C-C motif chemokine ligand 2/C-C receptor 2 is associated with glioma recurrence and poor survival

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Abstract. Several studies have explored the mechanisms of C-C motif chemokine ligand (CCL)2/CC receptor (R)2 function in tumorigenesis and inflammation. However, little is known about the role of CCL2/CCR2 in tumor recurrence, especially after radiotherapy. The present study aimed to determine the association between CCL2/CCR2 and glioma relapse. Moreover, the difference in the expression of CCL2/CCR2 between post-radiation and non-radiation recurrent glioma tissues was compared. A retrospective analysis of 80 patients with glioma who underwent tumor resection twice was performed. Primary group refers to glioma patients who received glioma resection surgery for the first time. Recurrent group refers to glioma patients who received glioma resection surgery after first relapse. In total, 10 patients with brain trauma who underwent partial resection of the normal brain as decompression treatment were used as controls. Protein expression levels of CCL2 and CCR2 were evaluated using immunohistochemistry. Prognostic analyses of patient survival using Kaplan-Meier curves and Cox regression models were performed. The expression levels of CCL2 and CCR2 were higher in recurrent glioma compared with the primary group. There was a positive correlation between tumor grade and protein expression of CCL2/CCR2. Furthermore, irradiation had a significant effect on CCR2 protein expression (P=0.014), but not on CCL2 protein expression (P=0.626). However, the expression of CCL2 and CCR2 showed no significant difference between primary and secondary glioblastoma. After adjusting for sex, radiotherapy and location of tumors in these gliomas, CCL2 was a prognostic factor for disease-free and overall survival (OS) times, as well as age and tumor grade. In the multivariate Cox modeling for glioma, CCR2 was significantly associated with OS rather than DFI. The significant correlations between CCL2/CCR2 expression and glioma tumor grade suggested that CCL2/CCR2 has a role in glioma progression. Combined with previous *in vitro* experiments, it was proposed that irradiation (radiotherapy)-induced expression of CCL2 is transient, while irradiation-induced expression of CCR2 is lasting. Therefore, CCL2/CCR2 is a potential therapeutic target for patients with glioma.

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Introduction

Glioma is the most common tumor type in the central nervous system, and glioblastoma multiforme (GBM; grade IV according to the the 2016 WHO Classification of Glioma) is the most lethal, with a median survival of 14.6 months (1-4). The median recurrence period of GBM is 6-12 months, and that of anaplastic glioma (grade III) is 18-36 months (5). Studies have shown that the malignancy and invasiveness of glioma is increased after recurrence (4,6). Considering that the recurrence and prognosis of malignant gliomas largely depends on the extent of invasion of tumors into normal brain parenchyma, brain radiotherapy with enlarged field is the recommended treatment to target the maximum number of tumor cells possible. However, clinical studies show that, even if patients with glioma received extended resection and field radiotherapy, ~80% of malignant glioma recurrence is located within 2-3 cm of the resected margin (7,8).

In addition to residual tumor cells and radioresistance, the tumor microenvironment also plays an important role in tumor recurrence (9). There are numerous different stromal cells aside from tumor cells in the tumor lesions, including include endothelial cells as well as inflammatory cells, that constitute tumor microenvironment (10). Recently, more and more studies have revealed that the tumor microenvironment plays an important role in the process of tumorigenesis, tumor

development, chemo- and radio-resistance and tumor recurrence (11-15). However, until now, the underlying mechanisms for these processes in glioma were unclear.

The C-C motif chemokine ligand (CCL)2, one of the most important members of the chemokine family of proteins, was the first C family chemokine to be cloned and identified (16). CCL2 is highly expressed in several human central nervous system tumors, such as glioma, meningioma and Schwannoma (17-19). Production of CCL2 in tumor microenvironments can be stimulated by IL-1β and TNF-α (17). In normal brain tissues, CCL2 is primarily secreted by endothelial cells, fibroblasts, microglia, astrocytes and monocytes and its expression is relatively low (17). CCL2 functions by binding to the CC receptor (R)2, leading to accelerated Ca^{2+} influx, cAMP inhibition, and phospholipase C and phosphoinositol-3 kinase activation (20). The activation of CCL2/CCR2 signaling recruits monocytes from the bloodstream through the vascular endothelium and regulates the routine immunological surveillance of brain tissues (21).

Several studies have revealed that CCL2/CCR2 signaling is a promising target in patients with tumors and inflammation (16,22-24). However, little is known on the role of CCL2/CCR2 in tumor recurrence, especially after radiotherapy. Our previous study proposed that after radiotherapy, irradiated brain tissues promote the ability of tumor cells to invade and metastasize by secreting cytokines and proteases, which may partly result in the recurrence and progression of glioma (25). The present study investigated the expression of CCL2 and CCR2 in primary and recurrent glioma and analyzed the association with glioma relapse. The difference in the expression of CCL2/CCR2 between post-radiation and non-radiation recurrent glioma was also compared.

Materials and methods

Patients and tissue specimens. The records of 80 patients with glioma who underwent two glioma resections in Qilu Hospital of Shandong University (Jinan, China) between January 1995 and December 2015 were analyzed. The tissue specimens were evaluated by two pathologists experienced in glioma pathology. All specimens were collected retrospectively from primary tumors and their corresponding recurrent tumors. All patients were treated according to the guidelines used in our institutions. The present study complied with national regulations, and was approved by The Institutional Ethics and Investigation Committee of Qilu Hospital, Shandong University [approval no. KYLL-2015(KS)-068].

The inclusion criteria were as follows: i) All the cases were solitary lesions and pathologically diagnosed as glioma (2016 WHO classification of tumors of the central nervous system (3)); ii) both the first and second operations were performed in Qilu Hospital; iii) cranial MRI was performed after the first operation with detection of enhanced new lesions, or >25% larger than before, iv) the expected survival time was >8 weeks and v) the Karnofsky score (KPS) was >60 (26). Patients with irradiation necrosis were excluded. According to whether the patients received radiotherapy after the first operation, they were divided into two groups: Post-radiation group (first operation, radiotherapy, recurrence, second operation) and non-radiation group (first operation, recurrence, second operation). 33 cases of primary (*de novo*) and 24 cases of secondary GBM (progressed from low-grade or anaplastic glioma) were included in this study. In addition, 10 non-tumor brain specimens were collected from patients with brain trauma who underwent partial resection of normal brain as decompression treatment for severe head injuries (Qilu Hospital of Shandong University) between January 2013 and December 2015. The clinicopathological characteristics of patients are listed in Table SI. Informed consent was provided by all individual participants included in the study (consent of patients <18 years old was provided by their guardians). Follow-up was performed over the phone and the deadline was April 2019. The time between surgery and disease recurrence was defined as the disease-free interval (DFI). The overall survival (OS) time of the primary tumors was calculated from the date of the operation following primary diagnosis to the date of death or last follow-up.

Immunohistochemistry. Paraffin sections (4 μm) were deparaffinized with xylene and ethanol gradient (100, 95 and 80%) methods at 65˚C for 30 h and treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidase, and then microwaved (98˚C) in 10 mM sodium citrate buffer (pH 6.0) to unmask the epitopes. After being blocked with goat serum (Beyotime Institute of Biotechnology) for 30 min at 37˚C, the sections were incubated with the rabbit anti-CCL2 antibody [1:100 in buffer (1% BSA, 99% PBS, pH 7.4); cat. no. ab73680] and the rabbit anti-CCR2 antibody [1:250 in buffer (0.75% glycine, 1.21% Tris, 10% glycerol) cat. no. ab155321] (both Abcam,) overnight at 4˚C. At the same time, the controls were treated similarly, except the primary antibody was replaced by PBS. After being washed three times with PBS, the sections were developed with DAB for ~1 min and counterstained with hematoxylin for 30 sec at room temperature. At last, the sections were examined and images were captured using a light microscope equipped with a digital camera (DM2000; Leica Microsystems GmbH) and stained cells were manually counted in five randomly selected fields of vision (original magnification, x400). Immunostaining intensity higher compared with the average background could be observed in the cytoplasmic staining. The mean number of cells expressing CCL2 and CCR2 was recorded. Immunohistochemically staining results were interpreted independently by two pathologists who were blinded to the clinical parameters of the individual cases. The scoring method used was described by Li *et al* (27) to determine positive CCL2 and CCR2 staining, The score of staining intensity of absent, low, medium and high was quantified as 0, 1, 2 and 3, respectively. The score of extent of staining (0, ≤25, ≤50 and ≤100%) was also classified as 0, 1, 2 and 3, respectively. The product of staining intensity and extent of staining was multiplied as the immunoreactive score (IRS), which ranged from 0 to 9. Then, the scores were divided into four groups: 0, 1-2, 3-5 and 6-9, corresponding to the absent, low, medium and high scores, respectively. Based on IRS, slides with scores of ≥3 were classified as positive expression, while slides with scores <3 were classified as absent expression.

Statistical analysis. The independent Student's t-test was used to analyze the continuous variables. The inconsistency rate for

Table I. Inconsistency rate of tumor grade and protein expressions of CCL2/CCR2 between primary and recurrent astrocytoma.

	Recurrent tumor					
	Grade		CCL2		CCR2	
Primary tumor	Low	High	Negative	Positive	Negative	Positive
Low/Negative, n (%)	10 (12.50)	21 (26.25)	8 (10.00)	21 (26.25)	15 (18.75)	26 (32.50)
High/Positive, n (%)	1 (1.25)	48 (60.00)	4 (5.00)	47 (58.75)	6 (7.50)	33 (41.25)
Total change, n (%)	22 (27.50)		25 (31.25)		32 (40.00)	
P-value	$1.097x10^{-5}$		0.001		0.001	

All data were analyzed using McNemar's test.

CCL2 is (21+4)/80=31.25%, and inconsistency rate for CCR2 is (26+6)/80=40.00%. Qualitative data were analyzed using χ^2 tests, including grade and location, and Fisher's exact test was used when n<5 as appropriate. McNemar's test was used to compare changes in grade and protein expression between primary diagnosis and recurrence. Spearman's rank correlation method was used for correlation analysis. The total survival curve was drawn by Kaplan-Meier survival function, and log rank tests were used for statistical analysis. When there was cross-over of survival curves, a weighted test, such as ABS permutation, was used instead. In addition, Cox multivariate regression analysis was performed to determine independent prognostic factors. P<0.05 was considered to indicate a statistically significant difference. All calculations were performed using SPSS version 17.0 (SPSS Inc.).

Results

Patient distribution, survival and recurrent status. To explore the expression pattern of CCL2/CCR2 in glioma, protein expression was analyzed and clinical data were obtained. In total, 80 patients with glioma were included in the present study. Overall, two patients were lost during follow-up and no patients were still alive on the last day of follow-up. The status of these patients with respect to age, sex, adjuvant radiotherapy, histological grade, location of tumors, DFI and OS is listed in Table SI. The average age was 42.90 years (range, 9-77 years). Mean DFI and OS time were 30.16 months (range, 2.10-105.80) and 60.19 months (range, 6.00-169.20), respectively. There was a significant difference in tumor grade ($P=1.097x10^{-5}$) when between primary and recurrent gliomas (Table I), which showed increased malignancy after tumor relapse. Among the 49 patients with high tumor grade in the first operation phase, only one patient (2.04%) developed low grade recurrent tumors. Non-brain tumor specimens from 10 patients with brain trauma were used as control, the characteristics of whom are presented in Table SII.

Inconsistency rate of protein expression of CCL2/CCR2 between primary and recurrent gliomas. Protein expression of CCL2 and CCR2 in patients with glioma and brain trauma are presented in Fig. 1. The immunoreactivity of all proteins studied was mainly localized in tumor cells, but a considerable proportion of cases were localized in stromal cells. The inconsistency rate of CCL2/CCR2 between primary and recurrent tumors was 31.25 and 40.00%, respectively (Table I). The frequency of CCL2 and CCR2 expression was higher in the recurrent gliomas (85.00 vs. 63.75% for CCL2, P=0.001; 73.7% vs. 48.75% for CCR, P=0.001) compared with in the primary group (Table I).

Correlation between tumor grade and protein expression of CCL2/CCR2. CCL2 and CCR2 showed significant positive correlations with tumor grade in the recurrent tumors ($P=4.014x10^{-5}$ and $P=9.763x10^{-5}$, respectively), as well as in the primary tumors (P=0.006 and P=0.019, respectively; Table SIII).

Effect of radiotherapy on CCL2/CCR2 protein expression. There was no significant difference in the baseline of clinical characteristics between glioma with and without radiotherapy after the first operation (Table II). No difference was observed between irradiation and histological grade, location of tumor or CCL2 protein expression during the second operation (P=0.433, P=0.302 and P=0.108, respectively; Table III). However, irradiation was significantly associated with CCR2 expression in recurrent glioma (P=0.020; Table III). It was suggested that irradiation-induced expression of CCL2 is transient, while irradiation-induced expression of CCR2 is lasting.

Difference of CCL2/CCR2 protein expression between primary and secondary GBM. GBM can be divided into *de novo* (primary GBM) or progression from low-grade or anaplastic glioma (secondary GBM) (3). Hence, the present study investigated the difference of CCL2/CCR2 protein expression between 33 cases of primary and 24 cases of secondary GBM. There was no significant difference in the baseline of clinical characteristics between primary GBM during the first operation and secondary GBM during the second operation (Table IV). In addition, no significant difference was observed for the protein expression of CCL2 and CCR2 between primary and secondary GBM (P=0.214 and P=0.346, respectively).

Impact of CCL2/CCR2 protein expression on patient survival. Kaplan-Meier analysis and results of analyses for

Table II. Baseline of clinical characteristics between patients with glioma with and without radiotherapy during the first operation.

Characteristics	Radiotherapy		P-value
	No	Yes	
Mean age ± SD, years	42.76±13.80	42.98±13.22	0.944
Sex, n (%)			0.227
Male	13 (16.25)	30 (37.50)	
Female	16 (20.00)	21 (26.25)	
Location of tumor [n (%)]			0.216[a]
Frontal lobe	15 (18.75)	29 (36.25)	
Temporal lobe	4 (5.00)	13 (16.25)	
Parietal lobe	4 (5.00)	4 (5.00)	
Occipital lobe	1 (1.25)	3 (3.75)	
Others	5 (6.25)	2 (2.50)	
Grade [n (%)]			0.148[a]
I	3 (3.75)	4 (5.00)	
II	13 (16.25)	11 (13.75)	
III	4 (5.00)	12 (15.00)	
IV	9 (11.25)	24 (30.00)	
Expression of CCL2, n (%)			0.472
Negative	12 (15.00)	17 (21.25)	
Positive	17 (21.25)	34 (42.50)	
Expression of CCR2, n (%)			
Negative	13 (16.25)	28 (35.00)	0.386
Positive	16 (20.00)	23 (28.75)	

[a]R x C contingency table χ^2 test for grade and location.

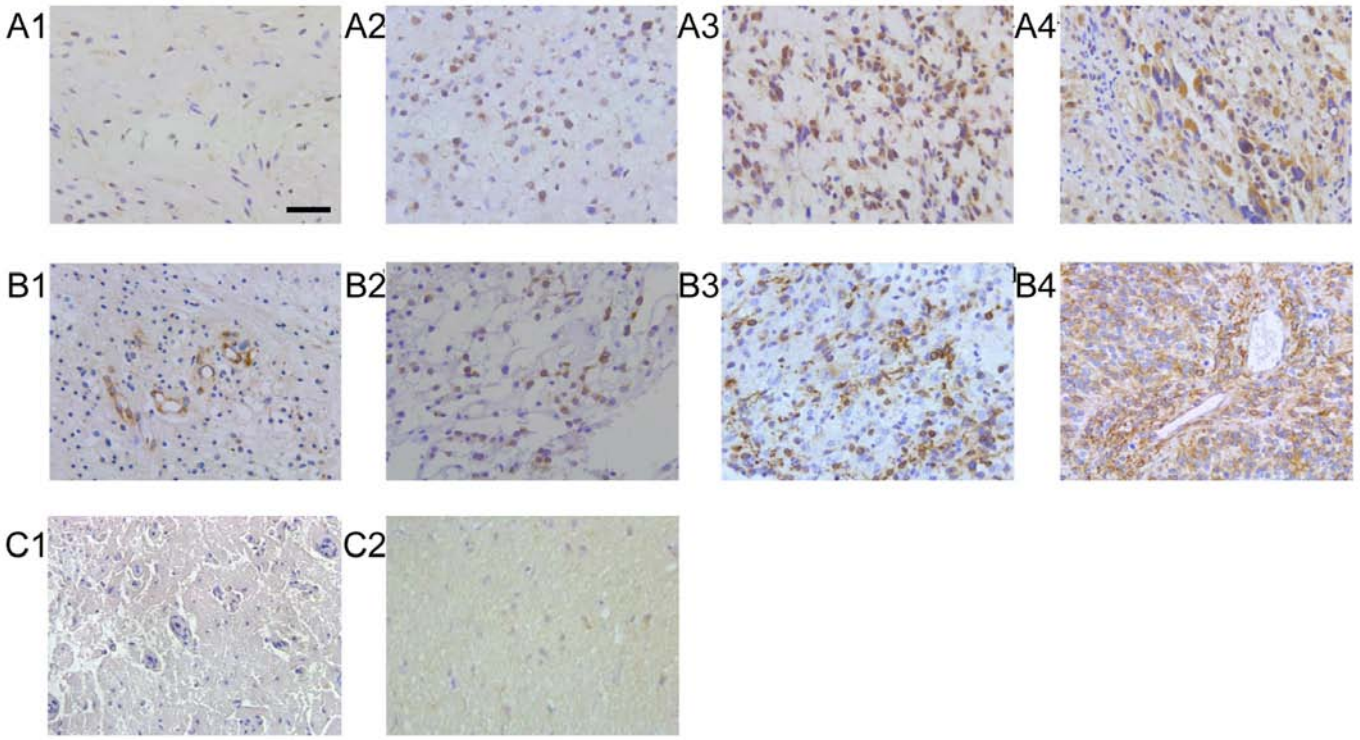

Figure 1. Representative results for the immunohistochemistry of CCL2 and CCR2 in primary glioma. CCL2 in glioma of WHO grade (A-1) I, (A-2) II, (A-3) III and (A-4) IV. CCR2 in glioma of WHO grade (B-1) I, (B-2) CCR2 II, (B-3) III and (B-4) C IV. CCL2 and CCR2 in normal tissues as normal control (C1 and C2). Scale bar, 200 µm. CCL, C-C motif chemokine ligand; CCR, C-C motif receptor; WHO, World Health Organisation.

Table III. Effect of radiotherapy on markers of recurrent astrocytoma.

Characteristics	Radiotherapy		χ^2	P-value
	No	Yes		
Histological grade, n (%)[a]			2.750	0.433
I	1 (1.25)	1 (1.25)		
II	5 (6.25)	4 (5.00)		
III	4 (5.00)	12 (15.00)		
IV	19 (23.75)	34 (42.50)		
Location of tumor, n (%)[a]			4.747	0.302
Frontal lobe	17 (21.25)	29 (36.25)		
Temporal lobe	4 (5.00)	13 (16.25)		
Parietal lobe	3 (3.75)	1 (1.25)		
Occipital lobe	1 (1.25)	4 (5.00)		
Others	4 (5.00)	4 (5.00)		
Expression of CCL2, n (%)[a]			-	0.108
Negative	7 (8.75)	5 (6.25)		
Positive	22 (27.50)	46 (57.50)		
Expression of CCR2, n (%)			5.379	0.020F
Negative	12 (15.00)	9 (11.25)		
Positive	17 (21.25)	42 (52.50)		

[a]Analyzed using Fisher's exact test. All other variables were analyzed using χ^2 tests.

Table IV. Difference of CCL2/CCR2 expression between primary and secondary glioblastoma.

Characteristics	Primary glioblastoma	Secondary glioblastoma	P-value
Mean age ± SD, years[a]	46.21±15.03	45.13±8.54	0.731
Sex, n (%)			0.157
Male	20 (60.61)	10 (41.67)	
Female	13 (39.39)	14 (58.33)	
Location of tumor, n (%)[b]			0.196
Frontal lobe	16 (48.49)	15 (62.50)	
Temporal lobe	6 (18.18)	6 (25.00)	
Parietal lobe	5 (15.15)	2 (8.33)	
Occipital lobe	3 (9.09)	0 (0.00)	
Others	3 (9.09)	1 (4.17)	
Expression of CCL2, n[b]			0.214
Negative	4 (12.12)	0 (0.00)	
Positive	29 (87.88)	24 (100.00)	
Expression of CCR2, n[b]			
Negative	9 (27.27)	4 (16.67)	0.346
Positive	24 (72.73)	20 (83.33)	

[a]Analyzed using t-tests. [b]Analyzed using Fisher's exact test. Sex was analyzed using a χ^2 test.

DFI are shown in Table V. It was revealed that age, tumor grade and the protein expression of CCL2/CCR2 were significant prognostic factors in gliomas using Kaplan-Meier analysis (Fig. S1). To exclude possible confounding factors, multivariate Cox analysis was used to predict DFI considering multiple variables simultaneously. After adjusting for sex, radiotherapy and location of tumors, the significant prognostic factors for DFI were age [hazard ratio (HR)=1.846;

Table V. Prognostic values of the clinicopathological parameters and markers.

	DFI					
		Mann-Whitney U		Cox		
Characteristics	Survival analysis P-value	Z	P-value	HR	95% CI	P-value
Age, years	$4.186x10^{-4}$[a]	-2.739	0.006	1.846	1.046-3.257	0.034
<50						
≥50						
Sex	0.594[a]					
Male						
Female						
Radiotherapy	0.526[a]					
Yes						
No						
Grade	$7.263x10^{-6}$[a]	-4.079	$4.500x10^{-5}$	2.247	1.301-3.882	0.004
I-II						
III-IV						
Location	0.359[a]					
Frontal						
Temporal						
Parietal						
Occipital						
Others						
CCL2	0.001[a]	-3.898	$9.700x10^{-5}$	1.663	1.012-2.731	0.045
Negative						
Positive						
CCR2	0.026[b]	-2.224	0.026	1.133	0.694-1.849	0.618
Negative						
Positive						

[a]K-M, Kaplan-Meier; [b]Two-stage test; Cox, Cox regression; HR, hazard ratio; CI, confidence interval.

95% confidence interval (CI), 1.046-3.257; P=0.034), tumor grade (HR =2.247; 95% CI, 1.301-3.882; P=0.004) and expression of CCL2 (HR=1.663; 95% CI, 1.012-2.731; P=0.045) (Table V).

Using the Kaplan-Meier survival analysis model, it was demonstrated that age, tumor grade and protein expression of CCL2/CCR2 were significant prognostic factors for OS in gliomas (Fig. S2 and Table SIV). In Cox multivariate modeling, after adjusting for sex, radiotherapy and location of tumors, expression of CCL2 and CCR2 remained significant prognostic factors for OS (HR=1.879; 95% CI, 1.092-3.236; P=0.023 and HR =1.744; 95% CI, 1.033-2.945, P=0.037, respectively; Table SIV), as well as age and tumor grade (Table SIV).

Discussion

As one of the most important methods in the treatment of glioma, radiotherapy has been used in the clinic for over a century (28). But recurrence still occurs, even after whole brain radiotherapy. An increasing number of studies have shown that irradiation promotes malignant behaviors, including increased proliferation, invasion and migration (4,29).

Recently, the tumor microenvironment has attracted more and more attention regarding research into tumorigenesis, tumor development, antitumor resistance and tumor relapse (11-13). Morganti *et al* reported that single high-dose (10 Gy) γ-ray irradiation altered the microenvironment of brain tissue and induced increased infiltration of peripheral $CCR2^+$ macrophages (30). A further study showed that CCL2 secreted by glioma cells induced microglia to infiltrate into tumor tissues, while microglia also secreted CCL2, which resulted in an amplifying effect for the recruitment of microglia (31). CCL2 is upregulated at both the mRNA and protein levels in the serum and tumor tissues of patients with glioma (21).

CCR2, the main receptor of CCL2, is overexpressed in most malignant glioma cells (20). The present study showed that there was significant positive correlation between CCL2/CCR2 and tumor grade in primary and recurrent glioma. CCL2 attracted microglia/macrophages, T lymphocytes, basophils, NK cells and hematopoietic progenitor cells to migrate and infiltrate into tumor tissues, all of which

participate in a variety of tumor pathological processes, such as stimulating tumor proliferation and migration (32). CCL2 induces microglia/macrophages migration into glioma and indirectly induces the invasion and migration by secreting cytokines (33). An *in vitro* co-culture study showed that high expression of CCR2 in tumor cells directly promotes perineural invasion, a phenomenon in which cancer cells, especially prostate cancer, invaded the nerves and then reached other sites via the nervous system (34). Zhu *et al* reported that intraperitoneal injection of monoclonal antibodies against CCL2 significantly prolonged the survival of glioma xenograft mice (35). Therefore, CCL2/CCR2 signaling is closely associated with the progression of glioma.

GBM, the most lethal type of glioma, is classified into primary and secondary GBM (3). The two subgroups are histologically indistinguishable; however, more and more studies have distinguished between these using genetic, epigenetic, and molecular profiles (36,37). Telomerase reverse transcriptase promoter mutation, PTEN tumor suppressor gene mutation and high-level gene amplification of EGFR are hallmarks in primary GBMs, while mutations of isocitrate dehydrogenase, mitochondrial 1/2, TP53 and transcriptional regulator ATRX are more common in secondary GBMs (38). However, until now, little was known about the difference of chemokines in primary and secondary GBM. The present study analyzed the expression of CCL2/CCR2 in primary and secondary GBM. However, the results revealed that there was no significant difference.

Targeting CCL2/CCR2 has been shown to be effective in tumor chemosensitization [18], but whether it has a radiosensitizing effect is largely unknown. Recently, more and more studies reported that CCL2/CCR2 is involved in irradiation-induced damage of brain tissues and irradiation-induced malignant behaviors, such as increased migration and invasion (39-41). Therefore, the combination of radiotherapy and inhibition of CCL2/CCR2 may be promising to improve the prognosis of patients with glioma.

Overall, the present study revealed a significant correlation between CCL2 and CCR2 expression and glioma tumor grade. Furthermore, irradiation affected the expression of CCR2, but not CCL2. Hence, CCL2/CCR2 has potential as therapeutic target for patients with glioma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ and ZJ conceived the study and designed most of the experiments. WZ and XZ interpreted the data and wrote the manuscript. QY performed most of the statistical analyses and generated all graphs and tables. JZ had primary responsibility for patient characterization and management. JZ, LM and WZ performed the immunohistological analysis. All authors discussed the results and approved the final submitted version. All authors read and approved the final manuscript. WZ and QY confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

All procedures performed were in accordance with the Declaration of Helsinki and the study was approved by The Institutional Ethics and Investigation Committee of Qilu Hospital, Shandong University [approval no. KYLL-2015(KS) -068]. All patients provided informed written consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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