

RESEARCH COMMUNICATION

Correlation of Delta-like Ligand 4 (DLL4) with VEGF and HIF-1 α Expression in Human Glioma

Zhiqiang Li*, Jiang Wang, Lingling Gong, Zhihua Wen, Chengshi Xu, Xiaodong Huang

Abstract

To investigate the potential role of the notch ligand delta-like ligand 4 (DLL4) in glioma angiogenesis, we examined whether its expression correlates with that of vascular endothelial growth factor (VEGF) and hypoxia-induced factor-1 α (HIF-1 α). Eighty-two specimens of human glioma and 7 of normal brain tissue were subjected to immunohistochemical analysis for DLL4, VEGF and HIF-1 α expression. Statistical analysis were performed to determine if protein expression correlated with clinicopathological parameters, including histological type, pathological grade, and microvessel density (MVD), determined using CD34-labelling. Expression of DLL4, VEGF and HIF-1 α was very strong in gliomas, relative to normal tissues, linked with the malignant grade. Moreover, DLL4 staining positively correlated with VEGF and HIF-1 α expression and with MVD. Thus our results indicate that DLL4 represents a potential biomarker and therapeutic target for glioma angiogenesis.

Keywords: Glioma - angiogenesis - delta-like ligand 4 - VEGF - HIF-1 α

Asian Pacific J Cancer Prev, **12**, 215-218

Introduction

Angiogenesis, the development of new blood vessels, is thought to be the main mechanism by which new vascular networks are formed in tumors. During the last three decades, intensive research has been performed to characterize the angiogenesis process and many angiogenesis-related factors or genes have been identified (Jouanneau, 2008).

Malignant gliomas exhibit many vessel-related pathological features (Yamanaka and Saya, 2009). These features include marked endothelial proliferation, and tortuous disorganized vessels of higher permeability, larger diameters and thicker basement membranes than vessels found in normal tissues. Aberrant microvasculature typically appears as glomeruloid tufts, proliferations of microvessels consisting of multilayered mitotically active endothelial and perivascular cells (Jain et al., 2007).

Proteins such as vascular endothelial growth factor (VEGF), matrix metalloproteinases and hepatocyte growth factor and its receptor have been identified to play important roles in the process of glioma angiogenesis (Chu et al., 2005; Li et al., 2005). Due to obvious and aggressive vascular proliferation and very poor prognosis, many antiangiogenic drugs were rushed for approval in clinical trials for glioma patients. Unfortunately, under experimental and clinical conditions antiangiogenic therapy has led to increased invasion and higher recurrence rates (Thurston and Kitajewski, 2008). Thus it is urgent to gain a deeper understanding of glioma angiogenesis in

order to find new antiangiogenic targets.

Delta-like ligand 4 (DLL4) is the most recently identified member of the Notch ligand family (Shutter et al., 2000) and plays an important role in vascular development. To date, VEGF and DLL4 are the only two genes involved in developing vasculature whose haploinsufficiency would lead to major vascular defects and embryonic lethality (Hellstrom et al., 2007). In addition, DLL4 is found to be highly expressed in experimental and human tumor tissues such as human clear-cell renal cell carcinomas (Patel et al., 2005) and colon (Jubb et al., 2009), bladder (Patel et al., 2006), and breast cancers (Shi and Harris, 2006). Blocking DLL4 promotes nonproductive angiogenesis which results in an inhibition of tumor growth and a decrease in tissue perfusion accompanied by an increase in vascular density. Furthermore, certain xenografts resistant to anti-VEGF therapy are reported to be sensitive to anti-DLL4 (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). Based on these studies, DLL4 is regarded as a potential target of antiangiogenic therapy. However, few studies have investigated DLL4 expression in human glioma and its relationship with glioma angiogenesis.

The aim of this study was to examine the expression of DLL4 in human gliomas and analyze its correlation with the expression of VEGF, the key modulator of tumor angiogenesis, and hypoxia-induced factor-1 α (HIF-1 α), an important hypoxia-inducible transcription factor involved in tumorigenesis.

Department of Neurosurgery, Zhongnan Hospital of Wuhan University, Wuhan, China *For correspondence : lifenzhi@yahoo.com.cn

Materials and Methods

Patients and tissue samples

Human specimens of 82 primary gliomas and 7 normal brain tissues from patients with severe craniocerebral trauma who underwent internal decompression were resected at the Department Of Neurosurgery, Zhongnan Hospital of Wuhan University in Wuhan, China. Surgeries were performed from 2002 to 2008 and ethical approval was obtained for scientific use of all human tissues from the medical ethics committee of Zhongnan Hospital. The glioma specimens were from 45 males and 37 females, and their ages ranged from 18 to 69 years with a median age of 47.6 years.

All tissues were fixed in buffered 4% paraformaldehyde (pH 7.4) and embedded in paraffin casts before histologic sections were prepared. During the histopathological examinations, the tumors were sorted and graded independently by two experienced neuropathologists using the brain tumor classification of the World Health Organization (2007) (Louis et al., 2007) as follows: 17 cases were diagnosed as diffuse astrocytoma (grade II; A II), 14 as oligodendrogloma (grade II; O II), 13 as anaplastic astrocytoma (grade III; A III), 14 as anaplastic oligodendrogloma (grade III; O III) and 24 cases as glioblastoma multiforme (grade IV; GBM).

Immunohistochemistry

Immunohistochemistry experiments were performed as described previously (Gesuete et al., 2009; Kang et al., 2010) with some modification. All paraffin-embedded tissues were serially sectioned; sections were 4 μ m. Immunohistochemical staining was carried out using the biotin-streptavidin method. Briefly, paraffin-embedded tissue sections were dewaxed and rehydrated through an alcohol series, and then endogenous peroxidase activities were blocked. After non-specific sites were saturated with 5% normal goat serum, the sections were incubated sequentially in the primary antibodies, a biotinylated secondary antibody and biotin-peroxidase complex. Finally, the sections were counterstained with hematoxylin. Staining without primary antibody in parallel served as negative control. The primary antibodies used were as follows: DLL4 (polyclonal, 1:250, Abcam, Cambridge, UK), VEGF (monoclonal, 1:50, Santa Cruz, CA, USA), HIF-1 α (monoclonal, 1:100, Santa Cruz), and CD34 (monoclonal, 1:50, Dako, Carpinteria, CA).

Evaluation of DLL4, VEGF and HIF-1 α staining

The number of tumor or endothelial cells exhibiting DLL4, VEGF or HIF-1 α staining was counted under a light microscope at a magnification of $\times 200$. Five random fields were examined for each tumor specimen. The expression was classified as negative (-) if <10% of cells were positive, and otherwise as positive. A semiquantitative grading scale was adopted: weak immunoreactivity (10–25%; +), moderate immunoreactivity (25–50%; ++), strong immunoreactivity ($\geq 50\%$; +++).

Determination of microvessel density (MVD)

MVD was counted under a light microscope at a

magnification of $\times 400$ of CD34-labelled microvessels in areas showing the most intense vascularization, initially located at low magnification. Each positive endothelial cell (EC) or group of cells was counted as an individual vessel. The mean vessel count from three fields was used as the number of microvessels.

Statistical analysis

Data were presented as mean \pm standard error (SE). All data were analyzed using Statistical Package for the Social Sciences (SPSS) 13.0 software. The chi-square (χ^2) test and Spearman's rank correlation (r_s) analysis were performed. A probability (P)-value less than 0.05 was considered statistically significant.

Results

Expression pattern of DLL4, VEGF, HIF-1 α in gliomas

According to the results of immunohistochemical staining, DLL4 was primarily distributed in the cytoplasm of tumor vascular ECs in all categorical glioma. An occasional immunoreactivity of DLL4 protein was observed in sparse tumor cells in diffuse astrocytoma and oligodendrogloma, but some tumor cells in anaplastic astrocytoma, oligodendrogloma and GBM, especially those in perinecrotic areas, showed positive cytoplasmic staining for DLL4 protein. Notably, DLL4 protein was also detected in ECs in invading nestin lesions (Figure 1).

The immunoreactivity of VEGF was mainly observed in the cytoplasm of tumor cells in both astrocytic and oligodendrocyte gliomas, and a variable positive staining was also observed in some ECs of tumor blood vessels and extracellular matrix. Unlike VEGF, the immunoreactivity of HIF-1 α was only observed in the nucleus or cytoplasm of tumor cells in astrocytic and oligodendrocyte gliomas, but not in ECs and extracellular matrix. As it was for DLL4, positive VEGF and HIF-1 α staining were also remarkable in perinecrotic areas. No immunostaining for these 3 proteins was observed in parenchymal or vascular cells in any of the 7 normal brain tissues.

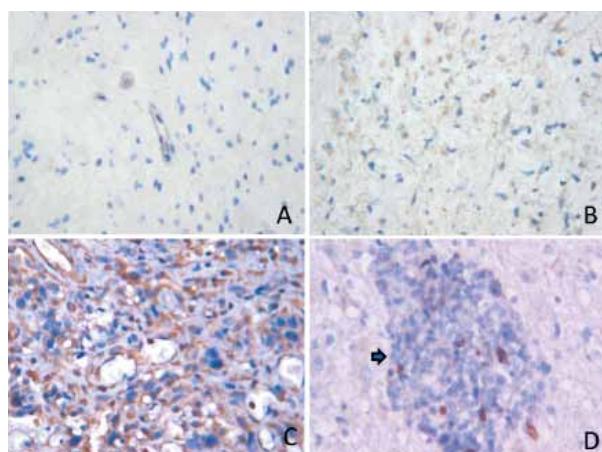


Figure 1. Immunohistochemical Staining of DLL4 in Human Brain Gliomas. Note lack of staining in normal brain tissue (A). In astrocytoma (B) and GBM (C), DLL4 immunostaining (brown) was primarily distributed in the cytoplasm of vascular ECs and occasionally tumor cells. The same was found for invading nestin lesions (D)

Expression of Delta-like Ligand 4 (DLL4) Correlates with that of VEGF and HIF-1 α in Human Glioma

Between the expression of DLL4 and HIF-1 α , a positive correlation was also observed ($r_s = 0.244, P = 0.027$).

Discussion

Glioma is characterized by rapid growth, intense angiogenesis, vascular malformations and a tendency to recur. Because of the abundant vessels existed in glioma, the translation of antiangiogenic therapy from laboratory to the clinic is most noteworthy for glioma treatment.

Notch signaling is an evolutionarily conserved signaling pathway important for intercellular communication and cell fate decision. Four Notch receptors (Notch1, 2, 3, 4) and 5 ligands (Jagged 1 and 2, and Delta-like 1, 3, and 4) have been identified in mammals (Yamanda et al., 2009). An optimal range of Notch signaling is required for vascular development, not only for embryonic vasculature but also in tumor angiogenesis. Notch1, Notch4, Jagged1, and DLL4 are reported to participate in tumor angiogenesis. DLL4 is the most recently identified Notch ligand which could interact with Notch1 and Notch4 (Yamanda et al., 2009).

To our knowledge, in this study we are the first to examine DLL4 expression in human glioma tissues using immunohistochemistry. Our results showed that DLL4 expression is upregulated in human categorical gliomas compared with normal brain tissues, and the immunoreactivity of DLL4 is stronger in high grade tumors than low grade tumors. Our results are consistent with other studies which showed that DLL4 is highly expressed in human tumors. For example, DLL4 expression was observed preferentially in the endothelium of 71% of colon cancers, but not in the endothelium adjacent to normal mucosa (Jubb et al., 2009). The expression of DLL4 was also significantly upregulated in human superficial and invasive bladder cancer, clear-cell renal cell carcinoma, breast cancer, and pancreatic cancer (Buchler et al., 2005; Patel et al., 2005; Patel et al., 2006; Shi and Harris, 2006). In transgenic livers, Hainaud et al., (2006) showed that DLL4 was gradually upregulated as hepatocarcinoma progressed, and expressed in tumor sinusoidal ECs. Furthermore, in the present study DLL4 expression was also detected in ECs in invading nestin lesions, suggesting that DLL4 may play an important role in the early stage of glioma invasion.

Because VEGF is considered the key proangiogenic factor during glioma angiogenesis and the cross-talk between VEGF and DLL4 in ECs during vascular differentiation has been reported (Liu et al., 2003; Thurston and Kitajewski, 2008; Benedito et al., 2009), we evaluated the association of DLL4 with VEGF and microvessel number. A significant positive correlation was revealed between DLL4 and VEGF, and between DLL4 and MVD. As we know, angiogenesis requires a tightly coordinated balance between EC sprouting and the maintenance of existing vascular tubes. This equilibrium has been shown to be controlled by DLL4 expression in endothelial tip cells, which activates Notch signaling and thereby suppresses sprouting in adjacent ECs (Benedito et al., 2009). Accordingly, impaired DLL4/Notch expression or function leads to excessive but nonproductive sprouting

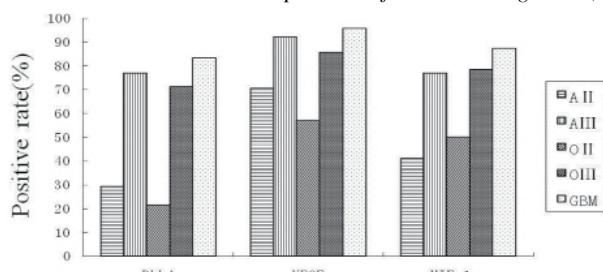


Figure 2. DLL4, VEGF, HIF-1 α Expression in Different Histological and Malignant Gliomas. No significant difference in DLL4, VEGF or HIF-1 α expression was observed between astrocytic tumors and oligodendroglial tumors ($P > 0.05$). However, positive staining for DLL4, VEGF and HIF-1 in high grade glioma (AIII n=13, OIII n=14, GBM n=24) were significantly increased compared with those in low grade glioma (AII n=17, OII n=14) ($P < 0.01$)

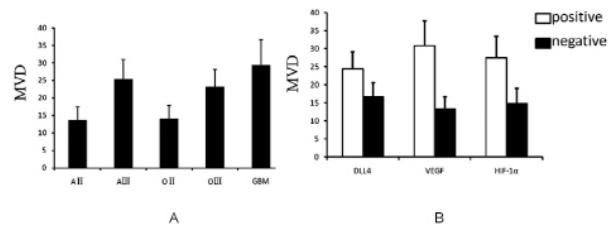


Figure 3. MVD in Glioma with Different Pathological Grade and Different Expression of DLL4, VEGF and HIF-1 α . A, in high grade glioma (AIII n = 13, OIII n = 14, GBM n = 24), MVD was significantly higher than in low grade glioma (AII n = 17, OII n = 14) ($P < 0.05$). B, in DLL4 (n = 48), VEGF (n = 67) and HIF-1 α (n = 56) positive staining gliomas, MVD was also higher than in DLL4 (n = 34), VEGF (n = 15) and HIF-1 α (n = 26) negative staining gliomas ($P < 0.05$)

Correlation of DLL4, VEGF, and HIF-1 α expression with pathological grade

In 58.5% (48/82) of all glioma specimens, DLL4 immunostaining was observed in the cytoplasm of tumor vascular ECs, while only in 15.9% (13/82) was DLL4 staining seen in tumor cells. VEGF staining in either tumor cells or tumor vascular ECs was seen in 81.7% (67/82) of the glioma cases. In 68.3% (56/82) of glioma specimens, HIF-1 α positive staining was observed only in tumor cells. The frequency and intensity of DLL4, VEGF and HIF-1 α expression varied by grades of primary gliomas. As the degree of malignancy of the primary glioma increased, positive staining rates for DLL4, VEGF and HIF-1 α increased significantly (Figure 2). No significant difference in DLL4, VEGF and HIF-1 α expression was observed between astrocytic and oligodendroglial tumors.

Relationship of DLL4, VEGF and HIF-1 α expression with MVD

MVD increased significantly as the malignancy of the primary glioma increased. A relationship emerged between the MVD and the expressions of DLL4, VEGF and HIF-1 α . MVD was higher in tumors that stained positive for DLL4, VEGF and HIF-1 α , compared to those that stained negative (Figure 3).

Correlation of DLL4 with VEGF and HIF-1 α in gliomas

A significant positive correlation existed between the expression of DLL4 and VEGF ($r_s = 0.473, P < 0.001$).

because too many ECs respond to VEGF (Benedito et al., 2009). Therefore, it is very important for functional glioma angiogenesis to keep a balance between DLL4 and VEGF. Our results provide further support that these may together regulate the vascular quantity and quality of glioma through cross-talk in signaling pathways.

Besides abundant microvessels, regional necrosis is another common pathological feature in glioma tissues and emerging evidence has suggested that hypoxia is an important modulator in the process of glioma angiogenesis (Jensen, 2009). In a previous study we observed that hypoxic-, but not normoxic-, conditioned media partially blocked endothelial-like cell apoptosis induced by hypoxia. Hypoxic-conditioned media also promoted the cord formation of endothelial-like cells seeded on Matrigel (Xu et al., 2010). It is well known that intratumoral hypoxia can trigger a series of downstream factors such as VEGF through HIF-1 α . Our results showed the positive association of DLL4 with HIF-1 α , indicating that DLL4 expression in glioma may be regulated by hypoxia. Indeed Diez et al., (2007) demonstrated that in various EC lines hypoxic conditions led to the induction of the Notch ligand DLL4 by HIF-1 α .

Angiogenesis emerges as a prospective target for glioma therapy. However, antiangiogenic agents targeting VEGF or its receptors, which have shown strong efficacy in experimental models, did not demonstrate exciting results in clinical trials, implying that other factors are involved. The role of DLL4 in glioma angiogenesis and its correlation with VEGF and HIF-1 α make DLL4 a potentially important target of antiangiogenic therapy. Antitumor effects of DLL4 blockade were recently reported (Noguera-Troise et al., 2006; Ridgway et al., 2006) and characterized by nonproductive angiogenesis, in which thin, dense, and nonperfused vessels with few pericytes were observed. Thus DLL4 blockade could be effective for tumors resistant to anti-VEGF therapy.

Acknowledgments

This work was supported by a grant for young talents from the Hubei Provincial Health Department of China (QJX2008-32). We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

References

- Benedito R, Roca C, Sorensen I, et al (2009). The notch ligands DLL4 and Jagged1 have opposing effects on angiogenesis. *Cell*, **137**, 1124-35.
- Buchler P, Gazdhar A, Schubert M, et al (2005). The Notch signaling pathway is related to neurovascular progression of pancreatic cancer. *Ann Surg*, **242**, 791-800, discussion 800-791.
- Chu SH, Yuan XH, Jiang PC, et al (2005). [The expression of hepatocyte growth factor and its receptor in brain astrocytomas]. *Zhonghua Yi Xue Za Zhi*, **85**, 835-8.
- Diez H, Fischer A, Winkler A, et al (2007). Hypoxia-mediated activation of DLL4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate. *Exp Cell Res*, **313**, 1-9.
- Gesuete R, Storini C, Fantin A, et al (2009). Recombinant C1 inhibitor in brain ischemic injury. *Ann Neurol*, **66**, 332-42.
- Hainaud P, Contreras JO, Villemain A, et al (2006). The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions. *Cancer Res*, **66**, 8501-10.
- Hellstrom M, Phng LK, Hofmann JJ, et al (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, **445**, 776-80.
- Jain RK, di Tomaso E, Duda DG, et al (2007). Angiogenesis in brain tumours. *Nat Rev Neurosci*, **8**, 610-22.
- Jensen RL (2009). Brain tumor hypoxia: tumorigenesis, angiogenesis, imaging, pseudoprogression, and as a therapeutic target. *J Neurooncol*, **92**, 317-35.
- Jouanneau E (2008). Angiogenesis and gliomas: current issues and development of surrogate markers. *Neurosurgery*, **62**, 31-50; discussion 50-32.
- Jubb AM, Turley H, Moeller HC, et al (2009). Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer. *Br J Cancer*, **101**, 1749-57.
- Kang YJ, Digicaylioglu M, Russo R, et al (2010). Erythropoietin plus insulin-like growth factor-I protects against neuronal damage in a murine model of human immunodeficiency virus-associated neurocognitive disorders. *Ann Neurol*, **68**, 342-52.
- Liu ZJ, Shirakawa T, Li Y, et al (2003). Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol*, **23**, 14-25.
- Li Z, Yuan X, Wu T (2005). Expression of VEGF, MMPs and its relation with vascular ultrastructure in primary and recurrent gliomas. *Chin J Neuromed*, **4**, 994-7.
- Louis DN, Ohgaki H, Wiestler OD, et al (2007). The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*, **114**, 97-109.
- Noguera-Troise I, Daly C, Papadopoulos NJ, et al (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature*, **444**, 1032-7.
- Patel NS, Dobbie MS, Rochester M, et al (2006). Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer. *Clin Cancer Res*, **12**, 4836-44.
- Patel NS, Li JL, Generali D, et al (2005). Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res*, **65**, 8690-7.
- Ridgway J, Zhang G, Wu Y, et al (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature*, **444**, 1083-7.
- Scehnet JS, Jiang W, Kumar SR, et al (2007). Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood*, **109**, 4753-60.
- Shi W, Harris AL (2006). Notch signaling in breast cancer and tumor angiogenesis: cross-talk and therapeutic potentials. *J Mammary Gland Biol Neoplasia*, **11**, 41-52.
- Shutter JR, Scully S, Fan W, et al (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev*, **14**, 1313-8.
- Thurston G, Kitajewski J (2008). VEGF and Delta-Notch: interacting signalling pathways in tumour angiogenesis. *Br J Cancer*, **99**, 1204-9.
- Yamanaka R, Saya H (2009). Molecularly targeted therapies for glioma. *Ann Neurol*, **66**, 717-29.
- Yamada S, Ebihara S, Asada M, et al (2009). Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade. *Blood*, **113**, 3631-9.
- Xu X-Z, Li Z-Q, Wen Z-H (2010). Glioma-conditioned medium blocks endothelial cells' apoptosis Induced by hypoxia and promotes its angiogenesis via up-regulation of u-PA/u-PAR. *Chinese J Cancer Res*, **22**, 119-25.