Correlation of high delta-like ligand 4 expression with peritumoral brain edema and its prediction of poor prognosis in patients with primary high-grade gliomas

Xian-xin Qiu, MD,^{1,2} Chen-hong Wang, MD,¹ Zhi-xiong Lin, MD,^{1,2} Na You, MD,¹ Xing-fu Wang, MD,³ Yu-peng Chen, MD,³ Long Chen, MD,¹ Shui-yuan Liu, MD,¹ and De-zhi Kang, MD, PhD²

¹Tumor Invasion Micro-ecological Laboratory, Fujian Medical University; and Departments of ²Neurosurgery and ³Pathology, The First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, China

OBJECT Peritumoral brain edema (PTBE) is a common phenomenon associated with high-grade gliomas (HGGs). In this study, the authors investigated the expression of Notch delta-like ligand 4 (DLL4) and its correlation with PTBE and prognosis in patients with an HGG.

METHODS Tumors from 99 patients with HGG were analyzed for DLL4 expression using immunohistochemistry. PTBE on preoperative MR images and the relationship between PTBE and DLL4 expression were evaluated. The effect of DLL4 on patient prognosis was assessed by using Kaplan-Meier survival and Cox proportional hazard models.

RESULTS Immunohistochemistry results revealed that the expression of DLL4 was distributed primarily within the cytoplasm of tumor vascular endothelial cells and seldom detected in tumor cells. DLL4 expression was correlated positively with the degree of edema ($r = 0.845$ and $p < 0.001$, Spearman's test). In addition, DLL4 was an independent predictor of prognosis in patients with HGGs ($p = 0.001$).

CONCLUSIONS DLL4 expression was correlated positively with the degree of PTBE and was an independent unfavorable prognostic indicator in patients with HGG.

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KEY WORDS peritumoral brain edema; high-grade glioma; Notch delta-like ligand 4

HIGH-GRADE glioma (HGG) is the most common and most lethal primary malignant brain tumor in adults, with a median overall survival (OS) of 1–2 years and a 5-year survival rate of 5%.^{28,43} Peritumoral brain edema (PTBE) in patients with HGG is a frequent and characteristic feature and may be fatal because of its neurological sequelae.

PTBE, at initial diagnosis, has been found by multivariate analysis to be an independent prognostic indicator for glioblastoma.^{33,37} In general, PTBE is associated with an incomplete blood-tumor barrier and vasogenic cerebral edema.^{22,42} However, the exact mechanism through which the pretreatment of PTBE leads to worsening clinical out-

come is unclear but is likely to be multifactorial in origin.⁴²

Vascular endothelial growth factor (VEGF), an essential angiogenic regulator and a strong inducer of vascular permeability, has been proposed as a major factor in the formation of PTBE associated with HGG.^{4,8,29} The inhibition of VEGF markedly reduces edema in patients with HGG.³² Hypoxia can stimulate VEGF secretion through the activation of hypoxia-inducible transcription factors.^{11,17,20} In addition, many other signaling pathways are involved in the modulation of VEGF secretion.^{16,18}

Several studies have found that delta-like ligand 4 (DLL4), 1 of the 5 known transmembranous Notch ligands, participates in tumor angiogenesis and cross-talk

ABBREVIATIONS DLL4 = delta-like ligand 4; HGG = high-grade glioma; HR = hazard ratio; MGMT = O⁶-methylguanine-DNA methyltransferase; OS = overall survival; PBS = phosphate-buffered saline; PI = positive index; PTBE = peritumoral brain edema; T2WI = T2-weighted image; TTP = time to progression; VEGF = vascular endothelial growth factor.

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with the VEGF pathway, thereby regulating tumor neovascularization.^{19,23,31,41} In breast cancer, colon cancer, and HGG, DLL4 expression is correlated positively with VEGF.^{14,15} Moreover, El Hindy et al.⁹ found that a higher level of DLL4 mRNA was potentially related to a higher degree of PTBE in patients with glioblastoma. However, whether DLL4 upregulation is more pronounced in patients with HGG with more severe edema has not been established. Moreover, the effect of DLL4 on the outcome of patients with HGG is unclear.

The purpose of this study was to determine the pattern of DLL4 expression in various HGG tissues and to establish whether PTBE levels correlate with DLL4 expression in patients with HGG. In addition, survival times in defined subpopulations of patients (as they relate to the level of DLL4 expression) were investigated to determine whether DLL4 has prognostic potential.

Methods

Patients and Tumor Tissues

Informed consent was obtained from each patient according to the research proposals approved by the local ethics committee of the Fujian Medical University.

A total of 99 patients with primary HGG were selected from consecutive patients who underwent tumor resection at the First Affiliated Hospital of Fujian Medical University between 2006 and 2012. Patient demographics and characteristics are shown in Table 1.

Eligibility criteria included written informed consent and the availability of tumor tissue, preoperative and postoperative MRI, and follow-up data. All specimens were obtained from resection and classified according to the World Health Organization (WHO) classification of brain tumors.²⁴

Clinical information was obtained by review of the medical records detailing radiographic results, by telephone or written correspondence, and by review of death certificates. Follow-up information for all the patients was collected every 2 months by telephone calls or questionnaire letters, last obtained in March 2014.

Treatment and Clinical Outcome Assessment

Extensive resection was performed after diagnosis, and adjuvant therapy (radiotherapy and chemotherapy) was administered to every enrolled patient. No patient received

any experimental antitumor vaccination or any steroids before the first diagnosis of edema. Postoperatively, every patient received radiotherapy to limited fields (2 Gy per fraction, once per day, 5 days/week, 60-Gy total dose) and adjuvant temozolomide (150–200 mg/m² of body surface area on Days 1–5) given at 4-week intervals. The duration of adjuvant cycles of temozolomide was individualized to each patient depending on his or her tumor response and clinical status.

Follow-up for all the patients in this study was performed in accordance with a strict protocol. Postoperatively, each patient was observed at 3-month intervals during the 1st year and at 6-month intervals thereafter. A patient was considered to have tumor progression if the lesion was revealed by imaging studies. “Pseudoprogressive” lesions were not included in the analysis.³⁶ For deceased patients, the underlying cause of death was classified according to data available on their death certificate, and deaths not related to the tumor were excluded from survival analysis.

The end point of the study was OS, which was measured from the day of surgery (equivalent to the day of diagnosis) until death of the patient. Data on survival beyond the end of the observational period (last follow-up visit) were considered censored observations.

Measurement of Preoperative PTBE on MRI

All preoperative MRI scans, acquired at initial diagnosis, were obtained at the First Affiliated Hospital of Fujian Medical University (1.5-T scanner; slice thickness 5 mm). Spin-echo MRI sequences included axial and sagittal T1-weighted images (with and without contrast enhancement) and axial T2-weighted images (T2WIs). PTBE was defined as a region of increased T2 signal intensity at the tumor margin. Accordingly, preoperative PTBE in patients with HGG was measured on the first diagnostic MRI scan and classified using the following simple criteria: edema that extended < 1 cm from the tumor margin was defined as minor, and edema that extended > 1 cm from the tumor margin was defined as major³⁷ (Fig. 1); the morphological classification of PTBE was determined according to the method of Hartmann et al.¹⁰ (i.e., as ring [the shape of an abnormal high signal was similar to a circle on the T2WIs, without sprawling in all directions] or irregular [the shape of an abnormal high signal tended to appear irregular on the T2WIs]) (Fig. 1).

TABLE 1. Characteristics of all 99 patients with HGG

WHO Grade & Histology Result	Sex (M/F)	Median Age in Yrs (range)	PTBE Grade (minor/major)	Median TTP in Mos (range)	Median Survival in Mos (range)	Alive at LO (n [%])
Grade III	16:17	47.0 (19–75)	18/15	16.0 (6–44)	32.0 (12–73)	11 (33.3)
AA	9:3	48.8 (19–67)	7/5	15.0 (9–43)	23.0 (15–54)	5 (41.7)
AO	5:8	47.2 (26–75)	6/7	25.5 (8–43)	34.5 (12–43)	3 (23.1)
AOA	2:6	42.8 (25–56)	5/3	19.0 (6–44)	35.5 (28–73)	3 (37.5)
Grade IV	34:32	54.8 (18–79)	17/49	6.0 (1–39)	10.0 (4–52)	8 (12.1)
GB	32:29	54.8 (18–79)	17/44	6.5 (2–39)	10.0 (4–52)	7 (11.5)
GS	2:3	61.2 (47–73)	0/5	5.5 (1–8)	10.0 (4–11)	1 (20.0)

AA = anaplastic astrocytoma; AO = anaplastic oligodendroglioma; AOA = anaplastic oligoastrocytoma; GB = glioblastoma; GS = gliosarcoma; LO = last observation; TTP = time to progression.

FIG. 1. Measurement of PTBE on preoperative T2WIs. The images show PTBE extending < 1 cm from the tumor margin, defined as minor edema (A), and extending > 1 cm from the tumor margin, defined as major edema (B). We classified the morphology of PTBE as ring (C) or irregular (D).

Immunohistochemistry

Tumor specimens were fixed in 10% formaldehyde and embedded in paraffin for histological sectioning. Paraffin-embedded sections were deparaffinized in xylene and dehydrated in graded alcohol. Immunohistochemistry studies for the DLL4 protein were performed using a rabbit monoclonal antibody (1:200) (ab7280, Abcam). In addition, we screened for other molecular markers, including Ki-67 (1:100) (clone Mib1; Dako), *O*⁶-methylguanine-DNA methyltransferase (MGMT) (1:50) (Santa Cruz Biotechnology, Inc.), and p53 (1:100) (clone M7001; Dako). Antigen retrieval was performed in citrate buffer at pH 6.0. The sections were incubated overnight at 4°C with the primary antibody. They then were rinsed with phosphate-buffered saline (PBS) and incubated with the horseradish peroxidase-conjugated secondary antibody, followed by a rinse in PBS, incubation with 3,3'-diaminobenzidine staining, and counterstaining with hematoxylin blue. The

negative control sections were incubated with PBS in concentrations equal to those of the primary antibody.

Evaluation of Immunohistochemical Staining

Two pathologists, blinded to the pathological diagnoses and clinical data, observed the immunohistochemical staining results. The number of positive endothelial or tumor cells was counted using a light microscope at a magnification of ×200. In each tumor specimen, 5 fields were examined. The expression was classified as low if < 10% of the cells stained positive; otherwise, the expression was classified as high.²¹ The positive index (PI) was defined as the percentage of immunopositive cells (ratio of positively stained cells to the total number of cells per slide, multiplied by 100). Ki-67 expression was scored as a percentage by counting the immunostained nuclei in 400 cells in the most positive area.³⁵ For MGMT scoring, a count of < 25% stained tumor cells was defined as low expression, and a count of ≥ 25% was defined as high expression.²⁶ Tumors were considered p53 deficient if the immunoreaction stained the nuclei of ≥ 10% of cells.^{5,35}

Statistical Analysis

All data were analyzed by using SPSS 19.0 software. Associations between DLL4 expression and categorical variables were analyzed by using the chi-square test or Spearman's rank correlation (*r*) analysis, as appropriate. Survival curves were constructed using the Kaplan-Meier method, and survival differences were evaluated by the log-rank test. Cox proportional hazard modeling of factors potentially related to survival and tumor progression were performed to identify which factors had significant influence. Differences with a *p* value of ≤ 0.05 were considered statistically significant.

Results

DLL4 Expression in HGGs

Expression of DLL4 protein was assessed by immunohistochemistry in a panel of 99 HGGs. Immunohistochemical staining showed that controlled staining without primary antibody was negative and that DLL4 expression was observed primarily in the cytoplasm of endothelial cells of glomeruloid and nonglomeruloid blood vessels within the HGGs (Fig. 2). In all endothelial subpopulations, the distribution of DLL4 expression was similar in glomeruloid vascular proliferation (*n* = 61 cases) and in nonglomeruloid endothelia (both individually distributed [*n* = 62 cases] and in clusters [*n* = 58 cases]). It is interesting to note that positive cytoplasmic staining of DLL4 was seldom detected in tumor cells (*n* = 11 cases).

Several patterns of DLL4 expression by tumor cells, including perivascular tumor cells (*n* = 8 cases), tumor cells adjacent to necrosis (*n* = 9 cases), and scattered tumor cells (*n* = 11 cases), were noted (Fig. 2). The high DLL4 expression rate in Grade IV gliomas (74.2% [49 of 66]) was higher than that in Grade III gliomas (48.5% [16 of 33]), but there were no significant differences within the same pathological grade (Table 2).

FIG. 2. Cellular expression of the DLL4 protein in HGG specimens. Positive staining of DLL4 is distributed primarily in the cytoplasm of tumor endothelial cells: single nonglomeruloid vessels (white arrows) (A), nonglomeruloid vessels in a cluster (red arrows) (B), and glomeruloid vessels (yellow arrows) (C). Positive cytoplasmic staining of DLL4 is seldom detected in some tumor cells: adjacent necrotic (black arrows) (D), perivascular (orange arrows) (E), and scattered tumor (green arrows) (F) cells. Immunohistochemistry, magnification $\times 400$. Figure is available in color online only.

Correlation of DLL4 Expression With Edema

As shown in the example in Fig. 1, the morphology, degree, and maximum extent of PTBE were assessed in the preoperative MR images of all 99 of the patients. The MR images of 35 (35.4%) patients showed minor edema, whereas major edema was seen in 64 (64.6%) patients. The mean maximum extent of PTBE in all the MR images was 18.2 ± 11.9 mm. Regarding edema morphology, there were 30 cases of the ring-shaped edema pattern and 69 cases of irregularly shaped edema.

The correlation between DLL4 expression and PTBE was also analyzed. The median PI of the DLL4 protein in major edema was 23.5 (95% CI 21.3–25.9), which is higher than the PI of 7.2 (95% CI 5.9–8.5) found in minor edema ($p < 0.001$; Fig. 3 left). The maximum extent of PTBE in HGGs increased with increasing DLL4 expression ($r = 0.845$ and $p < 0.001$, Spearman's test; Fig. 3 right).

The distribution of edema characteristics in the various DLL4 expression subgroups is listed in Table 3. There

was a weak correlation between edema morphology and DLL4 expression ($r = 0.280$; $p = 0.005$). The mean maximum extent of edema in the low-DLL4-expression group (23.5 ± 11.1 mm) was significantly higher than that in the high-DLL4-expression group (7.4 ± 3.8 mm; $p < 0.001$). In addition, the degree of PTBE detected by preoperative MRI was associated positively with DLL4 expression ($r = 0.845$; $p < 0.001$).

Effect of DLL4 Expression on Prognosis

Follow-up information was available for all of the patients. During the follow-up period, 80 patients (80.8%) with HGG had died. Of those 80 deaths, 78 were tumor related and 2 were not tumor related.

The time to progression (TTP) was measured from the date of surgery until the date of tumor progression as revealed by imaging studies. Kaplan-Meier curves showed that the median TTP for patients with high DLL4 expression was 7.0 months (95% CI 5.7–8.3), which is significantly shorter than the 13.0 months (95% CI 10.5–15.5)

TABLE 2. Frequencies of DLL4 immunohistochemistry/WHO grades of different cell types in the 99 HGGs

Cell Type	No. of Patients (%)					Total
	AA/III	AO/III	AOA/III	GB/IV	GS/IV	
Endothelial	5 (5.1)	6 (6.1)	5 (5.1)	45 (45.5)	5 (5.1)	66 (66.7)
Single nonglomeruloid vessel	5 (5.1)	6 (6.1)	3 (3.0)	42 (42.4)	5 (5.1)	61 (61.6)
Nonglomeruloid vessel in cluster	4 (4.0)	5 (5.1)	3 (3.0)	42 (42.4)	4 (4.0)	62 (62.6)
Glomeruloid vessel	5 (5.1)	5 (5.1)	3 (3.0)	41 (41.4)	4 (4.0)	58 (58.6)
Tumor	2 (2.0)	3 (3.0)	2 (2.0)	2 (2.0)	2 (2.0)	11 (11.1)
Perivascular	2 (2.0)	1 (1.0)	2 (2.0)	2 (2.0)	1 (1.0)	8 (8.1)
Adjacent necrosis	2 (2.0)	2 (2.0)	2 (2.0)	2 (2.0)	1 (1.0)	9 (9.1)
Scatter tumor cells	2 (2.0)	3 (3.0)	2 (2.0)	2 (2.0)	2 (2.0)	11 (11.1)

FIG. 3. Correlation between DLL4 and PTBE in patients with HGG. **Left:** The median PI of DLL4 protein in major edema is 23.5 (95% CI 21.3–25.9), which is higher than that in minor edema (7.2 [95% CI 5.9–8.5]; $p < 0.001$). **Right:** The maximum extent of PTBE in patients with HGG increases with increased DLL4 expression ($r = 0.845$ and $p < 0.001$, Spearman's test).

shown in those patients with low DLL4 expression ($p = 0.001$; Fig. 4).

Kaplan-Meier curves were used to assess tumor-related survival in patients with HGG. The 2 deaths not related to the tumor were excluded from analysis. Patients with high DLL4 expression had a median OS of 10.0 months (95% CI 8.6–11.4) compared with 17.0 months (95% CI 13.2–20.8) for those with low DLL4 expression (Fig. 4; $p = 0.001$).

The Cox multivariable proportional hazard model was also used to determine the independent prognostic significance of DLL4 expression. In addition to clinical characteristics, many other molecular markers, including Ki-67, MGMT, and p53, were included in the multivariable analysis to improve interpretation of the results. The expression levels of Ki-67, MGMT, and p53 are shown in Fig. 5. These results confirm that high DLL4 expression (survival hazard ratio [HR] 2.83 [95% CI 1.53–5.78]; $p = 0.001$) was a predictor of shorter TTP independent of age, sex, WHO grade, degree of PTBE, and levels of Ki-67, MGMT, and p53 expression. Similar results were obtained for OS and DLL4 expression (HR 3.51; 95% CI 1.78–6.49; $p = 0.001$; Table 4).

Discussion

Our data confirm that DLL4 expression is correlated positively with PTBE in patients with HGG, suggesting that DLL4 is a potential regulator of edema. To our knowledge, our study results provide the first direct evidence that DLL4 is an independent prognostic factor in HGG. By using an immunohistochemical technique, we assessed the pattern and level of DLL4 protein expression in patients with HGG. We found that positive staining of DLL4 was distributed primarily within the cytoplasm of tumor vascular endothelial cells and was seldom detected in HGG tumor cells. The high DLL4 expression rate in Grade IV gliomas was higher than that in Grade III gliomas. We also analyzed the correlation of DLL4 expression with PTBE. Our results indicate that the degree of edema is correlated positively with DLL4 expression.

HGG, characterized by severe PTBE and high neovascularization, is the most common primary cancer of the central nervous system. Despite advances in diagnosis and

treatment, the prognosis for patients with HGG is still very poor.²⁷ In those with HGG, perilesional edema can cause severe neurological signs and symptoms and high morbidity and mortality rates. However, the molecular mechanisms underlying the formation of PTBE are complex and multifactorial. Tumor angiogenesis is a requisite process for acquiring an adequate blood supply for tumor growth and progression, and it has been implicated in the development of perilesional edema by highly permeable tumor vessels and increased levels of angiogenic factors.

VEGF is a pivotal molecular mediator of PTBE in patients with glioma,^{4,38} and antiangiogenic therapy targeting the VEGF pathway dramatically reduces the degree of PTBE.¹³ VEGF interacts with many pathways as part of a complex network, including Notch, hypoxia-inducible factor, and aquaporin 4 (AQP4), to modulate angiogenesis and glioma-related edema. However, to date, *VEGF* and Notch *DLL4* are the only 2 genes that have been implicated in vascular defects.¹² In addition, a positive relationship between DLL4 and VEGF was detected in human HGG.²¹

The correlation between the greater degree of edema and the higher rate of DLL4 expression may be attributed to elevated VEGF levels and improved perfusion of tumor vessels in gliomas.⁹ The vessels within the tumor have

TABLE 3. Correlation of DLL4 expression with the degree of PTBE in the 99 HGGs

Edema Characteristics	DLL4 Expression (PI range 1.1–43.9)		p Value
	Low	High	
Morphology (no. of patients)			0.005*
Ring	16	14	
Irregular	17	52	
Maximum extent (mm)			<0.001†
Mean	7.4	23.5	
SD	3.8	11.1	
Degree of edema (no. of patients)			<0.001*
Minor	27	8	
Major	6	58	

* Spearman's test.

† Kruskal-Wallis test.

FIG. 4. The effect of DLL4 expression on survival and TTP in patients with HGG. Kaplan-Meier survival plots show a correlation of DLL4 expression with survival (**left**) and TTP (**right**), respectively. Figure is available in color online only.

increased permeability associated with disruption of the blood-brain barrier, which results in vasogenic cerebral edema.³⁴ This explanation is in line with the reduction in the degree of PTBE seen with antiangiogenic therapy.^{13,32}

Furthermore, our study results indicate that the outcome in patients with a high level of DLL4 is worse than that in patients with low DLL4 expression. A Cox multivariable proportional hazard model revealed that DLL4 was a predictor of tumor progression and survival independent of age, sex, WHO grade, degree of PTBE, and levels of Ki-67, MGMT, and p53 expression. Ki-67, MGMT, and p53 are commonly used in clinical molecular pathology, and it is not surprising that our results confirm the prognostic value of these 3 markers.^{1–3,7,25,30,40} The mechanisms by which DLL4 affects the outcome in patients with HGG may be multiple: 1) as noted above, the role of DLL4

in tumor angiogenesis is related, in part, to perilesional edema and may influence tumor cell growth;⁴⁴ 2) DLL4 plays a vital role in the maintenance of glioma stem cells, which have generally been considered the initiating cells of glioma, and the knockdown of DLL4 decreases glioma stem cell population in vitro and inhibits cocultured glioma neurosphere propagation in vivo;⁴⁴ and 3) the Notch pathway interacts with several signaling molecules and pathways, including Ras, p53, and epidermal growth factor receptor, each of which is important in tumor initiation and development.^{6,39}

Our study had several limitations. In this preliminary clinical observation, we did not establish whether DLL4 was the cause of edema or a response to other molecules that are active in precipitating edema. Moreover, the study was also limited by its study population and its retrospec-

FIG. 5. The expression of Ki-67, MGMT, and p53 in patients with HGG. **A:** Ki-67, low expression (< 30%; the median value of 30.3% was the cutoff). **B:** MGMT, low expression (< 25%). **C:** p53, low expression (< 10%). **D:** Ki-67, high expression (≥ 30%). **E:** MGMT, high expression (≥ 25%). **F:** p53, high expression (≥ 10%, defined as p53 deficient). Immunohistochemistry, magnification ×100. Figure is available in color online only.

TABLE 4. Cox proportional hazard regressions for survival and TTP in patients with HGG

Variable*	Survival		TTP	
	HR (95% CI)	p Value	HR	p Value
Sex	1.08 (0.84–1.43)	0.611	1.03 (0.78–1.23)	0.581
Age	1.73 (1.02–2.91)	0.026	1.48 (1.00–2.34)	0.054
DLL4 expression	3.51 (1.78–6.49)	0.001	2.83 (1.53–5.78)	0.001
Degree of PTBE	1.98 (1.12–3.17)	0.012	1.84 (1.12–3.11)	0.018
WHO grade	3.29 (1.23–6.01)	0.004	2.68 (1.14–5.29)	0.008
Ki-67 expression	1.08 (1.00–1.23)	0.008	1.09 (1.00–1.25)	0.012
MGMT expression	1.35 (0.89–2.85)	0.025	1.33 (0.83–2.81)	0.032
p53 expression	1.42 (0.85–2.98)	0.014	1.39 (0.81–2.76)	0.038

* The variables were compared in the following ways: sex, male versus female; age, ≥ 60 years versus < 60 years; DLL4 expression, high versus low; degree of PTBE, major versus minor; WHO grade, IV versus III; Ki-67 expression, $\geq 30\%$ versus $< 30\%$ (the median value of 30.3% was the cutoff); MGMT expression, high versus low; p53 expression, $\geq 10\%$ (p53 deficient) versus $< 10\%$.

tive nature. Thus, additional studies concerning the interaction between DLL4 and other molecular events in PTBE are needed to develop an efficient strategy for treatment.

Conclusions

This study showed that positive staining of DLL4 was distributed primarily within the cytoplasm of tumor vascular endothelial cells in HGGs and was seldom detected within tumor cells. DLL4 expression was correlated positively with the degree of PTBE and was an independent unfavorable prognostic indicator in patients with HGG.

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Author Contributions

Conception and design: Lin, Qiu. Acquisition of data: Qiu, CH Wang, You, XF Wang, YP Chen, L Chen, Liu. Analysis and interpretation of data: Qiu, CH Wang, You, XF Wang. Drafting the article: Qiu, CH Wang. Critically revising the article: Lin, Qiu, You, Kang. Reviewed submitted version of manuscript: Lin, Qiu, Kang. Approved the final version of the manuscript on behalf of all authors: Lin. Statistical analysis: Qiu, CH Wang, XF Wang, YP Chen, L Chen, Liu, Kang. Administrative/technical/material support: Lin, YP Chen, L Chen, Kang. Study supervision: Lin, XF Wang, YP Chen, L Chen.

Correspondence

Zhi-xiong Lin, Department of Neurosurgery, the First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian 350005, China. email: lzx1967@sina.com.