EphA2, vascular endothelial growth factor, and vascular endothelial growth factor correlate with adverse outcomes and poor survival in patients with glioma

Likui Shen, MDab, Ran Sun, MDb, Shifeng Kan, MDc, Zhimin Wang, MDa, Zhengquan Yu, MDb,*

Abstract
Purpose: To assess expression levels of Ephrin type-A receptor 2 (EphA2), vascular endothelial growth factor (VEGF), and von Willebrand factor (vWF), and assess their potentials as prognostic biomarkers to predict the risk of poor survival in patients with primary lower grade glioma.

Method: The study included 75 patients with histopathologically confirmed primary glioma (World Health Organization Grade IV). All patients underwent combined surgery and postoperative radiotherapy for the management of primary glioma. Immunohistochemical analysis was performed to evaluate expression levels of EphA2 and VEGF. Evaluation of tumor microvessel density was also performed at angiogenesis hot spots due to tumor growth. Main outcomes of the study were the prognostic efficiencies of EphA2, VEGF, and vWF in primary low-grade glioma, as well as whether their expression levels were associated with cancer progression.

Results: Of the patients with glioma, 67% had very strong expression of EphA2. Overall survival was inversely correlated with the expression of EphA2. Regarding VEGF expression, 38 patients (51%) had strong expression, 29 patients (39%) had weak expression, and 8 patients (11%) had no expression. Strong VEGF expression was associated with poor prognosis and poor survival.

Conclusion: EphA2, VEGF, and vWF could be considered prognostic markers for assessment of primary glioma.

Abbreviations: CNS = central nervous system, EphA2 = Ephrin type-A receptor 2, MVD = microvessel density, PBS = phosphate buffered saline, VEGF = vascular endothelial growth factor, vWF = von Willebrand factor, WHO = World Health Organization.

Keywords: ephrin type-A receptor 2, glioma, poor outcome, vascular endothelial growth factor, von willebrand factor

1. Introduction
Glioma is the most prevalent neoplasm of the central nervous system (CNS), which is classified by the World Health Organization (WHO) as Grades I to IV neoplasia, based on its differentiation.[1] Glioblastoma multiforme is a destructive type of glioma and the most common neoplasm among brain tumors.[1] Treatment includes surgical resection of the neoplasm, along with chemotherapy and radiotherapy.[1]
The rate of glioma has markedly increased in the past 20 years, thereby increasing the mortality and morbidity of glioma worldwide[2-3], despite the use of relatively new diagnostic techniques, the 3-year survival rate remains poor.[2,3] Many prognostic factors such as isocitrate dehydrogenase 1, p53, and Ki-67 have been introduced; however, there is continuing demand for biomarkers with high accuracy, good sensitivity, and good specificity. Such biomarkers will enable glioma to be diagnosed during early stages of disease, thereby producing better clinical monitoring.[4]

Vascular endothelial growth factor (VEGF) is a main mediator of angiogenesis and a potent cell-specific mitogen.[5,6] It is also involved in metastasis and carcinogenesis. VEGF has emerged as a prognostic factor in several carcinomas including gastric cancer, colon cancer, oral cancer, and cancer involving the CNS. However, the diagnostic efficiency and predictive value of VEGF in patients with glioma have not been clarified thus far.[6] Current studies focus on interactions between blood vessels and glioma cells; there is evidence that VEGF plays a very important role in the evolution of glioma. However, it remains unclear whether VEGF can be used as a potential biomarker in glioma.

The erythropoietin-producing human hepatocellular carci-
oma (Eph) receptor belongs to the tyrosine kinase family, which has 16 members. These receptors are classified into subfamily ligands A and B. Both ligands of Eph can attach to cell membranes and facilitate cell-to-cell contact.[6] The Erythropoi-
etin-producing human hepatocellular receptor is involved in several biological processes including tissue generation, guidance of axons, and angiogenesis during embryonic develop-
ment.[7] Notably, Erythropoietin-producing human hepatocellular recep-
tors are known to be overexpressed in many carcinomas.[7-10] Furthermore, many studies have revealed expression between Ephrin type-A receptor 2 (EphA2) and the pathogenesis of many tumors of the CNS. EphA2 is overexpressed in glioma, compared with normal brain tissues, and may be useful as a biomarker for the development of glioma vaccines.[7,9] Thus far, the prognostic significance of EphA2 has not been determined.

Glioma is frequently accompanied by thromboembolism and coagulopathies.[10] Thus, many anticoagulant therapies have been used as management strategies to treat patients with glioma; however, there remains a risk of increased cerebral bleeding.[10] The most common complication in patients with glioma is venous thromboembolism, which is a potential prognostic sign in these patients. Thrombin is a growth stimulant for glioma. The results of previous studies involving animal models suggest that intracerebral infusion of thrombin inhibitor can relieve cerebral edema, as well as neurologic signs and symptoms[11]; these effects may be mediated by the reduction of glioma cell proliferation. Von Willebrand factor (vWF) is an additional adhesive pro coagulant molecule that aids in platelet adhesion between sub endothelial and endothelial surfaces.[11,12] vWF is found primarily in endothelial cells; it is also expressed in non-endothelial carcinomas including glioma, and is therefore considered a prognostic factor for glioma.[12]

In this study, we assessed the expression levels of EphA2, VEGF, and vWF to determine their potentials as prognostic biomarkers for adverse outcomes, as well as their abilities to predict risk and poor survival in patients with primary lower grade glioma. In addition, we evaluated microvessel density (MVD) and clinical parameters among patients with primary lower grade glioma.

2. Patients and methods

In total, 100 patients were recruited for this study; 75 patients exhibited histo-pathologically confirmed primary glioma Grade IV, according to WHO classification,[7] and were therefore enrolled in the study. The control group comprised 25 individuals with normal brain tissue. Informed consent was obtained from all included patients. Ethical approval was also obtained from the Soochow University Ethical cum Research board under the vide letter no SU/2015-65/N/12. Patients underwent combined surgery and postoperative radiotherapy, as well as chemotherapy, for the management of primary glioma. Patients were followed up for 2 months. At 3 months postoperatively, additional radiological investigation was performed to determine the presence of complications or relapse. WHO criteria were used to assess disease progress, including tumor size and shape; these aspects were also evaluated by computed tomography and magnetic resonance imaging.[7,9] For the control group, twenty-five samples without any brain tumors or lesions were obtained from autopsy; in these samples, both cortex and white matter were included.

2.1. Immuno-histochemical analysis

Immuno-histochemical analysis was performed using paraffin-embedded processed tissue. Slides were then stained with Rotihistol dye, rinsed with alcohol, and rinsed with phosphate-buffered saline (PBS). Subsequently, slides were rinsed with 3% H2O2 for a minimum of 20 minutes at room temperature, then blocked in 5% blocking serum for 1 hour. EphA2 polyclonal antibody (diluted 1:1000) was then added to the slide overnight at a temperature of 4°C. For negative controls, the primary antibody was applied with 1x PBS. To enhance signal efficiency, goat anti-rabbit IgG antibody was added for 30 minutes, followed by streptavidin-horseradish peroxidase for 35 minutes. 3,3’-Diaminobenzidine solution was added to develop the signal; slides were then counterstained with hematoxylin dye for 20 seconds.

2.2. Assessment of EphA2 expression

EphA2 expression levels in tumor cells were evaluated at 400x magnification. EphA2 expression was evaluated as a score between 0 and 3; a score of 3 was considered to indicate positive staining. Scoring was performed by three independent clinicians who were experts in this field and were blinded to the patients’ clinical data.

2.3. Evaluation of tumor MVD at angiogenesis hot spots due to tumor growth

Tumor-related angiogenesis was evaluated by MVD in vascularized areas. All slides were scanned at low magnification (approximately 100x) to identify 4 hot spots of MVD. Then, each hot spot was further evaluated at a magnification of 200x to determine the number of stained microvessels per field. Stained vWF-positive blood vessels with all lumen and all stained clusters with and without laminate were regarded as individual microvessels. The final count was regarded as the average number of vessels from the 4 fields assessed at high magnification (200x). The MVD at hot spots was evaluated to reveal the most vascularized areas of the tumor. Each slide was evaluated at low
magnification (100x) to identify 4 hot spots of MVD. All evaluated hot spots were assessed at high magnification (approximately 200x). VWF-positive blood vessels with and without lumen spaces were regarded as individual microvessels. The final microvessel score was the average of the vessel counts from the 4 fields assessed at high magnification (200x). MVD was related to poorer survival in glioma based on the cut value of the MVD which is kept at 50 per 200 × field

2.4. Assessment of VEGF expression

For analysis of VEGF in patients with glioma, immunohistochemistry, Western blotting, and VEGF staining were performed. For VEGF staining, 5-μm-thick sections of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized with rehydrated alcohol; they were then incubated in 3% hydrogen peroxide in PBS to block endogenous peroxide for 15 minutes. All sections were incubated at room temperature for 25 minutes with protein blocking solution (5% horse serum and 1% goat serum in PBS, pH 7); they were then incubated with anti-VEGF antibody overnight at a temperature of 4°C.

To optimize the final staining protocol for each of the 4 antibodies used in the sample, each antibody was initially tested with various pretreatments and dilutions. Thus for 30 minutes, individual tissue parts were incubated with primary antibodies: anti-VEGF-A, rabbit polyclonal antibody, 1:250 dilution (A-20, SC-152, Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-VEGFR-1, rabbit polyclonal antibody, 1:100 dilution (Ab-1, RB-1527-P, Thermo Fisher Scientific, Fremont, CA). Different IHC staining protocols were tested for VEGFR-2, including different blocking steps, but it was not possible to obtain optimal staining results. The levels of VEGF expression and MVD were assessed as described in the section of the immunohistochemical analysis above.

2.5. Genotyping

A Citogene Blood Kit was used to extract DNA from blood samples, in accordance with the manufacturer’s instructions. The Sequenom MassARRAY iPLEX Gold platform was used to generate DNA sequences and perform genotyping with allele-specific primers. MassARRAY assay design software was used to design the primers. Primers were designed to specifically amplify the entire coding sequence of VWF, including intron-exon boundaries, 3.5 kb upstream of exon 1, and 0.8 kb downstream of the C-terminal stop codon. The primer sequences are should be made available on request. The quality of genotyping was assessed by duplicate analysis of 10% of the sample, which revealed a 100% rate of agreement. Sequence data were compared with the VWF reference sequence NC_000012.1.

2.6. VWF polymorphisms testing

Using dye-terminator chemistry, polymerase chain reaction (PCR) amplification and semiautomated bidirectional sequencing was carried out using a series of primers explicitly designed to amplify the entire VWF coding sequence, including intron-exon boundaries, 3.5 kb upstream of exon 1, and 1 kb downstream of the C-terminal stop codon, except the pseudogene sequence.13,14,15 Exon 28 PCR was performed on subjects and the resulting fragment was cloned into the Topo TA cloning vector to explore potential linkage of the 3 SNPs of interest on 1 allele (Invitrogen). Sequencing of Exon 28 from the resulting colonies was then performed on purified DNA. ELISA with 2 different mAbs for capture and a rabbit polyclonal Ab was used for VWF:Ag detection. VWF:R Co was measured using formalin-fixed platelets and standard concentrations of ristocetin on a BCS (Dade Behring). 0.65% agarose multimer gels were used for the assessment of VWF multimers. FVIII expression was measured using a 1-stage clotting assay. VWF-CB was measured using type III human placental collagen (Southern Biotechnology Associates) for capture and a rabbit polyclonal Ab for detection of VWF binding.

2.7. Association between the VWF gene and poor survival of glioma

For patients diagnosed with glioma, the association between VWF and poor survival was evaluated using the Genomic Data Commons and The Cancer Genome Atlas. To evaluate genomic data, the following data portals were used: GDC Data Portal (https://portal.gdc.cancer.gov/); Oncomine (https://oncomine.org/); cBioportal (http://cbioportal.org). The cumulative analysis showed that the cumulative 1-year survival was significantly shorter in patients with VWF levels >200IU/dL than in those with VWF levels.

2.8. Statistical analysis

A Kaplan-Meier curve was used to evaluate the association between V WF and overall survival. An R2 web-based application was used to determine the optimal cutoff. This method divided the total sample size and into equal-sized groups, based on gene expression. An ribonucleic acid sequence-based expression method was used to quantify the expression of a gene as fragments per kilobase per million. Statistical analyses were performed using SPSS software. $P < .05$ was considered to be statistically significant. The log-rank test was used to compare survival rate distributions. The chi-squared test was used to evaluate the association between EphA2 and MVD. Student t-test was used to analyze VEGF in terms of overall survival. The chi-squared test and Fisher exact test were used to assess the relationships of VEGF expression with clinico-pathologic parameters. Analysis of variance tests were used to compare continuous variables among groups.

3. Results

In total, 75 patients with recurrent or primary glioma Grade IV were analyzed in this study. The mean patient age was 60.1 years (range, 35–74 years). Sixty-five patients relapsed after surgery and died of the disease within 24 months.

3.1. Association between EphA2 and glioma

EphA2 expression was analyzed in the cytoplasm of tumor cells and endothelial cells (Fig. 1A and B). Twenty-five control individuals did not exhibit any EphA2 expression. The highest expression levels were present in a highly vascularized tumor bed and around endothelial cells near the tumor. All patients with glioma showed various intensities of EphA2 staining; 67% of the patients had very strong expression of EphA2. Over expression of EphA2 was found specifically in areas with the highest tumor MVD (Fig. 1C and D). A significant association was detected between EphA2 and MVD ($P < .05$) (Table 1). High expression of...
EphA2 was also observed in patients with recurrent disease, compared to those with primary disease, although this difference was not statistically significant (Fig. 1E and F) (Table 1).

Overall survival was inversely related to EphA2 expression. Patients who had low or moderate expression (score of < 3) exhibited a survival rate of 22–24 months. In contrast, patients who had high expression (score of 3) exhibited a survival rate of 10–13 months ($P < .05$) (Fig. 2). Increased EphA2 expression was significantly associated with death due to disease ($P = .004$ by log-rank test) in patients who had low and moderate expression (score of 3).

### 3.2. Association between vWF and glioma

Survival versus vWF gene expression is shown in Figure 3. Patients with glioma who had lower vWF gene expression...
exhibited better survival than patients who had higher vWF expression. Patients who had over-expression of vWF exhibited poor survival. Therefore, vWF expression has an inverse relationship with the survival rate ($P < .05$). In addition, patients with lower grade gliomas who had lower vWF gene expression had significantly better survival than patients who had higher vWF gene expression (hazard ratio, 0.89; 95% confidence interval, 0.32 to 0.82; 197 months vs 164 months $P = .01$ log rank test).

3.3. Association between VEGF expression and glioma

The association between VEGF expression and glioma was analyzed by immunostaining for VEGF and MVD in all 75 patients. The expression was categorized as strong, weak, or negative. Regarding VEGF expression, 38 patients (51%) had strong expression, 29 patients (39%) had weak expression and 8 patients (11%) had no expression (Fig. 4) (Table 2). Kaplan-Meier analysis of overall survival showed that strong VEGF expression was associated with poor prognosis and poor survival (Fig. 4). The median MVD count was 28.5 (range, 6.7–75.5). Forty patients had an MVD < 20.5; 35 patients had a high MVD (Table 2). The results revealed no significant differences in MVD counts with respect to all clinicopathologic characteristics; however, the MVD counts increased with age. Furthermore, the median MVD count was nearly identical in patients who had high VEGF expression and those who had low VEGF expression ($29.0 ± 16.7v.27.9 ± 18.6; P = .887a$) (Table 2). The overall survival durations of patients with glioma with strong and weak

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**Table 1**

| EphA2 Expression | Low/Moderate | High | $P$ |
|------------------|--------------|------|-----|
| MVD Low/Moderate | 20           | 5    | $<.05$ |
| MVD High        | 5            | 45   | $<.05$ |

EphA2 = Ephrin type-A receptor 2, MVD = Microvessel density, $P < .05$ was considered significant.

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Figure 2. Kaplan-Meier curve depicting overall survival based on high or low EphA2 expression. Increased EphA2 expression was significantly associated with death due to disease ($P = .004$ by log-rank test) in patients who had low and moderate expression (score 3).

Figure 3. Kaplan-Meier curve depicting overall survival based on vWF expression. Patients with lower grade gliomas who had lower vWF gene expression exhibited significantly better survival than those who had higher vWF gene expression (hazard ratio, 0.89; 95% CI, 0.32–0.82; $P = .01$ log rank test).

Figure 4. Kaplan-Meier curve depicting overall survival based on VEGF expression.
VEGF expression were 5 months and 21 months, respectively; these were significantly different. VEGF expression was a predictor of outcome.

4. Discussion

EphA2 is known to be overexpressed in many cancers, including lung, ovary, oral, and gastric.[17] A prior study showed that EphA2 was very strongly associated with cancer progression and metastasis, as well as poor clinical outcome.[18] However, the association between EphA2 and brain tumors was not previously investigated. In the present study, we found that higher EphA2 expression in both primary and recurrent glioma was associated with poor patient outcomes. Our findings are consistent with those of a study by Hatano et al.[19] which revealed overexpression of EphA2 in patients with primary glioblastoma-multiforme. A study by Liu et al.[9] showed that EphA2 expression was inversely proportional to the survival of patients with glioma, which was also consistent with our study results.

The present study revealed overexpression of EphA2 in highly vascularized tumors. Angiogenesis of the tumor was measured by determination of MVD. The tumor MVD is considered an important biomarker of malignant changes and can be used to predict the prognosis of patients with glioma. The results of some studies have suggested that the MVD has very limited prognostic value in patients with glioma. We also found that the MVD was not significantly associated with EphA2 expression.

The majority of authors observed that the microvascular density (MVD) had an important role in tumor progression but also in the survival rate.[20,21] The quantification of angiogenesis was made in the majority of studies with the classical hot-spot. The angiogenesis could be identifying with the panendothelial markers CD31 and CD34, and also with CD105.[20–22] CD31 and CD34 show the vascular status of cancer progress but they do not indicate the angiogenic intensity because they mark both neoformed vessels and normal, preexistent vessels in neoplastic and nonneoplastic tissues. CD105 seems to be more specifically for the endothelial cells of neoformed vessels. Its expression increased in the same time with the neoangiogenic progression.[20–22] However, this study has not taken any antibody for MVD such as CD31, CD34, CD105. This study also revealed that vWF marks the mature vessels only, however immature vessels or neo formed CD105-positive vessels were not evaluated in this study, these points has been incorproared in the limitation section of the study.

In 1865, Trousseau first reported a strong relationship between malignant tumor and coagulation characteristics.[23] An association between vWF expression and the presence of malignant tumors has since been reported; however, very few studies have shown an association between vWF expression and glioma. vWF promotes platelet adhesion and aggregation in malignant melanomas; it also enhances the metastasis of gastric adenocarcinomas. When the aortic valve is constricted, the flow of the blood through the valve is augmented to maintain cardiac output. Therefore, narrowing of the walls and a high rate of flow increases the shear stress of blood.[24] This stress causes uncoiling of vWF, which also occurs during tissue damage.[25] The inverse association between the survival rate and vWF expression is another sign of this phenomenon. Our study results also revealed that vWF expression might be an important biomarker in patients with glioma. Notably, the risk of venous thromboembolism is high in patients with glioma; this risk continues into the postoperative period, and is consistent with our findings regarding the association of vWF expression and thromboembolic events with poor outcome and poor survival in patients with glioma.

Regarding the association between VEGF and glioma, a prior study revealed that VEGF expression is strongly associated with glioma and metastasis.[21] All patients in our study were classified as WHO Grade IV; as in the prior study, we found an association between VEGF and adverse outcomes. However, a previous study regarding tumors of various histological grades showed that VEGF expression and the MVD were higher in poorly differentiated tumors than in well-differentiated tumors.[19] Our findings are consistent with those of other investigators who found that plasma VEGF levels were associated with disease progression in patients with neuroendocrine tumors.

Limitations: Long term prospective trial with larger sample size has been further needed to prove the findings. In future, there shall be a single parameter/score to define all the 3 parameters. The major limitation of the study was that the study has not taken any antibody for MVD such as CD31, CD34, CD105. This study also revealed that vWF marks the mature vessels only, however immature vessels or neo formed CD105-positive vessels were not evaluated in this study.

Table 2

| Characteristic                | Weak (n = 29) | Strong (n = 38) | None (n = 8) | P   | Low (n = 40) | High (n = 35) | P   |
|------------------------------|--------------|----------------|-------------|-----|-------------|--------------|-----|
| Sex                          |              |                |             |     |             |              |     |
| Male                         | 15           | 20             | 5           |      | 25          | 22           | .286|
| Female                       | 14           | 18             | 3           |      | 15          | 13           | .643|
| Age (yr), median 56          |              |                |             |     |             |              |     |
| ≤56                          | 14           | 17             | 5           |      | 24          | 22           | .643|
| >56                          | 15           | 21             | 16          |      | 13          | 13           | .062|
| Lymph node metastasis        |              |                |             |     |             |              |     |
| N0                           | 1            | 1              | 5           |      | 5           | 5            | .877|
| N1–4                         | 26           | 37             | 35          |      | 30          |              |     |
| Distant metastasis           |              |                |             |     |             |              |     |
| M0                           | 14           | 22             | 28          |      | 20          |              | .089|
| M1                           | 15           | 26             | 12          |      | 15          |              | .810|

MO = no metastasis, M1 = distant metastasis, MVD = microvessel density, N0 = no nodal involvement, N1–4 = nodal involvement with various degree. VEGF = vascular endothelial growth factor. P < .05 was considered significant.
5. Conclusion

The present study showed that EphA2, VEGF, and vWF could be useful as prognostic markers for assessment of primary glioma, further, EphA2 also for recurrent glioma. These biomarkers were associated with carcinogenesis and cancer progression. The association between overexpression of EphA2 and poor outcome in patients with glioma could be used to screen the candidates of tyrosine kinase inhibition therapy. Our study also revealed that vWF expression might be an important risk biomarker in patients with glioma.

Author contributions

Likui Shen Design of the study, establishment of aims and objectives, collection of data.
Ran Sun Collection of data, analysis of results, and writing of the manuscript.
Shifeng Kan: Laboratory investigations and report collection, writing of the manuscript.
Zhimin Wang Statistical analysis and review of the literature.
Zhengquan Yu Collection of data, coordination of study, and submission of manuscript.
Conceptualization: Zhengquan Yu, Ran Sun.
Data curation: Zhengquan Yu, Likui Shen, Shifeng Kan.
Formal analysis: Zhimin Wang.
Investigation: Likui Shen.
Methodology: Ran Sun.
Project administration: Zhengquan Yu, Likui Shen.
Resources: Shifeng Kan.
Software: Shifeng Kan.
Supervision: Zhengquan Yu, Zhimin Wang.
Validation: Ran Sun.
Visualization: Shifeng Kan.
Writing – original draft: Zhengquan Yu, Zhimin Wang.
Writing – review & editing: Zhengquan Yu.

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