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CTLA-4 (CD152) gene polymorphism at position 49 in exon 1 in Graves' disease in a Polish population of the Lower Silesia region

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
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Summary

Introduction:

Graves' disease (GD) is an autoimmune disease believed to be caused by a combination of environmental and genetic factors. The gene encoding cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is one of the candidate genes for conferring susceptibility to thyroid autoimmunity. The aim of the study was to investigate the association between the exon 1 *CTLA-4* gene polymorphism A(49)G and susceptibility to GD and Graves' ophthalmopathy (GO) as well as its severity in a Polish population of the Lower Silesia region.

Materials and Methods:

We analyzed the A(49)G exon 1 *CTLA-4* gene polymorphism in 99 unrelated Polish patients with GD, of whom 50 had clinically evident GO (NOSPECS class III and higher), and 154 matched healthy subjects from the Lower Silesia region. Genomic DNA was isolated from whole frozen blood using the NucleoSpin^R Blood kit. A/G transition was genotyped by polymerase chain reaction followed by labeling with the SnapShot kit of PE Applied Biosystems and detected using an ABI PRISM 310 capillary genetic analyzer.

Results:

The distribution of *CTLA-4* exon 1 A(49)G genotype, allele, and phenotypic frequencies did not differ between patients with GD and healthy subjects. There was a significantly lower frequency of the AA genotype in the group of patients with clinically evident GO than in patients without severe GO (22% vs. 43%; $p=0.02$, $OR=2.6$).

Conclusions:

Our results showed that the AA genotype in patients with GD is associated with a lower risk of GO severity.

Key words:

CTLA-4 • A(49)G • Graves' disease • Lower Silesia

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INTRODUCTION

Graves' disease (GD) is a T cell-mediated autoimmune disease of the thyroid gland characterized by hyperthyroidism, a diffuse goiter and, in some cases, ophthalmopathy and pretibial myxedema¹⁵. The course of Graves' ophthalmopathy (GO) is unpredictable, and a sudden worsening of GO can occur at any time. Hyperthyroidism is a direct result of the stimulating effect of an autoantibody directed against the thyroid-stimulating hormone receptor (TSHR)¹⁵.

Increasing evidence supports the suggestion that GD occurs in genetically susceptible individuals and that a number of genetic loci contribute to the development of disease. Genome screening has provided evidence for the linkage of three human chromosomal regions, designated GD-1 (chromosome 14), GD-2 (chromosome 20), and GD-3 (X chromosome), for GD^{6, 38, 39}. In family studies the transmission disequilibrium test showed the presence of a linkage disequilibrium between GD and HLA haplotype DRB1*0304-DQB1*02-DQA1*0501²³. Another candidate is the *CTLA-4* gene coding for cytotoxic T lymphocyte-associated antigen-4 (CTLA-4; CD152). Many studies have revealed the role of CTLA-4 in down-regulating T cell activation and maintaining immunologic homeostasis²⁷.

Lymphocytes of non-obese diabetic mice, an animal model of autoimmune diabetes, express CTLA-4 defectively¹³. CTLA-4-deficient mice show a severe lymphoproliferative disorder which is characterized by massive lymphocytic infiltration, multiorgan tissue destruction, and early lethality^{37, 42}. Administration of anti-CTLA-4 monoclonal antibody exacerbated autoimmune responses in a murine model of multiple sclerosis²⁵. These observations raised the possibility that alterations in the *CTLA-4* gene may lead to autoimmunity in humans. In fact, several observations showed that specific *CTLA-4* gene polymorphisms confer susceptibility to several autoimmune diseases³¹. The following three *CTLA-4* polymorphisms have been the most frequently studied for possible associations with various autoimmune diseases in humans: at position –318 from the ATG start codon in its promotor region¹⁶, at position 49 in exon 1^{20, 33}, and the (AT)_n repeat within the 3'-untranslated region of exon 4^{29, 35}. Ueda et al.⁴¹ extended these studies and found several additional *CTLA-4* gene polymorphic variants contributing to autoimmune tissue destruction.

In our study, we examined whether there is an association between the *CTLA-4* exon 1 gene polymorphism at position 49 and susceptibility to GD and GO

as well as its severity in a Polish population in the Lower Silesia region.

MATERIALS AND METHODS

The study included 99 unrelated, randomly selected Polish patients with GD (80 women and 19 men) living in the Lower Silesia area with ages between 20–82 years (mean 50±11 years). Diagnosis of GD was based on the presence of hyperthyroidism, diffuse goiter, detectable thyrotropin receptor autoantibodies, and/or increased radio-iodine uptake. The severity of ophthalmopathy was assessed according to the NOSOECs classification⁴⁴.

Patients with proptosis, extraocular-muscle dysfunction, exposure keratitis, and optic neuropathy (NOSPECS class III and higher) were considered as having clinically evident disease. Patients were categorized according to their highest NOSOECs class: class 0 consisted of 10 patients, class I of 19, class II of 20, and class III and higher of 50 patients. The control group consisted of 154 age- and sex-matched healthy subjects from the same area.

Amplification of genomic DNA

Genomic DNA was isolated using the NucleoSpin[®] Blood kit (Marcherey-Nagel, Germany) from whole frozen blood. 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 designated. The designation was based on the complete *CTLA-4* gene sequence derived from the NCBI Sequence Viewer. Allele identification was achieved by polymerase chain reaction (PCR) amplification of 0.5–1.0 ng of genomic DNA using the the Biometra UNO-Thermblock (Biometra, Germany). PCR was carried out in a total volume of 10 µl, containing 0.1 µM of each primer and TaqMasterMix (Qiagen, Germany) with 1 unit *Taq* DNA polymerase, 1 × PCR buffer (containing 15 mM of MgCl₂), and 200 µM of each dNTP. The PCR profile was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles denaturation at 95°C for 1 min, annealing for 1 min at 60°C, extension for 1 min at 72°C, and a final extension for 10 min at 72°C. The amplified DNA was 142 bp in length.

Minisequencing

The amplified product was purified of dNTPs and primers using the QIAquick PCR Purification Kit (Qiagen, Germany) to avoid participation in the subsequent primer-extension reaction. Details of minise-

quencing methods were previously described by Boćko et al.⁸

Electrophoresis and detection

The products of the SNaPshot reaction were analyzed on an ABI PRISM 310 Genetic Analyzer equipped with GeneScan Analysis Software version 3.1 of PE Applied Biosystems (USA). Electrophoresis and detection were described in details previously⁸.

Statistical analysis

Allele, phenotype, and genotype frequencies were compared between groups using Fisher's exact test. A *p* value of <0.05 was considered significant.

RESULTS

The distribution of *CTLA-4* exon 1 A(49)G of allele, phenotype, and genotype frequencies did not differ between patients with GD and healthy subjects, nor between GD patients without evident, with evident GO, and controls (Table 1). No significance differences in the distribution of the *CTLA-4* exon 1 A(49)G genotype, allele, and phenotypic frequencies were also found between patients without and with evident GO, although a trend towards higher frequencies of the AA allele in the group of patient without evident GO was observed (*p*=0.06; Table 2). There was a significantly higher frequency of the AA genotype versus the AG+GG genotype in patients without severe GO (genotype AA: 43% vs. 22%, Fisher's exact test *p*=0.02, OR=2.6; Table 3).

DISCUSSION

CTLA-4 (CD152) is a CD28 homologue expressed on activated T cells and, upon interaction with CD80

or CD86 on antigen-presenting cells, exerts a down-regulatory effect on T cell-mediated immune responses^{10, 27}. In addition, CTLA-4 signaling mediates antigen-specific apoptosis of T cells and suppresses autoreactive proliferation of T lymphocytes^{19, 36}. Therefore, this immunomodulatory protein is significant in regulating and maintaining self-tolerance. Breakdown of the tolerance may result in the induction of autoimmunity. T lymphocyte-mediated destruction of host cells is one of the common pathogenic autoimmune mechanisms, which include thyroid autoimmunity^{26, 36}.

Table 2. Genotype, phenotype and allele frequencies of the *CTLA-4* A(49)G gene polymorphism in patients with Graves' disease without and with evident Graves' ophthalmopathy (GO)

	Patients		p value
	without evident GO n = 49 (%)	with evident GO n = 50 (%)	
Allele frequencies			
A ⁴⁹	63 (64.3)	51 (51.0)	0.063
G ⁴⁹	35 (35.7)	49 (49.0)	
Phenotype frequencies			
A-positive	42 (85.7)	40 (80.0)	0.322
A-negative	28 (57.1)	39 (78.0)	
Genotype frequencies			
A ⁴⁹ A ⁴⁹	21 (42.9)	11 (22.0)	0.085
A ⁴⁹ G ⁴⁹	21 (42.9)	29 (58.0)	
G ⁴⁹ G ⁴⁹	7 (14.2)	10 (20.0)	

Table 3. Genotype frequencies of *CTLA-4* A(49)G gene polymorphism in patients with Graves' disease without and with evident Graves' ophthalmopathy (GO)

Patients	n	Genotype		OR
		AA	AG+GG	
Without evident GO	49	21 (42.9)	28 (57.1)	2.6
With evident GO	50	11 (22.0)	39 (78.0)	
p = 0.03				

Table 1. The *CTLA-4* gene exon 1 polymorphism in patients with and without evident Graves' ophthalmopathy (GO) and healthy subjects

	Patients total (I) n = 99 (%)	Patients		Controls (IV) n = 154 (%)	p value		
		without evident GO (II) n = 49 (%)	with evident GO (III) n = 50 (%)		I:IV	II:IV	III:IV
Allele frequencies							
A ⁴⁹	114 (57.6)	63 (64.3)	51 (51.0)	184 (59.7)	0.696	0.476	0.131
G ⁴⁹	84 (42.4)	35 (35.7)	49 (49.0)	124 (40.3)			
Phenotype frequencies							
A-positive	82 (82.8)	42 (85.7)	40 (80.0)	134 (87.0)	0.834	0.680	0.434
A-negative	67 (67.7)	28 (57.1)	39 (78.0)	104 (67.5)			
Genotype frequencies							
A ⁴⁹ A ⁴⁹	32 (32.3)	21 (42.9)	11 (22.0)	50 (32.5)	0.637	0.312	0.244
A ⁴⁹ G ⁴⁹	50 (50.5)	21 (42.9)	29 (58.0)	84 (54.5)			
G ⁴⁹ G ⁴⁹	17 (17.2)	7 (14.2)	10 (20.0)	20 (13.0)			

The *CTLA-4* locus has been found to be a strong genetic marker of GD^{12, 40}.

An association between the exon 1 *CTLA-4* A(49)G gene polymorphism and GD was found in previously published data on the Polish population⁷, European Caucasians in Germany¹⁸, the UK^{21, 41}, and Caucasians in the United States^{37, 47}. However, these results were not confirmed in studies on the Japanese^{1, 4}, Hong Kong Chinese²², African American¹¹, French¹⁷ and UK populations²². In our study we could not find differences in the frequencies of the AA, AG, and GG genotypes at position A(49)G between patients and healthy subjects.

GO is considered to be an autoimmune inflammatory disorder affecting the extraocular muscles and the orbital fatty/connective tissue²⁴. The effects of inflammation, mediated through the release of cytokines, include the proliferation of fibroblasts and adipocytes in the orbital soft tissues. The recruitment of T cells to the orbits of affected patients may result from expression of the target of the aberrant immune response in GD: the TSHR in the orbits of patients with this ophthalmic complication⁴³.

There is controversy concerning an association between *CTLA-4* gene polymorphisms and GO^{2, 5, 7, 9, 28, 45, 46}. Similarly to the studies performed by Vaidya et al.⁴⁵ in UK population, we showed a significantly higher frequency of the AA genotype in GD patients without evident GO compared with patients with evident GO. In contrast, such association was not found by others^{2, 5, 7, 9, 28, 46}.

The functional significance of the *CTLA-4* gene polymorphism is not fully clarified. It has been found that

the A(49)G single nucleotide polymorphism results in the substitution of the threonine with alanine at codon 17 of the leader sequence. This sequence serves as a signal peptide to direct the secreted protein to the endoplasmic reticulum¹². The codon 17 amino acid variation can affect a conformation of the leader peptide that leads to an “altered address” of intracellular CTLA-4 trafficking. This might result in an altered transition of CTLA-4 molecules between intracellular pools and the cell surface. Recent studies have shown different staining patterns of the intracellular distribution of CTLA-4 between patients with GG genotype and individuals homozygous for A at position 49³². In addition, a relationship between A(49)G variation and the strength of down-regulation of T cell activation has been shown^{30, 32}. Another explanation of how the A/G polymorphism may affect CTLA-4 function has been provided by Anjos et al.³ These authors reported that the G allele in the signal peptide was associated with incomplete product glycosylation, and a lower ratio of cell-surface to total cellular CTLA-4³.

As other autoimmune disorders, GD is a polygenic disease. This means that a combination of genetic factors influences the risk of disease; moreover, different etiologic factors may be involved in GD and GO, such as microbiological agents and environmental factors^{14, 34}.

The contradictory reports from different centers may be due to the genetic heterogeneity of the different ethnic groups and yet unknown environmental factors which influence the onset of the disease. It cannot be excluded that the etiologic factors involved in GD and GO may differ among different populations in geographically distinct regions.

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