



Title	Identification of PEPT2 as an important candidate molecule in 5-ALA-mediated fluorescence-guided surgery in WHO grade II/III gliomas
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1           **Identification of PEPT2 as an Important Candidate**  
2           **Molecule in 5-ALA-Mediated Fluorescence-Guided Surgery**  
3                       **in WHO Grade II/III Gliomas**

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23

24 **Abstract**

25 Purpose: 5-aminolevulinic acid (5-ALA) fluorescence-guided surgery (FGS) appears to be a promising  
26 treatment for glioma. However, 5-ALA-mediated fluorescence cannot always be detected in grade II/III  
27 gliomas. We hypothesized that gene expression patterns in the Protoporphyrin IX (PpIX) synthesis  
28 pathway may be associated with intraoperative fluorescence status of grade II/III gliomas, and then  
29 attempted to identify the key molecule of 5-ALA-mediated fluorescence.

30 Methods: Using 50 surgically obtained specimens, which were diagnosed as grade II and III gliomas,  
31 we analyzed gene expression within the PpIX synthesis pathway to identify candidate molecules  
32 according to intraoperative 5-ALA-mediated fluorescence status. The most likely candidate gene was  
33 selected and confirmed by protein expression analysis. To evaluate the biological function of the  
34 molecule in PpIX synthesis, functional analysis was performed using specific, small interference  
35 (si)RNA in the SW-1783 human grade III glioma cell line.

36 Results: Among the genes involved in the porphyrin synthesis pathway, the mRNA expression of  
37 *Peptide transporter 2 (PEPT2)* in FGS fluorescence-positive gliomas was significantly higher than that  
38 in fluorescence-negative gliomas. Protein expression of PEPT2 was also significantly higher in the  
39 fluorescence-positive gliomas, which was confirmed by western blot analysis and immunofluorescence  
40 analysis. The siRNA-mediated downregulation of the mRNA and protein expression of PEPT2 led to  
41 decreased PpIX fluorescence intensity, as confirmed by fluorescence spectrum analysis.

42 Conclusions: The results suggest PEPT2 is an important candidate molecule in 5-ALA-mediated FGS  
43 in grade II/III gliomas. As the overexpression of PEPT2 was associated with higher PpIX fluorescence  
44 intensity, PEPT2 may improve fluorescence-guided resection in grade II/III gliomas.

45 **Key words:** 5-aminolevulinic acid; Glioma; Fluorescence-guided surgery; Protoporphyrin IX

## 47 **Introduction**

48 Glioma is the most frequent primary intra-axial brain tumor. Surgery, combined with  
49 chemotherapy and radiotherapy, is the standard treatment; however, glioma remains incurable due to its  
50 high recurrence rate and invasiveness [1]. Patients with glioma can benefit from the maximum safest  
51 resection [2, 3]. However, surgeons often have difficulty distinguishing tumor tissue from normal tissue  
52 and in recognizing infiltrating glioma cells in normal tissues adjacent to tumor tissue intraoperatively.  
53 In addition, > 90% of recurrent gliomas occur within 2–3 cm of the borders of the original tumor lesion  
54 [4-7].

55 In recent years, 5-aminolevulinic acid (5-ALA) fluorescence-guided surgery (FGS) appears to be a  
56 promising treatment for glioma with documented survival benefits [8-10]. 5-ALA can be absorbed by  
57 glioma cells and then metabolized to Protoporphyrin IX (PpIX). Accumulated PpIX can lead to  
58 pronounced fluorescence when excited by violet–blue light, which assists in identifying the infiltrating  
59 area and increases the extent of tumor resection. However, the marginal region of tissue containing  
60 infiltrating glioma cells exhibits vague fluorescence due to insufficient PpIX accumulation [11]. In  
61 addition, 5-ALA-mediated fluorescence cannot be detected in all cases, particularly in the World Health  
62 Organization (WHO) grade II/III gliomas [12].

63 The molecular mechanisms underlying the accumulation of PpIX mediated by 5-ALA remain to  
64 be fully clarified. If the mechanisms can be elucidated, the fluorescence intensity mediated by 5-ALA  
65 in glioma may be managed by surgeons, and guide future maximum resection.

66 Compared with glioblastoma multiforme (GBM), a higher proportion of grade II/III cases do not  
67 exhibit 5-ALA-mediated fluorescence in tumor tissue. Therefore, the present study aimed to investigate  
68 the gene expression patterns of the PpIX synthesis pathway according to the 5-ALA-mediated

69 fluorescence status of grade II/III gliomas, and then identify the candidate molecules influencing the  
70 fluorescence.

71

## 72 **Methods and materials**

### 73 *Surgical excision of glioma specimens*

74 Since 2008, every patient who had suspected glioma in Hokkaido University Hospital (Sapporo,  
75 Japan) was administered with 5-ALA (20 mg/kg) orally 2–3 h prior to surgery. The fluorescence  
76 mediated by 5-ALA was visualized under a surgical microscope (OPMI-Pentero; Carl Zeiss) with  
77 high-powered, violet–blue LED light (CCS, Inc., Kyoto, Japan) during surgery. Surgically obtained  
78 glioma specimens emitting deep-red fluorescence or pink fluorescence were classified as  
79 fluorescence-positive. By contrast, glioma specimens without fluorescence were classified as  
80 fluorescence-negative. The fluorescence intensity status of each specimen was discussed and evaluated  
81 by two neurosurgeons (S.Y. and H.K.) during the procedure and recorded in the surgical records. If no  
82 fluorescence was detected in the tumor tissue, the tissue was cryopreserved and classified as  
83 fluorescence-negative. If fluorescence was partly detected in the excised tumor tissue, the fluorescing  
84 region was cryopreserved and the tissue was classified as fluorescence-positive. The surgical specimens  
85 were cryopreserved at -80°C.

86 In the present study, surgical specimens from archives in neurosurgery department in Hokkaido  
87 University Hospital were selected according to the following criteria: (1) obtained at primary tumor  
88 resection without any adjuvant therapy, including chemotherapy or radiotherapy; (2) histologically  
89 diagnosed as grade II or III gliomas based on WHO 2007 criteria, which included diffuse astrocytoma,  
90 oligodendroglioma, oligoastrocytoma, anaplastic astrocytoma, anaplastic oligodendroglioma, and

91 anaplastic oligoastrocytoma; classification and pathologic diagnosis of gliomas were made by a  
92 certified pathologist; (3) cryopreserved tissue samples were assigned to a corresponding intraoperative  
93 fluorescence status. The mutation status of isocitrate dehydrogenase (IDH) was also investigated.  
94 Mutation hotspots at codon 132 of IDH1 and codon 172 of IDH2 were screened using Sanger  
95 sequencing. In addition, 1p/19q loss of heterozygosity status was analyzed using a multiplex  
96 ligation-dependent probe amplification procedure. The tumors were re-diagnosed according to the  
97 WHO 2016 criteria according to the IDH and 1p19q status. In addition, all patients received magnetic  
98 resonance imaging (MRI) with contrast enhancement preoperatively. According to the enhancement  
99 pattern, the tumors were classified as enhanced tumors and non-enhanced tumors. Tumors that  
100 exhibited apparent enhancement, including a heterogeneous or ring-like pattern, were defined as an  
101 enhanced tumor. Tumors without obvious enhancement were defined as a non-enhanced tumor.

102

### 103 *Quantitative real-time polymerase chain reaction (qPCR) analysis*

104 As a control reference, two sets of commercially available human brain total RNA were obtained  
105 (Life Technologies; Clontech). The glioma total RNA was extracted from the frozen specimens using  
106 an All Prep DNA/RNA Mini kit (QIAGEN) based on the manufacturer's instructions. cDNA was  
107 synthesized from total RNA using the PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara  
108 Biotechnology Co., Ltd., Dalian, China). Several genes within the PpIX synthesis pathway were  
109 selected, including *ALAD*, *ALAS1*, *ABCG2*, *ABCB6*, *CPOX*, *FECH*, *HO-1*, *PEPT2*, and *UROS*, to  
110 identify candidate molecules in 5-ALA-mediated grade II/III glioma fluorescence. The gene primers  
111 are listed in Table S1. Reverse transcription-qPCR analysis was performed with LightCycler 96 (Roche  
112 Diagnostics, Basel, Switzerland) and the PCR product specificities were confirmed via melt curve

113 analysis. To normalize the target transcript, *GAPDH* was used, which is one of the most stably  
114 expressed housekeeping genes for endogenous control. The PCR experiments were run in triplicate.  
115 The  $-\Delta\Delta CT$  equation was applied to calculate the relative expression of tumor samples with the average  
116 value of the normal brain as a reference control.

117

### 118 *Immunofluorescence analysis.*

119 The protein expression of PEPT2 was detected by immunofluorescence staining using the  
120 paraffin-embedded tumor sections. The sections were incubated with rabbit polyclonal PEPT2 antibody  
121 (Abcam, 1:100) as the primary antibody in 0.5% BSA for 1 h at room temperature. Phosphate-buffered  
122 saline (PBS) was used in the negative control instead of the primary antibody. Alexa Fluor® 594 goat  
123 anti-rabbit antibody (Life Technologies, 1:200) in 0.5% BSA was used as the secondary antibody. The  
124 nuclei were stained with DAPI (Invitrogen). Staining was observed using the KEYENCE BZ-X700  
125 fluorescence microscope with a 20X objective.

126

### 127 *Western blot analysis*

128 The proteins were extracted from five fluorescence-negative grade II/III gliomas and five  
129 fluorescence-positive grade II/III gliomas. The proteins were loaded on a Blot™ 4%–12% Bis–Tris Plus  
130 gel (Invitrogen™), electrophoresed, and fractionated at 200 V for 30 min in SDS running buffer, and  
131 then transferred onto a 0.2- $\mu$ m-pore nitrocellulose membrane (Invitrogen™). Following blocking with  
132 2% ECL Prime blocking agent (GE Healthcare) in PBS-Tween 20 for 1 h at room temperature, the  
133 membrane was incubated with primary antibodies against PEPT2 (Abcam,1:500) and  $\beta$ -actin (Santa  
134 Cruz Biotechnology, Inc., 1:1,000) with gentle shaking at 4°C overnight, followed by incubation with a

135 horseradish peroxidase-conjugated, goat anti-mouse secondary antibody (Santa Cruz Biotechnology,  
136 Inc., 1:5,000) or goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., 1:5,000) at room  
137 temperature for 2 h. Finally, the proteins were visualized using the enhanced chemiluminescence  
138 method (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

139

#### 140 *RNA interference experiments*

141 The commercially available SW-1783 human grade III glioma cell line (ATCC®) was used in the  
142 present study. The SW-1783 cells were maintained in DMEM-high glucose (NacalaiTesque),  
143 supplemented with 10% fetal bovine serum (FBS, Life Technologies), in a 5% CO<sub>2</sub>-humidified  
144 incubator at 37°C. The SW-1783 cells were plated in 6-well plates. Each well of cells was transfected  
145 with 2,500 ng of *PEPT2* Silencer® Select Pre-designed small interfering (si)RNA (cat. no. s13065, Life  
146 Technologies) or Silencer® Select Negative Control #1 siRNA (Life Technologies) using Lipofectamine  
147 3000 (Invitrogen™) according to the manufacturer's instructions. The cells were harvested 24 h  
148 following transfection and subjected to RT-qPCR and western blot analyses to examine the silencing  
149 efficiency.

150

#### 151 *PpIX fluorescence spectrum analysis*

152 The cells were randomly divided into four groups: Negative control (NC) siRNA, NC siRNA +  
153 5ALA, PEPT2 siRNA, and PEPT2 siRNA + 5ALA. The 5-ALA (Cosmo Bio) was stored at 4°C in the  
154 dark and dissolved in DMEM-high glucose (NacalaiTesque), supplemented with 10% FBS, to a final  
155 concentration of 200 µg/ml. For the NC + 5ALA group and the PEPT2 siRNA + 5ALA group, the  
156 culture medium was replaced with a medium containing 5-ALA 24 h following transfection, followed

157 by incubation for 4 h at 37°C. For the NC siRNA group and PEPT2 siRNA group, the culture medium  
158 was replaced with fresh DMEM-high glucose, supplemented with 10% FBS without 5-ALA, at 24 h  
159 post-transfection. Following incubation for 4 h, the cells were washed with PBS (NacalaiTesque) and  
160 collected using 0.05% trypsin with 0.5 mM ethylenediaminetetraacetic acid in 15-mL conical tubes  
161 (FALCON®). The liquid supernatant was discarded following centrifugation at 1,000 revolutions per  
162 min. The fluorescence spectrum was detected by VLD-EX (SBI Pharma) in the dark room.  
163 Subsequently, the illuminant with optical fiber was placed into the 15-mL conical tubes. The distance  
164 between the bottom of the conical tube and the illuminant was maintained at ~2 cm. The excitation  
165 wavelength was 406 nm. Visual images were captured using a camera (ILCE-A5000, Sony). The  
166 quantified fluorescence spectrum was presented on the screen of the VLD-EX machine.

167

### 168 *Statistical analysis*

169 SPSS 22.0 and R statistical software, version 3.4.1, were used to conduct all statistical analyses.  
170 Continuous variables were compared using the one-way analysis of variance. The Least Significant  
171 Difference test was used to compare differences between groups. Categorical variables are expressed as  
172 frequency (percentage) and were compared using the Chi-square ( $\chi^2$ ) test.  $P < 0.05$  was considered to  
173 indicate a statistically significant difference. Hierarchical clustering analysis, presented as a heat map,  
174 was performed using R software to demonstrate the distinguishable mRNA expression profiles among  
175 the samples. “hclust” was used to compute the hierarchical clustering analysis, with the Ward method  
176 as the method of clustering, and the Euclidean distance as the distance metric. The results were  
177 visualized using dendrograms and heatmaps.

178

## 179 **Results**

### 180 *Patient characteristics*

181 A total of 50 grade II/III glioma specimens, including grade II (N = 22) and grade III (N = 28)  
182 specimens, were matched to the above criteria. The tumors were re-diagnosed according to the WHO  
183 2016 criteria. The types and the numbers of the grade II and grade III gliomas are listed in Table S2.  
184 The numbers of cases in terms of the grade of the glioma and 5-ALA-mediated fluorescence status are  
185 listed in Table 1. In the grade II gliomas, only 2/22 cases (9%) were detected with fluorescence. By  
186 contrast, in the grade III gliomas, 19/28 cases (68%) were detected with fluorescence and 9 cases  
187 (32%) showed no fluorescence. The fluorescence status was significantly influenced by histological  
188 malignancy ( $p < 0.001$ ). The correlation between IDH status and 5-ALA-mediated fluorescence status is  
189 shown in Table 2. Gliomas with IDH mutations were predominantly fluorescence-negative (25/36  
190 cases; 69%), whereas gliomas without IDH mutations were predominantly fluorescence-positive (10/14  
191 cases; 71%). The difference was statistically significant ( $p = 0.009$ ). The correlations between MRI  
192 contrast enhance status and the 5-ALA-mediated fluorescence status of the tumors are shown in Table  
193 S3. The non-enhanced tumors were predominantly fluorescence-negative (23/34 cases; 68%), and the  
194 difference was statistically significant ( $p = 0.044$ ).

195

### 196 *mRNA expression of genes in the PpIX synthesis pathway*

197 The relative mRNA expression levels, according to 5-ALA-mediated fluorescence status, are  
198 shown in Fig. 1a. Compared with those in the normal brain, the mRNA expression of levels of *ABCG2*  
199 ( $p < 0.05$ ), *ABCB6* ( $p < 0.05$ ), and *HO-1* ( $p < 0.05$ ) were significantly higher in the  
200 fluorescence-negative grade II/III gliomas, and the mRNA expression levels of *PEPT2* ( $p < 0.05$ ),

201 *ABCG2* ( $p < 0.01$ ), *ABCB6* ( $p < 0.01$ ), and *HO-1* ( $p < 0.001$ ) were significantly higher in the  
202 fluorescence-positive grade II/III gliomas. Compared with the fluorescence-negative grade II/III  
203 gliomas, the mRNA expression levels of *PEPT2* ( $p < 0.05$ ), *ALAD* ( $p < 0.01$ ), *ABCG2* ( $p < 0.05$ ),  
204 *ABCB6* ( $p < 0.01$ ), *CPOX* ( $p < 0.05$ ), *HO-1* ( $p < 0.05$ ), and *UROS* ( $p < 0.001$ ) were also significantly  
205 higher in the fluorescence-positive gliomas. There were no significant differences in the mRNA  
206 expression of *ALAS1* or *FECH* between the groups.

207 According to the PpIX synthesis pathway (Fig. 1b), *PEPT2* is responsible for transporting 5-ALA  
208 and *PEPT2* is an upstream molecule in the PpIX synthesis pathway. Therefore, the data obtained  
209 suggested that the overexpression of *PEPT2* affected the fluorescence intensity mediated by 5-ALA in  
210 grade II/III gliomas. In the IDH mutant specimens, the mRNA expression levels in the PpIX synthesis  
211 pathway were similar in all grade II/III gliomas (Fig. S1a). The IDH wild-type grade II/III gliomas also  
212 exhibited similar mRNA expression tendency (Fig. S1b). The present study also compared the mRNA  
213 expression of *PEPT2* between MRI enhanced grade II/III gliomas and MRI non-enhanced grade II/III  
214 gliomas. The mRNA expression of *PEPT2* in the enhanced tumors was higher than that in the  
215 non-enhanced tumors, although this difference did not reach statistical significance (data not shown).

216 Hierarchical clustering of the expression patterns demonstrated there are two major clusters (Fig.  
217 1c). Cluster 1 contained 19 tumors, and cluster 2 contained 31 tumors. In terms of the 5-ALA-mediated  
218 fluorescence status, the majority of fluorescence-negative tumors (16/28 tumors; 57%) and three of the  
219 21 fluorescence-positive tumors (14%) belonged to cluster 1. The majority of the fluorescence-positive  
220 tumors (18/21 tumors; 86%) belonged to cluster 2. In addition, in terms of the IDH status, only one out  
221 of the 14 IDH mutant gliomas (7%) belonged to cluster 1. In terms of MRI contrast enhancement status,  
222 only one of the 16 MRI contrast enhanced tumors (6%) belonged to cluster 1.

223

224 *Protein expression of PEPT2 measured by immunofluorescence and western blot*  
225 *analysis*

226 According to the results of the RT-qPCR analysis of the PpIX synthesis pathway, the present study  
227 focused on the overexpression of PEPT2, as it may be important in the accumulation of PpIX following  
228 the administration of 5-ALA in grade II/III gliomas. PEPT2, also known as SLC15A2, is widely  
229 expressed in glial cells and mediates the uptake of peptide substrates. PEPT2 polyclonal antibody was  
230 used to detect protein expression by immunofluorescence in five fluorescence-positive grade II/III  
231 gliomas and five fluorescence-negative grade II/III gliomas. The results of the protein expression of  
232 PEPT2 are shown in Fig. 2a. The immunofluorescence results of three fluorescence-positive cases and  
233 three fluorescence-negative cases are shown in Fig. 2a. Compared with the fluorescence-negative grade  
234 II/III gliomas, a higher expression of PEPT2 was presented in the fluorescence-positive grade II/III  
235 gliomas.

236 The present study also investigated the relative protein expression levels of PEPT2 and compared  
237 them between five fluorescence-negative grade II/III gliomas and five fluorescence-positive grade II/III  
238 gliomas using western blot analysis. Consistent with the product datasheet of primary PEPT2 antibody,  
239 bands at a size of 90 kDa were observed, and the predicted band was identified at a size of 82 kDa. The  
240 results (Fig. 2b, c) demonstrated that the protein expression of PEPT2 in the fluorescence-positive  
241 grade II/III gliomas was significantly higher than that in the fluorescence-negative grade II/III gliomas  
242 ( $p < 0.01$ ). These results revealed that the overexpression of PEPT2 might be important in the  
243 5-ALA-mediated PpIX fluorescence intensity of gliomas.

244

245 *RNA interference and PpIX fluorescence spectrum analysis.*

246 In order to further confirm the exact function of PEPT2, the present study aimed to inhibit the  
247 expression of PEPT2 in the SW-1783 cell line using siRNA. PEPT2 mRNA and protein expression was  
248 successfully downregulated by ~50% at 24 h post-transfection with specific siRNA (Fig. 3a–c). The  
249 fluorescence spectrums of the four groups (NC siRNA + 5ALA, NC siRNA, PEPT2 siRNA + 5ALA,  
250 and PEPT2 siRNA) were detected using VLD-EX in the dark room. The peak wavelength of 636 nm  
251 represents the PpIX fluorescence spectrum. The results demonstrated that the downregulation of PEPT2  
252 led to decreased fluorescence intensity (Fig. 3d, e) in the SW-1783 cells. This result suggested that  
253 5-ALA-mediated fluorescence may be influenced by the expression of PEPT2 in grade II/III gliomas.

254

## 255 **Discussion**

256 Patients with glioma may benefit from the maximum safe tumor resection [2,3]. However,  
257 surgeons may have difficulty distinguishing tumor tissue from normal tissue during surgery.  
258 5-ALA-mediated FGS has become a useful surgical technique in glioma resection and improves the  
259 overall survival of patients with malignant glioma [8-10]. However, fluorescence cannot be detected in  
260 all cases of glioma, particularly in grade II/III tumors [12]. In the present study, in the grade II gliomas,  
261 only 2/22 cases (9%) were detected with fluorescence. By contrast, in the grade III gliomas, 19/28  
262 cases (68%) were detected with fluorescence and 9 cases (32%) showed no fluorescence (Table 1).  
263 Therefore, the 5-ALA-mediated FGS in grade II/III gliomas may be useful.

264 The FGS mechanism mediated by 5-ALA in gliomas remains to be fully elucidated. In particular,  
265 compared with GBMs, there has been no previous investigation of the molecular mechanism  
266 underlying 5-ALA-mediated FGS in grade II/III glioma. Fluorescence-positive and

267 fluorescence-negative cases may show different gene expression patterns. Therefore, the present study  
268 was performed to investigate the differences in gene expression patterns between fluorescence-positive  
269 grade II/III gliomas and fluorescence-negative grade II/III gliomas. If the underlying genetic  
270 mechanism of the fluorescence of PpIX mediated by 5-ALA can be clarified, the fluorescence intensity  
271 may be managed by neurosurgeons in the future.

272 The present study first attempted to identify candidate genes with an effect on 5-ALA-mediated  
273 PpIX fluorescence intensity. The mRNA expression levels of genes in the PpIX synthesis pathway were  
274 compared among normal brain tissues, fluorescence-negative grade II/III gliomas, and  
275 fluorescence-positive grade II/III gliomas. The results demonstrated that mRNA expression levels of  
276 *ALAD* ( $p < 0.01$ ), *ABCG2* ( $p < 0.05$ ), *ABCB6* ( $p < 0.01$ ), *CPOX* ( $p < 0.05$ ), *HO-1* ( $p < 0.05$ ), *PEPT2* ( $p$   
277  $< 0.05$ ), and *UROS* ( $p < 0.001$ ) were significantly higher in the fluorescence-positive grade II/III  
278 gliomas than the fluorescence-negative grade II/III gliomas (Fig. 1a).

279 Among the above candidate genes, the present study focused on PEPT2 as it is an upstream  
280 molecule in the PpIX synthesis pathway (Fig. 1b). In the central nervous system (CNS), PEPT2 can  
281 remove peptide/mimetic drugs from the cerebrospinal fluid to the plasma. PEPT2 is also responsible  
282 for the uptake of peptide/mimetic drugs from brain extracellular fluid into brain cells, which is  
283 important in regulating drug metabolism in the CNS [13-15].

284 PEPT2 protein consists of 729 amino acids, and the core molecular size is 81,940 Da. PEPT2 is a  
285 high-affinity and low-capacity transporter, which is widely expressed in the brain, lung, kidney, eye,  
286 and mammary gland [16, 17]. PEPT2 is located in the cell membrane and is responsible for the  
287 selective transportation of peptides, amino acids, and drugs [18-20]. PEPT1 and PEPT2 are responsible  
288 for 5-ALA uptake. However, the affinity of PEPT2 to the same substrates is higher than that of PEPT1,

289 which is mainly expressed in the intestine [21, 22]. In general, > 400 dipeptides and 8,000 tripeptides,  
290 including 20 essential L- $\alpha$ -amino acids and the majority of D-enantiomers, can be  
291 sequence-independently transported by PEPT2. In addition, numerous peptide-like drugs, including  
292  $\beta$ -lactam antibiotics, angiotensin-converting enzyme inhibitors, and peptidase inhibitors can be  
293 mediated and transported by PEPT2 substrate [18, 20, 23].

294 Previous functional investigations of PEPT2 have focused predominantly on its transportation and  
295 absorption effect. In the kidney, PEPT2 is almost entirely responsible for the reabsorption of peptides  
296 and peptidomimetics [18, 23-25]. PEPT2 in the lung is located in alveolar type II pneumocytes, the  
297 bronchial epithelium, and the endothelium of small vessels, and is responsible for delivering peptides  
298 and peptidomimetics [26, 27]. Of note, it has been demonstrated that PEPT2-null mice are fertile and  
299 healthy. Therefore, the exact function of PEPT2 requires further investigation [28, 29].

300 In the present study, it was hypothesized that PEPT2 may be key in the uptake of 5-ALA and  
301 5-ALA-mediated PpIX fluorescence in grade II/III gliomas. The protein expression of PEPT2 was  
302 compared between fluorescence-negative grade II/III gliomas and fluorescence-positive grade II/III  
303 gliomas. The results demonstrated that the protein expression of PEPT2 in fluorescence-positive grade  
304 II/III gliomas was significantly higher than that in fluorescence-negative grade II/III gliomas ( $p < 0.05$ ),  
305 which suggested that the levels of PEPT2 may affect the fluorescence intensity of PpIX. To further  
306 investigate the exact function of PEPT2, the mRNA and protein expression of PEPT2 were inhibited in  
307 a grade III glioma cell line and the PpIX fluorescence spectrum was detected. The results demonstrated  
308 that the downregulation of PEPT2 decreased fluorescence intensity. These findings suggest that the  
309 expression of PEPT2 is important for the 5-ALA-mediated fluorescence intensity of PpIX.

310 Previous studies have demonstrated that the overexpression of ABCB6 can enhance the

311 5-ALA-mediated fluorescence of PpIX in human glioma [30]. ABCB6 is a transporter in the PpIX  
312 metabolic pathway. This result is consistent with the RT-qPCR results in the present study, which  
313 showed that the mRNA of *ABCB6* was overexpressed in fluorescence-positive grade II/III gliomas. In a  
314 study conducted by Takahashi *et al* [31], the mRNA expression levels of *PEPT2*, *ABCB6*, and *ABCG2*  
315 appeared to be relatively lower in samples with a high level of fluorescence, which is inconsistent with  
316 the results of the present study. This may be due to the samples used in the previous study being  
317 glioblastomas and metastatic brain tumors, which may exhibit gene expression patterns distinct from  
318 those of grade II/III gliomas. Hu *et al* [32] found that PEPT2 reduced the neurotoxicity of 5-ALA,  
319 which indicates that the expression of PEPT2 may also influence the efficacy of 5-ALA-mediated  
320 photodynamic therapy. This also suggests that PEPT2 is important in glioma treatment. Therefore,  
321 future studies may examine the role of PEPT2 in glioma photodynamic therapy.

322 In conclusion, the present study is the first, to the best of our knowledge, to demonstrate that  
323 PEPT2 is an important gene/protein in 5-ALA-mediated FGS in grade II/III gliomas. The  
324 overexpression of PEPT2 was associated with a higher fluorescence intensity of PpIX in grade II/III  
325 gliomas. These results may provide clues to improve the surgical treatment of grade II/III gliomas in  
326 the future.

327

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330 review.

331

332 **Compliance with Ethical Standards**

333 *Conflict of interest*

334 The authors declare no conflict of interest.

335

336 *Ethical approval*

337 This study was approved by the local Ethics Committee at Hokkaido University Hospital (Sapporo,

338 Japan; 017-0032). All procedures performed in the present study were in accordance with 1964

339 Helsinki Declaration and its later amendments.

340

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445

446 **Figure legends**

447 **Fig. 1** Relative mRNA expression levels, PpIX/heme biosynthesis, and metabolism pathway,  
448 hierarchical clustering of the expression patterns. **a** Relative mRNA expression levels of *ALAS1*, *ALAD*,  
449 *ABCG2*, *ABCB6*, *CPOX*, *FECH*, *HO-1*, *PEPT2*, and *UROS* of all grade II/III gliomas in three groups.  
450 Data are presented as the mean  $\pm$  standard error of the mean with three replicates for each glioma  
451 specimen. #P < 0.05 compared with the normal brain group, ##P < 0.01 compared with the normal  
452 brain group, ###P < 0.001 compared with the normal brain group, \*P < 0.05 compared with the  
453 fluorescence-negative group, \*\*P < 0.01 compared with the fluorescence-negative group, \*\*\* P <  
454 0.001 compared with the fluorescence-positive group. **b** PpIX/heme biosynthesis and metabolic  
455 pathway. Multiple enzymes and transporters are involved in this pathway. The enzymes are marked  
456 with rectangles. **c** Heatmap summary and hierarchical clustering for *ALAD*, *ALAS1*, *ABCG2*, *ABCB6*,  
457 *CPOX*, *FECH*, *HO-1*, *PEPT2*, and *UROS* of the 50 specimens. Samples are depicted in rows and RNAs  
458 are depicted in columns. Red indicates high expression, and green indicates low expression. ABCB10,  
459 ATP binding cassette subfamily B member 10; ABCC1, 2 or 3, ATP binding cassette subfamily C  
460 member 1, 2 or 3; PBzR, peripheral benzodiazepine receptor; FLVCR1, feline leukemia virus subgroup  
461 c receptor 1; SLC25A38, solute carrier family 25 member 38.

462

463 **Fig. 2** Protein expression of PEPT2 measured by immunofluorescence analysis and western blot  
464 analysis. **a** Protein expression of PEPT2 measured by immunofluorescence analysis. PBS was used in  
465 negative control instead of primary antibody. PEPT2 protein was detected with Alexa Fluor® 594 goat  
466 anti-rabbit secondary antibody (red). Nuclei were stained with DAPI (blue). Protein expression of  
467 PEPT2 in fluorescence-positive grade II/III gliomas was higher than in fluorescence-negative grade

468 II/III gliomas. **b** Western blotting of five fluorescence-positive grade II/III gliomas and five  
469 fluorescence-negative grade II/III gliomas using antibody against PEPT2.  $\beta$ -actin was used as a loading  
470 control. **c** Comparison of relative protein expression levels of PEPT2 between five  
471 fluorescence-positive grade II/III gliomas and five fluorescence-negative grade II/III gliomas based on  
472 densitometric analysis of western blots. Data are presented as the mean  $\pm$  standard error of the mean of  
473 three replicates for each glioma specimen. \* $p < 0.05$  compared with the fluorescence-negative grade  
474 II/III gliomas.

475

476 **Fig. 3** RNA interference experiments and PpIX fluorescence spectrum analysis. **a** Western blotting of  
477 SW-1783 cells transfected with PEPT2 siRNA or negative control siRNA using antibody against  
478 PEPT2.  $\beta$ -actin was used as a loading control. **b** Semi-quantitative analysis of western blots. Data are  
479 presented as the mean  $\pm$  standard error of the mean of three replicates for each experimental condition.  
480 **c** mRNA expression of *PEPT2* in SW-1783 cells transfected with PEPT2 siRNA or negative control  
481 siRNA. \*\* $p < 0.01$  compared with SW-1783 cells transfected with negative control siRNA. **d** PpIX  
482 fluorescence spectrum of the four groups (NC siRNA + 5ALA, NC siRNA, PEPT2 siRNA + 5ALA,  
483 and PEPT2 siRNA). **e** Visual images of the fluorescence of PpIX in the four groups. The density of  
484 cells in each sample was  $\sim 1 \times 10^6$ .

485

486

487 Table 1. 5-ALA-mediated FGS fluorescence status in relation to the grading of grade II/III gliomas.

Fluorescence status	Grade II glioma	Grade III glioma	Total
Fluorescence-positive	2	19	21
Fluorescence-negative	20	9	29
Total	22	28	50

488 5-ALA, 5-aminolevulinic acid; FGS, fluorescence-guided surgery.

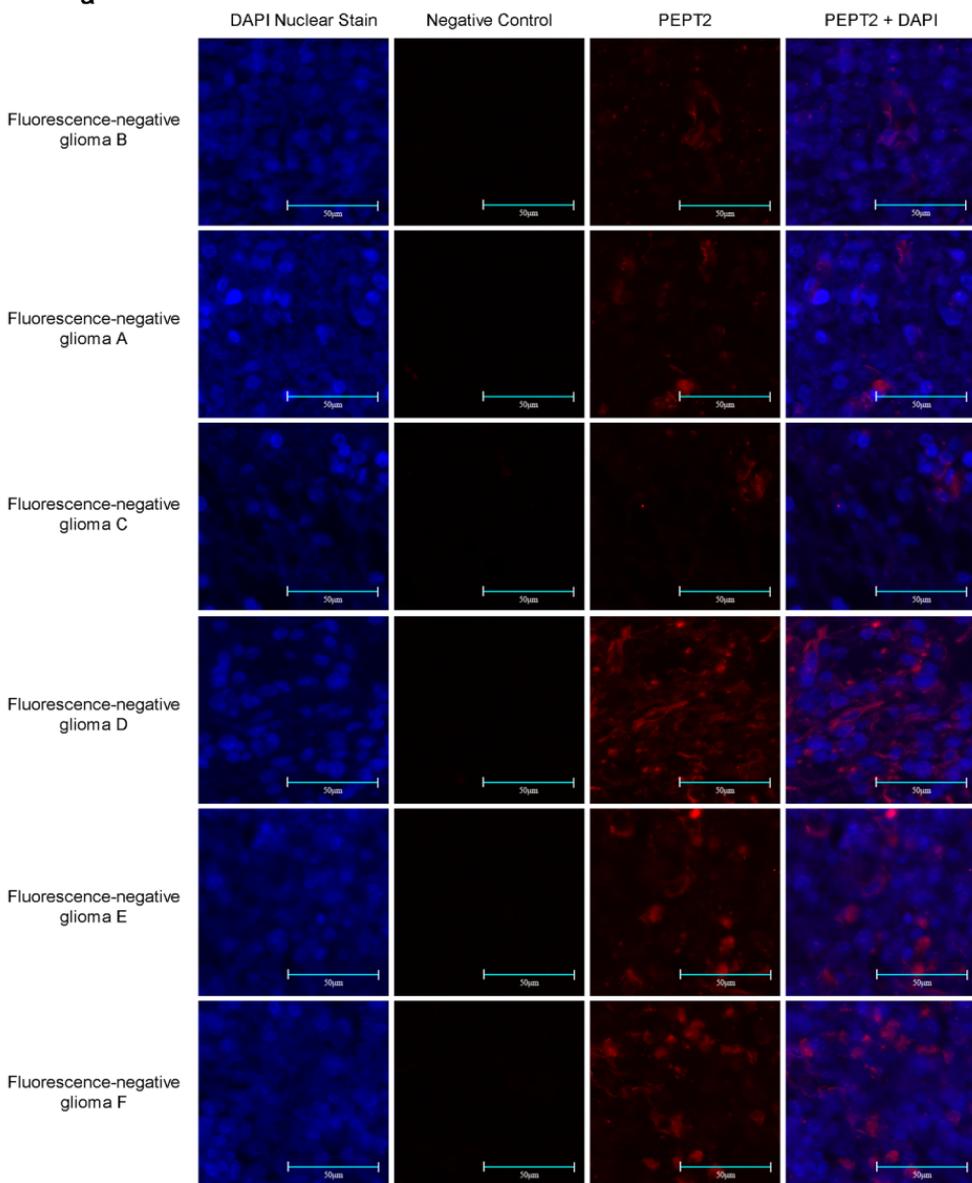
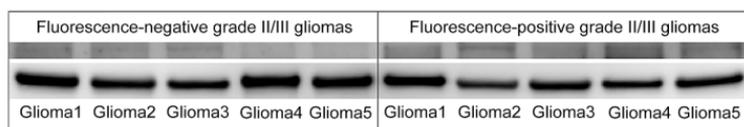
489

490 Table 2. 5-ALA-mediated fluorescence status in relation to the IDH status of 50 grade II/III gliomas.

Fluorescence status	IDH mutant glioma	IDH wild-type glioma	Total
Fluorescence-positive	11	10	21
Fluorescence-negative	25	4	29
Total	36	14	50

491 5-ALA, 5-aminolevulinic acid; IDH, isocitrate dehydrogenase.



**a****b****c**