Expression and prognostic impact of immune modulatory molecule PD-L1 in meningioma

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Abstract

While immunotherapy may offer promising new approaches for high grade meningiomas, little is currently known of the immune landscape in meningiomas. We sought to characterize the immune microenvironment and a potentially targetable antigen mesothelin across WHO grade I-III cases of meningiomas, and how infiltrating immune populations relate to patient outcomes. Immunohistochemistry was performed on tissue microarrays constructed from 96 meningioma cases. The cohort included 16 WHO grade I, 62 WHO grade II, and 18 WHO grade III tumors. Immunohistochemistry was performed using antibodies against CD3, CD8, CD20, CD68, PD-L1, and mesothelin. Dual staining using anti-PD-L1 and anti-CD68 antibodies was performed, and automated cell detection and positive staining detection algorithms were utilized. Greater degree of PD-L1 expression was found in higher grade tumors. More specifically, higher grade tumors contained increased numbers of intratumoral CD68−, PD-L1+ cells (p = 0.022), but did not contain higher numbers of infiltrating CD68+, PD-L1+ cells (p = 0.30). Higher PD-L1+/CD68− expression was independently predictive of worse overall survival in our cohort when accounting for grade, performance status, extent of resection, and recurrence history (p = 0.014). Higher expression of PD-L1+/CD68− was also present in tumors that had undergone prior radiotherapy (p = 0.024). Approximately quarter of meningiomas overexpressed mesothelin to levels equivalent to those found in pancreatic carcinomas and malignant mesotheliomas. The association with poor survival outcomes in our study suggests that PD-L1 may play a significant biologic role in the

aggressive phenotype of higher grade meningiomas. Thus, immunotherapeutic strategies such as checkpoint inhibition may have clinical utility in PD-L1 overexpressing meningiomas.

Keywords

Meningioma; PD-L1; B7-H1; Immunotherapy; Checkpoint; Lymphocyte; Macrophage

Introduction

Meningiomas represent the most common primary intra-cranial tumor found in adults, comprising roughly 36 % of such tumors [1]. Although most meningiomas are classified by WHO as grade I, higher grade subsets exist, and grade II atypical meningiomas and grade III anaplastic/malignant meningiomas constitute 20–35 % and approximately 3 % of all meningiomas respectively [2]. Regardless of grade, the primary treatment modality for symptomatic and enlarging meningiomas currently is surgical resection. Higher WHO grade tumors exhibit aggressive clinical behavior, with grade II meningiomas having median progression free survival of approximately 7 years, and grade III tumors displaying frank malignant characteristics with overall survival of less than 3 years [2]. For these higher grade meningiomas, radiotherapy is commonly utilized as an adjuvant; however, robust prospective evidence supporting its use, and its exact role in the postoperative setting is currently lacking [3]. In addition, when these tumors recur after having failed surgery and radiotherapy, treatment options are severely limited and the clinical outcomes extremely poor [4]. Thus, there is a clear need for new effective therapies to serve as adjuvant or salvage agents for atypical and anaplastic meningiomas.

Refining our understanding of the complex interaction between the immune system and malignancies has led to the development of exciting novel immunotherapeutic approaches. Inhibitors of immune checkpoint pathways cytotoxic T lymphocyte antigen 4 (CTLA-4), and programmed death 1 (PD-1)/PD-ligand 1 (PD-L1) have shown promise for even advanced systemic malignancies such as metastatic melanoma, and recently was granted approval from the FDA [5–7]. While immunotherapy also offers promising potential avenues for meningiomas, little is currently known of the immune microenvironment in these tumors. Earlier studies have discovered evidence of macrophages and small populations of both T and B lymphocytes [8–11]; however, their significance and their relationship to clinical behavior have remained largely unclear. A recent report by Du et al. demonstrated that there were significantly fewer infiltrating T lymphocytes, including CD4+ and CD8+ T cells and cells expressing PD-1 within anaplastic meningiomas compared to lower grade tumors [12]. In addition, they found that PD-L1 is expressed in meningioma both at the protein and mRNA levels, and the levels of expression were higher in anaplastic cases [12]. Their analysis however failed to detect a significant relationship between PD-L1 levels and survival outcomes in their cohort consisting mostly of grade I meningiomas. We sought to further explore the prognostic significance of PD-L1 in meningiomas, investigate how PD-L1 expression relates to the infiltrating immune cell populations, including CD68+ macrophages in a cohort enriched for grade II and III cases.

Furthermore, we tested we tested for the presence of a potentially targetable antigen mesothelin in meningiomas. Mesothelin is a cell surface protein whose exact role remains unclear, but is likely involved in cell adhesion [13]. It is the target of focus of an early phase clinical trial in pancreatic carcinoma, ovarian carcinoma, and malignant mesothelioma utilizing chimeric antigen receptor (CAR) T-cells targeted against mesothelin (clinicaltrials.gov, NCT#02159716). To explore whether mesothelin could serve as a potential targetable antigen in meningioma, mesothelin expression was also studied in our samples.

Materials and methods

Meningioma cases treated at the University of California, San Francisco Medical Center between 1989 and 2012 were selected based on the availability of sufficient tissue for immunohistochemical analysis as well as available clinical follow-up. In an attempt to optimize the translational potential for higher grade meningiomas, the cohort was enriched to capture all grade II and III encountered in the study period that had sufficient tissue. A random sample of grade I cases treated in the same time period was included for comparison in approximately equivalent numbers as grade III cases. Clinical information such as gender, tumor location, type of surgery, extent of resection, and treatment history was obtained from review of the medical records. Consultation cases were excluded. Recurrence was defined as the emergence of a new radiologically defined lesion or lesions that required treatment. All pathologic diagnosis was confirmed by two of the authors (GR and AP) according to the criteria by the WHO. Approval for the study was obtained from the University of California San Francisco Committee for Human Research (Approval #10-03204).

Tissue microarray

Tissue microarrays (TMA) were constructed using a semiautomatic tissue microarrayer (TMArryer, Pathology Devices, Westminster, MD). In brief, recipient blocks made using 5 % agarose (A9539, Sigma Aldrich, St Louis, MO) in disposable base molds ($37 \times 24 \times 5$ mm; 62352-37, VWR, Radnor, PA). Agarose wafers were processed overnight in a Leica ASP tissue processor (Leica Biosystems, Buffalo Grove, IL) and subsequently embedded using conventional methods. Tissue from donor paraffin-embedded blocks of the select cases was sampled using a 1.5 mm punch (02110006; Pathology Devices, Westminster, MD). Duplicates were prepared, with each block containing 96 samples and including randomly selected control tissues. Recipient blocks were re-embedded and sectioned at 5 micrometer thickness for immunohistochemistry.

For mesothelin staining experiments, normal tissue TMA sections were obtained from Folio (cat# ARY0HH0213, Powell, OH), normal peritoneum and pleura TMAs from Pantomics (cat# GIP541, LUP541, Richmond, CA), multiple tumor TMA from Pantomics (cat# MTU391, Richmond, CA) malignant mesothelioma from MyBioSource (cat# MBS640483, San Diego, CA).

Immunohistochemistry

The following primary antibodies were used in this study: CD3 (1:25, Clone: F7.2.38, Dako, Carpinteria, CA), CD8 (1:50, Clone C8/144B, Dako, Carpinteria, CA), CD20 (1:20, ab9475, Abcam, Cambridge, MA) CD68 (1:50, ab49777, Abcam, Cambridge, MA), PD-L1 (1:200, cat #13684, Cell Signaling Technology, Danvers, MA), mesothelin (1:50, sc-271540, Santa Cruz Biotechnology, Dallas TX).

Slides were deparaffinized in xylene and rehydrated in serial baths of graded ethanol (100 %, 95 %). Heat induced epitope retrieval was performed with a citrate buffer (Target Retrieval Solution Citrate pH6, Dako, Carpinteria, CA) in a decloaking chamber (Biocare Medical, Concord, CA) at 20 psi for 10 min, followed by 30 min of cooling at room temperature. Endogenous peroxidase activity was quenched in 3 % hydrogen peroxide for 10 min at room temperature. Blocking of non-specific binding of primary antibody was performed using a solution of 5 % normal goat serum (Cell Signaling Technology, Danvers, MA). Slides were incubated with primary antibodies at 4° overnight. The sections were then incubated at room temperature for 1 h with secondary horseradish peroxidase conjugated goat antibodies (Signal-Stain Boost IHC Detection Reagent HRP Mouse and HRP Rabbit, Cell Signaling Technology, Danvers, MA). Visualization was performed with chromagen diaminobenzidine (DAB) (SignalStain DAB Substrate Kit, Cell Signaling Technology, Danvers, MA). Counterstaining was performed with hematoxylin (Hematoxylin QS, Vector Laboratories, Burlingame, CA), and the slides were mounted with a cover slip (Cytoseal 60, Thermo Scientific, Waltham, MA).

For dual target staining, following primary antibody incubation, the slides were incubated with secondary goat anti-mouse and goat anti-rabbit antibodies, conjugated to the fluorochromes Alexa Fluor 488 and Alexa Fluor 555, respectively, at 1:200 dilution for 1 h at room temperature (Lot 1557766 and Lot 1600203, Life Technologies, Carlsbad, CA). The slides were cover-slipped with a mountant containing DAPI (ProLong Gold antifade reagent with DAPI, Life Technologies, Carlsbad, CA). All staining was completed in duplicate.

Image acquisition

Stained sections were imaged with Zeiss Axio Imager 2 microscope (Zeiss, Oberkochen, Germany). Image acquisition was completed while using identical camera settings of exposure time and white balance throughout all sections stained with the same antibody, with the aid of an automated scanning software, TissueFAXS (TissueGnostics, Vienna, Austria).

Image analysis

Quantification of positive staining was performed with the image analysis software StrataQuest (TissueGnostics, Vienna, Austria). In brief, for sections stained for each target epitope, a set of analysis algorithms were generated by a trained user. First, a cell detection algorithm identified nucleated cells based on hematoxylin or DAPI staining, which incorporated nuclear size, shape, and intensity of the signal. The next layer of analysis was designed to detect the intensity of staining in the DAB color or the FITC/Texas Red channels associated with each detected cell. Tabulation of positive cells in each TMA was determined

by a threshold set by the user based on the detection of signal intensity. Once each algorithm was generated and tested to appropriately identify positive cells, it was applied to all TMA images to calculate a total number of positive cells per area, as well as percentage of positively staining cells in the entire sample.

Statistical analysis

Expression of different markers between groups was compared using the *t*-test or ANOVA. Survivals were estimated using the Kaplan-Meier method. Comparisons of survivals used the Log-rank test. Mutivariate cox proportional hazards were used for modeling risks of progression or death based on prognostic variables. Splits for continuous predictive variables were generated using classification and regression trees. Statistical analyses were performed using JMP software (SAS, Cary, Canada).

Results

Patients

The cohort included 96 total patients with16 grade I, 62 grade II, and 18 grade III tumors. The median age was 60 at diagnosis, and 57 patients (59 %) were female. Twenty-five cases (26 %) were recurrent cases, with 23 cases (24 %) having received prior radiation therapy as adjuvant treatment for their meningioma. Table 1 summarizes the patients' baseline characteristics and prior therapies.

Infiltrating immune cell populations

Our immunohistochemistry analysis confirmed the presence of T lymphocytes in majority of cases. We found a median of 114 CD3+ lymphocytes per core ($64/mm^2$), and median of 126 CD8 + lymphocytes per core ($71/mm^2$) across all grades of tumors (Table 2). B lymphocytes were also present, but in much smaller numbers, with median of 3.5 CD20+ cells per core ($2/mm^2$). We did not note a difference in numbers of infiltrating any subpopulation of lymphocytes based on the grade of tumor, or on prior treatment history. CD68+ infiltrating macrophages were more abundant, and there was median of 3752 CD68+ cells per core ($2120/mm^2$).

Expression of PD-L1 in meningioma

PD-L1 expression was calculated as a percentage of positively staining cells across the core. Across all grades, the mean percent of PD-L1+ cells was 3.51 % (Range 0–85.4 %, Table 2). Higher expression of PD-L1 was found in higher grade tumors. On average, PD-L1 expression was found in 0.91 % of cells for grade I, 2.63 % of cells for grade II, and 8.84 % of cells for grade III meningiomas (ANOVA, p = 0.039, Fig. 1). Previous treatment with prior radiotherapy was also correlated with high PD-L1 expression; tumors that had undergoing prior radiotherapy contained an average of 6.79 % PD-L1+ cells, while tumors that did not had 2.50 % (Student's *t*-test, p = 0.041, Fig. 1). PD-L1 expression did not appear to have significant direct or inverse relationship to the degree of infiltrating CD3+, CD8+, CD20+ or CD68+ cells.

PD-L1 expression in infiltrating macrophages

To clarify the source of PD-L1 expression in meningioma and address the possibility of infiltrating macrophage populations being a significant source of PD-L1 within the tumor, we performed costaining experiments for PD-L1 and CD68 (Fig. 2). Although the majority of PD-L1+ cells were also negative for CD68 across all cores, suggesting that PD-L1 is likely expressed by the tumor cells themselves, in a subset of tumors, most of the PD-L1+ cells were also CD68+. In 36 samples (43.3 %), the CD68+ infiltrating macrophage population was the main source of PD-L1 expression within the tumor tissue, i.e. more than 50 % of the total PD-L1+ cells was also CD68+.

PD-L1 expression is predictive of poor overall survival

The median overall survival of the entire cohort was 11.8 years. A cox proportional hazards model was performed accounting for potential prognostic variables such as age, performance status, extent of resection, grade, recurrent status, along with numbers of lymphocytic/ macrophage infiltrates and level of PD-L1+ cells. On univariate analysis, Karnofsky Performance Scale (KPS), grade, and proportions of PD-L1+ cells and PD-L1+/CD68− cells were significant predictors of overall survival, while percent of PD-L1+/CD68+ cells was not. In addition, presence of PD-L1+/CD68− cells was more predictive of worse survival than total PD-L1 + levels (HR per 1 % expressing cells change: 1.037 vs. 1.015, respectively), suggesting that the prognostic impact of PD-L1 seem primarily driven by PD-L1 expression on CD68− meningioma cells. When accounting for age, KPS, grade and extent of resection in a multivariate model, PD-L1+/CD68− expression remained significantly predictive of worse overall survival (HR 1.263 per 1 % of cells, $p = 0.014$, Table 3). When the same multivariate model was built using all PD-L1 + cells, total PD-L1 + population was no longer predictive of overall survival ($p = 0.179$).

Classification and regression trees analysis revealed that presence of >0.12 % of PD-L1+/ CD68− cells as the most significant cut-off predicting worse overall survival (log rank, $p = 0.027$, Figure S1).

CD20+ cell infiltration predicts favorable progression free survival

Median progression free survival was 6.4 years. Cox proportional hazards modeling utilizing same prognostic variable as above revealed grade and CD20+ infiltrates as significant predictors of progression free survival in univariate modeling. Multivariate analysis accounting for grade and extent of resection revealed infiltration with CD20+ cells was independently predictive of improved progression free survival (HR 0.966 per CD20+ cell in core, $p = 0.041$, Table 3).

Mesothelin as a potential targetable antigen

The median percent of cells staining positively for mesothelin across all cores was 3.93 %; however, ten percent of cores contained >35.7 % of mesothelin positive cells (Figure S2). Using same staining conditions, sections of normal organs and multiple types of solid organ malignancies were also stained for mesothelin. Among normal organs, the percent of cells staining positive for mesothelin ranged from 0.87 to 31.8 %. Organs with >10 % staining included pleura (31.8 %), lymph node (21.6 %), stomach (16.2 %) and lung (15.4 %).

Positive staining was found 0.2–49.3 % of solid organ tumors. Tumors with the most mesothelin expression were ovarian adenocarcinoma (49.3 %), colon adenocarcinoma (19.9 %), stomach adenocarcinoma (14.7 %), pancreatic carcinoma (13.8 %), and mesothelioma (12.2 %). In our meningioma samples, the 75th percentile of mesothelin positive staining was 17 %, indicating that a quarter of the tumors expressed mesothelin levels near those found in pancreatic carcinoma and mesothelioma. Supplementary Table 1 summarizes the results of mesothelin staining.

Discussion

Our results demonstrate presence of infiltrating immune cells in meningioma, with CD68+ cells in relative abundance, fewer CD3+ and CD8+ lymphocytes, and rare CD20+ lymphocytes. Although the relative distribution of tumor infiltrating immune cells is consistent with those seen in other tumors of the central nervous system, such as glioblastoma, primary CNS lymphoma, and brain metastases [14–17], it is difficult to say how the degree of immune cell infiltration in meningiomas compare to those in these other tumors, as the method of detection varied. Traditional method of quantifying tumor infiltrating lymphocytes involves a semi-quantitative four-tiered system based on manual blinded assessment of low and high powered fields [18]. Our analysis minimizes biases by sampling the entire tissue section, and provides an objective fully quantitative count of the immune cell population using a software algorithm. Thus, the results obtained in our analysis is a count of cells across an area of tissue, rather than a category of "sparse" to "very dense" infiltration.

We also confirmed previous findings that PD-L1 is present in meningiomas, and more expression is found in higher grade tumors. In addition, PD-L1 expression, particularly among cells that were negative for CD68, was predictive of poor overall survival in our cohort. Prior investigation of PD-L1 expression in meningioma by Du et al. did not detect any prognostic impact of PD-L1 expression [12]. One main difference in our analysis was the inclusion of double staining of PD-L1 with CD68, and this analysis revealed that only the PD-L1 expression on CD68− cells was an independent predictor of survival outcome. On the other hand, total PD-L1 expression itself was not an independent prognostic indicator of outcome, which was consistent to findings of Du et al. [12].

This finding may also be explained in part by the types of cases included for analyses. The cohort evaluated by Du et al. included consecutive meningioma cases of all grades at a single institution, and hence 67 % of their TMA consisted of grade I tumors [12]. If the degree of PD-L1 expression is predictive of poor survival only among grade II/III tumors, this effect may not be detectable in a series consisting of mostly grade I tumors, as the prognostic impact maybe diluted. In addition, in a cohort of mostly grade I tumors, it may be likely that there were many censored samples in the survival analysis. If this is the case, it may explain why our analysis was able to detect a prognostic impact of PD-L1 expression, as the cases represented in our TMA were enriched for grade II/III tumors.

PD-1/PD-L1 axis-driven immunosuppression has been implicated in multiple tumor types, including tumors of the central nervous system such as glioblastoma, chordoma, and primary

central nervous system lymphoma [17, 19–21]. In many of these cases, PD-L1 expression was also observed on tumor infiltrating macrophages, including in malignant gliomas. In malignant gliomas, PD-L1 expressing tumor infiltrating macrophages are associated with worse outcomes, and are able induce an immunosuppressive phenotype by inducing T cell apoptosis [22, 23]. Our study discovered PD-L1 expressing CD68+ tumor infiltrating cells in a significant subset of meningiomas cases. However, the biologic impact of these CD68+/PD-L1+ cells seems unclear as their presence was not correlated with pathologic grade nor was it prognostic of overall or progression free survival in our cohort. On the other hand, we cannot be certain that the CD68−/PD-L1+ population seen within tumor tissue solely represents tumor cells. Dual staining with meningioma specific markers including vimentin and EMA were attempted, however, appropriate antibody combinations and staining conditions could not be achieved to allow for confident reading of PD-L1 expression signal.

In our series, PD-L1 expression was also significantly higher in recurrent tumors and after prior radiation therapy. This association was likely driven by the increase in PD-L1+/CD68− cells, as the degree of PD-L1+/CD68+ cells did not significantly differ based on prior radiotherapy. This finding was not surprising, as radiotherapy has been shown to upregulate PD-L1 in other tumor types, likely through release of interferon-gamma [24]. In a recent report, Moliterno et al., based on a retrospective series, observed that *de novo* grade III tumors seem to have more favorable survivals compared to those that progressed from a lower grade tumor, independent of extent of resection [25]. In our cohort, although prior treatment status correlated with higher PD-L1 expression, PD-L1 expression on CD68− cells was significantly predictive of poor overall survival while recurrent status alone was not. It would be of interest to see if the *de novo* cases in Moliterno et al.'s series indeed contained lower levels of PD-L1. Nonetheless, the association with decreased overall survival suggests that PD-L1 may play a biologically significant role in the aggressive phenotype of meningiomas that recur after radiotherapy. In addition, it may be worth exploring whether PD-1/PD-L1 axis blockade would have clinical utility in radiation failed tumors, and whether combination therapy with radiotherapy would be able to abrogate the potential impacts of this PD-L1 upregulation.

Given that CD20 + cells were detected with relative infrequency in the vast majority of samples studied, its association with progression free survival was somewhat a surprising finding. Prior studies have also described the rare presence CD20+ B cells within meningiomas, and consistent with Du et al.'s experience, we also did not detect a relationship between CD20+ infiltrates and tumor grade [12]. In addition, the ultimate biologic impact of infiltrating CD20+ cells is made less clear by our finding that presence CD20+ cells was not predictive of improved overall survival. Still, antibody mediated immunity may have a larger role in the immune-tumor interaction in meningiomas, given the lack of a blood brain barrier around these tumors, compared to other locations in the central nervous system. In a canine model of meningioma, Andersen et al. successfully induced an antibody mediated response to an autologous tumor cell lysate vaccine [26]. In their analysis, infiltration of plasma cells were also seen post vaccination, and isolated tumor-reactive antibodies were capable of cell-mediated induction of antibody-dependent killing of autologous tumor cells. Further characterization of the infiltrating B cell population,

including their activation status, is clearly needed to gain further insights into the impact of the humoral immune system's role in meningioma progression and proliferation.

Mesothelin was explored as a potential targetable antigen in meningioma, as an early phase clinical trial is currently exploring the use of CAR T cells targeted against mesothelin for pancreatic carcinoma, ovarian carcinoma, and malignant mesothelioma (clinicaltrials.gov, NCT#02159716). Mesothelin is also expressed in many normal organs, with higher levels in connective tissue such as pleura and the gastrointestinal system. The exact role of mesothelin remains unclear, as knockout mice develop and reproduce normally [27]. Based on our experience, tumors originating from solid organs generally seem to upregulate mesothelin expression relative to normal levels found in that particular organ. Mesothelin has been shown to be also expressed to varying degrees in meningiomas of all grades, while very few normal meninges expressed mesothelin [13]. Our analysis confirms these previous findings, and overall levels of expression appears similar to the previously published reports. Most tumors showed little to no staining, but a small subset had significant levels of expression, in up to 99 % of cells. Approximately a quarter of the tumors expressed mesothelin levels near those found in pancreatic carcinoma and mesothelioma (~15 %). Thus, in a subset of meningiomas with high mesothelin expression, a similar strategy of targeting mesothelin could be potentially utilized.

Our analysis used an automated quantification of all stained sections. The StrataQuest imaging analysis software, once trained, allowed for objective quantification of positive staining. In addition, the software applied the same algorithm of cell detection and positive staining quantification to all cores on the TMA. We feel that this method provided a rapid, reproducible and unbiased method of quantification of large number of samples when compared to manual counting or scoring.

Limitations of our analysis include those inherent to TMA based studies, such as the possibility that the cores may miss the intratumoral heterogeneity with regards to the immune microenvironment. Selection bias may have also been introduced by enriching our cohort for grade II/III tumors and cases that had sufficient tissue to generate cores.

Conclusion

In our cohort PD-L1 expression, specifically by CD68− cells, and not in CD68+ infiltrating cells, was associated with higher grade tumors and prior treatment with radiotherapy. The presence of PD-L1+/CD68− cells was independently prognostic of poor overall survival in our study, suggesting PD-L1 in this population may play a significant biologic role in the aggressiveness of higher grade and radiotherapy failed meningiomas. Thus, immunotherapeutic approaches such as checkpoint inhibitors of PD-1/PD-L1 axis, or targeting mesothelin through vaccines or engineered T cells may have clinical utility for aggressive high grade tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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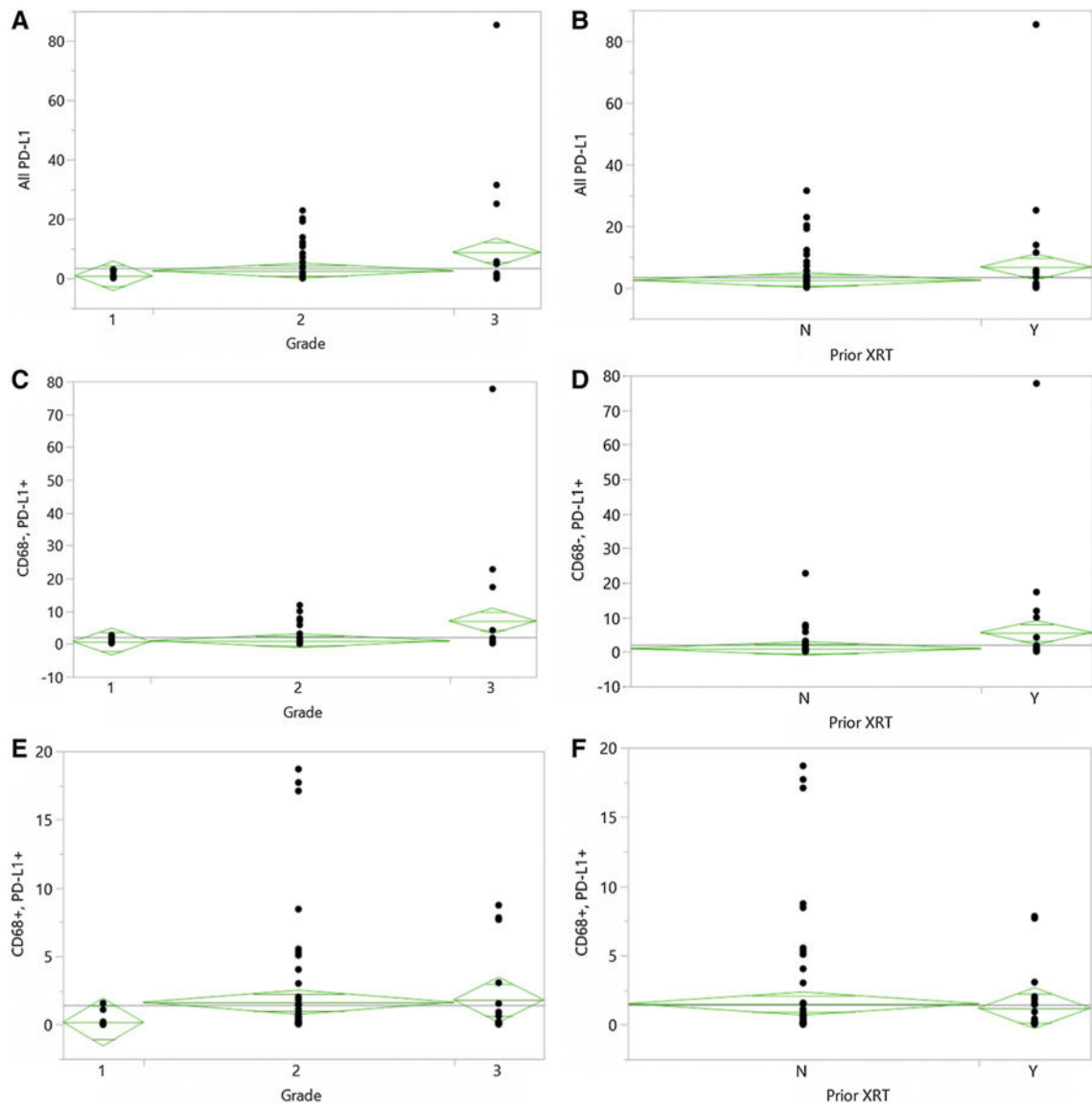
Fig. 1.
PD-L1 Expression based on tumor grade and prior radiotherapy. On average, PD-L1 expression was found in 0.91 % of cells for grade I, 2.63 % of cells for grade II, and 8.84 % of cells for grade III meningiomas (ANOVA, p = 0.039, **a**). Tumors that had undergone prior radiotherapy contained an average of 6.79 % PD-L1+ cells, while tumors that did not had 2.50 % (Student's *t*-test, p = 0.041, **b**). There were significantly greater numbers of PD-L1+/CD68− cells in higher grade tumors (ANOVA, p = 0.022, **c**), and tumors that had undergone prior radiotherapy (*t*-test, p = 0.024, **d**). There was no significant difference in numbers of infiltrating PD-L1+/CD68+ cells between tumors of different grades (ANOVA, p = 0.30, **e**), and based on prior radiotherapy (*t*-test, p = 0.71, **f**). Boxes represent mean and standard error
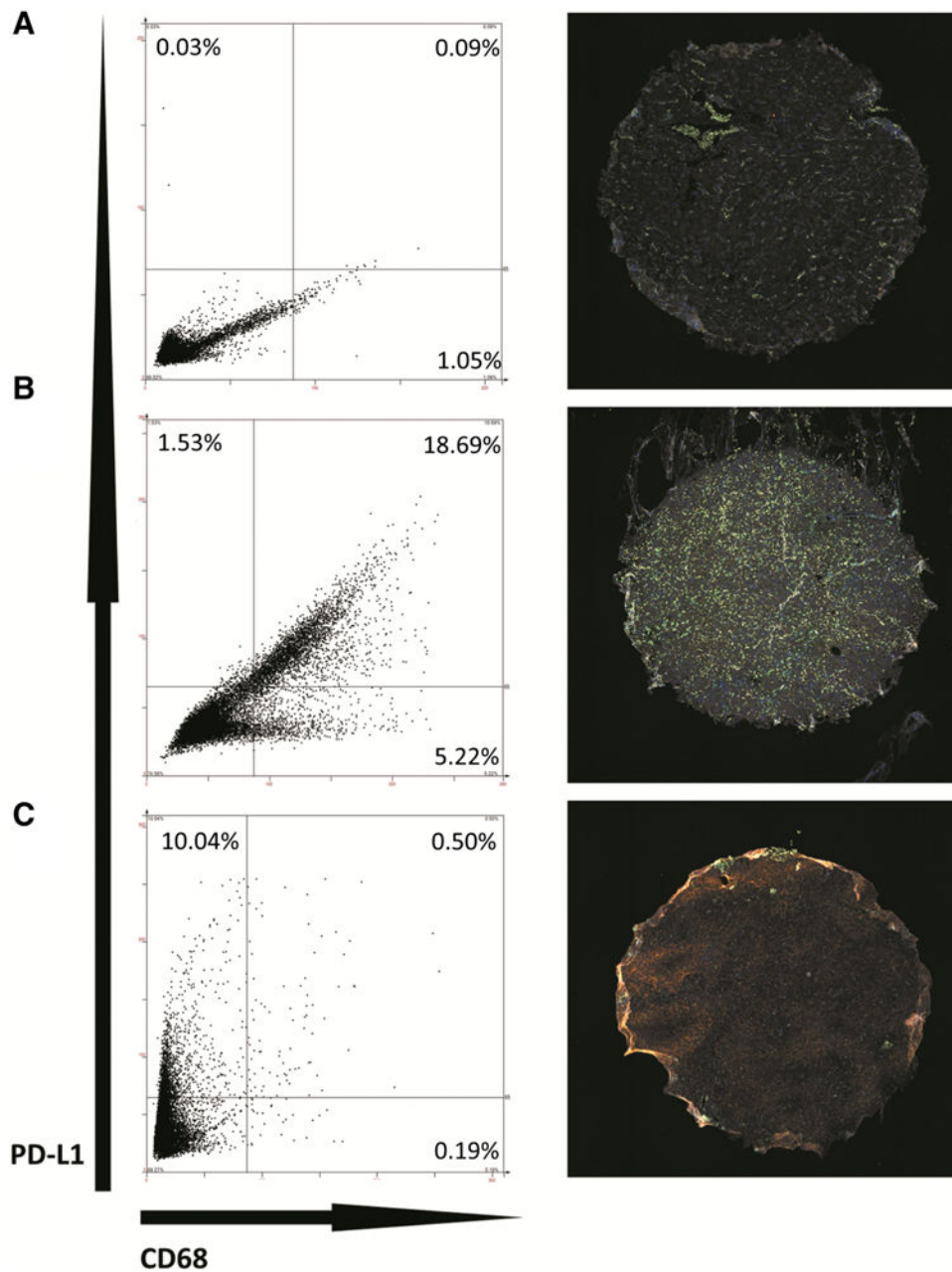
Fig. 2.
Patterns of PD-L1 Expression. Immunohistochemistry of double staining for PD-L1 and
CD68 in representative WHO grade I (**a**), grade II (**b**), and grade III (**c**) tumors. Left panels
show scatterplot generated by StratQuest image analysis software, based on mean staining
intensity in FITC (CD68, x-axis) and Texas Red (PD-L1, y-axis) channels within each
identified cell. Each *dot* represents one cell. *Dividing lines* within the *plot* represent intensity
threshold used to consider positive signal in each channel. Although the scales of the axes
appear different across the three representative samples, same staining intensity thresholds
were used for all cores to determine positivity in each channel. Right panels show
corresponding stained sections: DAPI (nuclear stain) in *blue*, FITC (CD68) in *green*, and

Texas Red (PD-L1) in *orange*. Sample **a** shows representative tumor with very low overall PD-L1, with minority of cells expressing CD68. Sample **b** shows a significant PD-L1 + population that is also CD68+, as well as a separate population of CD68+/PD-L1− cells. Sample **c** demonstrates PD-L1 expression that is predominantly within CD68− cells. Core diameter is 1.5 mm

Table 1

Clinical characteristics of patients

Characteristic	Entire population (n = 96)	
	n	**%**
Age at diagnosis, median (range)	59.8 (18.7–86.5)	
Gender		
F	57	59.4
M	39	40.6
Karnofsky performance status		
90	3	3.12
80	19	19.8
70	23	24.0
<70	19	19.8
Unknown	32	
Grade		
I	16	16.7
II	62	64.6
III	18	18.8
Recurrence status		
Recurrent tumor	25	26.0
Newly diagnosed tumor	71	74.0
Prior radiotherapy		
Yes	23	24.0
No	73	76.0
Extent of resection		
Gross total resection	36	43.3
Subtotal resection	47	56.6

Baseline clinical characteristics of patients whose tumors were included in the TMA

Table 2

Degree of infiltrating immune cell populations

	Mean (range)	25th percentile	Median	75th percentile
	Cells per core			
CD3+	194 (0–1512)	50	114.5	254
CD8+	188 (0–956)	52.75	126.5	244.75
CD20+	11.0 (0–220)	1	3.5	8
CD68+	4204 (596–13,781)	2337.25	3752	5163
	Percent of cells staining positive			
PD-L1+	3.5 (0–85.4)	0.063	0.41	1.9
PD-L1+/CD68+	1.43 (0–18.7)	0	0.90	5.14
PD-L1+/CD68−	2.08 (0–77.69)	0.01	0.105	0.713
Mesothelin	13.9 (0.03–99.97)	1.66	3.93	17.1

Numbers of CD3, CD8, CD20, and CD68+ cells per core, and percentage of totals cells positive for PD-L1, cells double positive for PD-L1 and CD68, cells positive for PD-L1 but negative for CD68, and cells positive for mesothelin as calculated by StrataQuest analysis software

Table 3

Multivariate Analysis of Predictive Variables for Survival Outcomes

Overall survival			Progression free survival	
Per unit HR (95 % CI)		p	Per unit HR (95 % CI)	p
KPS	1.014 (0.943, 1.025)	0.559	Not included in model	–
EOR				
GTR	1 [ref]	0.207	1 [ref]	0.439
STR	1.905 (0.708, 5.747)		0.738 (0.342, 1.597)	
Grade				
1	1 [ref]	0.222	1 [ref]	<0.001
2	0.654 (0.165, 4.421)		1.29×10^{10} (5.684, 4.45×10^{64})	
3	3.6×10^{-10} (0, 1.554)		4.77×10^{10} (18.95, 3.42×10^{65})	
CD20+	Not included in model	–	0.966 (0.887, 0.999)	0.041
PD-L1+/CD68−	1.263 (1.067, 1.496)	0.014	Not included in model	–

CI confidence interval, *GTR* gross total resection, *HR* hazard ratio, *ref* reference, *STR* subtotal resection