Expression of GLUT1 on Pseudopalisade and Perivascular Tumor Cells is an Independent Prognostic Factor for Patients with Glioblastomas

Running Head: GLUT1 is a prognostic factor for glioblastomas

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Abstract

Glioblastomas are highly aggressive brain tumors with a particularly poor prognosis. Potentially related is glucose transporter-1 (GLUT1/SLC2A1), a uniporter expressed by various carcinomas that may be involved in malignant neoplasm glycometabolism, including glioblastomas. GLUT1 is essential to the central nervous system depend on glucose for glycometabolism. To clarify the exact role of GLUT1 in glioblastoma, we assessed the expression and localization of GLUT1 in patient samples by immunohistochemistry and *in situ* RNA hybridization. This revealed that GLUT1 was mainly expressed on perivascular and pseudopalisade tumor cell membranes. All samples expressed GLUT1 to some degree, with 30.8% showing stronger staining. Based on these data, samples were divided into high and low expression groups, although *SLC2A1* mRNA expression was also higher in the high GLUT1 expression group. Kaplan-Meier survival curves revealed that high GLUT1 expression associated with lower overall survival (log-rank test, $p = 0.001$) and worse patient prognoses ($p = 0.001$). Finally, MIB-1 staining was stronger in high GLUT1 expression samples ($p = 0.0004$), suggesting a link with proliferation. We therefore hypothesize that GLUT1 expression in glioblastomas may enhance glycolysis, affecting patient prognosis. Examination of GLUT1 in patients with glioblastomas may provide a new prognostic tool to improve outcome.

Key words: Glioblastomas; Glucose transporter (GLUT); Angiogenesis; Warburg effect.

Introduction

The glucose transporter (GLUT) protein family contains various important molecules that use glucose diffusion gradients (and those of other sugars) for transport across the plasma membrane. They exhibit various substrate specificities, kinetic properties, and tissue expression profiles, depending on their role (1). The GLUT protein family belongs to a larger facilitator superfamily of membrane transporters (2), with 13 related members of the SLC2A (GLUT) protein family identified in humans (3). Structurally, these molecules are divided into three classes; GLUT1–4 (class 1); GLUT5, 7, 9, and 11 (class 2); and GLUT6, 8, 10, and 12 (class 3) (2). In the central nervous system, GLUT1 plays a critical role in cerebral glucose uptake and is the major GLUT isoform expressed in brain endothelial cells and brain astrocytes. It has been suggested that GLUT1 supplies glycolytically- derived lactate to neurons as a major fuel source (4, 5). The protein is a 492-amino-acid uniporter that is expressed by endothelial cells of the blood brain barrier (BBB). In humans, it is encoded by the *SLC2A1* gene (6) and the GLUT1 protein possesses 12 transmembrane segments, a single site of N-linked glycosylation, a relatively large, central, cytoplasmic linker domain, and also exhibits a topology where both the N and C terminals are positioned in the cytoplasm (6). In 1996, Younes et al. reported that GLUT1 expression in colorectal carcinomas associated with high incidence rates of lymph node metastasis (7). Subsequently, numerous

studies have reported contradictory evidence of the relationships between GLUT1 expression and prognosis in solid human tumors (8–14). Of note is a comprehensive meta-analysis of 2948 patients across 26 different studies that indicated that GLUT1 expression was a promising biomarker for prognosis, consistently showing an association with overall survival at three and five years (15). Among the tumor types evaluated, higher expression of GLUT1 in tumor tissues was also linked to worse overall survival at three and five years in oral squamous cell carcinomas and breast cancers (15). The expression of GLUT1 in astrocytomas and glioblastomas has also been reported to correlate with hypoxia-inducible factor-1 (HIF-1) in pseudopalisades, suggesting a link to hypoxia (16–20). However, the exact role of GLUT1 in tumorigenesis remains to be elucidated in highly aggressive glioblastomas. To address this, the present study analyzed the expression of GLUT1 in IDH wildtype, WHO grade IV glioblastomas and any correlation with patient prognosis.

Materials and Methods

Patient Samples

Surgically collected tissue samples from 52 patients with glioblastomas were analyzed in the present study. All tumor specimens were retrieved from the archives of Kurume University Hospital, Kurume, Japan between 2010 and 2016. The study was performed in accordance with the principles of

the Helsinki Declaration and was approved by the institutional ethics committee. All specimens were histologically diagnosed as IDH-wildtype glioblastoma using World Health Organization (WHO) criteria (21). Multivariate survival analysis was used to assess the significance of various prognostic factors, including complete resection, radiotherapy, age (>18 y), Karnofsky performance status (KPS) scores, and the expression of GLUT1.

Immunohistochemistry

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and processed using conventional histological and immunohistochemical methods. Sections 5-μm thick were stained with hematoxylin and eosin (HE) for histological evaluation. The remaining unstained serial sections were used for immunohistochemistry analysis following heat-induced antigen retrieval. These were stained with the appropriate antibodies and expression was evaluated with immunoperoxidase (ChemMate ENVISION kit/HRP[DAB]; DakoCytomation, Carpinteria, CA) using a Dako Autostainer Universal Staining System (DakoCytomation). The primary antibodies used were anti-GLUT1 (1:3200; Abcam, Cambridge, UK), anti-endoglin (CD105; 1:50; Novocastra, Newcastle, UK), and anti-Ki-67 (MIB-1; 1:100; Immunotech, Marseille, France). MIB-1 labeling indices were determined as the percentage of the nuclear area stained in areas of maximal labeling. GLUT1 expression in glioblastomas

was evaluated as both staining intensity and the number of stained cells by two observers in independent examinations. Samples that varied significantly between the two observers were re-evaluated to arrive at a consensus.

Fluorescence immunohistochemical staining

Fluorescence double immunostaining of GLUT1 and other markers (HIF1α, CD34, and Ki67) in tumor tissues was performed. Double staining was performed in a glioblastoma sample with anti-GLUT1 (1:100, Thermo, UK), anti-Ki67 (1:100, Dako, Denmark), and anti-CD34 (1:100, Leica, UK) antibodies followed by addition of anti-rabbit IgG-Texas Red (TR; sc-2780, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse IgG-Alexa488 (Life Technologies, Carlsbad, CA, USA). Assessment of staining co-localization was performed using the OPAL 7-color flHC Kit (Perkin Elmer, Watham, MA, USA) according to the manufacturer's protocols. Fluorescence was measured using a fluorescent microscope (BX51FL, Olympus, Tokyo, Japan) and a charged-coupled device camera (DP71, Olympus, Tokyo, Japan).

DNA Isolation and Mutation Analysis

The mutational statuses of *IDH1* and *IDH2* were determined by Sanger sequencing. Briefly, genomic DNA was isolated from the relevant formalin-fixed paraffin-embedded (FFPE) tissue blocks by

cutting 10-μm thick sections, followed by extraction using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). *IDH1* exon 4 and *IDH2* exon 4 were amplified by polymerase chain reaction (PCR) with the indicated primers (Table 1). The amplifying conditions for *IDH1* and *IDH2* were both an initial denaturing step of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, an annealing step at 60 °C for 30 s, and then extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were separated on 2% agarose gels by electrophoresis, excised, and then sequenced on an ABI PRISM 310 Genetic Analyzer (Life Technologies, Gaithersburg, MD). Analyses of sequence data were performed using GENETYX software Ver. 10 (GENETYX, Tokyo, Japan) and a reference sequence complementary to each gene.

RNAscope

SLC2A1 (*GLUT1*) mRNA *in situ* hybridization (ISH) was performed using an RNAscope 2.5 HD Assay Kit-BROWN (ACD, Newark, CA), according to the manufacturer's protocols. Tissue sections 2.5-μm thick were deparaffinized in xylene and dehydrated through an ethanol series. The sections were then incubated in citrate buffer (10 nmol/L, pH 6.0) and maintained at 100 °C to 103 °C for 15 min using a hot plate. They were then rinsed in deionized water and immediately treated with 10 μg/mL protease (Sigma-Aldrich, St. Louis, MO) at 40 °C for 30 min in a HybEZ hybridization oven (Advanced Cell

Diagnostics, Hayward, CA). Hybridization with target probes, preamplifier, amplifier, label probes, and chromogenic detection were performed as previously described for cultured cells. RNAscope scores and the heterogeneity of *SLC2A1* mRNA signals were estimated using the manufacturer's recommended protocols and a semiquantitative scoring system. The staining scores were 0; no staining or less than 1 dot to every 10 dots, staining score 1; 1–3 dots/cell, staining score 2; 4–10 dots/cell or very few dot clusters, staining score 3; >10 dots/cell and less than 10% positive cells with clusters, staining score 4; >10 dots/cell and 10% positive cells with clusters. The evaluation was performed by three observers (SK, YS, and KY) across independent examinations.

Cell culture

Human glioma cell lines T98, U87, and U251, were obtained from American Type Culture Collection (ATCC) in 2009. Authentication of the cell lines was unnecessary because cells were expanded by culturing them for less than two passages and stored at −80°C. Low-passage cells were used for experiments within 6 months after resuscitation. They were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100μg/mL streptomycin.

Cell viability and cell proliferation

Cell proliferation was evaluated in triplicates by a colorimetric WST-1 assay (Roche, Germany) according to the manufacturer's protocol. Practically, 1,000 cells of each population were seeded in 96-well plastic plates in 200 µL of culture medium supplemented with 0.1% FBS. The plates were incubated for 4 h at 37°C. Twenty microliters of WST-1 (10% of total volume) was added to the cells, and the cells were incubated. The plate was read using DS2® (Dynex, USA) by measuring the absorbance of the dye at 450 nm, with 600nm set as the reference wavelength. Averages of the absorbance values were calculated and plotted. The cells were treated with various concentrations of WZB-117 (Sigma-Aldrich, Germany), a specific GLUT-1 inhibitor.

Statistical Analysis

To reveal any correlation between the expression of GLUT1 in glioblastomas and patient prognoses, statistical analysis among the relevant groups was performed using JMP13 (SAS Institute Inc., Cary, NC). Survival rates were computed using Kaplan-Meier curves. Patients were excluded if they were lost to follow-up at the time of analysis. Cox proportional hazards regression models were used for multivariate analyses of overall survival. In addition, Kendal tau rank correlation tests were performed between GLUT1 expression, Ki-67 labeling indices, and the number of CD105 (endoglin) positive vessels in glioblastomas. Statistical significance was set at a threshold of $p < 0.05$.

Gene expression database

Information regarding SLC2A1 alterations and patient's survival time in glioblastomas was downloaded from the Cancer Genome Atlas (TCGA) Database, an open access database that is publicly available at http:///www.cbioportal.org [38, 39]. Six hundred and four glioblastoma cases (TCGA, Provisional) were selected. These cases were divided into two groups: SLC2A1 mRNA up-regulated or not altered. Gene altered cases were defined as that expression level of SLC2A was observed greater than standard-deviation from the mean. The proportion of up-regulated cases was calculated and overall survival and progression-free survival were estimated by Kaplan-Meier analysis. Furthermore, co-expression genes with SLC2A1 in glioblastomas were investigated by calculating Spearman's correlation coefficients. We selected VEGF as the neoangiogenetic marker, CD44 as the mesenchymal marker, PDGFRα as proneural marker, TGF-β as the epithelial mesenchymal transformation's marker and HIF1α as the hypoxic marker [40].

Result

Clinical Data

A summary of the clinical information for the 52 patients with glioblastomas is shown in Table 2. The median age at diagnosis was 65.9 (33–86) years and 18 cases (34.6%) were female, 34 cases

(65.4%) male. The median KPS at diagnosis was 74.0 (30–90) years. Tumors were localized to the frontal lobe in 21 cases, the temporal lobe in 18 cases, the parietal lobe in nine cases, and in other regions for the remaining four cases. All patients received surgical treatment, followed by chemoradiotherapy with temozolomide.

Expression of GLUT1

Our study showed that GLUT1 was expressed on tumor cell membranes in all 52 cases, supporting previous reports (13, 19). The intensity of staining could be divided into three categories, mild (grade 1; Fig. 1B), moderate (grade 2; Fig. 1C), or strong (grade 3; Fig. 1D). Six high power fields in each tissue samples were randomly selected and used to calculate the ratio of grade 2 to grade 3 assessments across the tumor sample. We defined a ratio of more than 20% between grade 2 and 3 as the high GLUT1 expression group, whereas ratios less than 19% were defined as a low expression group. Using these criteria, high expression of GLUT1 was detected in 16 cases and low expression was detected in 36 cases. Using an RNAscope system, *SLC2A1* mRNA was detected in glioblastoma tumor cells as either brown distinct points or clustered patterns (Fig. 2). A semi-quantitative four-level staining score was established for the RNAscope data. Samples defined as grades 3 or 4 (high expression) in the RNAscope study were the same as the GLUT1 high expression samples. In contrast, RNAscope staining

scores of grades 1 or 2 (low expression) were found in the GLUT1 low expression group. These data confirm that both GLUT1 and *SLC2A1* mRNA expression differ in glioblastomas, and that there is an association between protein and transcript.

Localization of GLUT1

We next assessed the localization of GLUT1-positive cells in tissues, revealing that GLUT1 cells were located at the perivascular pseudopalisading cells and in tumor cells at the boundary between tumors and normal brain tissue (Fig. 3). Especually, GLUT1-positive cells were more commonly found at the perivascular region, in addition to pseudopalisades and tumor boundaries (Fig. 3D). This suggests that GLUT1 expression associates with these migratory and hypoxic areas, indicating a link with glycometabolism. To show localized GLUT1 expression, double immunofluorescence was performed. Glioblastoma cells expressing GLUT1 were observed around CD34-positive blood vessels (Figure 5A). Furthermore, GLUT1 and HIF1α were co-expressed in pseudopalisading necrosis (Figure 5B). These findings supported the specific localization of GLUT1 expression in hypoxic area of glioblastomas.

Association Between GLUT1 Expression, Angiogenesis, and Proliferation

Next, CD105 staining was used as a marker to distinguish between normal vessels and malignant intratumoral neovascularization to assess any correlation between high GLUT1 expression and

angiogenesis. Averages of CD105-stained cell counts from three areas were recorded as CD105 intratumoral vessel density to compare the degree of angiogenesis. This showed that GLUT1 in the high expression group more frequently associated with CD105 positive vessels relative to the low GLUT1 expression group (Fig. 4A). The number of CD105 positive vessels positively correlated with the number of GLUT1-positive cells in the high expression tumors (correlation coefficient: 0.61). We also measured if there was any correlation between the expression of GLUT1 and tumor cell proliferation in glioblastomas using MIB-1 labeling indices. MIB-1 staining was immunohistochemically evaluated across continuous sections, revealing that the MIB-1 labeling indices in the high GLUT1 expression group were higher than that in the low expression group (Wilcoxon test: $p < 0.001$; Fig. 4B). Furthermore, double fluorescent immunostainings for GLUT1 and MIB-1 suggested that tumor cells with high proliferative potency expressed GLUT1 (Figure 5C). Together, these data indicate a link between GLUT1 angiogenesis and glioblastoma cell proliferation.

Cell proliferation and viability

GLUT1 expression of U87, U251, and T98 cells was confirmed by western blot analysis (data not shown). The viability of T98 and U87 with WZB-117 decreased in a concentration-dependent manner (Figure 6A, 6B), whereas that of U251 decreased at 5 μM of WZB-117 (Figure 6C).

Association Between GLUT1 Expression and Patient Survival

Finally, Kaplan-Meier survival curve analysis indicated that the high GLUT1 expression group had lower overall survival rates than the low expression group (log-rank test: p = 0.001; Fig. 6D). There were no differences between progression free survival rates in the high and low expression groups (data not shown). A further multivariate analysis showed that GLUT1 was an independent predictor for poor prognosis when compared to already known prognostic factors, such as age or KPS (Table 3; hazard ratio 5.59, 95% confident interval 2.22–14.4, p = 0.0003). This suggests that GLUT1 is a novel independent predictor for glioblastoma outcome.

Gene expression database

To validate the result of the present study, data from TCGA database regarding GLUT1 in glioblastomas was analyzed (refer to Supplemental data). Two hundred and forty nine cases (42%) showed up-regulated SLC2A1 in 591 glioblastomas from TCGA database. The overall survival of up-regulated cases tended to be shorter than the not altered cases (Log-rank test; p=0.375, Supplemental figure 1A), and the progression free survival of up-regulated cases was significantly shorter than the not altered cases (Log-rank test; p=0.01, Supplemental figure 1B). SLC2A1 correlated with VEGF (Spearman's correlation coefficient 0.63, Supplemental figure 2A, TGF-β (Spearman's correlation

coefficient 0.35, Supplemental figure 2B) and HIF1α (Spearman's correlation coefficient 0.33, Supplemental figure 2C). CD44 and PDGFRα did not show significant correlation with SLC2A1. These data from genome-wide analyses implied that GLUT1 was related with angiogenesis and hypoxia in glioblastoma as a whole tumor.

Discussion

Glioblastomas are extremely aggressive tumors of the central nervous system that have a particularly poor outcome. Previously, it has been shown that glioblastomas are in a relatively hypoxic state with a degree of undernutrition due to their high proliferative potency and invasive capacity (22, 23). This assessment has been supported by several studies that have reported hypoxic environments in glioblastomas (16–20, 24). Using these data and a reverse interpretation of the Pasteur effect, it can be inferred that the energy metabolism of glioblastomas under hypoxia would depend on the glycolytic system (25). Angiogenesis elicited by such hypoxia would result in an aerobic environment and the glycolytic system of these tumor cells would be enhanced by the Warburg effect (25). However, irrespective of whether tumor cells are under an aerobic or anaerobic condition, tumor cells require an enormous amount of glucose to meet their energy requirements. Our study indicates that glioblastoma cells up-regulate the GLUT1 transporter to promote glucose uptake in order to meet these high

requirements. The present study also suggests a correlation between GLUT1 expression and MIB-1 labeling indices, suggesting a link between cell proliferation and the transporter. GLUT1 expression was observed in high proliferative glioblastoma cells positive for MIB-1 (Figure 5C).

During our study, GLUT1 expression was evaluated in IDH wild-type glioblastomas using immunohistochemistry (to evaluate protein expression) and RNAscope *in situ* hybridization (to evaluate transcript expression). RNAscope allows single-molecule visualization in individual cells to be achieved through the use of a novel probe design strategy and a hybridization-based signal amplification system that suppresses background noise (26). Previously, a number of studies have reported a link between certain mRNAs and cancer. Among them, Wang et al reported the efficacy of the RNAscope technique for examining invasive breast carcinomas that compared it to real-time quantitative PCR (qPCR) and other US Food and Drug Administration (FDA)-approved methods, including fluorescence *in situ* hybridization (FISH) (27). Both RNAscope and qPCR data matched the FISH analysis in 97.3% of cases, showing that RNAscope is as accurate as qPCR and FISH (27). Importantly, the RNAscope approach can be used with archived FFPE tissues on glass slides and can be visualized either under a standard bright-field microscope (with chromogenic labels) or an epifluorescent microscope. A clear advantages of RNAscope is therefore its ability to visualize localized mRNA expression in samples. In

the current study, GLUT1 protein expression was found to localize to glioblastoma cells and this was supported by our RNAscope analysis of *SLC2A1* expression (Fig. 4). Highly stained samples in the GLUT1 high expression group had similarly high *SLC2A1* scores. Conversely, the GLUT1 low expression group had lower *SLC2A1* scores.

Our study also revealed that GLUT1 expression was mainly localized to perivascular tumor cells. There was also a positive correlation between GLUT1 expression and neoangiogenesis highlighted by CD105 (endoglin) staining. CD105 is a 180 kDa integral membrane glycoprotein that is an accessory component of the transforming growth factor-beta (TGF-β) receptor complex. Applying analysis of CD105 expression to distinguish between normal vessels and malignant neovascularization has previously been reported, supporting our interpretation (28–30). The protein is predominantly expressed on cellular lineages within the vascular system, although it is more highly expressed by proliferating endothelial cells that participate in tumor angiogenesis. This pattern may emerge due to relatively lower expression in the vascular endothelium of normal tissues (31, 32). Markowski et al. also reported that the expression levels of *HIF-1α*, *SLC2A1* (*GLUT1*), and *CD105* were higher in a group of patients with bladder cancer when compared to healthy subjects (30). The validation of the positive correlation of SLC2A1 and VEGF from TCGA database and GLUT1 expression around CD34 positive vessels

suggested GLUT1 was related with tumor neoangiogenesis. Combined, these data indicate that there is a link between GLUT1 and endoglin expression, suggesting that the glycolytic system that emerges during glioblastoma development is accelerated by neoangiogenesis.

In addition, our examination of the localization of GLUT1 within tumor tissues has revealed an association with pseudopalisade formation and microvascular proliferation, both pathological features of glioblastomas. The generation of pseudopalisades, hypercellular zones surrounding necrotic tissue, often results from the migration of glioma cells outwards from hypoxic areas due to vascular occlusion (33). Epithelial-mesenchymal transition (EMT) is the process by which epithelial cells lose cell polarity and adhesiveness and is one of the main mechanisms that exacerbates tumor migration in glioblastomas (31). The zinc finger E-box-binding homeobox (ZEB) proteins, ZEB1 and smad1-interacting protein-1 (also known as ZEB2), are another family of noteworthy transcription factor that are responsible for the mediation of epithelial-mesenchymal transition in numerous types of cancer including glioma (41). Chem et al. detected the expression of ZEB1 and ZEB2 in 91 cases of GBM with immunohistochemistry (42). The percentages of ZEB1 high expression and ZEB2 high expression were 31.9% (29/91) and 41.9% (36/91), respectively. They revealed that high expression of ZEB2 was significantly associated with lower survival rate of GBM patients (P=0.001). Justin V et al. revealed hypoxia-induced mesenchymal shift in

GBM primary material by showing co-localization of GLUT1, ZEB1 and the mesenchymal marker YKL40 in hypoxic regions of the tumor (43). These results suggested that GLUT1 may exist on downstream of ZEB1 pathway, further examination is needed in order to clarify the relationship GLUT1 and ZEB1. In past reports, GLUT1 has been found to associate with lymph node metastasis and is also highly expressed by breast cancer cells (10, 35). These studies also reported that GLUT1 expression contributed to increased tumor viability by promoting glycolysis. The results showing that GLUT1 is expressed in both hypoxic and proliferative area suggested that glioblastoma cells increased their proliferative potency via hypoxic stress.

These studies, in combination with our own analysis showing GLUT1 localization at tumor pseudopalisades, indicate a possible link to EMT. We therefore hypothesize that tumor malignancy in glioblastomas may be increased by GLUT1 through higher migration and invasiveness.

Finally, Kaplan-Meier survival curve analysis revealed that survival in the high GLUT1 expression group was lower than the low expression group. Our multivariate analysis also showed that GLUT1 was an independent predictor for worse prognoses compared to already known prognostic factors, including age and KPS. These data suggest that GLUT1 may act as an independent prognostic factor for glioblastoma due to an accelerated glycolytic system, likely through increased neoangiogenesis and the

Warburg effect. This would subsequently increase migration and invasiveness by promoting EMT. Previously, Phadngam et al. reported that GLUT1 acts downstream of the PI3K-Akt pathway (25) and several studies have suggested a link between GLUT1, HIF1α, and GSK3β, although the exact details remain unclear. For example, Azzalin et al. reported that various inhibitors of GLUT/SLC2A enhance the activity of the chemotherapeutic agents bis-chloroethylnitrosourea (BCNU) and temozolomide against high-grade gliomas (36). Chen et al. also reported that specific blockade of GLUT1 using WZB117 resensitizes breast cancer cells to adriamycin (37). In the present study, GLUT1 inhibition by WZB-117 decreased cell viability in cell lines. There is a great deal of scope for the therapeutic application of GLUT1 inhibitors against glioblastoma, although further experiments whether WZB-117 potentiate temozolomide and/or irradiation are required.

In conclusion, our study has revealed that GLUT1 is a biomarker and predictor for a worse prognostic outcome in patients with glioblastomas. This will assist the development and application of tools and treatment to better target vulnerable patients. However, recent advancements suggest that GLUT1 also has the potential to increase the efficacy of various anticancer agents. Therefore, our study also contributes to this aim by elucidating several important characteristics of GLUT1 expression in glioblastomas.

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Figure Legends

Figure 1.

Immunohistochemical intensity scores of GLUT1 staining in glioblastoma tumor cells. GLUT1 was found to be expressed at the tumor cell membranes in glioblastomas. The staining intensity scores were evaluated according to a four-grade system. (A) Negative cases showed no stain in tumor cells but positive in endothelium for GLUT1 (arrow), whereas tumor cell membranes were stained to varying degrees in positive cases and were divided into grade 1 (B, mild), grade 2 (C, moderate), and grade 3 (D, strong) staining.

Figure 2.

RNAscope *in situ* hybridization showing *SLC2A1* (*GLUT1*) mRNA expression in high GLUT1 expression samples. Tumor cells surrounding areas of necrosis or vessels that had (**A**) >10 dots/cell or (**B**) 10% positive cells with staining clusters (arrows) were assigned a score of 4. Tumor cells with (**C**) >10 dots/cell or (**D**) less than 10% positive cells with clusters (arrows) were given a score of 3.

Figure 3.

Localization of GLUT1 expression in glioblastoma tumor cells. (**A**) Immunohistochemical staining showed that GLUT1 was expressed predominantly perivascular region. GLUT1 staining was found

around vessels (arrow) and formed a mottled pattern. (**B**) GLUT1 was also expressed in pseudopalisades of the tumor cell and (**C**) boundary area.

Figure 4.

(**A**) Number of cases in each localized expression of GLUT1. GLUT1-positive cells were most often observed at the perivascular regions. GLUT1 positive cells were also seen in pseudopalisades and at the boundaries between tumor and normal brain tissue. Cases are overlapping. (**B**) Correlation between GLUT1 expression and angiogenesis. Cases with high GLUT1 expression possessed more CD105-positive vessels (Wilcoxon test $p < 0.001$). In addition, high GLUT1 expression positively correlated with the number of CD105-positive vessels (correlation coefficient: 0.61). (**C**) Correlation between GLUT1 expression and proliferative capacity. The MIB-1 labeling indices of the high GLUT1 expression group were higher than the low expression group (Wilcoxon test $p < 0.001$). In addition, high GLUT1 expression positively correlated with MIB-1 labeling indices (correlation coefficient: 0.51).

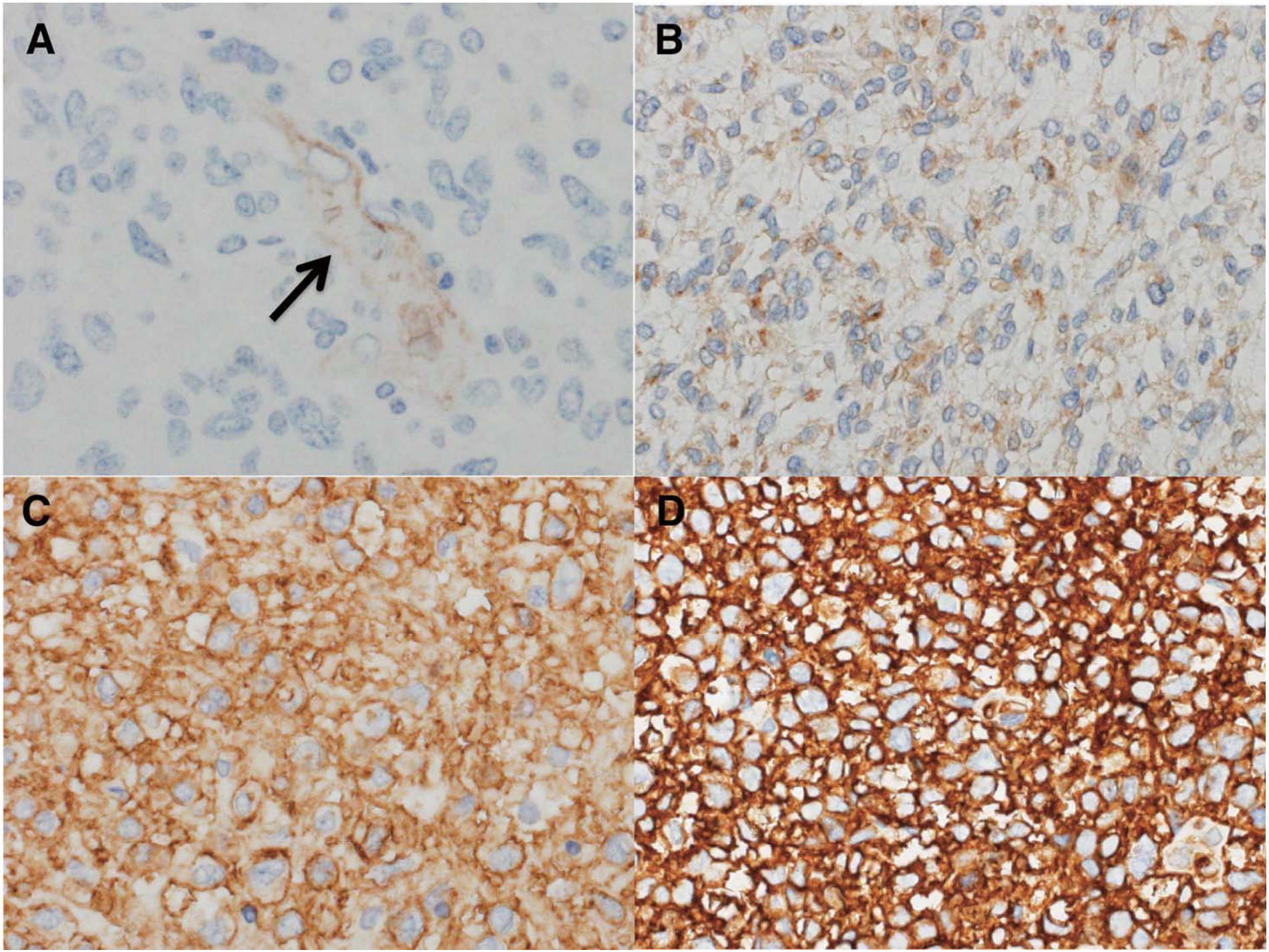
Figure 5.

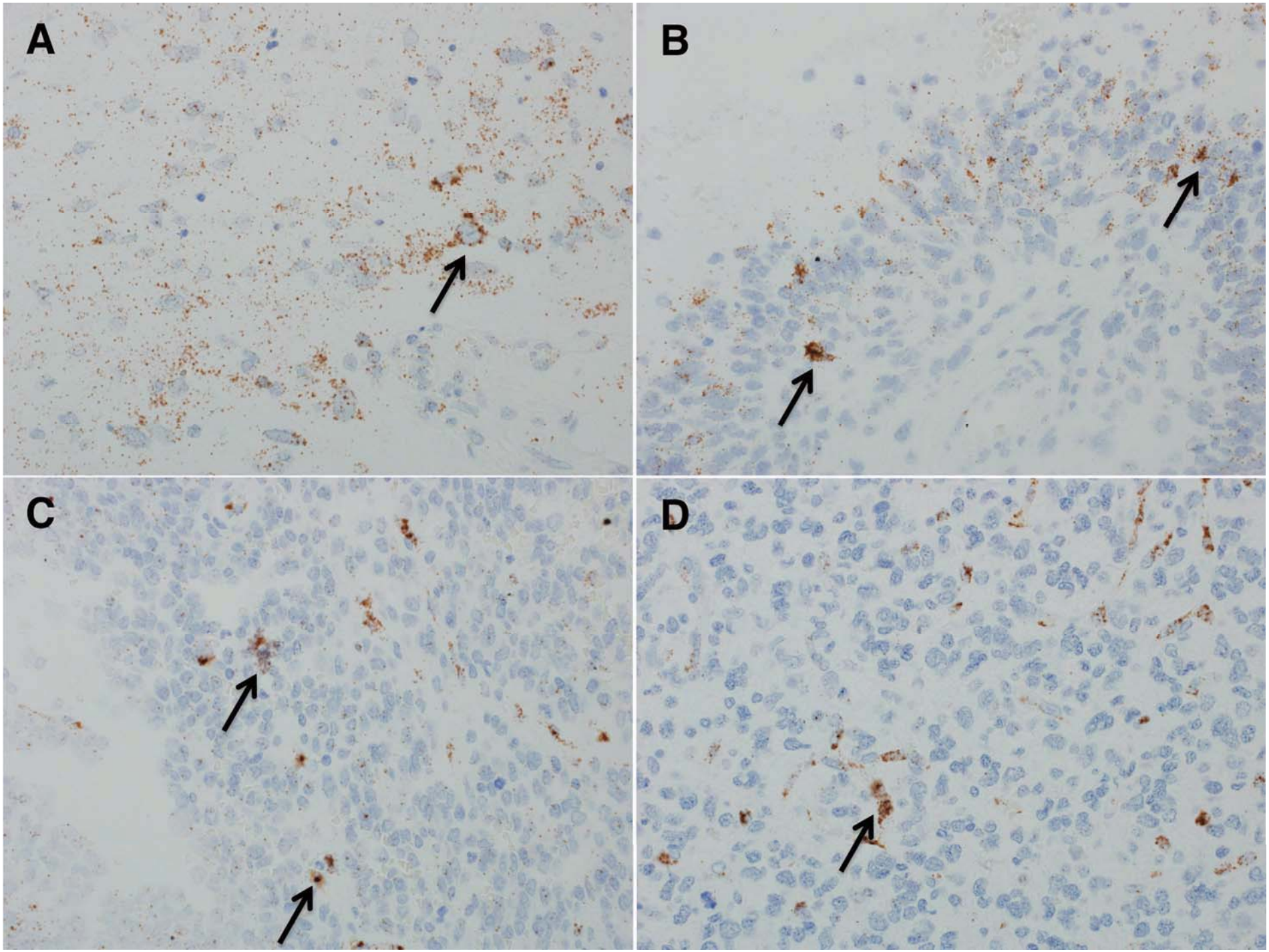
Double immunofluorescent staining of GLUT1 and other markers. (**A**) GLUT1 staining (left) and CD34 staining (center). Merged image (right) showed GLUT1 expression (red) around CD34 (green) positive

vessels. (**B**) GLUT1 staining (left) and HIF1α staining (center). Merged image (right) showed GLUT1 expression (red) around HIF1α (green) positive cells. (**C**) GLUT1 staining (left) and Ki67 staining (center). Merged image (right) showed co-expression of GLUT1 and Ki67 (green) in tumor cells.

Figure 6.

WST-1 cell proliferation assay and comparison of the Kaplan-Meier survival curves by GLUT1 expression. (**A-C**) U87, U251, and T98 cells were incubated in WZB-117 of indicated concentrations for 24 hours. Cell viability decreased in all tested cell lines in various manner. (**D**) Comparison of the Kaplan-Meier survival curves between the high and low GLUT1 expression groups showed that the high GLUT1 expression patient group had lower overall survival rates than the low expression group (log-rank test, $p = 0.001$).


A
B
C
D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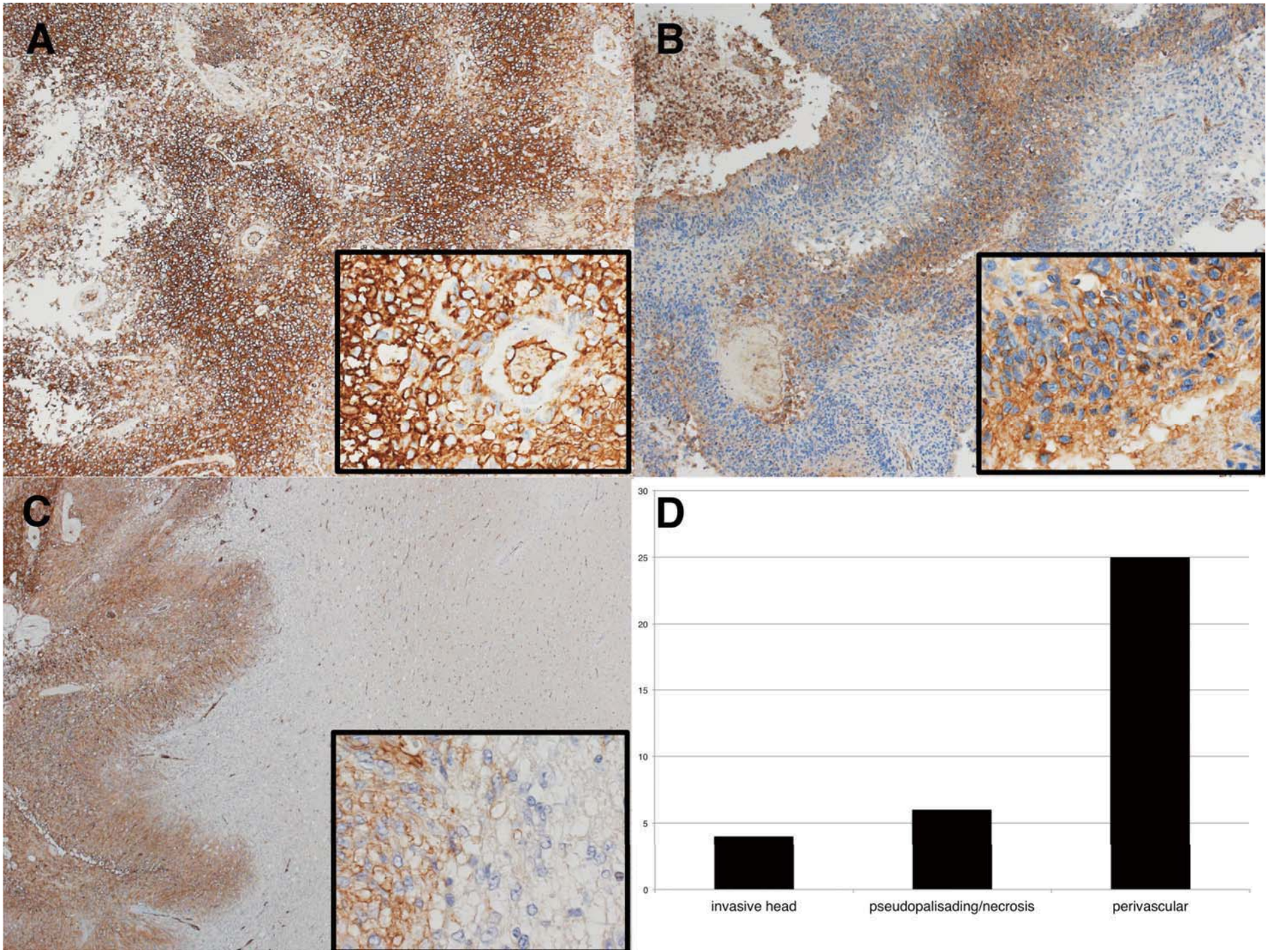
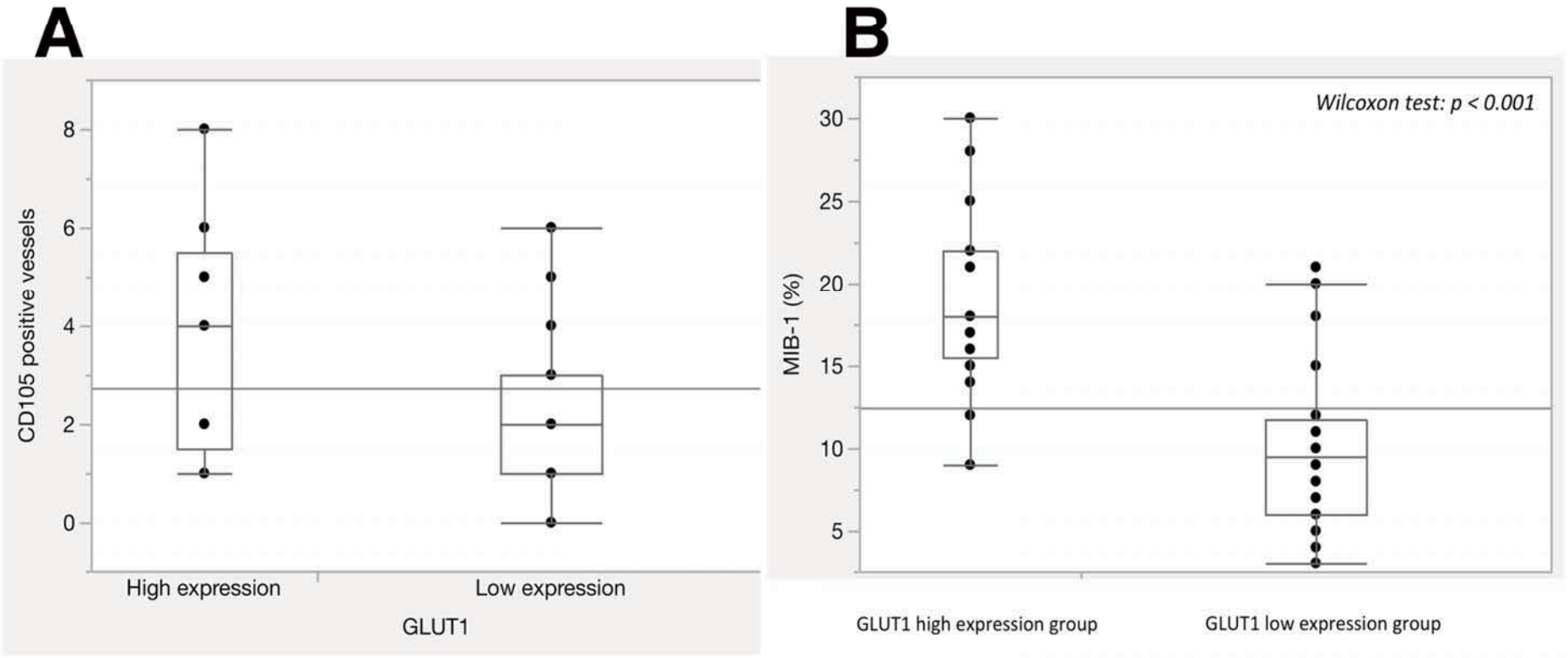
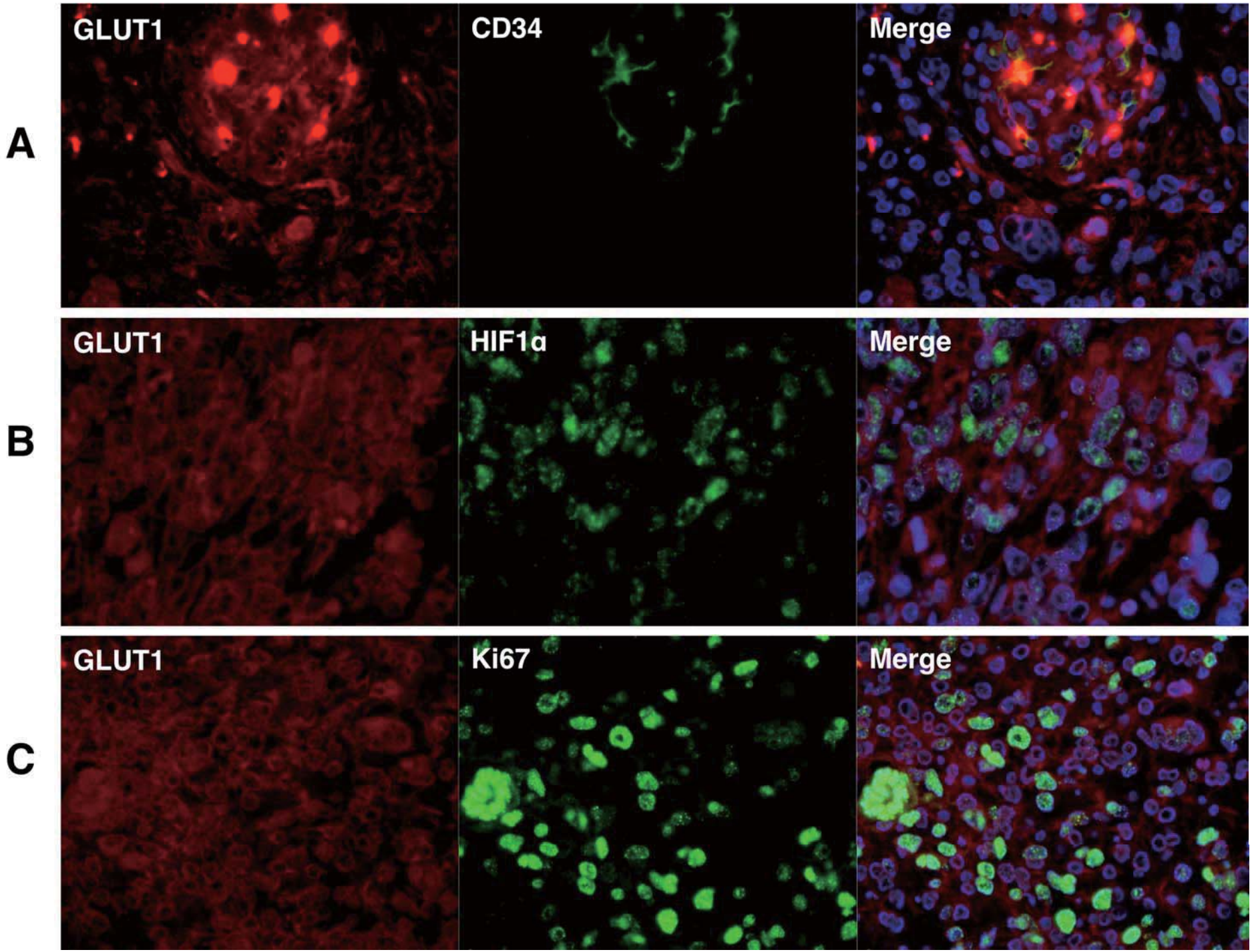
C
D


A
B
C
D
30
25
20
15
10
5
0
invasive head
pseudopalisading/necrosis
perivascular


A
CD105 positive vessels
8
6
4
2
0
High expression
Low expression
GLUT1
B
MIB-1 (%)
30
25
20
15
10
5
Wilcoxon test: p < 0.001
GLUT1 high expression group
GLUT1 low expression group


A
GLUT1
CD34
Merge
B
GLUT1
HIF1α
Merge
C
GLUT1
Ki67
Merge

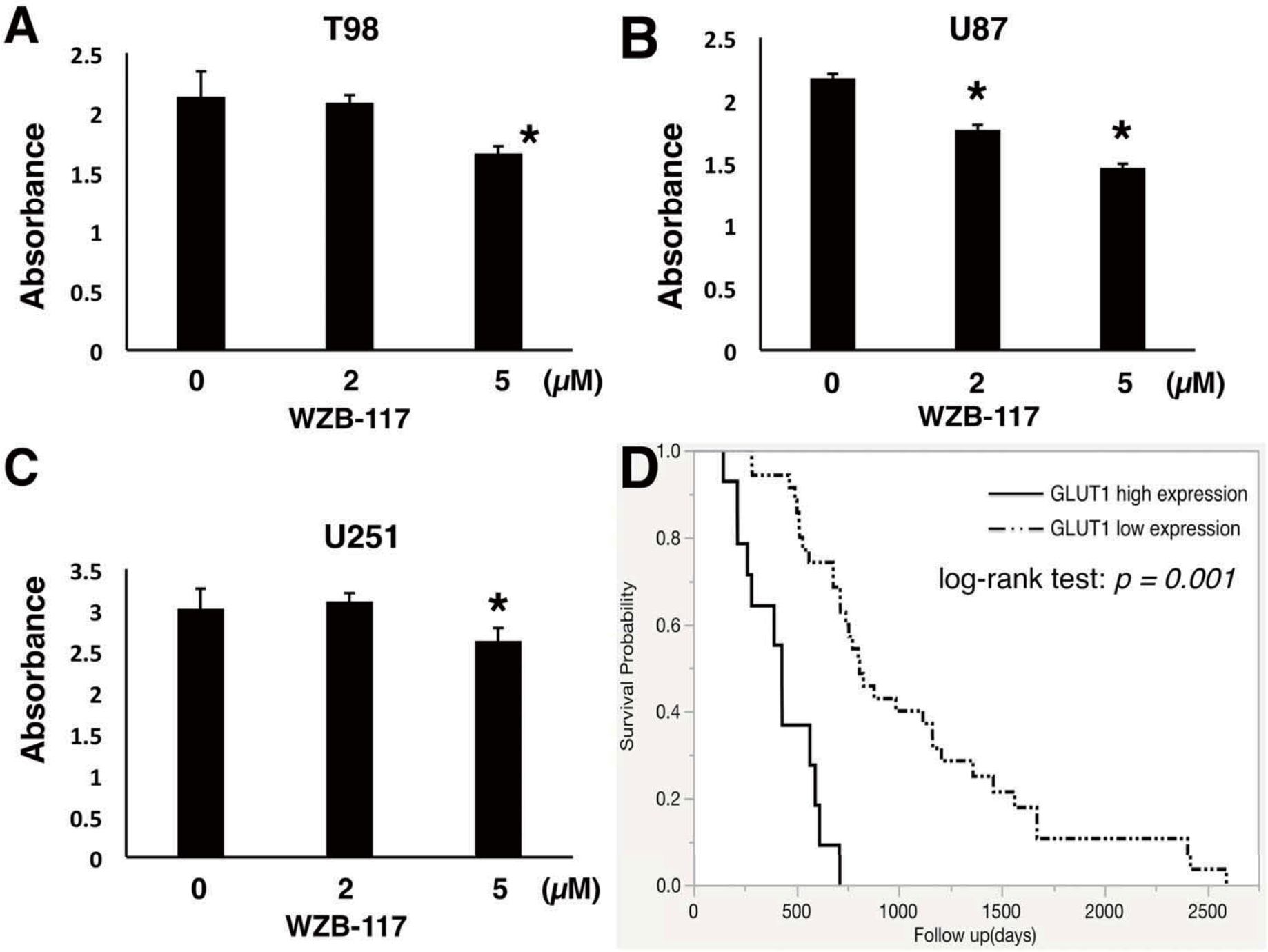

A
T98
Absorbance
2.5
2
1.5
1
0.5
0
*
0
2
5
(μM)
WZB-117
B
U87
Absorbance
2.5
2
1.5
1
0.5
0
*
*
0
2
5
(μM)
WZB-117
C
U251
Absorbance
3.5
3
2.5
2
1.5
1
0.5
0
*
0
2
5
(μM)
WZB-117
D
GLUT1 high expression
GLUT1 low expression
log-rank test: p = 0.001
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
2500
Follow up(days)

Table 1. Primer design and PCR product

Gene	Primer sequence	Product
IDH1	Forward: 5'-GGTTGAGGAGTTCAAGTTGAAACAAAT-3'	244bp
	Reverse: 5'-CACATACAAGTTGGAAATTTCTGGGCC-3'	
IDH2	Forward: 4'-GGGGTTCAAATTCTGGTTGAAAGATGG	289bp
	Reverse: 5'-TAGGCGAGGAGCTCCAGTCGGG-3'	

Table 2. Patients characteristics

Characteristics	n=52
Median age, years (range)	65.9 (33-86)
Sex, n (%)	
Male	34 (65.4%)
Female	18 (34.6%)
Localization, n (%)	
Frontal	21 (40.4%)
Temporal	18 (34.6%)
Parietal	9 (17.3%)
Others	4 (7.7%)
Median KPS at preoperation (range)	74.0 (30-90)
Surgical procedure, n (%)	
Gross total resection	33 (63.4%)
Partial resection	19 (36.6%)

Abbreviations; KPS, Karnofsky Performance Status

Table 3. Univariate and multivariate predictors of overall survival

	Univariate			Multivariate		
Variables	HR	95% CI	p value	HR	95% CI	p value
High expression of GLUT1	7.22	3.05-17.3	<0.0001	5.59	2.22-14.4	0.0003
KPS < 90	1.93	1.01-3.93	0.04	1.54	0.75-3.32	0.23
Age > 50	2.05	0.91-5.53	0.09	1.86	0.75-5.44	0.18
Except Gross total resection	1.13	0.58-2.12	0.69	1.04	0.50-2.06	0.89

Abbreviations; CI, confidence interval; KPS, Karnofsky Performance Status; HR, hazard ratio;

Supplemental data

Material and Methods

Gene expression database

Information regarding SLC2A1 alterations and patient's survival time in glioblastomas was downloaded from the Cancer Genome Atlas (TCGA) Database, an open access database that is publicly available at http:///www.cbioportal.org [38, 39]. Six hundred and four glioblastoma cases (TCGA, Provisional) were selected. Gene altered cases were defined as that expression level of SLC2A was observed greater than standard-deviation from the mean. These cases were divided into two groups: SLC2A1 mRNA up-regulated or not altered. The proportion of up-regulated cases was calculated and overall survival and progression-free survival were estimated by Kaplan-Meier analysis. Furthermore, co-expression genes with SLC2A1 in glioblastomas were investigated by calculating Spearman's correlation coefficients. We selected VEGF as the neoangiogenetic marker, NF1 as the mesenchymal marker, TP53 as proneural marker, TGF-β as the epithelial mesenchymal transformation's marker and HIF1α as the hypoxic marker [40].

Result

Gene expression database

To validate the result of the present study, data from TCGA database regarding GLUT1 in glioblastomas was analyzed. Two hundred and forty nine cases (42%) showed up-regulated SLC2A1 in 591 glioblastomas from TCGA database. The overall survival of up-regulated cases tended to be shorter than the not altered cases (Log-rank test; p=0.375, Supplemental figure 1A), and the progression free survival of up-regulated cases was significantly shorter than the not altered cases (Log-rank test; p=0.01, Supplemental figure 1B). SLC2A1 correlated with VEGF (Spearman's correlation coefficient 0.63, Supplemental figure 2A, TGF-β (Spearman's correlation coefficient 0.35, Supplemental figure 2B) and HIF1α (Spearman's correlation coefficient 0.33, Supplemental figure 2C). Other glioblastoma genes did not relate with SLC2A1. These data suggested that GLUT1 was related with angiogenesis and hypoxia in glioblastoma.

Supplemental Figure Legends

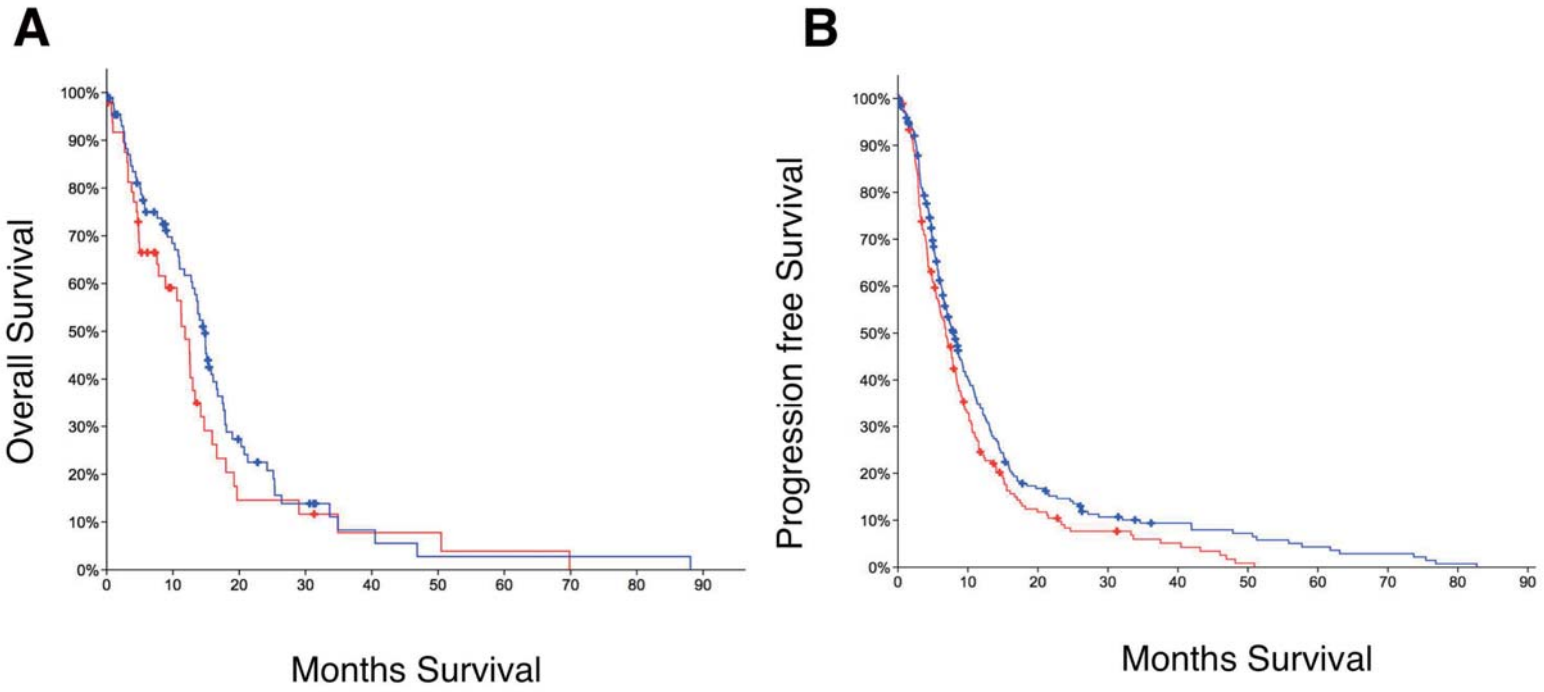
Supplemental Figure 1

Analysis of 591 glioblastomas from TCGA database. The overall survival of up-regulated cases was tended to be shorter than the not altered cases (Log-rank test; p=0.375, Supplemental figure 1A), and the progression free survival of up-regulated cases was significantly shorter than the not altered cases (Log-rank test; p=0.01, Supplemental figure 1B).

Supplemental Figure 2

Co-expression of genes and their correlation with SLC2A1 in glioblastomas from TCGA database. SLC2A1 expression was correlated with VEGF (Spearman's correlation coefficient 0.63, Supplemental figure 2A, TGF-β (Spearman's correlation coefficient 0.35, Supplemental figure 2B) and HIF1A (Spearman's correlation coefficient 0.33, Supplemental figure 2C).

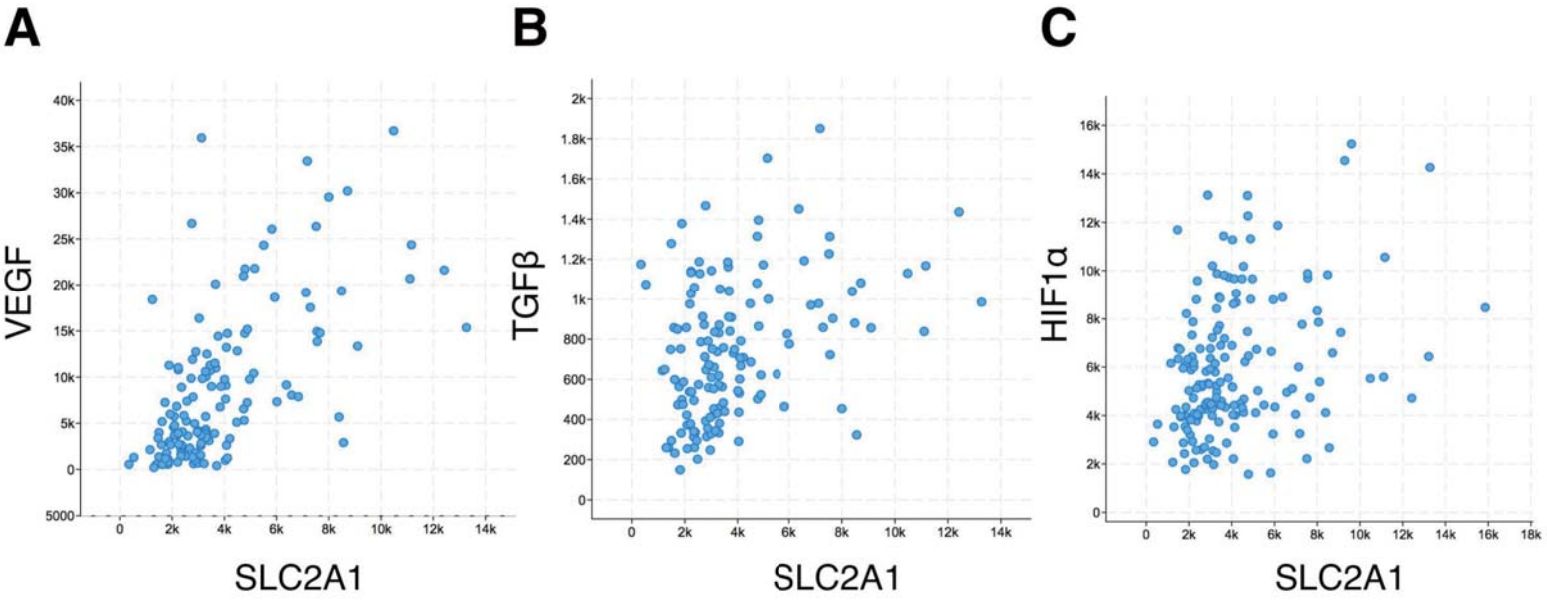
Supplemental figure 1. TCGA data analysis about GLUT1 up-regulated cases and not altered cases in glioblastomas.



A: The overall survival of up-regulated cases was tended to be shorter than not altered cases (Log-rank test; p=0.375).

B: Progression free survival of upregulated cases was significantly shorter than not altered cases (Log-rank test; p=0.01).

Supplemental figure 2. TCGA data analysis about correlation GLUT1 and other markers (VEGF, TGFβ, and HIF1α.



SLC2A1 had positive correlation with (A) VEGF (Correlation Coefficient: 0.62), (B) TGFβ (Correlation Coefficient: 0.35), and (C) HIF1α (Correlation Coefficient: 0.33).