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Human glioblastoma tumours and neural cancer stem cells express the chemokine CX3CL1 and its receptor CX3CR1

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ABSTRACT

Human gliomas represent an unmet clinical challenge as nearly two-thirds of them are highly malignant lesions with fast progression, resistance to treatment and poor prognosis. The most severe form, the glioblastoma multiforme, is characterised by a marked and diffuse infiltration through the normal brain parenchyma. Given the multiple effects of chemokines on tumour progression, aim of this study was to analyse the expression of the chemokine CX3CL1 and of its specific receptor CX3CR1 in 36 human surgical glioma samples, with different degrees of histological malignancy and in glioblastoma-derived neurospheres. Herein we show that both ligand and receptor are expressed at the mRNA and protein levels in most specimens (31/36). While receptor expression was similarly detected in low or high grade tumours, the uppermost scores of CX3CL1 were found in grades III–IV tumours: oligodendrogliomas, anaplastic astrocytomas and glioblastomas. Accordingly, the expression of CX3CL1 was inversely correlated with patient overall survival ($p = 0.01$). Glioblastoma-derived neurospheres, containing a mixed population of stem and progenitor cells, were positive for both CX3CR1 and for the membrane-bound chemokine, which was further up-regulated and secreted after TNF-IFNγ stimulation. Confocal microscopy of 3D neurospheres showed that the ligand was primarily expressed in the outer layer cells, with points of co-localisation with CX3CR1, indicating that this ligand–receptor pair may have important intercellular adhesive functions. The high expression of CXC3L1 in the most severe forms of gliomas suggests the involvement of this chemokine and its receptor in the malignant behaviour of these tumours.

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1. Introduction

Glioblastoma multiforme (GBM) is an aggressive tumour of the central nervous system, with high propensity to infiltrate throughout the brain, rendering it the most lethal primary brain tumour.[1,2] Despite different treatment modalities, 2-year survival is less than 30% and these results have remained unchanged over the last two decades.[2,3]

This poor prognosis has several causes: (i) glioblastomas are characterised by extensive dissemination of tumour cells within the normal brain areas, which renders total surgical resection virtually impossible without extensive neurological damage; (ii) these tumours are highly refractory to available chemotherapeutic and radiation treatments; (iii) clinicians and scientists have an insufficient understanding of the complex and aggressive nature of glioblastomas.

Established experimental evidence has demonstrated that tumour progression not only depends on the capacity of autonomous cell proliferation but also largely depends on external cues from the micro-environment.[4,5] In the reactive tumour stroma, now defined as inflammatory micro-environment, several growth factors, cytokines and proteolytic enzymes are expressed. Produced both by infiltrating macrophages, fibroblasts and tumour cells themselves, these mediators actively promote the angiogenic switch, tumour cell survival, proliferation and invasion of adjacent tissues.[6,7] Among the factors that contribute to the invasive phenotype of glioma cells, increasing attention has been directed to the chemokine system.

Several different chemokines are produced in the tumour milieu and constitute a paradigm of the cancer-related inflammation.[7–10] These chemotactic cytokines have a complex connection with tumour development. Chemokines were mostly studied for their potent effect on the recruitment of leucocytes at sites of inflammation or neoplasia; however, in the last decade it has become increasingly clear that tumours express also chemokine receptors and therefore neoplastic cells have the ability to mediate ligand-induced biological effects. It is now established that migrating malignant cells may exploit chemokine receptors to invade surrounding tissues and give distant metastasis.[7,8,11] In addition to cell mobilisation, chemokines enhance tumour cell resistance to apoptosis and/or proliferation and modulate angiogenesis and extra-cellular matrix turnover.[8,12–14]

Since one of the main features of GBM is its infiltrating potential[5] and given that several chemokines are produced in damaged brain areas to recruit immune cells and neural stem cells (NSCs), the present study aims to shed light on the putative role of the chemokine system in GBM progression. A number of studies have investigated the expression of chemokine receptors in gliomas. mRNA and protein expression for some specific receptors, like for instance CXCR3, CXCR4 and CXCL8 receptors has been reported.[15–17] In particular, the presence of CXCR4 has been associated with the most aggressive forms of gliomas and poor patient survival.[16,18,19]

Like other solid tumours, glioblastomas contain a small fraction of cells recognised as cancer stem cells (CSCs) or tumour-initiating cells (TIC), responsible for tumour origin, progression and recurrence *in vivo*.[20,21] A recent paper demonstrated that glioblastomas CSC growing as neurospheres highly express CXCR4 and this expression decreases in differentiated cells; moreover the stimulation with the specific ligand CXCL12 induces a significant proliferative response in CSC but not in corresponding differentiated cells.[19] These results suggest an important functional role of chemokines in cancer stem/progenitor cells.

One chemokine/receptor pair that has been rather poorly studied in tumours is CX3CL1 and CX3CR1. CX3CL1 was cloned from activated endothelial cells and neurons and originally termed Fractalkine or Neurotactin, respectively.[22,23] Unlike other chemokines, CX3CL1 is a transmembrane protein that can function as an adhesion molecule as well as a chemokine when cleaved by specific proteases. CX3CL1 is expressed in the nervous system mainly by neurons and astrocytes/glial cells, in inflammatory conditions.[24] CX3CL1 binds exclusively CX3CR1, a G-protein coupled receptor expressed mainly by leucocytes, including microglia in the brain.[25] Experimental studies highlighted the role of CX3CL1 in attenuating inflammation in the brain, thus indicating that the CX3CR1/CX3CL1 axis is a major player in the cross-talk between neurons and microglia, possibly contributing to the maintenance of homeostasis in the brain.[26–30] Only recently the receptor CX3CR1 was investigated in human malignancies and found to be expressed by cancer cells of prostatic and pancreatic carcinoma, and involved in tumour spread.[31,32] CX3CR1 was recently reported in established tumour cell lines originated from GBM,[33,34] in human glioma and in tumour-infiltrating leucocytes.[35,36]

Herein we show that both ligand and receptor are expressed in human glioma surgical samples, and that levels of CX3CL1 significantly correlated with severity of disease and inversely with overall survival. Glioblastoma-derived neurospheres, containing a mixed population of stem and progenitor cells, also express CX3CR1 and CX3CL1 and confocal microscopy images suggest that this axis is an important adhesive loop.

2. Materials and methods

2.1. *Patients and tissues specimen*

Tumours were collected from a series composed by 36 patients, 22 men and 14 females. All patients were operated by craniotomy and removal of the tumour at the Dept. of Neurosurgery, IRCCS Istituto Clinico Humanitas. Patients who were simply biopsied were excluded from the study. The histopathological diagnosis was made by a single pathologist (PC) and subsequently reviewed by a consultant neuropathologist. The lesions were classified along the WHO classification. In order to verify the differences in CX3CL1 and CX3CR1 expression in different oncotypes we analysed 8 cases of slowly growing tumours (3 cases of grade I astrocytomas and 5 cases of grade I and II oligodendrogliomas) and 28 cases of malignant tumours (4 oligodendrogliomas grade III, 2 cases of anaplastic astrocytomas grade III and 22 cases of glioblastoma grade IV). The overall survival, age of patients and tumour localisation were evaluated. All the patients gave their informed consent.

2.2. Neurosphere cultures

GBM-derived neurospheres (NS) are isolated from tumour samples immediately after surgery. Tumour brain tissue is processed according to the standard protocol used for neural stem cells (NSCs).[21] Cells are maintained in culture in a selective medium supplemented with 20 ng/ml EGF and 10 ng/ml FGF, in the absence of serum. These culture conditions select for a population of immature cells, namely stem and progenitor cells, while the differentiated counterparts do not survive. Neurospheres isolated from two different GBM patients were used in this study. These neurospheres were injected orthotopically in immunodeficient mice, generating serially transplantable, highly infiltrating tumours, histologically similar to the original human glioblastoma. Since extended period of neurospheres culture may induce significant alterations in cellular biology and gene expression, all analyses have been performed on neurospheres cultured only for few passages.

2.3. Immunohistochemistry for CX3CL1 and CX_3CR1

Formalin-fixed, paraffin-embedded tissues were deparaffinised in xylene, as described.[32] Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min at room temperature. For CX3CR1 staining, sections were exposed to an antigen retrieval procedure in sodium citrate buffer (pH 6.0), 98 °C for 30 min, before being incubated with specific antibody (Abcam, rabbit anti-human CX_3CR1, overnight at 4 °C), followed by a goat anti-rabbit secondary antibody (EnVision horseradish peroxidase rabbit/mouse, DakoCytomation) for 30 min at room temperature. For CX3CL1 staining, sections were directly incubated with specific primary antibody (R&D Systems, goat anti-human CX_3CL1, 1 h at room temperature), followed by a biotin-conjugated donkey anti-goat secondary antibody (SantaCruz Biotechnology®, Inc.) for 30 min at room temperature. Sections were subsequently incubated with avidin (ABC, vectastain) for 30 min at room temperature. After diaminobenzidine reaction (liquid DAB + Substrate Chromogen System, DakoCytomation), section was counter-stained with haematoxylin (Harris Hematoxylin, DiaPath). Immunoreactivity was scored semiquantitatively according to estimate the intensity of positive tumour cells (0, negative; 1, weak; 2, moderate; 3, strong).

2.4. Quantitative real-time PCR

Total RNA was isolated both from tissue specimens using TRI reagent (Ambion). Total RNA was quantified by Nanodrop Spectrophotometer ND-1000 and its quality was examined by 1.5% agarose gel electrophoresis. DNAse treatment (Turbo DNA-free™ kit, Ambion) was performed to avoid genomic DNA contamination. One microgram of total RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. Tumour samples as well as cell lines cDNA were analysed by SYBER Green-based Quantitative Real-Time PCR on ABI Prism® 7900HT Fast Real Time PCR System (Applied Biosystem). 18S was used as internal control to normalise sample. All gene specific primers were domestically designed. The sequence is indicated below:

18S:
Forward: 5′-CGC CGC TAG AGG TGA AAT TC-3′
Reverse: 5′-CTT TCG CTC TGG TCC GTC TT-3′
CX_3CL1:
Forward: 5′-TCT GCC ATC TGA CTG TCC TG-3′
Reverse: 5′-TGA TGT TGC ATT TCG TCA CA-3′
CX_3CR1:
Forward: 5′-GGG ACT GTG TTC CTG TCC AT-3′
Reverse: 5′-GAC ACT CTT GGG CTT CTT GC-3′

The threshold cycle Ct was automatically given by SDS2.2 software package (Applied Biosystem).

2.5. Elisa for CX3CL1 measurement

CX3CL1 levels in GBM-derived neurospheres supernatants were measured using human CX3CL1 DuoSet ELISA Development System (R&D Systems) according to manufacturer's instruction. Briefly, a 96-well flat-bottomed microplate (Costar) was coated with 4 μg/ml of captured antibody, diluted in carbonate buffer pH 9.6, overnight at room temperature. Each well was washed in washing buffer (0.05% Tween 20, PBS, pH 7.2–7.4) and blocked with 1% BSA in Washing Buffer for 2 h at RT. Wells were washed three times and then 50 μl of sample was added to each wells and incubated for 2 h at RT. Each sample was analysed in duplicate and threefold serial dilutions in Reagent Diluent (R&D System) (1% BSA in PBS, pH 7.2–7.4) were performed. A standard curve was performed using recombinant CX_3CL1, using twofold serial dilution in Reagent Diluent and a high standard point of 20 ng/ml. Each well was then washed in washing buffer and incubated with 50 μl of detection antibody for 2 h RT. Next, 50 μl of streptavidin-HRP was added to each well for 20 min at RT. Development was performed using the 3,30,5,50-tetramethyl-benzidine (TMB) Liquid Substrate System (SIGMA), and reaction was blocked by H_2SO_4 2N. The amount of CX3CL1 was evaluated by optical density using the VersaMax microplate reader (Molecular Devices) set to 450 nm.

2.6. Confocal microscopy

For immunofluorescence analysis, GBM-derived neurospheres were collected after 6 days of culture, washed in PBS and fixed in 4% PFA for 15 min at room temperature. After 2 washes in 2% bovine albumin serum (BSA) in PBS, neurospheres were incubated with the specific primary antibodies against CX3CL1 (R&D Biosystem, mouse anti-human CX_3CL1), CX3CR1 (Abcam, rabbit anti-human CX_3CR1) and nestin (Chemicon, mouse anti-human Nestin) in 2% BSA, 0.3% Triton X-100, 0.1% glycine, 5% Normal Goat Serum in PBS overnight at 4 °C. After three washes in washing buffer (0.2% BSA, 0.05% Tween 20 in PBS), neurospheres were incubated with specific secondary antibodies Alexa goat anti-mouse 647-conjugated and Alexa goat anti-rabbit 488-conjugated (Invitrogen, Molecular Probes) in washing buffer for 5 h at room temperature. DAPI (Invitrogen, Molecular Probes) was used

Table 1 – Clinical characteristics of glioma patients and immunohistochemistry scores of CX3CL1 and CX3CR1 in tumour tissues.

Sex	Age	Location	Histology	Grade	CX3CL1 score	CX3CR1 score	Radio therapy	Chemo therapy	Free interval	Overall survival	Death = 1
M	26	Frontal	Astrocytoma	I	0	1	No	No	22	142	0
F	30	Frontal	Astrocytoma	I	0	1	No	No		40	
M	41	Frontal	Oligodendroglioma	I	0	1	No	No	17	60	0
M	23	Frontal	Oligodendroglioma	II	0	2	No	No	33	37	1
M	35	Temporal	Astrocytoma	II	1	0	No	No	15	34	1
M	59	Frontal	Oligodendroglioma	II	1	0	Yes	No	7	27	1
M	40	Temporal	Oligodendroglioma	II	1	1	No	No	16	47	1
M	63	Parietal	Oligodendroglioma	II	1	1	Yes	Yes		53	0
F	69	Temporal	Anaplastic astrocytomas	III	1	1	Yes	Yes	6	8	1
M	77	Temporal	Oligodendroglioma	III	1	1	Yes	Yes		39	0
F	63	Temporal	Anaplastic astrocytomas	III	2	1	Yes	No		69	0
M	53	Frontal	Oligodendroglioma	III	2	0	Yes	Yes	3	6	1
F	71	Frontal	Oligodendroglioma	III	3	1	Yes	Yes	11	46	1
M	49	Frontal	Oligodendroglioma	III	3	0	Yes	Yes	30	33	0
F	74	Temporal	GBM	IV	0	2	No	No	3	6	1
M	61	Frontal	GBM	IV	1	0	Yes	Yes		9	1
F	61	Frontal	GBM	IV	1	1	Yes	Yes	8	16	1
M	59	Temporal	GBM	IV	1	0	Yes	Yes	12	17	1
F	59	Temporal	GBM	IV	1	1	Yes	Yes		30	0
M	75	Frontal	GBM	IV	2	1	Yes	No	3	4	1
F	41	Frontal	GBM	IV	2	1	Yes	Yes		67	0
F	62	Frontal	GBM	IV	2	0	Yes	No	2	5	1
M	77	Frontal	GBM	IV	2	1	Yes	Yes	5	9	1
M	71	Parietal	GBM	IV	2	0	Yes	Yes		4	1
M	55	Temporal	GBM	IV	2	2	No	No		3	1
F	60	Temporal	GBM	IV	2	1	Yes	Yes		27	1
M	66	Parietal	GBM	IV	2	0	Yes	No		6	1
F	72	Frontal	GBM	IV	2	1	Yes	Yes		9	1
F	58	Parietal	GBM	IV	2	2	Yes	Yes	13	24	1
M	62	Temporal	GBM	IV	2	1	Yes	Yes	6	10	1
M	64	Temporal	GBM	IV	2	3	Yes	Yes	7	14	1
F	77	Frontal	GBM	IV	3	2	Yes	Yes	18	21	1
M	58	Temporal	GBM	IV	3	1	Yes	Yes	12	18	1
M	73	Frontal	GBM	IV	3	1	Yes	Yes	14	16	1
F	28	Temporal	GBM	IV	3	3	Yes	Yes	39	59	1
M	68	Frontal	GBM	IV	3	0	No	No		6	1

to stain nuclei. Neurospheres were mounted with Fluor Preserve™ Reagent (Calbiochem) and analysed with a laser scanning confocal microscope (FluoView FV1000; Olympus). Images were acquired with an oil immersion objective (40× 1.3 NA Plan-Apochromat; Olympus). For image analysis, Imaris X64 6.2.1 software (Bitplane, AG) was used.

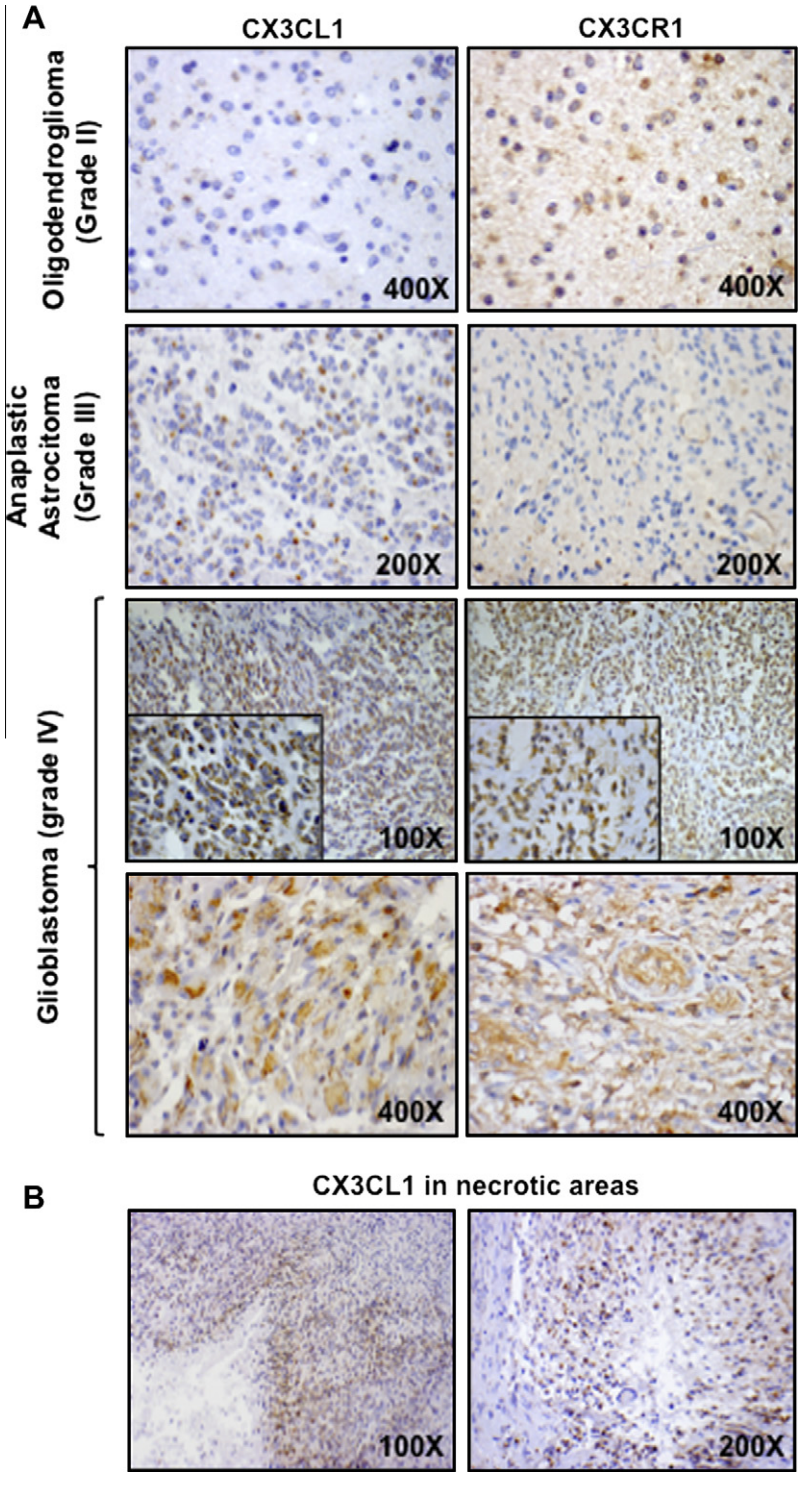

Fig. 1 – Immunohistochemistry of CX3CL1 and CX3CR1 in human glioma. (A) Immunohistochemistry of CX3CL1 (left panels) and of CX3CR1 (right panels) on paraffin sections of tumour tissues. CX3CL1 immunopositivity increases with tumour grade. Oligodendroglioma (grade II) showing faint expression of CX3CL1; anaplastic astrocytomas (grade III) expressing CX3CL1 with 'dot like' staining; in two cases of glioblastoma (grade IV) CX3CL1 is strongly expressed. Expression of the receptor CX3CR1 (right panels) is heterogeneous, with low grade tumours staining positive and grade III tumours with faint expression. CX3CR1 in glioblastoma tumours showed the highest immunopositivity. (B) Strong expression of CX3CL1-positive cells around areas of necrosis in two samples of glioblastoma. Optical magnification (OM) is shown, insets: ×400.

2.7. Statistical analysis

The significance of differential gene expression in tumour tissues and NTSCs was determined using the non-parametric Mann–Whitney *U*-test. To evaluate the differences in CX3CL1 and CX3CR1 expression during tumour progression, one-way ANOVA test was performed. Kaplan–Meier survival curve was used to correlate CX3CL1 and CX3CR1 expression with patients' clinical out come. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Expression of the chemokine CX3CL1 and of CX3CR1 receptor in human gliomas

Thirty-six patients undergoing surgery for primary brain tumours were included in this study. The lesions were classified according to the WHO classification: 8 cases were grade I and II (3 astrocytomas and 5 oligodendrogliomas), 28 cases were grade III and IV (4 oligodendrogliomas, 2 anaplastic astrocytomas and 22 glioblastoma multiforme). The clinical characteristics of the patients are listed in Table 1.

Expression of the chemokine CX3CL1 and the receptor CX3CR1 was detected by immunohistochemistry on paraffin-embedded sections of tumour tissues. Score of positivity was calculated as a combination of staining intensity and percentage of positive cells. A progressive increase in the CX3CL1 expression was observed with increasing tumour grade. In grade I gliomas, CX3CL1 was always negative and faintly positive in grade II samples, as shown in the representative picture of an oligodendroglioma (Fig. 1A left). CX3CL1 immunopositivity clearly increased in grade III anaplastic astrocytomas (Fig. 1A left), in grade III olidendrogliomas and was stronger in GBM (grade IV) (Fig. 1A left). Interestingly, around

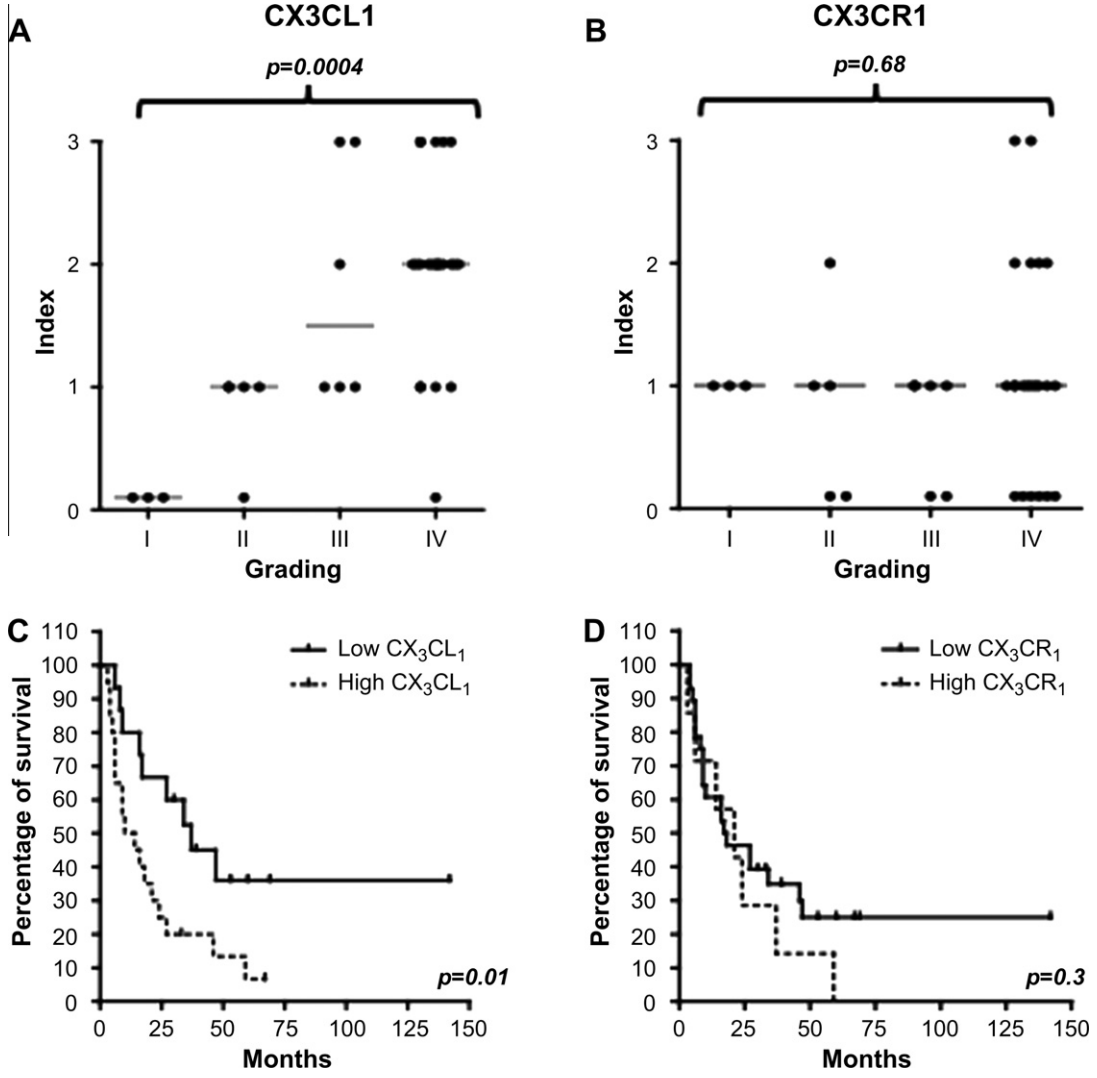

Fig. 2 – Correlation between CX3CL1 and CX3CR1 and clinical outcome. Expression of CX3CL1 and CX3CR1 in tumoural tissues was correlated with patients' clinical outcome. As shown, CX3CL1 expression strongly increases with tumour progression. Moreover, percentage of survival in patients with higher expression of CX3CL1 is significantly reduced in comparison with percentage of survival in patient with low CX3CL1 expression. On the contrary, CX3CR1 expression does not change with tumour progression. Moreover, CX3CR1 expression does not influence patients' clinical outcome.

the areas of necrosis, a typical feature of GBM, tumour cells were markedly expressing the chemokine (Fig. 1B).

Expression of the receptor CX3CR1 was more heterogeneous and, in contrast to ligand immunopositivity, did not significantly change along tumour grading. While some low grade tumours stained positive (oligodendroglioma depicted in Fig. 1A, right), other tumours of higher grade showed faint expression. Nevertheless, high grade GBM usually showed the highest receptor immunopositivity (Fig. 1A, right).

Expression of both CX3CL1 and CX3CR1 was confirmed at mRNA levels in selected tumour specimens obtained at surgery (data not shown).

3.2. *Correlation of CX3CL1 and CX3CR1 scores with clinical outcome of patients*

Data obtained by immunohistochemistry were analysed to find a correlation between CX3CL1 and CX3CR1 expression with clinical outcome. As shown in Fig. 2A, there was a statistically significant correlation between high CX3CL1 expression and tumour severity ($p = 0.0004$), while no correlation can be observed with CX3CR1 ($p = 0.68$; Fig. 2B). A significantly shorter overall survival was associated with high CX3CL1 expression ($p = 0.01$; Fig. 2C), but not with receptor positivity ($p = 0.3$; Fig. 2D).

In a further analysis, we evaluated CX3CL1 scores and patient survival only in grades III–IV tumours ($n = 28$). Patients with no or low expression of CX3CL1 (score 0, 1, $n = 7$) did not have a statistically different survival compared to patients with score 2, 3 ($n = 21$), although a tendency to worse prognosis was observed in the high CX3CL1 group ($p = 0.27$, data not shown).

3.3. *Expression of CX3CL1 and CX3CR1 in GBM-derived neurospheres*

GBM-derived neurospheres (NS) were isolated from tumour samples immediately after surgery and cultured in serum free medium with specific mitogens (epidermal growth factor and fibroblast growth factor).[21] We analysed the expression of both CX3CL1 and CX3CR1 in neurospheres isolated from two different GBM patients. CX3CL1 and CX3CR1 mRNA were expressed in both samples, although at different levels (Fig. 3A). CX3CL1 is enriched in neurospheres derived from both GBM, while the expression of the receptor varies between the two samples.

The neurosphere supernatants were collected and analysed by Elisa assay for the detection of the soluble chemokine. Both neurosphere cultures secreted constitutively CX3CL1, whose levels were increased after TNF/IFNγ stimulation (Fig. 3B). In NSB, CX3CL1 reached the considerable concentration of 25 ng/ml.

Next, we analysed the membrane chemokine expression and the distribution of the ligand/receptor pair in the neurospheres by confocal microscopy. Interestingly, in both samples the ligand was primarily expressed in the cells of the outer layer of the neurospheres whereas the receptor was present in both the outer and inner cells (Fig 4C–F and M–P). All neurosphere cells were positive for nestin (stem cell marker), ruling out the risk that anti-CX3CL1 and anti-CX3CR1 Ab could not reach the indoor compartment (Fig. 4A, B and I, L). Points of co-localisation were clearly visible (panels G, H and Q, R). These findings suggest an involvement of CX3CL1 and its receptor in an adhesive loop determining aggregation and 3D spatial conformation.

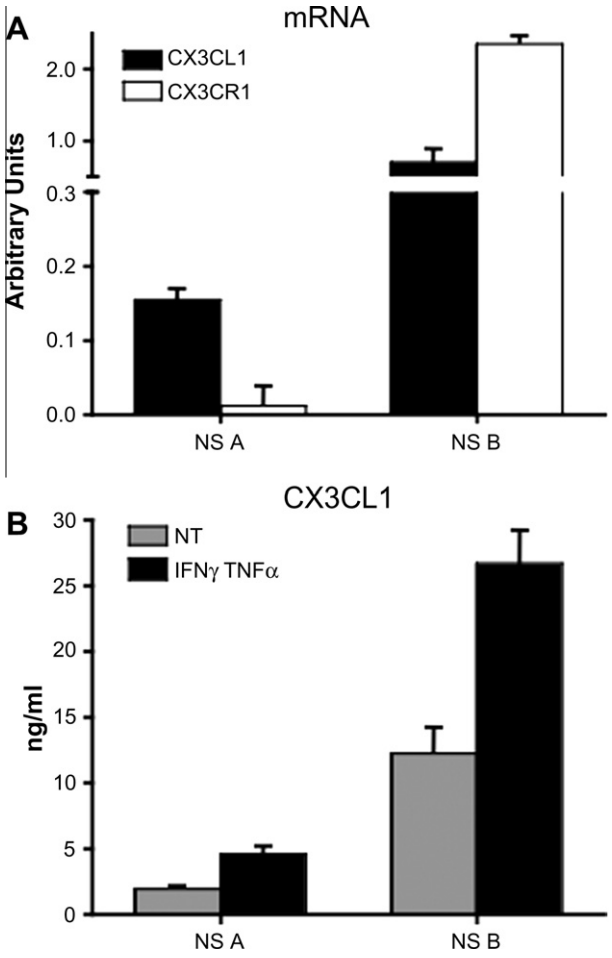

Fig. 3 – CX3CL1 and CX3CR1 expression in GBM-derived neurospheres. Levels of CX3CL1 and CX3CR1 mRNA were evaluated in neurospheres obtained from two GBM patients. CX3CL1 mRNA is highly expressed in both NS-A and NS-B, while CX3CR1 mRNA is strongly expressed only in NS-B. CX3CL1 protein expression was also evaluated by ELISA assay. Neurospheres were stimulated with TNFα and IFNγ for 24 h, and CX3CL1 expression was measured in the supernatant.

4. Discussion

In this study we have investigated the expression of the chemokine receptor pair CX3CL1 and CX3CR1 in primary human glial-derived tumours and in neurospheres isolated from GBM patients. The results demonstrate that glioma tumours express the ligand and the receptor both at mRNA and protein levels. High grade tumours significantly expressed CX3CL1 at higher intensity. In line with the finding that high grade tumours have poor prognosis, CX3CL1 scores were significantly associated with shorter overall survival ($p = 0.01$). In contrast,

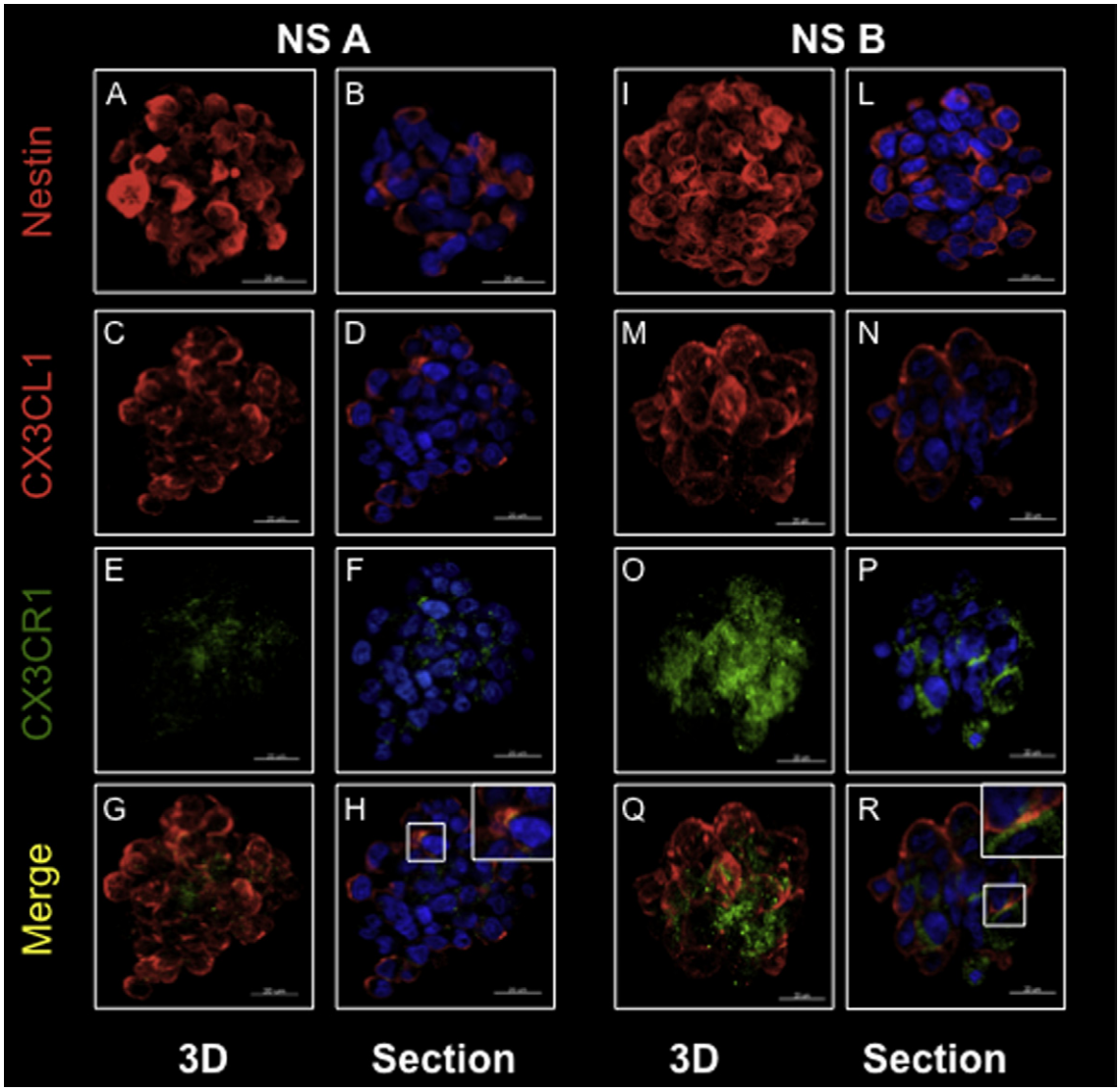

Fig. 4 – CX3CL1 and CX3CR1 co-localisation in GBM-derived neurospheres. Neurospheres derived from (A–H) NS-A and (I–R) NS-B were incubated with α-Nestin (stem cell marker), α-CX3CL1 and α-CX3CR1 antibodies. The expression of Nestin (A–B; I–L), CX3CL1 (C–D; M–N), CX3CR1 (E–F; O–P) and double staining for CX3CL1/CX3CR1 were visualised in both 3D images and in single sections. Magnifications in H and R show several points of co-localisation between CX3CL1 and CX3CR1. Bars, 20 μm.

the receptor was similarly expressed in low or high grade tumours and was not associated with overall survival.

CX3CL1 is one of the most expressed chemokines produced by activated neurons and glial cells,[23,24] while the receptor is primarily expressed by the specialised macrophages of the microglia.[26,27,29] A few number of studies documented exceptions to this rule: in different species and conditions also neurons as well as astrocytes have been found to express CX3CR1.[37] The pathophysiological significance of CX3CL1 expression in the brain and especially its strong up-regulation by GBM is unclear. Experimental evidence points to the CX3CR1/CX3CL1 loop as a major player in the cross-talk between neurons and microglia, with a suggested role in neuroprotection under experimental conditions of brain inflammation/injury.[26] CX3CL1 reduces the glutamate-mediated excitotoxicity of rat hippocampal neurons and supports neuronal survival, through the activation of the ERK1/2 and PI3K/Akt pathways.[28] Important microglia functions, such as mobilisation of intracellular Ca2+, chemotaxis, Fas-mediated apoptosis and LPS-induced activation, are down-regulated *in vitro* by CX3CL1.[38,39]

In CX3CR1-deficient mice, systemic injection of lipopolysaccharide caused enhanced neuron loss compared to wild type mice.[29] Also in a toxic model of Parkinson disease and in a transgenic model of amyotrophic lateral sclerosis, $Cx3cr1^{-/-}$ mice had more extensive neuronal damage.[29] Overall these findings indicate that CX3CL1 may control microglial-mediated neurotoxicity and provides a checkpoint to limit neuro-inflammation.[29,30,40] The higher expression of this chemokine in glioblastomas may be a mean to reduce and compensate cancer-related inflammation and tissue injury. Of interest, we noticed a marked up-regulation of the ligand around areas of necrosis, which is typical of GBM. Necrotic debris may directly stimulate CX3CL1 production, or indi-

rectly via inflammatory cytokines released with necrotic cells. Indeed TNF, which is released in the tumour milieu of glioblastomas, is a known stimulus for the production of CX3CL1.[29]

Another possibility is that CX3CL1 up-regulation is of particular benefit for neoplastic cells, and that high CX3CL1-producing tumour cells have a selective advantage. This chemokine, unlike others, has adhesive properties when expressed on the cell membrane. In this study we observed by confocal microscopy a direct chemokine–receptor interaction in 3D cultured neurospheres obtained from glioblastoma specimens. A similar observation was recently reported using *in vitro* established glioma cell lines.[34]

Previous studies have shown that CX3CR1-signalling activates downstream beta-1 integrins and focal adhesion kinases.[32,41] It is now well established that adhesion molecules not only mediate the physical interaction with the surrounding micro-environment, but they are also crucial determinants for cell survival, especially under sub-optimal conditions. Indeed chemokines have long been known to regulate both proliferation and survival of cancer cells under defined conditions.[19,42] For CX3CL1 in particular, we found that it rescues CX3CR1-expressing pancreatic cancer cells from apoptosis.[32]

Several adhesion molecules have been investigated in glioma tumours, for instance αvβ3 is expressed on the disorganised tumour-associated vessels of glioblastoma, as well as also on neoplastic cells.[43] Expression of α6β1 is associated with increased tumourigenesis of glioma.[44] Integrins and related signalling components (e.g. αvβ3, AKT) have recently raised much interest as potential target of therapeutic intervention in human glioma. Disruption of integrins by RGD peptides, or inhibition of AKT signalling reduces migration and invasion of glioblastoma cells.[45,46] Collectively, these results point to an important pro-tumour role of integrins in these tumours, and it is tempting to speculate that the CX3CR1–CX3CL1 axis may work on top of integrin-mediated adhesion.

Another important pro-tumour function of chemokines is the activation of matrix proteases in the tumour micro-environment.[8] CX3CL1 produced by endothelial cells was shown to activate MMP9.[47] MMPs and other proteolytic enzymes play a major role in matrix degradation, which facilitates neoplastic cell invasion and neo-angiogenesis.[7] The higher ligand expression in glioblastoma, with no concomitant up-regulation of the receptor, points to the possibility that CX3CL1 exerts also receptor-independent effects.

Of interest, we demonstrate in this study that cancer stem and progenitor cells isolated from GBM surgical specimens already express both ligand and receptor, indicating that the presence of the CX3CL1/CX3CR1 loop is an early event in the tumourigenesis process. Notably, another chemokine receptor, CXCR4, has been reported in glioblastoma progenitor cells.[19] CXCR4-positive cells have been visualised at the invasion front in human glioblastoma and the blockade of CXCR4 reduces the *in vitro* migration capacity of glioma cells.[17] Thus, elements of the chemokine system are involved in relevant pathogenetic mechanisms of brain tumours and may be considered as important tools to target invasive glioblastomas, including tumour-initiating cells.

Conflict of interest statement

None declared.

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