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Polymorphisms in CD28, *CTLA-4*, *CD80* and *CD86* genes may influence the risk of multiple sclerosis and its age of onset

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ABSTRACT

CD28/CTLA-4–CD80/CD86 molecules play an important role in the regulation of T cells activation. Defects in proteins involved in this pathway may lead to the development of autoimmune diseases in which T cells are involved.

In this case–control study (336 multiple sclerosis (MS) patients and 322 controls) we investigated the possible association of eleven polymorphisms in *CD28*, *CTLA-4*, *CD80* and *CD86* genes with susceptibility to MS and/or its progression. We also took into account *HLA-DRB1*15:01* status. Moreover, this study aimed to determine the possible gene-gene interactions between examined SNPs associated with the susceptibility to MS and its outcome.

Our investigation revealed that in *HLA-DRB1*15:01* negative individuals, G allele in rs231775A > G of *CTLA-4* gene was associated with higher risk of multiple sclerosis. Additionally, the association of rs2715267T > G of *CD86* gene with MS susceptibility was detected. In details, carriers of G allele at this polymorphic site possessed higher risk of MS in comparison to TT homozygotes. On the other hand, the lower risk of MS was observed in individuals carrying A allele at the rs1599795T > A polymorphic site of *CD80*. Furthermore, the analysis revealed an interaction between three polymorphisms: rs3116496T > C (*CD28*), rs6641T > G (*CD80*) and rs17281995G > C (*CD86*), associated with the age of MS onset.

1. Introduction

The optimal activation of T lymphocytes requires two signals: the first results from the interaction of T cell receptor (TCR) with antigen presented by MHC on the surface of antigen presenting cell (APC); the second is delivered from the interaction of costimulatory receptors on T lymphocytes with ligands presented on the surface of APCs. The absence of second signal may lead to anergy and/or apoptosis of T lymphocytes, which may contribute to failure in initiation of an effective immune response (Chitnis and Khoury, 2003). CD28–CD80/CD86 pathway provides the strongest costimulatory signal, whereas CTLA-4 is a negative regulator of T cell activation. The balance between activation (CD28–CD80/CD86) and inhibition (CTLA-4–CD80/CD86) processes is of great importance to the maintenance of immune tolerance. Defects in protein involved in these pathways may lead to the development of autoimmune diseases in which T cells are involved, such as multiple sclerosis (MS) (Sansom, 2000).

MS is a chronic disease of the central nervous system leading to demyelination and axonal damage in the brain and the spinal cord (Pender and Greer, 2007). It is estimated that the disease affects around 2.5 million people worldwide. MS most commonly starts in young adults, between 20 and 40 years of age. Moreover, women are affected twice as often as men, which is characteristic of many autoimmune diseases (Sawcer et al., 2014). The pathomechanism of MS is complex and its course and progression can be various. About 85% of patients experience relapsing–remitting (RR) course of the disease characterized by relapse followed by recovery period (Inglese, 2006). Most of them evolve into a secondary progressive (SP) phase characterized by a steady increase in disability within 25 years (Inglese, 2006, Weinshenker et al., 1989). About 10–15% of patients experience primary progressive (PP) form, defined by the accumulation of disability from the onset of the disease (Inglese, 2006).

Here we investigated the possible association of eleven polymorphisms in *CD28*, *CTLA-4*, *CD80* and *CD86* genes with susceptibility to multiple sclerosis and/or progression of this disease. Moreover, this

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study aimed to determine the possible gene-gene interactions between examined SNPs associated with the susceptibility to MS and its outcome. We also took into account *HLA-DRB1*15:01* status, which was determined in our earlier studies (Wagner et al., 2013, Wisniewski et al., 2013) According to the literature data stratification for this well-established MS risk factor is expected to help to reveal the associations of other polymorphisms with MS susceptibility (Bronson et al., 2010, Gyllenberg et al., 2014). This fact was also confirmed in our earlier study (Wagner et al., 2013).

2. Materials and methods

2.1. Study population

336 patients — 226 females and 110 males, with clinically definite MS according to the McDonald criteria (Polman et al., 2011) were included in this study. All patients were under the charge of Department of Neurology, Wroclaw Medical University. Degree of disability and the rate of its progression were scored using Kurtzke's Expanded Disability Status Scale (EDSS) and MS Severity Score (MSSS), respectively (Kurtzke, 1983, Roxburgh et al., 2005). 250 patients presented relapsing remitting course of multiple sclerosis, while 86 secondary progressive form of the disease. We did not include patients with primary progressive course, since we did not collect sufficient number of samples to perform powerful statistical analysis. The detailed characteristics of patients are presented in Supplementary Table 1.

Controls were 322 blood donors (138 females and 184 males) with no history of inflammatory disease. The study was approved by the ethics committee of Wroclaw Medical University and written informed consent was obtained from all participants.

2.2. Selection of single nucleotide polymorphisms

SNPs examined in this study were selected on the basis of the literature data and in silico analysis performed using SNPinfo Web Server (Xu and Taylor, 2009) (please see Supplementary Table 2). This analysis encompassed the gene-transcribed sequences and 3000 bp upstream and 3000 bp downstream regions. Prediction of SNP's function was performed on the basis of data for the CEU population (Utah residents with Northern and Western European ancestry) from the International HapMap Project and for the European population from the NCBI dbSNP database. Moreover, in order to choose TagSNPs, linkage disequilibrium (LD) blocks were estimated.

2.3. DNA isolation and genotyping

Genomic DNA was isolated from whole blood using Invisorb Blood Midi Kit (Stratec Molecular) according to the manufacturer's protocol.

Nine of the examined SNPs were genotyped by applying polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Primer sequences, annealing temperatures and restriction enzymes used in this study are listed in Supplementary Table 3. Genotyping of rs3116496T > C of *CD28* gene and rs1129055G > A of *CD86* gene was carried out using allelic discrimination method with the TaqMan SNP Genotyping Assays (Life Technologies, C_25922478_10 and C_7504226_10, respectively).

*HLA-DRB*15:01* status was determined by genotyping the rs31355588 G > A, as described by Wisniewski et al. (Wisniewski et al., 2013). These data were generated in our earlier studies (Wagner et al., 2013, Wisniewski et al., 2013) and they were taken into consideration in the analyses performed here.

Accuracy of genotyping methods for all SNPs was verified through direct sequencing of few samples representing homozygotes of two types and heterozygotes for each investigated SNP. These samples were used as the reference samples in following genotyping experiments.

2.4. Statistical analysis

Chi-square test, χ^2_{df}, was used to test the null hypothesis that cases and controls have the same distribution of genotype counts. In case of small numbers the distribution of the test statistics was estimated numerically. *Odds ratio* (OR) and confidence interval for it at 1-α = 0.95 level (CI95%) was computed as the measure of effect size. Homogeneity of two odds ratios was tested with Breslow–Day test. Median and mean were used as the location parameters. In case of median S_n statistics was computed as the measure of variability: $S_n = med\{med|x_i - x_j|; j = 1 \ldots n\}$ (Rousseeuw and Croux, 1993). Additionally 1st and 3rd quartile, minimal and maximal observation were reported. Departure from Hardy–Weinberg equilibrium (HWE) was tested with chi-squared test and measured as $f = \frac{p_{CC} - p_C^2}{p_C(1-p_C)}$, where p_C and p_{CC} are allele *C* and genotype *CC* frequencies while $f < 0$ and $f > 0$ corresponds to deficiency and excess of homozygotes, respectively, and $f = 0$ in case of HWE. Haplotype frequencies (HFs) among SNPs were estimated with *maximum likelihood* function (Excoffier and Slatkin, 1995). Measure for the estimation of pair-wise linkage disequilibrium (LD) was squared correlation between two SNPs, r^2 (Abdallah et al., 2003). For two SNPs $r^2 = \sum_i^2 \sum_j^2 \frac{D_{ij}^2}{p_i q_j}$, where p_i and q_j are the population allele frequencies of the *i*th allele on locus *A* and the *j*th allele on locus *B*, $D_{ij} = x_{ij} - p_i q_j$, and x_{ij} is the frequency of the haplotype with alleles *i* and *j* on loci *A* and *B*, respectively. Chi-square statistic was calculated to test that $D_{ij} = r^2 = 0$ between two SNPs: $\chi^2_{df=2} = \sum_i^2 \sum_j^2 \frac{nD_{ij}^2}{p_i q_j}$. Likelihood ratio statistics, LRS ~ χ^2, was used to test for differences in haplotype frequencies between cases and controls, $LRS = 2(LL_{\text{Cases}} + LL_{\text{Controls}} - LL_{\text{Combined}})$.

To control type I error in case of many tests for differences between SNPs genotypes of cases and controls global (omnibus) chi-square test was performed first to test the hypothesis, that there were no differences between cases and controls in any SNP, opposite to the alternative, that genotype frequencies in cases and controls were different at least in one SNP. Because of correlation between SNPs distribution, global chi-square statistics was estimated numerically. MS progression and its association with genetic factors was modeled as two-dimensional variable $\mathbf{x}_i = (EDSS_i, MSSS_i) \in \Re^2$ and tested with λ-Pillai statistic. Age of onset was described by mean, standard deviation (SD) and range [min-max]. Linear regression was used to test relations between selected polymorphisms and age of onset including interactions between them. *Bootstrap* approach (B = 5999) was employed to estimate model's coefficients (differences between the means) and 95% confidence intervals for them. Determination coefficient R^2 is total variation of response variable explained by the model.

Table 1
Genotypes distribution of *CTLA-4* rs231775A > G polymorphism in multiple sclerosis patients and controls in dependence on the presence of *HLA-DRB1*15:01* allele.

	Cases		Controls		OR	CI95%
	N	%	N	%		
CTLA-4 rs231775A > G in *HLA-DRB1*15:01* (−) individuals						
AA	52	29.5	97	39.6	1[a]	
AG	87	49.4	109	44.5	**1.49**	**0.96; 2.31**
GG	37	21.0	39	15.9	**1.77**	**1.01; 3.10**
∑	176	100.0	245	100.0		
CTLA-4 rs231775A > G in *HLA-DRB1*15:01* (+) individuals						
AA	55	34.4	21	27.3	1[a]	
AG	80	50.0	42	54.5	0.73	0.39; 1.36
GG	25	15.6	14	18.2	0.68	0.30; 1.55
∑	160	100.0	77	100.0		

$\chi^2_{df=2} = 6.934$; p = 0.031.
The statistically significant values are bolded.
[a] Reference group.