



Expression patterns of carbonic anhydrase IX and immune response biomarkers in pediatric gliomas and glioneuronal tumors

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Abstract

Purpose Carbonic anhydrase IX (CAIX), a hypoxia-induced enzyme, contributes to tumor acidosis and may modulate anti-tumor immunity. Its role in pediatric central nervous system (CNS) tumors remains poorly understood. This study examined CAIX and immune biomarker expression in pediatric gliomas and glioneuronal tumors (GNTs) and their associations with clinical outcomes.

Methods CAIX and selected immune biomarkers were evaluated using immunohistochemistry in 34 pediatric patients (ages 0–17 years) with low-grade gliomas (LGGs; $n=21$), GNTs ($n=6$), and high-grade gliomas (HGGs; $n=7$).

Results CAIX was expressed in 58.8% of tumors, with no statistically significant difference between the combined LGG/GNT and HGG groups. CD3 (87.9%), CD8 (81.8%), CD44 (93.9%), CD68 (100%), CD163 (75.8%), and CCR2 (69.7%) were the most frequently detected biomarkers, whereas PD-L1 (15.2%), PD-1 (0%), and CD206 (24.2%) were rarely detected. CAIX correlated positively with CD44 ($p=0.01$) and CD206 ($p=0.009$). Strong CD68 expression ($>30\%$) was associated with significantly better 2-year event-free survival (EFS) than lower expression (91.7% vs. 53.8%; $p=0.011$). No significant associations between EFS and CAIX or other immune biomarkers were observed (all $p>0.05$).

Conclusion Pediatric gliomas and GNTs frequently express CAIX and monocyte/macrophage-related biomarkers but exhibit a low immune checkpoint profile (PD-1 and PD-L1). Unlike findings from adult studies, CAIX was neither enriched in HGGs nor associated with a worse prognosis. Strong CD68 expression may represent a candidate target for combination therapeutic strategies, although validation in a larger, homogeneous pediatric CNS tumor cohort is needed.

Keywords Pediatric gliomas · Pediatric glioneuronal and neuronal tumors · Immunohistochemistry · Carbonic anhydrase IX · Immune biomarkers

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Introduction

Pediatric central nervous system (CNS) tumors are the most common solid tumors in childhood. Despite advances in multimodal therapy, they remain a leading cause of cancer-related mortality in children and are associated with pronounced neurocognitive late effects [1–4]. Therefore, the development of novel therapeutic strategies remains an important clinical priority.

Hypoxia promotes tumor progression and treatment resistance, making hypoxia-related molecular targets attractive candidates for precision anticancer therapies [5]. Carbonic anhydrase IX (CAIX) is a hypoxia-inducible, tumor-associated transmembrane metalloenzyme that regulates intracellular pH, supports tumor invasion, and contributes

to tumor microenvironment acidification [5, 6]. In adult cancers, including CNS tumors, strong CAIX expression is predominantly associated with high-grade tumors and worse prognosis [6, 7]. These associations have prompted the development of novel therapeutic approaches, including anti-CAIX-directed antibodies, small-molecule inhibitors, and cellular therapies [8–10]. However, most brain tumor studies analyzing CAIX expression used mixed adult–pediatric cohorts encompassing gliomas, ependymomas, medulloblastoma/primitive neuroectodermal tumors (MB/PNETs), and meningiomas. Such mixed cohorts may not accurately reflect pediatric tumor biology, as pediatric CNS tumors differ from those in adults in terms of tumor mutational burden (TMB) and the immune landscape [11–16].

Hypoxia further shapes antitumor immunity by suppressing tumor-infiltrating lymphocytes, polarizing tumor-associated macrophages (TAMs), and modulating PD-1/PD-L1 signaling [5, 10, 17, 18]. Pediatric tumors, which typically exhibit low TMB and reduced immunogenicity, respond less robustly to immune checkpoint inhibitors than adult tumors [19–23]. Nevertheless, CAIX has rarely been studied alongside broad immune biomarker panels in purely pediatric CNS tumor series [6, 11, 14, 24–30], despite the value of such studies in characterizing tumor behavior, refining prognosis, and informing more effective therapeutic strategies.

In this study, CAIX protein expression and tumor-associated immune biomarkers were examined in pediatric low-grade gliomas (LGGs), high-grade gliomas (HGGs), and glioneuronal and neuronal tumors (GNTs), and their potential impact on survival outcomes was assessed.

Materials and methods

CAIX and immune biomarker expression were evaluated in formalin-fixed, paraffin-embedded (FFPE) tumor tissues from pediatric patients with LGGs, HGGs, and GNTs. The institutional database was screened for patients aged 0–17 years diagnosed with CNS tumors between January 2000 and February 2022; only those diagnosed with glioma or GNT in 2010 or later were eligible. During the prospective period (March 2022 to July 2025), patients with newly diagnosed pediatric CNS tumors were enrolled. Tumor classification followed the 4th (2007), revised 4th (2016), or 5th (2021) edition of the WHO Classification of Tumors of the Central Nervous System [31–33]. Before 2020, diagnosis was based on histology and immunohistochemistry (IHC) for IDH1 and ATRX (9 cases), supplemented by karyotyping in an additional five cases. From 2020 onward, the Heidelberg CNS Tumor Methylation Classifier [34] was used alongside histology, IHC, RNA sequencing, and karyotyping. Overall,

34 glioma and GNT cases were included. The patient selection flowchart is illustrated in Fig. 1.

Data were locked and analyzed on August 31, 2025. A specialized neuro-oncology radiologist reviewed magnetic resonance imaging scans and assessed treatment response, tumor progression, and recurrence using the Response Assessment in Pediatric Neuro-Oncology criteria [35]. Primary outcomes were event-free survival (EFS), defined as the time from diagnosis to progression, relapse, or death, and overall survival (OS), defined as the time from diagnosis to death. Follow-up was defined as the interval from diagnosis to data lock. Survival status as of August 31, 2025 was verified through the Lithuanian Cancer Registry, which links the State Register of Deaths and Their Causes with the National Population Registry to track outcomes.

Immunohistochemistry

FFPE blocks were sectioned at 2 µm and stained at the National Center of Pathology in Vilnius. A board-certified neuropathologist assessed CAIX and immune biomarkers (CD3, CD8, CD44, CD68, CD163, PD-1, PD-L1, CD206, and CCR2). IHC was performed on Dako and Ventana Benchmark Ultra platforms using standard protocols for deparaffinization, heat-induced epitope retrieval, and peroxidase blocking. Details of antibody clones, detection systems, antigen retrieval, incubation, amplification, and hematoxylin counterstaining are provided in Supplementary Information 1 (SI1). The UltraView detection system (REF code (92) 760–500) was used.

Immunoreactivity for CAIX and immune biomarkers was recorded as positive (1) or negative (0). For CAIX, staining intensity and the percentage of positive tumor cells were assessed in pediatric glioma and GNT tissues. CAIX scoring methods have been previously described [12, 13, 15, 36–38]. The H-score system (percentage of positive cells × staining intensity) was applied [28, 38]. Staining intensity was graded on a scale of 0–3 (0, no staining; 1, weak; 2, moderate; 3, strong) (SI2). Based on H-score distribution, CAIX expression was classified as 0 (absent), 1 (weak, <10), 2 (moderate, 10–30), and 3 (strong, >30), reflecting heterogeneous expression.

For immune biomarkers, at least 2,000 tumor cells were visually assessed to determine the percentage of membranous immunoreactivity. CD3, CD8, PD-1, PD-L1, and CD206 were graded as 0 (absent), 1 (<3%), 2 (3%–5%), or 3 (>5%). CD68, CD163, and CCR2 were scored as 0 (absent), 1 (<10%), 2 (10%–30%), or 3 (>30%). CD44 was evaluated using the same H-score approach as CAIX and categorized as 0 (absent), 1 (<100), 2 (100–200), and 3 (>200). All assessments were performed blinded to the clinical data.

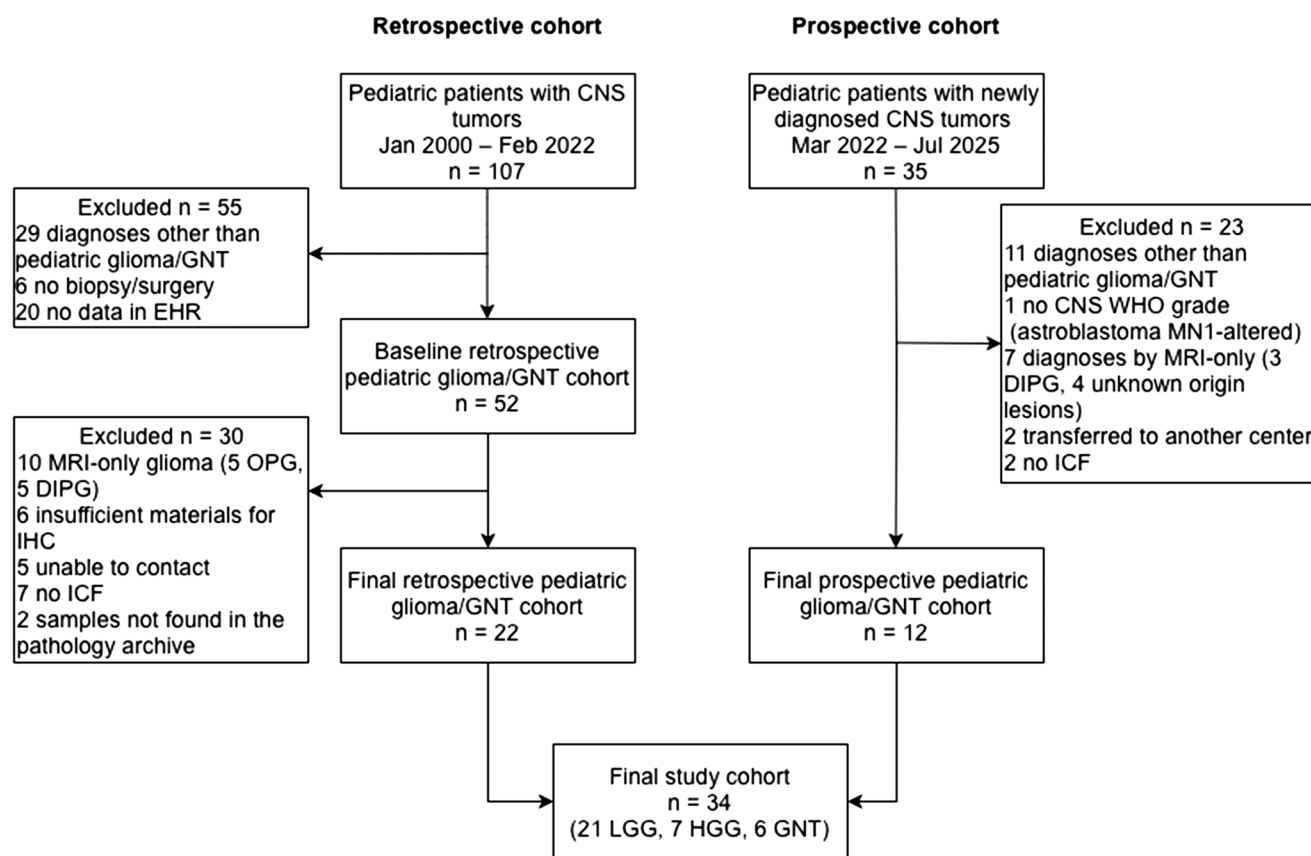


Fig. 1 Flowchart of patient selection for pediatric glioma and GNT diagnosis. Abbreviations: CNS, central nervous system; DIPG, diffuse intrinsic pontine glioma; EHR, electronic health records; Feb, February; GNT, glioneuronal and neuronal tumor; HGG, high-grade glioma;

ICF, informed consent form; IHC, immunohistochemistry; Jan, January; Jul, July; LGG, low-grade glioma; Mar, March; MRI, magnetic resonance imaging; OPG, optic pathway glioma; WHO, World Health Organization

Statistical analysis

Descriptive statistics were used to summarize tumor characteristics. The Shapiro–Wilk test was used to assess the normality of continuous variables. Continuous and ordinal variables were compared using the Mann–Whitney *U* test, and associations were evaluated using Spearman’s rank correlation coefficient. Fisher’s exact test was used to compare categorical variables. OS and EFS were estimated using the Kaplan–Meier method, with univariate analyses performed to evaluate factors associated with survival outcomes. A two-sided *p*-value of <0.05 was considered statistically significant. Analyses were conducted using IBM SPSS Statistics 30.0 and R (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics

Thirty-four patients with pediatric gliomas and GNTs were included. Twenty-seven (79.4%) had LGGs/GNTs, and seven (20.6%) had HGGs. For analysis, LGGs and GNTs were combined as a low-grade group (grades 1–2), and HGGs were analyzed as a high-grade group (grades 3–4). CAIX expression was assessed in all 34 cases, whereas the full immune biomarker panel was available in 33 (97.1%) cases due to insufficient residual tissue material in one LGG/GNT sample. Four deaths (11.8%) occurred, all in the HGG group (57.1%); three due to disease progression and one due to postoperative complications. Baseline clinical characteristics are summarized in Table 1.

Table 1 Characteristics of patients and tumor samples in the cohort and in low- and high-grade groups

Patient and tumor sample characteristics	Total glioma/ GNT N = 34	LGG/GNT Grades 1–2 N = 27	HGG Grades 3–4 N = 7
Tumor type by diagnosis and CNS WHO grade, n (%)			
LGG	21 (61.8)	21 (77.8):	-
- Pilocytic astrocytoma (G1)		17 (81.0%)	
- Subependymal giant cell astrocytoma (G1)		2 (9.5%)	
- LGG, unclassified (G1/G2)		1 (4.8%)	
- Diffuse astrocytoma, IDH-mutant (G2)		1 (4.8%)	
GNT	6 (17.6)	6 (22.2):	
- Ganglioglioma (G1)		4 (66.7%)	-
- Dysembryoplastic neuroepithelial tumor (G1)		1 (16.7%)	
- Low-grade glioneuronal and neuronal tumor, unclassified (G1/G2)		1 (16.7%)	
HGG	7 (20.6)	-	7 (100):
- Anaplastic oligodendroglioma (G3)			1 (14.3%)
- Diffuse midline glioma, H3 K27-altered (G4)			2 (28.6%)
- Diffuse hemispheric glioma, H3 G34-mutant (G4)			1 (14.3%)
- Diffuse pediatric-type high-grade glioma, H3-wildtype, and IDH-wildtype (G4)			1 (14.3%)
- Infant-type hemispheric glioma (G4)			1 (14.3%)
- Glioblastoma, IDH-wildtype (G4)			1 (14.3%)
Specimens by patient cohort, n (%)			
Retrospective	22 (64.7)	18 (66.7)	4 (57.1)
Prospective	12 (35.3)	9 (33.3)	3 (42.9)
Investigated biomarkers (IHC), n (%)			
CAIX	34 (100)	27 (100)	7 (100)
Immune biomarkers	33 (97.1)	26 (96.3)	7 (100)
Survival status, n (%)			
Alive	30 (88.2)	27 (100)	3 (42.9)
Dead	4 (11.8)	0	4 (57.1)
Tumor specimens, n (%)			
Diagnostic (primary)	32 (94.1)	27 (100)	5 (71.4)
Relapse/progression (secondary)	2 (5.9)	0	2 (28.6)
Sex			
Males, n (%)	19 (55.9)	14 (51.9)	5 (71.4)
Females, n (%)	15 (44.1)	13 (48.1)	2 (28.6)
Age at diagnosis (years), median (IQR)			
	9.4 (6.2,12.3)	8.2 (6.1,12.2)	11.6 (9.4,12.2)
Tumor localization, n (%)			
Supratentorial	22 (64.7)	16 (59.3)	6 (85.7)
Infratentorial	10 (29.4)	9 (33.3)	1 (14.3)
Spinal	2 (5.9)	2 (7.4)	0
Follow-up time (years), median (IQR)			
	4.1 (1.6, 8.8)	5.2 (2.2, 9.2)	1.1 (0.4, 2.2)
First-line treatment			
GTR	16 (47.1)	16 (59.3)	0
Bx/PR	12 (35.3)	10 (37.0)	2 (28.6)
Bx/PR + CT	1 (2.9)	1 (3.7)	0
Bx/PR + RT + CT	4 (11.8)	0	4 (57.1)
GTR + CT	1 (2.9)	0	1 (14.3)
No adverse events after the first-line treatment, n (%)			
Complete response	11 (32.4)	10 (37.0)	1 (14.3)
Partial response	3 (8.8)	3 (11.1)	0
Stable disease	5 (14.7)	5 (18.5)	0
First adverse events, n (%)			
Progressive disease	8 (23.5)	3 (11.1)	5 (71.4)
Relapse	6 (17.6)	6 (22.2)	0

Table 1 (continued)

Patient and tumor sample characteristics	Total glioma/ GNT N = 34	LGG/GNT Grades 1–2 N = 27	HGG Grades 3–4 N = 7
Death*	1 (2.9)	0	1 (14.3)
Time to the first adverse event (years), median (IQR)	0.6 (0.3, 2.3)	2.3 (0.5, 2.7)	0.4 (0.1, 0.5)

Abbreviations: Bx, biopsy; CT, chemotherapy; CNS, central nervous system; GNT, glioneuronal and neuronal tumors; GTR, gross total resection; HGG, high-grade glioma; IHC, immunohistochemistry; IQR, interquartile range; LGG, low-grade glioma; PR, partial resection; RT, radiotherapy; WHO, World Health Organization

*Early death due to postoperative complications

Immunohistochemical expression of CAIX and immune biomarkers

Positive rates, median values, interquartile ranges (IQRs), and four-tier staining intensity scores for CAIX and immune biomarkers are summarized in Table 2 for the entire cohort and for each subgroup. Figure 2A–C presents the expression levels.

CD3 and CD68 expression levels were significantly higher in the LGG/GNT group than in the HGG group ($p=0.009$ and $p=0.002$, respectively; Mann–Whitney U test) (Fig. 2B). Strong CD68 staining ($>30\%$) was also significantly associated with the LGG/GNT group ($p=0.013$; Fisher's exact test) (Fig. 2C). No other biomarkers showed statistically significant differences between groups (all $p>0.05$).

Correlation analysis revealed moderate positive correlations between CAIX and CD44 ($R=0.44$, $p=0.010$) and between CAIX and CD206 ($R=0.45$, $p=0.009$), based on Spearman's rank correlation analysis (Fig. 2D). Figure 2E illustrates the correlations between immune biomarkers.

Finally, an assessment was conducted to determine whether marker expression was associated with basic clinical characteristics. A higher proportion of CCR2-positive cells was observed in supratentorial tumors compared with infratentorial or spinal tumors ($p=0.019$; Mann–Whitney U test). CAIX and the remaining immune biomarkers showed no significant differences according to sex, age at diagnosis (≤ 8 vs. >8 years), or tumor location (all $p>0.05$).

Impact of CAIX and immune biomarkers on outcomes in pediatric gliomas and GNTs

At a median follow-up of 5.2 years (IQR, 2.2–9.2), no deaths occurred among the 27 patients with LGGs/GNTs. In the HGG group, the median follow-up was 1.1 years (IQR, 0.4–2.2), during which four of seven patients (57.1%) died. Kaplan–Meier analysis showed significantly worse EFS and OS in patients with HGGs (both $p<0.001$) (Fig. 3A, B). Biopsy or partial resection was associated with poorer OS ($p=0.016$) but not EFS ($p=0.079$) (Fig. 3C, D).

Among patients with LGGs/GNTs, three (11.1%) experienced disease progression and six (22.2%) experienced relapse, with a median time from diagnosis to any event of 2.3 years (IQR, 0.5–2.7). In the HGG group, five of seven patients (71.4%) experienced progression as the first adverse event, and one patient (14.3%) died 3 days postoperatively due to complications. The median time from diagnosis to an adverse event was 0.4 years (IQR, 0.1–0.5) (Table 1).

Strong CD68 expression ($>30\%$) was associated with improved EFS, with a 2-year EFS rate of 91.7% (95% CI, 77.3–100.0) compared with 53.8% (95% CI, 35.6–81.4) in patients without strong expression ($p=0.011$). CAIX and other immune biomarkers were not significantly associated with EFS (all $p>0.05$). Kaplan–Meier curves for EFS based on CAIX and selected immune biomarkers are shown in Fig. 3E. No significant differences in OS were observed according to biomarker expression (all $p>0.05$), consistent with the low number of deaths ($n=4$). Additional EFS data are provided in SI3.

Univariate Cox regression analysis showed that strong CD68 expression ($>30\%$) in pediatric gliomas and GNTs was associated with improved EFS (HR=0.18, 95% CI 0.04–0.80, $p=0.02$), with events defined as progression, relapse, or death from any cause. High CNS WHO grade (grades 3–4) was associated with poorer EFS across the entire cohort (HR=5.49; 95% CI, 1.89–15.91; $p=0.002$). CAIX expression, other immune biomarkers, and clinical variables (sex, age, tumor location, and extent of surgery) were not significantly associated with EFS (all $p>0.05$) (SI4). Multivariate analysis was not performed because of the small cohort size.

Discussion

CAIX expression and immune biomarkers compared with adult literature

Adult studies have identified CAIX as a common and prognostically adverse marker in CNS tumors. In adult glioblastoma (GBM), astrocytic, and oligodendroglial tumors, CAIX positivity reaches approximately 70%, increases with

Table 2 CAIX and immune biomarker expression in pediatric glioma and GNT samples

	Total glioma/GNT	LGG/GNT(Grades 1–2)	HGG(Grades 3–4)	<i>p</i> -value [#]
CAIX expression				
Immunohistochemistry (FFPE)	N = 34	N = 27	N = 7	
CAIX positivity (H-score >0), n (%)	20 (58.8)	17 (63.0)	3 (42.9)	0.4 ¹
Median expression (IQR)	4.5 (0.0, 30.0)	5.0 (0.0, 32.5)	0 (0.0, 12.5)	0.4 ²
None (H-score 0), n (%)	14 (41.2)	10 (37.0)	4 (57.1)	0.6 ¹
Weak (H-score <10), n (%)	5 (14.7)	5 (18.5)	0	
Moderate (H-score 10–30), n (%)	7 (20.6)	5 (18.5)	2 (28.6)	
Strong (H-score >30), n (%)	8 (23.5)	7 (25.9)	1 (14.3)	
Immune biomarker expression				
Immunohistochemistry (FFPE)	N = 33	N = 26	N = 7	
CD44 positivity (H-score >0), n (%)	31 (93.9)	24 (92.3)	7 (100)	>0.9 ¹
Median expression (IQR)	200.0 (150.0, 270.0)	200.0 (185.0, 277.5)	180.0 (105.0, 220.0)	0.3 ²
None (H-score 0), n (%)	2 (6.1)	2 (7.7)	0	0.5 ¹
Weak (H-score <100), n (%)	4 (12.1)	2 (7.7)	2 (28.6)	
Moderate (H-score 100–200), n (%)	15 (45.5)	12 (46.2)	3 (42.9)	
Strong (H-score >200), n (%)	12 (36.4)	10 (38.5)	2 (28.6)	
CD3 positivity (% > 0), n (%)	29 (87.9)	24 (92.3)	5 (71.4)	0.2 ¹
Median expression (IQR)	2.0 (1.0, 5.0)	3.0 (1.0, 5.0)	1.0 (0.5, 1.0)	0.009 ²
None (0%), n (%)	4 (12.1)	2 (7.7)	2 (28.6)	0.056 ¹
Weak (<3%), n (%)	15 (45.5)	10 (38.5)	5 (71.4)	
Moderate (3%–5%), n (%)	10 (30.3)	10 (38.5)	0	
Strong (>5%), n (%)	4 (12.1)	4 (15.4)	0	
CD8 positivity (% > 0), n (%)	27 (81.8)	22 (84.6)	5 (71.4)	0.6 ¹
Median expression (IQR)	1.0 (1.0, 3.0)	1.0 (1.0, 3.0)	1.0 (0.5, 1.0)	0.053 ²
None (0%), n (%)	6 (18.2)	4 (15.4)	2 (28.6)	0.3 ¹
Weak (<3%), n (%)	17 (51.5)	12 (46.2)	5 (71.4)	
Moderate (3%–5%), n (%)	8 (24.2)	8 (30.8)	0	
Strong (>5%), n (%)	2 (6.1)	2 (7.7)	0	
CD68 positivity (% > 0), n (%)	33 (100)	26 (100)	7 (100)	-
Median expression (IQR)	30.0 (25.0, 40.0)	32.5 (30.0, 40.0)	20.0 (13.0, 22.5)	0.002 ²
None (0%), n (%)	0	0	0	0.013 ¹
Weak (<10%), n (%)	3 (9.1)	1 (3.8)	2 (28.6)	
Moderate (10%–30%), n (%)	17 (51.5)	12 (46.2)	5 (71.4)	
Strong (>30%), n (%)	13 (39.4)	13 (50.0)	0	
CD163 positivity (% > 0), n (%)	25 (75.8)	18 (69.2)	7 (100)	0.2 ¹
Median expression (IQR)	5.0 (1.0, 20.0)	5.0 (0.0, 23.8)	6.0 (5.0, 17.5)	0.7 ²
None (0%), n (%)	8 (24.2)	8 (30.8)	0	0.2 ¹
Weak (<10%), n (%)	10 (30.3)	6 (23.1)	4 (57.1)	
Moderate (10%–30%), n (%)	11 (33.3)	8 (30.8)	3 (42.9)	
Strong (>30%), n (%)	4 (12.1)	4 (15.4)	0	
PD-1 positivity (% > 0), n (%)	0	0	0	-
Median expression (IQR)				
PD-L1 positivity (% > 0), n (%)	5 (15.2)	4 (15.4)	1 (14.3)	>0.9 ¹
Median expression (IQR)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	>0.9 ²
None (0%), n (%)	28 (84.8)	22 (84.6)	6 (87.7)	0.7 ¹
Weak (<3%), n (%)	1 (3.0)	1 (3.8)	0	
Moderate (3%–5%), n (%)	2 (6.1)	2 (7.7)	0	
Strong (>5%), n (%)	2 (6.1)	1 (3.8)	1 (14.3)	
CD206 positivity (% > 0), n (%)	8 (24.2)	8 (30.8)	0	0.2 ¹
Median expression (IQR)	0.0 (0.0, 0.0)	0.0 (0.0, 1.0)		-
None (0%), n (%)	25 (75.8)	18 (69.2)	7 (100)	0.5 ¹
Weak (<3%), n (%)	5 (15.2)	5 (19.2)	0	
Moderate (3%–5%), n (%)	1 (3.0)	1 (3.8)	0	
Strong (>5%), n (%)	2 (6.1)	2 (7.7)	0	
CCR2 positivity (% > 0), n (%)	23 (69.7)	16 (61.5)	7 (100)	0.07 ¹

Table 2 (continued)

	Total glioma/GNT	LGG/GNT(Grades 1–2)	HGG(Grades 3–4)	<i>p</i> -value [#]
Median expression (IQR)	3.0 (0.0, 15.0)	1 (0.0, 15.0)	10.0 (4.5, 12.5)	0.3 ²
None (0%), n (%)	10 (30.3)	10 (38.5)	0	0.083 ¹
Weak (<10%), n (%)	9 (27.3)	6 (23.1)	3 (42.9)	
Moderate (10%–30%), n (%)	10 (30.3)	6 (23.1)	4 (57.1)	
Strong (>30%), n (%)	4 (12.1)	4 (15.4)	0	

[#]Statistical analysis: First row *p*-value compares positive vs. negative; second row *p*-value compares median expression; third row *p*-value compares staining intensity categories (none–strong), all between LGG/GNT and HGG groups

^{1,2}Statistical tests: ¹Fisher's exact test; ²Mann–Whitney *U* test

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; GNT, glioneuronal and neuronal tumor; HGG, high-grade glioma; IQR, interquartile range; LGG, low-grade glioma

WHO grade, and predicts a poorer prognosis [12, 13, 24, 36, 37]. Elevated CAIX expression has also been reported in adult ependymomas, craniopharyngiomas, and meningiomas, where it frequently co-occurs with other hypoxia-related markers and is associated with shorter survival [11, 14, 39]. Conversely, CAIX was detected in 59% of pediatric gliomas and GNTs in our study, with no higher frequency in HGGs. This finding likely reflects the predominance of LGGs/GNTs in the entire cohort. Methodological differences may also contribute to discrepancies between studies. In the present study, CAIX assessment was based on the H-score system, which differs from the percentage-based scoring approaches used in several adult CNS tumor studies [12, 36, 37]. The H-score system, originally applied in adult meningioma studies, incorporates staining intensity and the percentage of positive cells [28] and has also been used in GBM studies [13]. Similarly, a four-tier scoring system has been employed in studies of MB/PNETs and GBMs [15, 37]. Nordfors et al. reported that MB/PNET cohorts spanning a broad age range with predominantly pediatric (median age, 7.4 years; range, newborn–68 years) did not show a significant increase in CAIX expression, with positivity observed in only 23% of cases [15], which is consistent with the findings of the present study. Nevertheless, in that cohort, CAIX expression was associated with poorer prognosis in both univariate and multivariate analyses. Overall, these results suggest that CAIX may be biologically relevant in pediatric CNS tumors; however, its expression appears less pronounced and more dependent on cohort composition than in adult populations [16].

The immune microenvironment in pediatric gliomas and GNTs is monocyte/macrophage-rich and immune checkpoint-poor

Hypoxia can reshape local and systemic immune responses [40]. The analyzed specimens showed marked enrichment of CD68-positive monocytes/macrophages. CD68, a widely used monocyte/macrophage marker, was expressed in all tumors, and most tumors also expressed CD163, a marker

of M2-polarized macrophages. These findings indicate a prominent myeloid component in pediatric gliomas and GNTs, similar to that observed in adult gliomas [27, 30, 41]. Conversely, expression of immune checkpoint proteins was low. PD-1 was absent in all pediatric gliomas and GNTs, whereas PD-L1 expression was detected in only 15% of IHC samples. This differs from adult gliomas, in which PD-L1 expression is more frequent and tends to increase with tumor grade [22, 26, 29]. The low checkpoint profile is consistent with the low mutational burden of pediatric CNS tumors [16, 19, 20] and indicates that a PD-1 or PD-L1 blockade is unlikely to be effective in this population. Additionally, CCR2-positive cells were more frequently observed in supratentorial tumors than in infratentorial or spinal tumors, suggesting that tumor location may influence their recruitment.

CAIX expression was positively associated with CD44, a cell-adhesion molecule, and CD206, a macrophage mannose receptor; both markers are associated with M2 macrophage polarization [27, 42, 43]. In adult meningiomas, CD44 expression has been linked to higher tumor grade and poorer clinical outcomes [28]. This co-expression pattern suggests that hypoxic regions within pediatric CNS tumors may coincide with extracellular matrix remodeling and M2-like macrophage polarization. These regions may represent potential targets for combination therapeutic strategies that simultaneously modulate hypoxia and TAMs.

Strengths

This study focused exclusively on pediatric gliomas and GNTs, thereby minimizing potential confounding arising from age-related biological differences often present in mixed pediatric–adult CNS tumor cohorts. Both hypoxia-related and broad immune-related biomarkers were evaluated, enabling the identification of co-expression patterns, including associations between CAIX and CD44 as well as CAIX and CD206. Additionally, LGGs/GNTs, which represent the most common pediatric CNS tumors, comprised most of the cohort. Although these tumors are often nonfatal,

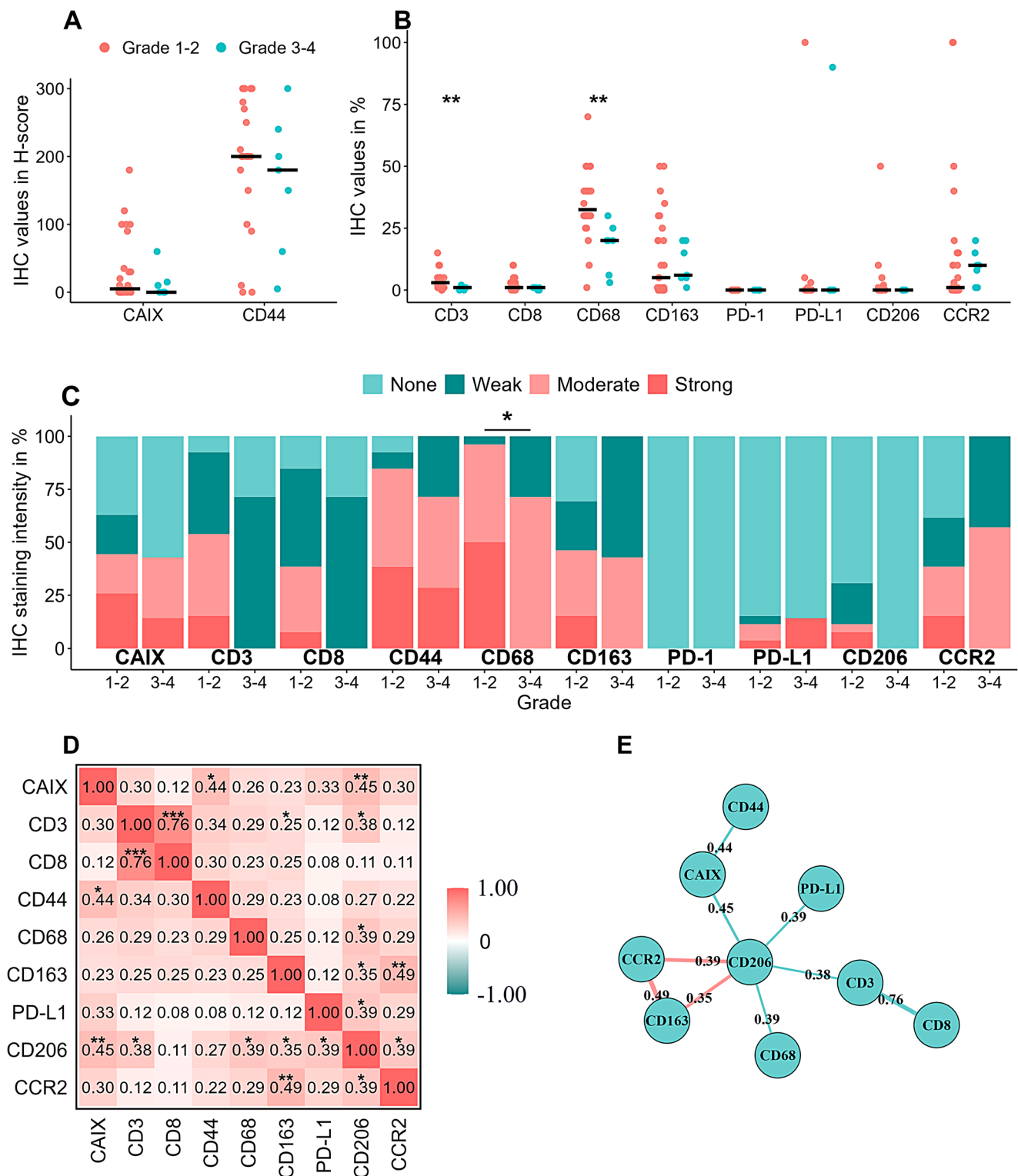


Fig. 2 Expression and correlation of CAIX and immune biomarkers. **(A)** CAIX and CD44 expression assessed using H-score. **(B)** Immune biomarker expression was assessed as a percentage of positive cells. **(C)** Distribution of CAIX and immune biomarker staining intensities in LGG/GNT (grades 1–2) and HGG (grades 3–4). **(D)** Strength of correlations between CAIX expression and selected immune biomarkers in pediatric gliomas and GNTs. **(E)** Network map showing significant

correlations (orange line) among immune biomarkers. *Correlation coefficient (R) is interpreted as follows: 0–0.19, negligible; 0.2–0.39, weak; 0.40–0.69, moderate; 0.70–0.89, high; and 0.90–1.0, very high.* Significance levels: panels 2a and 2b, ** $p < 0.01$ (Mann–Whitney U test); 2c, * $p < 0.05$ (Fisher’s exact test); 2d, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Spearman’s rank-order correlation test)

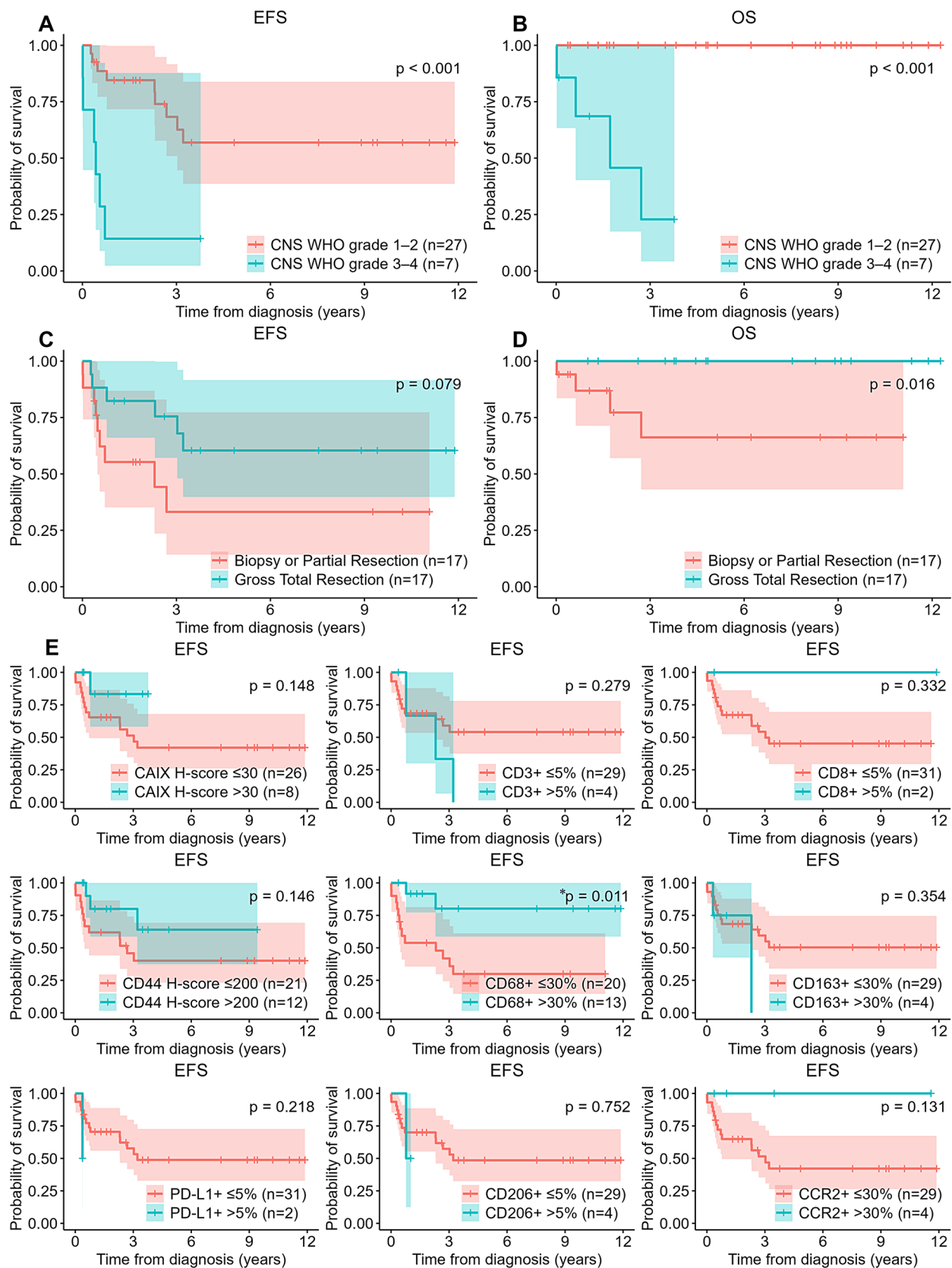


Fig. 3 Kaplan–Meier curves of (A) EFS and (B) OS by CNS tumor type: LGGs/GNTs (grades 1–2) versus HGGs (grades 3–4); (C) EFS and (D) OS by extent of tumor resection: GTR versus Bx/PR in pediatric

gliomas/GNTs; (E) EFS by CAIX and immune biomarker expression in pediatric gliomas/GNTs. *** $p < 0.001$; * $p < 0.05$ (log-rank test)

they may progress in up to one-third of patients and can substantially affect quality of life [44, 45]. Survival analyses also provided initial estimates of clinical relevance.

Limitations

First, the retrospective, single-center design and heterogeneous cohort of pediatric gliomas and GNTs may have introduced selection bias and limited the generalizability of the findings. The WHO classification of CNS tumors has evolved across editions (2007, 2016, and 2021) [31–33], with increasing incorporation of molecular alterations and refinement of diagnostic subtypes. The diverse histopathological diagnoses and small subgroup sizes (7 HGGs and 6 GNTs) further limited statistical power. Finally, variability in CAIX and immune biomarker scoring systems across studies limits direct comparisons with published findings [12, 15, 23, 25–29, 36, 37].

Second, despite histopathological differences, LGGs and GNTs were analyzed together because both are primary neuroepithelial CNS tumors and are typically classified as WHO grade 1–2. They share similar low-grade clinical behavior, favorable prognosis, and overlapping molecular features, including activation of the mitogen-activated protein kinase pathway [46]. Nevertheless, biological variability between these tumor types may have influenced tumor behavior and microenvironmental characteristics. To assess the impact of this grouping, analyses were repeated after excluding GNTs (LGGs, $n=21$ vs. HGGs, $n=7$). No significant differences were observed between the original LGG/GNT versus HGG comparison and the LGG-only versus HGG comparison (all $p>0.05$). A direct comparison between LGGs and GNTs also showed no significant differences in immune biomarker positivity, median values, or expression intensity (all $p>0.05$). However, CAIX expression differed significantly between LGGs and GNTs, with higher median expression (H-score 62.5 vs. 1.0, $p=0.02$) and stronger staining intensity (66.7% vs. 14.3%, $p=0.02$) in GNTs than in LGGs.

Third, previous glioma studies have linked CAIX expression to poorer prognosis, particularly in adult GBM-enriched cohorts [13, 37]. Therefore, higher CAIX expression was expected in the HGG group with an impact on worse survival outcomes. However, only one of the seven HGGs in our cohort was an IDH-wild-type GBM, and CAIX expression was not associated with adverse clinical outcomes (Fig. 3E). Notably, these analyses were not adjusted for tumor grade because of the small number of HGG cases. Our study showed that CAIX expression may also occur in low-grade pediatric CNS tumors, although this has not been systematically studied in pediatric-only low-grade cohorts, where survival outcomes are generally favorable. Given the

limited cohort size and biological heterogeneity, these findings should be interpreted with caution.

Fourth, strong CD68 expression ($>30\%$) was associated with improved EFS. As CD68 positivity and strong expression were more common in LGGs/GNTs than in HGGs, this association may partly reflect the more favorable prognosis of low-grade tumors. However, the HGG subgroup comprised only seven patients. Stratified Cox regression analysis revealed that strong CD68 expression remained associated with improved EFS in patients with LGGs/GNTs. Each 1% increase in CD68 positivity reduced event risk by 5.3% (HR=0.947, 95% CI 0.89–1.00, $p=0.039$), whereas no significant association was observed in HGGs (HR=0.983, 95% CI 0.91–1.06, $p=0.659$). Notably, a GBM study reported lower CD68 expression than CD163 expression [46], consistent with the lower CD68 expression observed in HGGs than in LGGs/GNTs in this cohort. This suggests that CD68 expression may reflect a favorable myeloid microenvironment rather than merely serving as a surrogate marker of tumor grade in predominantly pediatric glioma and GNT cohorts. However, validation in larger, histologically homogeneous pediatric glioma cohorts is required.

Conclusions

CAIX was detected in a substantial proportion of pediatric gliomas and GNTs. However, unlike adult gliomas, CAIX expression was neither enriched in HGGs compared with LGGs/GNTs nor associated with adverse prognosis. The CNS tumor microenvironment was characterized by abundant monocyte/macrophage infiltration and low PD-1 and PD-L1 expression. Furthermore, CAIX expression correlated with adhesion and M2-like markers (CD44 and CCR2), suggesting overlap between hypoxic and immunosuppressive niches in pediatric gliomas and GNTs. Survival analysis revealed an association between strong CD68 expression and improved EFS. Nevertheless, interpretation of these findings is limited by the predominance of LGGs/GNTs, the limited HGG cases, and cohort histopathological heterogeneity. Therefore, CAIX and immune-related biomarkers remain promising targets for further study in larger, molecularly and histologically homogeneous pediatric CNS tumor cohorts using standardized biomarker assessment and integrated therapeutic approaches.

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Author contributions All authors contributed to the study conception and design. M.K. conceptualization, methodology, formal analysis, investigation, visualization, data curation, writing - original draft, D.P. investigation, methodology, S.R., R.R., D.R. resources, A.P., A.L., S.G. conceptualization, visualization, writing - review & editing, D.M. conceptualization, I.A. resources, G.T. formal analysis, J.R. conceptualization, methodology, visualization, writing - review & editing, supervision.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval This study was approved by the Vilnius Regional Committee of Biomedical Research (Approval No. 2020/10-1274-753).

Consent to participate Informed consent was obtained from parents or legal guardians for all participants under 18 years of age. Participants aged 12 years and older were also given the opportunity to provide assent and, where applicable, to sign the consent form.

Consent to publish No separate informed consent for publication of individual details, images, or videos was required, as the manuscript contains no information that could identify any patient.

Competing interests The authors declare no competing interests.

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