CCR5 chemokine receptor gene polymorphisms in ocular toxoplasmosis

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ABSTRACT

C−C chemokine receptor type 5 (CCR5) is a chemokine receptor that influences the immune response to infectious and parasitic diseases. This study aimed to determine whether the CCR5Δ32 and CCR5 59029 A/G polymorphisms are associated with the development of ocular toxoplasmosis in humans. Patients with positive serology for *Toxoplasma gondii* were analyzed and grouped as 'with ocular toxoplasmosis' (G1: n = 160) or 'without ocular toxoplasmosis' (G2: n = 160). A control group (G3) consisted of 160 individuals with negative serology. The characterization of the CCR5Δ32 and CCR5 59029 A/G polymorphisms was by PCR and by PCR-RFLP, respectively. The difference between the groups with respect to the mean age (G1: mean age: 47.3, SD ± 19.3, median: 46 [range: 18–95]; G2: mean age: 61.3, SD ± 13.7, median: 61 [range: 21–87]; G3: mean age: 38.8, SD ± 17.9, median: 34 [range: 18–80]) was statistically significant (G1 vs.G2: p-value < 0.0001; t = 7.21; DF = 318; G1 vs.G3: p-value < 0.0001; t = 4.32; DF = 318; G2 vs. G3: p-value < 0.0001; t = 9.62; DF = 318). The Nagelkerke r^2 value was 0.040. There were statistically significant differences for the CCR5/CCR5 (p-value = 0.008; OR = 0.261), AA (p-value = 0.007; OR = 2.974) and AG genotypes (p-value = 0.018; OR = 2.447) between G1 and G2. Individuals with the CCR5/CCR5 genotype and simultaneously the CCR5-59029 AA or AG genotypes have a greater risk of developing ocular toxoplasmosis (4% greater), which may be associated with a strong and persistent inflammatory response in ocular tissue.

1. Introduction

Ocular toxoplasmosis (OT) is the most common cause of posterior uveitis. Its severity may vary according to the immune system of each patient and the reactivation of latent parasites within the retina triggering necrotizing retinopathy and leading to visual impairment (de-la-Torre et al., 2014). The lesions usually heal within two to four months in immunocompetent patients leaving a hyper-pigmented scar. In more than 70% of cases of patients seeking an ophthalmologist, OT lesions that have healed are associated with other injuries (Maenz et al., 2014). Some years ago, we demonstrated that OT represents 27% of ocular diseases among patients from the northwestern region of São Paulo State, Brazil (Ferreira et al., 2014).

CCR5 is a chemokine receptor expressed on several cells with immune function whose role consists in the recruitment and mobilization of cells to sites of inflammation (Silva-Carvalho et al., 2016). The CCR5Δ32 polymorphism, characterized by a deletion of 32 nucleotides, results in a low expression of a non-functional protein on the cell surface (Gupta and Padh, 2015; Silva-Carvalho et al., 2016). Studies have demonstrated that CCR5-deficient murine animals have increased susceptibility to *T. gondii* infection as well as an increase in the number of parasites in the liver and intestine (Bonfá et al., 2014). Individuals with

Table 1

Characteristics and Frequencies of genotypes and alleles of the polymorphisms of the *CCR5* gene in individuals with ocular toxoplasmosis without ocular toxoplasmosis and controls.

	OT (G1) (n = 160)		Without OT (G2) (n = 160)		Controls (G3) (n = 160)		p
Mean age (± SD)	47.3 ± 19.3[a,b]		61.3 ± 13.7[a,c]		38.8 ± 17.9[b,c]		p-value < 0.0001[*]
Min/Max	18–95		21–87		18–80		
Median	46		61		34		
	n	%	n	%	n	%	
Genotypes							
CCR5/CCR5	141	88.1	148	92.5	144	90.0	
CCR5/CCR5Δ32	19	11.9	12	7.5	16	10.0	
Alleles							
CCR5	301	94.1	308	96.2	304	95.0	
CCR5Δ32	19	5.9	12	3.8	16	5.0	
Genotypes							
CCR559029 A/A	48	30.0	39	24.4	47	29.4	
CCR559029 A/G	81	50.6	80	50.0	81	50.6	
CCR559029 G/G	31	19.4	41	25.6	32	20.0	
Alleles							
CCR559029 A	177	55.3	158	49.4	175	54.7	
CCR559029 G	143	44.7	162	50.6	145	45.3	

OT = Ocular toxoplasmosis.

a = G1xG2.; b = G1xG3; c = G2xG3.

  [*] G1xG2; G1xG3; G2xG3 p-value < 0.0001.

the AA genotype, which relates to the CCR5 promoter polymorphism 59029, show higher CCR5 expression on the leukocyte surface when compared to the other genotypes (Oliveira et al., 2015).

These polymorphisms have been correlated with susceptibility to various infectious diseases including HIV and inflammatory diseases such as osteomyelitis, preeclampsia, rheumatoid arthritis and systemic lupus erythematosus (Rao et al., 2014; Silva-Carvalho et al., 2016; Gupta and Padh, 2015; Souza et al., 2015). The aim of this study was to investigate possible associations of the *CCR5*Δ32 (rs333) and *CCR5* 59029 A/G (rs1799987) polymorphisms with the development of OT in humans.

2. Materials and methods

2.1. Ethics information

All individuals, who agreed to participate in this research, were informed about the nature of the study and were required to sign an informed consent form authorizing the use of their samples. The study was approved by the Research Ethics Committee of the Medicine School in São José do Rio Preto (case #1980/2009).

2.2. Sample selection and clinical diagnosis

This study enrolled 320 immunocompetent patients with serologically diagnosed toxoplasmosis (IgG anti-*T. gondii* antibodies) matched by gender, being treated in the Retinopathy Outpatient Service of Hospital de Base of the Medicine School in São José do Rio Preto (FUNFARME) and in the Medical Services Outpatient Clinic (AME) in São José do Rio Preto. Patients were grouped as 'with OT' (G1; n = 160) or 'without OT' (G2; n = 160). Patients 'without OT' had other ocular diseases without any evidence of OT. In order to verify the frequency of the alleles in the study population, a control group (G3) was formed of 160 healthy volunteer blood donors from the blood bank of São José do Rio Preto, whose serology results for antibodies against toxoplasmosis were negative.

The clinical evaluation of subjects was conducted by two experienced physicians using an indirect binocular ophthalmoscope (Binocular Ophthalmoscope ID10, Topcon Corporation, USA), and all were classified according to the ETDRS criteria (ETDRS, 1985).

2.3. Inclusion/exclusion criteria

The inclusion criteria of the patient groups were positive laboratory diagnosis of toxoplasmosis, the presence of ocular scars/lesions (G1) or without ocular scars/lesions due to toxoplasmosis (G2), and being a resident in a municipality in the northwestern region of the state of Sao Paulo. The inclusion criteria for the control group (G3) were negative laboratory diagnosis for toxoplasmosis and living in the same geographical region as the patients.

The exclusion criteria for all groups were other infectious and parasitic diseases, blood dyscrasia, and the use of oral anticoagulants.

2.4. Laboratory analysis

IgG anti-*T. gondii* antibodies were confirmed by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions, performed in duplicate (ETI-TOXO-G PLUS; DiaSorin S.p.A. Italy).

Genomic DNA was attained from peripheral blood using a commercial kit for silica column extraction (QIAamp1DNA Blood Mini Kit, QIAGEN, the Netherlands) following the manufacturer's instructions.

Identification of the deletion of 32 base pairs of the *CCR5* gene (CCR5Δ32) was achieved using the polymerase chain reaction (PCR) technique. The methodology used to identify the CCR5 59029 A/G polymorphism in the promoter region of the *CCR5* gene was polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The A/G alleles were identified by the presence of a restriction site for the Bsp1286I enzyme (FastDigest, Fermentas-Thermo Scientific). The PCR conditions were previously described in detail (de Oliveira et al., 2015).

2.5. Statistical analysis

Genotype and allelic frequencies were obtained by direct counting. Statistical calculations were performed using GraphPad Instat software (version 3.06). The chi-square test was used to compare proportions between groups, adopting a level of significance of 5%. The mean ages were compared using the *t*-test. The Hardy–Weinberg equilibrium was verified using the ARLEQUIN program version 3.11 (http://cmpg.unibe.ch/software/arlequin3/). A binary logistic regression test (stepwise method) was performed using the SPSS program (IBM, version 23)

to verify the risk factors associated with the development of OT.

3. Results

Four hundred and eighty subjects were analyzed. Table 1 shows the characteristics of individuals with toxoplasmosis and control subjects. The differences between groups in respect to the mean age were statistically significant (G1 vs.G2: p-value < 0.0001; t = 7.21; DF = 318; G1 vs. G3: p-value < 0.0001; t = 4.32; DF = 318; G2 vs. G3: p-value < 0.0001; t = 9.62; DF = 318).

In all groups, the distribution of the genotypes of the CCR5Δ32 and CCR5 59029 A/G polymorphisms were in Hardy-Weinberg equilibrium (p-value ≥ 0.05). No statistical difference was found both for alleles and for genotypes of the *CCR5* gene (homozygotes and heterozygotes) between individuals with OT and controls.

Statistical analysis of the risk factors for the CCR5 polymorphisms identified statistically significant differences for the CCR5/CCR5 (p-value = 0.008; OR = 0.261), AA (p-value = 0.007; OR = 2447) and AG (p-value = 0.018; OR = 2.477) genotypes between G1 and G2. The Nagelkerke r^2 value was 0.040. Multivariate logistic regression analysis showed that individuals in G1 and G2 who presented the CCR5/CCR5 genotypes and simultaneously AA or AG had a higher risk of developing OT (equivalent to 0.04 times or 4% greater). There was no statistically significant differences in the distribution between genders within the groups.

4. Discussion

This study tested the hypothesis that the CCR5Δ32 and CCR5 59029A/G polymorphisms of the *CCR5* gene are associated with the development of eye damage due to toxoplasmosis. Two groups of patients, both of which had clinical and serologic diagnoses of the disease, were selected in order to investigate the potential effect of the association of these polymorphisms on the presence and absence of ocular disease. A group of individuals without infection (G3) was also selected to measure the frequency of these polymorphisms in a healthy population. To our knowledge, this is the first study that analyzed the frequencies of these two polymorphisms of the *CCR5* gene in the development of OT in humans.

At first glance, data from this study seem to indicate that both polymorphisms are not associated with the development of OT. The differences in frequencies of both the analyzed polymorphisms between the groups of patients and controls were not statistically significant. However, after the multivariate logistic regression analysis we observed that the individuals of G1 and G2 who presented the CCR5/CCR5 genotypes and simultaneously the CCR5-59029 AA or AG genotypes have a higher risk of developing OT (equivalent to 0.04 times or 4% greater).

Bonfá et al. (2014), using a murine model, observed that homozygous mice for the *CCR5* gene (⁻/⁻) showed marked susceptibility to infection, presenting 100% mortality in up to 16 days after inoculation of *T. gondii*, while 70% of the wild-type animals (controls) survived until the 30th day after infection. In addition, histological tests of the liver and small intestine showed that a greater amount of DNA of the parasite was identified in CCR5⁻/⁻ animals compared to control animals.

The *CCR5* gene encodes the receptor of the same name, which, in addition to being expressed in different cell types, allows the binding of the CCL3, CCL4 or CCL5 chemokines (Blanco and Ochoa-Callejero, 2012; Carpenter et al., 2014; Ortiz-Alegría et al., 2010). There is evidence that binding of these chemokines to CCR5 is crucial to trigger the immune response against different microorganisms (Blanco and Ochoa-Callejero, 2012; Gupta and Padh, 2015; Kikumura et al., 2012; Oliveira et al., 2014; Wong and Fish, 2003). A fundamental role in protecting against intracellular and extracellular parasites has been attributed to CCR5, because it modulates the initial immune response against the parasite.

Experimental studies show that CCR5 plays an important role in controlling natural killer (NK) cell activity in tissues infected by *T. gondii*. There is evidence that animals with CCR5 deficiency have decreased inflammatory response with higher parasitemia and consequently higher death rates (Khan et al., 2001, 2006; Kikumura et al., 2012). According to Kikumura et al. (2012), CCR5 is one of the main molecules in the retina of animals during *T. gondii* infection; this is justified as the levels of CCR5 expression have reached a peak in the retina and brain of the murine animals by the 28th day of infection.

Activation of the immune response against parasites involves the recruitment of a variety of cells including neutrophils, T lymphocytes, macrophages, dendritic cells and inflammatory monocytes. The CCR5 receptor may be expressed by resident cells or by those that migrate to the tissue after stimulation by pro-inflammatory cytokines such as interleukin-12 (IL-12), IFN-γ and TNF, or when in contact with the pathogenic agent (Moser and Loetscher, 2001). Thus, the mechanisms that control replication of the parasite and the subsequent inflammation can indeed be partly due to the cells recruited by CCR5.

Neutrophils, Macrophages and dendritic cells are essential in the initial phase of immune response because they are IL-12 secreting cells; this cytokine is essential for resistance against infection (Denkers et al., 2003; Scott and Hunter, 2002). IL-12 stimulates NK cells to produce IFN-γ and to promote the development of Th1 cells that produce IFN-γ (Gazzinelli et al., 1994). The interaction of parasite antigens with the CCR5 chemokine receptor induces IL-12 synthesis in mature dendritic cells, with this being a major pathway of IFN-γ-dependent resistance to infections (Aliberti et al., 2000). Intraocular immune response is suppressed under normal circumstances, but there is experimental evidence that *T. gondii* infection promotes the production of factors such as IFN-γ that suppress the immune privilege of this organ. Thus, factors similar to the response that occurs in other tissues are involved in the development of eye injuries (Gazzinelli et al., 1994; Kijlstra and Petersen, 2014; Maenz et al., 2014; Roberts and McLeod, 1999).

Human studies reveal that a mutant allele of the *CCR5* gene, with a 32 base pair deletion, results in reduced cell surface expression of CCR5, decreasing the efficiency of the immune response in these individuals (Blanpain et al., 2000; Vallochi et al. Al., 2008). We observed that individuals who had this deletion of 32 base pairs in heterozygosity did not have an increased risk of developing ocular toxoplasmosis, since this alteration appears to protect ocular tissue by the reduced expression of the CCR5 chemokine and consequently lower immune and inflammatory response. Additionally, Vallochi et al. (2008) did not observe any association between the CCR5Δ32 allele and ocular toxoplasmosis in Brazilian patients, as this is a rare variant, thereby corroborating the results found in this study.

Machado et al. (2014) reported that the immune response may partially promote the development of ocular lesions resulting from *T. gondii* infection and the mechanisms involved may be associated with the pathogenesis and protective effects that control tissue damage. The increased frequency of circulating NK cells and proinflammatory monocytes in children infected with *T. gondii*, especially those who have active ocular lesions, are indicative of an intense and persistent proinflammatory response.

Clinical diagnosis of OT is suspected based on the results of the dilated fundus examination which is used to verify the presence of exudative lesions and scars characteristic of this disease (Pleyer et al., 2014; Vasconcelos-Santos, 2012). However, the analysis of serum to detect the presence of IgG anti-*T. gondii* antibodies indirectly ensures that all patients in G1 and G2, that is, with and without OT, were exposed to the parasite (Dhakal et al., 2015; Martins et al., 2015).

Although studies reported that the clinical diagnosis alone leads to a considerable number of false positive results as confirmed by biological ocular fluid tests (de-la-Torre et al., 2007, 2009; Vasconcelos-Santos 2012; Ozgonul and Besirli, 2017), in this study all the patients with clinical diagnoses by two ophthalmologists, were also submitted to

serology tests using the ELFA technique.

The average age of the seropositive patients (G1 and G2) was higher than the mean age of the individuals with negative serology (G3). This finding is easy to understand because the greater the host's age, the greater is the chance of exposure to this infection. In addition, the average age of the patients with OT (G1) was lower than the mean age of those without evidence of the disease (G2). This observation is consistent with previously published data (Ayo et al., 2016; Ayo et al., 2015; Ferreira et al., 2014; Hofhuis et al., 2011). Infection with *T. gondii* occurs at any time in life but a significant number of patients are infected congenitally (Kijlstra and Petersen, 2013; Pfaff et al., 2014). However, there are no diagnostic tests that reliably define at what time the infection occurred. The disease can also manifest in childhood and the resulting scars tend to be persistent. Therefore, it is not unusual to find population analyzes reporting the presence of this disease in young age groups (Gonzalez Fernandez et al., 2016; Arantes et al., 2015; Dubey et al., 2012; Maenz et al., 2014; Nogareda et al., 2014; Hofhuis et al., 2011).

Individuals with the CCR5/CCR5 genotype and simultaneously the CCR5-59029 AA or AG genotypes have a greater risk of developing ocular toxoplasmosis, which may be associated to a strong and persistent inflammatory response in the ocular tissue.

Author's contribution

CCBM corresponding author and head of the FAMERP Toxoplasma Research Group. CCBM, LCM were responsible to concept and design of the study. MP, GCAJr, FBF, APB, performed the inclusion of patients with ocular toxoplasmosis, sample collection, and develop the ophthalmological clinical evaluation and clinical analyses. GMFJ, APO, ALG, FHM performed the laboratory tests. CCBM, LCM, GMFJ, CMA performed the data analysis. CCBM, LCM, GMFJ, CMA wrote the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest

The authors do not have any conflict of interest with the present work.

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