J Mol Neurosci (2014) 53:500–505
DOI 10.1007/s12031-013-0214-2

Higher Circulating Levels of Chemokine CCL20 in Patients with Multiple Sclerosis: Evaluation of the Influences of Chemokine Gene Polymorphism, Gender, Treatment and Disease Pattern

A. Jafarzadeh · S. Bagherzadeh · H. A. Ebrahimi · H. Hajghani · M. R. Bazrafshani · A. Khosravimashizi · M. Nemati · F. Gadari · A. Sabahi · F. Iranmanesh · M. M. Mohammadi · H. Daneshvar

Received: 20 August 2013 / Accepted: 15 December 2013 / Published online: 7 January 2014

Abstract Chemokines play an important role in the autoimmune diseases. The aim of this study was to investigate the levels of CCL20 and a polymorphism [-786C>T (rs6749704)] in the chemokine gene in patients with multiple sclerosis (MS). The blood samples were collected from 135 MS patients and 135 healthy subjects as a control group. The patients have relapsing-remitting (RRMS; n=65), primary progressive (PPMS; n=47), secondary progressive (SPMS; n=35) or progressive relapsing (PRMS; n=14) patterns. The serum levels of CCL20 were measured by ELISA. The DNA was analyzed for CCL20 polymorphism using PCR–RLFP. The mean serum levels of CCL20 in the MS group were significantly higher than in the healthy group (P<0.001). In patients with a SPMS pattern, the frequency of CT genotype at rs6749704 (24.3 %) was significantly lower as compared to patients with other patterns (42.8 %; P<0.04). No significant differences were observed between subjects with different genotypes in rs6749704 regarding the CCL20 levels. The mean serum levels of CCL20 in both newly diagnosed and previously diagnosed patients was significantly higher than in the healthy group (P<0.05 and 0.001, respectively). The mean serum levels of CCL20 in patients with RRMS, SPMS and PPMS patterns were significantly higher than in the healthy group (P<0.004, P<0.04, and 0.05, respectively). The levels of CCL20 in untreated patients and in patients who received interferon-β, methylprednisolone or the combination of interferon-β plus methylprednisolone were higher as compared to the control group (P<0.05, P<0.03, P<0.005, and P<0.05, respectively). These results showed higher levels of CCL20 in patients that represent that the chemokine may play an important role in the pathogenesis of MS. The rs6749704 polymorphism was an associated SPMS pattern. The levels of CCL20 were not influenced by gender, disease pattern and treatment.

Keywords Multiple sclerosis · CCL20 · Gene polymorphism · Treatment

A. Jafarzadeh · H. A. Ebrahimi · F. Gadari · A. Sabahi · F. Iranmanesh
Neurology Research Center, Department of Neurology, Kerman University of Medical Sciences, Kerman, Iran

A. Jafarzadeh (✉) · S. Bagherzadeh · H. Hajghani · A. Khosravimashizi · M. Nemati · M. M. Mohammadi · H. Daneshvar
Department of Immunology, Medical School, Kerman University of Medical Sciences, Kerman, Iran
e-mail: Jafarzadeh@kmu.ac.ir

A. Jafarzadeh
Department of Immunology, Medical School, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

M. R. Bazrafshani
Department of Medical Genetic, Medical School, Kerman University of Medical Sciences, Kerman, Iran

Introduction

The MS is a neurodegenerative central nervous system (CNS) disease and its clinical course is defined as RRMS, PPMS, PRMS and SPMS (Anlar 2009). The immune system plays key roles in the pathogenesis of MS (Buc 2013). It has been reported that the MS is a Th1 / Th17 mediated autoimmune disease (Jadidi-Niaragh and Mirshafiey 2011; Rostami and Ciric 2013). Upon activation, Th17 cells produce a group of cytokines including IL-17A, IL-17 F, IL-21, and IL-22, which are considered to be the dominant T cells involved in

autoimmune diseases such as MS (Jadidi-Niaragh and Mirshafiey 2011; Kuchroo and Awasthi 2012).

The infiltration of leukocytes into the CNS is an essential step in the neuropathogenesis of MS that is controlled by chemokines (Holman et al. 2011). Chemokines are a group of small polypeptides, which attract various types of leukocytes to sites of inflammation. Chemokines play an important role in immunity as well as in inflammatory and autoimmune disease (Raman et al. 2011).

The chemokine CCL20 is expressed in a variety of tissues and immune cells in response to various cytokines (Lee et al. 2013; Tesmer et al. 2008). The receptor for CCL20, known as CCR6, and accordingly, CCL20, acts as a chemoattractant for CCR6-expressing cells, including Th17 cells (Zhang et al. 2013). It has been reported that Th17 cells express CCR6 and its ligand CCL20 (Maddur et al. 2012), indicating that Th17 cells might regulate their own recruitment to inflamed tissues in an autocrine manner that may have an important role in sustaining Th17-mediated inflammation.

The CCL20 changes and its gene polymorphism have not been investigated in MS patients adequately. Accordingly, this study was conducted to evaluate the serum levels of CCL20 and a single nucleotide polymorphism [-786C>T (rs6749704)] in the promoter region of the chemokine gene in patients with MS to clarify any association.

Materials and Methods

Subjects

Blood samples were collected from 135 MS patients in Shefah Hospital of Kerman (a city located in the southeast of Iran). The expert neurologists confirmed the MS, according to the clinical and paraclinical findings based on McDonald's criteria (McDonald et al. 2001). The patients with RRMS were in the silent stage and the last attack happened two months before the sampling. Healthy controls were recruited among blood donors and interviewed with regard to CNS disorders, and none of them had any history of CNS or any other relevant disease. All controls were basically healthy, with no acute or chronic illnesses. This study was approved by the Ethical Committee of Kerman University of Medical Sciences. A peripheral blood sample (2–4 ml) was obtained from all participants and the sera was separated and stored at −70 °C until analysis.

DNA Extraction

Peripheral blood was collected in EDTA pre-coated tubes and then genomic DNA was extracted by a commercial kit (Bioneer, South Korea). Extracted DNA samples were stored at −20 °C for further use.

Polymorphism Genotyping

CCL20 gene polymorphism at rs6749704 was analyzed by the polymerase chain reaction–restriction length polymorphism (PCR–RFLP) method. PCR reaction mixture was made up of the addition of the following reagents to a 0.2 ml microcentrifuge tube on ice: 2.5 μl of Taq DNA polymerase buffer (10×), 0.5 μl of MgCl2 (stock concentration 1.5 mM), 0.5 μl of each dNTP [dATP, dCTP, dGTP, and dTTP (stock concentration of 10 mM)], 1 μl of each primer [forward primer: TTTGACATTTGCTGTGCTGAC and reverse primer: GGCTCAAACCTCAGCTTCAC (stock concentration of 25 ng/μl)], 1 μl of prepared DNA, and sterile double-distilled water to a final volume of 25 μl. The amplification was performed with the following program:

One cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 64 °C for 20 s and 72 °C for 30 s. The amplified PCR product of CCL20 gene covers rs6749704 with a molecular size of 214 bp. The Rsa I (Fermentase, Finland) restriction enzyme has merely a restriction site on this region; thus, the fragment will be digested into two 129- and 85-bp fragments following digestion. In the case of a heterozygotic form (C/T), three different fragments with 214, 129, and 85 bp are then visible. In the homozygotic form, a 214-bp fragment [without any digestion (C/C)] or two 129 and 85 bp [digesting both alleles (T/T)] was then observed. The digested products were electrophoresed on a 2.5 % agarose gel after adding 4 μl of loading buffer (Cinnagen, Iran) and studied on a Chemi-Doc model XRS (Bio-Rad, USA) after staining with ethidium bromide.

Chemokine Assay

The serum levels of CCL20 were measured by commercial ELISA kits (R&D systems, UK) according to the manufacturer's guidelines. The sensitivity of the assay was 2 pg/ml.

Statistical Analysis

Differences in variables were analyzed using Student t, ANOVA and $\chi 2$ tests as appropriate and P values of less than 0.05 were considered significant. The data were analyzed by a statistical software (SPSS version 15, Chicago, IL, USA).

Results

The mean ages of patients and the control group were 35.72 ±7.90 years and 36.50±7.79 years, respectively (P=0.41). The gender distribution of patients was 106 (78.50 %) female and 29 (21.50 %) male, and in the control group was 100 (74.10 %) female and 35 (25.90 %) male (P=0.47).

Table 1 Serum levels of chemokine CCL20 in MS and healthy groups according to gender

Group	Sex	No.	CCL20 levels[a] (mean±SD)	*P* value
MS	Male	29	72.96±14.75	0.86
	Female	106	77.49±12.84	
	Total	135	76.52±10.54	
Healthy control	Male	35	32.65±6.30	0.82
	Female	100	33.36±2.69	
	Total	135	33.18±2.56	

[a] The serum levels of cytokine expressed as Pg/ml

The Levels of CCL20 in MS and Control Groups

The mean serum levels of CCL20 in MS patients were significantly higher than those in the control group ($P<0.001$). In both MS and control groups, no significant differences were observed between men and women regarding the levels of CCL20. In men and women patients the levels of CCL20 were significantly higher as compared to healthy subjects with same gender ($P<0.01$ and $P<0.001$, respectively) (Table 1).

The Levels of CCL20 According to Chemokine Gene Polymorphisms

There were no significant differences between MS and healthy groups with respect to the percentages of the different genotypes at rs6749704 (Table 2). In both MS and healthy groups, no significant differences were observed among subjects with various genotypes at rs6749704 with respect to the levels of CCL20 (Table 3). However, in MS patients with CC, CT, and TT genotypes, the serum levels of CCL20 were significantly higher as compared with controls with the same genotypes ($P<0.03$, 0.01 and 0.01, respectively).

The Levels of CCL20 in Newly and Previously Diagnosed MS Patients

The serum levels of CCL20 in both newly diagnosed and previously diagnosed patients were significantly higher than those in the healthy group ($P<0.05$ and 0.001, respectively).

Table 2 The frequencies of the genotypes of CCL20 gene polymorphism in MS patients and controls

Genotypes	MS patients No. (%)	Healthy subjects No. (%)	*P* value
CC	9 (6.7 %)	9 (6.7 %)	1.00
CT	51 (37.8 %)	54 (40.0 %)	0.70
TT	75 (55.6 %)	72 (53.3 %)	0.71
C	69 (25.5 %)	72 (26.6 %)	0.76
T	201 (74.4 %)	198 (73.3 %)	0.76

Table 3 Serum levels of CCL20 in MS and healthy groups according to the genotypes of chemokine gene polymorphisms

Group	Genotype	No.	CCL20 levels[a] (mean±SD)	*P* value
MS	CC	9 (6.7 %)	124.15±40.47	0.90
	CT	51 (37.8 %)	82.45±17.94	
	TT	75 (55.6 %)	66.77±13.71	
	C	69 (25.5 %)	88.71±16.39	0.45
	T	201 (74.4 %)	73.12±10.90	
Healthy	CC	9 (6.7 %)	30.91±5.97	0.82
	CT	54 (40.0 %)	34.46±4.40	
	TT	72 (53.3 %)	32.50±3.45	
	C	72 (26.6 %)	33.95±3.86	0.73
	T	198 (73.3 %)	32.34±2.72	
Total	CC	18 (6.7 %)	77.53±22.84	0.44
	CT	105 (38.9 %)	57.77±9.26	
	TT	147 (54.4 %)	49.99±7.31	
	C	141 (26.1 %)	60.66±8.57	0.46
	T	399 (73.9 %)	53.23±5.74	

[a] The serum levels of cytokine expressed as Pg/ml

The mean serum levels of CCL20 in previously diagnosed patients were higher than in newly diagnosed patients, but the difference was not significant. In both newly and previously diagnosed patients no significant differences were observed between men and women regarding the levels of CCL20 (Table 4).

The Levels of CCL20 in MS Patients According to Disease Pattern

The serum levels of CCL20 in patients with RRMS, SPMS and PPMS patterns were significantly higher than controls ($P<0.004$, $P<0.04$, and 0.05, respectively). The mean levels of CCL20 in patients with PRMS were higher than controls, but the difference was not significant ($P=0.16$). In MS groups,

Table 4 Serum levels of chemokine CCL20 in newly and previously diagnosed MS patients according to gender

Group	Sex	No.	CCL20 levels[a] (mean±SD)	*P* value
Newly diagnosed MS patients	Male	12	86.51±26.91	0.48
	Female	35	60.35±19.82	
	Total	47	67.03±16.22	
Previously MS patients	Male	17	63.40±16.85	0.34
	Female	71	85.95±16.49	
	Total	88	81.59±13.69	
Healthy	Male	35	32.65±6.30	0.82
	Female	100	33.36±2.69	
	Total	135	33.18±2.56	

[a] The serum levels of cytokine expressed as Pg/ml

Table 5 Serum levels of chemokine CCL20 in MS patients according to disease patterns

Group	Diseases form	No.	CCL20 levels[a] (mean±SD)	*P* value
MS patients	RRMS	65	77.43±14.57	0.75
	SPMS	37	90.73±27.46	
	PPMS	19	59.75±13.08	
	PRMS	14	57.49±16.26	
	Total	135	76.52±10.54	
Healthy	–	135	33.18±2.56	

[a] The serum levels of cytokine expressed as Pg/ml

no significant differences were observed among patients with various patterns regarding the levels of CCL20 (Table 5).

The Chemokine Gene Polymorphism in MS Patients According to Disease Pattern

There were no significant differences between MS patients with various disease patterns regarding the frequencies of genotypes and alleles at rs6749704. The frequency of CT genotype in patients with SPMS (24.3 %) was significantly lower as compared to patients with other MS patterns (42.8 %; $P<0.04$). The frequency of CT genotype in patients with SPMS (24.3 %) was also lower than that observed in the healthy control group, but the difference did not reach statistical significance ($P=0.07$) (Table 6).

The Levels of CCL20 in MS Patients According to Treatment

The serum levels of CCL20 in patients who were treated with interferon-β, methylprednisolone or the combination of interferon-β plus methylprednisolone were significantly higher than in the healthy group ($P<0.03$, $P<0.005$, and 0.05, respectively). The levels of CCL20 in untreated patients were also significantly higher than in the healthy group ($P<0.05$). No significant differences were observed among patients with different treatments regarding the levels of CCL20 (Table 7).

Table 7 Serum levels of chemokine CCL20 in MS patients according to treatment

Group	Treatment	No.	CCL20 levels[a] (mean±SD)	*P* value
MS patients	Interferon	55	75.13±19.14	0.78
	Methylprednisolone	15	102.44±20.53	
	methylprednisolone+ Interferon	10	91.15±38.11	
	No treatment	47	67.03±16.22	
Healthy control	–	135	33.18±2.56	

[a] The serum levels of cytokine expressed as Pg/ml

Discussion

Our results showed higher levels of CCL20 in MS patients as compared to the healthy group. The CCL20 is produced by a variety of cells including endothelial cells, neutrophils, NK cells, Th17 cells, B cells, dendritic cells, Langerhan's cells and macrophages in response to stimulators such as IL-1α, IL-β, IL-6, IL-17, IL-21, IFN-γ and TNF-α (Lee et al. 2013; Tesmer et al. 2008). The participation of these cells has been reported in the pathogenesis of the MS diseases by several groups of investigators (Hoglund et al. 2013; Jadidi-Niaragh and Mirshafiey 2011). The higher expression of the mentioned cytokines has been also observed in MS patients (Kallaur et al. 2013; Wang et al. 2013), which can be considered as an account for the induction of the CCL20 production. Furthermore, astrocytes, in response to cytokines such as TNF-α, IL-1β, IL-6 and IL-17, produce CCL20 that induce the T cell recruitment to the CNS (Ambrosini et al. 2003; Meares et al. 2012). Interestingly, the steady expression of the CCL20 by the epithelial cells of the brain is thought to be important for the initial entry of Th17 cells into the CNS (Reboldi et al. 2009). Moreover, the IL-9 receptor is constitutively expressed on astrocytes and the IL-9 induces the production of CCL20 by astrocytes (Zhou et al. 2011). The IL-9–induced CCL20 expression in astrocytes causes a second wave of Th17 cell migration into the inflamed sites and exacerbates the disease (Zhou et al. 2011). The neutralizing antibodies against IL-9 inhibit disease in the latter phase of experimental autoimmune

Table 6 The frequencies of the genotypes of CCL20 gene polymorphism in MS patients according to their disease patterns

Diseases form	Chemokine gene polymorphisms			Total	Alleles	
	CC	CT	TT		C	T
RRMS	4 (6.2 %)	29 (44.6 %)	32 (49.2 %)	65 (100.0 %)	37 (28.4 %)	93 (71.5 %)
SPMS	4 (10.8 %)	9 (24.3 %)	24 (64.9 %)	37 (100.0 %)	17 (22.9 %)	57 (77.0 %)
PPMS	1 (5.3 %)	8 (42.1 %)	10 (52.6 %)	19 (100.0 %)	10 (26.3 %)	28 (73.6 %)
PRMS	0 (0.0 %)	5 (35.7 %)	9 (64.3 %)	14 (100.0 %)	5 (17.8 %)	23 (82.1 %)
Healthy	9 (6.7 %)	54 (40.0 %)	72 (53.3 %)	135 (100.0 %)	72 (26.6 %)	198 (73.3 %)

encephalitis (EAE), which supports this interpretation (Zhou et al. 2011).

The major role of Th17 in the pathogenesis of EAE has been demonstrated. The IL-23 KO mice were found to be resistant to EAE (Rodgers and Miller 2012). IL-23 is important for the differentiation and expansion of the Th17 cells. Furthermore, the adoptive transfer of myelin-specific Th17 cells into the naive mice was sufficient for the induction of EAE (Rodgers and Miller 2012). Accordingly, our results represent that CCL20 (as a Th17 chemokine) may play an important role in the pathogenesis of MS disease. Accordingly, the targeting of the CCL20 or CCR6 may be suitable targets for consideration of MS treatment in future investigations.

In our study, there were no significant differences between MS and healthy groups with respect to the genetic variations at rs6749704. However, the frequency of CT genotype in patients with SPMS was significantly lower than in other MS patterns. The frequency of CT genotype in patients with SPMS was also lower than in the control group, with a borderline significance. These data indicate that rs6749704 may have a relationship with some patterns of MS disease. More studies with a larger sample size can be conducted in this field. Moreover, in both MS and healthy groups, no significant differences were observed between subjects with CC, CT, and TT genotypes at rs6749704 regarding the levels of CCL20. These data also demonstrated that the levels of CCL20 were not influenced by rs6749704 polymorphism.

Our results also demonstrated that the serum levels of CCL20 in both newly diagnosed and previously diagnosed patients were significantly higher than in the healthy group. As mentioned before, CCL20 plays an important role in migration of Th17 cells to inflamed tissues (Zhang et al. 2013). After the Th17 cells' entrance into the lesion areas, they secrete many inflammatory cytokines, causing the creation of a cytokine cascade by inflammatory cells. This cytokine environment promotes the inflammatory process and stimulates the production of CCL20, including the trafficking of more Th17 cells to the inflammatory lesion. These explanations provide a potential interpretation for the continuous presence of Th17 in the lesions through a positive feedback loop by CCL20. Therefore, CCL20 may participate in both induction/initiation and maintenance phases of MS disease.

Our findings showed higher serum levels of CCL20 in patients with RRMS, SPMS and PPMS patterns. The serum levels of CCL20 were also higher in patients with PRMS as compared to controls, but the difference was not significant (P=0.16). The latter may be due to the small sample size in the PRMS group. The higher frequency of Th17 cells and their cytokines in patients with RRMS, SPMS and PPMS patterns, which were reported by other investigators (Christensen et al. 2013; Matsushita et al. 2013), are in agreement with our findings. These results suggest that the CCL20 may participate in the pathogenesis of all patterns of MS.

Our findings also showed that the serum levels of CCL20 in untreated patients and in treated patients with interferon and/or methylprednisolone were significantly higher than in the healthy group. No significant differences were observed between patients with different treatment programs regarding the levels of CCL20. Accordingly, the levels of CCL20 were not influenced by treatment programs. Higher serum levels of CCL20 have been observed in remission than during relapse in RRMS and the methylprednisolone treatment did not influence the chemokine levels, which is in agreement with our results (Kalinowska-Lyszczarz et al. 2011). Moreover, it has been reported that the serum levels of CCL2, CXCL11 and IL-18 were not influenced by treatment with methylprednisolone (Rentzos et al. 2008; Szczucinski et al. 2007). Therefore, treatment with interferon and/or methylprednisolone did not reduce the CCL20 levels in MS patients. This encourages more studies for developing novel treatments. The targeting of the CCL20 or its receptor CCR6 may be suitable targets for consideration of the MS treatment in future investigations.

In conclusion, the results of the present study showed higher levels of CCL20 in MS patients. This may represent that chemokine plays an important role in the pathogenesis of the disease. The rs6749704 polymorphism was associated with a SPMS pattern. The serum levels of CCL20 were not influenced by gender, disease patterns and treatment programs.

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