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Prognostic value of indoleamine 2, 3-dioxygenase-1 expression in glial tumors

Munir KAYA¹ , Asude AKSOY² , Gokhan ARTAS³ , Metin KAPLAN⁴

¹Department of Internal Medicine, Medical Faculty, Firat University, Turkey

²Department of Medical Oncology, Healthy Sciences University, Elazig Fethi Sekin City Hospital SUAM, Turkey

³Department of Pathology, Medical Faculty, Firat University, Turkey

⁴Department of Neurosurgery, Medical Faculty, Firat University, Turkey

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Az indolamin 2, 3-dioxigenáz-1 expressziójának prognosztikai értéke gliadaganatokban

Kaya M, MD; Aksoy A, MD, PhD; Artas G, MD; Kaplan M, MD

Correspondent:

Dr. Asude AKSOY,
Department of Medical
Oncology, Healthy Sciences
University, Elazig Fethi Sekin
City Hospital SUAM; Elazig,
Turkey.
Phone:
+90 0424 600 6000 6669,
e-mail:
asudeaksoy@hotmail.com,
<https://orcid.org/0000-0002-5609-9658>

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Background and purpose – Gliomas are the most common primary malignant central nervous system tumors in adults, exhibiting a poor prognosis. Indoleamine 2, 3-dioxygenase-1 (IDO-1) has important functions in cancer immunotherapy due to its role in escaping cancer cells from the immune system. In this study we purposed to evaluate the correlation between IDO-1 expression and clinicopathological parameters in gliomas, and whether IDO-1 can be a prognostic marker.

Methods – n=75 patients in total, n=25 patients with low grade glial tumors (LGG, grade 1-2), n=25 patients with high grade glial tumors (HGG, grade 3-4), and n=25 persons with normal brain tissue as control group were included in this study. IDO-1 expression was categorized by using immunohistochemical staining in biopsy specimens as high (H) and low (L) groups among the patients with gliomas. We used a 95% percent confidence interval and $p < 0.05$ to analyze the association between the degree of IDO-1 expression, clinicopathological characteristics, and survival rates in glioma patients.

Results – In HGG, IDO-1 levels were higher than in control brain tissue and LGG ($p < 0.001$). The mean overall survival (OS) was longer in the L-IDO-1 group (64.53 ± 3.34) in months (95% CI: 57.969-71.098) compared to the H-IDO-1 group (43.74 ± 4.36) in months, (95% CI: 35.218-52.330) ($p < 0.05$).

Háttér és cél – A gliomák a leggyakoribb, rossz prognózzal rendelkező, primer rosszindulatú központi idegrendszeri daganatok felnőtteknél. Az indolamin 2, 3-dioxigenáz-1 (IDO-1) fontos funkcióval rendelkezik a rák immunterápiájában, mivel a rákos sejtek immunrendszer elől való elmenekülésében játszik szerepet. Ebben a tanulmányban az volt a célunk, hogy felmérjük az IDO-1-expresszió és a klinikopatológiai paraméterek közötti összefüggést gliomákban, és hogy az IDO-1 használható-e prognosztikai markerként.

Módszerek – Összesen n = 75 beteg, n = 25 beteg alacsony fokú gliadaganattal (LGG, 1–2. fokozat), n = 25 beteg magas fokú gliadaganattal (HGG, 3–4. fokozat), és n = 25 személy normál agyszövetű kontrollcsoportként került be a vizsgálatba. A biopsziás minták immunhisztokémiai festése révén, az IDO-1 expressziója alapján a gliomás betegeket magas (H) és alacsony (L) csoportba soroltuk. 95%-os konfidenciaintervallumot és $p < 0,05$ értéket használtunk az IDO-1-expresszió mértéke, a klinikopatológiai jellemzők és a gliomás betegek túlélési aránya közötti összefüggés elemzésére.

Eredmények – HGG-ben az IDO-1 szintje magasabb volt, mint a kontroll agyszövetben és LGG-ben ($p < 0,001$). A teljes túlélés (OS) középértéke hosszabb volt az L-IDO-1 csoportban ($64,53 \pm 3,34$ hónap; 95%-os CI: 57,969–71,098), mint a H-IDO-1 csoportban ($43,74 \pm 4,36$ hónap; 95%-os CI: 35,218–52,330) ($p < 0,05$).

Conclusion – IDO-1 expression is an independent prognostic biomarker to predict OS and progression in HGG. IDO-1 can be evaluated as an alternative instrument for precision medicine in the treatment of gliomas.

Keywords: glioma, indoleamine 2,3-dioxygenase 1, prognosis, survival

Következtetés – Az IDO-1-expresszió független prognosztikai biomarker, ami HGG esetén előre jelzi az OS-t és a progressziót. Az IDO-1 a precíziós orvoslás alternatív eszköze lehet a gliomák kezelése során.

Kulcsszavak: glioma, indolamin 2,3-dioxigenáz-1, prognózis, túlélés

Cancer cases are growing drastically over the world nowadays. Central nervous system (CNS) tumors are among the top 19 most prevalent malignancies, according to GLOBOCAN 2018 statistics. Primary CNS tumors include 1.6% of all new cancer diagnoses¹. In adults, gliomas are the most frequent primary malignant CNS tumors. They are highly heterogeneous and can occur anywhere in CNS. Gliomas represent 75% of primary CNS tumors in adults, and more than half of them are glioblastoma multiforme (GBM)^{1, 2}. Glial tumors have a poor prognosis with limited treatment options. Investigations into the genomic structure of cancer have finally revealed that the molecular changes underlying CNS tumor biology are very diverse. The World Health Organization (WHO) has recognized the importance of molecular characteristics in defining brain tumor entities. In the last revision of recommendations for brain tumor classification in 2021, the WHO proposed to use distinct sets of molecular markers to complement the histological definition of gliomas. The mutation status of isocitrate dehydrogenase (IDH 1/2) significantly differed between low-grade gliomas, secondary GBM, and primary (de novo) GBM. High-grade gliomas constitute 15% of primary brain tumors. GBM is the most malignant of these³⁻⁶. IDH wild GBM is the most common of primary brain cancers and has a worse prognosis.

Various genetic alterations are known to influence many tyrosine kinase receptors and associated pathways, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor alpha (PDGFRA), met proto-oncogene, receptor tyrosine kinase (MET), B-Raf proto-oncogene serine/threonine kinase (BRAF), the phosphatidylinositol-3 kinase (PI3K), and cyclin-dependent kinase 2 (CDK2) A/B⁷. Today, human EGFR 2 (Her2) expression in breast cancer, Kirsten rat sarcoma virus (K-ras), and BRAF mutation status in colon cancer, EGFR, anaplastic lymphoma kinase (ALK), ROS proto-oncogene 1, receptor tyrosine kinase (ROS) mutation status, and programmed death-ligand 1 (PD-L1) expression levels in lung cancer have become an important touchstone for treatment⁸⁻¹³. Although targeted therapies for CNS malignancies are not as common as they are for

other cancers, medicines like selumetinib in patients with plexiform neurofibromatosis and larotrectinib in patients with neurotrophic tyrosine receptor kinase (NTRK) gene fusions-positive CNS tumor have shown encouraging outcomes^{14, 15}.

Increased IDO-1 and kynurenine (Kyn) inhibits natural killer (NK) cell function and prevents the activation of effector T cells and Treg cells; thus, they cause immunosuppression by activating tolerogenic dendritic cells (Tol-DC) and myeloid-derived suppressor cells¹⁶. Increased levels of IDO-1 and Kyn have been observed to inhibit natural killer (NK) cell function, stop the activation of effector T cells and Treg cells, and have a function in immunosuppression activating tolerogenic dendritic cells (Tol-DC) and myeloid-derived suppressor cells. Tryptophan (Trp) metabolites are toxic to lymphocytes. They promote the differentiation of regulatory T cells, help suppress CD4+ T cells, increase regulatory T cells' action, modulate monocytes and lymphocytes' immune functions, promote apoptosis of effector T cells, and the proliferation of regulatory T cells. Trp metabolites stimulate the cell cycle of tumor cells and acquiring multidrug resistance against chemotherapeutic agents^{17, 18}.

IDO-1 overexpression increases Kyn concentration relatively compared to Trp. Therefore, in some cancers, researchers have used the Kyn/Trp ratio as a prognostic marker¹⁹.

There are many ambiguous areas in malignant gliomas' etiopathogenesis, and there are minimal sufficiently extensive studies regarding IDO-1 expression in glial tumors²⁰.

Consequently, we investigated the role of IDO-1 in glial tumors and whether it can be a prognostic marker.

Methods

Selection of cases

Our study was conducted in the departments of medical oncology, pathology, and neurosurgery following the approval of the board of ethics at our university. According to the Declaration of Helsinki, written informed consent

was obtained from all patients whose tissues were used in the study. The study included 75 cases that underwent neurosurgery at our university faculty of medicine between the years 2014–2019. The participants comprised patients who were operated for their LGG (n=25 grade 1–2), HGG (n=25 grade 3–4), and a control group (n=25). The control group consisted of brain tissues from patients operated for various reasons (brain hemorrhage, trauma). We obtained the clinical information of patients from the electronic data center of the hospital and excluded any patients with missing data.

Immunohistochemistry

All pathology preparations were re-evaluated by an independent pathologist. He sliced 4–6 µm pieces from paraffin blocks and transferred them on polylysine slides. The IDO-1 primary antibody (anti-mouse IDO-1 Antibody, 122402, Bio legend, USA) was incubated for 60 minutes at room temperature in a humid environment. Following the application of the primary antibody and PBS washing for 3x5 minutes, the tissues were incubated for 30 minutes at room temperature in a humid environment with a secondary antibody (biotinylated Goat Anti-Polyvalent (anti-mouse/rabbit IgG), TP-125-BN, Lab Vision Corporation, the USA). The preparations were viewed, analyzed, and photographed using a Leica DM500 microscope (Leica DFC295). Histochemical scores were generated based on the prevalence of staining (0.1: <25%, 0.4: 26–50%, 0.6: 51–75%, 0.9: 76–100%) and severity of intensity (0: none, +0.5: very little, +1: little, +2: medium, +3: severe). Histochemical score = Prevalence X Intensity²¹.

The mean value for the Ki-67 values of the tissue immune histochemistry of the patients included to the research was computed. The Ki-67 measurement had a mean value of 32. We categorized Ki-67 values as < 32 % low (L) Ki-67 group and ≥ 32 % high (H) Ki-67 group^{22–24}.

Statistical analysis

All descriptive and inferential statistical analyses were carried out using the IBM Statistical Package for the Social Sciences (SPSS) version 22.0 software (Chicago, IL, USA). The Kolmogorov-Smirnov test determined if variables with constant measurement values were suitable for normal distribution. The Log-Rank test under Kaplan-Meier analysis was used for survival studies. The cut-off value of IDO-1 was calculated by ROC analysis. The Cox Regression test was used for multivariate analysis. Threshold values for the specified variables were determined using the Youden index. The results within the 95% confidence interval (CI) were found to be statistically significant ($p < 0.05$).

Results

IDO-1 expression in glial tissue by immunohistochemistry

The patients' median follow-up duration was 48.0 (19.0–80.0) months. **Table 1** shows the general clinical characteristics of the cases.

IDO-1 was expressed in all glial tumor tissues and control brain tissue. H-grade glial tumors expressed statistically significantly higher level of IDO-1 immunohistochemically in their cytoplasm, compared to the control brain tissue and LGG, as shown in **Table 2**, and **Figure 1A–C**. There was no difference in IDO-1 expression between patients diagnosed with LGG and normal brain tissue ($p > 0.05$).

We determined the cut-off value of IDO-1 in patients with glial tumors by the ROC (Receiver Operation Characteristics) curve. For IDO-1, the AUC (area under the curve) value = 0.807 ± 0.049 ($p < 0.001$, CI 95%: 0.712–0.903), and the cut-off value were calculated to be 0.35 with a sensitivity of 70% and specificity of 68% (**Figure 2**). < 0.35 IDO-1 was defined as low (L-IDO-1), and ≥ 0.35 IDO-1 was defined as high (H-IDO-1).

Survival analysis

In terms of the correlation between IDO-1 expression and the baseline characteristics of the patients, there was a moderate, statistically significant positive correlation between age, grade, Ki-67 values, and IDO-1 expression. There was a moderate, statistically significant negative correlation between OS and IDO-1 expression (**Table 3**).

The mean OS was 64.53 ± 3.34 months (95% CI: 57.969–71.098) for the L-IDO-1 group and 43.74 ± 4.36 months (95% CI: 35.218–52.33) for the H-IDO-1 group. The longer mean OS value was statistically significant in the L-IDO-1 group ($p = 0.033$) (**Figure 3A**).

In terms of the effects of IDO-1 levels on progression-free survival (PFS), we determined that the patients in the L-IDO1 group had statistically longer PFS of 60.50 ± 3.48 months (95% CI: 53.665–67.343), compared to the PFS of the H-IDO-1 group (36.56 ± 6.12 months; 95% CI: 24.566–48.567), ($p < 0.001$) (**Figure 3B**).

Looking at the mean OS according to the grades, while the mean OS of LGG was 65.83 ± 2.12 months (95% CI: 61.676–69.991), the mean OS of HGG was 30.38 ± 4.0 months (95% CI: 22.545–38.225). We detected longer OS in low-grade tumors, and it was statistically significant ($p = 0.001$) (**Figure 4**).

For the relationship between Ki-67 levels and OS values, while the mean OS was 63.59 ± 2.99 months (95% CI: 57.302–69.501) in the L-Ki-67 group, the mean OS was 25.05 ± 2.50 months (95% CI: 24.927–40.429) in the H-Ki-67 group. We found a longer OS with statistical significance in the L-Ki-67 group ($p = 0.001$).

Table 1. *The relationship between baseline characteristics and IDO-1 expression*

Variables	n (%)	p-value	IDO-1*	p-value [§]
Age		0.001 [#]		0.001
< 65	38 (76%)		1.18 (0.20-1.80)	
≥ 65	12 (24 %)		1.25 (0.45-1.80)	
Gender		0.001 [#]		0.002
Woman	20 (40 %)		1.21 (0.20-1.80)	
Man	30 (60 %)		1.21 (0.20-1.80)	
Tumor size		0.001 [#]		0.147
L (< 4 cm)	23 (46 %)		0.78 (0.2-1.8)	
H (≥ 4 cm)	27 (54%)		0.77 (0.2-1.8)	
Tumor location		0.001 [#]		0.475
R (right)	26 (52%)		0.80 (0.20-1.80)	
L (left)	24 (48%)		0.75 (0.20-1.80)	
ECOG performance		0.001 [#]		0.001
Good	39 (78%)		0.64 (0.20-1.80)	
Poor	11 (22%)		1.24 (0.80-1.80)	
Ki-67*		0.001 [#]		0.029
L (< 32)	33 (66 %)		0.45 (0.20-1.80)	
H (≥ 32)	17 (34 %)		1.07 (0.20-1.80)	
Grade		0.001 [#]		0.001
L (Grade 1, 2)	25 (33.3 %)		0.34 (0.2-0.6)	
H (Grade 3, 4)	25 (33.3 %)		1.21(0.8-1.80)	
Resection		0.001 [#]		0.001
Total	45 (90 %)		0.74 (0.20-1.80)	
Subtotal	5 (10 %)		1.12 (0.80-1.20)	
Progression		0.001 [#]		
(+)	20 (40 %)		1.1 (0.3-1.8)	0.001
(-)	30 (60 %)		0.52 (0.2-1.8)	
CT status		0.001 [#]		0.001
(+)	9 (18%)		1.03 (0.3-1.8)	
(-)	41(82%)		0.39 (0.2-1.2)	
Use of antiepileptic		0.001 [#]		0.170
(+)	35 (70 %)		0.86 (0.2-1.8)	
(-)	15 (30%)		0.57 (0.2-1.2)	

[#] Kolmogorov-Smirnov test used, [§]: Independent T test used, *median(min-max), CT: Chemotherapy, ECOG Performance: Eastern Cooperative Oncology Group, Good (0,1,2), Poor (3,4), (+): Present, (-): Absent

With a univariate analysis about the factors affecting survival, the presence of progression, poor performance, and H-IDO-1 were determined to be statistically significant in affecting OS. In the multivariate Cox regression analysis, cases with poor performance, the presence of progression, and H-IDO-1 levels affected survival negatively as independent prognostic factors (**Table 4**).

Discussion

Gliomas are the most common primary malignant tumors affecting the CNS. Gliomas comprise approximately 30% of all primary brain tumors^{1,25}. Due to poor prognosis and short surveillance of GBMs, studies are focused on targeted treatment methods that may be effective for

Table 2. The relationship between IDO-1 expression and control group

		IDO-1*	p-value
Group	Control	0.29 (0.10-0.60)*	
Low grade (1, 2)		0.34 (0.20-1.80)*	0.149
High grade (3, 4)		1.21(0.45-1.80)*	0.001

*Histoscore median(min-max), T test used

this malignant tumor. According to the most recent categorization, patients with GBM who are IDH 1/2 wild have a poorer prognosis. IDH wild and IDH mutant gliomas have wildly different gene expression profiles. IDH wild GBM ratios rise in advanced aging, similar to the rise in IDO-1 expression in IDH wild GBM ratios^{6, 26, 27}.

In general, the aggressiveness of gliomas increases with aging. The immune system's responsiveness to any stimulation may modify as people grow old. In the CNS parenchyma, increasing number of senescent cells, neuroinflammation, and vascular permeability all have negative influence on glioma prognosis. Immune checkpoint inhibitor ligands like PD-L1, released cytokines like IL-10 and transforming growth factor (TGF)- β influence the complicated interplay between the stroma and the antigen-presenting cells in the tumor microenvironment. Tumor-infiltrating T cells in GBM increase the expression of immune-suppressive factors such as IDO-1 and PD-L1²⁷.

IDO-1 is a cytosolic heme (Fe²⁺) group enzyme that catalyzes the first and most time-consuming step in tryptophan (Trp) degradation²⁸. Trp metabolites such as L-Kin, L-Hydroxyquinurea, 3-hydroxanthranilic acid, quinolinic acid, and picolinic acid influence IDO-1 function. These compounds restrict the transmission and growth of intracellular pathogens²⁹. IDO-1 expressed in tissue macrophages limits the growth of effector T cells via IFN γ . The buildup of Trp metabolites has been demonstrated to cause immune suppression by increasing the development of regulatory T cells (Treg) and causing effector T cell death³⁰. Trp metabolites are toxic to lymphocytes and boost the action of regulatory T cells (Treg) by inhibiting CD4⁺ T cells and other immune cells. Trp metabolites accelerate the cell cycle of cancer cells, causing them to develop multidrug resistance to CT drugs^{17, 18}. Although the mechanism of IDO-1-induced immune-suppression is uncertain, increased IDO-1 and Kyn levels have been shown to inhibit NK cell function and effector T cell activation. The immune system supports tumor growth by suppressing antitumor T cells in the tumor microenvironment³¹. This weakness in the immune system has led to the development of immune-modulatory strategies in cancer treatment.

Ogawa et al. used two distinct inhibitors of IDO-1 – 1-MT (1-methyl tryptophan) and epacadostat – to study the effect of IDO-1 on colon cancer cells (INCB024360)³². They observed that 1-MT blocked tumor cell turnover and suppressed apoptosis-induced and colonic pre-neoplastic lesions in rats.

On P815 mouse tumor models, Uyttenhove et al. examined IDO-1 expression in several primary human cancers¹⁶. They found that IDO-1 was expressed at a very high rate of 9/10 in GBM tumor tissues, similarly to our results. The study showed that after IDO-1 transfection, these tumor cells became immune-resistant and showed this effect by preventing T cells' accumulation specific to the tumor microenvironment. They demonstrated that

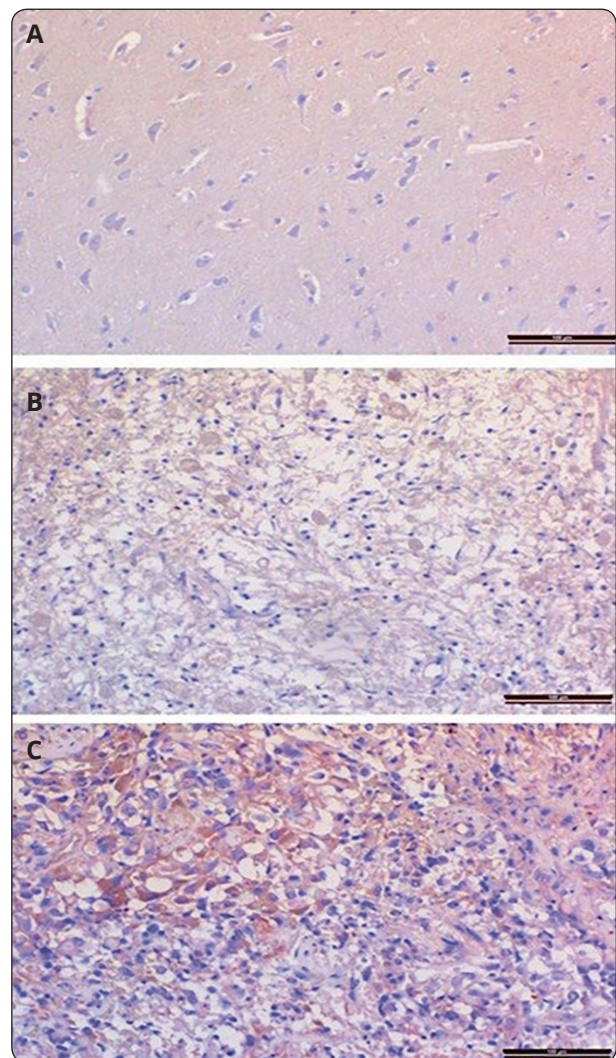


Figure 1. Histopathological evaluation for indoleamine 2, 3-dioxygenase-1 expression (IDO1), Haematoxylin & Eosin (HE) X100, Immunoperoxidase X 100. **A:** IDO-1 intensity in control brain tissue, **B:** IDO-1 intensity in low-grade glial tumor, **C:** IDO-1 intensity in high-grade glial tumor

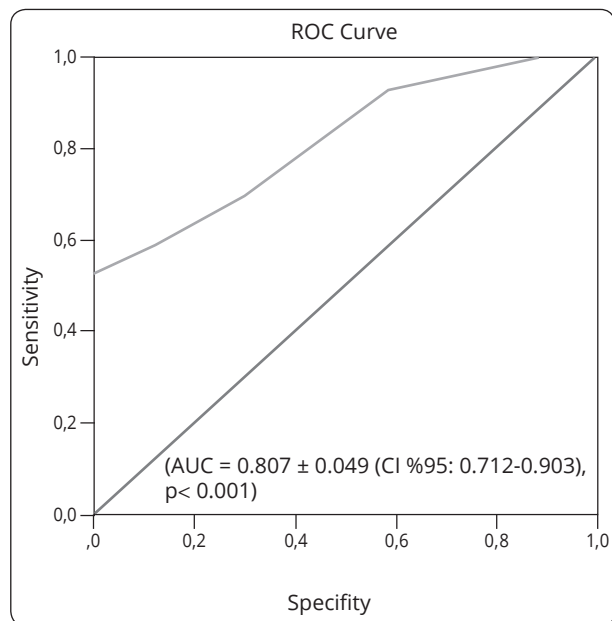


Figure 2. The receiver operating characteristic (ROC) curve for cut-off to the value of IDO-1

the tumors with high IDO-1 expression levels act by reducing their growth rate in vitro without affecting their proliferation rate. However, T cells have Trp-sensitive checkpoints, working in an exceptional situation in the opposite direction and blocking their proliferation. In mice inoculated with P815 antigens, partial resistance

Table 3. The correlation between basal characteristics of patients and IDO-1 expression

Parameter	r	p-value
Age	0.543	0.001
Grades (L/H)	0.797	0.001
Ki-67* (L<%32<H)	0.678	0.001
OS	-0.481	0.001

*median (min-max), L: Low grade, H: High grade

developed, and IDO-1 inhibitors were used to address the problem.

Curti et al. found that injecting splenic IDO-1 into acute myeloid leukemia cells (AML) boosted Treg cells in the bone marrow and CD4+, CD25+ T cells on the AML cell surface³³. They showed that when they treated IDO-1 expressing AML cells with IDO-1 inhibitor, CD4+ and CD25+ T cells on the AML cell surface turned into CD4+ and CD25- T cells. As a consequence, they demonstrated that IDO-1 may be used to evade immune regulatory systems.

Researchers observed that significant quantities of IDO-1 were expressed in colon cancer patients' cells, leading to a loss of tryptophan. They demonstrated that IDO-1 allows tumor cells to escape from the immune system by decreasing CD8 T cells' number and increasing the T cell (Treg) count³⁴.

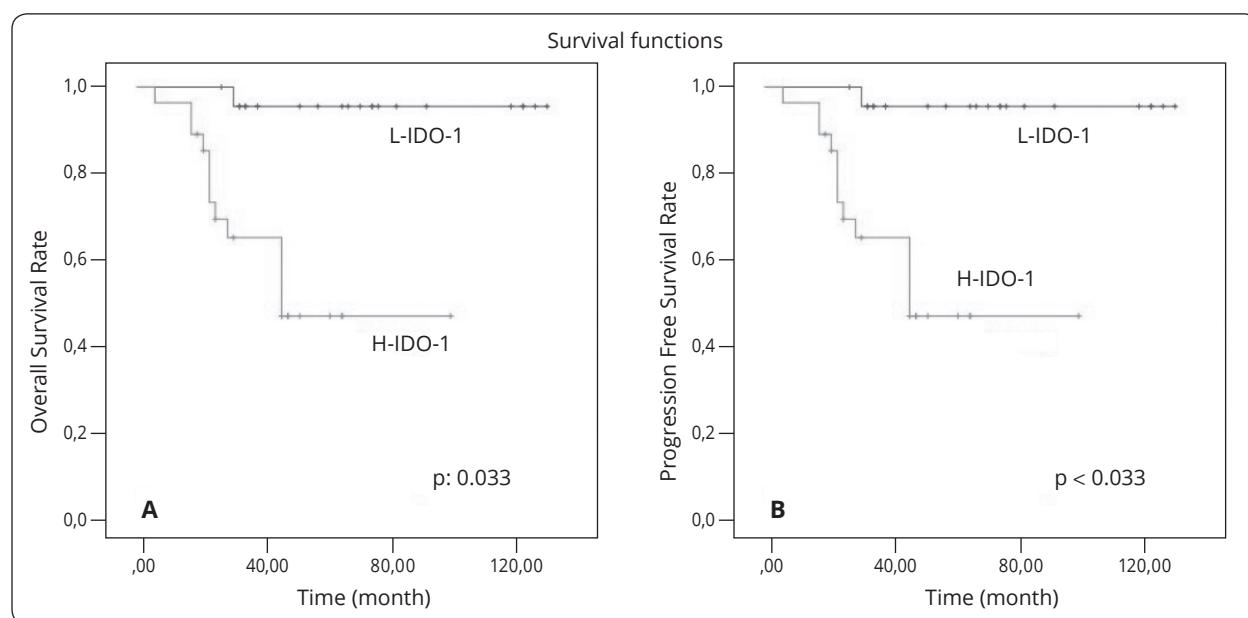


Figure 3. Kaplan-Meier curves for survival. **A:** Overall survival (OS) Kaplan-Meier curves stratified by indoleamine 2,3-dioxygenase-1 expression (IDO-1) groups. **B:** Progression-free survival (PFS) Kaplan-Meier curves stratified by indoleamine 2,3-dioxygenase-1 expression (IDO-1) groups

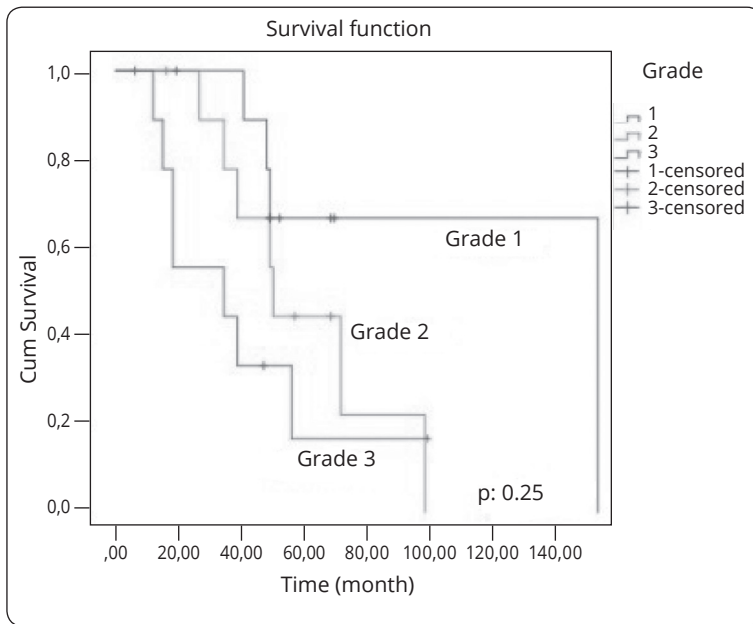


Figure 4. Overall survival (OS) Kaplan–Meier curves stratified by grades of tumor

Immunotherapies have recently been at the forefront of medicine. The topic of how to boost immunity in immune-compromised tumors has been discussed in several publications. *Spranger* et al. designed a study on how this can boost the immune system's strength³⁵. They were able to immunize the tumor microenvironment further using dual immune-modulatory agents (IDO-1 inhibitor + α -PD-L1) (IDO-1 inhibitor + anti-CTLA-4). As a result, the tumor microenvironment had more efficient and functional T cells.

Using the same rationale, while pembrolizumab's efficacy in non-small cell lung cancer (NSCLC) was only 10% in another research, combining it with a powerful and selective oral IDO-1 inhibitor raised response rates

to approximately 35%³⁶. IDO-1 inhibitors improved the immune system's performance.

There are studies on the relationship between IDO-1 expression and angiogenesis. IDO-1 expression enhanced angiogenesis in fibroblasts and vascular endothelial cells³⁷. *Nonaka* et al. compared IDO-1-expressing tumor cells and normal tumor cells in vivo and in vitro and showed more neovascularization in IDO-1 expressing tumor cells³⁸. We'll probably hear about trials with IDO-1 inhibitors and anti-VEGF/anti-EGFR factors soon.

Our results were similar to the studies exhibiting that high IDO-1 expression is associated with poor prognosis and short survival in patients with solid tumors such as ovarian, breast, colorectal, non-small cell lung, and cervical cancers^{39–44}.

Patients with high Ki-67 levels diagnosed with glioma, non-small cell lung cancer, and breast cancer have a shorter relapse-free lifespan^{23, 24, 45, 46}. Similar to the literature, our study found a statistically significant inverse relationship between H-Ki-67 levels and survival. This current study observed that all glial tumors express IDO-1, and detected that exceptionally high-grade glial tumors express more significant and higher levels of IDO-1 compared to low-grade glial tumors. High IDO-1 levels were associated with poor prognosis and shorter OS. We detected high IDO-1 levels in patients whose cancer progressed.

Our research has some limitations. To begin with, the number of identified glioma patients was limited. Second, we only used immunohistochemistry on tumor tissue. The third weakness is that we assessed glioma patients without categorizing them based on their molecular sub-analysis. We might have addressed more objective

Table 4. The factors affecting survival according to univariate and multivariate analysis

Variables	Univariate Analysis			Multivariate Analysis		
	p-value	HR	%95 CI	p-value	HR	%95 CI
ECOG (good/poor)	0.001	10.095	3.384-30.117	0.001	65.833	5.018-863.654
Progression (+/-)	0.001	4.648	1.454-14.856	0.008	0.052	0.006-0.462
IDO-1 (L/H)	0.033	6.542	0.854-50.090	0.035	50.250	1.318-1915.88
Age (<65 $\geq$)	0.340	2.915	1.010-8.414	0.408	1.904	0.414-8.750
Ki-67* (L /H)	0.001	8.948	1.999-40.043	0.172	0.163	0.012-2.197

HR: Hazard Ratio, CI: Confidence Interval, ECOG (Performance): Eastern Cooperative Oncology Group: Good (0,1,2), Poor (3,4), L: Low grade, H: High grade, *: median (min-max)

findings on the proliferative processing mechanism in gliomas if we had additional data on IDO-1 levels in the blood.

Conclusion

High IDO-1 expression according to immunohistochemistry may be an independent prognostic biomarker in predicting OS and PFS in HGG. As a result, we believe IDO-1 might be targeted to treat GBM. In GBM second-

line therapy, studies with a larger patient group and a comparison to anti-VEGF medications will provide new light on the unknown.

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