Journal of Sciences, Islamic Republic of Iran 22(2): 111-116 (2011) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Association of *CCR2* Gene but not *CCR5* Gene Polymorphisms with Alzheimer's Disease

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Received: 26 January 2011 / Revised: 13 April 2011 / Accepted: 12 June 2011

Abstract

Chemokines participate in the regulation of immune and inflammatory responses by interacting with their specific receptors on related immune and inflammatory cells such as B-lymphocytes, T-lymphocytes and antigen-presenting cells. Chemokines and their receptors are therefore considered to mediate inflammation and tissue damage in autoimmune disorders. The recent studies have revealed the genotypes of chemokine receptors (CCR) and their related polymorphisms in a number of autoimmune and infectious diseases. We used the polymorphic DNA markers (CCR2-64I) and (CCR5Δ32) to study the association of CCR2 and CCR5 gene mutations with Late-onset Alzheimer's disease (LOAD) and the relation between clinical features and genotypes in affected individuals. A total of 160 patient samples and 163 healthy controls from west northern Iran (Eastern Azerbaijan) were genotyped for the two polymorphisms by the PCR-RFLP method and genotype frequencies were statistically determined. No significant linkage was determined between CCR5Δ32 and the disease of interest. However the gene CCR2 was appeared to be significantly linked to the disease, as it could be concluded from statistical analysis.

Keywords: Alzheimer's disease; CCR2; CCR5

Introduction

Alzheimer is the most common neurodegenerative disease affecting 20-30 million individuals worldwide [1]. The aged people which are the growing portion of population are at risk of this disease [2]. The hallmark of neurodegeneration in Alzheimer patients is an up regulation of inflammatory cytokines in reactive microglia cells in brain [3,4]. This process may play an important role in the accumulation of microglia cells at A-beta site in senile plaque [5]. It has been shown that a number of chemokines and their related receptors are upregulated in brain tissue of Alzheimer patients. MCP-1 (Monocyte chemoattractant protein-1) and RANTES

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(Regulated upon Activation Normal T-cell Expressed and Secreted), which serve as in vitro potent microglial and macrophage chemoattractant, are produced by astrocytes and oligodendrocytes when stimulated with amyloid peptides [6]. Furthermore the levels of these chemokines are increased in cerebrospinal fluid (CSF) of patients with AD compared with healthy age matched controls. The increasing expression of MCP-1 and RANTES related receptors (CCR2 & CCR5 respectively) in brain tissue of patients suggest that these molecules might play a role in the regulation of brain immune response in AD. These receptors also involve in recruitment of leucocytes to inflammatory site [7]. Genes for CCR2 & CCR5 are characterized by the presence of CCR5Δ32 & CCR2-64I polymorphisms. CCR5 encoded by the *CMKBR5* gene located on chromosome 3p21.3. A 32 bp deletion in the open reading frame of the CCR5 gene induce a frame shift mutation causing a premature stop codon within the third extra cellular domain and results in a truncated protein product that is not a functional receptor [8-13]. The CCR2 gene is located at the same location as CCR5 (3p21.3) separated by a 19 Kb DNA sequence from each other. A valine to isoleucine substitution at codon 64 within the first membrane region of CCR2 is associated with nonfunctional receptor [14]. In contrast to CCR5, this polymorphism is frequent in African-American and Asian populations [15-17].

Based on the above findings implying the potential importance of receptors of CCR2 and CCR5 chemokines in the pathogenesis of AD and different frequency of related polymorphisms of the genes in various populations, we studied the distribution of the CCR2-64I & CCR5Δ32 in 160 AD patients as well as in 163 age matched controls, in order to determine whether they influence the susceptibility or exert a protective effect on development of the disease in eastern Azerbaijan of Iran.

Materials and Methods

Sample Preparation

The study included 160 AD patients (women & men, age range between 65-99, the mean age 76.06±7.75 yr) and 163 healthy controls. All Alzheimer subjects were diagnosed by expert clinicians according to the MMSE criteria [18]. The sporadic form of the disease was ensured where no affected individuals were present in first degree relatives of subjects, and the age of onset was above 65 years. The Control group included 163 healthy individuals, with the same ethnicity to subject group (women & men, age range between 65-89 years, the mean age 75.29±6.75 yr), which were randomly selected from a distinguish lab.

Experimental Methods

Blood specimens were collected in sterile tubes containing EDTA, and the DNA was extracted using the salting out method. The CCR5Δ32 genotype was determined by PCR without RFLP. The PCR reaction was prepared in a total volume of 25 µl, containing 0.1 micrograms of genomic DNA, 0.01 µg each of primers (forward: 5′TCTCCCAGGAATCATCTTTACC3′, reverse: 5′AGCCCTGTGCCTCTTCTTC3′), 2.5 µg of 10×PCR buffer (670 mM Tris-HCl pH 8.8, 160 mM (NH4)2SO4, 0.1%Tween-20), dNTP mix (10mM each), 50 mM MgCl2, Taq DNA polymerase (5000u/ml). The primer designing was carried out using online primer 3 program and Ensembl Genome Browser for blasting. After denaturation of template DNA at 94°C for 5 minutes, 35 cycles of PCR reactions were optimized and performed by denaturation at 94°C for 1 minutes, annealing at 56.4°C for 1 min and extension at 72°C for 1 min. A further extension was performed at 72°C for 5 min. The PCR product was fractionated on an 8% acrylamide gel and visualized flowing to staining by AgNO3. The PCR produced a 220-bp product from the wild-type allele and a 188-bp product from the deleted allele (Fig. 1).

CCR2-64I genotypes were determined according to the previously described PCR conditions with slight modification. The PCR reaction was prepared in a final volume of 25µl, containing 0.1 micrograms of genomic DNA, 0.01 µg each of primers (forward: 5′TTT GTGGGCAACATGATGG3′, reverse: 5′GCACATTGC ATTCCCAAAG3′), 2.5µl of 10×PCR buffer (670mMTris-HCl pH 8.8, 160 mM (NH4)2SO4, 0.1%Tween-20), dNTP mix (10mM each), 50 mM MgCl2, Taq DNA polymerase (5000 u/ml). The primer designing and blasting was done as described previously. The cycling conditions were as follows: initial denaturation at 94°C for 4 min followed by 31 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 45 sec and final extention at 72 °C for 5 min. The PCR products were digested using 2unit/µl of Bsej1 restriction enzyme in a total volume of 25µl, containing 5µl PCR product in supplied buffer. The mixture was incubated at 65°C for 12-16 h. The digested PCR product was fractionated on 8% polyacrylamid gel and visualized after staining by AgNo3. Electrophoresis of digested PCR products created 171- and 152-bp fragments for CCR2 wild type and 64I alleles, respectively (Fig. 2).

Data Analysis

Statistical analysis was performed using the Sigma Stat 2.0 software. Allelic and genotypic frequencies were obtained by direct counting. Hardy–Weinberg equilibrium was tested by using a $\chi 2$ goodness-of-fit test. Fisher's exact test was used for differences in genotypes and haplotypes between the groups. Statistical significance was set at $P< 0.05$. The odds ratio (OR) was calculated at 95% CI.

Results

A total of 323 individuals were enrolled in the present study to evaluate the association of desired polymorphisms in CCR2 AND CCR5 genes with T2D using PCR-RFLP procedure. The patient and control groups were matching by age, gender and education status. Table 1 shows the results obtained from statistical analysis of mentioned variables, indicating to non-significant differences between the two groups.

The PCR products obtained from amplification of target sequence within CCR5 gene has been demonstrated in Figure 1. The homozygote individual for wild allele shows a single band of 220bp, where the heterozygotes showed two bands with 220bp (wild) and 188bp (mutated). The allele and genotype frequency of CCR5 has been shown in Table 2. No significant difference was observed between the case and control groups for the polymorphic region of the CCR5 gene. Figure 2 illustrates the DNA fragments related to CCR2 gene following to PCR-RFLP reaction. Homozygote normal samples were left uncut revealing a DNA fragment of 171 bp, homozygote mutant samples were cut showing a single 152bp and the heterozygotes showed both DNA fragments. Only 1.3% of AD patients were homozygous for the CCR2-64I allele indicating to strong association of decreased frequency of CCR2-64I polymorphism with AD (Table 3). Because CCR2 and CCR5 are located in a 19Kb of DNA space of together, they are closely linked on chromosome 3. The co-segregation or independent assortment of the two markers was studied in case and control groups. CCR2-64I carriers were never found to be homozygous for the CCR5Δ32, confirming almost complete linkage disequilibrium [12].

Discussion

On the basis of the data obtained in previous studies Aβ deposition is associated with a local inflammatory response, which is initiated by the activation and migration of microglia in inflammatory sites [13,19-21]. In addition, the recruitment of microglial cells in senile plaques is induced by chemokines and their up regulated receptors in AD brain [3,4,22] Also, it has been demonstrated that CCR5 receptor is over expressed on microglial Cells of AD brain and many of this reactive microglia were found to be associated with amyloid peptides [7]. In conclusion, Aβ deposits stimulate the expression of a range of pro inflammatory genes. On the other hand, new studies have demonstrated that the most highly induced genes in human postmortem brain microglia stimulated with Aβ belonge to the chemokine family [23]. However, it has been suggested that CCR5 receptor develop plaque atherosclerosis. This receptor is expressed on macrophages, Th1 and Th2 lymphocytes, coronary endothelial cells and aortic smooth muscle cells [21, 24-29]. Recent genetic studies showed that the CCR5Δ32 variant of gene CCR5 protects individuals from early acute myocardial infarction (AMI) and severe CHD. For this reasons, the allelic variant CCR5Δ32 may have a protective role against AMI as a consequence of an attenuated inflammatory response [30].

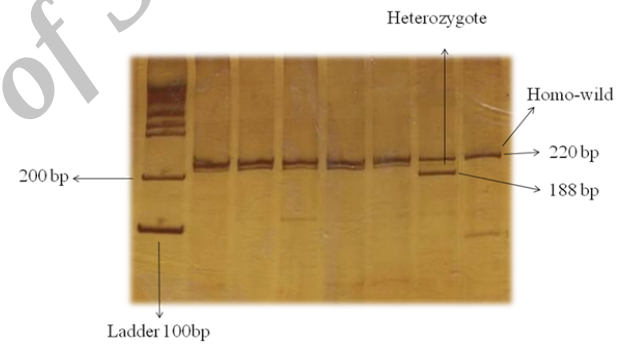

Figure 1. Polyacrylamide gel demonstrating the PCR product of CCR5 gene in homozygote normal and heterozygote individuals.

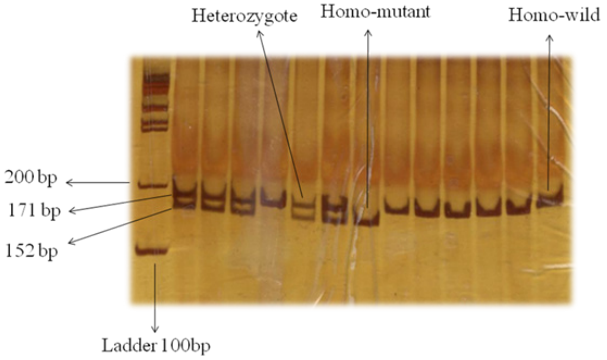

Figure 2. Polyacrylamide gel demonstrating the PCR-RFLP product of CCR2 gene in homozygote and heterozygote individuals.

Table 1. Comparison of mean age, sex and education levels between AD cases and control subjects using *t*-test and χ^2 test analysis

		All individuals	
		AD patients (n=160)	Control subjects (n=163)
Age		76.06±7.75[a]	75.29±6.75[a]
Sex(F/M)[b]		94/66	95/68
Education	Illiterate	33.8%	32.5%
levels	Primary school	21.9%	20.9%
	Secondary school	12.5%	11.7%
	Diploma	22.5%	23.9%
	Academic	9.4%	11.0%

[a] Mean±S.D., [b] Female/Male

Table 2. CCR5 allele and genotype frequencies (%) in AD patients and healthy controls

	All individuals			
CCR5 frequencies	AD patients *n*=160	Healthy controls *n*=163	OR(95CI) for significant difference	P-value
Allele				
wt	300(93.75)	312(95.71)	0.67(0.33-1.35)	
Δ32	20(6.25)	14(4.29)		
Genotype				0.26
wt/wt	140(87.5)	149(91.4)		
wt/Δ32	20(12.5)	14(8.6)		
Δ32/Δ32	0(0)	0(0)		

Values are expressed as *n* (%), P=0.26 for AD patients vs. healthy controls (Fisher's exact test)

Table 3. CCR2 allele and genotype frequencies (%) in AD patients and healthy controls

	All individuals			
CCR2 frequencies	AD patients *n*=160	Healthy controls *n*=163	OR(95CI) for significant difference	P-value
Allele				
wt	291(90.94)	225(69.02)	4.50(2.87-7.05)	
64I	29(9.06)	101(30.98)		
Genotype				<0.01
wt/wt	133(83.1)	83(50.9)		
wt/64I	25(15.6)	59(36.2)		
64I/64I	2(1.3)	21(12.9)		

Values are expressed as *n* (%), P<0.01 for AD patients vs. healthy controls (Fisher's exact test)

It has been shown that in HLA-DR4 positive Russian population the CCR5Δ32 is associated with multiple sclerosis (MS), indicating that it may influence the severity of the disease [31]. The previous studies have also revealed that, CCR2-64I frequency is decreased in patients with pulmonary sarcoidosis compared with controls, while CCR5Δ32 allelic frequency is significantly increased in the same population, suggesting a specific role in disease protection and susceptibility, respectively [13]. These receptors are also well-known as an important co-receptor for the entrance of HIV into the host cells [13,32]. The regulated on activation normal T expressed and secreted protein (RANTES) is a ligand for the CCR5. Also CCR2 is a receptor for the beta-chemokine monocyte chemo attractant protein-1 (MCP-1) which specifically mediates monocyte chemotaxis [33].

There are growing evidences that, basic molecular aspects of chemokine biology differ with race [15]. The frequency of the CCR5Δ32 allele is high in northern European populations (11~15%), these frequencies are very low in Greece, Saudi Arabia, Pakistan, and India (1~3%) [34,35]. Furthermore, the CCR5Δ32 allele has not been found in China, Japan, Korea, and Thailand [35, 36]. Incontrast, the CCR2-64I allele is more frequent in Africa and Asia than in northern Europe [32]. Immigration, genetic admixtures, and the founder effect might be responsible for differences which are observed in the CCR polymorphisms [36].

The results obtained from the present study indicate that in our study population occurrence of the CCR2-64I polymorphism is decreased in AD patients and thus is associated with a low risk of developing the disease (P <0.001). The low frequency of the genotype 64I/64I in AD patients proves real protective effect of this polymorphism of AD. The statistic analysis of the data obtained in our study revealed no significant difference in genetic distribution of CCR5 polymorphism between the case and control group (OR=0.67, 95% CI: 0.33-1.35). Also, the haplotypes of CCR5Δ32/Δ32 have never been observed, either in AD or in control group.

In our study the haplotypes of CCR2-64I/64I, CCR5+/Δ32; CCR2+/64I, CCR5Δ32/Δ32 and CCR2-64I/64I, CCR5Δ32/Δ32 were not simultaneously observed either in patients or controls, confirming that the mutant alleles of the two genes are almost in a complete linkage disequilibrium. The similar results have been reported by others [37].

A possible association between decreased frequency of the CCR2-64I and AD have to be further elucidated in larger case control studies and, moreover, if this association will be confirmed, the mechanism by which the CCR2-64I polymorphism could exert such a

protective effect on the development of AD should be studied in detail, considering both the possible involvement of other chemokines or chemokine receptors and the complex interactions with other genetic and environmental factors that are unique to each ethnicity [38]. It can be concluded that, the presence of CCR5Δ32 has no significant influence on the occurrence of the disease.

Acknowledgements

We are grateful for valuable cooperation of Danesh laboratory in providing the control samples. We thank Dr. Parvin Javadi (Khuban old people's home, Tabriz) and Dr. Shoukoh Mosavipoor (Mehr old people's home, Tabriz) for their kind collaboration. The study was supported by a grant from Deputy for Research, Tabriz University of Medical Sciences.

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