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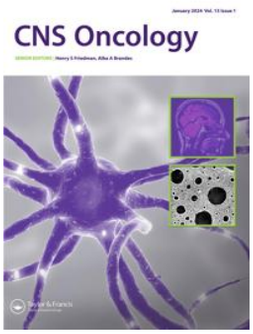

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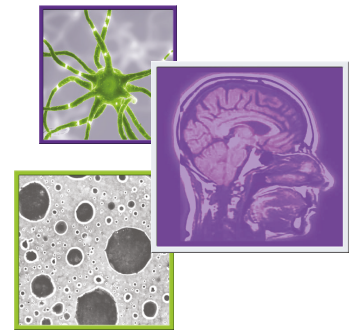

Distribution of tumor-infiltrating immune cells in glioblastoma

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Aim: Evaluation of features related to infiltrating immune cell level in glioblastoma. **Methods:** Tumor-infiltrating lymphocytes (TILs) through H&E staining, and TILs (CD3, CD4, CD8 and CD20) and macrophage (CD68 and CD163) levels through immunohistochemistry were evaluated through digital analysis. **Results:** CD68 (9.1%), CD163 (2.2%), CD3 (1.6%) and CD8 (1.6%) had the highest density. Higher $CD4^+$ was associated with unmethylated MGMT ($p = 0.016$). Higher $CD8^+$ was associated with larger tumoral size ($p = 0.027$). Higher $CD163^+$ was associated with higher age ($p = 0.044$) and recursive partitioning analysis = 4. Women ($p < 0.05$), total resection ($p < 0.05$), MGMT-methylation ($p < 0.001$), radiotherapy ($p < 0.001$), chemotherapy ($p < 0.001$) and lower $CD4^+$ ($p < 0.05$) were associated with longer overall survival. **Conclusion:** Macrophages are more frequent than TILs. Some subsets are associated with clinical features.

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Keywords: biomarker • glioblastoma • macrophages • MGMT • overall survival • prognosis • tumor-infiltrating lymphocytes

The outcome for most glioblastoma patients is lethal. However, retrospective series have identified a small subset of patients with longer survival, and some clinicopathological features and scores like recursive partitioning analysis (RPA) have demonstrated a prognostic role in glioma patients [1–4].

Glioblastoma patients' survival has been improved under a scheme of postoperative radiation with alkylating chemotherapy and epigenetic silencing of the MGMT as a prognostic biomarker has been associated with increased survival [1,2,5–10]. Additional therapies against gliomas, including immunotherapy, are currently under intensive research [11]

Tumor-infiltrating lymphocytes (TILs) have been demonstrated to have a prognostic and predictive role in different malignancies [12–14]. Although the brain is an immunologically isolated organ, the presence of lymphocytes has been documented in gliomas.The role of lymphocytes in the brain has not been fully studied, since previous small studies only investigated certain subpopulations of TILs, depending on the methodologies they used [15–27].

In the present study, we investigated the association between the density of monocyte TILs, CD3, CD4, CD8, CD20 lymphocyte subsets and CD68 and CD163 macrophages over clinicopathological features including MGMT-promoter methylation status and prognosis in 43 glioblastoma cases.

Future Medicine

Materials & methods

Study population

We examined patient files and pathology reports of the 43 glioblastoma cases who underwent neurosurgical resection at the Department of Neurosurgery at Instituto Nacional de Enfermedades Neoplasicas from January 2008 to July 2013. The histological diagnosis was established and verified by a neuropathologist (S. Casavilca) according to the 2007 WHO classification guidelines.

Tumor size was calculated based on preoperative MRI or CT scan as follows: longest diameter, widest diameter and thickness (number of layers). Clinicopathologic features were summarized in Table 1.

TIL evaluation through H&E

Evaluation of TILs was performed, as previously reported, through their distribution (focal, multifocal or diffuse) and intensity (mild, moderate or marked) as well as its presence in perivascular area (absent, mild or definite) through the whole slides of the 43 resected glioblastoma tissues [15,28]. The whole evaluation was performed by manual eyeballing by two institute pathologists (S Casavilca and J Sanchez).

Tissue microarrays & immunohistochemistry

Tissue microarrays and immunohistochemical staining were performed using the 43 glioma tissue samples as previously described [29]. Briefly, tissue microarrays were constructed with a tissue microarrayer (Quick-Ray Manual Tissue Microarrayer; Unitma Co. Ltd, Seoul, Korea). Each tumor was sampled from representative areas using a 6 mm punch, yielding composite array blocks comprising a total of eight tissue cores.

Paraffin-embedded specimens were cut into 3 μm sections. After deparaffinization with xylene and rehydration, antigen retrieval was performed by microwave treatment in 10 mmol/l sodium citrate buffer (pH 6.0) for 20 min. The endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Nonspecific binding was blocked for 10 min using a protein-blocking buffer. The sections were washed in phosphate-buffered saline. Diluted primary antibodies against CD3 (IS503, Dako, Glostrup, Denmark), CD4 (IS649, Dako), CD8 (IS623, Dako), CD20 (IS604, Dako), CD68 (IS613, Dako) and CD163 (clone MRQ-26; Bio SB, Inc., CA, USA) were applied to the tissue microarray and incubated overnight at 41°C. For negative controls, the primary antibodies were replaced by normal mouse serum. Human normal tonsil was used as positive control. Samples were then incubated with the horseradish peroxidase labeled secondary antibody in the immunohistochemical kit (DakoKIT-5930, MaxVision, Fu Zhou, PR China) for 30 min at room temperature. Diaminobenzidine was used for color development and hematoxylin as counterstain. Results were visualized and photographed under a light microscope (Olympus BX-63; Olympus Optical Co., Ltd, Tokyo, Japan). The immune cells were digitally quantified using TissueMorph software (Visopharm, Hoerlson, Denmark).

Evaluation was performed by examining each section using at least five different high-power fields (40 objective and 10 eyepiece) with the most abundant TIL areas. Percentage of infiltrating immune system cell was calculated by the rate of absolute number of positive staining cells/ total number of cells multiplied by 100. The whole process was supervised by two institute pathologists (S Casavilca and J Sanchez; Figure 1).

MGMT promoter methylation analysis by methylation-specific PCR

Methylation-specific PCR was performed to detect MGMT promoter methylation in 31 glioblastomas tumor samples. Briefly, for DNA extraction, six slides (10-μm-thick sections) from tissue samples were processed and purified according to the commercial GeneJET FFPE DNA Purification kit (Thermo Scientific, IL, USA). 1 μg of the DNA was denatured by NaOH and modified by sodium bisulfite by EpiJET Bisulfite Conversion Kit (Thermo Scientific, PA, USA), following the manufacturer's directions. Methylation-specific PCR was performed as described previously [30] using primer sequences for MGMT as follows: 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′ (forward) and 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′ (reverse) and methylated specific primers: 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′ (forward) and 5′-GCA CTC TTC CGA AAA CGA AAC G-3′ (reverse). In all reactions, a negative control was added, in which the sample was replaced with water. Positive methylation controls: *in vitro* methylated normal blood lymphocytes *in vitro* with CpG Methyltransferase. The PCR reaction products were analyzed by electrophoresis on agarose gels (6%) and stained with a solution of SYBR Safe DNA gel stain (Invitrogen, Life Technologies, CA, USA). The unmethylated or methylated DNA amplicon size was 93 bp and 81 bp, respectively.

Table 1. Clinical features and 3-year overall survival.

Features	n (%)	3-year OS	p-value
Age (years)			
Median (range)	47/(8–74)		0.273
<48	23 (53.5)	11.20%	
≥48	20 (46.5)	5.00%	
Gender			**<0.05**
Female	21 (48.8)	18.10%	
Male	22 (51.2)	4.50%†	
Karnofsky (%)			0.7
≤80	15 (34.9)	6.70%†	
>80	28 (65.1)	13.10%	
Seizures			0.454
No	29 (67.4)	8.50%	
Yes	14 (32.6)	7.10%	
Focal neurologic signs			0.76
No	21 (48.8)	17.10%	
Yes	22 (51.2)	5.00%‡	
Resection			**<0.05**
Subtotal	22 (51.2)	4.50%§	
Total	21 (48.8)	17.10%	
Tumor size (cm)			0.739
Median/range	5 (2–7)		
<5	19 (44.2)	7.90%	
≥ 5	24 (55.8)	8.30%	
Pathological subtype			–
Multiform	31 (72.1)	7.00%	
Small cells	2 (4.7)	50.00%	
Giant cells	2 (4.7)	0.00%	
Oligodendroglial component	4 (9.3)	25.00%	
PNET component	3 (7.0)	0.00%	
Gliosarcoma	1 (2.3)	0.00%	
Perivascular			0.794
Absent	31 (77.5)	8.10%	
Mild	3 (7.5)	33.30%¶	
Definite	6 (15.0)	16.70%	
TIL intensity			0.873
Mild	28 (71.8)	4.60%	
Moderate	10 (25.6)	10.00%	
Marked	1 (2.6)	0.00%	
TIL distribution			0.107
Diffuse	15 (38.5)	6.7%	
Focal	9 (23.0)	29.6%	
Multifocal	15 (38.5)	6.70%††	
Methylation status of MGMT promoter			**<0.001**
1	16 (51.6)	6.30%#	
2–3	15 (48.4)	13.30%	

†OS estimated at 21 months.
‡OS estimated at 25 months.
§OS estimated at 18 months.
¶OS estimated at 12 months.
††OS estimated at 22 months.
#OS estimated at 14 months.
‡‡OS estimated at 17 months.
OS: Overall survival; RPA: Recursive partitioning analysis; TIL: Tumor-infiltrating lymphocyte.

Table 1. Clinical features and 3-year overall survival (cont.).

Features	n (%)	3-year OS	p-value
RPA			0.142
3	15 (34.9)	18.70%	
4	28 (65.1)	3.60%	
Radiotherapy			**<0.001**
No	10 (23.3)	10.00%	
Yes	33 (76.7)	7.20%	
Chemotherapy			**<0.001**
No	14 (32.6)	7.10%	
Yes	29 (67.4)	8.10%	
CD3			0.295
<1.6%	21 (51.2)	11.40%	
≥1.6%	20 (48.8)	5.00%	
CD4			**<0.05**
<0.032%	19 (48.7)	15.80%	
≥ 0.032%	20 (51.3)	10.00%‡‡	
CD8			0.059
<1.6%	19 (51.4)	12.60%	
≥ 1.6%	18 (48.6)	5.60%	
CD20			0.43
<0.03%	19 (50)	6.60%	
≥ 0.03%	19 (50)	5.30%	
CD68			0.748
<9.1%	14 (50)	7.10%	
≥ 9.1%	14 (50)	10.70%†	
CD163			0.156
<2.2%	18 (50)	13.90%	
≥ 2.2%	18 (50)	5.60%	

†OS estimated at 21 months.
‡OS estimated at 25 months.
§OS estimated at 18 months.
¶OS estimated at 12 months.
††OS estimated at 22 months.
#OS estimated at 14 months.
‡‡OS estimated at 17 months.
OS: Overall survival; RPA: Recursive partitioning analysis; TIL: Tumor-infiltrating lymphocyte.

Statistical analysis

The age, preoperative KPS and tumor size were used as continuous variables whereas all the other covariates as categorical variables. Tumor resection was defined as follows: gross total resection and partial removal. MGMT promoter methylation status was dichotomised into methylation versus unmethylation.

Association between clinicopathological features and immune cell density was performed comparing median percentage of every immune cell: CD3, CD4, CD8, CD20, CD68 and CD163 through chi-square test of independence or Fisher's exact test. Cox regression was used to estimate different levels of hazard ratios according to the number of CD3-, CD4-, CD8-, CD20-, CD68- and CD163-positive cells in glioblastomas and adjusted for age, sex, preoperative Karnofsky performance status (KPS), tumor size, degree of resection and MGMT promoter methylation.

Progression-free survival was defined as the date of diagnosis to the first image confirming recurrence and overall survival (OS) as diagnosis of death or last visit at the clinic. Kaplan–Meier survival analysis was used to determine the distribution of OS and progression-free survival along the time, and the p-values were calculated using log-rank test. Immune cell levels were divided in higher or lower than the median value and a additional score combining the CD3 and CD8 levels regarding their median value (low vs high) was performed for evaluating the impact on OS.

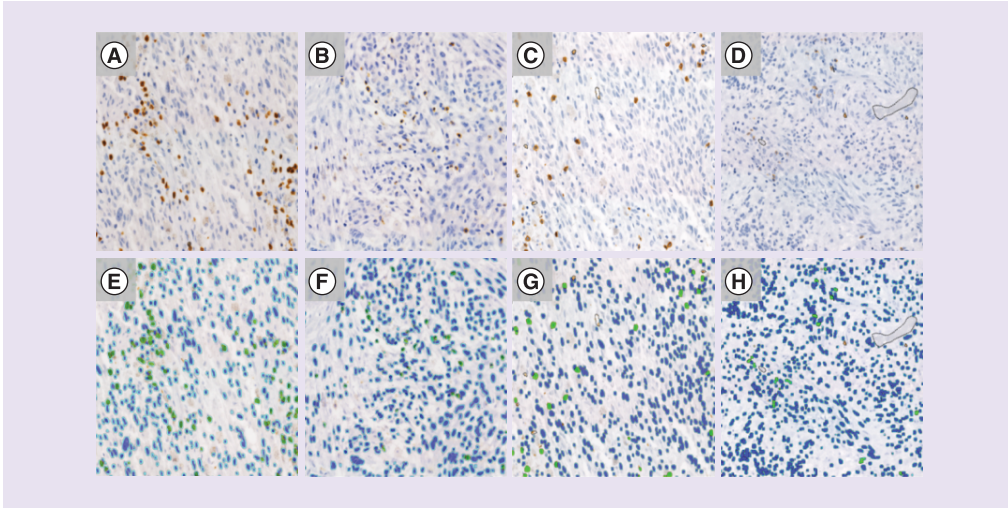

Figure 1. Tumor-infiltrating lyphocytes in glioblastoma. (A, B, C, D) $CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$TIL infiltration in glioblastoma. **(E, F, G, H)** Digital image analysis of $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ staining by TissueMorph software (Visiopharm) showing positive (green) and negative (blue) cells.

Results were performed by statistical softwares R and SPSS 20.0 (IBM, NY, USA). A two-tailed p-value of 0.05 was regarded as significant. Research methodology and information analysis were performed through strengthening the reporting of observational studies in epidemiology endorsement [31].

Results

Clinicopathological features

Clinical and pathological features of this glioblastoma cohort (n = 43) are shown in Table 1. The median age was 47 (range 8–74) years and 51.2% of patients were male. The Karnofsky scores at the diagnosis time were dichotomized at cutoff of 80 (high > 80 [65.1%]). Seizures were found in 32.6%. The median tumor size was 5 cm (range 2–7 cm). Complete resection was performed in 21 (48.8%) patients, both radiotherapy and chemotherapy were administered in 65.1% patients and only radiotherapy in 11.6%. Median survival after diagnosis was 14 months and 41 (95.3%) patients deceased during follow-up. Female sex ($p = 0.009$), level of resection ($p < 0.001$), MGMT promoter methylation ($p < 0.001$), radiotherapy ($p < 0.001$), chemotherapy ($p < 0.001$) and TIL CD4 level ($p < 0.001$) were significantly associated with OS in the Kaplan–Meier analysis. Age ($p = 0.273$), Karnofsky score ($p = 0.700$), RPA ($p = 0.142$) and tumor size ($p = 0.739$) were not associated with OS (Figure 2).

Tumor-infiltrating lymphocyte evaluation through H & E

The TIL intensity was assessed as mild (71.8%), moderate (25.6%) or marked (2.6%). The TIL distribution was classified as focal (23.1%), multifocal (38.5) or diffuse (38.5%). Perivascular TIL infiltration was absent (77.5%), mild (7.5%) or definite (15.0%). TIL intensity was not associated to age ($p = 0.060$), sex ($p = 0.798$), tumor size ($p = 0.956$), preoperative KPS ($p = 1.000$), MGMT-promoter methylation ($p = 0.406$) nor degree of resection ($p = 0.648$). TIL distribution was associated with gender ($p = 0.002$) but not with age ($p = 0.097$), preoperative KPS ($p = 1.000$), tumor size ($p = 0.916$), MGMT promoter methylation ($p = 0.775$) and degree of resection ($p = 0.773$). TIL presence in perivascular area was not associated with age ($p = 0.451$), sex ($p = 1.000$), preoperative KPS ($p = 0.453$), tumor size ($p = 0.133$), MGMT promoter methylation ($p = 0.236$) nor degree of resection ($p = 0.457$). Neither TIL distribution ($p = 0.107$), TIL intensity ($p = 0.873$) nor TIL presence in perivascular area ($p = 0.794$) was associated with OS (Table 2).

Infiltrating immune system cells evaluation through immunohistochemistry.

The median percentage of CD3, CD4, CD8, CD20, CD68 and CD163 in five high-power fields was 1.6% (0.1–19.1%), 0.032% (0–5.5%), 1.6% (0.016–12.1%), 0.03% (0–4.7%), 9.1% (2.2–42%) and 2.2% (0–26.5%), respectively (Figure 1).

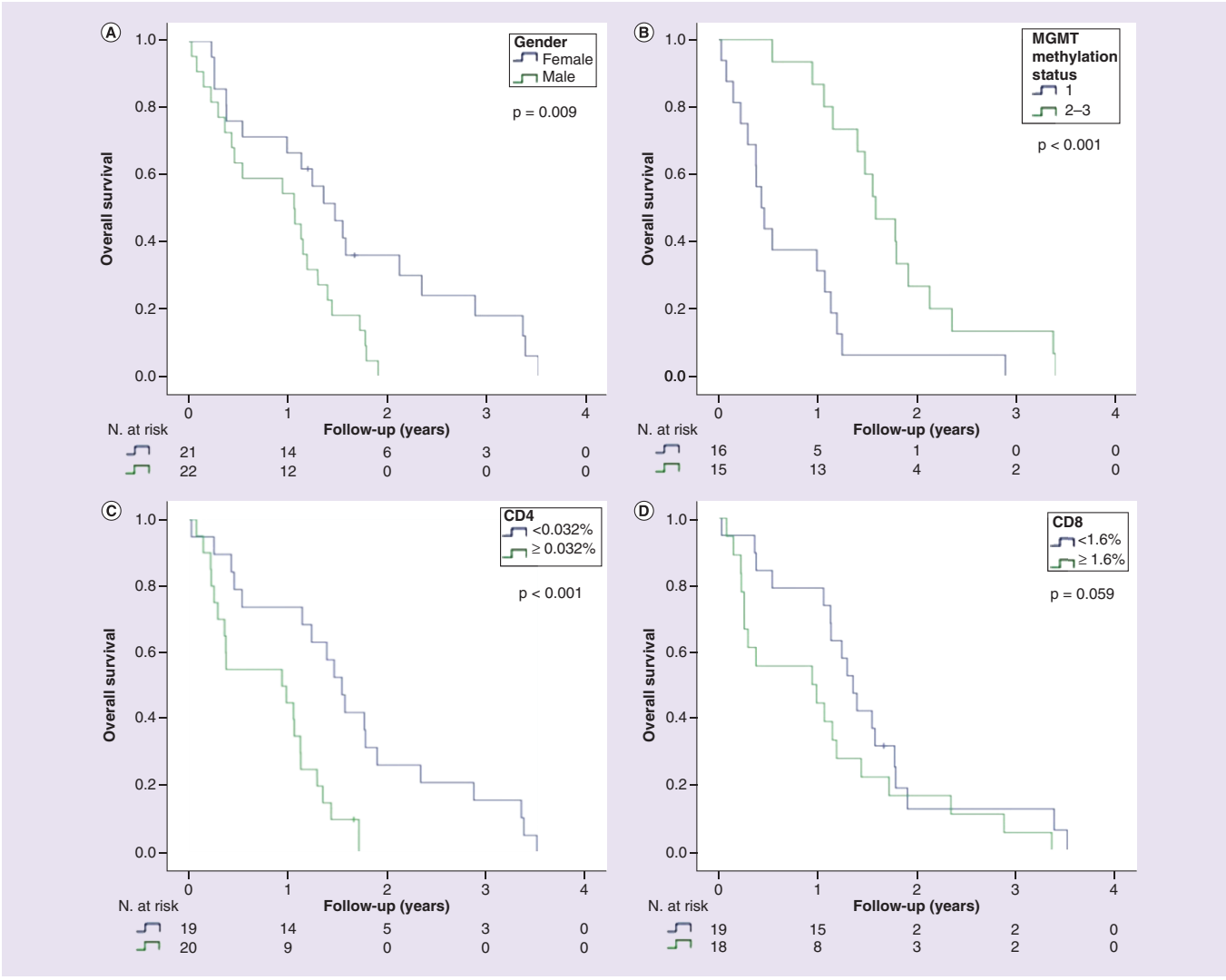

Figure 2. Clinicopathological features associated with survival. Estimated overall survival curve according to gender **(A)**, MGMT methylation status **(B)**, CD4 **(C)** and CD8 level **(D)**.

Level of CD4 TIL higher than the media was associated with unmethylated MGMT promoter ($p < 0.05$), level of CD8 TIL higher than the media was associated with larger tumor size ($p = 0.027$), level of CD20 TIL higher than the media was associated with presence of perivascular TIL infiltration and level of CD163 macrophages higher than the media was associated with older age and RPA = 4 (Table 3).

Level of CD3 ($p = 0.295$), CD8 ($p = 0.059$), CD20 ($p = 0.430$), CD68 ($p = 0.748$) and CD163 ($p = 0.156$) were not associated with overall OS. Only CD4 TIL was associated with OS in univariate analysis ($p < 0.05$) and was kept significant in multivariate analysis (< 0.05). Combination of CD3 (high vs low) and CD8 (high vs low) TIL confirmed that those with higher levels of both CD3 and CD8 TIL tend to have the shortest survival ($p = 0.287$) (Figure 2).

Discussion

Different clinicopathological features have been shown to have a significant association with longer survival [1,2,5–10]. Female gender, gross total resection and methylated MGMT status have been confirmed in our institutional cohort as prognostic factors.

In this study, we showed that presence of lymphocytes inside glioblastomas was rare. Therefore, slight and defined perivascular TILs were found in 7.5 and 15% cases, respectively. Moderate or high intensity was found in 28.2%

Table 2. Association of tumor infiltrating lymphocytes intensity, distribution and perivascular state with clinical features.

Features	Total n = 39[†]		p-value	Diffuse, n = 15 (%)	Total n = 39[‡]		p-value	Total n = 40[§]		p-value
	Mild, n = 28 (%)	Moderate + marked, n = 11 (%)			Focal, n = 9 (%)	Multifocal, n = 15 (%)		Absent, n = 31 (%)	Mild + definite, n = 9 (%)	
Age (years)			0.060				0.097			0.451
Median (range)	53 (98–74)	44 (21–68)		53 (36–73)	37 (13–74)	46 (8–72)		51 (8–74)	47 (37–73)	
<48	11 (57.9)	8 (42.1)		4 (21.1)	6 (31.6)	9 (47.4)		14 (70.0)	6 (30.0)	
≥ 48	17 (85.0)	3 (15.0)		11 (55.0)	3 (15.0)	6 (30.0)		17 (85.0)	3 (15.0)	
Gender			0.798				0.002			1.000
Female	14 (73.7)	5 (26.3)		10 (52.6)	7 (36.8)	2 (10.5)		16 (80.0)	4 (20.0)	
Male	14 (70.0)	6 (30.0)		5 (25.0)	2 (10.0)	13 (65.0)		15 (75.0)	5 (25.0)	
Karnofsky			1.000				1.000			0.453
≤ 80	10 (71.4)	4 (28.6)		6 (42.9)	3 (21.4)	5 (35.7)		12 (85.7)	2 (14.3)	
>80	18 (72.0)	7 (28.0)		9 (36.0)	6 (24.0)	10 (40.0)		19 (73.1)	7 (26.9)	
Resection			0.648				0.773			0.457
Subtotal	13 (68.4)	6 (31.6)		8 (42.1)	5 (26.3)	6 (31.6)		16 (84.2)	3 (15.8)	
Total	15 (75.0)	5 (25.0)		7 (35.0)	4 (20.0)	9 (45.0)		15 (71.4)	6 (28.6)	
Promotor MGMT			0.406				0.775			0.236
1	13 (81.2)	3 (18.8)		7 (43.8)	3 (18.8)	6 (37.5)		13 (81.2)	3 (18.8)	
2/3	8 (61.5)	5 (38.5)		6 (46.2)	1 (7.7)	6 (46.2)		8 (57.1)	6 (42.9)	
Tumor size (cm)			0.956				0.916			0.133
Median (range)	5 (2–7)	5.1 (3–7)		5 (3.3–7)	4 (2–5.3)	5 (3.2–7)		4.7 (2–7)	5.9 (3.3–6.3)	
<5 cm	13 (72.2)	5 (27.8)		6 (33.3)	5 (27.8)	7 (38.9)		17 (89.5)	2 (10.5)	
≥ 5 cm	15 (71.4)	6 (28.6)		9 (42.9)	4 (19.0)	8 (38.1)		14 (66.7)	7 (33.3)	

[†]Four cases do not specify TIL intensity category.
[‡]Four cases do not specify TIL distribution category.
[§]Three cases do not specify perivascular state.
TIL: Tumor-infiltrating lymphocyte.

and diffuse TIL distribution was found in 38.5%. However, no association with clinicopathological features was found.

Bertrand and Mannen *et al.* were credited as the first to describe TIL presence in gliomas and found that 63 out of 172 (36.6%) astrocytoma cases harbored perivascular lymphocytes, however, most of these cases were weak and were not associated with a better outcome [24].

The follow-up studies with less than 100 gliomas cases confirmed that 28–31% gliomas cases harbored perivascular infiltrating lymphocytes, although no correlation between the presence of TILs and the clinical prognosis was found [18,25,32–34].

TIL subpopulations contained more T-lymphocyte subtype (median CD3: 1.6%) than B-lymphocyte subtype (median CD20: 0.03%), and almost all T-lymphocytes were CD8+. Macrophages were more frequent than lymphocytes (median CD68: 9.1%) and activated macrophages represented almost a third of them (median CD163: 2.2%) in our glioblastoma patient cohort. In our study, necrosis was ubiquitously present in glioblastoma, and necrotic tissues have been previously described as highly infiltrated by macrophages. This association could explain the relatively high density of macrophages in our glioblastoma series.

Compared with other reported malignancies, the densities of immune cell subpopulation in our study were low, which can be explained by the blood–brain barrier and specific local microenvironmental features. These low densities makes it necessary to select only areas with TIL conglomerates for subpopulation analysis [13,14].

The low CD3 TILs were associated with larger tumor size, the low CD4 TILs were associated with methylated MGMT promoter status and low CD8 TILs had a trend to be associated with methylated MGMT promoter status in our univariate analysis (and CD4 in multivariate analysis). Consistent with the association between methylated

Table 3. Relationship between immune cell concentration (%) and clinicopathological features.

Features	CD3 (n = 41)	CD4 (n = 39)	CD8 (n = 37)	CD20 (n = 38)	CD68 (n = 28)	CD163 (n = 36)
Median (range)	1.59 (0.1–19.1)	0.032 (0–5.5)	1.6 (0.016–12.1)	0.03 (0–4.7)	9.06 (2.2–42)	2.22 (0–26.5)
Age (years)	n = 41 NS	n = 39 NS	n = 37 NS	n = 38 NS	n = 28 NS	n = 36†
– <48	1.59/(0.1–15.6)	0/(0–3.1)	1.62/(0–11.4)	0.06/(0–3.5)	9.56/(3.1–42)	1.44/(0–15.6)
– ≥ 48	1.69/(0.2–19.1)	0.04/(0–5.5)	1.59/(0.1–12.1)	0.02/(0–4.7)	7.58/(2.2–37)	3.54/(0–26.5)
Gender	n = 41 NS	n = 39 NS	n = 39 NS	n = 38 NS	n = 28 NS	n = 36 NS
– Female	1.21/(0.1–15.6)	0.01/(0–5.5)	1.59/(0–10.2)	0.03/(0–0.4)	5.94/(2.2–37)	1.45/(0–26.5)
– Male	2.19/(0.1–19.1)	0.14/(0–3.1)	1.64/(0.1–12.1)	0.03/(0–4.7)	11.07/(4–42)	3.51/(0–16.3)
Karnofsky	n = 41 NS	n = 39 NS	n = 37 NS	n = 38 NS	n = 28 NS	n = 36 NS
– ≤80	1.14/(0.1–12.8)	0.03/(0–3.1)	0.98/(0.1–9)	0.03/(0–3.5)	7.95/(2.2–37)	3.6/(0–13)
– >80	2.08/(0.1–19.1)	0.03/(0–5.5)	1.88/(0–12.1)	0.04/(0–4.7)	10.3/(3.1–42)	1.51/(0–26.5)
Resection	n = 41 NS	n = 39 NS	n = 37 NS	n = 38 NS	n = 28 NS	n = 36 NS
Subtotal	2.08/(0.1–19.1)	0.09/(0–5.5)	1.84/(0.1–11.9)	0.04/(0–4.7)	9.48/(4.3–42)	3.54/(0–15.6)
Total	1.21/(0.1–15.6)	0/(0–1.9)	0.53/(0–12.1)	0.02/(0–0.4)	8.63/(2.2–31.2)	1.58/(0–26.5)
Tumor size (cm)	n = 41 NS	n = 39 NS	n = 37†	n = 38 NS	n = 28 NS	n = 36 NS
<5	1.12/(0.1–19.1)	0.04/(0–1.1)	0.98/(0.1–11.9)	0.02/(0–4.7)	8.63/(2.2–37)	1.01/(0–13)
≥5	2.96/(0.1–15.6)	0.03/(0–5.5)	2.19/(0–12.1)	0.04/(0–1)	10.3/(2.9–42)	2.69/(0–26.5)
Methylation status of MGMT promoter	n = 30 NS	n = 28†	n = 26 NS	n = 28 NS	n = 20 NS	n = 25 NS
– 1	3.27/(0.1–13.4)	0.04/(0–5.5)	1.88/(0.2–12.1)	0.1/(0–4.7)	11.46/(2.2–42)	4.63/(0.2–15.6)
– 2/3	1.2/(0.1–19.1)	0/(0–1.5)	0.32/(0.1–11.9)	0.01/(0–0.4)	8.63/(3.1–31.2)	1.37/(0–26.5)
RPA	n = 41 NS	n = 39 NS	n = 37 NS	n = 38 NS	n = 28 NS	n = 36†
– 3	1.52/(0.1–15.6)	0/(0–1.6)	2.05/(0–11.4)	0.07/(0–0.4)	10.3/(3.1–42)	0.35/(0–15.6)
– 4	1.59/(0.1–19.1)	0.09/(0–5.5)	1.59/(0.1–12.1)	0.03/(0–4.7)	7.95/(2.2–37)	3.6/(0–26.5)
Perivascular	n = 38 NS	n = 36 NS	n = 34 NS	n = 35†	n = 26 NS	n = 33 NS
– Absence	1.12/(0.1–19.1)	0.03/(0–5.5)	0.98/(0–12.1)	0.02/(0–1)	7.77/(2.2–37)	2.22/(0–16.3)
– Presence	4.63/(0.4–15.6)	0.27/(0–1.6)	3.15/(0.1–10.2)	0.35/(0–4.7)	17.34/(3.9–42)	2.72/(0–26.5)

†Significant at the level $p < 0.05$.
NS: Not significant; RPA: Recursive partitioning analysis.

MGMT and longer survival, we found that lower levels of CD4 TILs were predicted and lower levels of CD8 also had a trend of predicting better patient outcomes in our study.

Farmer *et al.* demonstrated that $CD8^+$ and $CD4^+$ TILs consisted of 41 and 42% of TILs in nine high-grade gliomas, respectively [35].

Hitchcock and Morris assessed the presence of TILs through immunohistochemistry in five low-grade and 15 high-grade astrocytomas and found that both low- and high-grade tumors harbored an average of 4.5–4.2%, 9.6–9.4% and 26–36% of $CD4^+$, $CD8^+$ and macrophages, respectively [36].

Kuppner *et al.* evaluated TILs through immunohistochemistry in seven glioblastoma specimens and found scarce lymphocytes. Absence of slight, moderate and markedly intense of CD3 staining were found in four specimens, one and two specimens, respectively. Absence of rare and moderate intense of CD4 expression was found in five and two specimens, respectively. Absence of rare, moderate and markedly intense CD8 expression was found in four, two and one specimen, respectively [37].

El Andaloussi and Lesniak studied TILs in ten glioblastomas and six control brain specimens. The analysis of single cell tumor suspensions by Flow cytometry revealed that lymphocytes represented approximately 17% (12–21%) of cells whereas less than 1% was found in control brain samples. $CD3^+$ TIL were 25% and approximately 6% were $CD4^+$ $CD25^+$ and displayed a tumor suppressor function *in vitro*. Of the $CD4^+$ cells in TILs, 55% expressed FoxP3, a Treg-specific protein critical to regulatory T-cell development and function [19].

Hussain *et al.* evaluated 50 glioblastoma tumors and found that the predominant immune cells were microglia and macrophages. They also demonstrated that infiltrating $CD8^+$ TIL were phenotypically $CD25^-$ and most $CD4^+$ TIL were $CD25^+$ $FOXP3^+$ [38].

Consistent with our results, Kim *et al.* evaluated 67 glioblastomas cases and found that CD8 were more frequent than CD4 (6.8 vs 1.5 cells in average). They found a relationship between high levels of CD8 TIL and longer survival, but not for $CD3^+$ TIL [22].

Sayour *et al.* evaluated 57 glioblastoma cases and found that the CD8 TIL absolute count was more than three-times than the $CD4^+$, and FoxP3 TIL represented less than a third of $CD4^+$ TIL. Absolute numbers of TIL did not predict outcome, but the increased ratio of CD3 and CD8 over FoxP3 TIL was positively correlated with survival outcomes [39].

Yang *et al.* evaluated 108 glioblastoma cases and found that higher $CD8^+$ TIL cells are associated with longer survival (intermediate or extensive T-cell infiltrates in long-term survivors vs short-term survivors was 38 vs 20%, respectively; $p < 0.006$) [40].

Madkouri *et al.* evaluated 186 glioblastoma tumors and found that CD163 macrophage infiltrates were highly and homogeneously stained and $CD8^+$ TIL infiltrates were less frequent and preferentially located in perivascular area. Higher $CD8^+$ TIL infiltrates were associated with good prognosis [41].

Differences in the effect of $CD4^+$ TIL presence in previous glioblastoma studies and our results are probably the result of their double-edged immunological sword. On one hand, CD4 helper T cells perform critical roles in the recruitment, activation and regulation of many facets of the adaptive immune response including activation of CD8 TIL cells [42]. Therefore, the presence of CD4 helper T cells has been associated with better survival in different malignancies including breast cancer. On the other hand, CD4 Tregs can dampen anti-tumor immunity and promote tumor progression [43]. Tregs are unfavorable prognostic markers in patients with breast cancer [44], hepatocellular carcinoma [45] and pancreatic cancer [46]. Our findings of an inverse association between CD8 TIL density and survival coincide with the trend of inverse association with MGMT methylation status. This could indicate that tumor epigenetic alterations can modulate anticancer immune activity.

In conclusion, tumor infiltrating immune cells are not common in glioblastomas and demonstrate that immune factors like the density of infiltrating lymphocyte subsets can affect the outcome of glioblastoma patients with current standard therapies.

Future perspective

Many strategies are currently being developed for modulating host immune activity against different malignancies; however, they are active only in some patients, carry toxicities and are expensive. We expect that more effective immunomodulators will be developed in the near future and will demonstrate anticancer activity in some glioblastoma cases. However, it will be necessary to identify those patients who will obtain the best benefit from these agents, and a signature combining levels of immune cells could be a predictive and prognosis biomarker. An objective methodology to quantify infiltrating immune cell like that used in this research is expected to be standard in the close future.

Summary points

- Median survival in glioblastoma is short.
- Clinical features can identify glioblastoma patients with longer survival.
- Density of immune cell inside glioblastoma lesion is low.
- Most frequent immune cell in glioblastoma is macrophages.
- Density of CD8 and CD163 was associated to aggressiveness features and density of CD4 to survival.

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Ethical conduct of research

The conduct of this survey was approved The Institutional Review Board of INEN (#051–2016-CRP-DI-DICON/INEN), and written informed consent was obtained from each glioma tissue donor who agreed to the use of the tumor tissue and clinical data for future research when possible.

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