Commentary: preclinical efficacy of immune-checkpoint monotherapy does not recapitulate corresponding biomarkers-based clinical predictions in glioblastoma by Garg et al. (2017)

Lijie Zhai, Erik Ladomersky, Kristen L. Lauing, Meijing Wu, Denise M. Scholtens, Rohan Savoor, Bin Zhang, Jennifer D. Wu, Craig Horbinski, Rimas V. Lukas, David C. Binder, and Derek A. Wainwright

ABSTRACT
Preclinical modeling and gene expression analyses have yielded distinct observations for the role of immune checkpoint, IDO1, in glioblastoma (GBM). Accordingly, our recent work differs with Garg et al. (2017) with respect to IDO1 among preclinical and bioinformatic GBM datasets. Here, we discuss the methodological differences that affected study interpretation, and potentially, future clinical decision-making for IDO1-targeting approaches against GBM.

Glioblastoma (GBM; WHO grade IV glioma) is the most common, malignant, primary central nervous system cancer in adults. Even with the aggressive current standard of care treatment which includes maximal surgical resection, followed by treatment with radiotherapy, temozolomide, and tumor-treating fields, the median GBM patient survival is only 15–20 months. Recent cancer immunotherapy efforts, with an emphasis on the inhibition of immune checkpoints, programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), have demonstrated success in treating aggressive malignancies including melanoma, non-small cell lung-1, and renal cell-cancers. Although many randomized trials have yet to report results, the first phase III clinical trial (Checkmate-143) evaluating PD-1 mAb (nivolumab) in recurrent GBM patients demonstrated a failure to improve overall survival (OS) as compared to the treatment with anti-VEGF-A mAb (bevacizumab; n = 369 total patients enrolled). These results suggest important immunological differences for single agent immune checkpoint inhibitory approaches between GBM and cancers arising outside of the central nervous system (CNS); thus sparking next generation efforts to discover more effective strategies for GBM eradication. In parallel with immunotherapeutic efforts, the identification of prognostic biomarkers that are capable of stratifying GBM patient survival, is another area of highly active research.

Indoleamine 2, 3-dioxygenase 1 (IDO1) is a potently immunosuppressive mediator that decreases the effectiveness of immunome-based anti-cancer strategies. IDO1 is an interferon-inducible catabolic enzyme that converts the essential amino acid, tryptophan (Trp), into its downstream metabolite, known as kynurenine (Kyn). IDO1 utilizes multiple molecular mechanisms to promote immunosuppression, including the canonical pathway of Trp metabolism, as well as noncanonical effects that are independent of enzyme activity. IDO1 protein levels and/or metabolic activity have also been prognostically useful in several types of cancer, although immunohistochemistry (IHC) has been more controversial in this assessment. Based on our work confirming that, in situ hybridization reliably detects IDO1 mRNA transcripts in human GBM tissue, as compared to the poor sensitivity of antibody-based IHC, we evaluated IDO1 RNA-Seq data from The Cancer Genome Atlas (TCGA) for its potential prognostic value in low- and high-grade glioma. We discovered that, high IDO1 levels independently associate with decreased patient survival in WHO grade II, III, and IV (GBM) glioma. Additionally, we discovered that, IDO1 expression progressively increases with glioma grade and is maximal in GBM. We also found a positive correlation between high mRNA expression for markers of cytotoxic T cells, CD3E and CD8A, with high IDO1 levels in patient-resected GBM. The capability of human T cells to directly increase intratumoral IDO1 expression was further supported by the evaluation of humanized mice reconstituted with human immune cells (NSG-SGM3-BLT) and intracranially-engrafted human patient-derived GBM xenografts (PDX). Notably, the depletion of human CD4+ and CD8+ T cells from NSG-SGM3-BLT mice with PDX, significantly decreased intratumoral IDO1 expression levels. Further underscoring the critical role of IDO1 as a therapeutically target,
we recently determined that, IDO1 enzyme inhibition synergizes with radiotherapy (RT) and PD-1 blockade to achieve durable GBM control in syngeneic mice. Interestingly, no survival benefit was achieved by single or dual agent treatment of RT, anti-PD-1 mAb, and IDO1 enzyme inhibitor, highlighting the requirement for all three agents to achieve an optimal immune-mediated control of GBM.

Given the large number of preclinical and clinical studies evaluating new immunotherapeutic strategies and biomarkers, there is an increasing likelihood for discrepancy among study conclusions as investigators report observations under non-redundant conditions and with different methodologies. It is therefore important to discuss these discrepancies for consolidating and appreciating our current state of knowledge, and thus to best inform the design of next-generation clinical trials. Here, we discuss our findings in relation to the recent study, "Preclinical efficacy of immune-checkpoint monotherapy does not recapitulate corresponding biomarkers-based clinical predictions in glioblastoma", by Garg et al. 2017; Oncoimmunology. 16

**Prognostic profile of IDO1 mRNA expression in GBM – selection of TCGA datasets**

Despite similarly-focused studies by Zhai et al. 15 and Garg et al., 16 which collectively evaluated the prognostic potential of GBM IDO1 expression across the TCGA and REMBRANT databases, the conclusions of each investigation varied. While Garg et al. documented a null prognostic profile for IDO1 gene expression in GBM patients, our study indicated an increased IDO1 mRNA level with negative prognostic outcomes. One critical factor potentially contributing to this discrepancy is the GBM TCGA databases used among these independent studies. In our analysis, we investigated the Illumina HiSeq RNA-Seq dataset (n = 172) as of April 13, 2017. In contrast, Garg et al. used the Affymetrix HT Human Genome U133a microarray platform dataset (n = 540). The selection of different TCGA datasets, obtained from different detection platforms, may significantly influence the associated outcomes following data analysis utilizing different methodologies. First, although studies have demonstrated a high correlation between the gene expression data obtained from microarray and RNA-Seq assays, past investigations have also revealed discordant gene expression patterns between these 2 platforms, which are mainly attributable to different detection principles and different data processing/normilization methods. When combined, these variables can lead to different IDO1 mRNA levels for the same patient sample as detected by 2 independent platforms. As shown in Figure 1, the Spearman correlation analysis indicates that, at least 15% of the 155 overlapped samples between the Affymetrix HT Human Genome U133a microarray and RNA-Seq platform, displays discrete distribution, even though the overall correlation is statistically significant (p < 0.001). Another variance between the two TCGA datasets is the sample size difference [(n = 540) for microarray data and (n = 172) for RNA-Seq data, respectively]. As shown in Figure 1, among the patient samples with complete information for survival analysis, there are only 155 overlapping samples between the microarray and RNA-Seq datasets. Therefore, the information from the extra 368 patients in the microarray data could also change the final statistical analysis outcomes, and depending on whether they possess an equally distributed molecular subtype (ie. MGMT promoter methylation, mIDH status, newly-diagnosed vs. recurrent), could associate with different patient outcomes. Additional underlying parameters that may contribute to different conclusions are associated with the different TCGA GBM databases, tumor purity, and ratio of tumor cells versus non-tumor cells. Previous studies have indicated that non-tumor cells, such stromal cells and infiltrating immune cells, play an important role in gliomagenesis, malignant progression, and resistance to treatment. Thus, different tumor purities among individual GBM patients could affect gene expression distinctly, resulting in a substantial influence on gene expression and prognostic profile associations. This hypothesis was supported by a recent study focusing on glioma purity analysis. 17 Zhang et al. separated 1,105 glioma cases from the TCGA database, and 310 glioma cases from the CGGA database, into low and high tumor purity groups, followed by re-evaluation of the prognostic value using previously established stratifying factors and a multivariate Cox regression model. Strikingly, most recognized prognostic indicators varied depending upon different purity conditions. Given that IDO1 can be expressed both by GBM and non-tumor cells, a consideration for tumor purity is
an important retrospective factor that possibly contributes to gene expression differences across bioinformatic databases.

**Prognostic profile of IDO1 mRNA expression in GBM – selection of cutoff value**

The discrepant prognostic significance for IDO1 in GBM also reflects another critical difference between our survival analyses, with that performed by Garg et al., and relates to the method for determining IDO1 expression cutoff values for stratification of patient survival outcomes when using Kaplan-Meier (KM) analysis. While Garg et al. utilized the median as a threshold in their analyses, we employed an optimal cutoff determined by maximizing the significance assessed by the log-rank test via Cutoff Finder. Both methods have been used in numerous investigations, with a lack of consensus in the biostatistics community for defining the optimal method to determine the associated gene expression level with overall survival. Given the lack of consensus in cutoff value methodology used for KM analysis, it is crucial to validate the prognostic significance of a biomarker with additional, more powerful analytical tools, such as the multivariate Cox regression model that, controls for other prognostically-relevant variables capable of skewing univariate analysis. While no such analysis was performed by Garg et al., we demonstrated that, IDO1 expression is prognostic in GBM patients when controlled for additional clinicopathologic parameters (age, gender, tumor molecular subtype, radiation, and chemotherapy) (Table 1 in ref. 15).

**IDO1 in gliomagenesis – comparison between normal brain and GBM**

The study by Garg et al. analyzed the TCGA and REMBRANT databases and concluded that, IDO1 expression is identical between normal brain and GBM ([n = 11] in normal brain and [n = 202] in GBM, respectively, Figure 3(c,f) in ref. 16). Due to the lack of normal brain samples in the RNA-Seq TCGA GBM dataset, our study analyzed the AffyU133 microarray TCGA GBM cohort and determined that, IDO1 mRNA levels are significantly higher in GBM patients as compared to normal human brain [4.12 ± 0.0436 (n = 528) and 3.769 ± 0.115 (n = 10), respectively; \( P = 0.0147 \); Suppl. Figure 3(b) in ref. 15]. Despite these discordant conclusions, it’s important to note that, Garg et al. determined that, “a small subset of patients with GBM tumors (5–10%) tend to show higher IDO1 expression than their normal counterparts” (Figure 3(c) in ref. 16). Also notable, the Project Betastasis portal utilized by Garg et al. only evaluated samples with molecular subtype information (classical, mesenchymal, neural, and proneural) for comparing GBM and normal brain samples, resulting in a substantially smaller sample size (n = 202) for statistical analysis. In contrast, our study utilized all the GBM samples with IDO1 expression values, thereby providing optimal statistical power. Notably, the normal brain suffered from a small sample size across both studies. Nevertheless, our analysis of IDO1 expression among different WHO glioma grades indicates that, IDO1 is positively correlated with glioma malignancy (Figure 2(a) in ref. 16), supporting the hypothesis that, IDO1 plays a pathogenic role in malignant glioma.

| Variables                      | Total No. of Patient Events | Death No. | %    | Median | 95% CI        | P    | HR | 95% CI | P     |
|-------------------------------|----------------------------|-----------|------|--------|---------------|------|----|--------|-------|
| Age at diagnosis, year        |                            |           |      |        |               |      |    |        |       |
| < 50                          | 33                         | 21        | 63.6 | 14.5   | [12.7, 21.9]  | 0.146|    |        |       |
| ≥ 50                          | 115                        | 81        | 70.4 | 13.0   | [10.4, 15.4]  |      |    |        |       |
| Sex                           |                            |           |      |        |               |      |    |        |       |
| Male                          | 99                         | 65        | 65.7 | 13.0   | [11.3, 15.1]  | 0.814|    |        |       |
| Female                        | 49                         | 37        | 75.5 | 14.7   | [9.86, 17.9]  |      |    |        |       |
| Tumor Subtypes                |                            |           |      |        |               |      |    |        |       |
| Classical                     | 39                         | 27        | 69.2 | 14.0   | [11.8, 16.1]  | 0.656|    |        |       |
| Mesenchymal                   | 51                         | 35        | 68.6 | 11.3   | [10.3, 15.9]  |      |    |        |       |
| Neural                        | 25                         | 20        | 80.0 | 14.9   | [5.39, 18.0]  |      |    |        |       |
| Proneural                     | 33                         | 20        | 60.6 | 14.7   | [10.9, 22.2]  |      |    |        |       |
| Chemotherapy                  |                            |           |      |        |               |      |    |        |       |
| Yes                           | 117                        | 74        | 63.2 | 14.5   | [13.0, 16.1]  | < 0.0001| |        |       |
| Radiation therapy             |                            |           |      |        |               |      |    |        |       |
| Yes                           | 128                        | 83        | 64.8 | 14.7   | [13.0, 15.9]  | < 0.0001| | [4.39, 12.80] | < 0.0001| |
| No                            | 20                         | 19        | 95.0 | 2.24   | [0.953, 3.75] |      |    |        |       |
| IDO1                          |                            |           |      |        |               |      |    |        |       |
| Low                           | 106                        | 71        | 67.0 | 14.9   | [11.7, 16.1]  | 0.016| 1.82| [1.17, 2.81]| 0.0076| |
| High                          | 42                         | 31        | 73.8 | 12.3   | [10.3, 14.0]  |      |    |        |       |
IDO1 expression coincidently increased with both others showed no relationship, including the REMBRANDT analysis by Garg et al., or even a poorer prognosis when CD8+ T cell levels are increased intratumorally. Collectively, these findings strongly indicate that, IDO1 is an important immune checkpoint with expression characteristics that both rely on- and regulate the function of GBM infiltrating lymphocytes. Thus, the positive correlation between IDO1, Tregs, and CD8+ TILs, is incongruent with a mathematically null result.

Concluding remarks

The advent of high-throughput sequencing and other omics-related techniques has led to an exponential increase of various bioinformatics databases, which provides enormous data for researchers to explore. The lack of consensus for data processing and methodologies to statistical analyze bioinformatic studies has further increased the likelihood of different outcomes across independent databases. The inconsistent conclusions reflect caveats and challenges for bioinformatically-focused cancer research, and can provide confusion to the literature, rather than an intended clarity of facts. This also highlights an urgent need to standardize bioinformatic approaches through collaboration between computational scientists, statisticians, and neuro-oncologists.

Regardless of the discrepant observations between ours’ and the study by Garg et al., IDO1 is a pivotal immune checkpoint in GBM, has been demonstrated to be pathogenic in multiple experimental animal modeling studies of GBM, and has been re-confirmed to be maladaptive using bioinformatic analysis of GBM patient data.  

Notwithstanding, there are still unaddressed, IDO1-specific questions worthy of further investigation. One such consideration pertains to GBM with syngeneic allografts in immunocompetent old mice and conducted with well-established intracranial brain tumors, may better resemble the potent immunosuppressive microenvironment that is representative of a human GBM treated with immune checkpoint monotherapy.
the fact that, neither our analysis nor that by Garg et al. determined the prognostic significance of IDO1 in recurrent GBM, which possesses distinct immuno-pathology, and associates with a shorter survival time as compared to tumors isolated from newly diagnosed subjects. By selecting the ‘days to tumor recurrence’, as defined in the TCGA GBM clinical dataset, we identified 99 cases of recurrent GBM with IDO1 expression values in the AffyU133 microarray, but only 15 associated cases in the RNA-Seq dataset. Using the microarray data, we investigated whether IDO1 expression is associated with GBM recurrence. Subjects with a higher level of IDO1 expression [(mRNA level ≥ 4.467 (n = 23)] had a median of 186 days to recurrence, as compared to 251 days for subjects with lower intratumoral IDO1 expression levels [(mRNA level < 4.467 (n = 76)) (p = 0.03, Figure 2). This finding confirms that, higher IDO1 expression is positively associated with a faster rate of GBM recurrence. In addition to the data mining-based IDO1 expression analysis, studies on IDO1-targeted immunotherapy against GBM also raise challenging questions. The recent Phase III clinical trial failure of anti-PD-1 mAb monotherapy to improve recurrent GBM patient survival suggests that, a combination approach is likely required to overcome the strong immunosuppressive GBM microenvironment. As determined in our recent preclinical GBM therapy study,7 only trimodal, but not dual- or mono-therapeutic treatment with RT, anti-PD-1 mAb and IDO1 enzyme inhibition, led to a durable survival benefit in a well-established model of GBM. These data highlight both the synergy that occurs when therapeutically targeting IDO1 in brain cancer, as well as the underperformance of IDO1 blockade when not properly incorporated into the optimal therapeutic cocktail. In the end, IDO1 inhibition remains as an attractive treatment strategy against GBM given its low associated toxicity13, high prognostic value,5,15,34, and synergy7,32 when combined with other therapeutic modalities.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Funding
This work was supported by NIH grants R00 NS082381 (D.A.W.); R01 NS097851-01 (D.A.W.); National Cancer Institute P50 CA221747 Project 2 (D.A.W. and R.V.L.), and T32 CA0070085 (E.L.).

ORCID
Erik Ladomerysky  http://orcid.org/0000-0002-2008-1907
Kristen L. Lauing  http://orcid.org/0000-0002-4408-1821
David C. Binder  http://orcid.org/0000-0003-4400-5455
Derek A. Wainwright  http://orcid.org/0000-0001-7232-4264

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