nature portfolio

Peer Review File

Turning attention to tumor–host interface and focus on the peritumoral heterogeneity of glioblastoma

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Wang et al. investigate the tumor microenvironment (TME) in the peritumor brain zone (PBZ), a region that is known for glioma recurrence. Multi-regional sampling of the PBZ was based on preoperative MRI, intraoperative surgical navigation with HoloLens glasses, and microscopic imaging. Two regions in the PBZ zones were identified by differences in cerebral blood flow (CBF) with higher CBF zone noted as “germinal interface (GI)” and lower CBF zone noted as “other peritumoral interfaces (OPI). Single cell analyses comparing these two zones show that GI exhibited more neovascularization, increased infiltration of macrophages and T-lymphocytes and increased “stem-like” tumor cells. Overall, the authors concluded that the GI zone is the region for GBM invasion and recurrence and potential critical area for PBZ-targeting therapy after surgical resection. There were some findings in the manuscript that build upon the characterization of the tumor microenvironment in glioma from what is already known. However, there were some aspects of novelty that are important for the field including the identification of heterogenous regions of the PBZ zone and cellular composition of the TME that may contribute to the aggressiveness / progression of recurrent diseases. However, several weaknesses are noted that should be addressed to enhance the novelty and clarity of the results.

1. Figure 1 & Supplemental Figure 1: The authors begin by sharing multiparametric MRI slices where they delineate a peritumoral brain zone and argue for the existence of a special “germinal interface” within the PBZ. The primary feature of the GI is an increased cerebral blood flow. For one, the nomenclature here is misleading. Nowhere in the paper is there evidence that seeding, or selection of tumor clones occurs in this region named “germinal interface”. Besides this, there is insufficient evidence in the early figures that this region of elevated CBF can be robustly identified across patients. This figure would be strengthened by including volumetric analyses of this region of elevated CBF across multiple patients. The authors should measure the volume of the elevated CBF region and calculate: A. the proportion of the PBZ that is occupied by the elevated CBF region across multiple scans. B. the proportion of the elevated CBF zone to tumor volume across multiple scans.
2. The CD31 stain in figure 3a does not show appropriate structure. The authors need to repeat this stain to visualize blood vessel architecture.
3. To support figure 4b, additional immune cell stain for M1/M2 markers should be included. For example, multiplex IHC analysis for macrophages including M1/M2 marker stains such as CD86 and Arginase-1, will validate the authors’ claim generated from scRNAseq data that M2 polarization occurs in GI. Additionally, markers for neutrophils should include myeloperoxidase stain in addition to CD66b to differentiate pathologically activated neutrophils from nonspecific granulocytes. The authors should change M1/M2 to pro-inflammatory / pro-inflammatory phenotypes.
4. Figure 7d poorly shows DAPI positive cells in the OPI and GI cells, which suggests poor tissue quality, low laser intensity, or poor image processing. Appropriate images showing clear DAPI signal with CD133 and Nestin stains of the same region should be provided. The authors should also include more specific tumor GSC markers, such as OLIG2 and SOX2 in Figure 7d.
5. The study should include spatial transcriptomic analysis of the GI and OPI regions to show the close proximity of cell types (astrocytes, endothelial and macrophages) involved in the “cross-talk” signals generated from Figure 6. The computational analysis does not sufficiently support the “specialized cell-cell communication” from these cell types in the GI.
6. The manuscript is poorly written and needs further grammatical editing.

Reviewer #2

(Remarks to the Author)
Wang and colleagues present an interesting study focused on understanding the regional biology of the invasive front of glioblastoma tumors. The design of the study incorporates a deliberate sample acquisition of invasive tumor populations based on distinct advanced MRI features incorporating DSC perfusion. This is a clinically relevant approach that can provide biologically meaningful insights for a tumor region that is historically understudied yet critical in tumor recurrence. Findings have translational potential. However, there are important concerns and clarifications needed with the manuscript to support the conclusions drawn, which potentially temper enthusiasm, as detailed below:

1. The authors need to explicitly clarify the PBZ relative to areas of enhancement. Previous studies have defined invasive peritumoral regions as non-enhancing on T1+C MRI (PMID: 20093527; PMID: 22711606; PMID: 37770427; PMID: 28255030). Please frame the current manuscript in the context of these prior studies and clarify whether any of the PBZ (e.g., GI and OPI) biopsies were taken from areas of enhancement, non-enhancement or both. If both, please define the number of biopsies from enhancement and non-enhancement, and whether there was a difference in the number of GI samples and OPI samples taken from areas of contrast enhancement? Is this what is meant by a “blurry boundary’ of GI on page 6? For instance, supplementary figure 1 shows multiple examples of the GI ROIs (red dotted lines) encompassing areas of enhancement on T1+C. This can be seen with supplementary Fig 1a (first row, cystic, second column T1+C), the red dotted ROI encircles an area of enhancement. Similarly, the third row of Supplementary fig 1a (cystic-solid, second column T1+C) also shows an area of enhancement within the red dotted ROI demarcating GI. Supplementary Fig 1c shows that the ROI defined for GI for the Temporal lobe case (first Row) for the DTI image (4th column from the left) appears to be in the ventricle (occipital horn), with subependymal enhancement on the T1+C. Similarly, the recurrence case (3rd row) GI ROI appears to be within enhancing tumor on the T1+C (3rd column from the left). The authors should include MRI values for T1+C measurements (taking into account normalization and bias correction) for the biopsy samples from GI and OPI regions, to be included in supplementary data/table.

2. Given the potential similar T1+C characteristics between GI and tumor core, the results and discussion should be expanded address the comparison of MRI features and molecular features between GI and Tumor core, and discuss how the GI represents a distinct entity from the tumor core, and not just the periphery of the tumor core.

3. Page 5: The authors should report the quantitative perfusion MRI measurements (e.g., CBF) from each biopsy location, from the regions of interest used to determine biopsy locations. please specify size of the ROIs used for feature extraction. this should be included in supplementary data table for review. were there particular thresholds used to determine "high" vs. "low" regions? Were "intermediate" regions ever sampled?

4. On page 30 (methods), please provide greater detail of the post-processing methodology for perfusion MRI that would allow for reproduction/replication of the CBF maps.. Please provide greater description of the methods including denoising, manual correction, and CBF generation. Please provide details of functool software, including version.

5. On page 31, did the authors document values for CBF from the enhancing tumor core? If these data are available, an analysis should be performed to compare sequencing profiles from high CBF from the GI zone (from PBZ) versus high CBF from the enhancing tumor core. CBF values from each biopsy should be included in a supplementary data/table.

6. Page 6: Given that relative cerebral blood volume (rCBV) is a commonly used metric in neuro-oncology, and can be measured with the DSC technique employed in this study, can the authors please provide justification for pursuing CBF measurements and not rCBV, or rationale for not pursuing both? Given the focus of the microvasculature, the study could benefit from an evaluation of a broader panel of perfusion MRI metrics including rCBV, to help characterize the blood supply of the GI and OPI regions. Did they correlate rCBV with molecular markers of angiogenesis, hypoxia, etc, and how does this align with previous reports (PMID: 20093527, PMID: 22711606). Were other vascular perfusion metrics evaluated, such as PSR, K2, MTT, etc? (PMID: 37770427)

7. Page 30: please provide more information on the PWI method, including Gradient echo or spin echo? Echo Planar Imaging? Flip angle? Contrast dosage and type? Number of phases of dynamic imaging? Was preload dose employed?

8. Page 30: was CBF normalized to an internal control?

9. While ASL maps were referenced, details of the ASL technique and post processing are absent from the methods section. Please describe, and include how the ASL maps were used in guiding biopsy localization. How were potential discrepancies between DSC derived CBF and ASL derived CBF handled? If ASL was used to guide biopsies, these values should be included in a supplementary data/table for each biopsy.

10. Perfusion metrics have been shown to correlate with tumor cell density. this is further supported by greater number of tumor cells in the GI compared to OPI. Can the authors please provide quantitative measures of tumor purity from the GI and OPI biopsies? How does tumor purity from GI compare with tumor core?

11. Page 6: In Supplementary Fig 1 c, the more posterior ROI defined for GI for the Temporal lobe case (first Row) for the DTI image (4th column from the left) appears to be in the ventricle (occipital horn), which would explain the absence of

identifiable "fiber tracts" in this region. Please provide an axial T1+C image to replace the sagittal for the Frontal lobe case (end row, 3rd colum from the left). The recurrence case (3rd row) GI ROI appears to be within enhancing tumor on the T1+C (3rd column from the left).

12. The authors should report the quantitative diffusion MRI measurements (e.g., FA) to support the assertion of destruction of the nerve fiber bundles.

13. Fig 3b: typo in lableing “aear” should be “area”?

14. Please provide details of Cd31 labeled vascular quantification and the methodology for generating the figure 3b insert. What software were used. Were areas of necrosis included in the total area? On page 7, the authors describe “numerous dilatations and thickened dysmorphic vessels in the tumor core and abundant neovasculature in the GI area”. Can the authors give more details on how these qualitative assessments were made, and if there are quantitative metrics that support this? For example, did the authors evaluate average vessel size in tumor core, GI, and OPI? Did they evaluate vessel number in addition to total area? Values contributing to this analysis should be included in supplementary data/tables.

15. Page 17: it’s not clear what the authors are intending to report in regards to Ki-67 comparison between GI and tumor core with the following statement”GI is the special zone which with more powerful proliferation potential in PBZ.” are they stating this comparing GI to OPI? Or GI to tumor core? Are they stating that GI proliferative indices are approaching those of tumor, but different from OPI? Figure 5f: please define the single “*” and double “**”.

16. “Germinal interface” is a confusing term. The rationale for this naming scheme is not given. The terminology raises into question if this tumor region encompasses a focus on developmental pathways, or alternatively whether the biology of this region is related to other lymphoid tissue such as a germinal center, etc. Please re-name the GI to clarify this region based on the identification within non-enhancing tumor displaying higher perfusion imaging metric. Similarly, please re-name the OPI to clarify this region based on the identification within non-enhancing tumor displaying lower perfusion.

17. Page 5. Define CBF and ASL-MRI for first use

18. Page 5. Patient selection for GBM tumor inclusion – there is no comment on IDH-status, or these are confirmed IDH-WT tumors? There are 4 recurrent tumors, while the rest are primary tumors. What are the characteristics of the tumor cohort that was selected for single cell?

19. Page 6 – define what is meant by different “types of GBM”

20. Page 9, 25. Given the GI was selected based on increased MRI perfusion metrics, is it surprising that there were a greater number of microvessels seen in this study? This finding is helpful to confirm the region obtained during sampling but should be otherwise downplayed as novel in and of itself in the discussion. Similarly, is it surprising to find increased VEGF and hypoxia signaling pathways in the regions with increased vasculature? These are helpful confirmatory findings but do not add to the novelty of the study. Please cite the reference PMID: 22711606 and PMID: 20093527 and compare this study's findings with previously published results, particularly in the context of image-localized biopsies from the non-enhancing margin (similar to the PBZ described here) and the correlates of advanced MRI with tumor aggressiveness.

21. Page 10. The rationale to explore differential gene expression profiling specifically within the endothelial cells across regions is not defined. Please clarify. Moreover, how do the authors confirm the identified GO pathway signatures (PI3K-Akt, and HIF1) are activated pathways in the vascular endothelial cells specifically? Of note, from Fig 5e the upregulated pathways identified in the GI (PI3K-Akt and HIF1) are the same noted attributed to endothelial cells in Fig 3d/e. Please clarify.

22. Page 13. Please note CD-68 staining will highlight macrophage lineage, thus can include the microglia population. Please also clarify this for Fig 4b – how do you reconcile increased CD68 in GI when the single cell data of immune composition suggested higher microglia population in OPI (fig 2d) given this marker should evaluate for both resident microglia and peripheral monocyte/macrophages.

23. Page 17. Figure 5g. Is the differential expression of the MHC class I genes and ligands significant? Please include test of significance to support the biological conclusions. Note that supplemental 6a highlights MHC class II complexes, so the biological conclusions regarding tumor-immune interactions here appear weak.

24. Page 19. The authors identified a cell-cell network computationally. The validation of these findings via spatial analysis of the tissue regions is suggested to support the claims.

25. Discussion, Page 6. Regarding the correlate of fiber tracts. The authors observed a decrease in number and destruction of fiber tracts in the GI zone vs a pushing phenomenon in the OPI. The authors postulate this may be due to increased tumor cell invasion in the GI leading to tract destruction. Can the authors reconcile this hypothesis with well-known mechanisms of invasion where nerve fibers are not described as destroyed (ie amoeboid pattern or perineural tumor cell invasion etc)?

26. Discussion. What are the correlations of the 2 non-enhancing PBZ tumor regions to known GBM subtype classifications? (TCGA and Pathway-based/Garafano)?
Recommend having English grammatical review for inconsistencies and spelling errors.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The study addresses an original and clinically important issue of cellular composition of various peritumoral brain zones (PBZ) and their relevance to tumor recurrence. About 90% of glioblastoma (GBM) recurrences occur at the peritumoral brain zone (PBZ) which has not been studied in such details as a tumor core. In this study, two PBZ tissues and one tumor tissue were sampled from each of 26 GBM patients based on the sophisticated preoperative imaging. Using the advanced tumor and blood flow imaging along with histology they defined special regions in the PBZ, named the “germinal interface” (GI). The GI exhibited more neovascularization than the other peritumoral interfaces (OPI). They focused on scRNAseq analysis of cells in those areas (from 10 cases) and the characteristics of infiltrating immune/tumor cells with various techniques. Their study demonstrates that there are one or more GI with increased infiltration of macrophages and T lymphocytes compared with that in OPI. The GI, OPI, tumor areas differ in a number of tumor cells. They conclude that the results show more tumor cells in the GI than in the OPI, and massive substantial differences in the gene expression profiles of these tumor cells. They speculate that GI may be the key area of for tumor recurrence and PBZ-targeting therapy after surgical resection. Altogether, this is a very interesting study which provides novel information about GBM heterogeneity and is important to the field. The authors are aware of and recognize the main limitations of the study. Technically, it is well done with proper analytical methods, however some of the differences are not analyzed statistically, which is a minor drawback. In general, he methodology is sound and the work meet the expected standards in your field. Some details should be provided in the methods for the work to be reproduced. Some clarification and a space for the improvements is indicated.
Major comments:
1. The authors wanted to focus on properties of cells in the GBM peritumoral brain zone (PBZ). However, it is difficult to define unambiguously those zones due to a considerable variability in surgical resections that dependent on a quality of tumor visualization and the tumor location (a vicinity of vital areas may limit resection). They attempt to dissect PBZ into specific areas such as “germinal interface” (GI) that exhibit more neovascularization than the other peritumoral interfaces (OPI). While this is an interesting observation, differentiating features of those areas are not well explained and their distinction might be very subjective. Boundaries of safe resection are variable and patient dependent. If one adds an additional variable resulting from various extents of resection, the resulting data are hard to be compared. While they used modern methods to visualize tumors and adjacent areas, they should relate/discuss their definition of areas to previous work. Lemèe and coworkers (Lemée, Clavreul, & Menei, 2015) defined 4 regions of interest identified as the necrotic zone, the florid tumor zone, the interface zone (IZ), and the PBZ. In contrast, Laytysheva et al., used radiological imaging and defined the PBZ as the area extended outside the contrast-enhancing (CE) tumor core, composed by three different layers: the peri-enhancing zone, extended 5 mm over the peritumoral CE ring; the near zone, between 5 and 10 mm; and the far zone, between 10 and 15 mm, from CE region (Latysheva et al.,2020). Here they define the PBZ (consisting of GI and OPI) as the tissue surrounding the contrast-enhancing lesion at a distance of < 1 cm. The authors should discuss how their definition of the PBZ in relation to the concepts in the field. I am not convinced that <1 cm from the contrast-enhancing lesion is PBZ and not the tumor.
2. The authors wrote that: “GI was characterized by a disorganized tissue structure, abundant mitotic figures, widespread infiltrating neoplastic cells and numerous newly formed vessels.” I would say it is a histological characteristics of the tumor border , not peritumoral zone.
3. The mean age of GBM patients is 56.5 ±13.1 years, which is younger than typical mean age of GBM patient indicates a relatively young population. Could this affect the results?
4. The authors conclude that ”the GI provides the tumor blood supply and is the main region of tumor/immune cell infiltration” however this conclusion is not fully supported by the evidence. The GI is defined as “high blood supply region”, so it should not be concluded based on the defining features. Defining GI was based mostly on areas of relatively high perfusion.
5. The authors studied the integrity of fiber bundles in the PBZ by DTI-based tractography in 8 patients. But it is not clear if the noted decrease in and destruction of fiber tracts in the GI zone was overlapped with areas characterized as “high blood supply regions”.
6. Fig.3. They wrote “ The proportions of macrophages and T cells in the GI were higher than those in the OPI” . How was it in GI versus tumor?
7. Immunohistochemistry was also used to show “macrophage, CD3+ T cell and neutrophil infiltration in the GI, OPI and Tumor regions”. The performed staining for CD68 shows glioma associated myeloid cells)GAMs) as it does not differentiate microglia from monocytes/macrophages.
8. Data presented in the Supplementary fig 3 “The distribution of individual cell types in each sample”) show a relatively high percentage of T cells in some GBMs which is surprising. Moreover, they found higher % T cells in the GI. These potential differences have to be quantify and statistically analyzed. With such huge variability in GBMs, differences between GI and OPI might hard to detect. Is t-SNE plot from integrated samples or representative one.
9. Fig.5g shows “Expression of major histocompatibility complex (MH 293 C) class I genes and genes coding for ligands of PD1 (CD274 and PDCD1LG2) and CTLA4 (CD80 and CD86) in 3 regions”. But no changes are clearly visible and statistical analysis would be required here to make any meaningful statement.

10. Supplementary fig 4. Indicate a number of samples used for the analysis of MMP9 expression.
11. Fig.7s. Staining for CD131 is poorly visible. What is quantified as GSCs? Double positive for nestin and CD131? It is not clear.
12. For evaluation of scRNAseq providing the information on a number of cells sequenced (per sample) and after discarding those of low quality would be important.
13. The authors discuss “Interestingly, pseudo-time trajectory analysis of macrophages revealed that macrophages in PBZ were during the differentiation that toward to pro-tumorigenic phenotype. Further, they were in the process of M1 or M2 polarization in different patients”. This is irrelevant discussion as the study does not differentiate between infiltrating monocytes and mature macrophages and detecting intermediate states/phenotypes may reflect various contribution off monocyte infiltration.
14. Some generalization such as “tumor invasion and recurrence potentially occur in the GI” should be avoided as higher expression of the MMP9 protein does not signify higher invasion and the it would require comparison of post-recurrence MRI images to support the notion that GI are areas of recurrence. The prevalence of GSCs is not sufficient considering vague identification of those cells.
15. Ethics approval letter is in Chinese, so it is hard to understand if it refers to the study.

Minor comments:
• Some authors statements are not correct: “Glioblastoma (GBM) is the most common primary tumor of the central nervous system, with a median survival time of 15-20 month”. Glioblastoma (GBM) is the most frequent primary CNS tumor in adults. Median survival is 15 months.
• Gene/RNA names should be in italics.
• The authors use abbreviations without showing full names (“PWI, may be useful to evaluate tumor cell infiltration in PBZ for example, the ADC…”)
• Correct cluster37, it should be cluster 37.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors nicely addressed my concerns. The manuscript content and write-up is improved.

For figure 1C, I would recommend to remove the OR images to protect the patient and surgical staff identity, either replaceing just with “wording” or a cartoon figure.

Reviewer #2

(Remarks to the Author)
We thank the authors for their work in revising the manuscript. The authors have addressed our prior concerns and the current manuscript is much improved. We have no further concerns.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
I found the revised version a considerable improvement of the manuscript, most shortcomings were eliminated and figures, results and discussion much improved. The authors reasonably addressed concerns of this and other reviewers. In particular, I do appreciate their efforts to more precisely define and name peritumoral zone (PTZ) areas. They you explained better differentiating features of those areas (PBZ and tumor zone) and tried to put some numbers referring to the radiologically defined areas, the regional CBF value (measured with ASL) and ROIs of each biopsy location in the revised manuscript, which provides references for other researchers to replicate the findings. They added the information from multiplexed IHC staining. They discussed extensively the issue of PTZ definition in the context of other studies on this topic and included some of the aspects into the revised Discussion. They also admitted to some limitations to their research and troubles to defining boundaries between PTZ and tumor border. They calculated the proportions of macrophages and T cells in various areas, and added IHC analysis for microglia (TMEM119) as requested. The authors dealt well with the concerns regarding defining myeloid subsets, which is not easy considering the shortage of discriminating markers.

Their responses to other reviewer comments are reasonable and additional data and explanations clarify some points raised by reviewers.

I share the author’s view that much of the interesting things happen in the PTZ and more precise dissection of the

functionalities of cells in these regions is required and would bring clinically important information.