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Lack of Association between an Exon 1 *CTLA-4* Gene Polymorphism A(49)G and Multiple Sclerosis in a Polish Population of the Lower Silesia Region

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Abstract. Multiple sclerosis (MS) a chronic inflammatory demyelinating disease of the central nervous system is believed to have a T cell-mediated autoimmune etiology. The cytotoxic T lymphocyte antigen 4 (*CTLA-4)* gene is a strong candidate for the involvement in autoimmune diseases because *CTLA-4* plays an important role in the downregulation of the early and late stages of T cell activation and the maintenance of peripheral T cell tolerance. To examine the genetic association of the *CTLA-4* gene locus with MS, we analyzed an exon 1 *CTLA-4* gene polymorphism A(49)G in 102 unrelated Polish MS patients in the Lower Silesia region and 101 age- and sex-matched healthy subjects. The distribution of *CTLA-4* exon 1 A(49)G genotype, phenotype and allele frequencies did not differ between patients with MS and healthy subjects.

Key words: *CTLA-4*; gene polymorphism; A(49)G; multiple sclerosis; Lower Silesia.

Introduction

Multiple sclerosis (MS) is a debilitating inflammatory disease of the central nervous system (CNS). The prevailing hypothesis of MS etiology is that it is a T cell-mediated autoimmune disorder in which myelin-specific components are the targets of the immune response. Alternatively, an immune response directed against an oligodendrocyte-infected virus may lead to demyelination.

The familial occurrence of MS and the higher concordance rate in monozygotic twins (25%) compared with dizygotic twins (2.3%) indicate the importance of genetic factors[7]. The only genetic factor clearly confirmed to be of importance in MS has been identified on chromosome 6p21 in the HLA region as the HLA class II haplotype Dw2[6]. However, many MS patients express class II haplotypes that are not associated with increased MS risk. Together, this evidence suggests that no single MS susceptibility locus is necessary and sufficient to cause disease. More likely is that the genetic etiology results from the action of several genes. Candidate susceptibility genes may be genes encoding proteins regulating the immune response. The cytotoxic T lymphocyte antigen 4 (*CTLA-4*) gene is a strong candidate gene for involvement in autoimmune diseases. There is evidence to suggest that CTLA-4 is an important negative regulator of T cell responses in-

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volved in the maintenance of peripheral T cell tolerance[11].

It has been shown that administration of CTLA-4 antibodies exacerbates the severity of experimental autoimmune encephalomyelitis (EAE), the murine model for MS[16].

Polymorphism of the *CTLA-4* gene (chromosome 2q33) could thus have effects upon the immune response. Of the several *CTLA-4* gene polymorphisms, three have been most frequently studied, namely a dinucleotide microsatelite (AT)n marker at position 642 of the 3'-untranslated region of exon 4: *CTLA-4* 3'(AT)n[18] in the promoter region *CTLA-4* C(–318)T[3], and the single nucleotide polymorphism in exon 1 *CTLA-4* A(49)G[10].

Several diseases of presumed autoimmune etiology, including Graves' disease[2, 5], Hashimoto's thyroiditis[2, 17], insulin dependent diabetes mellitus[1, 15] and Addison disease[4, 22], have been associated with *CTLA-4* gene polymorphism. The are few reports on *CTLA-4* gene polymorphism in SM which gave inconsistent results[8, 9, 12, 20].

In our study we examined whether there is an association between the *CTLA-4* exon 1 gene polymorphism at position 49, which results in amino acid exchange (Thr to Ala) in the leader sequence[10], and susceptibility to MS in a Polish population in the Lower Silesia region.

Materials and Methods

The study included 102 unrelated, randomly selected, Polish MS patients (73 patients with relapsing--remitting and 29 with secondary progressive MS) living in the Lower Silesia area aged between 19 to 61 years (mean 39.0 years). All patients had clinically and laboratory supported definite MS according to the Poser criteria[19]. The control group consisted of 101 healthy age- and sex-matched healthy subjects.

Amplification of genomic DNA. DNA from 7–14 μl of frozen venous blood was extracted by the Chelex® method[23]. To amplify the targeted sequence of DNA in exon 1 of the *CTLA-4* gene from chromosome 2, the primers F 5'-TTG CCT TGG ATT TCA GCG GCA CAA-3' and R 5'-CAC CTC CTC CAT CTT CAT GCT CC-3' were designed. The designation was based on the complete *CTLA-4* gene sequence derived from NCBI Sequence Viewer.

Allele identification was achieved by PCR amplification of 3.2–12.7 ng of genomic DNA by using the Biometra UNO-Thermoblock. PCR was carried out in a total volume of 36.8 μl containing 0.1 μM of each primer, 0.6 U of DyNAzyme II polymerase (Finnzymes, Espoo, Finland), 200 μM of each dNTP with 10 × PCR buffer of PE Applied Biosystems (containing 15 mM of $MgCl_2$) and 0.16 mg/ml BSA (Promega, Madison, USA).

The PCR profie was as follows: initial denaturation at 96ºC for 2 min followed by 30 cycles of 94ºC (annealing for 1 min at 60ºC, extension for 1 min at 72ºC) and final extension for 10 min at 72ºC. The amplified DNA was 142 bp in length.

Minisequencing. The amplified product was purified of dNTPs and primers using the QIAquick PCR Purification Kit of Qiagen to avoid participation in the subsequent primer-extension reaction. For the minisequencing reaction, the commercial kit SNapShot of PE Applied Biosystems was used.

The ABI PRISM® SNaPshot ddNTP Primer Extension Kit detects single nucleotide polymorphisms (SNPs) by extension of an unlabeled SNP primer with fluorescently labeled dideoxynucleotides ([F]ddNTPs). The ddNTPs are labeled with four different colors: A(dR6G – green), C(dTAMRA – yellow), G(dR110 – blue) and T(dROX – red). The SNP primer 5'-GGC TCA GCT GAA CCT GGC T-3', whose design was based on the sequence from the NCBI Sequence Viewer, binds to a complementary sequence. AmpliTaq DNA Polymerase FS adds a single ddNTP to the 3' end of the primer on an amplified and purified template. After the extension and labeling reaction, the unincorporated ddNTPs were removed by enzymatic treatment using SAP (Sigma, Deisenhofen, Germany) to avoid comigration of [F]ddNTPs with the fragment of interest.

Electrophoresis and detection. The products of the SNaPshot reaction were analyzed on the ABI PRISM® 310 Genetic Analyzer equipped with GeneScan Analysis Software version 3.1 of PE Applied Biosystems. To run a sample on the ABI PRISM® 310, the GS POP-4 (1 ml) E module of PE Applied Biosystems is required. The Gene Scan E Run Module encodes optimal injection and electrophoresis parameters. For a precise spectral overlap between dyes, the Gene Scan E matrix file was created, using the ABI PRISM® dRhodamine Matrix Standards Kit of PE Applied Biosystems. 1.0 μl of the SNaPshot product was denatured (95°C for 5 min) in 10 μl of HD Formamide (Sigma) and subjected to electrophoresis with laser excitation/fluorescence detection. On the electrophoregram the alleles are shown as peaks (20 bp), and the color of the peak differs according to the different nucleotide at the place of interrogation (Fig. 1, 2).

Statistical analysis. Statistical analysis was performed using the χ^2 test.

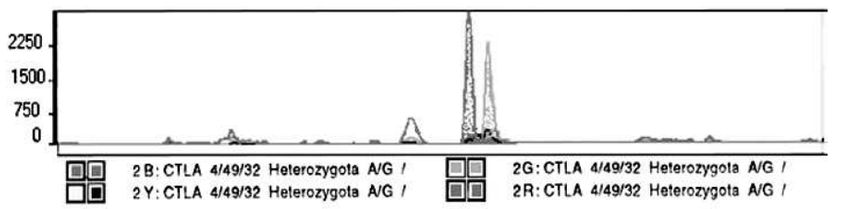

Fig. 1. Heterozygote detection on a ABI PRISM® 310 DNA Capillary Genetic Analyzer, using the SnaPshot™ Multiplex Kit

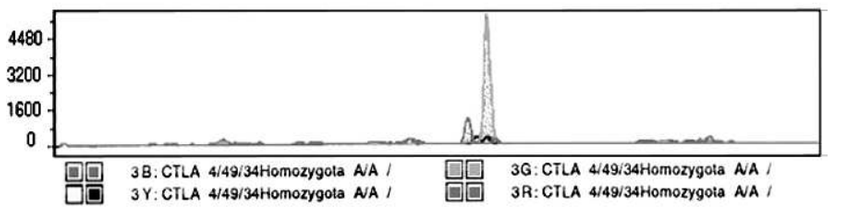

Fig. 2. Homozygote detection on a ABI PRISM® 310 DNA Capillary Genetic Analyzer, using the SnaPshot™ Multiplex Kit

Results

The distribution of *CTLA-4* exon 1 genotype, phenotype and allele frequencies did not differ between patients with MS and healthy subjects (Table 1).

The distribution of *CTLA-4* exon 1 A(49)G genotype, allele, and phenotypic frequencies did not differ significantly between MS patients and control subjects. No differences in the distribution of *CTLA-4* exon 1 A(49)G genotype, allele and phenotype frequencies between patients with relapsing-remmitting and secondary progressive MS were found (data not shown).

Discussion

During the past decade there has been a growing appreciation of the importance of inhibitory regulator pathways in normal and disease-related cellular immune functions. Characterization of the molecular mechanisms underlying negative immune regulation has revealed the essential role of inhibitory pathways in the maintenance of immune homeostasis. The intimate relationship which exists between positive and negative regulation during the generation of immune responses is shown by the functional profiles of the homologous proteins CD28 and CTLA-4 (CD152). Despite their structural similarities and shared specificities for ligands B7-1 (CD80) and B7-2 (CD86), the two proteins mediate opposing effects on T cell activation. The CD28/B7 interaction serves as a positive costimulator in the context of T cell receptor engagement by major histocompatibility complex/antigen complexes. In contrast, the CTLA-4 receptor downregulates CD28 effects by delivering a negative signal upon B7 engagement and is expressed on the cell surface 24–38 h after T cell stimulation. It has been shown that CTLA-4 is primarily detected in the perinuclear Golgi vesicles, and that CTLA-4 engagement is sufficient for directing CTLA-4 expression/localization to the cell surface[13] This may indicate that early expression of CTLA-4 on the cell surface might out-compete CD28 and deliver

Table 1. The *CTLA-4* gene exon 1 polymorphism in MS patients and controls

	Patients n=102	Controls n=101	p value
Allele frequencies			
G^{49}	85 (41.7%)	79 (39.1%)	ns
A^{49}	119 (58.3%)	123 (60.9%)	ns
Phenotype frequencies			
G^{49} -positive	71 (69.6%)	66 (65.3%)	ns
G^{49} -negative	88 (86.3%)	88 (87.0%)	ns
Genotype frequencies			
A^{49} A^{49}	31 (30.4%)	35 (34.6%)	ns
A^{49} G^{49}	57 (55.9%)	53 (52.5%)	ns
G^{49} G^{49}	14 (13.7%)	13 (12.9%)	ns

inhibitory signals that would prevent T cell activation. CTLA-4 also terminates the function of previously activated T cells by inhibition of IL-2 production and proliferation (reviewed in[11]).

The strongest evidence of the inhibitory role of CTLA-4 has been demonstrated in CTLA-4-deficient mice. CTLA-$4^{-/-}$ mice develop a fatal lymphoproliferative disorder characterized by a massive expansion of lymphocytes, subsequent lymphocytic infiltration into nonlymphoid tissues, and death by 3 to 4 weeks of age[24]. This could point at a possible role of CTLA-4 in autoimmune diseases[14, 21].

In our study we did not find differences in the frequencies of the *CTLA-4* exon 1 polymorphism between controls and Polish patients with MS in the Lower Silesia region.

In a recent study, RASMUSSEN et al.[20] also did not find significant differences in the distribution of genotypes or haplotypes of the *CTLA-4* gene between MS patients populations of white Caucasians from Denmark and of Shanghai Chinese origin. A Japanese study also failed to detect an association between MS and *CTLA-4*[8].

Conversely, association between the A/G exon 1 heterozygous genotype of *CTLA-4* 49G and MS in European Caucasians from Norway, including strong association with the relapsing-remitting form of the disease, has been reported[9]. LIGERS et al.[12] observed significantly more patients homozygous for the 49G allele in exon 1 than control subjects in a Swedish population. The numbers of individuals in the Norwegian[9] and Swedish[12] studies were considerably higher than in our study (296 Norwegian MS patients vs. 271 controls; 378 Swedish MS patients vs. 162 control subjects). However, since we found almost identical genotypic frequencies in patients and controls, inclusion of a larger number of individuals would probably not change the results of our study.

As other autoimmune disorders, MS is a polygenic disease. This means that combinations of genetic factors influence risk of disease moreover, different etiologic factors may be involved in MS, such as microbial agents and environmental factors[14]. Contradictory results reported from different centers may be due to genetic heterogeneity between the different ethnic groups and still unknown enviromental factors influencing the unset of the disease.

It cannot be excluded that the etiologic factors involved in MS may differ among different populations in geographically distinct regions.

In conclusion, we did not find evidence that *CTLA-4* is implicated in susceptibility to MS in a Polish population in the Lower Silesia area.

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References

1. ABE T., TAKINO H., YAMASAKI H., OZAKI M., SERA Y., KONDO H., SAKAMAKI H., KAWASAKI E., AWATA T., YAMAGUCHI Y. and EGUCHI K. (1999): CTLA-4 gene polymorphism correlates with the mode of onset and presence of ICA512 Ab in Japanese type 1 diabetes. Diabetes Res. Clin. Pract., **46**, 169–175.
2. AWATA T., KURIHARA S., IITAKA M., TAKEI S., INOUE I., ISHII C., NEGISHI K., IZUMIDA T., YOSHIDA Y., HAGURA R., KUZUYA N., KANAZAWA Y. and KATAYAMA S. (1998): Association of CTLA-4 gene A-G polymorphism (IDDM12 locus) with acute-onset and insulin-depleted IDDM as well as autoimmune thyroid disease (Graves' disease and Hashimoto's thyroiditis) in the Japanese population. Diabetes, **47**, 128–129.
3. DEICHMANN K., HEINZMANN A., BRUGGENOLTE E., FORSTER J. and KUEHR J. (1996): An Mse I RFLP in the human CTLA-4 promotor. Biochem. Biophys. Res. Commun., **225**, 817–818.
4. DONNER H., BRAUN J., SEIDL C., RAU H., FINKE R., VENTZ M., WALFISH P. G., USADEL K. H. and BADENHOOP K. (1997): Codon 17 polymorphism of the cytotoxic T lymphocyte antigen 4 gene CTLA-4 in Hashimoto's thyroiditis and Addison's disease. J. Clin. Endocrinol. Metab., **82**, 4130–4132.
5. DONNER H., RAU H., WALFISH P. G., BRAUN J., THORSTEN S., FINKE R., HERWIG J., USADEL K. H. and BADENHOOP K. (1997): CTLA-4 alanine-17 confers genetic susceptibility to Graves' disease and type 1 diabetes mellitus. J. Clin. Endocrinol. Metab., **82**, 143–146.
6. DYMENT D. A., SADOVNICK A. D., EBERS G. C. and SADNOVICH A. D. (1997): Genetics of multiple sclerosis. Hum. Mol. Genet., **6**, 1693–1694.
7. EBERS G. C. and SADOVNICK A. D. (1994): The role of genetic factors in multiple sclerosis susceptibility. J. Neuroimmunol., **54**, 1–17.
8. FUKUZAWA T., YANAGAWA T., KIKUCHI S., YABE I., SASAKI H., HAMADA T., MIYASAKA K., GOMI K. and TASHIRO K. (1999): CTLA-4 gene polymorphism may modulate disease in Japaese multiple sclerosis patients. J. Neurol. Sci., **171**, 49–55.
9. HARBO F., CELIUS G., VARTDAL F. and SPURKLAND A. (1999): CTLA-4 promoter and exon 1 dimorphism in multiple sclerosis. Tissue Antigens, **53**, 106–110.
10. HARPER K., BALZANO C., ROUVIER E., MATTEI M. G., LUCIANO H. F. and GOLDSTEIN P. (1991): CTLA-4 and CD28 molecules are closely related in both mouse and humans as to sequence, message expression, gene structure, and chromosomal location. J. Immunol., **147**, 1037–1044.
11. KOSMACZEWSKA A., CISZAK L., BOĆKO D. and FRYDECKA I. (2001): Expression and functional significance of CTLA-4, a negative regulator of T cell activation. Arch. Immunol. Ther. Exp., **49**, 39–45.
12. LIGERS A., XU C., SAARINRN S., HILLERT J. and OLERUP O. (1999): The CTLA-4 gene is asociated with multiple sclerosis. J. Neuroimmunol., **97**, 182–190.
13. LINSLEY P. S., BRADSHAW J., GREENE J. L., PEACH R., BENNETT K. L. and MITTLER P. S. (1996): Intercellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. Immunity, **4**, 535–543.

14. NOSERWORTHY J. H., UCCHINETTI C., RODRIGUEZ M. and WAINSHENKER B. G. (2000): Multiple sclerosis. N. Engl. J. Med., **343**, 938–952.
15. OSEI-HYIAMAN D., HOU L., ZHIYIN R., ZHIMING Z., YU H., AMANKWAH A. A. and HARADA S. (2001): Association of a novel point mutation (C159G) of the CTLA4 gene with type 1 diabetes in West Africans but not in Chinese. Diabetes, **50**, 2169–2171.
16. PERRIN P. J., MALDONADO J. H., DAVIS T. A., JUNE H. C. and RACKE K. M. (1996): CTLA-4 blockage enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. J. Immunol., **157**, 1333–1336.
17. PETRONE A., GIORGI G., MESTURINO C. A., CAPIZZI M., CASCINO I., NISTICO L., OSBORN J., DI MARIO U. and BUZZETTI R. (2001): Association of DRB1 04-DQB1 0301 haplotype and lack of association of two polymorphic sites at CTLA-4 gene with Hashimoto's thyroiditis in an Italian population. Thyroid, **11**, 171–174.
18. POLYMEROPOULOS M. H., XIAO H., RATH D. S. and MERRIL C. R. (1991): Dinucleotide repeat polymorphism at the CTLA-4 gene. Nucleic Acids Res., **19**, 4018.
19. POSER C. M., PATY D. W., SCHEINBERG L., MCDONALD W. I., DAVIS F. A., EBERS G. C., JOHANSON K. P., SIBLEY W. A., SILBERBERG D. H. and TOURTELLOTTE W. W. (1983): New diagnostic criteria for multiple sclerosis guidelines for research protocols. Ann. Neurol., **13**, 227–231.
20. RASMUSSEN H. B., KELLY M. A., FRANCIS D. A. and CLAUSEN J. (2001): CTLA-4 in multiple sclerosis. Lack of genetic association in a European Caucasian population but evidence of interaction with HLA-DR2 among Shanghai Chinese. J. Neurol. Sci., **184**, 143–147.
21. THOMPSON C. B. and ALLISON J. P. (1997): The emerging role of CTLA-4 as an immune attenuator. Immunity, **7**, 445–450.
22. VAIDYA B., GEATCH H. R., PERROS P., BALL S. G., BAYLIS P. H., CARR D., HURREL S. J., JAMES R. A., KELLY W. F., KEMP E. H., YOUNG E. T., WEETMAN A. P., KENDALL-TAYLOR P. and PEARCE S. H. S. (2000): Association analysis of the cytotoxic T lymphocyte antigen-4 (CTLA-4) and autoimmune regulator-1 (AIRE-1) genes in sporadic autoimmune Addison's disease. J. Clin. Endocrinol. Metab., **82**, 688–691.
23. WALSH P. S., METZGER D. A. and HIGUHI R. (1991): Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques, **10**, 506–518.
24. WATERHOUSE P., PENINGER J. M., TIMMS E., WAKEHAM A., SHAHINIAN A., LEE K. P., THOMPSON C. B., GRIESSER H. and MAK T. W. (1995): Limphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science, **270**, 985–989.

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