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THE ALA/ALA GENOTYPE OF PPARγ PRO12 ALA POLYMORPHISM IS ASSOCIATED WITH LATE ONSET OF MULTIPLE SCLEROSIS

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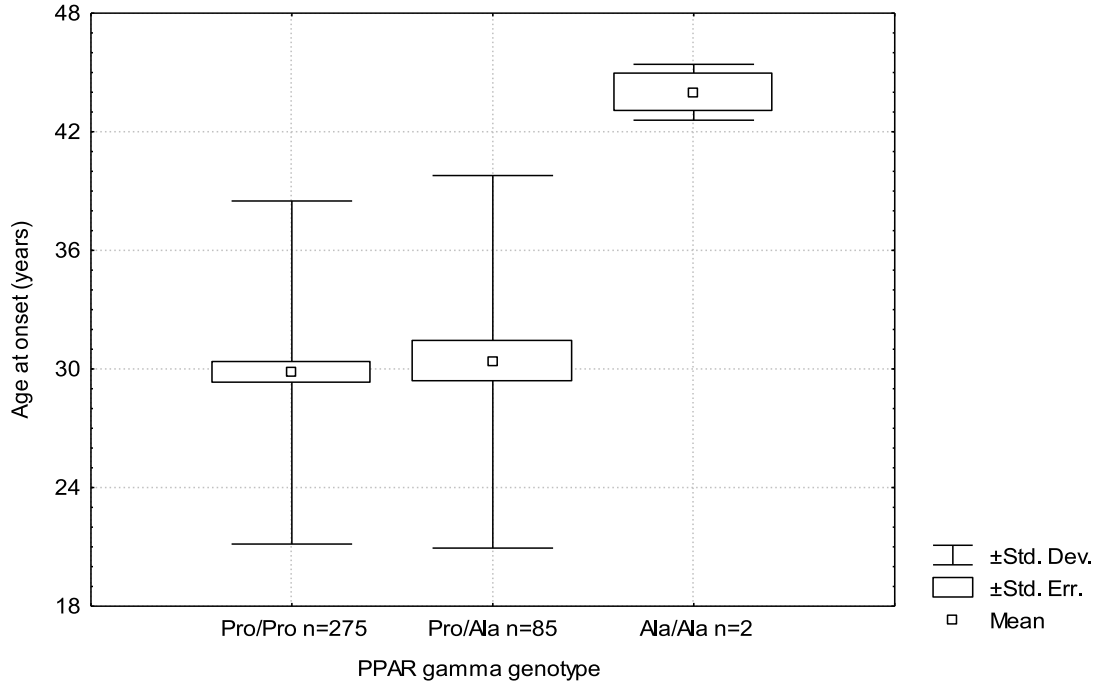
Abstract - The function of peroxisome proliferator-activated receptor γ (PPARγ) in immune regulation, as well as in anti-inflammatory and anti-proliferative actions towards T lymphocytes, has been reported. A potential role of PPARs in multiple sclerosis (MS) was suggested. The aim of this study was to investigate if there is an association of PPARγ-2 Pro12Ala polymorphism with MS in 361 patients from Serbia. The genotype and allele frequencies of Pro12Ala polymorphism were not significantly different between controls and patients, or between females and males. In contrast to controls, we detected a rare Ala/Ala genotype in patients with MS. We found that there is a significant association of Ala/Ala genotype with older age at onset (ANOVA, p=0.07; LSD post-hoc, Ala/Ala vs. Pro/Ala, p=0.03, Ala/Ala vs. Pro/Pro p=0.02). It would be useful to validate our results in other populations, as well as to perform follow-up of the disease progression in regard to PPARγ genotypes.

Key words: PPARγ, gene polymorphism, multiple sclerosis, age at onset

INTRODUCTION

Multiple sclerosis (MS) is a polygene prototype inflammatory autoimmune disorder of the central nervous system (CNS) (Compston et al., 2002). The etiology of the disease is not completely understood, but it has been proposed that the MS phenotype results from the interaction of genetic makeup and environmental factors.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, and they function as gene regulators in a ligand-dependent manner. We can distinguish three subtypes of PPAR – PPARα, PPARβ and PPARγ, which are encoded by different genes. The function of PPARγ was primarily described in the context of glucose and lipid metabolism (Desvergne et al., 1999). Apart from its role in metabolism, an important function of PPARγ in immune regulation has been reported (Glass et al., 2006). The role of PPARγ in anti-inflammatory and anti-proliferative actions towards T lymphocytes had been shown previously (Schmidt et al., 2004). Recently, emphasis was placed on their function as inhibitors of the T_H17 lymphocyte subset (Klotz et al., 2009a). The effects of PPARγ were also studied in experimental allergic encephalomyelitis (EAE), an animal model for MS. It has been reported that a heterozygous PPARγ-deficient mouse exhibits an exacerbated MS phenotype accompanied by increased T-cell expansion and pronounced immune responses (Natarajan et al., 2003). It was also shown that the activation of PPARγ by specific agonists reduces symptoms of EAE (Fein-



ANOVA, p=0.07; LSD post-hoc, * Ala/Ala vs. Pro/Ala, p=0.03, # Ala/Ala vs. Pro/Pro p=0.02

Fig. 1. PPARγ Pro12Ala genotypes and age at onset of MS

stein et al., 2002). This demonstrated the importance of PPARγ in the regulation of CNS autoimmunity, emphasizing the role of this family of transcription factors in limiting the progression and prevention of autoimmune diseases of the CNS.

The PPARγ gene is located on chromosome 3p25.2 (Greene et al., 1995; Beamer et al., 1997). It gives rise to four alternative splicing transcripts (γ1, γ2, γ3 and γ4), which are transcribed from four different promoters and differ in their first exons. All of the transcripts yield the same protein, except for the γ2 transcript which encodes for 30 additional amino acids on the N-terminus (Ackert-Bicknell et al., 2006). The PPARγ1 variant is widely expressed while PPARγ2 is expressed specifically in adipose tissue (Tontonoz et al., 1994).

The Pro12Ala substitution in the PPARγ2 gene was originally described by Yen et al. (Yen et al., 1997). This is a CCA-to-GCA missense mutation located in codon 12 of exon 1, the region of the gene that codes the domain which enhances ligand-independent activation (Werman et al., 1997). Pro12Ala exchange results in reduced transcriptional activity (Deeb et al., 1998) and diminished binding efficiency toward PPAR responsive elements (PPRE) (Masugi et al., 2000). This polymorphism was previously extensively studied in association with phenotypes such as diabetes mellitus type 2 and metabolic syndrome.

The potential role of PPARs in MS has been suggested but after a thorough search through the literature and existing databases with the results of MS genetic studies (http://www.msgene.org, http://t1dbase.org), we found that only one study had been undertaken to assess the association of PPARγ-2 Pro12Ala (rs1801282) polymorphism with MS (Klotz et al., 2009b). The aim of this work was to perform a second, larger study in patients with multiple sclerosis from Serbia.

MATERIALS AND METHODS

Subjects

Three hundred and sixty one (n=361) unrelated patient with relapsing-remitting (RR) and secondary progressive (SP) multiple sclerosis of Serbian origin were recruited from the Neurology Clinic of the Military Medical Academy (MMA), Serbia. All patients fulfilled the criteria for clinically definite MS (Polman et al., 2011) and the course of the disease was determined according to the clinical data (Lublin et al., 1996). Disease severity was estimated in accordance with the Multiple Sclerosis Severity Score (MSSS) (Roxburgh et al., 2005), representing the Expanded Disability Status Scale (EDSS) (Kurtzke et al., 1883) corrected for disease duration. Global MSSS values were calculated according to the clinical data at the time when the blood samples for genetic analysis were taken. The patients were not undergoing immunomodulatory treatment at the time of MSSS estimation.

The MS patients group consisted of 60.4% females and 39.6% males. The female-to-male ratio was 1.5. The mean age in the patient group was 38.5±10.5 years, while the mean age at disease onset was 30.0±8.9 years. The control group consisted of 103 healthy female and 90 male volunteers, members of the MMA staff, aged 40.3±14.2 and of the same ethnic origin as the MS patients. The Ethical Committee of the MMA approved the study. Each participant gave written informed consent to participate in the study.

Genetic analysis

Genomic DNA was isolated from peripheral blood samples collected with EDTA and purified by the proteinase K/phenol extraction method (Kunkel et al., 1997).

The Pro12Ala polymorphism was analyzed by PCR using an ABI 2700 Thermal cycler (Applied Biosystems, Foster City, CA). The sequences of primers were 5'-TCTGGGAGATTCTCCTATTGGC–3' (forward primer) and 5'–CTGGAAGACAAACTACAAGAG–3' (reverse primer) (Hara et al., 2000). The forward primer contained one nucleotide mismatch, making it possible to use the restriction enzyme *Hin*6I for the detection of Pro12Ala polymorphism. The PCR reaction was carried out in a 25 μl reaction mixture containing 200 ng of genomic DNA, 0.15 μM of each primer, 200 μM of each dNTP (Fermentas, Lithuania) and 1U of *DreamTaq* polymerase (Fermentas, Lithuania). The reaction mixtures were incubated at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s. The PCR products were digested with *Hin*6I at 37°C overnight. The Pro allele gave one 154 bp fragment, whereas the Ala allele gave 133 bp and 21 bp fragments. The digestion products were loaded onto an 8% polyacrylamide gel for genotyping and run for 2 h in an electric field of 12 V/cm. The gels were stained with silver nitrate and visualized using a GDS8000 gel documentation system (Ultra Violet Products, Inc., Upland).

Statistical analysis

Statistical analysis was performed using Statistica software package version 5 (Stat Soft Inc., 1997). Estimation of differences in the distribution of both allele and genotype frequencies between control and patient groups, as well as deviation from the Hardy-Weinberg equilibrium, were performed by the Pearson chi-square ($\chi 2$) test. For an analysis of the effects of genotype on descriptive and clinical parameters, such as age at onset, disease duration and MSSS values, we used the analysis of variance (ANOVA) followed by a post-hoc test. Differences with two-tailed alpha probability (p) < 0.05 were considered significant in all tests.

RESULTS

Patients

The 218 female and 143 male patients with clinically definite MS were included in this study (72.3% of RR with disease duration of 7.4±5.7 years and 28.7% of the SP patients with disease duration of

Table 1. Genotype and allele frequencies of PPAR gamma Pro12Ala polymorphism in MS patients and controls

Genotype	Overall			Females			Males		
	Controls n=193 (%)	MS n=361 (%)	p (χ^2)	Controls n=103 (%)	MS n=218 (%)	p(χ^2)	Controls n=90 (%)	MS n=143 (%)	p(χ^2)
Pro12Pro	155 (80.3)	275 (76.1)	0.31	81 (79.4)	166 (76.2)	0.51	74 (82.2)	109 (76.2)	0.32
Pro12Ala	38 (19.7)	84 (23.3)		22 (21.6)	50 (22.9)		16 (17.8)	34 (23.8)	
Ala 12Ala	0 (0.0)	2 (0.6)		0 (0.0)	2 (0.9)		0 (0.0)	0 (0.0)	
Allele									
Pro12	0.90	0.88	ns	0.89	0.88	ns	0.91	0.88	ns
Ala12	0.10	0.12		0.11	0.12		0.09	0.12	

14.7±7.3). The mean age in the patient group was 38.5±10.5 years, while the mean age at disease onset was 30.0±8.9 years. The mean MSSS in all patients was 4.5±2.2 (in the RR group it was 4.1±2.2, while in the SP group it was 6.5±2.5). We deliberately selected subjects in the control group who were not younger than the patients so that the possibility of younger, healthy subjects to develop MS in the future would be minimal.

Genotypes and alleles in MS patients and controls

The prevalence of Pro12Ala polymorphism of the PPARγ-2 gene in MS patients and controls is shown in Table 1. We did not find any deviations from Hardy-Wainberg equilibrium, either in the control group or among the patients. The genotype and allele frequencies of Pro12Ala polymorphism were not significantly different between the control subjects and the patients (Table 1). We also checked for the genotype distribution according to gender, and found no difference in females compared to the males, or in female and male patients compared to the controls of the same gender.

Genotypes and clinical parameters

We analyzed the possible association of Pro12Ala genotypes with disease course, age at onset and MSSS. The genotype distribution was not significantly different between RR and SP patients (76.7%, 22.5%, 0.8% vs. 76.2%, 23.8%, 0%, respectively). There was no association of genotypes with MSSS (data not shown). We found a significant association of the Ala/Ala genotype with an older age at onset (ANOVA, p=0.07; LSD post-hoc, Ala/Ala vs. Pro/Ala, p=0.03, Ala/Ala vs. Pro/Pro p=0.02, (Fig. 1)). All patients with Ala/Ala genotype were in the RR group, with mean disease duration of 3.0±2.8 years (not significantly different from other genotypes).

DISCUSSION

The present study examined PPARγ-2 Pro12Ala gene polymorphism as a potential risk factor for MS. We did not find an association of Pro12Ala polymorphism with susceptibility to multiple sclerosis. It was previously suggested that there is a north-to-south gradient in the frequency of the 12Ala allele, as well as the heterogeneity of its effect on DMT2, according to the same gradient. The effect size tended to be lower in the south (Ludovico et al., 2007). Similarly, one of the most striking epidemiological characteristics of MS is the uneven distribution of the disease worldwide, which means decreasing prevalence in a north-to-south gradient in the northern hemisphere. The 12Ala allele frequency in the healthy participants of the Serbian population is in the same range as in

other Caucasians, and follows the north-to-south gradient, with 12Ala frequency similar to the Italian population (Scacchi et al., 2007), significantly lower compared to the Finnish, Swedish and Danish populations (Altshuler et al., 2000; Hansen et al., 2005). As within the controls, we found slightly lower 12Ala frequency among patients with multiple sclerosis compared to the sole previous study in the German population (0.12 vs. 0.15, respectively) (Klotz et al., 2009b). Nonetheless, an important finding is that we did not find any subject homozygous for 12Ala, either in the control population or in T2DM patients from Serbia (Soskic et al., 2010); in the MS patients we found 0.6%. Nevertheless, neither we nor the authors of the previous study (Klotz et al., 2009b) have found significant association of this polymorphism with MS susceptibility.

The main and only significant finding in our study was the association of the Ala/Ala genotype with an older age at MS onset in comparison to genotypes that carry the Pro12 allele. This result is in complete accordance with the previous study of PPARγ-2 Pro12Ala polymorphism with MS, which also showed significantly later disease onset in Ala/Ala genotype in a population of German patients with MS (Klotz et al., 2009b). Although the Ala/Ala homozygote is very rare in a population and in both studies of MS, the difference in age at onset was significant and reproducible. It is reasonable to assume that if there is an effect of the PPARγ gene on MS, it is at the level of disease modulation rather than in disease susceptibility.

It had been proven that the presence of pro-inflammatory cytokines (a state that is similar to the state of relapse in MS) causes a reduction of PPARγ-2 expression in mouse adipocytes (Tanaka et al., 1999), and reduces the expression levels of PPARγ in peripheral blood mononuclear cells taken from MS patients (Klotz et al., 2005). Transient transfection assays demonstrate that the Ala-variant exhibited altered the binding activity to PPARγ-responsive DNA elements, as compared to the wild type of PPARγ (Deeb et al., 1998; Masugi et al., 2000). It was also suggested that the Ala/Ala genotype could be involved in a protection from the inflammation-induced loss of PPARγ, thus favoring the balance towards anti-inflammatory signaling (Klotz et al., 2009b). So far, without further follow-up of the patients with Ala/Ala genotype, we cannot discuss whether there is an influence on disease severity and its course. It would be interesting to carry out such research, since there is a short disease duration (3±2.8) and the patients are still in the RR phase. Of course, a higher number of patients should be involved in the follow-up, but only if the study were of a multicenter kind, because the Ala/Ala is rare.

In current pharmacogenomic research of inflammatory related diseases such as MS, PPARγ has a very important role (Drew et al., 2008). Treatment with PPARγ agonists was observed to have protective effects in a mouse model of neuroinflammation (Jeongl et al., 2011). Also, it was shown that the prostanoid PGD_2 dehydration product 15-deoxy $-\Delta^{12,14}$ 15d-PGJ_2, a ligand for PPARγ (Forman et al., 1995; Kliewer et al., 1995), mediates immunological response by reducing inflammatory response (Clark et al., 2000). An *in vitro* study which directly dealt with MS, proved that the synthetic PPARγ ligand pioglitazone, the investigational drug GW347845 and ciglitazon, inhibit the proliferation and secretion of pro-inflammatory cytokines of peripheral blood mononuclear cells derived from MS patients (Schmidt et al., 2004). It was recently proposed to use the PPARγ-mediated T cell-intrinsic molecular mechanism that selectively controls Th17 differentiation in mice and in humans. The authors suggested that it is amenable to pharmacologic modulation and thus concluded that PPARγ represents a promising molecular target for specific immunointervention in Th17-mediated autoimmune diseases such as MS (Klotz et al., 2009a).

Nevertheless, we can conclude that we replicated the significant association of Ala/Ala genotype with delayed MS onset in a specific southeastern European population. As literature data supports the idea that PPARγ has a role in neuroinflammation and in MS, it would be worthwhile to validate our results in other populations, especially in those with high-

er frequency of 12Ala allele, as well as to perform multicentric follow-up of the disease progression in regard to PPARγ genotypes in order to address this question.

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