The Intricate Expression of CC Chemokines in Glial Tumors: Evidence for Involvement of CCL2 and CCL5 but Not CCL11

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Abstract- Chemokines are biologically active peptides involved in the pathogenesis of various pathologies including brain malignancies. They are amongst primitive regulators of the development of immune responses against malignant glial tumors. The present study aimed to examine the expression of CC chemokines in anaplastic astrocytoma and glioblastoma multiform patients at both mRNA and protein levels. Blood specimens in parallel with stereotactic biopsy specimens were obtained from 123 patients suffering from glial tumors and 100 healthy participants as a control. The serum levels of CCL2, CCL5, and CCL11 were measured by ELISA and stereotactic samples subjected to western and northern blotting methods for protein and mRNA, respectively. Demographic characteristics were also collected by a researcher-designed questionnaire. Results of the present study indicated that, however, CCL2 and CCL5 are elevated in serum and tumor tissues of patients suffering from a glial tumor at both mRNA and protein levels, the CCL11 was almost undetectable. According to the findings of the present investigation, it could presumably be reasonable to conclude that chemokines are good predictive molecules for expecting disease severity, metastasis, and response to treatment.

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Introduction

Brain gliomas are among the most frequent primary brain tumors which approximately perceive 50% of symptomatic intracranial neoplasms (1). However, Anaplastic Astrocytoma (AA) is a malignant tumor, but it is well evidenced that its prognosis is better than the most malignant form of astrocytomas, Glioblastoma Multiform (GBM).These tumor types are characterized by several complicated conditions varied from uncontrolled cellular proliferation, necrosis, robust angiogenesis, and extensive infiltration of surrounding brain to resistance to apoptosis (2,3). The survival of AA and GBM patients is varied from 12 to 14 months, despite cytoreductive surgery which is followed by radiotherapy and chemotherapy as an adjuvant. In spite of considerable progressions in this field, underlying mechanisms for tumor development, genetics, biology and clinical behavior of glial tumors pathogenesis is not yet to be fully understood. In this context, here is not a therapeutic strategy to reduce the invasive and the proliferative ability of the glial cells (4,5). Chemokine and their corresponding receptors are nowadays considered as a therapeutic target for GBM. Chemokines were originally identified as a subfamily of cytokines which regulating the chemotaxis of leukocytes towards the injured tissues (6). They are classified into C, CX3C, CXC, and CC according to the position, presence or absence of conserved cysteine motifs in their biochemical structure (7). More than 50 Chemokines are

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identified to date (7,8). The CCL2, CCL5, and CCL11 (previously known as MCP-1, RANTES, and Eotaxin, respectively) fit in the CC subfamily of chemokines (8). Recruitment of macrophages and monocytes to the central nervous system (CNS) is facilitated by CCL2, and this chemokines has been reported to be overexpressed in GMBs. Rewardingly, the CCL2 receptor (especially CCR2A) is also expressed by the neoplastic cells. The CCR2A isoform presents at the cytoplasm of neoplastic GBM cells, and CCL2 inhibition demonstrated leads defective recruitment of glioma cell CCR2A expressing (9).

Intracellular cytokine analysis indicated that CCL2 is also overexpressed in both GBM cell lines (D-54, U-87, and U-251) and regulatory (Tregs) cells. Malignant GBM cells demonstrated to express more CCR4 (one of the CCL2 receptors) compared to healthy control participants. Surprisingly, this GBM-derived CCL2 was alleviated by chemotherapy reagents (e.g. temozolomide and carmustine) (3). The human astrocytoma cell line U373 also indicate to produce a CCL2 (as an inducible gene) via TNF-α mediated activation of NF-KB pathway. TNF-α and NF-KB regulate CCL2 production in turn leads to macrophages mediated neurodegenerative disease which occurs following neurodegeneration (5). The presence of the SP1 site (specify of constitutive genes) and AP1, NF-KB consensus elements in CCL2 gene is well defined. Both CCL2 and CXCL8 are massively produced by the GBM cell lines T98G in response to TNF-α and IL-1 when co-cultured with peripheral blood monocytes (10). In situ hybridization studies have revealed elevation of CCL2 in GMB patients and this CCL2 is produced by all involved cell types, including astrocytes, glial cells, and tumor-associated macrophages (11).

Moreover, likewise CXCL12, (the other member of CXC subfamily) CCL2 is also a constitutive and homeostatic chemokine which expression is regulated by GC box, SP1, and NF-Kß .These elements are characteristics of the housekeeping genes. Elevated CSF (cranial, spinal fluid) levels of CCL2 (10-fold) are reported in different neurological tumors such as benign gliomas. (R) In brief, according to the aforementioned introductory comments and compelling evidence regarding the fact that CC chemokines play a primary role in brain tumor growth through diverse biological effects including cell proliferation and angiogenesis. So, this project designed to examine whether or not these specific CC chemokines (CCL2, CCL5, and CCL11) are crucially involved in the pathogenesis of AA and GMB.

Materials and Methods

Specimens were collected from 123 patients suffering from glial tumors including 68 AA and 55 GBM (either from tumor tissues by stereotactic biopsy or peripheral blood) from 2006 to 2011 at a university hospital in Kerman province, Iran. The presence of malignant glial tumor was confirmed by an expert team of neurologist according to the patient's clinical and paraclinical findings. A blood sample was taken from a patient at the time of diagnosis and also post treatment. Tumor tissues were obtained using stereotactic biopsy during surgical resections by an expert neurosurgeon team. Healthy controls were selected from Rafsanjan population, matched for age and sex. The study protocol was approved by ethics committee of Rafsanjan University of Medical Sciences.

ELISA assays

Serum levels of CCL2, CCL5, and CCL11 were measured by ELISA (R&D systems, UK) in patients and healthy controls immediately after specimen collection. In brief, 96-well EIA/RIA plates were coated with a capturing monoclonal antibody against respected chemokines and were then blocked with a mixture of 1% bovine serum albumin in PBS. The resultant supernatants were diluted in reagent diluents (1% bovine serum albumin in PBS) and incubated for 24 hours at room temperature. Detection antibody was then diluted in reagent diluents and incubated for two hours at room temperature. The binding of antibody was detected with streptavidin-conjugated horseradish peroxides and developed with a substrate solution. A standard curve was generated for each set of samples assayed and was made from seven points of a twofold dilution series. Each standard or sample was assayed in duplicate. The sensitivity of kits was 2pg/ml and inter, and intra-assay assessments of the reliability of the kit were conducted.

RNA analysis

RNA Extraction

Tumor tissue was collected via stereotactic biopsy and subjected to lysis and then used for RNA analysis. Depending on the cells pellet, 0.5-1 ml. of Trizol® was added to the lysed tissues and immediately transferred to –80°C. The frozen Trizol® extracts were thawed at room temperature. 0.2 ml chloroform was added, and this was mixed vigorously for 15 seconds. The sample was then incubated at room temperature for 5 minutes

and centrifuged at 12000g for 15 minutes at 4°C. The upper aqueous layer (containing RNA) was transferred to a fresh tube. Then 0.5 ml isopropanol was added to the isolated aqueous layer and this mixture was incubated at room temperature for 10 minutes. The RNA was precipitated by centrifugation at 12,000g for 10 minutes at 4°C. The pellet was washed with 1ml 75% (v/v) ethanol and centrifuged at 7500g for 5 minutes at 4°C. The final pellet was air-dried for 10-15 minutes and was dissolved in 25-50 µl DEPC-treated water (volume added was dependent on the pellet size) at 60°C for 10 minutes. Samples were stored at -80°C until subsequent use.

Generation of cDNA (RT-Reaction)

To make complementary DNA (cDNA) reverse transcription reactions were performed using the following protocol:4µl of 5x strand buffer (125mM tris-HCl pH 8.3, 188 mMKCl, 7.5 mM MgCl2 25 mM DTT),1 µl of each dNTP [dATP, dCTP, dGTP, dTTP (stock concentration of 10mM in DEPC-treated water)], 4 µl of oligo-dT (stock concentration of 125 µg /ml),1 µl of RNA , 4 µl DEPC–treated water, 1.5 µl M-MLV reverse transcriptase enzyme.

After addition of M-MLV-reverse transcriptase and mixing, the tube was incubated for 60 minutes at 37°C. The resultant product was stored at -20°C for further use in PCR reactions.

Polymerase chain reaction (PCR)

To amplify DNA species, a PCR reaction mixture was made by addition of the following reagents to a 0.5 ml microcentrifuge tube on ice: 10 µl of Taq polymerase buffer (10x), 3 µl of MgCl2 (stock concentration 1.5mM), 2 µl of each dNTP [(dATP, dCTP, dGTP, dTTP) stock concentration of 10mM], 2 µl of each primer pair (Table 1 (forward and reverse), stock concentration of 25ng/ µl), 4 µl of cDNA. Sterile double distilled water has reached to a final volume of 99 µl.

Table 1. Indicates the sequence of primers used in the project to detect CC chemokine mRNA. AT= annealing temperature; F= forward primer; MT= melting temperature; R=reverse primer.

Gene Name	F&R	*Primers (5´ to 3´)*	*AT (°C)*	*MT(°C)*
CCL2	**F**	**GAATTCATGAACCCAAGTGCTGCTTT**		68
	R	**AACGTTTTACGGAGCTCTTTTTGACC**	64	68
CCL5	**F**	**TTCACTTCAAGAACATCCA**	51	56
	R	**GAGCATTGGTTAAAGAATATAAA**		56
CCL11	**F**	**AGTGTGCATTGACCCGAAATTA**	60	65
	R	**TTACAGCACGAAACAGTTGGC**		65

The above mixture was overlaid with 40-60 µl mineral oil. The PCR thermocycler was run with the following program: 94 °C for 5 minutes, 94 °C for 1 minute (denaturation), 1 minute at appropriate annealing temperature for annealing of different targets (Table 2), 72°C for 2 minutes (elongation). Denaturation, annealing, and elongation were set for 30 cycles and were followed by a final reaction at 72°C for 10 minutes (final elongation) and held in 4°C up to 24h. During the last 45 seconds of the first stage, 1µl of Taq polymerase was added to the mixture. In addition to appropriate annealing and melting temperatures. The amplified fragment of complementary DNA (cDNA) (approximately 50 ng) was ligated into pUAg cloning vector (100 ng) (Ingenious, R & D Systems Europe Ltd, Abingdon, UK) by incubation with T4 ligase (1 Weiss unit) and ligase reaction buffer (30 mMTris–HCl, pH 7.8 containing 10 mM MgCl2, 10 mM DTT, and 1 mM ATP) to a final volume of 10µL for 16 hours at 4°C. The vector was transformed into Escherichia coli XL-1 Blue and grown on Luria-Broth (LB) plates containing ampicillin (50µg/ml) overnight at 37°C. Colonies were selected and cultured overnight at 37°C in LB media containing ampicillin as before. From this overnight culture, the pUAg vector with the ligated cDNA fragment was isolated using the PlasmidMidi kit (Qiagen Ltd, Dorking, and Surrey, UK). The cDNA fragment was excised from the pUAg vector using the respected restriction enzymes (Boehringer Mannheim, Sussex, UK) for 1 hour at 37°C. The restriction digest was electrophoresed on a 1% agarose gel at 70 V for 1 hour. The cDNA fragment was excised from the gel and purified from the agarose using the Gel Extraction Kit (Qiagen) and sequenced using dye-terminator chemistry.

Northern blot analysis

The RNA (20 µg) was electrophoresed on a 1% agarose/17% formaldehyde gel before being transferred

and fixed onto Hybond-N™ nitrocellulose membrane (Amersham International, Aylesbury, UK). Part-length cDNA of IP-10/Mob-1 was randomly labeled with (α-32P) dATP (50 μCi) (ICN Biomedicals Ltd). To standardize the RNA loading, the filters were reprobed with 18S rRNA, which was labeled with (α-32P) dATP (20 μCi) using Nick translation. The membranes were prehybridized at 42°C in 50% (v/v) formamide containing 5× sodium-salt-phosphoric acid-EDTA 2× Denhardt's, 0.1% (w/v) sodium dodecyl sulphate (SDS), and 0.1 mg/ml heat denatured salmon sperm DNA. The membranes were then hybridized with one of the labeled cDNA oligonucleotide probes in fresh hybridization mixture at 42°C overnight. The membranes were washed twice for 15 min at room temperature with 2 × SSC/0.1% (w/v) SDS, before a final 20 minutes wash at 55°C with 0.1× SSC/0.1% (w/ v) SDS. Then the membranes were autoradiographed using intensifying screens at -70°C. Quantization of the hybridization intensity was by phosphorimager analysis.

Western blotting analysis

To analyze the expression of CCL2, CCL5, and CCL11, equal amounts of protein (35 g) were loaded and resolved on a 10% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking with 3%(w/v) milk in phosphate-buffered saline (PBS)/Twine (10 mMTris, pH 7.4 containing 140 mMNaCl, 0.1% (v/v) Tween 20), the nitrocellulose membrane was incubated overnight at 4°C in PBS/Tween containing3% (w/v) milk including anti-human primary Ab against CCL2 CCL5 AND CCL11 (R&D System, Minneapolis, MN, USA). Subsequently, anti-mouse immunoglobulin G (IgG) horseradish peroxidase-conjugated antibodies (Amersham life science, Amersham, UK) (diluted, 1:1000) were used accordingly, and the enhanced chemiluminescence (ECL) detection system (Amersham International) were used to define protein localization and amount. The membranes were treated with enhanced chemiluminescence reagents I and II (Amersham Life Science) and exposed to X-ray film (Amersham Life Science, UK). B-actin was used as an internal control to compare the data from different films.

The β-actin monoclonal antibody (Sigma, MO, USA) was gently shaken overnight at 4ºC. After incubation, the membranes were washed three times with PBS/tween for 10 min. Then, the membranes were exposed to the anti-mouse immunoglobulin G (IgG) horseradish-peroxidase-conjugated antibody (Amersham Life Science, Amersham, UK) for 1 h at room temperature and further, exposed to X-ray films.

Statistical analysis

All data are expressed as mean ± SEM. Comparisons of variables between two groups were performed using an unpaired Student's T test. Statistical significance was considered at $P<0.05$.

Table 2. Participants' characteristics;

Variable	controls	Patients(AA/GBM)
Age (Years) Mean ± SD	48 ± 12	53 ± 7
Sex: M/F	88/101	87/36
Familial history of cancer	NA	50.4%
Smoking behavior (Nonsmoker/Smoker)	-	73 /50
(AA/GBM)	-	68/55
Total	189	

AA= Anaplastic Astrocytoma, GBM= Glioblastoma Multiform, NA= Not Assessed

Results

During a 5 year study, a total of 123 AA and GBM patients diagnosed in Kerman and Khorasan e Razavi, provinces of Iran. Totally 55.28 % of patients were AA and 44.71% were suffering from GBM. Statistical analysis didn't illustrate a significant difference between pate patients and controls, regarding age and sex. Demographic characteristics are summarized in Table 2.

ELISA assays

ELISA assays demonstrated that the CCL2 level was 348.8 ± 8.56 pg/ml and 293.73 ± 5.7 pg/ml in patients and controls, respectively. Statistical analysis revealed a significant difference between patients suffering glial tumors with control ($P<0.05$). Analysis of current results demonstrated that serum concentrations of CCL5 were also elevated in these patients in compare to respected control (Figure 1a,b) ($P<0.05$). The mean concentration of CCL5 was 898.34 ± 39.82 pg/ml and 168.95 ± 17.81 pg/ml which shows a five-folds increase relative to its control level ($P<0.05$). Unlike CCL2 and

CCL5, we observed no significant difference between our patients and controls with regard to CCL11. However, CCL11 was increased, and its mean concentration was 171.28 ± 2.93 pg/ml and 142.45 ± 3.41 pg/ml in patients and controls, respectively, but it was not differed significantly (Figure 1c).

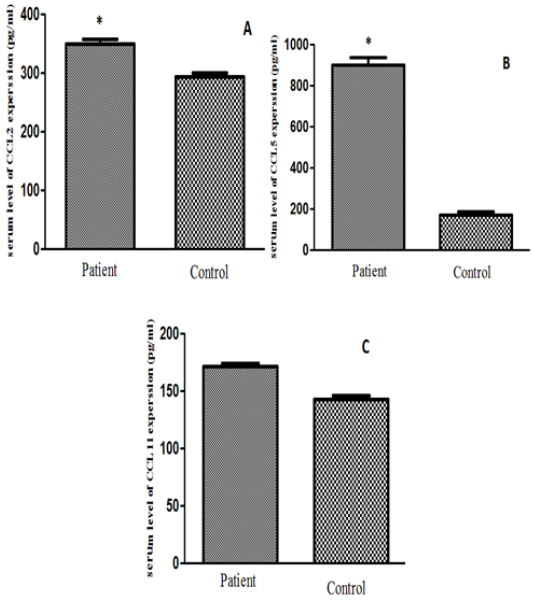

Figure 1. A) The level of CCL2, B) CCL5 and C) CCL11 in patients suffering from glial tumors and control subjects.

Data are expressed as mean ± SEM. *=Significant difference with control.

Western Blotting

Our Western blotting analyzes are in accordance with a result obtained by ELISA. As clearly presented in Figure 2B, however, CCL2 and CCL5 are expressed almost with the same levels in both AA and GBM patients, but they were very weakly expressed in NBT (Figure 2B). This is in contrast with regard to CCL11, and as is visible in Figure 1C this chemokine is very weakly expressed at the level which is almost could be undetectable in compare to the sharp bands of β-actin.

Northern Blotting

To confirm concordance between mRNA and protein levels of studied CC chemokines we employed northern blotting. Our northern blotting analysis is according to confirm chemokines protein levels achieved by western blotting (Figure 2A). With this regard, as is obvious from Figure 1 both CCL2 and CCL5 are increased in AA and GBM while CCL11 remained unchanged.

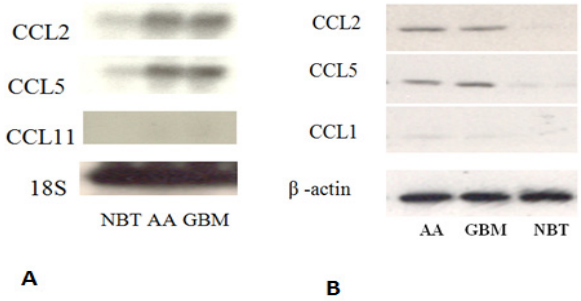

Figure 2. A) Northern blotting and B) western blotting analysis of the intratumor level of CCL2, CCL5, and CCL11 in Normal Brain Tissue, Anaplastic Astrocytoma, and Glioblastoma Multiform tumor tissues against 18S RNA and β-actin, respectively.
AA= Anaplastic Astrocytoma, GBM= Glioblastoma Multiform, NA= Not Assessed, NBT= Normal Brain Tissue

Discussion

In the present study, we sought to examine the role of CC chemokines CCL2, CCL5 and CCL11 in

malignant glial tumors. Current results showed that the serum levels of CCL2 and CCL5 are increased in AA and GBM and AA patients. We also demonstrated the elevated levels of intratumor mRNA and protein levels of both CCL2 and CCL5 while both message (mRNA) and protein levels of CCL11 was weakly expressed in tumor tissue. This may re-confirm the involvement of CCL2 and CCL5 but not CCL11in pathogenesis of glial tumor.On the other side, the expression of CCR2A has been reported in glial cells that may exhibit a role for CCL2 (its respected ligand) in the pathogenesis of GMB and AA via tumor cells migration as well as the host response to therapy (2). Specific receptors for CCL2 and CCL5 (CCR2 and CCR5) were reported to be expressed in a wide variety of tissues, including brain and various cell types, such as endothelial cells and embryonic germinal neuroepithelium, mature neurons, glial cells and microglia cells (12-14). It seems that the published data regarding the role of CCR5 and CCR3 is limited. Furthermore, present results together with other existing data may partially confirm a role for CCL2 in the pathogenesis of glial tumors particularly in the processes of glial cells migration and metastasis. Increased level of these pro-migratory proteins may aid either tumor cells to migrate and proliferate or chemoattract specific clone of lymphocyte to the tumor tissues for performing cell-cell interactions against tumor cells that it has been well evidenced these chemokines are involved in the pathogenesis of tumors (15).

In particular, our examinations on intra-tumor tissue by northern and western blotting analysis revealed that studied chemokines are elevated (at both mRNA and protein levels) probably meant that the expression of these chemokines is not exclusive to tumor cells and other tumor neighboring cell types (i.e. astrocytes, endothelial cell, *etc.*) may also involve in chemokine expression. Reasonably, increased intra-tumor levels of these inflammatory chemokines may probably be due to intratumor hypoxia and also a lack of nutrients, as it has already been reported that some other members of chemokine family were extremely expressed in central regions of the tumor (16).

The CCL2 gene comprises both NF-KB site (that is of a characteristic of stimulatory genes) and GC box (which is related to its tissue-specific and regulation and fits with properties of housekeeping genes). It has been demonstrated that both CCL8 and CCL2 gene expression in parallel with protein production by T98G cells were related to the membrane-associated IL-1alpha (17). Thus, these results together with Ueda A and colleagues findings indicate that CCL2 expression is probably controlled by at least these two distinct pathways in GBM and AA patients. Therefore, due to their vast expression, both intra-tumor and in serum these chemokines (CCL2 and CCL5) may probably be useful for differentiation of benign tumors from GBM and AA and also to predict the occurrence of tumor metastasis. Consistent with our results, Leungs and co-workers reported that recruitment of macrophages to the gliomas is regulated by both T-cell mediated up-regulation and constitutive expression of CCL2 (10). Elevated CCL2 and CCL5 expression probably are also related to their angiogenic properties (18). More recently, Bajetto-Oh and co-workers reported a CXCL12 mediated expression of CCL2 and CXCL8 in glioma cells co-expression of CCL2 and CXCL8 in Bajetto-Oh study and CCL2 and CCL5 in the present study may re-confirm the role played by them in vascular endothelial cell proliferation and then tumor neovascularization and formation of tumor vessel network (13,19).

Compelling documents demonstrating that chemokine/receptor axis could play a primary role in brain tumor growth via diverse biological effects, including cell proliferation, angiogenesis, and chemokine induction. It is now well established that GBMs are categorized within the most vascularized tumors. In AA and GBM, tumor tissues majority of lymphoid/myeloid cells are infiltrated into tumor tissues in response to tumor-derived chemokines. Therefore, the main sources of circulatory CCL2 and CCL5 in the circulation of our studied AA and GBM patients probably are activated macrophages and T cells, which in turn, may contribute to the tumor expansion. Odat and colleagues reported that the IL-1alpha-mediated CCL2 production by alprazolam is mainly due to inhibition of c- Rel/p65 and c-Rel/p50 binding to the CCL2 promoter region and Fas ligand on human glioma cells leads to the selective induction of chemokine expression, which involves the ERK1/ERK2 and p38 MAPK signaling pathways. Therefore, the Fas-Fas ligand system in human brain tumors may be involved not only in apoptotic processes but also in the provocation of angiogenic and proinflammatory responses via chemokine expression (4,20).

It is also related that a TAT-mediated increase in SP1 binding activities augments the binding of AP1 and NF-kB, leading to synergistic activation of the CCL2 promoter. These results indicate that CCL2 is produced by malignant glioma in vivo as well as in vitro and suggest that testing for CCL2 in parallel with serum in CSF may also be useful in the clinic to differentiate malignant glioma from benign glioma and to detect

subarachnoid dissemination of the tumor cells (5,6). The increased levels of these chemokines in these patients could probably also be due to the critical role played by them in the recruitment of cells involved in the neovascularization processes to glial tumors; that may aid neurogenesis and angiogenesis in these patients because of their neurogenesis and angiogenesis properties (21). Finally, elevation of these mediators may also be related to the critical role of chemokines and their receptors, (CCR2 and CCR5), in development, recruitment of neural progenitors, and homeostasis of neural progenitors in developing CNS tissues (22). Future studies are required to delineate further roles played by chemokine/receptor axis network in the development of glial tumors and metastasis.

It's also essential to tumor precisely determine whether if chemokines and their corresponding receptors are applicable for therapy of glial tumors alone or in association with tumor routine therapeutic methods. It may, in fact, be reasonable that speculate the disease severity and the event of relapse and probably a response to treatment (the following therapy) by determination of chemokines serum level, along with other clinical and paraclinical findings.

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