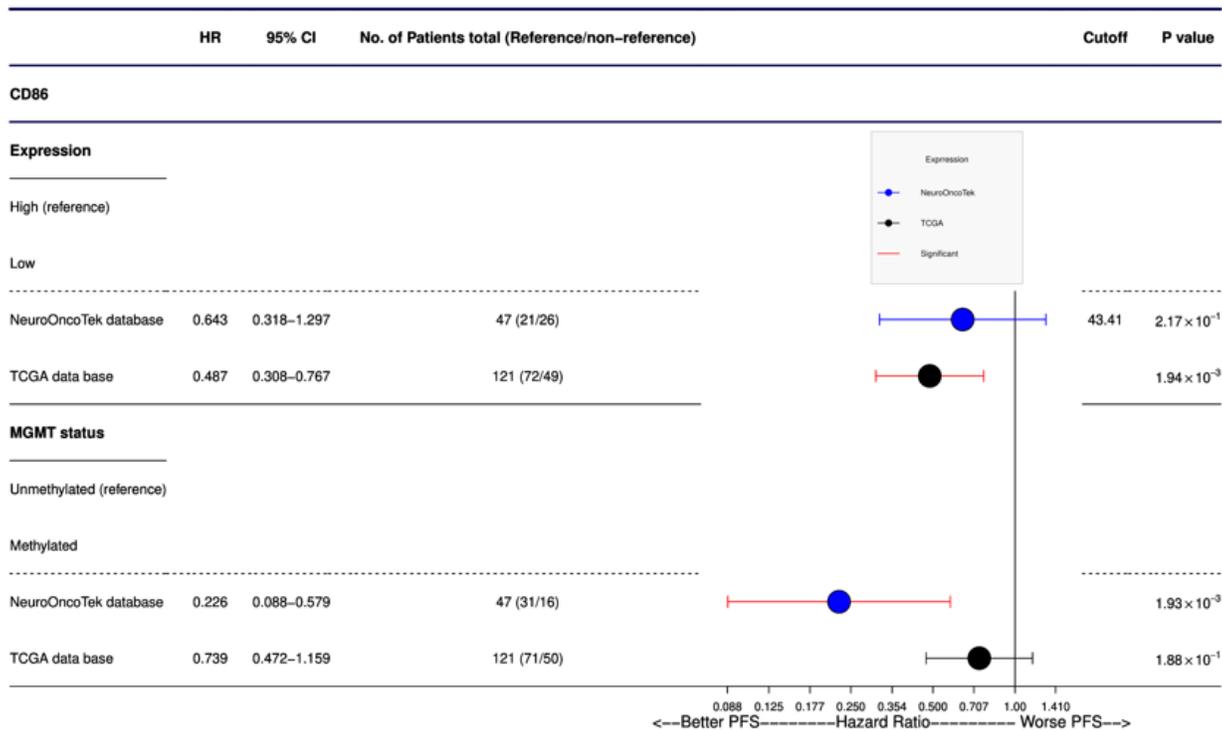


Figure 3

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 non-significant trend was observed in our ONT cohort

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SuppFigures.pptx](#)