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ROLE OF *CCR5* -2150 A>G AND Δ32 POLYMORPHISMS IN RHEUMATOID ARTHRITIS: A CASE-CONTROL STUDY IN A SICILIAN POPULATION.

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ABSTRACT

Rheumatoid arthritis (RA) is an inflammatory joint disorder whose progression leads to destruction of cartilage and bone. Chemokines, molecules able to induce chemotaxis in inflammation, are involved in RA pathogenesis. Aim of this study was to determine whether -2150 A>G and delta32 (Δ32) polymorphisms in the chemokine receptor 5 (CCR5) confer susceptibility to rheumatoid arthritis. Polymorphisms were assessed in 70 seropositive RA patients and 200 healthy individuals of Messina and province. About -2150 A>G polymorphism, a significant increase in AG genotype frequency was observed in controls than in patients, despite a not significant difference in allelic frequencies. Conversely, allelic and genotypic frequencies related to Δ32 polymorphism were significantly higher in controls group than in patients. Furthermore, in the patient group no individuals with Δ32/Δ32 genotype were found. These results suggest that CCR5 polymorphisms seem to play an important role in susceptibility to RA exerting a protective role in the disease..

1. Introduction

Rheumatoid arthritis (RA) is a complex autoimmune disease of the synovial joints. The typical features for RA includes the chronic inflammatory of synovial membrane and formation of a pannus, which leads to joint destruction finally [1]. Similar to other autoimmune conditions, RA is a heterogeneous disease with variable combinations of several genes polymorphisms that contribute to susceptibility to the disease [2].

Among genes involved in disease there are those for chemokines and their receptors, as chemokines are important mediators of the inflammatory response and play an important role in the pathophysiology of joint inflammation and destruction in RA [3]. Chemokines are small proteins with several functions including immune surveillance and immune cell recruitment. Their receptors, containing 7 transmembrane domains, are coupled to a trimeric signal-transducing G protein. For this reason they differ from other transmembrane proteins such as ATP - binding cassettes (ABC) proteins or Band 3 protein involved in ions and SO42-transport [4-6].

The chemokine receptor CCR5, mainly expressed on cells involved in the immune response, such as T-cells and macrophages, is composed of 352 amino acids [7]. It binds Rantes, macrophage inflammatory protein MIP 1 α and MIP 1 β, and β chemokines [8]. The protein contain seven trans-membrane domains, an amino terminal, a carboxyl tail terminal and three intracellular loops [9].

CCR5 is the principal co-receptor, with CD4, for macrophage-tropic strains of human immunodeficiency virus type 1 (HIV-1) involved in the initial phase of HIV infection via sexual transmission [10]. Trans-membrane domains and loops play an important role in both HIV interaction and chemokine binding [11,12]. Therefore amino acid modifications of CCR5 have important consequences for HIV infection, ligand binding affinity and receptor surface expression.

Several mutations were identified in the coding sequence of the CCR5 gene and well documented in relation to HIV infection and progression [13,14]. In particular a 32-bp deletion mutation (Δ32) which interrupts the coding region of the CCR5 chemokine receptor, causes a frame shift at aminoacid 185. Individuals homozygous for the mutation have a near total resistance to HIV-1 infection despite repeated exposure, while the onset of AIDS in heterozygotes who are HIV-1 infected is delayed by 2–3 years compared with CCR5 wild-type individuals [15]. The Δ32 mutation is mainly distributed in individuals of European descent, with a decreasing frequency from north to south [16]. A descending gradient (north-south) was also observed in Sicily, probably due to its ancient history [17].

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The mutated allele frequency in a cohort of Sicilian HIV-1 affected individuals was lower than in controls [18]. Furthermore, several single nucleotide polymorphisms (SNPs) were identified in the CCR5 promoter region able to reduce the level of CCR5 expression and the rate of progression to AIDS in HIV-1- infected patients [19]. A female-specific association of CCR5 promoter region polymorphisms with Löfgren's syndrome was found [20] being CCR5 expression as well as that of other genes estrogen-dependent [21,22].

About RA, no association was found between some polymorphisms in CCR5 3'-untranslated region and the disease [23], although alterations in this region may affect gene expression abolishing or introducing target sequences for miRNAs [24]. Conversely, CCR5 -2459 A/G polymorphism was associated with RA while nothing is known in the literature on the role CCR5 -2150 polymorphism in the disease.

Therefore, this study was carried out to evaluate the possible role of CCR5 -2150 and Δ32 polymorphisms in Rheumatoid arthritis in a cohort of Sicilian patients.

2. Methods

Patients and control population

A total of 70 individuals were recruited in the study from Messina and province. All the patients were rheumatoid factor (RF) positive based on erythrocyte sedimentation rate (ESR), serum level of C-reactive protein (CRP) and level of Rheumatoid factors (RFs). Their ages ranged from 20 to 80 years (mean 48 years). Disease duration ranged between 3 and 45 years (mean 29 years). 42 (57%) of the patients were female. As controls, 200 healthy donors, living in same geographical area for at least two generations, age and sex matched, with no history of inflammatory arthritis were included in the study. Demografic features of Rheumatoid arthritis patients and healthy controls in Sicilian population are shown in table 1. All participants were informed of the purpose of the study and agreed to take part.

Genotyping (Primary and polymerase chain reaction)

Eparinized peripheral blood was collected from patient and controls before any therapy. Genomic DNA was extracted by using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The nucleotide sequence of primers is shown in table 2. PCR was carried out in 50-µl volumes containing a 0.2 µm concentration of each primer. 1 U Taq Gold polymerase (PE Applied Biosystems), and 0.8 µg genomic DNA as template, under the following conditions: denaturation at 95 °C for 1.5 min, 1 min of annealing and extension at 72 °C for 50 s for 35 cycles, after an initial 10 min denaturation at 95 °C. The annealing temperature was optimized for each primer set. For -2150 A>G this numbering system is based on the first nucleotide of the CCR5 translational start site defined as 1 and the nucleotide upstream from that as -1, as originally described by Mummidi et al. (2000) [25] .

Direct sequencing

Molecular analysis was performed by Sanger sequencing. All PCR products were sequenced on a 3500 Genetic Analyzer (Applied Biosystems), using the BigDye Terminator v3.1 chemistry, following manufacturer's procedures.

Statistical analysis

Traditional statistical analyses, including genotypic and allelic analyses, were performed using "SNPator" web based software (http://www.snpator.org).

Arlequin 3.5.1.3 (http://cmpg.unibe.ch/software/arlequin35/) was used to test for Hardy Weinberg equilibrium (HWE). Statistical significance was defined as $P < 0.05$.

Features	RA patients	Control subjects
Number	70	200
Age (yrs)	48 (20-80)	53 (25-75)
Male/Female	28/42	90/110

Table 1 - Demografic features of Rheumatoid arthritis patients and healthy controls in Sicilian population.

dbSNP ID	Position	Forward Primer	Reverse Primer	Tm (C°)	Amplicon size (bp)
rs147420214	-2150 A>G	5'-CAACTCTTAAGATAATCAG-3'	5'-TACTCATCTCAGAAGCTA-3'	50	524
rs33	CCR5Δ32	5'-GTCTTCATTACACCTGCAGCTC-3'	5'-GTGAAGATAAGCCTCACAGCC-3'	56.5	198/166*

Position of SNP in the promoter region of the CCR5 gene was based on the first nucleotide of the CCR5 translational start site (designated as +1)
* For rs33 PCR gives an expected product of 198 bp in the case of the wild-type allele, and 166 bp in the case of the Δ32 allele.

Table 2 - Primer sequences used for PCR and sequencing reaction.

3. Results

The allele and genotype distribution of the *CCR5* -2150 A>G (rs147420214) and Δ32 (rs33) polymorphisms in RA and control individuals are showed in table 3.

With regard to -2150 A>G polymorphism, the frequencies of the A and G alleles were 43.0% and 57.0% in the controls, and 47.0% and 53.0% in the patient group. This difference was not statistically significant ($p=0.482$; $X^2=0.495$).

Conversely, a significant difference between controls and RA patients was observed in genotype distribution: the frequencies of the *CCR5* -2150 A/A, -2150 A/G and -2150 G/G genotypes were 10.0%, 66.0% and 24.0% in the controls and 21.0%, 50.0% and 29.0% in the patient group ($p=0.020$; $X^2=7.80$).

Different trend was observed for *CCR5*Δ32 polymorphism: significant difference in allelic frequencies and genotypic distribution was found between controls and patients group.

The frequencies of the w^+ and Δ32 alleles were 89.0% and 11.0% in the controls, and 96.4% and 3.6% in the patient group ($p=0.008$; $X^2=6.94$).

The frequencies of the CCR5 $w^{+/}$ w^+, $w^+/$ Δ32 and genotypes were 80.0%, 18.0% and 2.0% in the controls and 93.0% ($w^{+/}$ w^+) and 7.0% ($w^+/$ Δ32) in the patient group ($p=0.040$; $X^2=6.45$). No patients with Δ32/ Δ32 were found.

Polymorphism	Patient (N=70)	(%)	Control (N=200)	(%)	X^2	p-value
(rs147420214) -2150 A>G 59338 A>G						
AA	15	(21)	20	(10)		
AG	35	(50)	132	(66)	7.80	0.020
GG	20	(29)	48	(24)		
A	65	(47)	172	(43)	0.495	0.482 (NS)
G	75	(53)	228	(57)		
rs33 CCR5Δ32						
w^+/w^+	65	(93)	160	(80)		
$w^+/\Delta32$	5	(7)	36	(18)	6.45	0.040
$\Delta32/\Delta32$	0		4	(2)		
w^+	135	(96.4)	356	(89)	6.94	0.008
Δ32	5	(3.6)	44	(11)		

Genotypes and allelic frequencies were compared using 2 × 2 contingency tables yielding χ2
NS, not significant.

Table 3 - Genotypic and allelic frequencies for *CCR5*-2150 A>G and Δ32 polymorphisms in RA patients and healthy controls.

4. Discussion

In this study, we analyzed two SNPs in the CCR5 gene in a cohort of Sicilian Rheumatoid arthritis (RA) patients in order to identify their possible role in the disease. CCR5 -2150 A>G and Δ32 were analyzed performing a case-control study on 70 RA patients and 200 healthy controls. CCR5Δ32, characterized by a 32bp deletion in single coding exon of the gene, was widely studied in several populations [26] in relation to its role in HIV-1 infection. In fact, CCR5Δ32 does not produce a functional protein, explaining the near-complete protection against HIV-1 infection in individuals homozygous for the allele. However, other studies showed that CCR5Δ32 was not a protective factor mediating a partial resistance to HIV-1 infection [27]. Conversely, nothing is known about CCR5-2150 polymorphism localized in the promoter region of the gene, although several other polymorphisms have been linked at a reduced surface expression of CCR5 receptor [28].

In rheumatoid arthritis, leukocyte migra¬tion is mediated in part by chemokines and chemokine recep¬tors and plays an important role in the perpetuation of inflam¬mation in rheumatoid synovium [29] and CCR5 + monocytes are found in the synovial fluid [30].

Therefore, it is possible to hypothesize that polymorphisms in the CCR5 gene able to reduce the expression of the CCR5 receptor on leukocytes surface may exert a protective effect against rheumatoid arthritis. The results in our cohort of patients lead in this direction: about CCR5-2150A>G polymorphism, we found a frequency of AG genotype significantly higher in controls than in patients, despite the difference in frequencies of the mutated allele G in two groups was not significant.

About CCR5Δ32 significant differences were found both in genotypic and allelic frequency in both groups. No subjects homozygous for the CCR5Δ32 allele in patients group were found.

In conclusion, our results suggest a protective role of the two CCR5 polymorphisms in the pathogenesis of the disease.

It would be of interest to perform the same analysis in RA patients belonging to non-Italian populations in order to determine whether the frequencies of these polymorphisms have the same trend..

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