Title: **Endothelin B receptor expression in malignant gliomas: the perivascular immune escape mechanism of gliomas**

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Abstract

In order to clarify the role of endothelin B receptors (ETBRs) in gliomas, we analyzed cell cultures and surgical specimens of gliomas using RT-PCR and immunohistochemistry. RT-PCR measured the absolute expression of ETBR mRNA in twelve samples, which included gliomas that were classified using the World Health Organization (WHO) classification system Grade I to IV, as well as two glioblastoma cell lines (CCF-STTG1 and U87-MG). Using immunohistochemistry, 77 glioma specimens were evaluated for their expression of ETBR and infiltrating T lymphocytes, including an analysis of cytotoxic T cells (CTLs) and regulatory T lymphocytes (Tregs). The number of ETBR-positive vessels in the glioblastomas (Grade IV) was significantly higher than in other grades of gliomas (comparisons to Grade IV, Grade I: $p = 0.0323$, Grade II: $p = 0.0009$, Grade III: $p = 0.0273$). The ETBR expression rate (defined as the number of ETBR-positive blood vessels divided by the total number of blood vessels) in the glioblastomas was higher than the ETBR expression rate in the low-grade gliomas (compared to Grade IV, Grade I: $p = 0.0132$, Grade II: $p = 0.0018$, Grade III: $p = 0.0745$). In addition, the cases which had an ETBR expression rate of 50% or higher exhibited fewer infiltrating CTLs and more infiltrating Tregs compared to the cases with an ETBR expression rate <50% (CTLs: $p = 0.0342$; Tregs: $p = 0.0175$). *Isocitrate dehydrogenase 1* (*IDH-1*) mutations were identified in 21 cases, but there was no correlation between ETBR expression and *IDH-1* mutations for any WHO grade. These results suggest that ETBR expression during neo-angiogenesis may interfere with the homing of CTLs around the tumor and be

involved in the immune escape mechanism of gliomas.

Keywords: Cytotoxic T lymphocyte, Endothelin B receptor, Glioma, Immune escape mechanism

Running title: Endothelin B receptors in gliomas

Introduction

Endothelin (ET) is a vasoexcitor peptide that contributes to the morbidity of cardiovascular disorders. The ET family includes at least 3 peptides (ET-1, ET-2, and ET-3) and 2 receptors (endothelin A receptor: ETAR and endothelin B receptor: ETBR) [1, 2]. These peptides play key roles in various cardiovascular disorders. Recently, ET peptide and receptor expression has been identified in diverse tumor types, such as lung, prostate, ovarian, and colorectal cancer tissues. For example, ETBR expression was found in 7/7 cases of aggressive lung cancer, while ET-1 expression was identified in a case of metastatic colorectal cancer [3-7]. In neoplasms, the expression of ET family members is considered to be a factor in tumor progression. Recently, some investigators have demonstrated that these ET family members are expressed in brain tumors, particularly gliomas [8-10], and ETBRs are known to be expressed in gliomas [11]. This increased ETBR expression is correlated with the degree of malignancy in a variety of tumor types [11].

Kandalaft and Buckanovich *et al.* have also suggested that ETBRs are involved in the immune escape mechanism in human ovarian cancer tissue. In their studies, ETBR overexpression in the endothelium of tumor vessels promoted angiogenesis, suppressed intracellular adhesion molecule 1 (ICAM-1) expression in the endothelial cells, and inhibited T cell transendothelial migration and homing. There was also a correlation between the number of tumor-infiltrating lymphocytes (TILs) and ETBR as well as between ETBR expression on the tumor vessels and patient prognosis [12, 13].

Generally speaking, cytotoxic T cells (CTLs) work offensively for tumor cells, while regulatory T lymphocytes (Tregs) work defensively for tumor cells [14, 15]. The immune activity of CTLs and Tregs in response to the tumor attracts the attention of other immune cells. A previous report regarding central nervous system lymphoma identified a correlation between overall survival and TIL number [16]. Therefore, in our current study, we investigated the immune escape mechanism of gliomas, focusing on the role of ETBR. We sought to determine whether ETBR expression was correlated with both malignancy and the number of cytotoxic immune cells present in a given tumor. Our hypothesis is that ETBR expression and the associated decrease in cytotoxic immune cell presence in the tumor are causally related. In order to define the 'immune escape mechanism of gliomas', it is necessary to demonstrate the presence of ETBR in tumor-created vessels. CD105 (endoglin) is a marker of vascularization, particularly tumor vascularization [17, 18]. Therefore, we examined ETBR expression in tumor-created vasculature using double fluorescent staining for ETBR and CD105.

Methods

Case selection

Primary gliomas that were surgically resected at Kurume University Hospital between September 2000 and April 2011 were selected for this study. Subsequently, all specimens were histologically diagnosed according to the World Health Organization (WHO) criteria for tumors of the central nervous system [19]. More than 10 unlabeled sections with an area greater than 25 mm^2 were generated from paraffin blocks containing the tumors. The controls consisted of 6 brain tissue specimens from autopsy cases without a neurological disease diagnosis.

IDH-1 mutation is a driver mutation found in gliomas [20]. We screened for *IDH-1* mutations in order to classify glioma subtypes.

Total RNA was isolated from an additional 12 frozen sections, including tumors classified as WHO Grade I to IV. These 12 frozen sections were in freezer storage in our department and were unrelated to the 77 paraffin sections used for immunohistochemistry (described above). RNA was also isolated from two glioblastoma cell lines, CCF-STTG1 and U87-MG. For this RT-PCR, the cell lines were used after 2 passages after dissolution. In addition, COS-7 cells were used as a positive control [21], and water was used as a negative control.

This study was carried out in accordance with the principles of the Helsinki Declaration and was

approved by the ethics committee of Kurume University Institution.

Expression of ETBR mRNA

Cells and culture conditions

The U87-MG human glioblastoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and was maintained in DMEM. The CCF-STTG1 human glioblastoma cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and was cultured in RPMI-1640. The culture media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2. These two cell lines can be grown indefinitely. We evaluated the cell lines for mycoplasma infection, and infectious signs were not observed.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from different human glioma tissues using the acid guanidinium phenol chloroform method from Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The RNA samples were quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The cDNA was reverse transcribed from 2.5 μg total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) and was primed with oligo (dT) primers (GE Healthcare). Three microliters of cDNA was used for PCR with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). The primers for ETBR and beta-

actin are listed (Fig. 1a). RT-PCR was performed as described previously [22].

Immunohistochemistry

The surgically resected samples were fixed with buffered 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin or labeled with various antibodies for proteins of interest. All paraffin blocks were cut into 4-μm thick sections on a microtome. To examine the localization of ETBR, CTLs, and Tregs in the glioma tissues, a polyclonal rabbit anti-ETBR antibody (orb5103, Biorbyt, Cambridgeshire, UK), a monoclonal mouse anti-isocitrate dehydrogenase 1 (anti-IDH1) R132H (DIA-H09, Dianova, Hamburg, Germany), a monoclonal mouse anti-T-cell intracytoplasmic antigen (anti-TIA-1; a marker of CTLs) antibody (2G9, Immunotech, Marseille, France), and a monoclonal anti-Foxp3 (a marker of Tregs) antibody (kind gift of Professor Elias Campo, University of Barcelona, Spain) were used. All sections were de-paraffinized in xylene, and endogenous peroxidases were inactivated in 3% hydrogen peroxide for 5 min. The sections were incubated for 4 h at 4°C in a humidified chamber with primary antibodies against ETBR (1:600). The sections from the same cases were incubated with primary antibodies against IDH-1 R132H (1:100, 30 min), TIA-1 (1:400, 30 min), or Foxp3 (1:40, 180 min) at room temperature.

The TIA-1 and Foxp3 immunostaining were performed to investigate whether TIA-1-positive cells and Foxp3-positive cells surrounded the ETBR-positive blood vessels. The other sections were stained with a panel of antibodies, including CD4 (MBL, Tokyo, Japan; 1:30, 30 min), CD8 (Novocastra; 1:50,

30 min), and CD56 (Novocastra; 1:100, 30 min).

Fluorescence immunohistochemical staining for ETBR and CD105 (endoglin)

CD105 (endoglin) is a marker of tumor vascularization. Double fluorescent immunostaining for ETBR and CD105 was performed on 4 glioma samples. The secondary antibodies were anti-mouse IgG for CD105 (Alexa488, Life Technologies, Carlsbad, CA, USA) and anti-rabbit IgG-Texas Red for ETBR (sc-2780, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Assessment of immunoreactivity

Following the hematoxylin and eosin staining, we microscopically analyzed an area that showed the general structure of the tumor without necrosis. Although the tumor cells of gliomas stain for ETBR, this study focused on the tumor vessels and T cells. All blood vessels and ETBR-positive vessels in 10 high-power fields (HPFs) were counted. The diameter of one HPF was 0.5 mm, and the area of one HPF was 0.196 mm^2. The number of ETBR-positive blood vessels over the number of all measured blood vessels was defined as the "ETBR expression rate," and the number of ETBR-positive blood vessels was defined as "ETBR-expressing blood vessels." The numbers of tumor-infiltrating CTLs and Tregs in the same areas were counted similarly. The correlations among the ETBR expression rates, ETBR-expressing vessels, and the number of tumor-infiltrating T lymphocytes were analyzed. The immunohistochemical studies were performed by 2 observers (S.N. and Y.S.) using a fluorescence microscope (BX51FL, OLYMPUS, Tokyo, Japan) and a CCD camera (DP71, OLYMPUS). In each case, we used the average

value of the recordings of these 2 observers.

Statistical analysis

The average ETBR-positive vessel rates and the average numbers of tumor-infiltrating CTLs and Tregs were analyzed with JMP9 software (SAS Institute Inc., Cary, NC, USA). A Student's *t*-test was used to compare the average values. A p-value < 0.05 indicated statistical significance.

Results

RT-PCR detection of ETBR mRNA in gliomas

RT-PCR was performed to measure the absolute expression of ETBR mRNA, which revealed a product of 197 base pairs (bp) in all glioma cell lines (including CCF-STTG1 and U87-MG) as well as 3 cases of WHO Grade I glioma, a case of WHO Grade II glioma, 3 cases of WHO Grade III glioma, and 7 cases of WHO Grade IV glioblastoma. A clear beta-actin product (376 bp), which served as an internal standard, was seen in all cases (Fig. 1b).

ETBR expression on blood vessel endothelium in gliomas

Immunohistochemical staining for ETBR in glioma samples

Specimens were selected from 77 patients who did not receive chemotherapy or radiotherapy before tumor resection. Table 1a shows the WHO classification of the selected specimens. These patients were treated with standard-of-care approaches. The immunohistochemical staining for ETBR was performed

on all 77 glioma samples and 6 normal brain tissue samples. Some blood vessel endothelium in the glioma samples exhibited positive staining on the cellular membrane. However, all 6 normal brain tissue samples were negative for ETBR expression on the blood vessels (Fig. 2a). All observed blood vessels regardless of ETBR expression in the 10 HPFs were counted. The immunohistochemical staining revealed ETBR expression on some blood vessel endothelium. A representative image of positive ETBR staining in blood vessels in each case is shown in Figure 2 (Fig. 2b-e). The average number of ETBR-expressing vessels and ETBR-non-expressing vessels is shown in Table 1b. In the glioblastoma samples, the average number of ETBR-expressing vessels in the 10 HPFs was 8.03 (range: 0-19). The average number of ETBR-expressing blood vessels was 5.19 (range: 0-23) in WHO Grade III samples, 2.75 (range: 0-7) in WHO Grade II samples, and 4.13 (range: 0-9) in WHO Grade I samples (Table 1b). Significantly more ETBR-expressing blood vessels were seen in Grade IV samples compared to the other grades (compared to Grade IV, Grade I: $p = 0.0323$, Grade II: $p = 0.0009$, Grade III: $p = 0.0273$) (Table 1b). Although we observed no significant difference between Grade IV and Grade III samples regarding their ETBR expression rate (ETBR-expressing blood vessels/total blood vessels) in the 10 HPFs, the ETBR expression rate was significantly higher in the Grade IV samples than in the Grade I and Grade II samples (compared to Grade IV, Grade I: $p = 0.0132$, Grade II: $p = 0.0018$, Grade III: $p = 0.0745$) (Table 1c). When the samples were combined into either a low-grade glioma (LGG) group (Grade I and II samples) or a high-grade glioma (HGG) group (Grade III and IV samples), both the number of ETBR-

expressing blood vessels and the ETBR expression rate were significantly higher in the HGG group than in the LGG group (number of ETBR-expressing blood vessels: $p = 0.0036$, ETBR expression rate: $p = 0.0011$).

Comparison of tumor-infiltrating CTLs and ETBR expression

The double immunohistochemical staining for ETBR and TIA-1 in the 6 glioma samples (3 glioblastomas and 3 anaplastic astrocytomas) showed little CTL infiltration around ETBR-expressing blood vessels. However, these lymphocytes did infiltrate around non-ETBR-expressing blood vessels (Fig. 2f, g).

CTLs were seen in 68 of the 77 cases (average CTL counts: 13.8/10 HPFs, range: 0-83). When ETBR expression increased, the number of tumor-infiltrating CTLs decreased ($p = 0.0068$) (Fig. 3a). Because the median ETBR expression rate was 50.00%, we divided the cases into 2 groups, consisting of a high ETBR expression rate group (50% or more) and a low ETBR expression rate group (less than 50%). The number of the tumor-infiltrating CTLs in the high ETBR expression rate group was significantly lower than the low ETBR expression rate group ($p = 0.0342$) (Fig. 3b).

In 36 of the 77 cases, Tregs were not seen in the 10 HPFs (Tregs = 0/10 HPFs). Thus, our study did not reveal a significant correlation between the ETBR expression rate in gliomas and tumor-infiltrating Tregs ($p = 0.1524$) (average Treg counts: 5.09/10 HPFs, range: 0-85). However, the number of tumor-infiltrating Tregs was lower in the low ETBR expression rate group than in the high ETBR expression

rate group ($p = 0.0175$) (Fig. 3d).

We immunolabeled the 6 samples with the most TIA-1-positive cells with the typical natural killer (NK) cell marker CD56, the CTL and NK cell marker CD8, and CD4, a marker for immune cells such as T helper cells, monocytes, and macrophages. Within 4 of the 10 HPFs, CD56-positive cells were found within the tumors, whereas the number of CD8-positive cells was approximately the same as the number of TIA-1-positive cells. Thus, the TIA-1-positive cells were mostly CTLs. CD4 is a marker of macrophages or astrocytes, and CD4-staining was not observed in lymphocytic small cells (Fig. 2h).

IDH-1 mutation and its correlation with WHO grades

IDH-1 mutations were observed in 22 gliomas. None of the WHO Grade I gliomas was mutated (0%), whereas 9 of the 12 cases of WHO Grade II were mutated (75%). As shown in Table 1a, 10 of the 21 cases of WHO Grade III were mutated (47.6%), and 2 of the 36 cases of WHO Grade IV were mutated (5.6%). The average ETBR expression rate of mutated *IDH-1* HGG was 47.6%, and the average ETBR expression rate in non-mutated *IDH-1* (wild type) HGG was 53.9% ($p = 0.23$). There was no correlation between *IDH-1* mutation and the ETBR expression rates according to each WHO grade ($p > 0.05$).

Fluorescence immunohistochemical staining for ETBR and CD105 (endoglin)

All ETBR-positive blood vessels were CD105-positive, but not all CD105-positive blood vessels expressed ETBR (Fig. 4).

Discussion

The ET system is comprised of a strong vasoconstrictor peptide and its receptor [1, 2]. In addition, a link has been suggested between the ET system and pathological conditions, such as hypertension, arteriosclerosis, and cerebrovascular spasm after subarachnoid hemorrhage [1, 2, 23, 24]. Recently, ET-1 and ETBR expression has been reported in various organ tumors, and ET-1 and ETBR are thought to be involved in tumor growth and progression [3-7]. ETBR may mediate the immune escape mechanism in gliomas, but the exact mechanism is unclear. Therefore, these studies focused on the relationship between the tumor vessels and T cells.

The levels of ETBR expression in the vascular endothelium vary greatly, depending on the anatomical site of the blood vessel. In particular, ETBR expression has been reported in the brain, lung, heart, kidney, and adrenal glands [24]. However, the constitutive ETBR expression is not high; rather, ETBR expression is induced under particular conditions, such as low tissue perfusion states during renal disorders, myocardial ischemia, shock, and cerebrovascular disorders [25, 26]. Although no clear ETBR-expressing blood vessels in normal brain tissue samples were observed, we identified multiple ETBR-expressing blood vessels in the glioma tissue samples. Thus, the pathological state of gliomas may induce ETBR expression. Therefore, double fluorescent labeling of ETBR and endoglin (CD105), a marker of tumor neovascularization, was performed. Although endoglin is a vascular endothelial marker, this protein is expressed primarily in newly forming vessels [17, 18]. ETBR was only expressed in the

CD105-expressing blood vessels (tumor neovascularization), whereas ETBR was not expressed in blood vessels that did not express CD105 (non-tumor neovascularization). In previous reports, endoglin was characterized as a prognostic factor for gliomas [27, 28]. In addition, Sugita et al. demonstrated the relationship of endoglin between ETBR in primary CNS lymphoma (PCNSL) [18, 29]. Our result is consistent with these reports, and suggests that the immune escape mechanisms of glioma may develop as the gliomas infiltrate and destroy the brain parenchyma.

Because the bands of lanes 5-7, 13, 14 were thinner compared to other lanes, the RT-PCR was repeated 10 times, with reproducible results. The thinner band lanes may have been due to the variability in the patient samples, including differences in necrotic tissue and/or tissue cellularity.

Because there was larger variation in the CTL number, correlations between ETBR and tumor infiltrating CTLs were examined with continuous and discrete variables. The significant correlation of ETBR expression rate and the number of infiltrating CTLs was seen in the analysis of both the continuous variables ($p=0.0068$) and the discrete variables ($p=0.0004$). Therefore, ETBR is associated with the biological behavior of gliomas.

The TIA-1 protein is generally expressed by CTLs and NK cells [30]. Because an anti-TIA-1 antibody was used to assess CTLs in this study, it is possible that NK cells were also immunostained. Therefore, we labeled the 6 samples with the most TIA-1-positive cells with CD56 (the prototypical NK cell marker), CD8 (a CTL and NK cell marker), and CD4 (a marker for immune cells such as T helper

cells, monocytes, and macrophages). Very few CD4-positive and CD56-positive cells were found within the tumors, whereas the number of CD8-positive cells was approximately the same as the number of TIA-1-positive cells. Thus, TIA-1-positive cells were mostly CTLs.

Buckanovich *et al.* demonstrated that the ETBR expression in the vascular endothelium of human ovarian cancer tissue reduces the number of TILs [13]. In addition, ICAM-1 is expressed in the vascular endothelium and is involved in the adhesion of activated T cells to the vascular endothelium [31]. In the present study, we showed a correlation between high ETBR expression and reduced levels of tumor-infiltrating CTLs in the glioma samples. Double fluorescent labeling of ETBR and CTLs also demonstrated that CTLs tended to not adhere to the endothelium of ETBR-expressing blood vessels. Thus, the ETBR expression in glioma vascular endothelium correlated with glioma malignancy, suggesting ETBR expression may act to suppress the host's immune response against the glioma.

Treg cell differentiation is induced by Foxp3, suppressing the immune response [14, 15]. In recent years, Tregs have gained attention for their ability to suppress the immune response in cancer [32]. An investigation of Tregs and life expectancy in glioma patients found that although the Treg numbers are low, they are involved in tumor enlargement and shrinkage [33]. In the present study, we showed a relationship between ETBR expression in the vascular endothelium of gliomas and tumor-infiltrating Tregs. Because the number of Tregs is intrinsically low in many cases, our interpretation of this result may be controversial. Furthermore, Tregs elicit their immunosuppressive function by releasing

suppressive cytokines, and therefore, a low number of tumor-infiltrating Tregs does not exclude the possibility of an immunosuppressive effect. Kutlu *et al.* suggested that Tregs may function differently in cancer than during normal immune surveillance [34].

Although gliomas can express various factors, the correlation of ETBR expressing blood vessels and TILs was in the primary focus of this study. A correlation between ETBR expression and patient prognosis could not be done because this was a retrospective study, where many patients were not followed up. ETBR and/or TILs may not be an independent prognostic factor for gliomas, such as IDH-1[20]. Other previous reports suggest another immune escape mechanism exists in tumors [35, 36]. ETBR expression, which gliomas may use as an immune escape mechanism, is a microenvironmental change. Immunotherapy is currently one of the most promising types of therapy and is the subject of many clinical studies [36-37]. The main aim of these therapies is to maintain a high activation of immune cells. Conversely, few studies have focused on the blood vessels through which immune cells must pass when homing in on tumors. Kandalaft *et al.* has suggested that ETBR is a “new target” in cancer immune therapy [12]. In recent years, the blood vessel endothelium of glioblastomas has been reported to develop from stem-like cells and has different functions compared to normal vessels [38]. Our study also suggests that ETBR expression similarly suppresses T cell homing to tumors in human glioma tissue. The concomitant use of ETBR inhibition with certain cancer immunotherapies may lead to more effective treatments for gliomas in the future.

Disclosure and acknowledgement

We thank Professor Elias Campo and his department members for providing antibodies. The authors declare no conflicts of interest. This work was supported by JSPS KAKENHI Grant Number 24500427. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For this type of study, formal consent was not required.

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Figure Legends

Fig. 1 RT-PCR detection of ETBR mRNA in gliomas

(a) The PCR primers were as follows: for ETBR (*), 5′-TCT CCC TTA TTA TCC ACT GCT AAT G-3′ (sense) and 5′-TAA CTA TAG CCA CTT TAG GCA ACC A-3′ (antisense); and for beta-actin, 5′-TCG TGA TGG ACT CCG GTG AC-3′ (sense), 5′-TCG TGG ATG CCA CAG GAC TC-3′ (antisense). The PCR product sizes were 197 base pairs (bp) for ETBR and 376 bp for beta-actin.

(b) RT-PCR analysis of ETBR expression, showing a PCR product of 197 bp in all cases.

Lane 1, positive control, COS-7; lane 2, the glioblastoma cell line, CCF-STTG1; lane 3, the glioblastoma cell line, U87-MG; lanes 4-6, pilocytic astrocytoma (WHO Grade I) tissues; lane 7, oligoastrocytoma (WHO Grade II) tissue; lanes 8-10, anaplastic astrocytoma (WHO Grade III) tissues; lanes 11-15, glioblastoma (WHO Grade IV) tissues; lane 16, negative control, water. Beta-actin was used as an internal standard. A clear beta-actin product (376 bp) is seen for all cases used in this experiment.

Fig. 2 Immunohistochemical staining of normal and glioma samples for ETBR and lymphocytes

(a-e) Representative immunohistochemical staining image of ETBR-expressing blood vessels in normal and glioma sample tissues. Scale bars = 20 μm. (a) ETBR immunohistochemical staining of normal brain tissue. ETBR was not expressed on endothelial cells. (b) Pilocytic astrocytoma tissue. ETBR was expressed on endothelial cells and on 31.9% of all observed vessels. (c) Diffuse astrocytoma tissue. ETBR was expressed on endothelial cells and on 29.7% of all observed vessels. (d) Anaplastic astrocytoma tissue. ETBR was expressed on endothelial cells and on 45.9% of all observed vessels. (e) Glioblastoma tissue. ETBR was expressed on endothelial cells and on 56.5% of all observed vessels.

(f, g) Double immunohistochemical staining for ETBR and TIA-1 in glioma samples. ETBR immunostaining was visualized with diaminobenzidine (brown), and TIA-1 immunostaining was visualized with vulcan fast red (red). (f) A representative image of CTLs that did not infiltrate into the tumor through ETBR-expressing blood vessels. (g) A representative image of CTLs that infiltrated into

the tumor through non-ETBR-expressing blood vessels.

Scale bars = 20 μm. HPF = high-power field.

(h) The number of CD8-positive cells was approximately the same as the number of TIA-1-positive cells. Very few CD56-positive lymphocytes were found within the tumors. CD4 labels macrophages and astrocytes, and does not label lymphocytic small cells. These 4 photos were taken at approximately the same location in the same case of anaplastic astrocytoma.

Fig. 3 Comparison of tumor-infiltrating T lymphocytes and ETBR expression

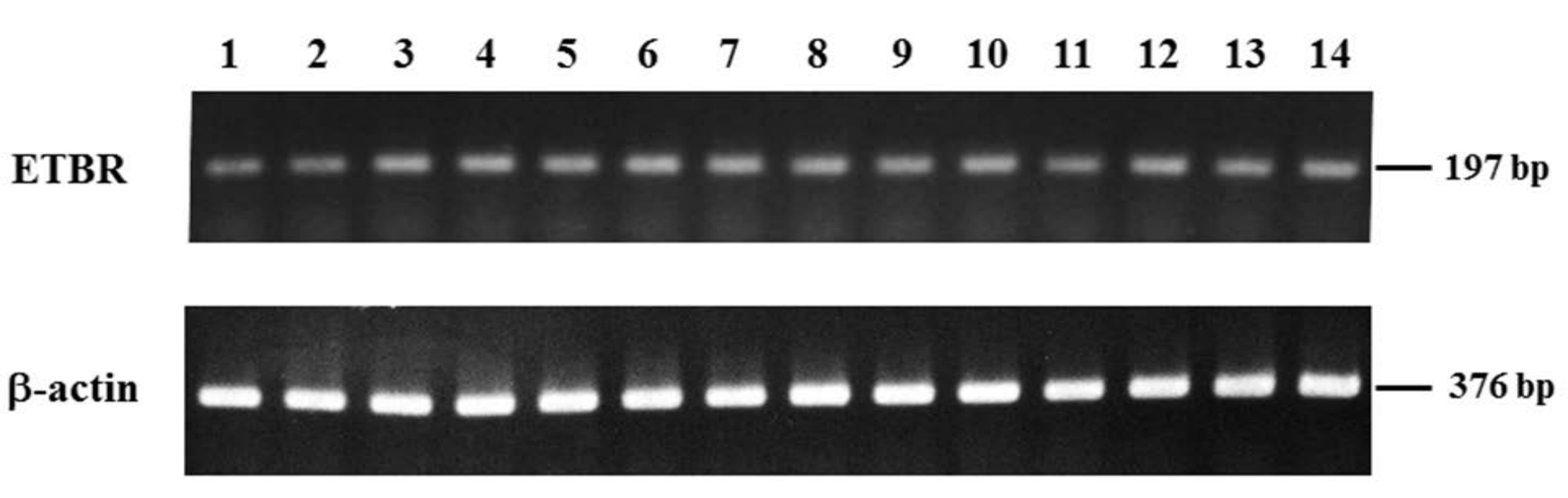
(a-d) Comparison of ETBR-expressing blood vessels of gliomas and tumor-infiltrating CTLs and Tregs. Comparison of the ETBR expression rate of gliomas and tumor-infiltrating cytotoxic T lymphocytes. (a) A comparison of the number of tumor-infiltrating CTLs and the ETBR expression rate suggested that fewer CTLs infiltrated the tumor as the ETBR expression increased ($p = 0.0068$). (b) Compared with cases with an ETBR expression rate of less than 50%, the number of tumor-infiltrating CTLs was lower in cases with an ETBR expression rate of 50% or more ($p = 0.0342$). (c) A comparison of the number of tumor-infiltrating Tregs and the ETBR expression rate did not reveal a relationship between these two factors ($p = 0.1524$). (d) Compared with cases with an ETBR expression rate of less than 50%, the number of tumor-infiltrating Tregs was higher in cases with an ETBR expression rate of 50% or more ($p = 0.0175$).

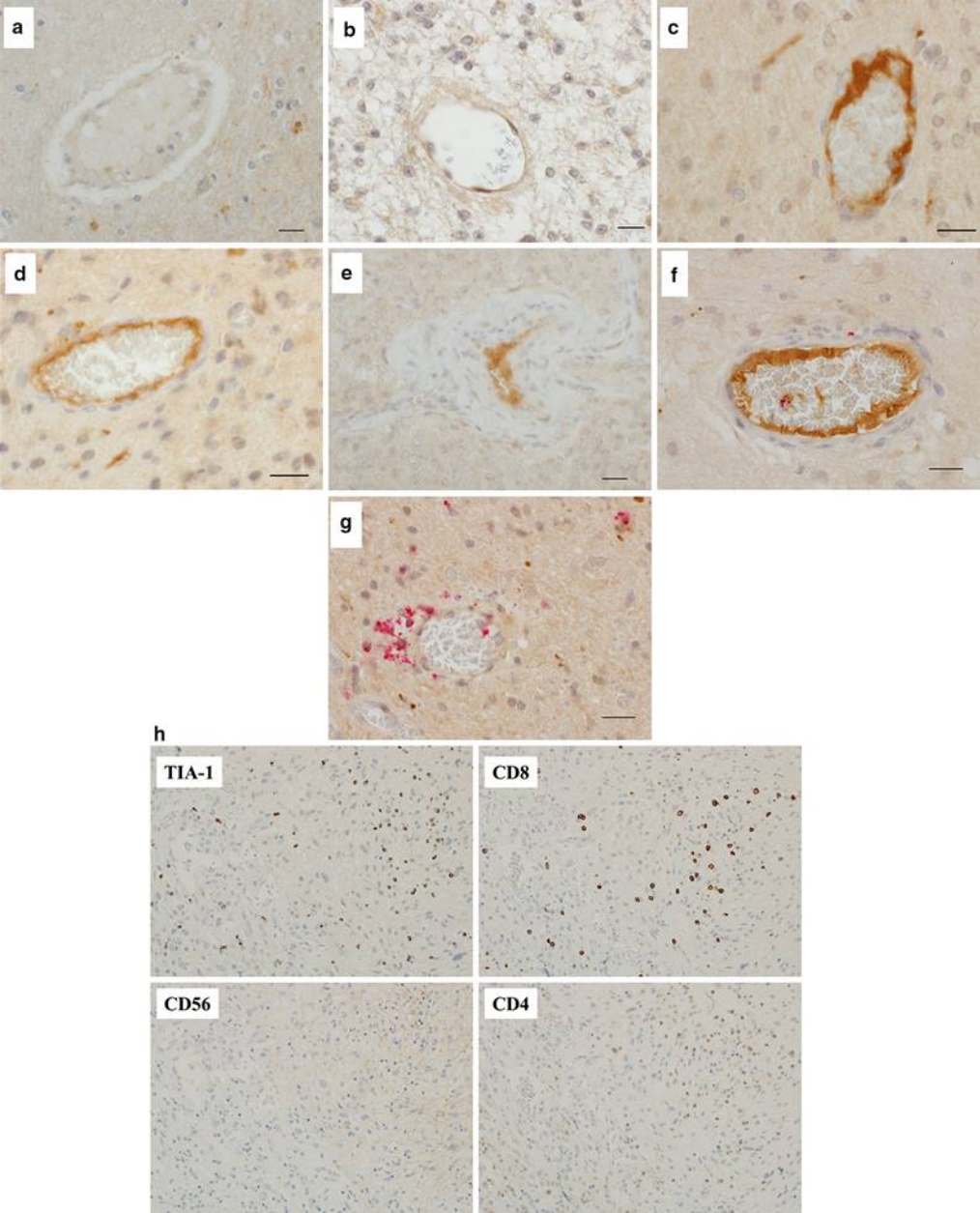
HPF = high-power field.

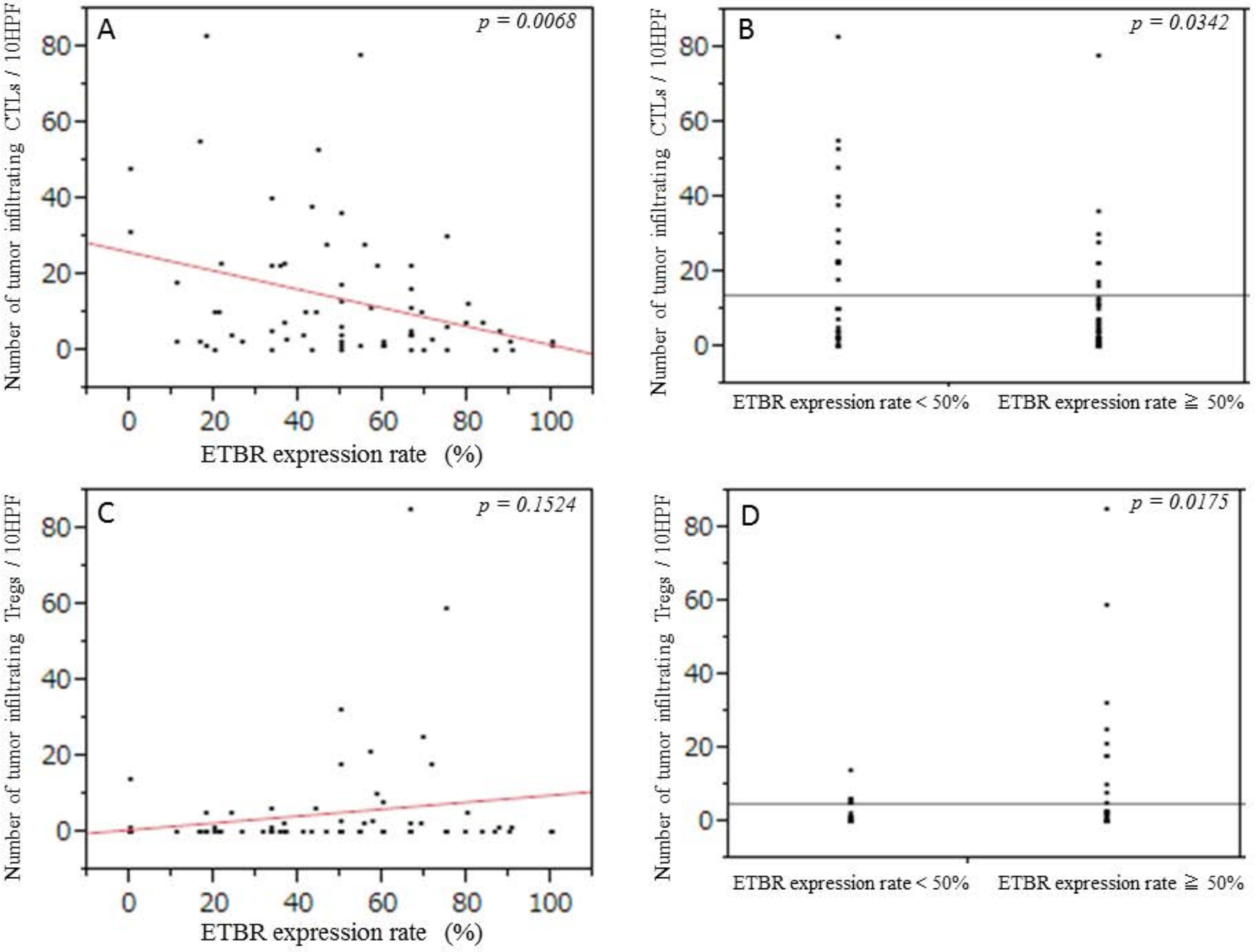
Fig. 4 Fluorescent double immunostaining of ETBR and CD105 (endoglin)

Fluorescent double immunostaining of ETBR (green; Alexa488-conjugated secondary antibody) and CD105 (red; Texas Red-conjugated secondary antibody). (A) A representative image showing ETBR expression in CD105-positive blood vessels. (B) A representative image showing that ETBR was not expressed in CD105-negative blood vessels. Far right panels are merged images, which show ETBR, CD105, and DAPI staining.

Scale bars = 20 μm.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
ETBR
197 bp
β-actin
376 bp


a
b
c
d
e
f
g
h
TIA-1
CD8
CD56
CD4


A
p = 0.0068
Number of tumor infiltrating CTLs / 10HPF
ETBR expression rate (%)
B
p = 0.0342
Number of tumor infiltrating CTLs / 10HPF
ETBR expression rate < 50%
ETBR expression rate ≧ 50%
C
p = 0.1524
Number of tumor infiltrating Tregs / 10HPF
ETBR expression rate (%)
D
p = 0.0175
Number of tumor infiltrating Tregs / 10HPF
ETBR expression rate < 50%
ETBR expression rate ≧ 50%

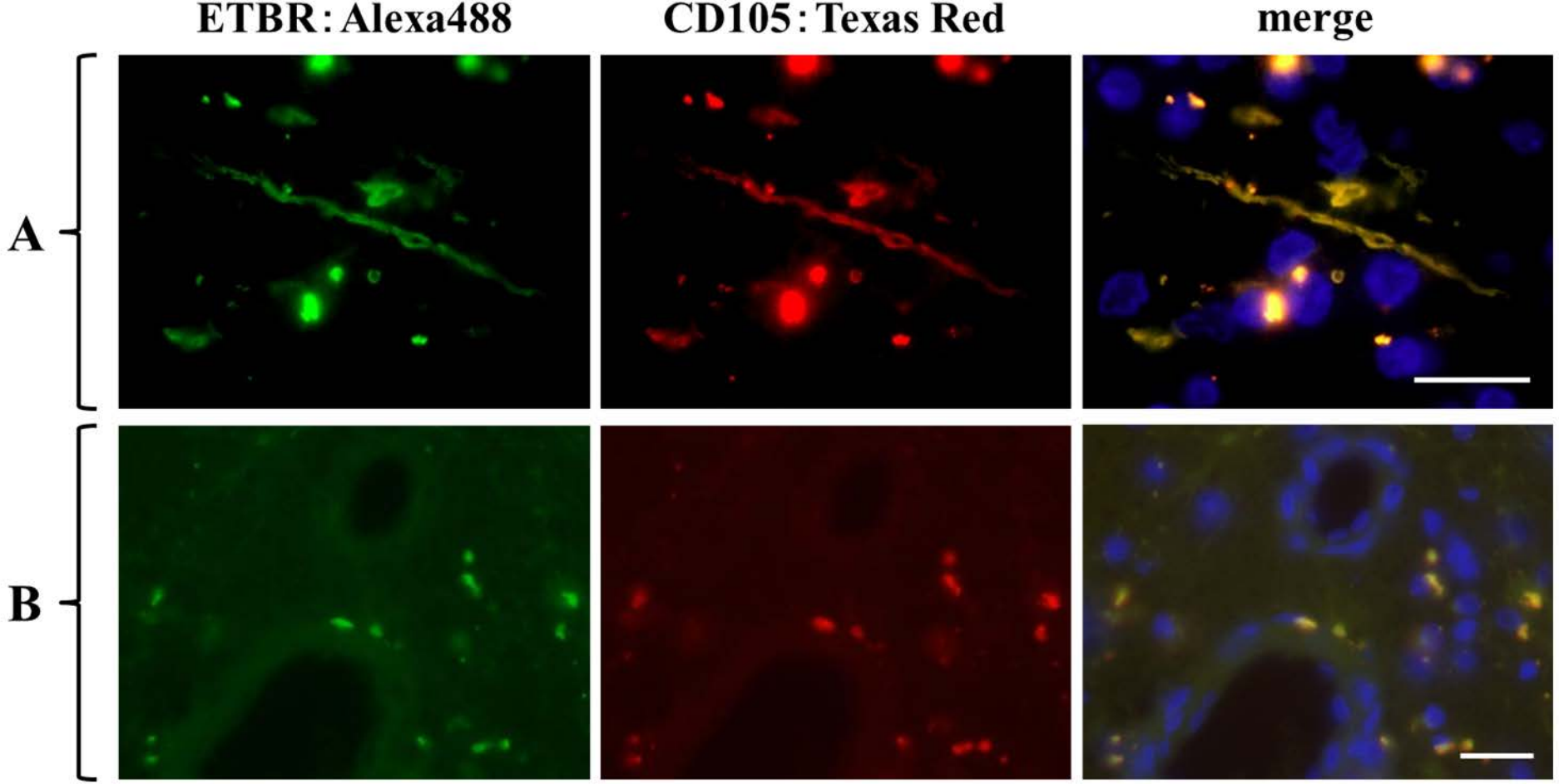

ETBR : Alexa488
CD105 : Texas Red
merge
A
B

Table1a. Brain tumor series analyzed in this study

WHO grading	Histological diagnosis	Number of cases (No. of IDH-1 mut)
Grade I	Pilocytic astrocytoma	7 (0)
	Ganglioglioma	1 (0)
Grade II	Diffuse astrocytoma	4 (2)
	Oligodendroglioma	4 (3)
	Oligoastrocytoma	4 (4)
Grade III	Anaplastic astrocytoma	8 (2)
	Anaplastic oligodendroglioma	11 (7)
	Anaplastic oligoastrocytoma	2 (1)
Grade IV	Glioblastoma	36 (2)
		Total 77 (21)

Table 1b. The number of ETBR-expressing blood vessels

WHO grading	Average number of ETBR-expressing and non-expressing vessels		counts of expressing vessels p value (compared to Grade IV)
	expressing vessels	non-expressing vessels	
Grade IV (n=36)	8.03 (range: 0-19)	6.40 (range: 0-19)	
Grade I (n=8)	4.13 (range: 0-9)	6.14 (range: 0-22)	p=0.0323
Grade II (n=12)	2.75 (range: 0-7)	7.40 (range: 1-25)	p=0.0009
Grade III (n=21)	5.19 (range: 0-23)	8.85 (range: 4-14)	p=0.0273

Table 1c. ETBR expression rate of gliomas

WHO grading	Average number of ETBR expression rate	p value (compared to Grade IV)
Grade IV (n=36)	56.5 (range: 0-100)	
Grade I (n=8)	31.9 (range: 0-54.5)	p=0.0132
Grade II (n=12)	29.7 (range: 0-75.0)	p=0.0018
Grade III (n=21)	45.9 (range: 0-100)	p=0.0745

ETBR epression rate: ETBR-positive vessels / all measured vessels