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SHORT REPORT

CTLA4 + 49 A/G and CT60 polymorphisms in Dutch coeliac disease patients

Martine J van Belzen[1,2], Chris JJ Mulder[3], Alexandra Zhernakova[1], Peter L Pearson[1], Roderick HJ Houwen[2] and Cisca Wijmenga*[,1]

[1]*Department of Biomedical Genetics, University Medical Centre Utrecht, Utrecht, The Netherlands;* [2]*Department of Paediatric Gastroenterology, University Medical Centre Utrecht, Utrecht, The Netherlands;* [3]*Department of Gastroenterology, VU University Medical Centre, Amsterdam, The Netherlands*

Coeliac disease is an autoimmune disorder, characterised by villous atrophy of the small intestine, which results from a T-cell-mediated response to gluten-derived peptides. The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is involved in the regulation of T-cell activation and the *CTLA4* + 49 A/G polymorphism in exon 1 has been implicated in several autoimmune disorders, including coeliac disease. However, this polymorphism was recently excluded as being the causal variant in Graves' disease, autoimmune hypothyroidism and type I diabetes mellitus. This causal variant was mapped to the 3′ region of *CTLA4*, with the CT60 polymorphism showing the strongest association. The aim of this study was to determine the role of the *CTLA4* gene in coeliac disease in the Dutch population. The + 49 A/G and CT60 polymorphisms were genotyped in a case–control cohort of 215 patients and controls. The frequency of the + 49 G-allele was increased in cases, although not significantly. However, the frequency of the CT60 G-allele was increased with borderline significance in coeliac disease patients ($P = 0.048$), although the genotype distributions did not show a significant difference between cases and controls. These results indicate the involvement of the *CTLA4* gene in coeliac disease development. The haplotype carrying the CT60 G-allele was shown to be associated with lower mRNA levels of the soluble CTLA-4 isoform, providing a possible mechanism for the T-cell-mediated destruction of the small intestine.
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Introduction

Coeliac disease is an autoimmune disorder and is strongly associated with the HLA region. The majority of the patients express the HLA-DQ2 protein and almost all remaining patients express HLA-DQ8. Coeliac disease is characterised by an inappropriate T-cell response to gluten in the small intestine. Gluten proteins are present in wheat, barley and rye, and gluten-derived peptides can bind to HLA-DQ2 and DQ8 on the surface of antigen-presenting cells. These complexes are recognised by gluten-specific T cells in the gut of coeliac disease patients, resulting in inflammation, crypt hyperplasia and villous atrophy.[1,2] The HLA association only partly explains the genetic contribution, so non-HLA genes must also be involved in coeliac disease.[3]

The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the cell designation 28 antigen (CD28) are involved in the regulation of T-cell activation. CD28 induces the T-cell response, whereas CTLA-4 has an inhibitory effect.[4]

***Correspondence: Dr C Wijmenga, Complex Genetics Group, Department of Biomedical Genetics, Stratenum, University Medical Centre Utrecht, PO Box 80030, Utrecht 3508 TA, The Netherlands. Tel. +31 30 2503810; Fax +31 30 253 8479; E-mail: t.n.wijmenga@med.uu.nl**
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Owing to their functions they are interesting candidate genes for autoimmune disorders. The genes encoding these proteins are both located within a 150 kb region on chromosome 2q33–34. The A/G polymorphism at position +49 in exon 1 of the *CTLA4* gene has been extensively studied in autoimmune disorders, and association with type I diabetes mellitus,[5] Graves' disease,[6,7] Hashimoto's thyroiditis[8] and multiple sclerosis[9] has been reported (see, for a review, Kristiansen *et al*[10]). It is likely that coeliac disease shares a common genetic background with other autoimmune diseases, so the *CTLA4* gene may be involved in coeliac disease as well. Indeed, association between the +49 A/G polymorphism and coeliac disease was reported in several populations.[11–13] However, a recent publication showed that the causal variant involved in Graves' disease, autoimmune hypothyroidism and type I diabetes mellitus is most probably located in the 6.1 kb 3′ region of the *CTLA4* gene, and the +49 A/G polymorphism could be rejected as a causal variant.[14] The most strongly associated polymorphism in this 6.1 kb region was CT60. We therefore investigated the contribution of the *CTLA4* gene to coeliac disease risk in the Dutch population by genotyping the +49 A/G and CT60 polymorphisms in a Dutch case–control cohort.

Subjects and methods

Subjects

A total of 215 independent, biopsy-proven coeliac disease patients of Dutch origin were available for this study. Their mean age was 39 years and 65% were female. The control group consisted of 215 sex- and age-matched independent Dutch individuals. The study was approved by the Medical Ethics Committee of the University Medical Centre Utrecht and written informed consent was obtained from all the participants.

Genotyping

Genotypes of the A/G polymorphism at position +49 of the *CTLA4* gene were determined by PCR-restriction fragment length polymorphism (PCR-RFLP) with the *Fnu*4HI enzyme. The fragment containing the polymorphism was amplified with primers 5′-TTG CCT TGG ATT TCA GTG GC-3′ and 5′-CTG CTG AAA CAA ATG AAA CCC-3′, resulting in a fragment of 175 bp. Digestion with *Fnu*4H1 of the product containing the G-allele resulted in fragments of 42 and 133 bp. The genotyping procedure was verified by sequencing of three samples with predicted genotypes AA, AG and GG, respectively. These three samples were included as positive controls in each of the five sample plates. The CT60 A/G polymorphism was genotyped using Assay-by-Design on the TaqMan 7900HT system (Applied-Biosystems).

Statistical analysis

Allele and genotype frequencies of the *CTLA4* +49 A/G and CT60 polymorphisms were determined in cases and controls. Differences in global allele and genotype distributions were tested for significance by means of a χ^2 test using 2×2 or 2×3 contingency tables, respectively. The genotype frequencies were tested for Hardy–Weinberg equilibrium by χ^2 analysis, with a value of $P<0.05$ considered as not in Hardy–Weinberg equilibrium. Odds ratios were calculated using the Woolf–Holdane correction. Haplotypes of the two polymorphisms were estimated using the SNPHAP program (D Clayton; available from http://www-gene.cimr.cam.ac.uk/clayton/software/).

Results

The +49 A/G polymorphism in the *CTLA4* gene could be genotyped in 210 cases and 208 controls (Table 1). The genotypes in the controls were in Hardy–Weinberg equilibrium ($\chi^2=0.48$, $P=0.49$). The frequency of the G-allele was slightly increased in the coeliac disease patients, but this difference was not significant. The distribution of the three genotypes was not significantly different between cases and controls. However, the GG-genotype was more prevalent in the coeliac disease patients, with a frequency of 18% in cases compared to a frequency of 11% in controls (OR 1.78, 95% CI 0.99–3.31, $P=0.038$).

The CT60 polymorphism could be genotyped in 215 cases and 213 controls (Table 2), and the genotypes in the controls were in Hardy–Weinberg equilibrium ($\chi^2=0.80$,

Table 1 Allele and genotype frequencies of the CTLA4 +49 A/G polymorphism

	Coeliac disease (N=210)	*Controls (N=208)*	*P-value (χ^2)*	*OR (95% CI)*
Allele			0.14 (2.17)	
A	256 (61%)	274 (66%)		
G	164 (39%)	142 (34%)		1.23 (0.92–1.65)
Genotype			0.12 (4.29)	
AA	83 (39%)	88 (42%)		
AG	90 (43%)	98 (47%)		
GG	37 (18%)	22 (11%)		1.78* (0.99–3.31)

*P-value for the GG-genotype *vs* the other genotypes: $P=0.038$ ($\chi^2=4.27$, 1 df).

Table 2 Allele and genotype frequencies of the CTLA4 CT60 polymorphism

	Coeliac disease (N = 215)	*Controls (N = 213)*	*P-value (χ^2)*	*OR (95% CI)*
Allele			0.048 (3.91)	
A	184 (43%)	211 (50%)		
G	246 (57%)	215 (50%)		1.31 (0.99–1.73)
Genotype			0.13 (4.13)	
AA	37 (17%)	49 (23%)		
AG	110 (51%)	113 (53%)		
GG	68 (32%)	51 (24%)		1.46* (0.94–2.30)

*P-value for the GG-genotype *vs* the other genotypes: $P = 0.076$ ($\chi^2 = 3.15$, 1 df).

$P = 0.37$). The G-allele was significantly more frequent in cases ($P = 0.048$), and the frequency of the GG-genotype was also increased in cases, although not significantly (OR 1.46, 95% CI 0.94–2.30, $P = 0.076$).

The two polymorphisms formed three haplotypes: +49A-CT60A (42% in cases *vs* 49% in controls), +49G-CT60G (39% in cases *vs* 34% in controls) and +49A-CT60G (19% in cases *vs* 17% in controls), respectively, which were not significantly differently distributed between cases and controls (data not shown).

Discussion

The *CTLA4* gene has been implicated as a general susceptibility gene for autoimmune diseases.[10] The +49 A/G polymorphism is the most extensively studied, as this is the only polymorphism in the *CTLA4* gene that alters an amino acid.[14] The G-allele encodes an alanine residue at codon 17 of the protein, whereas the A-allele encodes a threonine residue. Type I diabetes mellitus, Graves' disease, Hashimoto's thyroiditis and multiple sclerosis have all been associated with the G-allele. So the codon 17 alanine residue seems a common denominator for autoimmunity. However, the +49 A/G polymorphism was recently excluded as a causal variant and strongest disease association was observed with the CT60 A/G polymorphism.[14] Two very common *CTLA4* haplotypes were present: one protective haplotype with an A-allele at position +49 and at CT60, and one disease-susceptible haplotype with a G-allele at position +49 and at CT60. More importantly, the disease-susceptible haplotype encodes an alternatively spliced CTLA-4 mRNA, resulting in lower mRNA levels of the soluble CTLA-4 isoform compared to the protective haplotype.

The involvement of the *CTLA4* +49 A/G polymorphism in coeliac disease has been studied by several groups. Positive association with the A-allele was reported in French and Swedish/Norwegian populations,[11–13] but was absent in Italian, Tunisian and Finnish populations and in a combined sample of families from Northern Europe.[15–17] These conflicting results may be due to population-specific effects. However, association could also be absent due to the occurrence of a recombination event between the +49 A/G variant and the disease-causing variant in the 6.1 kb 3′ region of *CTLA4*. This would also explain the observed association between coeliac disease and the +49 A-allele in several populations, while other autoimmune disease were shown to be associated with the G-allele. Therefore, genotyping a polymorphism from the 6.1 kb region 3′ of *CTLA4*, like CT60, would be a better choice for studying the involvement of the *CTLA4* gene in disease susceptibility.

The +49 A/G and the CT60 polymorphisms were both genotyped in our cohort of Dutch coeliac disease patients and controls. Positive association between CT60 and coeliac disease was observed, with an increased frequency of borderline significance of the G-allele in cases ($P = 0.048$). The +49 G-allele was also increased in cases, but statistical significance was not reached due to the presence of the recombined +49A-CT60G haplotype in the Dutch population. The genotypes of both polymorphisms were not significantly differently distributed between cases and controls. The CT60 G-allele is associated with a slightly increased risk for coeliac disease in the Dutch population (OR = 1.31, 95% CI 0.99–1.73). A large effect of the *CTLA4* locus was not to be expected, as there was no evidence for linkage to the *CTLA4* region in our genome-wide screen in Dutch affected sibpairs.[18] However, support for linkage was present in Scandinavian populations and a combined sample of families from Northern Europe.[17] The CT60 G-allele may be associated with a larger risk in these populations.

This study indicates the involvement of the *CTLA4* gene in coeliac disease. These results should be confirmed in a larger data set, which would also allow more accurate risk estimations. Especially those populations in which association with the +49 A-allele was observed should be genotyped for the CT60 polymorphism. The risk conferred by the *CTLA4* gene is probably mediated by the disease-susceptible haplotype, associated with lower mRNA levels and possibly with lower protein levels of the soluble CTLA-4 isoform. This may result in a disruption in the regulation of the T-cell response by CTLA-4 and CD28, leading to a T-cell-mediated destruction of the gut. Comparison of mRNA levels of the soluble CTLA-4 isoform in small intestinal tissue from coeliac disease patients and normal controls

may provide more insight into the role of the *CTLA4* gene in coeliac disease pathogenesis.

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