European Journal of Human Genetics (2005) 13, 440–444

www.nature.com/ejhg

ARTICLE

A common CTLA4 haplotype associated with coeliac disease

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Coeliac disease is a common enteropathy with a strong inherited risk characterised by dietary wheat, rye and barley induced T-cell activation. Although there is replicated linkage to 2q33, results are inconsistent from association studies of the most promising candidate genes: the CD28/CTLA4/ICOS cluster. CTLA4 plays a key role in regulating T lymphocyte mediated inflammatory responses, and variants in the 3′ region influence development of diabetes and thyroid disease. We genotyped CTLA4 variants (−1722 C/T, −658 T/C, −318 C/T, +49 A/G, +1822 C/T, CT60 A/G) to tag all common haplotypes (>5% frequency) and an ICOS variant (IVS+173 C/T) in 340 white UK Caucasian coeliac disease cases. Strict ascertainment criteria for coeliac cases required both villous atrophy at diagnosis and positive serology. In total, 973 healthy controls were available for SNP, and 705 for CTLA4 haplotype, based association analyses. Coeliac disease showed weak association with the CTLA4 +1822T ($P=0.019$) and CT60 G ($P=0.047$) alleles. Strong association was seen with a common CTLA4 haplotype ($P=0.00067$, odds ratio 1.41) of frequency 32.7% in coeliac disease and 25.5% in healthy controls. A common CTLA4 haplotype shows strong association with coeliac disease, and contains multiple alleles reported to affect immunological function. Loss of tolerance to dietary antigens in coeliac disease may be mediated in part by heritable variants in co-signalling genes regulating T-cell responses.

European Journal of Human Genetics (2005) **13,** 440–444. doi:10.1038/sj.ejhg.5201357
Published online 12 January 2005

Keywords: coeliac; celiac; CTLA4

Introduction

Coeliac disease is a chronic inflammatory intestinal disease induced by dietary antigens in wheat, rye and barley with ~1% prevalence in the UK population.[1] Twin and family studies suggest a strong genetic component to coeliac disease susceptibility. Although disease is strongly associated with HLA-DQ2 (carried by 90% of N. European patients), which presents immunodominant wheat gliadin epitopes, DQ2 is also common in the healthy population (~30%) demonstrating that it is necessary but not sufficient for disease susceptibility. Non-HLA genes are likely to contribute a greater proportion of genetic susceptibility to disease.

Evidence for a coeliac disease susceptibility locus on chromosome 2q has been provided by five independent linkage studies,[2–6] centred around the immuno-regulatory 300 kb CD28/CTLA4/ICOS gene cluster. The CTLA4 gene plays a key role in regulating T lymphocyte mediated inflammatory responses, and contains several single-nucleotide polymorphisms (SNPs) known to alter function. A comprehensive study mapping the gene cluster suggested

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Received 13 May 2004; revised 16 November 2004; accepted 17 November 2004

variants in the 3′ region of CTLA4 influence autoimmune responses in type I diabetes, Graves' disease and autoimmune hypothyroidism.[7] It is therefore feasible that CTLA4 variants might also control whether DQ2 positive individuals develop T-cell mediated responses to gliadin and subsequent coeliac enteropathy. In total, 11 previous studies in coeliac disease have analysed markers around CTLA4,[2–5,8–15] generating ambiguous results with three of 11 studies reporting association with the +49 SNP, and one of three studies with the CT60 SNP, in addition to inconsistent microsatellite marker associations. These studies have typically genotyped only one or two SNPs (except[12]), have been relatively small, and have not comprehensively analysed CTLA4 haplotypes. Aiming to overcome these issues we therefore genotyped CTLA4 haplotype tagging SNPs (as described[16]) in a large white UK Caucasian coeliac disease/control cohort.

Methods

Subjects

Patients with coeliac disease ($n=340$) were recruited from specialist adult clinics in London (Hammersmith Hospital, St Bartholomew's Hospital) and Oxford (John Radcliffe Hospital). Strict criteria were used for case ascertainment, requiring both villous atrophy on pretreatment small intestinal biopsy and positive serology (IgA antiendomysial or antitissue transglutaminase antibody). In the case of IgA deficiency, or diagnosis prior to introduction of serological tests, we required both villous atrophy at diagnosis and biopsy evidence of improved intestinal histology on gluten-free diet (ie encompassing the revised ESPGAN criteria). Median age at diagnosis was 42.1 years (range 0.5–78.1, interquartile range 30.6–54.7), female to male ratio 2.76 : 1.

Healthy controls ($n=321$) were recruited from clinical and laboratory staff volunteers, and from the UK National Blood Transfusion Service. All cases and controls were adult and of white Caucasian ethnic origin. Approval was obtained from institutional Research Ethics Committees. Further controls, similarly ascertained (white UK Caucasian hospital staff/National Blood Transfusion Service donors), were available from published data produced by the Cambridge Institute for Medical Research ($n=652$ published for single SNPs and $n=384$ published for SNP haplotypes).[7,16]

Genotyping

Genomic DNA was extracted from frozen whole blood using the Puregene system (Gentra Systems, Minneapolis). Genotypes were obtained by PCR-RFLP methodology. SNPs were chosen based on previous reports, known functional effect, and to tag all common (>5% population frequency) CTLA4 haplotypes.[16] Table 1 shows genotyping primers and restriction enzymes. PCR products were digested overnight and analysed by agarose gel electrophoresis. This methodology was robust with 97.8% genotype success rate.

Statistical analysis

Allele and haplotype counts were compared by two-tailed 2×2 χ^2 test. Hardy–Weinberg equilibrium was assessed using χ^2 test comparing genotypes as expected under Hardy–Weinberg equilibrium (based on observed allele frequency) to the observed genotypes. Haplotypes were reconstructed in the unrelated cases and control data sets using PHASE version 2.0.2 software (utilising Bayesian methods to infer haplotypes). Relative risk associated with a rare allele was estimated as an odds ratio (OR) with a 95% confidence interval (CI).

Table 1 Primers and restriction enzymes for *CTLA4* PCR-RFLP genotyping

Reference SNP ID	*Gene/SNP primer*	*Sequence*	*Enzyme*
rs733618	CTLA4 −1722 C/T F	5′-AACACACAGCAGTGGCAGGGcCAG-3′	*Bst*NI[a]
	CTLA4 −1722 C/T R	5′-GCCCTTTCTGACTTCCACAG-3′	
rs11571317	CTLA4 −658 T/C F	5′-TCCTTCTGCAAAACCAGAGG-3′	*Aci*I
	CTLA4 −658 T/C R	5′-TCCCTTGACAGCTTTACAAATAAG-3′	
rs5742909	CTLA4 −318 C/T F	5′-TGGACTGGATGGTTAAGGATG-3′	*Mse*I
	CTLA4 −318 C/T R	5′-AGAAGGCACTTGAATAGAAAGC-3′	
rs231775	CTLA4 +49 A/G F	5′-GCTCAGCTGAACCTGGCT-3′	*Fnu*4HI
	CTLA4 +49 A/G R	5′-AAATCACTGCCCTTGACTGC-3′	
rs231779	CTLA4 +1822 C/T F	5′-TCAAAGGGATTGAGCAGATG-3′	*Bsm*AI
	CTLA4 +1822 C/T R	5′-TCCCATGCTCCTTTGTTCTC-3′	
rs3087243	CTLA4 CT60 A/G F	5′-GATTTCTTCACCACTATTTGGGATATtAC-3′	*Bsa*AI[b]
	CTLA4 CT60 A/G R	5′-AGATCAAAATGGCTGCAAGG-3′	
rs10932029	ICOS IVS+173 T/C F	5′-TACGCACCCAAAAGACAGTG-3′	*Dde*I
	ICOS IVS+173 T/C R	5′-AAAGGCAGCAATGCAAACTC-3′	

[a]The small type nucleotide c denotes a mismatch in the forward primer to create an *Bst*NI site for the CTLA4 −1722 G allele.
[b]The small type nucleotide t denotes a mismatch in the forward primer to create an *Bsa*AI site for the CTLA4 CT60 G allele.

Results

We first analysed SNP and haplotype frequencies in 321 healthy controls. All seven SNPs genotyped were in Hardy–Weinberg equilibrium in our healthy control cohort ($P>0.05$ for each SNP, data not shown). Published data in the same ethnic population (white UK Caucasian) had defined common SNP based haplotypes around CTLA4.[16] We genotyped the SNPs necessary to tag these haplotypes. No significant differences were seen at either CTLA4 SNP or haplotype frequency level between the two UK Caucasian control data sets (Table 2a and b, $P>0.05$ for each SNP/haplotype). A combined healthy control data set was therefore used for further analyses to increase statistical power.

At the single SNP level (Table 3a), positive association was seen with the CTLA4 +1822T allele and the CTLA4 CT60 G allele (ie these alleles were more common in coeliac disease than controls). The strength of association was similar after removing from the analysis 41 patients with co-existing autoimmune thyroid disease and/or type I diabetes (diseases showing association with CT60 G,[7] data not shown).

A single common CTLA4 haplotype showed strong association with coeliac disease ($P=0.00067$, OR 1.41, 95% CI 1.16–1.73, Table 3b). This association remained significant after highly conservative correction for multiple testing (Bonferroni × 13 SNP/haplotype tests, Pc = 0.0087). This effect was slightly stronger in earlier onset coeliac disease (patients below the median age at diagnosis) with haplotype frequency 34.7% in earlier onset cases *vs* 25.5% in healthy controls ($P=0.00062$, OR 1.56, 95% CI 1.21–2.01).

Discussion

The conclusive identification of genetic variants outside the HLA predisposing to coeliac disease has to date proved elusive. Five linkage studies have individually provided nominally significant evidence for a coeliac disease susceptibility locus on chromosome 2q,[2–6] and taken together the evidence is compelling.

The most promising candidates for this locus are found in a 300 kb region containing the co-signalling molecule cluster (ICOS, CTLA4, CD28),which has for some years been suspected of harbouring variants predisposing to coeliac disease, and to autoimmune disease in general. Recently, a comprehensive genetic mapping study of the entire 300 kb ICOS-CTLA4-CD28 locus reported variants 6.1 kb 3′ of CTLA4 associated with autoimmune thyroid disease and type I diabetes.[7] Regression analyses suggested that the CTLA4 CT60 A/G variant was the primary causal variant, and this variant also showed strongest association. The CT60G variant is associated with lower levels of soluble CTLA4 expression, and this polymorphism affects the ratio of trans-membrane to soluble mRNA splice forms of the CTLA4 gene, thereby possibly influencing disease susceptibility. Other studies have reported a functional role for the CTLA4 + 49 variant, in which the +49G (Ala19) allele is associated with incomplete glycosylation of the signal peptide, altered processing in the endoplasmic reticulum, and lower cell surface levels in transfected cells and also for the −318 promoter variant.[17] Interestingly, the NOD mouse, known to be predisposed to autoimmune disease, also has a defect in expression of a CTLA4 isoform,[7] and

Table 2 Healthy control (a) allele frequencies and counts of CTLA4 SNPs and (b) haplotype frequencies of CTLA4 SNPs

		−1722 C/T	*−658 T/C*	*−318 T/C*	*+49 G/A*	*+1822 T/C*	*CT60 A/G*
(a)							
Current cohort ($n=321$)	Allele frequency	7.0%	8.7%	9.1%	34.2%	33.8%	46.6%
	Minor/major allele counts	45/595	54/564	56/562	210/404	201/393	286/328
CIMR cohort ($n=652$)[a]	Allele frequency	7.4%	7.6%	9.2%	35.8%	34.9%	47.7%
	Minor/major allele counts	96/1202	92/1112	117/1157	465/835	442/826	616/676

CTLA4 SNP Haplotype						Haplotype designation[b]	Current cohort ($n=321$)	CIMR cohort[b] ($n=384$)
(b)								
−1722	−658	−318	+49	+1822	CT60			
A	C	C	A	C	A	A+G+J	38.0%	39.6%
A	C	C	A	C	G	F+H	9.9%	7.5%
A	C	C	G	T	G	B+I	26.8%	24.4%
A	C	T	A	C	G	C	9.1%	8.6%
A	T	C	A	C	A	D+K	8.3%	9.7%
G	C	C	G	T	G	E	7.0%	5.8%

[a]CIMR cohort data from Ueda *et al*.[7]
[b]Haplotype designation and CIMR cohort data from Johnson *et al*.[16]

Table 3 (a) CTLA4/ICOS allele counts and frequencies and (b) CTLA4 haplotype frequencies in coeliac cases and healthy controls

	Coeliac disease (n = 340)		*Healthy controls (n = 973)*			
SNP	Allele count (minor/major)	Allele frequency	Allele count (minor/major)	Allele frequency	*P-value*	*Odds ratio (95% CI)*
(a)						
CTLA4 −1722 C/T	36/640	5.3%	141/1797	7.3%	0.082	
CTLA4 −658 T/C	52/618	7.8%	146/1676	8.0%	0.84	
CTLA4 −318 T/C	67/605	10.0%	173/1719	9.1%	0.53	
CTLA4 +49 G/A	260/406	39.0%	675/1239	35.3%	0.081	
CTLA4 +1822 T/C	266/406	39.6%	643/1219	34.5%	**0.019**	1.24 (1.04–1.49)
CTLA4 CT60 A/G	289/385	42.9%	902/1004	47.3%	**0.047**	0.84 (0.70–1.00)
ICOS IVS+173 C/T	110/570	16.2%	102/538[a]	15.9%	0.91	

(b) CTLA4 haplotype						Haplotype designation[b]	Coeliac disease ($n = 340$)	Healthy controls ($n = 705$)	*P*-value	Odds ratio (95% CI)
−1722	−658	−318	+49	+1822	CT60					
A	C	C	A	C	A	A+G+J	34.8%	38.8%	0.11	
A	C	C	A	C	G	F+H	8.3%	8.6%	0.79	
A	C	C	G	T	G	B+I	32.7%	25.5%	**0.00067**	1.41 (1.16–1.73)
A	C	T	A	C	G	C	9.5%	8.8%	0.57	
A	T	C	A	C	A	D+K	7.4%	9.1%	0.19	
G	C	C	G	T	G	E	5.3%	6.3%	0.36	

[a]SNP ICOS IVS+173 C/T genotyped only in single cohort ($n = 321$).
[b]Haplotype designation from Johnson *et al*.[16]

recent data have suggested that the HLA-DQ8 transgenic mouse develops a coeliac disease/dermatitis herpetiformis like phenotype on the NOD background.[18]

Our data suggest that coeliac disease is most strongly associated with a haplotype of the CTLA4 gene, rather than any of the individual variants analysed. We cannot exclude that a (as yet unidentified) single true disease-causing allele in CTLA4 or another neighbouring gene might be carried on this haplotype. It is interesting however to speculate that a combination of functional variants inherited in *cis*, rather than any single variant alone, is required to predispose to coeliac disease. Our data are consistent with the weak association with CT60 G found in a recent Dutch study.[15] We could not replicate the association with ICOS (most significant for the IVS + 173 T variant tested here) reported in a Finnish population.[12] Neither could we replicate the association with the CTLA4 + 49 A allele reported in a few studies,[4,10,11] indeed in our data the G allele was slightly more common in coeliac disease (non-significant). Poor statistical power, lack of comprehensive linkage disequilibrium mapping, less than strict phenotyping and ethnic differences might explain the variability in previous studies of the CD28/CTLA4/ICOS cluster in coeliac disease.

The current data suggest that functional variation in the CTLA4 gene predisposes to coeliac disease. Further research in coeliac disease might include the search for disease predisposing genetic variants in other co-stimulatory molecules and immunoregulatory pathways (using large cohorts and haplotype tagging SNPs), as well as studies of the function of the disease predisposing CTLA4 haplotype.

Acknowledgements

This study was funded by the Wellcome Trust (DAvH holds a Clinician Scientist Fellowship). We thank all patients and healthy controls for providing DNA samples, Professor T Vyse's laboratory for equipment access, and the Wellcome Trust Centre for Human Genetics for access to computer facilities. We are grateful to the CIMR investigators for placing CTLA4 genotype/haplotype data in the public domain.

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