Original Article

Histologic characterization of the immune infiltrate in isocitrate dehydrogenase wild-type and mutant World Health Organization Grade II and III gliomas

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Abstract

Aim: This study aims to describe the immune infiltrations in low-grade glioma (LGG) with respect to their histological classification, isocitrate dehydrogenase 1 and 2 (IDH1/2) mutation status and survival. **Materials and Methods:** The IDH1/2 status (mutant or wild-type) of 66 World Health Organization Grade II and III gliomas were defined using next-generation sequencing or multiplex ligation-dependent probe amplification. The immune infiltrates of these tumors (46 mutant IDH, 20 wild-type IDH) were assessed immunohistochemically using a panel of antibodies (CD3, CD4, CD8, FOXP3, CD20, CD68, and CD163). Confirmatory analyses were performed on a cohort of lower grade gliomas from the Cancer Genome Atlas (TCGA). Statistical analyses were performed with Mann–Whitney *U*-tests and Kaplan–Meier survival estimates. **Results:** There was no relation between the amount of CD3+, CD4+, CD8+, or CD20+ lymphocyte infiltration and IDH mutation status in the tumors. FOXP3+ T regulatory cell infiltrates were rare, but more frequent in IDH1/2 wild-type tumors ($P = 0.046$). While the presence of these cells did not correlate with overall survival, *FOXP3* messenger RNA expression was associated with survival in a distinct cohort of LGG from the TCGA ($P < 0.05$). CD4+ lymphocyte infiltrates, on the other hand, tended to prevail in astrocytic tumors as compared to oligodendrogliomas ($P = 0.056$). While CD68 (M1) microglial/monocytic cells were equally abundant in IDH mutant and wild-type tumors, the presence of round, activated M1 CD68+ microglia significantly associated with a mutant IDH status ($P = 0.015$). **Conclusion:** FOXP3+ expression and activated CD68+ M1 cells associated with IDH status in LGG, and might contribute to their differential evolution.

Keywords: Immune cell infiltration, isocitrate dehydrogenase mutation, low-grade glioma, microglia

Introduction

The immune system, as a substantial component of the tumor microenvironment, has emerged as an integral player in the aggressiveness of glioma, and as a potential therapeutic target against the more malignant forms of these tumors.[1-3]

Isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations also play a central role in the biology of gliomas and define subcategories of tumors with a generally better prognosis than those that maintain a wild-type form of these enzymes.[4,5]

Recent reports suggest that mutated IDH may affect lymphocytic and monocytic immune cell infiltration into tumor tissue.[6-8] Wild-type tumors appear to attract more (regulatory) lymphocytes and express the checkpoint inhibitor programmed death ligand 1 (PDL-1) to higher levels than their mutant counterparts. These findings are however based on a heterogeneous series of lower grade World Health Organization (WHO II and III) and (mostly) Grade IV tumors and have primarily focused on the T-lymphocytic component of the immune infiltrates.

Using messenger RNA (mRNA) expression deconvolution techniques to study the immune signature of low-grade

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glioma (LGG) samples rather than histology, Gao *et al*.[9] also found that LGGs (IDH wild-type and mutant) from the EORTC 22033–26033 trial presented different immunophenotypic score (IPS) values (a score derived from immunophenotyping that is associated with response to checkpoint inhibitors in melanomas). They however did not attempt to correlate these findings with histological characterization of the infiltrates.

Altogether, the influence of IDH mutations on the nature of the immune infiltration in LGGs, and its potential role in the progression of these tumors, still remain to be defined in cohorts of LGGs (II and III). In this work, we set out to describe the immune infiltrate of a series of fully characterized 66 LGGs obtained from our surgical practice, and correlate it to the IDH and 1p/19q co-deletion status of the tumors and their prognosis.

Materials and Methods

Specimens/patient population

This study was carried out after approval by the Institutional Review Board (IRB 16–342). According to Dutch Regulations and the Ethical Committee of the University Medical Center of Utrecht, this retrospective study of archival tissue samples did not require the informed consent of the patients. A total of 267 adult patients (≥18 years of age) were operated (resection or stereotactic biopsy) for a LGG (WHO 2016 Grade II or III) at the university medical center of Utrecht between January 2011 and December 2016 (excluding 14 patients with pleomorphic xanthoastrocytoma, ganglioglioma, anaplastic pilocytic astrocytoma, and granular cell astrocytoma). The IDH ^{R132H} mutation status of these tumors was systematically analyzed by immunohistochemistry (IHC) between 2011 and 2014. Since October 2014, 70 tumors were analyzed for both IDH1 and IDH2 mutations by targeted next-generation sequencing (NGS) analysis using the Cancer Hotspot Panel and Ion Torrent Personal Genome Machine (Thermo Scientific). Twelve patients were also analyzed by multiplex ligation-dependent probe amplification (MLPA) for IDH1 and IDH2 mutations. Of this, a total of 82 genetically characterized patients, 9 were excluded because their index surgery had been performed at progression following a previous surgery (n = 5) or a prior cytotoxic treatment (n = 4). Formalin-fixed, paraffin-embedded tissue was unavailable for an additional 7 patients. As a result, a total of 66 patients were fully characterized by NGS or MLPA with respect to their IDH1 and IDH2 mutational status.

Fifty-three of the 66 tumors were also assessed for 1p19q deletion by MLPA, with a threshold for co-deletion set at >93% loss of all probes on both arms (kit P088; MRC-Holland, Amsterdam, The Netherlands). According to the 2016 WHO classification of brain tumors, co-deleted tumors were considered in the present paper as oligodendrogliomas, while all *IDH* wild-type gliomas and mutant *IDH* gliomas with intact or partially deleted 1p19q were labeled as astrocytomas. Clinical characteristics, progression-free survival (defined as the period between the date of diagnosis and first radiographic progression), and overall survival were obtained through a retrospective review of the medical records of the patients up to November 1, 2017.

Immunohistochemistry and staining procedure

The presence of tumor-infiltrating immune cells was analyzed on serial 4 μm sections of formalin-fixed paraffin embedded (FFPE) tissue blocks of each tumor. Representative areas of these blocks were selected by the senior pathologist (whole slide), based on the hematoxylin and eosin staining. The following antigens were used to define the subpopulations of the immune infiltrates: FOXP3 (Abcam ab20034): Regulatory T-cells; CD20 (Dako M755): B-cells; CD4 (Cell Marque104R-16): T4 lymphocytes; CD8 (Dako M7103): T8 lymphocytes; CD68 (Novocastra NCL-CD68-KP1): M1 monocytes/microglia; CD163 (Novocastra NCL-CD163): M2 monocytes/microglia.[10] FFPE samples were processed as follows: Antigen retrieval was achieved by incubation in citrate buffer for 12 min at 126°C. Following incubation with the primary and secondary antibodies, the signal was developed with 3,3'-diaminobenzidine. Nuclear counterstaining was performed with hematoxylin.

Applying a semi-quantitative scoring system [Table 1], infiltration of CD68+ and CD163+ cells was assessed independently by two investigators, one of whom is a senior neuropathologist. Scores of the remaining immune infiltration were assessed by one investigator and were randomly confirmed by the senior neuropathologist.

Statistical analysis

The relationship between marker expression and *IDH* mutation status was analyzed by Mann–Whitney tests for ordinal and interval IHC scores. Correlations between populations of infiltrating cells and patient survival were examined using Kaplan–Meier survival estimates with Gehan–Breslow

Table 1: Semi-quantitative scoring charts used for the histologic evaluation of tumor-associated immune cell infiltration

	0	1	2	3	4
T/B-cells	0 positive cells	Up to 1 perivascular layer positive cells	>1 perivascular layer of positive cells and/or an infiltrative pattern	>1 perivascular layer and 1–5 parenchymal cells	>1 perivascular layer and parenchymal clusters of positive cells
Microglia parenchymal increase (×30 magnification, 568 × 313 μ)	0 positive cells	1–15 positive cells	15–30 positive cells	>30 positive cells	N/A
Microglia parenchymal activation (×30 magnification, 568 × 313 μ)	0 positive cells or ramified type only	Partly ramified, partly activated (phagocytic and/or Gitter type)	Activated MG only (phagocytic and/or Gitter type)	N/A	

N/A: Not available, MG: Microglia

Generalized Wilcoxon analyses. The analyses were performed using SPSS 25.0 software (IBM Nederland, Amsterdam, The Netherlands).

Results

Sample characterization

Of the 66 tumors, 45 had an IDH1 mutation (43 IDH^{R132H}, 2 IDH^{R132C}) and one with an $IDH2^{R172\ L}$ mutation. Ten IDH mutant tumors showed a complete co-deletion of 1p and 19q. Three tumors with *IDH* mutations had not been assessed for 1p/19q co-deletion. Altogether, 53 tumors could be classified as astrocytomas (37 Grade II and 16 Grade III) and 10 as oligodendrogliomas (7 Grade II and 3 Grade III) [Table 2].

Tumor-infiltrating lymphocytes

CD4+ T-cells were found in 51/65 (78%) of the specimens [Table 3]. They were equally distributed between *IDH* mutant and wild-type tumors but tended to be more abundant (higher IHC score) in astrocytic tumors compared to oligodendrogliomas (P = 0.056, Mann–Whitney U-test). CD8+ T-cells infiltrated 47/65 (72%) tumors, irrespective of their histology or IDH1 mutation status. FOXP3+ regulatory T-cells were observed in only 4/66 (6%) tumors, all of which were astrocytomas. They were seen more often in wild-type than in mutant *IDH* tumors (P = 0.046, Mann–Whitney U-test). CD20+ B-cells were present in 24/66 (36%) of the tumors and did not show any preference for specific histologies.

CD68+ M1 monocytic/microglial cells were found in 63/66 (95%) tumors but at similar densities. There was a preponderance of active, phagocytic and/or Gitter cells in IDH mutant tumors [P = 0.015, Mann–Whitney U-test, Figure 1]. A significant difference in the activation of CD68 cells (M1 phenotype) was found in the astrocytoma tumors (P = 0.010, Mann–Whitney U-test). M2-phenotype CD163+ monocytic/microglial cells were restricted to 31/66 (47%) of the samples and did not show any different patterns of infiltration or activation among the tumor types or *IDH* status.

Survival analyses

The presence of FOXP3 positive cells within the tumors did not correlate with overall survival. This held true for both the complete series of patients and for the astrocytomas, to which these cells seemed to be restricted to. Given the small number of patients with FOXP3+ infiltrates in our series, we assessed survival of a supplementary cohort of 27 Grade II and III gliomas from the Cancer Genome Atlas (TCGA) repository (TCGA Research Network: http://cancergenome.nih.gov/), for whom the Agilent mRNA expression data of FOXP3 were available. Patients were stratified on the basis of a median split of their mRNA expression data (downloaded on May 3, 2014, and updated on December 7, 2018, for the corresponding clinical data). In this series of patients, tumors with a higher expression of FOXP3 presented with a poorer survival than those with a lower expression [Kaplan–Meier survival estimates, $P < 0.05$, Breslow test, $P < 0.05$, Figure 2].

No other characteristics of the immune infiltrate including activated M1 cell infiltrates (Activated CD68+ cells) and CD4+ lymphocytic infiltrate-correlated with overall survival. This held true for both histologic subtypes, as well as in multivariable analysis. More intense CD4 infiltrates tended

Table 2: Clinical characteristics of 66 low-grade glioma subjects including glioma subtype, WHO grade, and age

	Grade II	Grade III	Total	Median age at diagnosis (range)
IDH-mutant tumors	32	14	46	45 (18–83)
IDH-mutant astrocytic tumors	23	10	33	45 (18–83)
IDH-wild type (astrocytic) tumors	14	6	20	50.5 (33–78)
Oligodendrogliomas	7	3	10	48.5 (28–62)

IDH: Isocitrate dehydrogenase

Table 3: Scoring results of CD4+, CD8+, CD20+, CD68+, CD163+, and FOXP3+ -stained lower grade diffuse glioma sections

Antigen	Total (%)	Feature	Frequency (%)				
			0	1	2	3	4
FOXP3	4/66 (6.1)		62 (93.9)	4 (6.1)			
CD4	51/65 (78.5)		14 (21.5)	34 (52.3)	10 (15.4)	2 (3.1)	5 (7.7)
CD8	47/65 (72.3)		18 (27.7)	38 (58.5)	5 (7.7)	1 (1.5)	3 (4.6)
CD20	24/66 (36.4)		42 (63.6)	18 (27.3)	6 (9.1)	0 (0)	0 (0)
CD68	63/66 (95.5)	Increase	3 (4.5)	4 (6.1)	28 (42.4)	31 (47.0)	N/A
		Activation	16 (24.2)	38 (57.6)	12 (18.2)	N/A	N/A
CD163	31/66 (47.0)	Increase	35 (53.0)	20 (30.3)	2 (3.0)	9 (13.6)	N/A
		Activation	30 (46.9)	30 (46.9)	4 (6.3)	N/A	N/A

Total (%): Number of positive cases in each category (score ≥1)/total number of stained cases (percentage), Frequency (%): Number of positive cases for each score value, range 0-4 (lymphocytes), and range 0-3 (monocytes/microglia). N/A: Not available

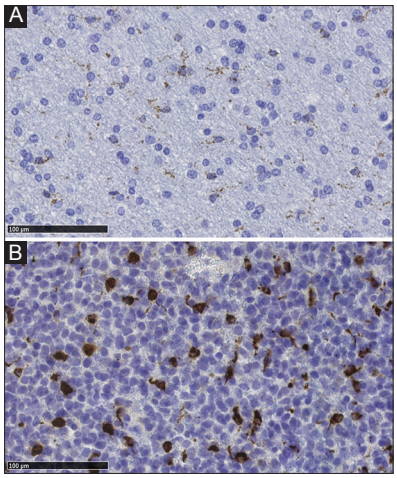

Figure 1: CD68 antigen (brown) staining in low-grade diffuse gliomas, at ×30 magnification. (A) Infiltration of mostly ramified CD68+ in isocitrate dehydrogenase wild-type astrocytoma; (B) Infiltration of active CD68+ in isocitrate dehydrogenase mutant astrocytoma

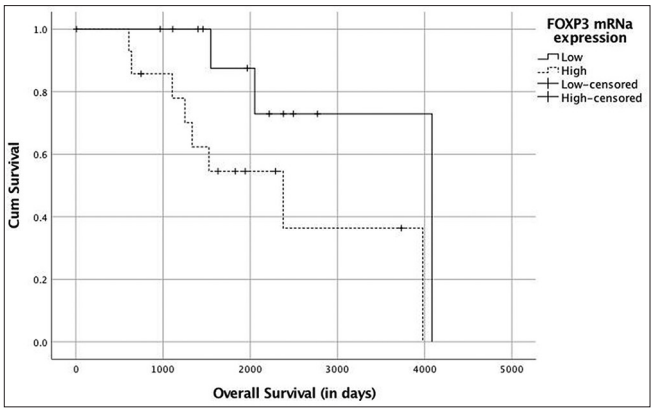

Figure 2: Kaplan–Meier survival estimates of low-grade gliomas of the cancer genome atlas stratified with respect to their expression of FOXP3+ messenger RNA in the tumor ($P = 0.044$)

to associate with a better progression-free survival ($P = 0.098$, univariate Cox analysis). In these analyses, only *IDH* mutation showed a significant positive association with progression-free survival.

Discussion

Here, we described the immune infiltrate in a series of 66 LGGs and correlated this to the *IDH 1*/2 and 1p/19q co-deletion status of the tumors and their prognosis.

FOXP3 expressing lymphocytes play a major role in the immune tolerance of tumors.[11] We found these cells to be restricted to a minority of tumors, which correlated with *IDH* wild-type status. Interestingly, we also found an association between FOXP3 expression and dismal prognosis in an independent cohort of Grade II and Grade III gliomas from the TCGA repository. These results suggest that immunosuppressive Treg cells may play a role in the progression of LGGs and might help predict their prognosis. In support of this view, Gao *et al*.[9] observed higher IPS scores (an immune gene expression signature that predicts the efficacy of checkpoint inhibitors) in *IDH* wild-type LGGs. Likewise, Berghoff *et al*.[7] observed an increased expression of the checkpoint regulator PDL-1 in *IDH* wild-type gliomas as compared to their mutant counterparts.

A relationship between the presence of IDH1/2 mutations and the presence of the other components of the lymphocytic immune cell tumor infiltration in gliomas has been suggested. As a general feature, these reports unanimously demonstrate a repressed lymphocyte recruitment in case of an *IDH* mutation.[6,7] In our series of tumors, however, we did not observe any correlation between the *IDH* mutational status and these infiltrates. This discrepancy can stem from the differential degree of homogeneity of the tumor series and analysis methods between the previous reports and ours. Our cohort of patients is indeed the largest published to date that only comprises of low-grade tumors. Interestingly, we observed denser CD4 + infiltrates in astrocytic tumors than in oligodendrogliomas, suggesting that these histological subtypes may elicit different forms of immune responses. This trend did nevertheless not reach statistical significance and needed to be confirmed in independent cohorts of patients.

Contrary to previous hypotheses, we did not find any difference in the distribution or activation of pro-oncogenic, inflammatory, and immunosuppressive M2 monocytic/microglial cell infiltrates between *IDH* wild-type and mutant tumors.[2] However, we found a significant association between *IDH* mutations and the morphology of CD68+ microglia: Active, phagocytic and/or Gitter cells were especially found in *IDH* mutant tumors whereas quiescent, ramified cells were found in *IDH* wild-type tumors. Activated M1 monocytic/microglial cells are immune-supportive and help the immune system fight neoplasms, and could contribute to the favorable prognosis and longer survival of *IDH* mutant tumors. If confirmed, this finding could pave the way to potentially novel therapeutic solutions against *IDH* wild-type tumors.

Our study has several limitations. First, the retrospective nature of data collection is vulnerable to selection bias, which we attempted to limit by using all available Grade II and III gliomas with a genetically defined IDH1 and IDH2 molecular status in our tissue repository. Second, variability in interpretation of IHC stains using a semi-quantitative approach to define the immune infiltrate is vulnerable to subjectivity, as the interpretations depend not only merely on the acuity of the investigator's eye and experience but also the staining methods and process.[12] We attempted to reduce this variability by integrating the scores of two independent observers regarding the infiltration of CD68+ and CD163+ cells, and regarding lymphocytes by random confirmation by the senior neuropathologist and by discussing (and resolving) any disagreement. Another limitation is the fact that the investigated tissue blocks or biopsies do not always represent the whole tumor and tumor-infiltrating cell density might vary between different areas of the tumor. Investigating only a

portion of tumor tissue might thus have influenced the results. Finally, the high percentage (75%) of censored patients, due to the favorable prognosis of LGGs and our short follow-up, decreases the power of our overall survival analyses.

Here, we find that IDH mutations associate with a more activated state of M1 monocyte/microglia cells and reduced infiltration by FOXP3+ cells. This suggests that the balance between immunosuppressive Treg cells and immune-supportive CD68+ Gitter cells play a key role in the progression of LGG. Whether the infiltration of LGGs by FOXP3+ and activated CD68+ M1 cells can be targeted to prevent the progression of these tumors or can be used as a clinically relevant prognostic tool deserves further research in a larger cohort study.

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Conflicts of interest

There are no conflicts of interest.

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