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Cytotoxic T Lymphocyte Antigen–4 +49A/G polymorphism does not affect susceptibility to autoimmune hepatitis

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Keywords
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Abbreviations
AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANA, antinuclear antibodies; ANOVA, analysis of variance; APC, antigen presenting cell; CTLA-4, cytotoxic T lymphocyte antigen-4; GATK, genome analysis toolkit; GWAS, genome-wide association study; HLA, human leucocyte antigen; HWE, Hardy–Weinberg equilibrium; IAIHG, international autoimmune hepatitis group; ICD, international classification of diseases; IgG, immunoglobulin G; IQR, interquartile range; LKM-1, liver kidney microsomal-1 antibodies; MS, multiple sclerosis; PCR, polymerase chain reaction; SLA, soluble liver antigen; SMA, smooth muscle antibodies; SNP, single nucleotide polymorphism.

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Abstract
Background & Aims: Single nucleotide polymorphisms (SNP) in the Cytotoxic T lymphocyte antigen-4 gene (*CTLA-4*) have been associated with several autoimmune diseases including autoimmune Hepatitis (AIH). In this chronic idiopathic inflammatory liver disease, conflicting results have been reported on the association with a SNP at position +49 in the *CTLA-4* gene in small patient cohorts. Here, we established the role of this SNP in a sufficiently large cohort of AIH patients. *Methods:* The study population consisted of 672 AIH patients derived from academic and regional hospitals in the Netherlands and was compared with 500 controls selected from the 'Genome of the Netherlands' project cohort. Genotype frequencies were assessed by PCR for patients and by whole genome sequencing for controls. *Results:* No significant differences in allele frequencies were found between patients and controls (G Allele: 40% vs 39%, $P = 0.7$). Similarly, no significant differences in genotype frequencies between patients and controls were found. Finally, there was no relation between disease activity and the G allele or AG and GG genotypes. *Conclusion:* The Cytotoxic T Lymphocyte Antigen-4 +49 A/G polymorphism does not represent a major susceptibility risk allele for AIH in Caucasians and is not associated with disease severity at presentation.

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Autoimmune hepatitis (AIH) is a relatively rare inflammatory liver disease of unknown aetiology (1). The diagnosis is based on the combination of clinical presentation, laboratory and histological findings and exclusion of viral and other causes. Diagnostic hallmarks include elevated serum Immunoglobulin G (IgG) and gamma globulins, auto-antibodies [AIH type-1: Antinuclear antibodies (ANA), smooth muscle antibodies (SMA), soluble liver antigen antibodies (SLA) and AIH type-2: liver kidney microsomal-1 antibodies (LKM-1)] and histologically proven interface hepatitis (1). Although the exact pathogenic trigger of AIH remains unknown, it is generally believed that disease occurs as the consequence of an exaggerated immune response in a genetically susceptible host (2). Indeed, several immune-related genes, including human leucocyte antigen (HLA) class-II molecules (HLA-DR3, -DR4 and -DR7), have been associated with the development of AIH in different populations (3). However, the presence of these genes is neither sufficient nor necessary to cause AIH (2).

A polymorphism at position +49 in the cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene has been described as a potential determinant of increased susceptibility to autoimmune diseases such as multiple sclerosis (MS), type 1 diabetes and autoimmune thyroiditis (4). The CTLA-4 molecule is expressed on the surface of activated T-cells and regulatory T-cells (T-regs) and acts as an inhibitory signal receptor in T-cell activation through binding to the B-7 ligands 1 and 2 (CD80 and CD86) on antigen presenting cells (APC) in competition with CD28. The *CTLA-4* +49 A/G single nucleotide polymorphism (SNP) results in an amino acid substitution of Threonine with Alanine at position 17 in the CTLA-4 protein (5). This is associated with a lower expression and function of the CTLA-4 protein (5–7). This polymorphism has been described as a non-HLA susceptibility determinant in Caucasian AIH patients (8). Similarly, Fan *et al.* found an association with homozygosity of the G allele and AIH susceptibility in Chinese patients (9). These studies suggest that the *CTLA-4*+49 G allele is an independent risk factor associated with susceptibility to AIH. It should be noted, however, that both study populations were small. Also, these associations were not confirmed in subsequent Japanese, Brazilian and German study populations, suggesting a varying risk among different ethnic populations (10–12). The aim of the present study was to assess the role of this polymorphism in a sufficiently large cohort of well-defined Caucasian AIH patients.

Materials and methods

Patients

Caucasian AIH patients with a clinical diagnosis of AIH were included from the Dutch Autoimmune Hepatitis Studygroup cohort, a nationwide collaboration of 31 centres in the Netherlands (8 academic medical centres and 23 general district hospitals; http://www.autoimmuunhepatitis.nl). AIH patients were identified by treating physicians and by searching the database for international classification of diseases (ICD) codes. The search was performed in local diagnostic registers in the departments of gastroenterology and hepatology as well as internal medicine. In all patients clinical and biochemical parameters were assessed to exclude other aetiologies such as alcohol, drugs and metabolic disorders. Viral hepatitis was excluded by serological testing. If performed, liver biopsy was used to establish diagnosis and the presence of fibrosis and cirrhosis. Available data on induction and maintenance therapy, as initiated and recorded by the treating physician, was retrospectively collected from the patient hospital records. Similarly, both clinical response to induction therapy and the occurrence of a relapse after treatment withdrawal were scored as assessed by the treating physician.

Control subjects were included from the Rainbow Project 'Genome of the Netherlands' (www.nlgenome.nl), a whole genome sequencing project with genetic data of 500 independent, healthy subjects (parents) and 266 children from the indigenous Dutch population (13, 14). In this study, only the genetic data from the parents were used. The institutional review boards of all participating centres and institutions approved the protocol. All participating patients and controls gave written informed consent.

Genotyping

Genomic DNA of AIH patients was isolated from peripheral blood mononuclear cells (PBMC's) with DNAzol® Genomic Isolation Reagent (Invitrogen Life Technologies BV, Bleiswijk, the Netherlands) according to the manufacturer's protocol. The *CTLA-4* +49 A/G (SNPdb: *rs231775*) genotypes in AIH patients were assessed by polymerase chain reaction (PCR), using a Taqman® Assay-by-Design (Applied Biosystems, Europe BV, Nieuwerkerk a/d IJssel, the Netherlands; assay number: C___2415786_20). The assays were performed according to the manufacturer's specifications and analysed using a ViiA 7 real-time PCR System (Applied Biosystems). The DNA samples were processed in 96-well plates (PE Applied Biosystems) with two negative controls per plate.

Control genotypes were assessed on the Illumina HiSeq 2000 platform at the Beijing Genomic Institute, Hong Kong. The raw control data were analysed with Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/), and Genome Analysis Toolkit (GATK, http://www.broadinstitute.org/gsa/wiki/index.php/Home_Page) software packages.

Statistical analysis

Genotypes and allele frequencies were tested for Hardy–Weinberg equilibrium (HWE) with χ^2-test (one

degree of freedom). Genotype distributions with a *P*-value > 0.05 were considered to be within HWE. Differences in *CTLA-4* +49 A/G allele frequencies and genotype frequencies between patients and controls were tested for statistical significance with the χ^2-test in 2 × 2 and 2 × 3 contingency tables, respectively. Differences in continuous variables between groups were tested for statistical significance with Students *t*-test, one-way analysis of variance (ANOVA) with post-hoc Bonferroni comparison or Mann–Whitney *U*-test. Log transformation of continuous variables was applied when appropriate. A *P*-value < 0.05 was considered statistically significant.

Results

Baseline characteristics

Overall, 672 Caucasian AIH patients (78% females) with a median International AIH Group diagnostic score of 18 (IQR: 15–20) (cut-off values: ≥12 probable AIH; ≥17: definite AIH) were included in this study (15). The median age at diagnosis was 44 years (IQR: 23–58) with a median follow-up time of 8.4 years (IQR: 5.4–14.9) prior to inclusion. Median alanine aminotransferase (ALT) and IgG levels at time of diagnosis were 328 U/L (IQR: 137–811) and 21.2 g/L (IQR: 16.2–30.0), respectively. Antinuclear antibodies (ANA) were positive (Titre: ≥ 1:40) in 377 of 560 (67%), whereas 310 of 526 (59%) patients had positive smooth muscle antibodies (SMA). Only 20 of 435 (5%) patients had positive LKM-1 antibodies. Sixty-eight (16%) of the 419 patients had undetectable ANA, SMA and LKM-1 antibodies. Recorded liver biopsy reports were available in 543 patients, showing fibrosis and cirrhosis in 282 (52%) and 65 (12%) patients respectively. A total of 570 (of 632 patients; 90%) received prednisone induction therapy followed by azathioprine in addition to or as replacement of prednisone maintenance therapy in 481 patients. Clinical and biochemical remission was reported in 541 of 562 patients (96%) during follow-up. A total of 193 of patients, however, experienced a relapse following treatment withdrawal as recorded by the treating physician.

Allele and genotype frequencies

The genotype calls of the *CTLA-4* +49 A/G SNP were successful in 667 of 672 AIH patients (99.3%) and in 498 of 500 controls (99.6%). The allele frequencies were in HWE in both patients (χ^2: 0.13; $P = 0.7$) and controls (χ^2: 0.12; $P = 0.7$).

There was no statistically significant difference in the distribution of allele frequencies between AIH patients and controls (G Allele: 40% vs 39%; $P = 0.7$) (Table 1). Also, no differences in genotype frequencies between patients and controls were identified (AA: 36% vs 38%; AG: 49% vs 47%; GG: 15% vs 15%, $P = 0.8$) (Table 1). Analysis of 485 ANA and/or SMA positive (type-1) AIH patients revealed similar results (AA: 37% vs 38%; AG: 47% vs 47%; GG: 16% vs 15%, $P = 1.0$). Separate analysis of the small cohort of LKM-1 antibody positive patients ($n = 20$) did not show a statistical significant difference in genotype frequencies either (AA: 25% vs 38%; AG: 50% vs 47%; GG: 25% vs 15%, $P = 0.4$).

Association of *CTLA-4* genotypes with clinical features

Genotype frequencies were distributed similarly between male and female groups. The alanine transaminase and IgG levels were somewhat higher in the homozygote AA and GG groups when compared with the heterozygote AG group, yet no statistical significance or trend was observed (Table 2). Autoantibody positivity for ANA, SMA and LKM-1 antibodies as well as 'seronegativity' was equally distributed among the genotypes (Table 2.). The frequency of fibrosis and cirrhosis at diagnosis was similar among all genotypes (Table 2). Also, treatment responses (reaction to induction therapy and relapse rates), did not differ significantly between genotype groups.

Discussion

In this study, we did not observe any significant differences in allele and genotype frequencies of the *CTLA-4* +49 A/G polymorphism between AIH patients and controls. These results contradict the initial reports by Agarwal *et al.* (2000) and Fan *et al.* (2004) in 155 and 62

Table 1. Allele and Genotype frequencies

	AIH (*N* = 667)	(%)	Controls (*N* = 498)	(%)	Table	*P*-value (χ^2)
Allele frequency						
A	804	(60)	609	(61)		
G	530	(40)	387	(39)	2 × 2	0.7 (0.15)
Genotype						
AA	240	(36)	188	(38)	2 × 3	0.8 (0.41)
AG	324	(49)	233	(47)	2 × 2 AA vs AG + GG	0.5 (0.38)
GG	103	(15)	77	(15)	2 × 2 GG vs AG + AA	1.0 (<0.001)
HWE						
P-value (χ^2, 1 df)	0.7 (0.13)		0.7 (0.12)			

AIH, autoimmune hepatitis; HWE, Hardy–Weinberg equilibrium; df, degrees of freedom.

Table 2. CTLA-4 + 49 A/G polymorphism and clinical features at presentation in AIH patients

	+49 A/G genotype			
Characteristics	AA	AG	GG	*P*-value
Patients, *n* (%)	240 (36)	324 (49)	103 (15)	
Age, years (IQR)	47 (29-59)	48 (30–60)	50 (36–59)	0.5
IAIHG-score, points (IQR)	18 (15–20)	18 (15–21)	18 (15–20)	0.7
Gender				
Female, *n* (%)	181 (75)	253 (78)	84 (81)	0.4
Biochemistry				
ALT, U/L (IQR)	384 (157–872)	298 (120–786)	350 (128–897)	0.2
IgG, g/L (IQR)	22.4 (16.4–32.0)	20.5 (15.5–28.8)	20.7 (15.9–27.8)	0.4
Serum auto-antibodies				
ANA $\geq$ 1:40, *n* (%)	135/200 (68)	181/269 (67)	57/87 (66)	0.9
SMA $\geq$ 1:40, *n* (%)	115/187 (61)	152/260 (58)	43/79 (54)	0.6
LKM-1 $\geq$ 1:40, *n* (%)	5/142 (4)	10/224 (5)	5/69 (7)	0.5
Seronegative, *n* (%)*	19/140 (14)	39/212 (18)	10/67 (15)	0.5
Histology				
Fibrosis, *n* (%)	105/200 (53)	137/267 (51)	40/76 (54)	1.0
Cirrhosis, *n* (%)	21/200 (11)	35/267 (13)	9/76 (12)	0.7
Treatment response				
Lack of response to induction therapy, *n* (%)	4/202 (2)	13/272 (5)	4/88 (5)	0.2

ALT, alanine transaminase; IAIHG, international autoimmune hepatitis group; IgG, immunoglobulin G; IQR, interquartile range; ANA, antinuclear antibodies; LKM-1, liver kidney microsomal antibodies, SMA, smooth muscle antibodies. Numbers between parentheses are in percentages (%) for proportional variables and interquartile range (…-…) for continuous variables.
*ANA, SMA and LKM-1 negative.

AIH patients, respectively, both suggesting that the G allele is associated with type-1 AIH (8,9). Another genotyping study, performed by Schott *et al.* (2007), could not confirm these findings in a German cohort of 127 Caucasian AIH patients (10). They argued that the difference in allele frequencies found by Agarwal *et al.* (2000) (41% vs 31%, $P = 0.03$) is likely because of an under-representation of the G allele in their controls (10). Although the study by Schott *et al.* (2007) was not sufficiently powered to reliably exclude such a statistical significant association, our overall as well as separate type 1 AIH analyses do confirm this conclusion.

In concordance with the results reported previously by Bittencourt et al. (2003), the *CTLA-4* +49 SNP was not associated with type-2 AIH patients in our study population.(12) It should be noted, however, that the number of type-2 AIH patients in this study was small ($n = 20$).

In a recent meta-analysis, incorporating the results of five studies with a total of 526 AIH patients and 631 controls from different ethnic backgrounds, Miyake *et al.* concluded that there might be ethnic differences in the AIH-associated susceptibility of the *CTLA-4* +49 G allele and that further studies in different populations were therefore needed (16). The cohort described in this study represents the largest well-defined AIH population investigated so far. Based on our findings, it is unlikely that the *CTLA-4* +49 A/G polymorphism represents a major susceptibility risk for AIH in Caucasians. This is underlined by a lack of association between the G allele and disease variables. The scope of this study was focused on one single nucleotide polymorphism, leaving other potential susceptibility loci inside and outside the *CTLA-4* gene unremarked. Therefore, we cannot exclude a potential additive or synergistic effect of the *CTLA-4* +49 A/G polymorphism to disease susceptibility in combination with *HLA-DR3, -DR4* or *-DR7* positivity. Indeed Agarwal *et al.* (2000) did find a higher frequency of the *CTLA-4* +49 G allele in HLA-DR3 positive patients (8). Since we could not confirm the independent association of the *CLTA-4* +49 G allele with AIH, we hypothesize that a *HLA-DR3-CTLA-4* +49 G association is not likely to exist in this cohort.

To study the genetic background of disease, particularly in autoimmunity, genome-wide association studies (GWAS) have been successful in identifying new candidate genes (17). Future studies on genomic variation in AIH should therefore use similar methods to open new ways to a better understanding of the complex aetiopathogenesis of AIH.

In conclusion, this study in a large cohort of AIH patients argues against the role of the *CTLA-4* gene in the susceptibility to AIH. Further genome-wide studies are mandatory to unravel the genetic susceptibility to AIH.

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