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Urinary D-asparagine level is decreased by the presence of glioblastoma

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Abstract

Gliomas, particularly glioblastomas (GBMs), pose significant challenges due to their aggressiveness and poor prognosis. Early detection through biomarkers is critical for improving outcomes. This study aimed to identify novel biomarkers for gliomas, particularly GBMs, using chiral amino acid profiling. We used chiral amino acid analysis to measure amino acid L- and D-isomer levels in resected tissues (tumor and non-tumor), blood, and urine from 33 patients with primary gliomas and 24 healthy volunteers. The levels of D-amino acid oxidase (DAO), a D-amino acid-degrading enzyme, were evaluated to investigate the D-amino acid metabolism in brain tissue. The GBM mouse model was created by transplanting GBM cells into the brain to confirm whether gliomas affect blood and urine chiral amino acid profiles. We also assessed whether D-amino acids produced by GBM cells are involved in cell proliferation. D-asparagine (D-Asn) levels were higher and DAO expression was lower in glioma than in non-glioma tissues. Blood and urinary D-Asn levels were lower in patients with GBM than in healthy volunteers ($p < 0.001$), increasing after GBM removal ($p < 0.05$). Urinary D-Asn levels differentiated between healthy volunteers and patients with GBM (area under the curve: 0.93, sensitivity: 0.88, specificity: 0.92). GBM mouse model validated the decrease of urinary D-Asn in GBM. GBM cells used D-Asn for cell proliferation. Gliomas induce alterations in chiral amino acid profiles, affecting blood and urine levels. Urinary D-Asn emerges as a promising diagnostic biomarker for gliomas, reflecting tumor presence and severity.

Keywords Biomarker, Chiral amino acids, D-amino acids, Glioma, Glioblastoma, Urine

Introduction

A glioma is a primary central nervous system tumor of varying grades, with the most aggressive form being glioblastoma (GBM; WHO grade 4) and is also the most common primary malignant brain tumor in adults with a poor prognosis [25]. Even with multimodal therapeutic approaches, the relative survival rate for GBMs remains low, with 6.9% of patients surviving 5 years after diagnosis [26]. Furthermore, since most patients with GBM are diagnosed using imaging modalities, such as CT and MRI, GBM detection is often delayed after symptoms appear. Previous research has indicated that early detection of GBM results in a better prognosis through timely therapeutic interventions [13]. These findings indicate

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the need to develop biomarkers for GBMs using versatile samples, such as blood and urine, collected during medical checkups of asymptomatic patients.

A hallmark of cancer is the reprogramming of energy metabolism [9]. Through this reprogramming, cancer cells efficiently use energy for survival and growth. However, studies on cancer metabolism have focused on carbon metabolism, including the glycolytic, citric acid, and tricarboxylic acid cycles. Recently, studies have clarified that the enhanced synthesis and uptake of amino acids by cancers result in their efficient proliferation [3, 6, 7, 11, 19]. The relationship between gliomas and amino acid metabolism has also attracted attention. Metabolomic studies using blood samples from patients with gliomas have reported changes in alanine (Ala) and other amino acids levels [5, 12, 29, 36]. Serine (Ser) is reportedly used for cancer growth and proliferation [1, 28]. Cancer uses asparagine (Asn) to promote metastasis [17]. Moreover, although a new treatment for gliomas using D-amino acid oxidase (DAO), a D-amino acid-degrading enzyme, is expected [4], the relationship between D-amino acids and cancer remains unclear. How gliomas differentiate and utilize these optical isomers is a topic of interest. Therefore, it is essential to construct chiral amino acid profiles of glioma tissue and liquid samples to elucidate novel chiral amino acid metabolism in patients with gliomas.

We have previously developed analytical techniques to separate amino acids into their D- and L-optical isomers [10, 21, 22]. In the present study, we hypothesized that glioma cells use D-amino acids for cell proliferation, affecting D-amino acid levels in the body. Previously, we reported that Ser, Ala, Asn, and proline (Pro) are the most abundant amino acids in human plasma and urine [10, 21, 22]. Thus, we focused on these four amino acids. This study aimed to identify novel biomarkers for gliomas, particularly GBMs, by constructing a chiral amino acid profile using liquid samples (plasma and urine) that can be easily used in clinical tests, in addition to brain tissues.

Methods

Study population

A total of 42 patients with first-onset brain tumors were enrolled. Among those, nine patients were excluded as they had different tumor types (central neurocytoma, hemangiopericytoma, pilocytic astrocytoma, cavernoma, cortical dysplasia or diffuse astrocytoma, malignant lymphoma, and metastases). Consequently, 33 patients with gliomas were enrolled in the study (see Supplementary Tables 1 and 2 [Online Resource 1]). Ten IDH-mutant gliomas and 16 IDH-wild gliomas were included.

Overall, 24 healthy volunteers were also enrolled after a thorough examination by a neurologist to confirm the absence of both neurological abnormalities and cognitive impairments (see Supplementary Table 1 [Online Resource 1]). Healthy volunteers were recruited from the participants who had joined a clinical trial that aimed to assess the effects of polyphenols on cognition in older adults without dementia (Clinical Study Number: jRCTs041180064 and UMIN000021596) [24].

Blood and urine sampling

Blood (plasma) and urine samples were collected from patients with gliomas and healthy volunteers. In patients with gliomas, these samples were collected after surgery. The collected samples were immediately stored on ice, followed by centrifugation at 3,000 rpm for 30 min in an ice-cold environment. Subsequently, the samples were stored at −80 °C until amino acid analysis. All samples were collected between 2019 and 2022 at Kanazawa University Hospital.

Brain tissues were obtained from all 33 enrolled patients. The number of blood and urine samples differed because some samples could not be collected before surgery. Blood and urine samples were collected from 26 to 18 individuals, respectively.

Removal of brain tissues by craniotomy

A craniotomy was performed to remove brain tumors and obtain brain tissues. Following treatment and an institutional review board-approved protocol, surplus tumor tissues were resected to obtain brain tissues for amino acid measurements. Vivid tumor samples were collected from tumor core areas without necrosis. The extent of brain tumor resection was based on the standard treatment, thereby eliminating the risk of adverse events directly related to the study.

Brain tissues for amino acid measurements consisted of non-tumor and tumor sections. The non-tumor tissues were distant from the excised tumor lesions, which were systematically removed for approach or extended resection of the lesion at the time of standard tumor resection using electrophysiologic functional monitoring and intraoperative awake brain mapping. Pathologists and neurosurgeons distinguished between tumor and non-tumor areas during surgery. The pathologist diagnosed the tumor area using rapid intraoperative pathological examination, while the neurosurgeon used intraoperative findings and postoperative hematoxylin and eosin staining of the excised brain tissues. Resected brain samples were immediately frozen at −80 °C to preserve the in vivo concentration of amino acids without being influenced by amino acid-degrading enzymes or tissue denaturation. The evaluation focused on the white matter, where gliomas frequently occur, to minimize the impact of the collection site. Although different brain regions exhibit varying amino acid levels [30], this study disregarded

the influence of brain regions since the non-tumor and tumor areas were essentially from the same brain region. All samples were collected between 2019 and 2022 at Kanazawa University Hospital.

Determination of chiral amino acids by 2D high-performance liquid chromatography

D- and L-amino acids were evaluated using a 2D high-performance liquid chromatography system (Nanospace SI-2 series, Shiseido, Tokyo, Japan), as previously described [8, 18]. Initially, NBD-amino acids were isolated in the first dimension using a microbore-ODS column prepared in a fused silica capillary (1000 mm × 0.53 mm i.d., 45 °C, Shiseido, Tokyo, Japan). The isolated fractions were subsequently transferred to the second dimension, which consisted of a narrow-bore enantioselective column, KSAACSP-001 S (250 mm × 1.5 mm i.d., 25 °C, prepared in collaboration with Shiseido), for the determination of D- and L-enantiomers. The mobile phases for the second dimension comprised mixed solutions of MeOH and MeCN containing formic acid. NBD-AAs were detected using fluorescence at 530 nm, with excitation at 470 nm.

Immunohistochemistry

We examined cases of central nervous system WHO grade 2 (9 cases), grade 3 (8 cases), and grade 4 (14 cases) using paraffin blocks from glioma cases stored in our laboratory, in addition to brain tissues from the cases in this study (see Supplementary Table 3 [Online Resource 1]). Histological diagnosis was determined according to the revised WHO criteria [34]. A non-tumor area was used as normal (10 cases). This procedure was performed in cases where sufficient tissue could be removed for staining.

In brief, 4-μm-thick tissue sections were stained using the standard hematoxylin and eosin staining technique. The slides were immunostained using the Envison^{+} System (Dako, Tokyo, Japan). Slides were autoclaved at 120 °C for 10 min in Target Retrieval Solution (pH 6.0; Dako, Glostrup, Denmark) after deparaffinization using Fast Solve. Quenching was performed using 3% hydrogen peroxide (H_2O_2) in methanol for 20 min, followed by blocking with 5% skim milk in TBS-T. Subsequently, sections were incubated overnight at 4 °C with a 1:200 dilution of DAO rabbit polyclonal antibody (HPA038654, Sigma, St Louis, MO, USA) and 1:200 dilution of serine racemase (SRR) (A-4) mouse monoclonal antibody (SC-365217, Santa Cruz, Dallas, TX, USA). The corresponding secondary antibody was applied at room temperature for 1 h after washing with TBS-T, and color development was achieved using 3,3-diaminobenzidine tetrahydrochloride (DAB Substrate Kit SK-4100, Vector, Akasaka, Japan) for 2–5 min. Hematoxylin was used for counterstaining (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Images were acquired using a BZ-X700 microscope (Keyence, Osaka, Japan).

The positive rate of tumor cells in three high-magnification fields of view under a microscope was counted for each molecule, and the average value was calculated.

Human GBM mouse model

Animal experiments were performed according to our previous study, following the same protocol approved by the institutional review board [32]. We generated a mouse brain tumor model of human GBM by transplanting 10×10^5 KGS01 cells, derived from a human patient-derived GBM cell line established at Kanazawa University, into the brains of nude mice (BALB/cSlc-nu/nu, Charles River Laboratories, Osaka, Japan) ($n=5$). Previous reports have confirmed that KGS01 cells are glioma-initiating cells capable of self-renewal in vitro and replicating the original tumor characteristics in a mouse xenograft model [2, 16]. The procedure involved drilling a hole into the skull, 3 mm outside the bregma, using a microdrill; next, stereotactic injection of glioma cells was performed to a depth of 3 mm below the dura mater. After 28 days, all mice were euthanized, and their brains were excised. The excised brains were stained with hematoxylin and eosin and that brain tumors were confirmed in all mice (see Supplementary Fig. 1 [Online Resource 2]).

Cell culture

KGS01 cells were cultured in a neurosphere formation medium containing DMEM/F12 (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with recombinant human epidermal growth factor at 20 ng/mL (Sigma–Aldrich, St. Louis, MO, USA), recombinant human basic fibroblast growth factor at 20 ng/mL (Sigma–Aldrich), MACS® NeuroBrew-21 supplement without vitamin A (Miltenyi Biotec, North Rhine-Westphalia, Germany), and GlutaMAX (Gibco) and 1% penicillin/streptomycin (Gibco).

Cell viability assay

Cell viability was assessed using an AlamarBlue assay (Bio-Rad Laboratories, Hercules, CA, USA). Neurospheres were dissociated into single cells using StemPro Accutase (Thermo Fisher Scientific), and the cells were seeded at a density of 3.0×10^3 cells/200 μL in a 96-well Costar ultra-low attachment plate (Corning) in asparagine-free medium (amino free DMEM/MEM essential amino acids/L-Alanyl-L-Glutamine/1%FBS/100uM Glycine medium) with 20 μL AlamarBlue. The cells were treated with different concentrations of D-Asn (only DMSO, 10 μM, 100 μM, and 1000 μM) after 4 h of incubation. The relative numbers of viable cells were

determined by measuring the absorbance using a microplate reader (Bio-Rad Laboratories) at 0 and 72 h after adding amino acids. The average fluorescence values from the six wells in each group were calculated and plotted. Cells were treated with various concentrations of D-Asn to evaluate the effect of D-amino acids on cell proliferation. In addition, the concentration of amino acids in the medium was measured at 0 and 72 h after adding D-Asn.

Study approval

The Ethics Committee of Kanazawa University Hospital approved this study, which was conducted according to the Declaration of Helsinki. The IRB number for patients with gliomas was No. 2893, and that for heathy controls was No. 2016−417. All participants provided written informed consent and were informed of their right to withdraw from the study at any time.

All animal experiments were performed according to the guidelines of Kanazawa University for animal care and approved by the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (registration number AP-214259).

Statistical analyses

Data are presented as mean ± standard error of the mean, calculated using SPSS Statistics version 23 (IBM Inc, Tokyo, Japan). Statistical analysis was performed using Student's *t*-test and the Mann–Whitney U test when comparing two groups. One-way ANOVA with Tukey's multiple comparison test was used for multiple group comparisons. Statistical significance was set at $p<0.05$.

Results

Chiral amino acid profile in non-glioma and glioma tissues

We examined whether the chiral amino acid profile differed between glioma and non-glioma tissues obtained at the time of tumor resection. D-Ser and D-Asn levels increased in glioma tissues compared with those in non-glioma tissues. The D-Ser level increased in WHO grade 3 tissues compared with that in non-glioma tissues ($p<0.05$). In contrast, the D-Asn level increased in WHO grade 3 and grade 4 tissues compared with that in non-glioma tissues ($p<0.01$) (Fig. 1A). Similarly, L-Ser, L-Ala, L-Asn, and L-Pro levels increased as the glioma grade increased ($p<0.05$) (Fig. 1B). The %D-Ser level significantly decreased in WHO grade 4 tissues compared with that in non-glioma tissues ($p<0.001$) (Fig. 1C). These findings demonstrate chiral amino acid selectivity in glioma tissues.

Expression of chiral amino acid-related metabolic enzymes in glioma tissues

Subsequently, we investigated potential alterations in D-amino acid-related metabolic enzymes such as DAO and SRR, known regulators of chiral amino acid levels. Specifically, D-Ser is synthesized from L-Ser through the action of SRR [35]. Increased L-Ser levels also induce increased D-Ser levels in the rat brain [31]. Additionally, DAO metabolizes D-amino acids [21]. These findings suggest that the altered levels of L- and D-forms of chiral amino acids were caused by changes in DAO and SRR expression levels in the brains of patients with gliomas.

We conducted immunohistochemistry of DAO and SRR in the brain to explore this hypothesis. DAO and

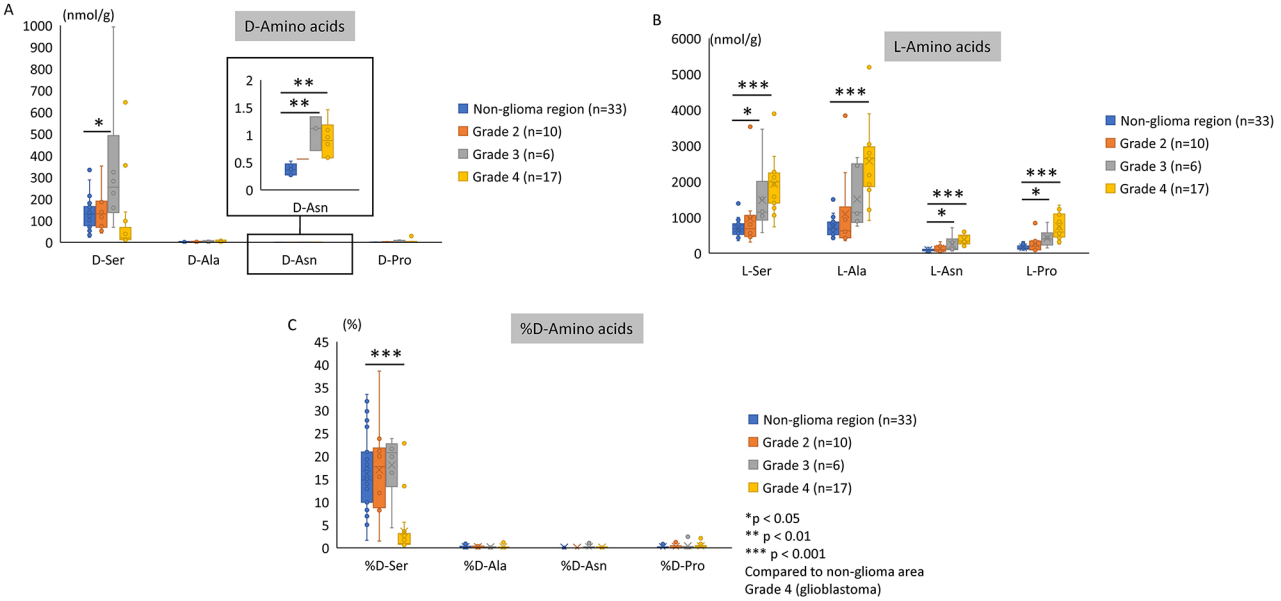


Fig. 1 Brain chiral amino acid profiles. D-amino acid levels (**A**), L-amino acid levels (**B**), and %D-amino acid levels (**C**) in the non-glioma and glioma areas (WHO grade 2–4 glioma). *$p<0.05$, **$p<0.01$, ***$p<0.001$, as analyzed with one-way ANOVA. Ser: serine, Ala: alanine, Asn: asparagine, Pro: proline

SRR were detected in astrocytes identified morphologically in the brain (Fig. 2A–L). The number of DAO-positive cells in WHO grade 3 ($p=0.05$) and grade 4 ($p<0.001$) tissues was lower than that in normal tissues (Fig. 2B, E, H, K, M). The number of SRR-positive cells was lower in WHO grade 2 tissues than in normal tissues, but no significant change was noted in grade 3 and 4 tissues compared with that in normal tissues (Fig. 2C, F, I, L, N). Theoretically, D-amino acid levels increase as DAO expression decreases. These data support the finding that D-Asn levels increased in gliomas (Fig. 1A).

Chiral amino acid profiles in liquid samples

In clinical investigations, blood and urine, which are easy to collect, are often used as samples. Therefore, we examined whether gliomas altered the chiral amino acid profile in plasma and urine.

In the plasma, D-Ser, D-Ala, and D-Asn levels decreased in patients with gliomas compared with those in healthy controls ($p<0.05$). Furthermore, D-Asn levels decreased in patients with WHO grade 4 compared with those in heathy controls (0.171 ± 0.045 vs. 0.216 ± 0.061 nmol/mL, $p<0.01$) (Fig. 3A). However, L-amino acid levels were similar between patients with gliomas and healthy controls (Fig. 3B). Moreover, %D-Ser, %D-Ala, and %D-Asn levels decreased in patients with gliomas compared with those in healthy controls ($p<0.05$). Furthermore, %D-Asn levels decreased in patients with WHO grade 4 compared with those in heathy controls (0.342 ± 0.104% vs. 0.452 ± 0.141%, $p<0.01$) (Fig. 3C).

In urine, creatinine-corrected D-Ser, D-Asn, and D-Pro levels decreased in patients with WHO grade 4 compared with those in heathy controls (1.379 ± 0.374 vs. 1.773 ± 0.535, $p<0.05$; 0.132 ± 0.042 vs. 0.227 ± 0.089, $p<0.001$; 0.001 ± 0.001 vs. 0.004 ± 0.005, $p<0.01$, respectively; Fig. 4A). Additionally, creatinine-corrected L-Ser, L-Ala, and L-Asn levels decreased in patients with gliomas compared with those in healthy controls (Fig. 4B). Creatinine-corrected L-Asn levels decreased in patients with grade 4 compared with those in heathy controls (0.907 ± 0.601 vs. 1.655 ± 1.363, $p<0.01$) (Fig. 4B). Although %D-Ser and %D-Ala decreased in patients with grade 2 gliomas compared with those in healthy controls, no change was noted between patients with grade 4 and healthy controls (Fig. 4C).

Renal function affects chiral amino acid levels in blood and urine [10, 14, 15, 21, 22]. However, no significant difference was observed in serum creatinine, an indicator of renal function, between heathy controls and patients with gliomas (see Supplementary Fig. 2 [Online Resource 2]). Additionally, creatinine-corrected values were used for urinary D-amino acids. Therefore, this result showed that patients with gliomas have a unique chiral amino acid profile in liquid samples.

Alteration of chiral amino acid profiles before and after GBM removal

We investigated changes in the chiral amino acid profile before and after GBM removal. Blood and urine samples were collected approximately 1 month after surgery. None of the patients were bedridden with tube feeding.

In the same patients, creatinine-corrected urinary D-Ser, D-Ala, and D-Asn levels increased after surgery compared with those before removal ($p<0.05$) (Fig. 5). In contrast, creatinine-corrected urinary L-amino acid levels except for L-Ala showed no change. No significant differences were noted in amino acid levels in plasma before and after GBM removal (see Supplementary Fig. 3 [Online Resource 2]).

Based on these results, we focused on urinary D-Ser and D-Asn as candidate biomarkers for gliomas (Figs. 4 and 5). D-Ala and L-Ala were excluded as candidate biomarkers based on the absence of differences in preoperative conditions between heathy controls and patients with GBMs (Fig. 4A), despite their significant alterations postoperatively (Fig. 5).

Evaluation of urinary D-Ser and D-Asn as glioma diagnostic biomarkers

The multidimensional data from the blood and urinary D-amino acid analysis results were plotted in a low-dimensional plot using the principal coordinate analysis (PCoA) to visualize diversity and similarity. For example, in Fig. 6A (left), the x-axis of the loading data demonstrates the difference between healthy individuals and all patients with gliomas. Deviation from the 0.0 value signifies higher discrimination ability. The y-axis indicates the influence of other factors, such as sex. Thus, the yellow circles indicate that U_D-Asn/Cre (creatinine-corrected urinary D-Asn) was the most effective factor in distinguishing heathy controls from all patients with gliomas. Figure 6A (right) shows the outcomes of distinguishing healthy individuals (red dots) from all patients with gliomas (blue dots) using these amino acids. Receiver operating characteristic (ROC) analysis using U_D-Asn/Cre showed an area under the curve (AUC) of 0.88 (sensitivity: 0.96 and specificity: 0.72), separating healthy controls from all patients with gliomas (Fig. 6B). Meanwhile, D-Ser/Cre mice showed a lower ability to discriminate between healthy controls and patients with gliomas than D-Asn mice (Fig. 6A). Therefore, we focused on urinary D-Asn/Cre.

Next, a sub-analysis was performed in which all patients with gliomas were subdivided by grade. U_D-Asn/Cre was a useful discriminating factor between healthy controls and patients with gliomas (Fig. 6C, D, E, F, G, H). The AUC for distinguishing healthy controls from WHO grade 3 patients with gliomas (AUC, 0.97; sensitivity, 0.88; specificity, 1.00) (Fig. 6E, F) and patients

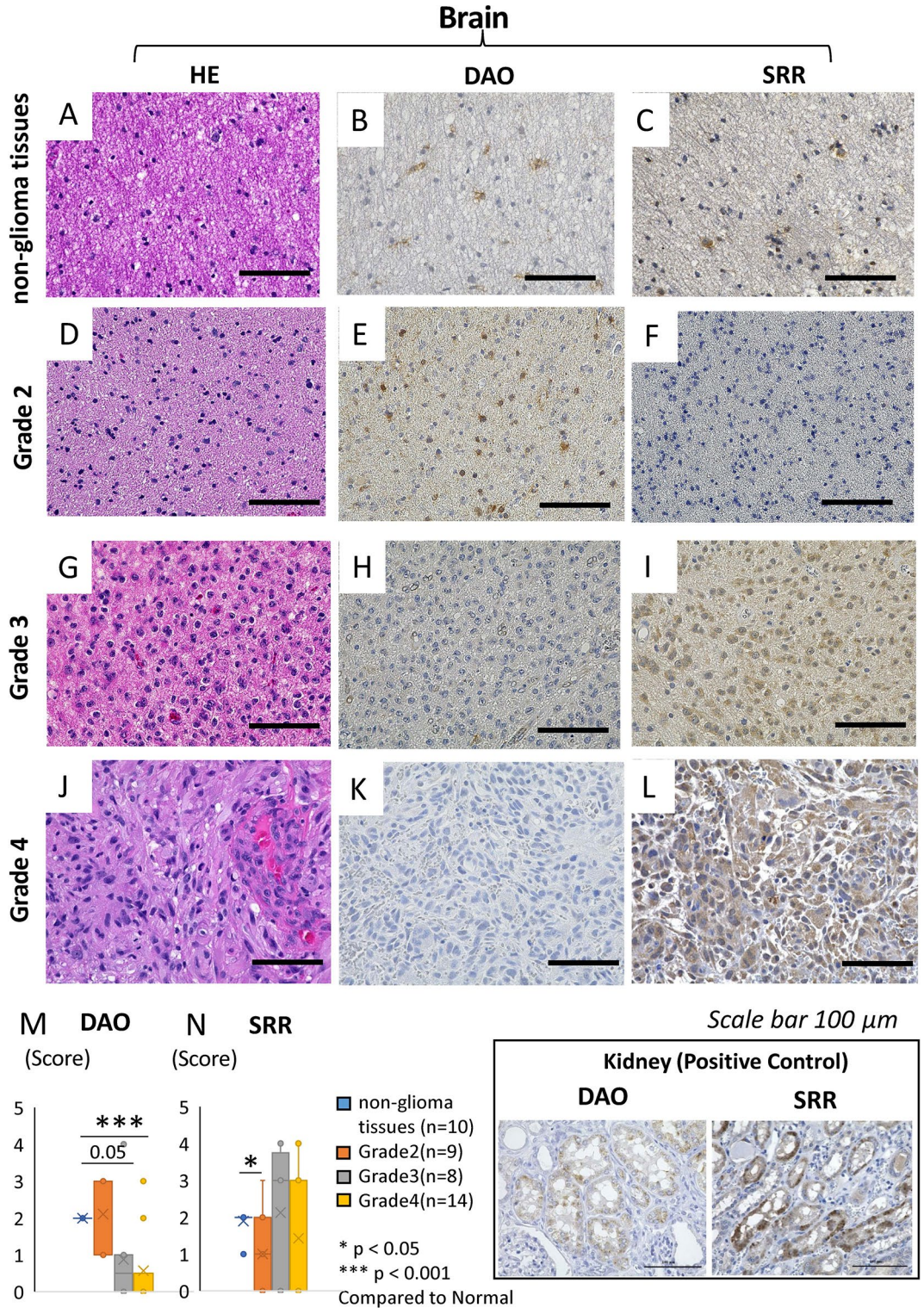


Fig. 2 Intra-brain expression of DAO and SRR. Hematoxylin and eosin staining (**A**, **D**, **G**, **J**); DAO (**B**, **E**, **H**, **K**), and SRR (**C**, **F**, **I**, **L**) were detected in the brain; and counts of DAO (**M**) and SRR (**N**)-positive cells. Positive rates were evaluated in the following five levels. Score 0: <5%, Score 1: 5–25%, Score 2: 25–50%, Score 3: 50–75%, Score 4: >75%. *$p < 0.05$, ***$p < 0.001$, as analyzed with a Mann–Whitney U test, compared with non-glioma tissues. DAO: D-amino acid oxidase, SRR: serine racemase

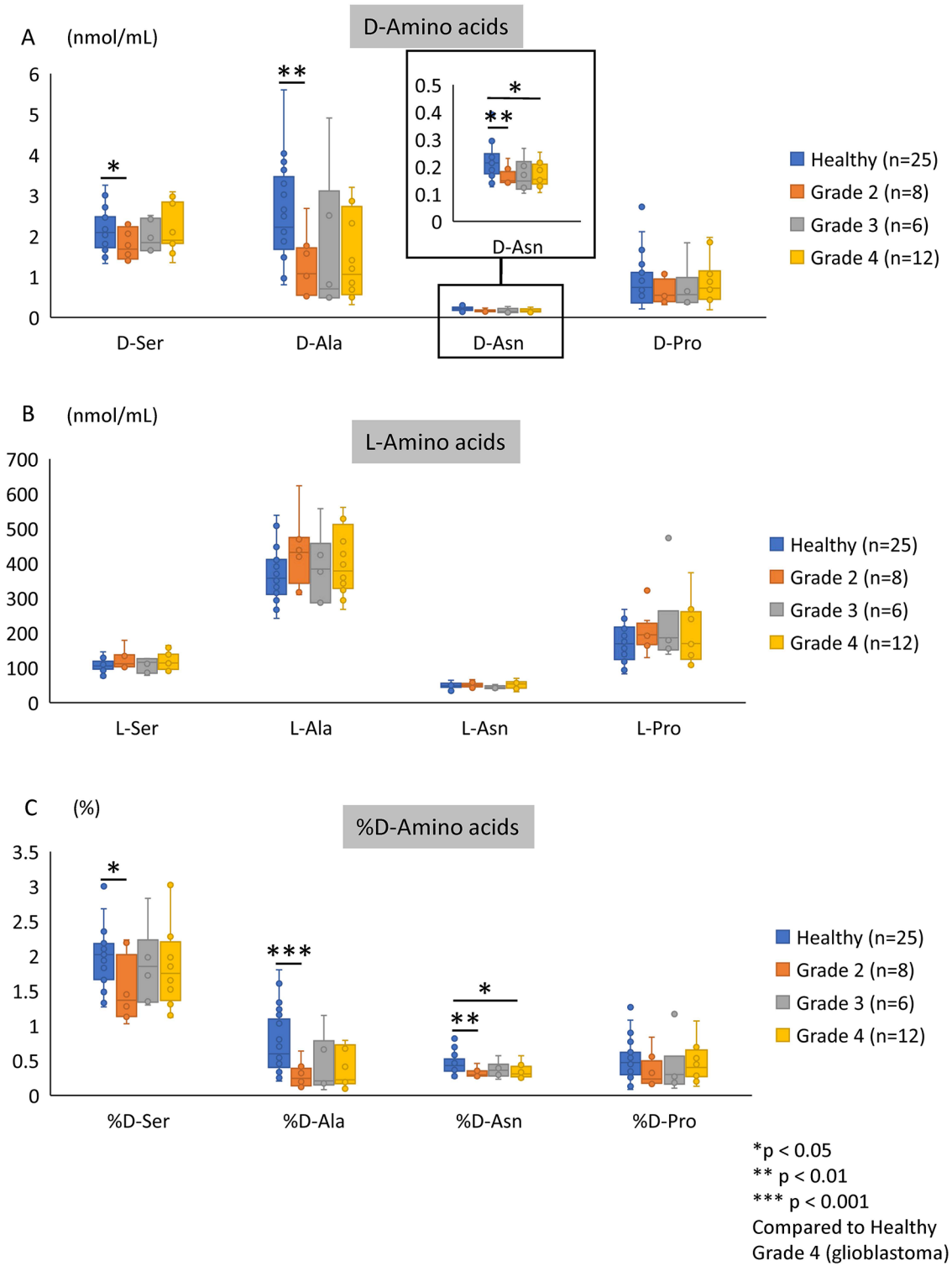


Fig. 3 Plasma chiral amino acid profiles. D-amino acid levels (**A**), L-amino acid levels (**B**), and %D-amino acid levels (**C**) in healthy controls and patients with gliomas (WHO grade 2–4 gliomas). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as analyzed with one-way ANOVA. Ser: serine, Ala: alanine, Asn: asparagine, Pro: proline

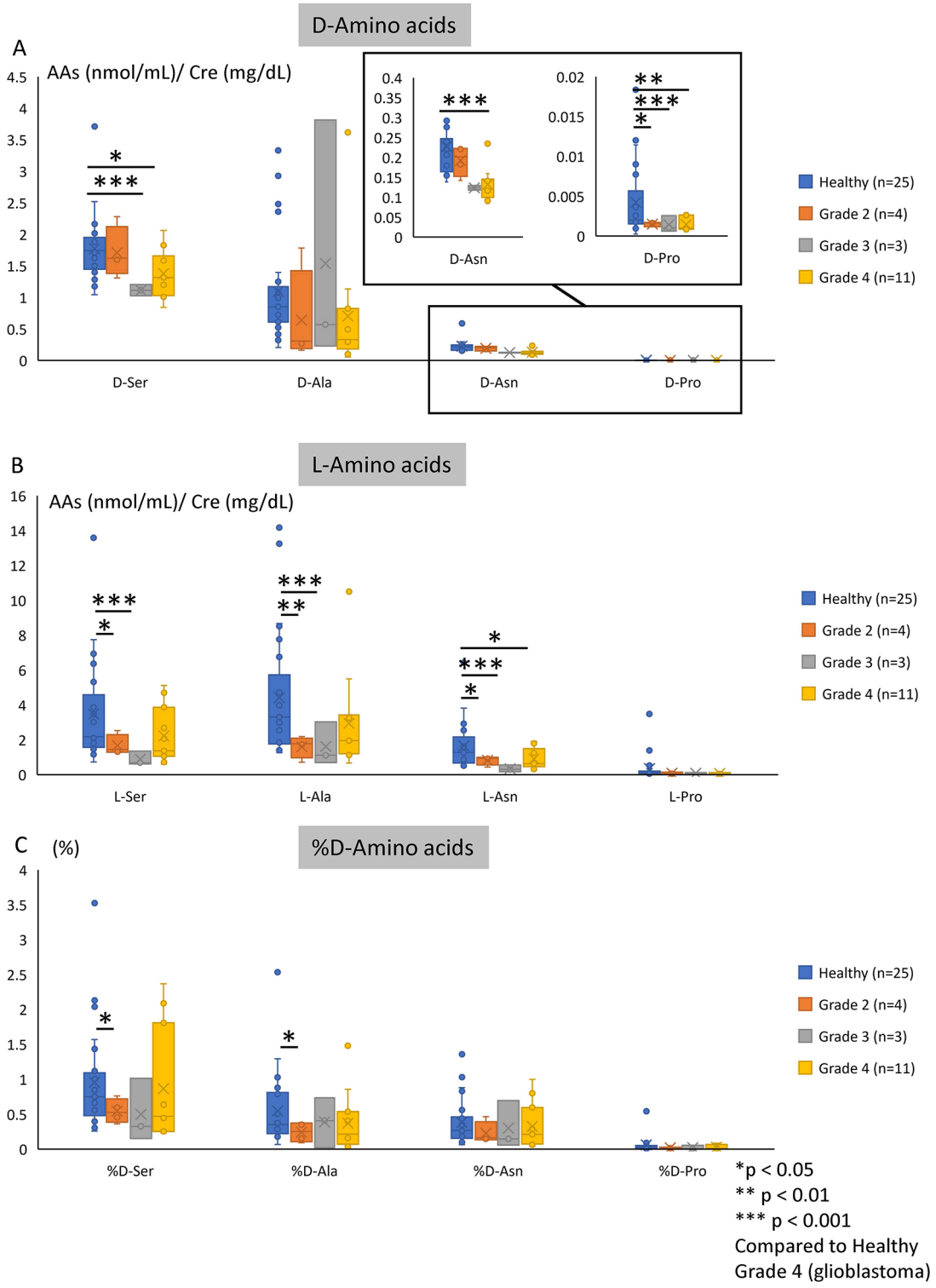


Fig. 4 Urine chiral amino acid profiles. D-amino acid levels (**A**), L-amino acid levels (**B**), and %D-amino acid levels (**C**) in healthy controls and patients with gliomas (WHO grade 2–4 gliomas). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as analyzed with one-way ANOVA. Ser: serine, Ala: alanine, Asn: asparagine, Pro: proline, AAs: amino acids, Cre: creatinine

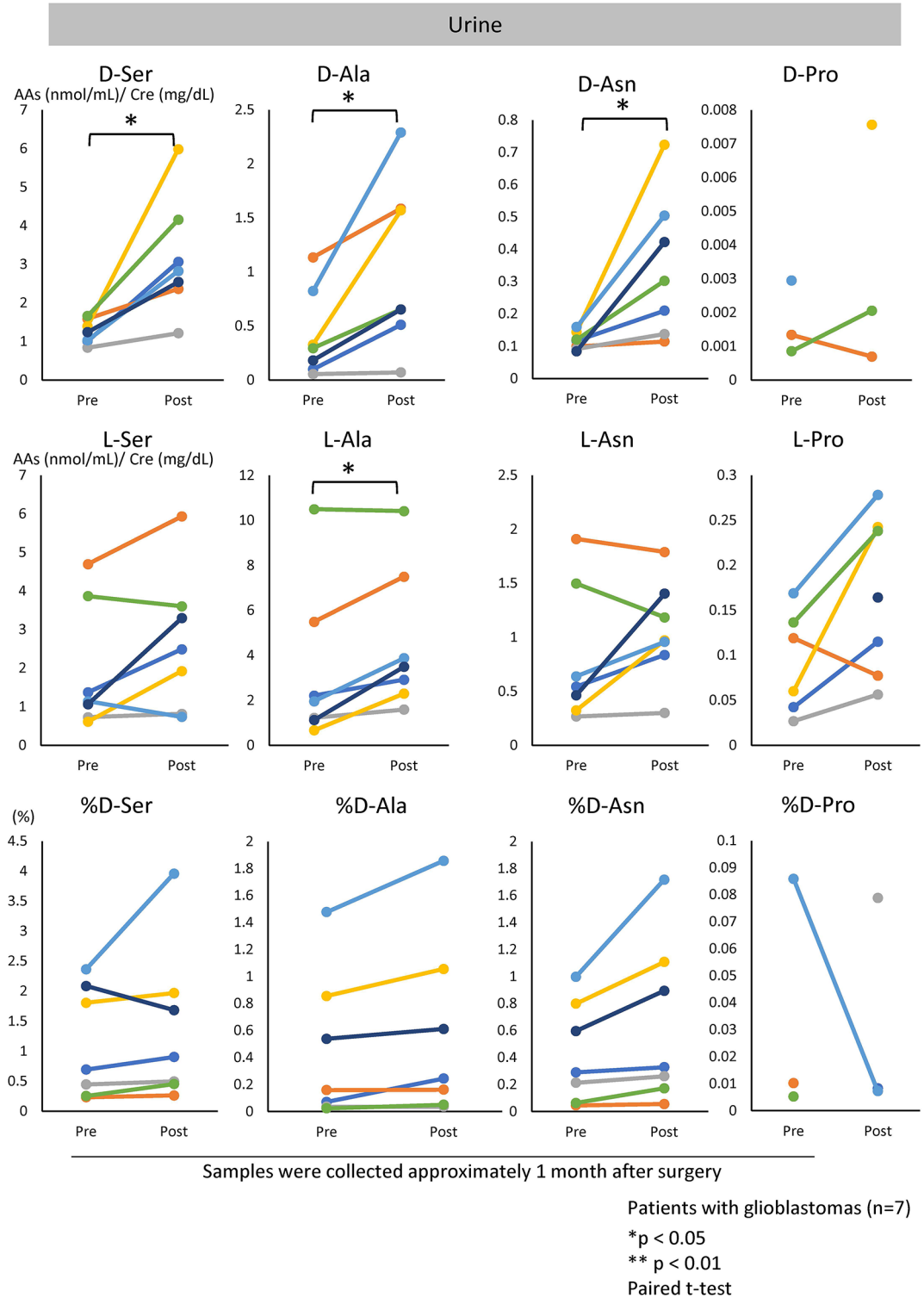
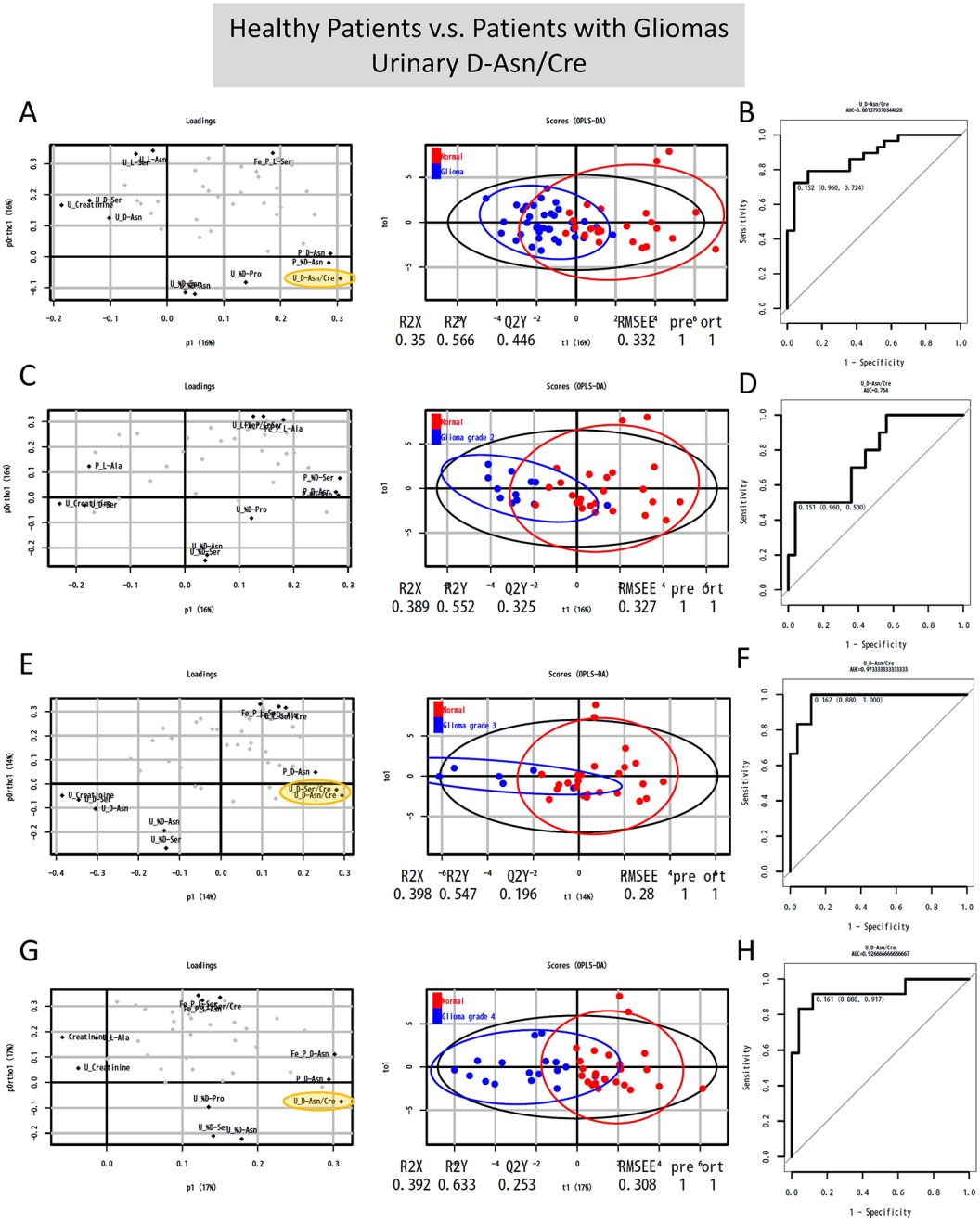


Fig. 5 Urine chiral amino acid profile before and after GBM excision. Chiral amino acid levels in patients with GBMs before and after surgery. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as analyzed with a paired *t*-test. Ser: serine, Ala: alanine, Asn: asparagine, Pro: proline, GBM: glioblastoma, AAs: amino acids, Cre: creatinine



Summary	AUC	Sensitivity	Specificity
Healthy patients v.s. All patients with gliomas	0.88	0.96	0.72
Healthy v.s. WHO grade 2	0.76	0.96	0.50
Healthy v.s. WHO grade 3	0.97	0.88	1.00
Healthy v.s. WHO grade 4 (glioblastoma)	0.93	0.88	0.92

Fig. 6 PCoA and ROC analysis in heathy controls and patients with gliomas. Comparison between heathy controls and all patients with gliomas using PCoA (**A**) and ROC analysis (**B**). Comparison between heathy controls and WHO grade 2–4 patients with gliomas using PCoA (**C**, **E**, **G**) and ROC analysis (**D**, **F**, **H**). PCoA: principal coordinates analysis, ROC: receiver operating characteristic

with GBMs (AUC: 0.93, sensitivity: 0.88 and specificity: 0.92) (Fig. 6G, H) was particularly high.

In contrast, U_D-Ser/Cre had lower AUC (0.7–0.8), sensitivity, and specificity than U_D-Asn/Cre (see Supplementary Fig. [Online Resource 2]).

Chiral amino acid profile in human GBM mouse models

Mouse models were created by transplanting human GBM-derived cells into the brain to confirm if brain tumors affect blood and urine chiral amino acid profiles. Five GBM mouse models were created, and four survived for 28 days. Blood and urine samples were collected from these mice before euthanasia (Fig. 7A). Chiral amino acid analysis was performed on samples collected before and after transplantation. Pathological examination confirmed glioma growth in mouse brains (see Supplementary Fig. 1 [Online Resource 2]). Chiral amino acid analysis was performed on normal and GBM mouse model brains, plasma, and urine.

D-Ser levels were higher in the GBM mouse model brains than in normal mouse brains ($p<0.05$), whereas D-Asn and D-Pro levels were below the detection sensitivity. The levels of all four L-amino acids tended to be higher in GBM mouse models than in normal mice (Fig. 7B). In plasma, D-Ala levels were higher in GBM mouse models than in normal mice ($p<0.05$). D-Asn levels tended to be lower in GBM mouse models than in normal mice (Fig. 7C). In urine, D-Asn levels were significantly lower in GBM mouse models than in normal mice ($p<0.05$), and D-Ser and D-Pro levels tended to be lower in GBM mouse models than in normal mice. The levels of all four L-amino acids were lower in GBM mouse models than in normal mice ($p<0.05$) (Fig. 7D).

The GBM mouse models exhibited a systemic chiral amino acid profile similar to that of human patients with gliomas. Notably, changes in urinary D-Asn levels before and after transplantation of glioma cells into the brain were similar to human results. These results showed that GBM affects chiral amino acid profiles in the brain, blood, and urine.

Relation between human glioma cell line KGS01 cells and D-Asn levels

Finally, we evaluated whether glioma cells directly regulate D-Asn levels. D-Asn levels increased in glioma tissues. Therefore, we examined how increased D-Asn levels affect glioma cells. When KGS01 cells were cultured in increasing D-Asn concentrations from 10 to 1000 μM, the cells proliferated in the presence of D-Asn (Fig. 8A).

Based on these results, we proposed a putative model of the D-Asn profile in patients with gliomas (Fig. 8B).

Discussion

This study found that glioma tissues can distinguish between D- and L-bodies of amino acids and use them. Furthermore, we confirmed that glioma tissues alter chiral amino acids such as D-Asn and D-Ser in the blood and urine. Notably, urinary D-Asn is a potential biomarker for diagnosing gliomas as it most accurately reflects their presence in the brain compared with other amino acids.

Chiral amino acid analysis of brain tissue samples from patients with gliomas revealed higher levels of D-Ser and D-Asn in glioma tissues than in non-glioma tissues. The D-Ser level increased in only WHO grade 3 gliomas, while the D-Asn level increased in WHO grade 3 and 4 gliomas (GBM). In other words, D-Asn levels reflected the presence of gliomas and their severity. Conversely, L-amino acids (L-Ser, L-Ala, L-Asn, and L-Pro) and D-Asn were more abundant in glioma tissues than in non-glioma tissues. Previous studies have reported that cancer uses various amino acids for growth, including glutamine [3], Ser [1, 19, 28], glycine [11], tryptophan [27], tyrosine, and phenylalanine [6]. The association between gliomas and amino acids has also been investigated [5, 12, 29, 36]. However, these reports did not directly measure amino acid levels in the brain. In addition, these reports did not distinguish between amino acid chirality. Therefore, whether D- or L-amino acids were altered in the brain was unclear. These results confirm that glioma tissues can distinguish between D- and L-amino acids and increase specific amino acid levels. Furthermore, the relationship between D-Asn, with trace in vivo levels, and gliomas was also clarified for the first time through the improved performance of our independently developed chiral amino acid analysis [10, 21, 22].

We investigated whether changes in chiral amino acid levels in glioma tissues in the brain are reflected in the blood and urine, which can be easily collected for testing. D-Asn and D-Ser levels in the blood and urine decreased in patients with gliomas compared with those in healthy controls. This phenomenon was also confirmed by the transplantation of KGS01 cells into mouse brains. Furthermore, urinary D-Ser and D-Asn levels increased to almost the same levels as those in healthy controls after glioma removal. These data suggest that these amino acids reflect the presence of gliomas in the brain.

The main possible mechanisms by which glioma cells affect blood amino acid levels are the involvement of amino acid transporters and the blood-brain barrier. In brain tumors, tumor-induced disruption of the blood-brain barrier [23] and upregulation of amino acid transporters [20] have been reported. Several cancers show upregulated expression of the amino acid transporters ASCT2 and LAT1, indicating increased uptake of amino acids by tumor cells [7]. These changes are assumed to promote amino acid uptake from the blood into the

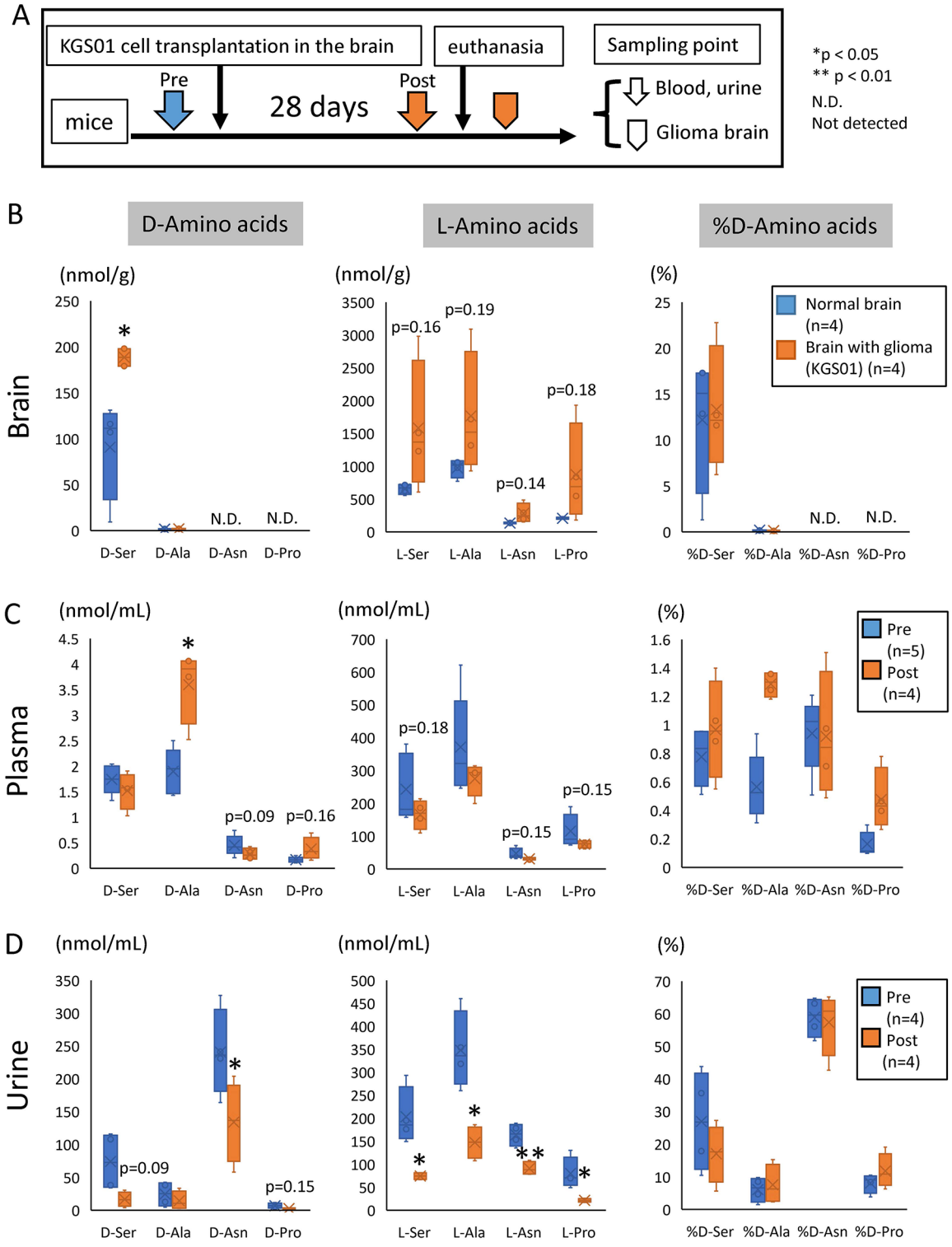


Fig. 7 Whole-body chiral amino acid profile of mice transplanted with human glioma cells (KGS01). Simplified protocol (**A**). Chiral amino acid levels in the brains of normal and transplanted mice (**B**). Chiral amino acid levels in the plasma (**C**) and urine (**D**) before and after transplantation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as analyzed with a Mann–Whitney U test. Ser: serine, Asn: asparagine

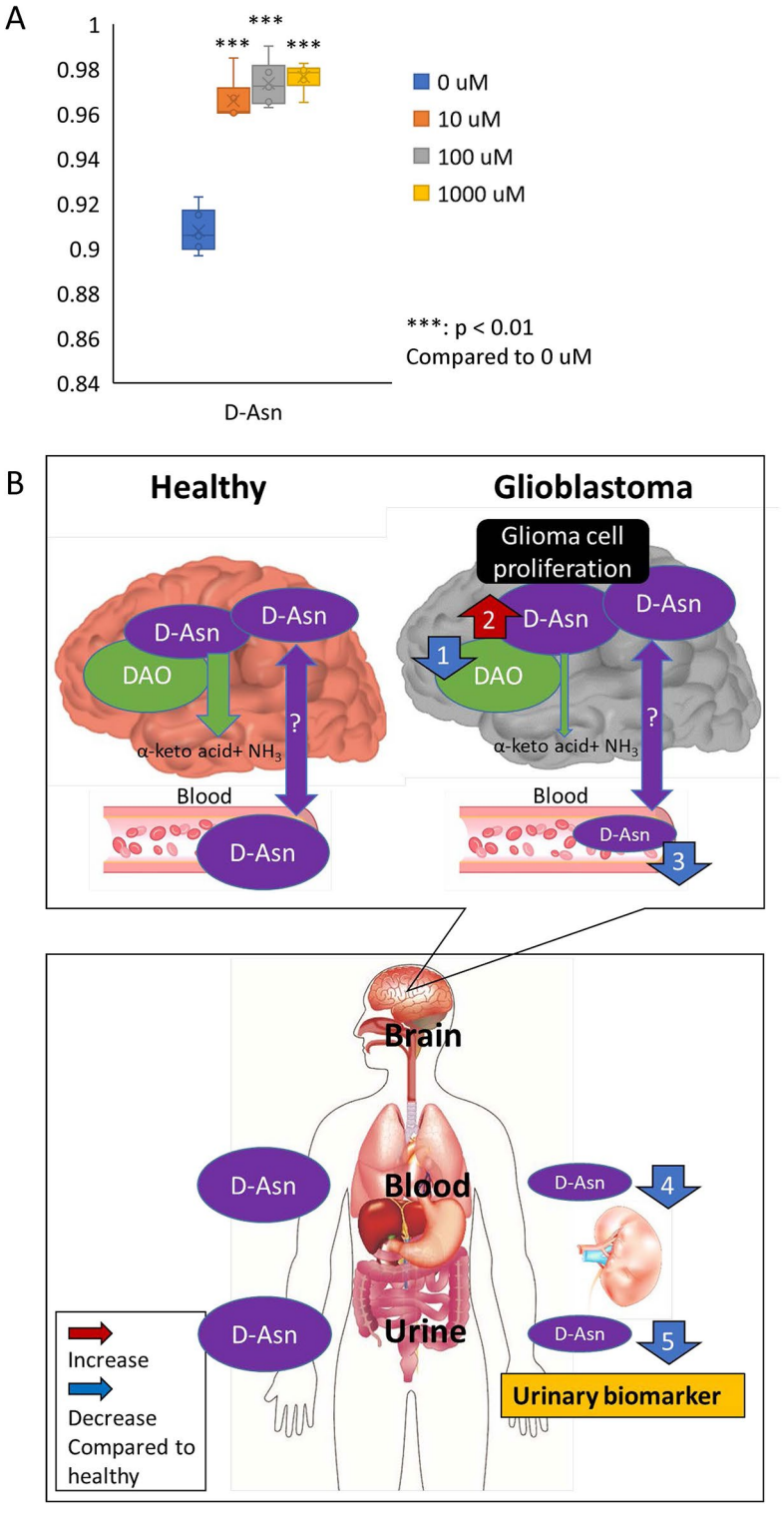


Fig. 8 Human glioblastoma cells (KGS01) utilize D-Asn for cell proliferation. KGS01 cells showed increased cell proliferation using D-Asn level-dependent manner (**A**). Differences in D-Asn profiles between heathy controls and patients with gliomas (**B**). (1) The number of DAO-positive cells in the brains of patients with glioblastomas decreased. (2) The level of D-Asn, the substrate of DAO, increased. (3) Glioma cells in the brain decrease D-Asn levels in the blood and urine. D-Asn uptake from the blood into the brain may be enhanced. 4,5. D-Asn is not reabsorbed by the kidneys and is excreted in the urine at nearly blood levels. Thus, D-Asn is a candidate biomarker reflecting the presence of gliomas in the brain. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as analyzed with a Mann–Whitney U test. Ser: serine, Asn: asparagine

brain, resulting in decreased blood amino acid levels. Furthermore, since blood amino acids are excreted into urine, the decreased blood amino acid levels are also thought to affect urinary amino acid levels. The kidneys reabsorb approximately 50% of D-Ser [14], while almost 100% of D-Asn is excreted into the urine [33]. Considering this renal reabsorption mechanism, urinary D-Asn is thought to reflect changes in brain and blood amino acids more accurately than urinary D-Ser. Urinary D-Asn was the most accurate discriminator between healthy controls and patients with gliomas.

This study demonstrates that glioma tissues in the brain alter blood and urinary D-amino acids. Other major factors reported to alter D-amino acids in the body include renal function and microbiota. Blood and urinary D-Asn and D-Ser levels are altered in patients with renal diseases [14, 15, 21, 22, 33]. Since D-Asn and D-Ser are excreted in the urine, their blood levels increase, and urinary levels decrease as renal function deteriorates. However, no significant differences were noted in renal function between the participants in this study.

Furthermore, the urinary amino acids were corrected for creatinine. Thus, the study results were largely unaffected by renal function. The gut microbiota has been reported as a major source of D-amino acids [10, 21, 22]. Although the microbiota was not evaluated in this study, changes in amino acid levels were observed at diagnosis and before and after removal of glioma tissues. Microbiota influence is considered small because samples were collected from the same patient before and after surgery. Therefore, this study newly found that glioma tissues in the brain affect blood and urinary D-Asn levels in addition to kidney diseases and microbiota.

Finally, we examined how glioma cells increase D-Asn levels in glioma tissues. The possible mechanisms by which D-amino acid levels change in tissues are mainly changes in the expression of metabolic enzymes and transporters. Few D-amino acid transporters were identified and excluded from this study. The enzymes identified as D-amino acid-metabolizing enzymes are DAO, SRR, and D-aspartate oxidase [21, 31, 35]. Of these, DAO and SRR are involved in D-Ser and D-Asn metabolism. Their expression in glioma tissues was evaluated by immunohistochemistry, and the number of DAO-positive cells decreased in WHO grade 3 and GBMs compared with that in normal tissues. It is also possible that IDH mutations affect amino acid metabolism. Our data showed there was no difference in urinary D-Asn/Cre between IDH mutant gliomas and IDH wild gliomas (data not shown). Additional studies are needed to validate it with large number of cases.

Conversely, no obvious change was observed for SRR. Therefore, in this study, the decrease in DAO-positive cells may have contributed to the increase in D-Asn levels. The decrease in DAO-positive cells may have increased D-Asn levels in glioma tissues. However, the detailed regulatory mechanism of D-Ser could not be clarified. Further studies are needed considering transporters and other factors.

The degree of DAO expression has been reported to be related to glioma cell proliferation [4]. Furthermore, a study reported on cancer therapy involving amino acid-related metabolic enzymes [17]. Therefore, we investigated whether D-Asn are related to glioma cell proliferation. D-Asn promoted the proliferation of cells derived from the glioma cell line KGS01.

The strength of this study lies in the involvement of medical specialists in participant selection, with neurosurgeons diagnosing patients with gliomas and neurologists diagnosing healthy volunteers with no brain abnormalities. However, this study also has some limitations. First, there were differences in age and sex between heathy controls and patients with gliomas. Second, the kinetics of D-Asn have not been evaluated in other cancers, and specificity remains an issue for the future. Third, although GBM cells proliferated with the addition of D-Asn in vitro, this experimental system was conducted in a special environment, amino acid-free medium. Therefore, it is necessary to verify the relationship between D-Asn and GBM in future experiments in which D-Asn is administered to mice in vivo.

Whole-body chiral amino acid analysis of heathy controls and patients with gliomas identified a new glioma biomarker candidate, namely, urinary D-Asn level. Urinary D-Asn most accurately distinguished gliomas, especially GBMs, from healthy controls through PCoA and ROC analysis. Unlike imaging tests and spinal fluid sampling, which are performed only at advanced medical institutions, tests using urine samples are expected to be used at various medical institutions. Thus, the study results hold promise for the early-stage screening of patients with asymptomatic gliomas and offer a new research avenue in chiral amino acids for glioma research.

In conclusion, glioma cells decreased the number of DAO-positive cells and increased D-Asn levels because of their active proliferation. Furthermore, glioma cells may proliferated using D-Asn. Thus, increased D-Asn levels in glioma tissues suggest the presence and proliferation of glioma cells. This finding may lead to establishing potential therapeutic targets in addition to biomarkers.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Author contributions
Y.N., M.K., and M.N. designed and performed the human experiments. Y.N., M.K., M.N., H.S., and T.Y. collected the samples and analyzed the clinical data. M.S. and K.O. recruited and verified healthy volunteers and collected samples, while Y.N. and T.I. performed the cell and animal experiments. M.M. performed HPLC quantification of the chiral amino acids. Y.N., M.K., M.N., T.W., and Y.I. wrote the manuscript, and M.N. and T.W. supervised the study. All authors read and approved the final version of the paper.

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Data availability
All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest
The authors have declared that no conflict of interest exists.

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