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# Immunohistochemical characterization of lymphangiogenesis-related biomarkers in primary and recurrent gliomas

## A STROBE compliant article

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**Abstract**

Glial tumors constitute the majority of primary intracranial brain tumors. The expression of specific markers of lymphangiogenesis in gliomas still remains unclear.

A total of 40 surgical specimens from 20 patients with recurrent gliomas were included in the study. The expression of D2-40, vascular endothelial growth factor (VEGF)-C, VEGF-D, and VEGF receptor-3 (VEGR-3) was detected by immunohistochemistry (IHC). The clinicopathologic data (p53 and Ki67) were also collected and analyzed.

At relapse malignant transformation rate was 65% (13/20 cases). D2-40, VEGF-C, VEGF-D, and VEGFR-3 were expressed in 20%, 30%, 60%, and 20% of primary and 45%, 30%, 75%, and 35% of recurrent glioma tumors ($P < .01$, $P = 1.00$, $P = .03$, $P = .03$). In 13 cases with increased malignancy grade, the expression of Ki67 and p53 were higher at relapse compared with the primary tumors ($P = .001$, $P = .045$). Multivariate survival analysis showed VEGF-D was an independent prognostic factor for malignant transformation (HR = 0.376, $P = .045$).

Glioma is easy to relapse with tumor progression. VEGF-D was an independent prognostic factor for malignant transformation.

**Abbreviations:** CNS = central nervous system, VEGF = vascular endothelial growth factor, VEGFR = vascular endothelial growth factor receptor.

**Keywords:** D2-40, glioma, Ki67, lymphangiogenesis, p53, vascular endothelial growth factor-C, vascular endothelial growth factor-D, vascular endothelial growth factor receptor-3

## 1. Introduction

Glial tumors constitute the majority (65%) of primary intracranial brain tumors. Despite advances in surgical, medical, and radiation therapies, the patients with high-grade gliomas showed poor prognosis.[1] In those patients, extensive vasogenic edema is associated with short survival.[2] Drugs against mediator of cerebral edema can treat edema caused by tumor or brain radiation necrosis. Clinical experience suggested that bevacizumab, a recombinant humanized monoclonal antibody against vascular endothelial growth factor (VEGF), provided clinical benefit by decreasing peritumoral edema.[3] Bevacizumab can inhibit angiogenesis and lymphangiogenesis. However, the expression of specific markers of lymphangiogenesis in glioma remains unclear.

The D2-40 has been shown to be a very sensitive and specific marker for lymphatic endothelium in most tissues.[4] VEGF-C and VEGF-D bind vascular endothelial growth factor receptor (VEGFR)-3, and they are key regulators in the formation and maintenance of lymphatic vessels in various solid tumors.[5,6] In physiologic adult tissues, VEGFR-3 is not expressed in the endothelium of blood vessels but highly expressed in the lymphatic endothelium.

In our present study, the clinicopathologic characteristics of primary and recurrent glioma were analyzed, and the distribution and expression level of D2-40, VEGF-C, VEGF-D, and VEGFR-3 in primary and recurrent gliomas were examined by immunohistochemistry (IHC).

## 2. Materials and methods

### 2.1. Study specimens

A total of 40 surgical specimens (20 primary and 20 recurrent specimens) from 20 recurrent glioma patients (8 males and 12 females) were included. The patients included in this study were enrolled from the Second Hospital of Shandong University

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between June 2011 and February 2016. The inclusion criteria were listed as follows: glioma was located in the noneloquent areas of the cerebral hemisphere, postoperative magnetic resonance imaging (MRI) scans were available to confirm total tumor resection, also MRI/computed tomography scans were used to determine the recurrence of glioma. Tumor type and grade were determined by Department of Pathology, according to the World Health Organization classification.[7,8] From those patients, 12 surgical specimens of adjacent normal brain tissue were available and were used as control. Tissues were collected during craniotomy and fixed in 10% buffered formalin. Patients of grades III and IV received standard radiotherapy, as follows: 30 to 33 fractions, 1.8 to 2.0 Gy/fraction, 5 days/wk, to a total dose 60 Gy using 6 to 10 MV X-rays in vitro. The patients were also given daily oral temozolomide (TMZ) 75 mg/m$^2$ during radiotherapy. Four weeks after radiotherapy, all of the patients received 6 cycles of TMZ. Each cycle lasted 5 days with 28 days interval between each cycle. About 150 mg/m$^2$ of TMZ was given for the first cycle for 5 days, followed by 200 mg/m$^2$ of drug for the rest of the cycles if no significant drug-related toxicities were observed. The patient's clinicopathologic data (p53 and Ki67, scored as a continuous variable, rounded to the closest 5th percentile) were also collected and analyzed.

This study was approved by the Human Research Ethics Committees from the Second Hospital of Shandong University. Written informed consent was obtained from all participants.

### 2.2. Immunohistochemistry

Five-micrometer-thick sections of human tumor tissues were deparaffinized and rehydrated. For D2-40, VEGF-C, VEGF-D, and VEGFR-3 staining, heat-induced epitope retrieval was performed by boiling the tissue section in Tris-EDTA buffer (pH 9.0) for 15 minutes, in microwave oven. After cooling to room temperature, the section was exposed to methanol (containing 3% $H_2O_2$) for 10 minutes to block endogenous peroxidase. D2-40 was detected with an anti-D2-40 monoclonal antibody (1:200 dilution, ab77854, Abcam, Cambridge, UK), VEGF-C was detected with an anti-VEGF-C monoclonal antibody (1:200 dilution, ab191274, Abcam), VEGF-D was detected with an anti-VEGF-D monoclonal antibody (1:200 dilution, ab155288, Abcam), and VEGFR-3 was detected with an anti-VEGFR-3 antibody (1:100 dilution, ab27278, Abcam) for 2 hours at room temperature in a humidified chamber. The slide was then washed with phosphate-buffered saline (PBS) and incubated with the secondary detection antibody polyperoxidase-labeled anti-rabbit/mouse IgG (ZSGB-BIO, Beijing, China) for 60 minutes in accordance to the manufacturer's instructions. 3,3-Diamino benzidine was used as a chromogen. The section was counterstained with hematoxylin and mounted in gelatin. Negative and positive controls for each immunohistochemical marker were included with every experiment. A lymphangioma tissue sample served as a positive control and replacement of the primary antibody by 0.01 mol/L PBS was used as a negative control.

### 2.3. Histologic assessment

Immunohistochemical staining of 10 randomly selected fields in each tumor specimen was evaluated under light microscope (200×). Evaluation of immunostaining was performed independently by 2 pathologists who were blind to the clinical data. In case of discordance between pathologists, a 3rd pathologist reviewed the result to achieve a consensus. Immunostaining was semiquantitatively recorded by estimating the proportion of positive cells and classified as follows: negative = 0%, "1+" = 1% to 25%, "2+" = 26% to 50%, and "3+" ≥ 50% positively stained cells.

### 2.4. Statistical analysis

Statistical analysis was performed with SPSS 19.0 and GraphPad. cameyo. Nonparametric Spearman correlation test was used to analyze the relationship between the pathologic grades and expression levels of D2-40, VEGF-C, VEGF-D, VEGFR-3, p53, or Ki67. Nonparametric Spearman correlation test was also used to assess relationship between the first disease-free survival time and the expressions of different markers. The expression levels of markers at diagnosis and relapse were analyzed by Wilcoxon matched-pairs signed rank. Multivariate prognostic analyze of glioma was performed using the Cox proportional hazards regression model. *P*-value of .05 or less was considered statistically significant.

## 3. Results

### 3.1. Clinicopathologic characteristics of patients

The clinicopathologic characteristics of 20 glioma patients are detailed in Table 1. The median age of patients at diagnosis was 43.5 years old (range: 40–48.75 years old), and the mean tumor-free survival was 1.65 ± 1.34 years. The most common clinical presentation was headache (n = 14), limb paralysis (n = 3), and epilepsy (n = 3). The mean tumor size was 4.3 ± 1.7 cm. There were 1 case of grade I, 6 cases of grade II, 9 cases of grade III, and 4 cases of grade IV at diagnosis but 2 cases of grade II, 2 cases of grade III, and 16 cases of grade IV at relapse. At relapse malignant transformation rate was 65% (13/20 cases).

### 3.2. Expression of D2-40, VEGF-C, VEGF-D, and VEGFR-3 in control, primary, and recurrent glioma tissues

There was no expression of D2-40, VEGF-C, VEGF-D, and VEGFR-3 in the adjacent normal brain tissue. D2-40-positive staining was observed in the cytoplasm and detected in 13 of 40 glioma specimens (Fig. 1A). VEGF-C-positive staining was mostly observed in the cytoplasm or/and weakly in the nucleus in 12 of 40 glioma specimens (Fig. 1B). VEGF-D expression was observed in the cytoplasm or/and in the nucleus in 27 of 40 glioma specimens (Fig. 1C). VEGFR-3-positive tumor cells were observed in the cytoplasm and nucleus in 11 of 40 glioma specimens (Fig. 1D). IHC showed that D2-40, VEGF-C, VEGF-D, and VEGFR-3 was expressed in 20%, 30%, 60%, and 20% of primary and 45%, 30%, 75%, and 35% of recurrent glioma tumors ($P < .01$, $P = 1.00$, $P = .03$, $P = .03$). The first disease-free survival time was not related to the expressions of D2-40 ($P = .78$), VEGF-C ($P = .64$), VEGF-D ($P = .11$), VEGFR-3 ($P = .31$), Ki67 ($P = .69$), and p53 ($P = .35$). There were 6 cases with negative staining for all lymphangiogenesis markers (all of D2-40, VEGF-C, VEGF-D, and VEGFR-3). There was no significant difference of the first disease-free survival time between them and others ($P = 1.665$).

Moreover, the pathologic grade was significantly related to the positive stainings for D2-40 ($P = .0060$), VEGF-D ($P = .017$), and VEGFR-3($P = .032$), and to the scores of Ki67 ($P < .001$) and p53 ($P = .003$), while was not significantly related to VEGF-C expression ($P = .059$).

**Table 1**

**The clinical and pathologic characteristics of glioma patients.**

| Case no | Age, y | Initial | | Recurrent | | First tumor-free survival, y |
|---|---|---|---|---|---|---|
| | | Pathologic types | Grades | Pathologic types | Grades | |
| 1 | 51 | Ganglioglioma | I | Glioblastoma | IV | 2 |
| 2 | 38 | Diffuse astrocytoma | II | Diffuse astrocytoma | II | 0.25 |
| 3 | 41 | Diffuse astrocytoma | II | Glioblastoma | IV | 0.75 |
| 4 | 24 | Diffuse astrocytoma | II | Glioblastoma | IV | 1 |
| 5 | 31 | Diffuse astrocytoma | II | Glioblastoma | IV | 6 |
| 6 | 42 | Diffuse astrocytoma | II | Glioblastoma | IV | 1 |
| 7 | 40 | Diffuse astrocytoma | II | Glioblastoma | IV | 3 |
| 8 | 45 | Anaplastic astrocytoma | III | Glioblastoma | IV | 1 |
| 9 | 49 | Anaplastic astrocytoma | III | Glioblastoma | IV | 0.5 |
| 10 | 41 | Anaplastic astrocytoma | III | Anaplastic astrocytoma | III | 0.5 |
| 11 | 48 | Anaplastic astrocytoma | III | Glioblastoma | IV | 1 |
| 12 | 40 | Anaplastic astrocytoma | III | Glioblastoma | IV | 3 |
| 13 | 57 | Anaplastic astrocytoma | III | Glioblastoma | IV | 1 |
| 14 | 44 | Anaplastic astrocytoma | III | Glioblastoma | IV | 2 |
| 15 | 39 | Anaplastic astrocytoma | III | Glioblastoma | IV | 3 |
| 16 | 43 | Anaplastic astrocytoma | III | Anaplastic astrocytoma | III | 2 |
| 17 | 53 | Glioblastoma | IV | Diffuse astrocytoma | II | 2 |
| 18 | 57 | Glioblastoma | IV | Glioblastoma | IV | 0.5 |
| 19 | 46 | Glioblastoma | IV | Glioblastoma | IV | 1.5 |
| 20 | 46 | Glioblastoma | IV | Glioblastoma | IV | 1 |

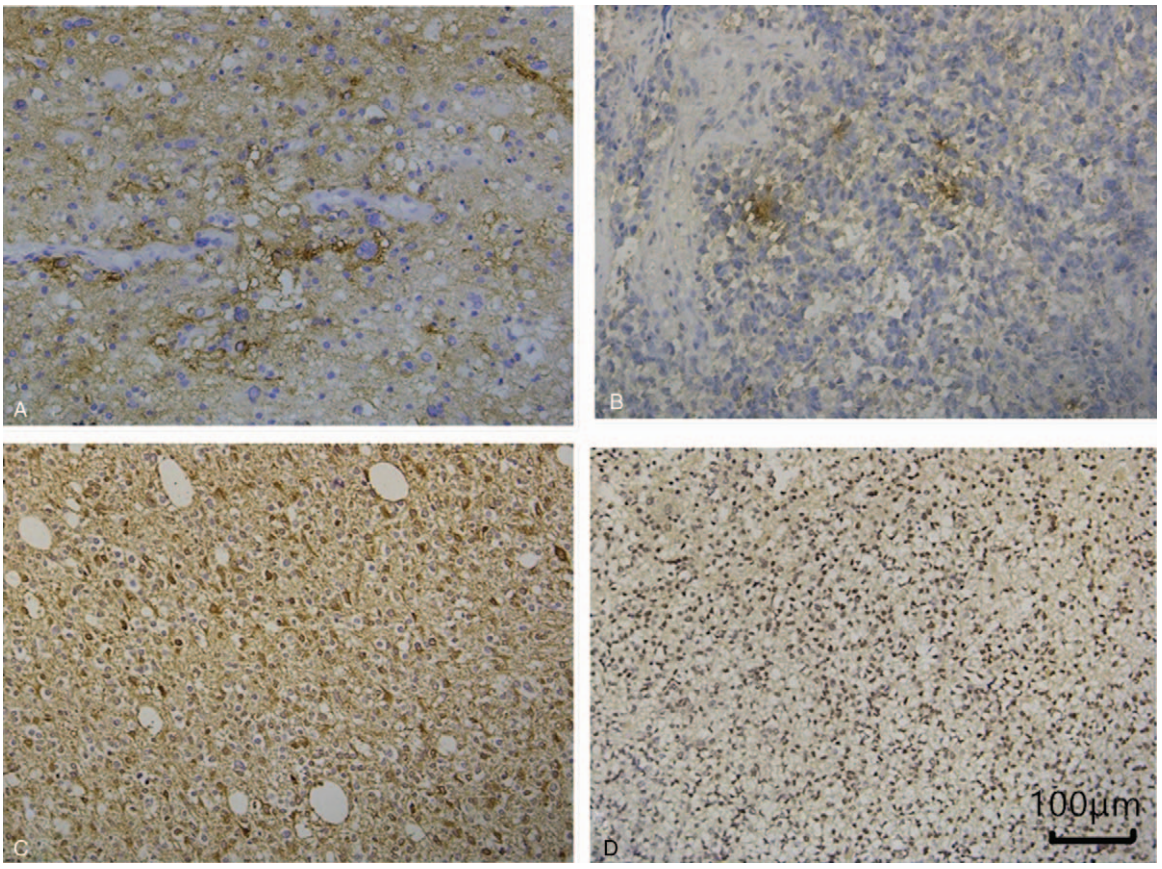


**Figure 1.** D2-40, vascular endothelial growth factor (VEGF)-C, VEGF-D, and vascular endothelial growth factor receptor (VEGFR)-3 expression in glioma tissues. A representative section from: (A) D2-40-positive glioma specimens IV (+). (B) VEGF-C-positive glioma specimens IV (++). (C) VEGF-D-positive glioma specimens IV (2 +). (D) VEGFR-3-positive glioma specimens IV (+).

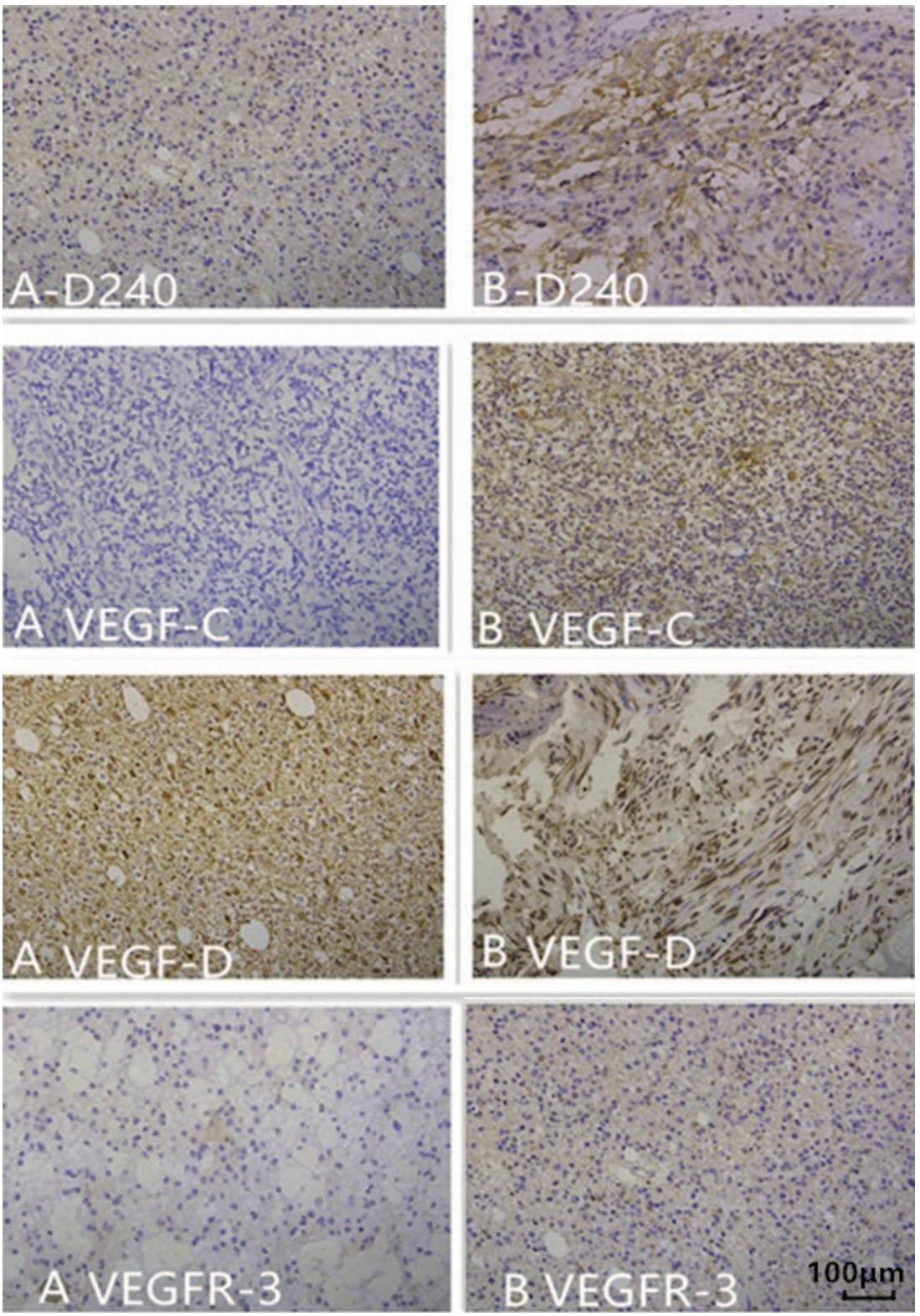


**Figure 2.** D2-40, vascular endothelial growth factor (VEGF)-C, VEGF-D, and VEGFR-3 expression in the primary (A) and recurrent specimens (B) from the same patient. (A-D240) A representative section from specimens of grade II (−). (B-D240) A representative section from specimens of grade IV (2+). (A VEGF-C) A representative section from specimens of grade III (−). (B VEGF-C) A representative section from specimens of grade IV (+). (A VEGF-D) A representative section from specimens of grade III (+). (B VEGF-D) A representative section from specimens of grade IV (2+). (A VEGFR-3) A representative section from specimens of grade III (−). (B VEGFR-3) A representative section from specimens of grade IV (+).

In 13 cases with increased malignancy, the expression levels of Ki67, p53, D2-40, VEGF-C, VEGF-D, and VEGFR-3 were increased at relapse (Fig. 2). Ki67 and p53 scores were significantly higher at relapse compared with primary tumor ($P = .001$, 10% [5–27.5%] vs 40% [35–55%] for Ki67; $P = .045$, 10% [0–25%] vs 25% [17.5–37.5%] for p53). There was significant difference in the D2-40 and VEGF-D expression between recurrent and primary tumors ($P = .031$, $P = .047$), but there is no significant difference in the VEGF-C and VEGFR-3 expression between recurrent and primary tumors ($P = .094$ and $P = .25$). A multivariate survival analysis, including expression of D2-40, VEGF-C, VEGF-D, VEGFR-3, Ki67, p53, primary tumor grade, and relapse tumor grade, showed that VEGF-D was an independent prognostic factor for malignant transformation (HR = 0.376, $P = .045$).

## 4. Discussion

Glial tumors account for approximately 1.5% of all cancers. As a rule, high-grade gliomas almost always grow back even after complete surgical excision, so they are called recurrent cancer of the brain. And gliomas at relapse usually transform to higher grade glioma. In our study, at relapse malignant transformation rate was 65%. There is a great need for biomarkers associated with prognosis. Overexpression of VEGF is associated with several CNS tumors. Multivariate survival analysis showed VEGF-D was an independent prognostic factor for malignant progression. Debinski et al found the activating protein-1 family of transcription factors play a potentially critical role in the progression of gliomas by eliciting uncontrolled upregulation of VEGF-D.[9] The VEGF-D system appears to be a very attractive target for new molecular diagnostics and rational molecular anticancer therapies.[10] Weickhardt et al also found patients with low expression of VEGF-D benefited from bevacizumab treatment, patients with higher VEGF-D expression received less benefit.[11] Clinical utilization of VEGF-D needs further evaluation.

In our study, we want to detect the status of the expression of lymphangiogenesis markers in primary and recurrent glioma tumors. It was reported endothelial cells in brain tumors may either acquire lymphangiogenic mechanisms or be derived from precursor cells carrying lymphatic abilities.[12] Case report by Frank et al indicated a lymphatic pathway of metastasis, demonstrated by histopathologic analysis.[13] We found the expression levels of Ki67, p53, D2-40, VEGF-D, and VEGFR-3 were increased at relapse. But the disease-free survival time was not related to the expressions of them. The anatomical relationship between malignant tumor and its vascular and lymphatic bed is an important factor influencing metastasis. Brain tumors are different from tumors of other parts of the body at metastasis and relapse. There may be unique barriers in brain, such as the dura mater and thickened basement membrane of blood vessels, they prevent lymphatic and hematogenous spread of intracranial tumors. The tumor cells in CNS lack extracellular matrix proteins for tumor dissemination. In addition, intracranial tumor has earlier and more serious symptoms and shorter median survival time before extracranial metastasis detected. The main goal of the current surgical treatment of GBM is maximal safe resection, removing as much as possible the tumor while preserving the neurologic function. The partial resection of glioma preserving functional cortex may affect the first disease-free survival time.

Staining for p53 is often performed to detect single-invading astrocytoma cells. Ki67, a marker of cell proliferation, represents an excellent biomarker to predict the aggressiveness of gliomas and patients' outcomes.[14,15] Despite involved in tumor angiogenesis, VEGF-C and VEGF-D are thought to stimulate the growth of lymphatic vessels because the expression of their specific receptor, VEGFR-3, has been demonstrated to be expressed in the lymphatic vessels. VEGFR-3, which is not expressed in normal brain, has been proposed as a marker for lymphatic endothelial cells,[16] and VEGFR-3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels.[17] VEGF-C has been shown to be expressed in the lymphatic vessels in the tumor microenvironment.[18] It is important in tumor biology elsewhere in the body, and the levels of VEGF-C correlate with increased lymph node metastasis and poor prognosis.[19–22] Glioblastomas, the most frequent high-grade gliomas, are aggressive, rapidly growing, destructive lesions, while low-grade gliomas are less aggressive in their behavior. In our study, the pathologic grade was significantly related to the expression of biomarkers (D2-40, VEGF-C, VEGF-D, and VEGFR-3, excepting VEGF-C), and to the score of p53 and Ki67. The expression of most markers (D2-40, VEGF-D, p53, and Ki67) was higher in patients with increased malignancy at relapse compared with the primary tumors. It is interesting, but not surprising that various genes not expressed in normal tissue might be expressed particularly in the higher grade tumors. These results should be more persuasive in a large number of patients. This study has limitations: pathologic markers such as IDH mutation, 1p/19q codeletion, and O6-methylguanine-DNA methyltransferase (MGMT) are not included in our study. They are important in the diagnosis and treatment of brain glioma. The patients selected in our study all underwent primary and recurrent gliomas surgery, bias in patient selection is a limitation.

## 5. Conclusion

Our results suggest that glioma is easy to relapse with tumor progression. VEGF-D was an independent prognostic factor for malignant transformation.

## Author contributions

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**Methodology:** Yuan Chen.
**Project administration:** Jun Jiang, Shun Wang.
**Resources:** Jun Jiang.
**Software:** Jun Jiang.
**Supervision:** Shun Wang, Chengwei Wang, Chuncheng Qu, Yuguang Liu.
**Validation:** Jun Jiang.
**Visualization:** Jun Jiang.
**Writing – original draft:** Jun Jiang, Shun Wang.
**Writing – review & editing:** Jun Jiang, Shun Wang, Chengwei Wang, Chuncheng Qu, Yuguang Liu.

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