

Expression of CXC-motif-chemokine 12 and the receptor C-X-C receptor 4 in glioma and the effect on peritumoral brain edema

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Abstract. The present study aimed to evaluate the association between CXC-motif-chemokine 12 (CXCL12)/C-X-C receptor 4 (CXCR4) expression and peritumoral brain edema (PTBE) in glioma patients. Immunohistochemical techniques were used to detect the expression of CXCR4 and CXCL12 in 58 glioma tissues. Magnetic resonance imaging was used to evaluate the extent and type of brain edema in preoperative glioma patients. The association between edema and CXCL12/CXCR4 expression was examined by χ^2 analysis. The prognostic significance of CXCL12 or CXCR4 was determined by log-rank tests and Cox's proportional hazards model. Expression of CXCL12 and CXCR4 was observed in vascular endothelial cells and tumor cells. The degree ($P=0.033$) and morphology ($P=0.033$) of PTBE were significantly associated with the level of CXCL12 expression in vascular endothelial cells. The degree ($P=0.001$) and morphology ($P=0.001$) of PTBE were associated with the level of CXCR4 expression in tumor cells. CXCR4-positive vascular endothelial cells were significantly associated only with the degree of edema ($P=0.030$). Therefore, the present study indicated that levels of CXCL12 expression in vascular endothelial cells and CXCR4 expression in tumor cells are associated with PTBE.

Introduction

Glioma, which is the most common intracranial tumor in adults, is characterized by high vascularization and invasive capacity, as well as peritumoral brain edema (PTBE) (1-4). In preoperative magnetic resonance imaging (MRI), the degree and morphological characteristics of PTBE vary between different

types of glioma (5). PTBE, which facilitates glioma cell invasion and significantly affects patient prognosis, is a major cause of the high disability and mortality rate of glioma (4,6). A previous study has demonstrated that glioma recurrence patterns are affected by the degree and morphological characteristics of PTBE (7). In addition, a study has identified that PTBE is an imaging feature of glioma cell invasion, a complex process regulated by specific signaling pathways (8). Previous studies have shown that the cell surface C-X-C receptor 4 (CXCR4), and its protein ligand, CXC-motif-chemokine 12 (CXCL12), are overexpressed in glioma tissues (9-11). The CXCR4-CXCL12 signaling pathway regulates glioma cell differentiation, invasion and metastasis (12,13). According to the law of tissue remodeling presented by Lin (14), tissue morphology is the result of the interactions among molecules within the tumor microenvironment. It was hypothesized that the CXCR4-CXCL12 signaling pathway affects the degree and morphological characteristics of PTBE. To the best of our knowledge, there are currently no studies describing the association between the CXCR4-CXCL12 axis and PTBE in glioma patients. Therefore, the present study investigated the association between CXCR4/CXCL12 and PTBE in glioma tissues.

Materials and methods

Study samples. The clinicopathological data of patients with supratentorial glioma ($n=58$; 41 male, 17 female) treated at the First Affiliated Hospital of Fujian Medical University (Fujian, China) between December 2007 and April 2014 were collected retrospectively. The median age was 44.57 ± 13.90 years (range, 17-75 years). The pathological diagnosis of each patient was confirmed by neuropathologists based on the brain tumor pathology classification standard of 2007 (15). All patients received postoperative chemoradiotherapy. Chemotherapy (at least four cycles) with temozolomide was administered at a dose of 150-200 mg/m²/day. Radiotherapy (60 Gy in 2 Gy fractions) was applied to the field of the tumor lesion plus a 2 cm margin, according to MRI at the time of the second chemotherapy cycle. The present study was approved by the Ethics Committee of Fujian Medical University (Fuzhou, China), and all enrolled patients provided written informed consent for inclusion in the present study.

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Immunohistochemistry. Glioma and adjacent non-tumor tissue acquired during surgery from the patients described previously were embedded in paraffin immediately and sectioned to 5 μ m using a microtome. Sections were mounted on glass slides, dewaxed and hydrated for immunohistochemistry. For CXCL12 and CXCR4, heat-induced epitope retrieval was performed by boiling the tissue sections in 10 mmol/l citrate buffer (pH 6.0) for 15 min in a microwave oven. Subsequent to cooling to room temperature, the sections were exposed to methanol containing 3% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase. CXCL12 was detected with an anti-human CXCL12 monoclonal antibody (5 μ g/ml; cat. no. MAB350; R&D Systems, Inc., Minneapolis, MN, USA) for 1 h at room temperature. CXCR4 was detected with an anti-human CXCR4 monoclonal antibody (0.5 μ g/ml; cat. no. MAB172, R&D Systems, Inc.) for 1 h at room temperature. The slides were then washed with PBS and incubated with the secondary detection antibody, polyperoxidase-anti-rabbit/mouse IgG (dilution, 1:1,000; cat. no. C0101, ZSGB-BIO, Beijing, China) for 50 min at room temperature. Then the slides were treated with diaminobenzidine (dilution, 1:1,000; cat. no. 070004; Cell Chip Biotechnology Co., Beijing, China) as per the manufacturer's protocol. The sections were counterstained with hematoxylin and mounted in gelatin. Gastric cancer and tonsil tissues were used as positive controls for CXCR4 and CXCL12 expression, respectively. Gastric cancer tissues were acquired from a patient with postoperative pathological diagnosis of gastric carcinoma. Tonsil tissues were acquired from a normal control. PBS was substituted for the primary antibodies as a negative control.

Histological assessment. Immunohistochemical staining of five randomly selected fields for each tumor specimen was evaluated under a light microscope (magnification, $\times 200$) and classified according to the method proposed by Zagzag *et al* (16): Low expression, staining $<5\%$ of structures; high expression, staining of $>5\%$ of structures. The results were evaluated independently by two pathologists. In cases of discordance, a third pathologist reviewed the results to achieve a consensus.

Imaging methodology and analyses. Preoperative MRI scans [T1 weighted image (T1WI), T2 weighted image (T2WI), axes, arrows and coronal enhanced images] were performed at the First Affiliated Hospital of Fujian Medical University. The boundaries of the contrast enhancement area on T1 weighted images were drawn. These maps were then compared with their respective T2 weighted images. According to Schoenegger *et al* (2), PTBE was classified according to degree as follows: Minor, edema extending ≤ 1 cm from the tumor margin (Fig. 1A and B) and major, edema extending >1 cm from the tumor margin (Fig. 1C and D). According to Hartmann *et al* (7), the PTBE morphology types were classified as: Ring form, circular region of increased T2 signal intensity (Fig. 1A and B); and irregular form, irregular regions of increased T2 signal intensity (Fig. 1C and D). Based on these definitions, PTBE types and degrees on preoperative MR images were classified by two experienced imageologists. Discrepancies in the classification were reviewed by a third imageologist until a consensus was achieved.

Analysis. Each patient was followed-up in outpatient visits or telephone conversations. The median progression-free survival time (PFS, months) was calculated from the date of surgery to the date of first tumor progression (or mortality or the latest follow-up). The overall survival (OS, months) time was defined as the period from the time of surgical resection to the latest follow-up (or mortality). PFS and OS times for patients without tumor progression or mortality ended with the latest follow-up.

Statistical analysis. Statistical analysis was performed with SPSS 19.0 for Windows (IBM SPSS, Armonk, NY, USA). The associations between age, sex, pathological grade, morphological types of PTBE, degrees of PTBE and CXCR4/CXCL12 expression levels were initially examined by χ^2 analysis or Fisher's exact test. Non-parametric tests were used to analyze the association between pathological grades and expression levels of CXCL12 or CXCR4. The associations between PFS and OS and CXCL12/CXCR4 expression levels were determined by log-rank tests and presented as Kaplan-Meier curves. In multivariate analyses, the COX proportional hazards model was adopted to assess the effects of CXCL12/CXCR4 expression levels on PFS and OS. In addition, hazard ratios as well as the corresponding 95% confidence intervals were calculated. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CXCL12 and CXCR4 expression in glioma tissues. Immunohistochemical staining demonstrated that CXCL12 and CXCR4 were located in the cell membrane and cytoplasm. Widespread staining of CXCL12, but not CXCR4, was also observed in adjacent normal brain tissues. In the 58 glioma tissue samples, CXCL12 expression was observed mainly in the vascular endothelial cells, with low levels of expression also observed in tumor cells. CXCL12 positive staining was detected in 52/58 glioma specimens. In vascular endothelial cells, CXCL12 expression was detected in glomeruloid vessels (Fig. 2A) and non-glomeruloid vessels (Fig. 2B), with low expression identified in 26 and high expression in 32 glioma tissue samples. In addition, CXCL12-positive endothelial cells were particularly high expression in areas of tumor adjacent to necrosis (Fig. 2C). In tumor cells (Fig. 2D), low CXCL12 expression was detected in 49 samples and high expression in 9 samples.

CXCR4 expression was detected in 52/58 glioma tissues, mainly in tumor cells (Fig. 2E and F), with particularly high expression in areas of tumor adjacent to necrosis (Fig. 2G). In addition, CXCR4 with low levels of expression also observed in vascular endothelial cells (Fig. 2H). Low CXCR4 expression in tumor cells was detected in 19 samples and high expression was detected in 39 samples. In vascular endothelial cells, low expression was detected in 36 samples and high expression was detected in 22 samples.

Notably, in tumor cells with high CXCR4 expression, the expression of CXCL12 in vascular endothelial cells was also high in the same specimens. It was also revealed that CXCR4 was highly expressed in tumor cells, and CXCL12 was lowly expressed in tumor cells in the same tissue. Similarly, in

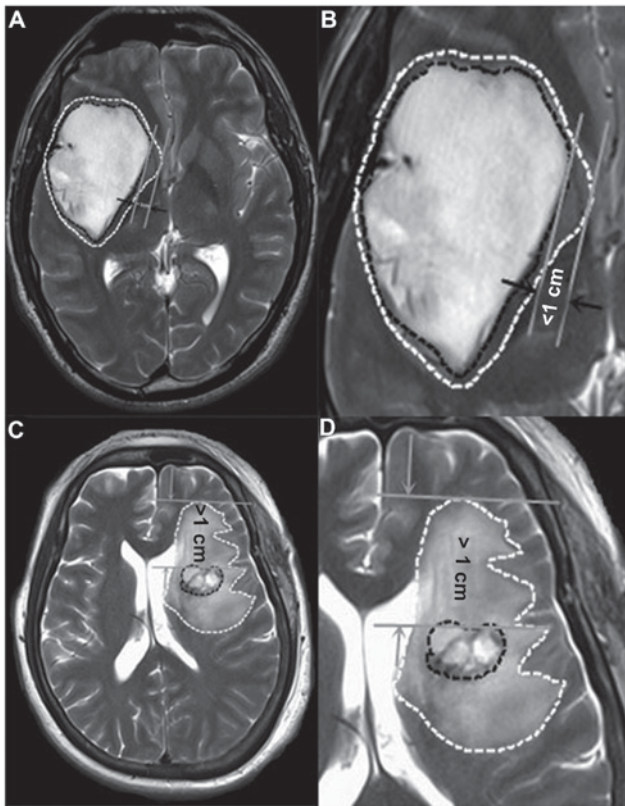


Figure 1. Measurement of peritumoral edema in magnetic resonance imaging T2 weighted images. (A) Circular edema extending <1 cm from the tumor margin, defined as minor edema in ring form. (B) Image A at $\times 10$ magnification. (C) Irregular edema extending >1 cm from the tumor margin, defined as major edema in irregular form. (D) Image C at $\times 10$ magnification.

vascular endothelial cells, high CXCL12 expression was associated with low CXCR4 expression in tumor cells.

Association between CXCL12 and CXCR4 expression and clinicopathological features. The associations between CXCL12 expression and clinicopathological features are listed in Table I. CXCL12 expression in vascular endothelial cells was significantly associated with age ($P<0.0001$) and pathological grade ($P<0.0001$), while the level of CXCL12-positive tumor cells was significantly associated only with pathological grade ($P=0.003$). However, no association was observed between patient sex and CXCL12 expression in endothelial or tumor cells.

CXCR4 expression in tumor cells was significantly associated with age ($P=0.002$) and pathological grade ($P<0.0001$). The level of CXCR4-positive vascular endothelial cells was also significantly associated with age ($P=0.016$) and pathological grade ($P<0.0001$). However, CXCR4 expression was not associated with patient sex in tumor or endothelial cells (Table II).

Association between CXCL12 and CXCR4 expression and PTBE. PTBE is one of the most important features of gliomas on preoperative MRI; therefore, the association between CXCL12 and CXCR4 expression and PTBE in gliomas was investigated. CXCL12 expression in vascular endothelial cells was significantly associated with edema degree ($P=0.033$) and edema morphology ($P=0.033$), while the level of

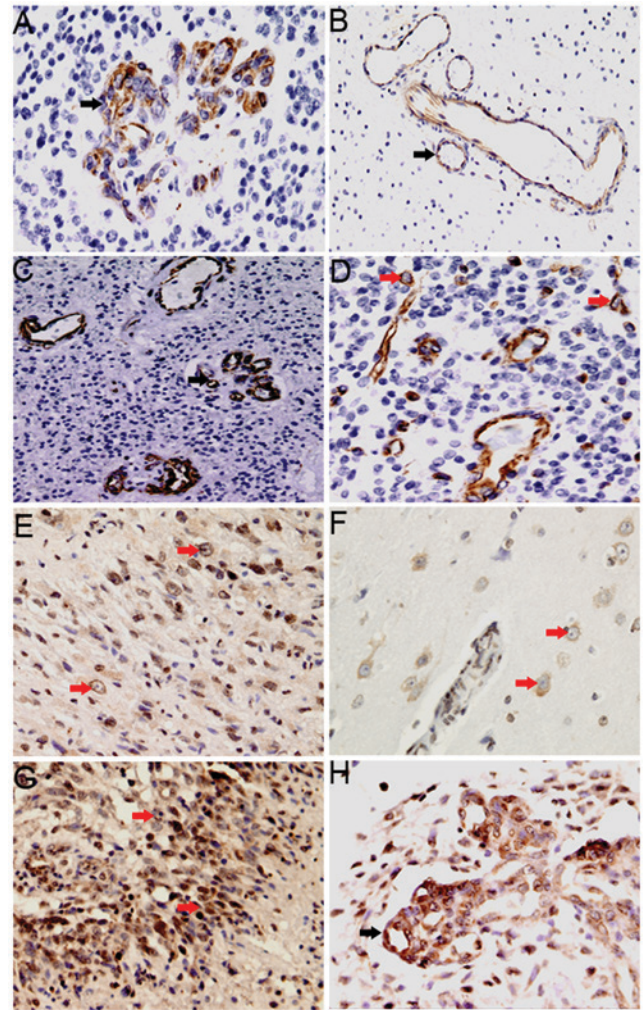


Figure 2. Degree and cellular distribution of CXCL12 and CXCR4 expression in glioma tissues. (A-D) CXCL12 expression in vascular endothelial cells (black arrow) and in tumor cells (red arrow). (A) CXCL12 expression in glomeruloid vessels (magnification, $\times 200$). (B) CXCL12 expression in the non-glomerular vessels of the peritumoral edema area (magnification, $\times 200$). (C) CXCL12 is expressed in vascular endothelial cells of the surrounding necrotic area (magnification, $\times 200$). (D) CXCL12 expression in tumor cells (magnification, $\times 200$). (E-H) CXCR4 expression in tumor cells (red arrow) and endothelial cells (black arrows). (E) CXCR4 expression in tumor cells (magnification, $\times 200$). (F) CXCR4 expression in perivascular tumor cells of the peritumoral edema area (magnification, 400). (G) CXCR4 expression in tumor cells in the surrounding necrotic area (magnification, $\times 200$). (H) CXCR4 expression in glomeruloid vessels (magnification, $\times 200$). CXCR4, C-X-C receptor 4; CXCL12, CXC-motif-chemokine 12.

CXCL12-positive tumor cells was not significantly associated with edema degree ($P=0.067$) or morphology ($P=0.167$; Table I).

For CXCR4, the degree ($P=0.001$) and morphology ($P=0.001$) of PTBE were positively-associated with the expression of CXCR4 in tumor cells. The level of CXCR4-positive vascular endothelial cells was significantly associated with edema degree ($P=0.030$), while no association was observed between edema morphology and CXCR4 expression in vascular endothelial cells ($P=0.106$; Table II).

Association between CXCL12 and CXCR4 expression and patient clinical outcome. To investigate the association between CXCL12 and CXCR4 expression and patient prognosis,

Table I. Association between CXCL12 and characteristics of patients.

Patients features	Tumoral CXCL12, n		P-value	Endothelial CXCL12, n		P-value
	Low	High		Low	High	
Age			0.473			<0.0001
<44.57 years	25	3		20	8	
≥44.57 years	24	6		6	24	
Gender			0.426			0.778
Male	36	5		19	22	
Female	13	4		7	10	
Pathological grade			0.003			<0.0001
II	19	1		16	4	
III	16	0		8	8	
IV	14	8		2	20	
Edema degree			0.067			0.033
Slight	23	1		15	9	
Severe	26	8		11	23	
Edema morphology			0.167			0.033
Ring form	24	2		16	10	
Irregular form	25	7		10	22	

CXCL12, CXC-motif-chemokine 12. P-values were calculated with χ^2 analysis.

Table II. Association between CXCR4 and characteristics of patients.

Patients features	Tumoral CXCL12, n		P-value	Endothelial CXCL12, n		P-value
	Low	High		Low	High	
Age			0.002			0.016
<44.57 years	15	13		22	6	
≥44.57 years	4	26		14	16	
Gender			1.000			0.773
Male	14	27		26	15	
Female	5	12		10	7	
Pathological grade			<0.0001			<0.0001
II	13	7		19	1	
III	4	12		9	7	
IV	2	20		8	14	
Edema degree			0.001			0.030
Slight	14	10		19	5	
Severe	5	29		17	17	
Edema morphology			0.001			0.106
Ring form	14	12		19	7	
Irregular form	4	28		16	16	

CXCR4, C-X-C receptor 4. P-values were calculated with χ^2 analysis.

corresponding PFS and OS were calculated from the study patients (Table III). The median PFS was 18.12 months (range, 1-50 months)

and the median OS was 25.33 months (range, 2-58 months). It was identified that high CXCL12 expression in tumor cells in

Table III. Cox proportional hazards regressions for PFS and OS in gliomas.

Variables	PFS		OS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≥ 44.57 vs. <44.57 years)	1.14 (0.46-2.84)	0.772	2.46 (0.80-7.59)	0.851
Gender (male vs. female)	1.66 (0.69-4.02)	0.258	1.11 (0.37-3.31)	0.119
Pathological grade (II vs. III vs. IV)	3.77 (1.88-7.58)	<0.0001	2.98 (1.12-7.49)	0.020
Edema (slight vs. severe)	12.66 (1.96-81.20)	0.007	0.50 (0.05-5.32)	0.564
Edema (ring form vs. irregular form)	0.16 (0.03-0.84)	0.030	3.43 (0.37-31.95)	0.278
CXCL12 expression in TC (high vs. low)	1.90 (0.75-4.95)	0.172	0.41 (0.11-1.51)	0.181
CXCL12 expression in EC (high vs. low)	0.85 (0.34-2.12)	0.719	3.15 (0.96-10.31)	0.058
CXCR4 expression in TC (high vs. low)	0.69 (0.26-1.84)	0.460	1.72 (0.47-6.27)	0.414
CXCR4 expression in EC (high vs. low)	0.74 (0.30-1.82)	0.513	0.47 (0.11-2.07)	0.315

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; CXCL12, CXC-motif-chemokine 12; CXCR4, C-X-C receptor 4; TC, tumor cell; EC, endothelial cell.

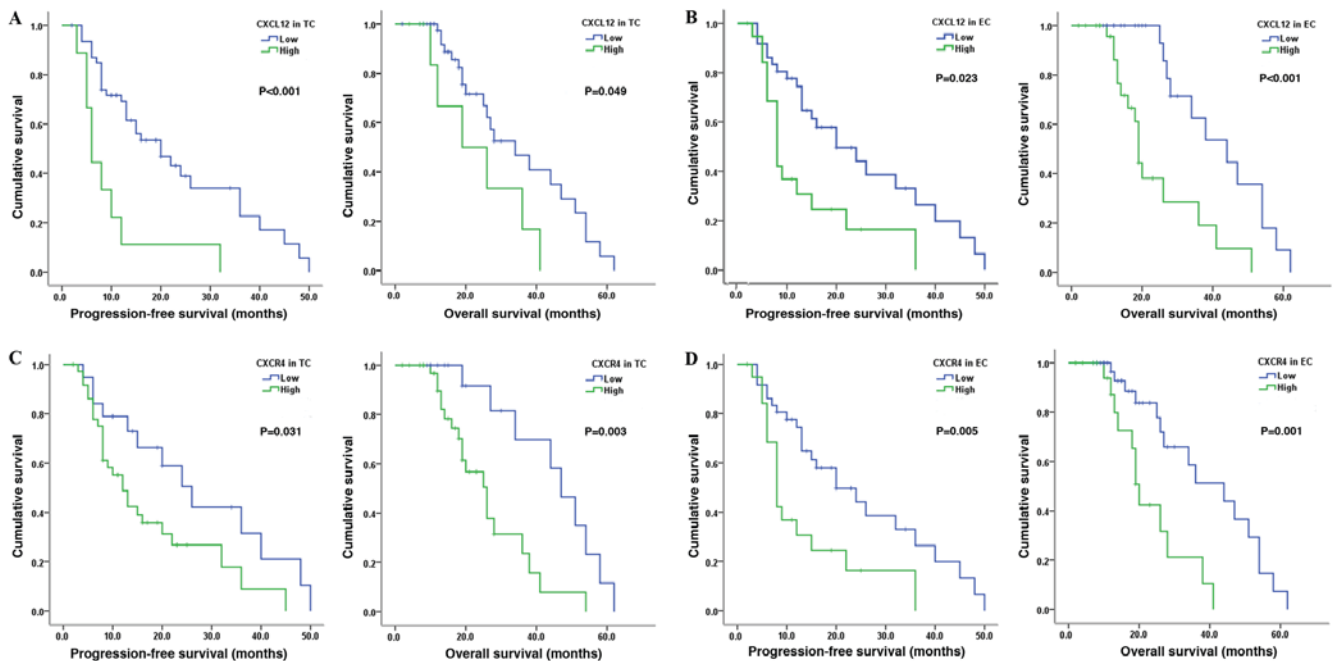


Figure 3. CXCL12 and CXCR4 expression affects PFS and OS in patients with glioma. Kaplan-Meier plots showing an association between CXCL12 expression and PFS and OS in (A) tumor cells and (B) endothelial cells. Kaplan-Meier plots showing an association between CXCR4 expression and PFS and OS in (C) tumor cells and (D) endothelial cells. TC, tumor cells; EC, endothelial cells; PFS, progression-free survival; OS, overall survival; CXCR4, C-X-C receptor 4; CXCL12, CXC-motif-chemokine 12.

patients with glioma was associated with reduced PFS ($P<0.001$) and OS ($P=0.049$; Fig. 3A). Similar results were observed in patients with high CXCL12 expression in vascular endothelial cells (PFS, $P=0.023$; OS, $P<0.001$; Fig. 3B). For CXCR4, the PFS ($P=0.031$) and the OS ($P=0.003$) were short in patients with high CXCR4 expression in tumor cells (Fig. 3C). Furthermore, a similar association between high CXCR4 expression in vascular endothelial cells and short PFS ($P=0.005$) and OS ($P=0.001$) times was observed (Fig. 3D). Multivariate analysis showed that, regardless of the level or site of expression, CXCL12 and CXCR4 were not independent prognostic factors in patients with glioma; only pathological grade was found to be an independent prognostic factor.

Discussion

PTBE is a type of vasogenic edema, which results from interactions among cytokines secreted by nests of tumor and endothelial cells (6). CXCR4 is an a chemokine G-protein coupled receptor with seven-transmembrane alpha helices and 352 residues (17). The associated ligand CXCL12, also termed stromal-derived factor-1a, has long been known to regulate lymphocyte chemotaxis and the development of lymphoid organ structure (18). The binding of CXCR4 and CXCL12 activates downstream signaling molecules, including extracellular signal-regulated kinase-1/2, mitogen-activated protein kinase, protein kinase B/phosphoinositide 3-kinase, nuclear

factor- κ B and c-Jun N-terminal kinase, which promote the differentiation and invasion of tumor cells (19-22). In addition, activation of the CXCL12-CXCR4 signaling pathway induces angiogenesis in glioma (23,24), and blockade of this pathway has been demonstrated to reduce the density of tumor angiogenesis (25,26). Therefore, the CXCR4-CXCL12 signaling pathway may affect the degree and morphological characteristics of PTBE.

In the present study, immunohistochemical staining demonstrated that CXCL12 was expressed mainly in vascular endothelial cells, while CXCR4 was mainly expressed in tumor cells, with particularly strong staining around the necrotic area. The degree and form of PTBE reflected the expression levels of CXCL12 in vascular endothelial cells and CXCR4 in tumor cells. In addition, CXCL12-positive endothelial cells were observed at the site of peritumoral edema and CXCR4-positive tumor cells were detected within the area of peritumoral edema or perivascular regions. These results indicated that the expression levels of CXCL12 in vascular endothelial cells and CXCR4 in tumor cells are associated with PTBE.

In addition, the association between CXCL12/CXCR4 expression and clinicopathological features was analyzed. Previous studies in patients with glioma have revealed an association between CXCR4 and clinicopathological features or patient survival time (10,27). In the present study, it was found that age was associated with CXCR4 expression in tumor cells and vascular endothelial cells. However, only patient age was associated with CXCL12 expression in vascular endothelial cells. These data are consistent with the observation by Schoenegger *et al* (2) that the incidence of PTBE is associated with patient age. In the present study, CXCL12/CXCR4 expression in tumor cells and vascular endothelial cells was associated with pathological grade. In addition, Kaplan-Meier survival curve analysis revealed significantly shorter PFS and OS for patients with high CXCL12 and CXCR4 expression in tumor cells and/or endothelial cells. However, multivariate analysis found that CXCL12 and CXCR4 expression in tumor and endothelial cells were not independent prognostic factors for patients with glioblastoma.

In summary, the present data indicated that the expression levels of CXCL12 in vascular endothelial cells and CXCR4 expression in tumor cells are associated with the degree and morphological characteristics of PTBE, while CXCL12/CXCR4 expression is not an independent prognostic factor for glioma patients. However, it should be noted that the present study is subject to the limitations of a retrospective study design, potentially inaccurate data collection that relied on patient memory and a small sample size. Large-scale randomized multicenter studies are required to analyze the function of the CXCL12-CXCR4 signaling pathway in patients with glioma.

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