Table 1 Clinical and biological features of CAD patients undergoing CABG

Clinical and biological characteristic	Value	
	Cases	Controls
Age, years	57.6 (8.2)	57.2 (5.8)
Gender, male/female	242/76	189/59
Angina pectoris:		
No, rarely/frequently on effort/resting	51/96/171	248/0/0*
NIDDM in case history, yes/no	69/249	0/248*
Smoking, ever smoked/never smoked	210/108	144/104
Hypertension		
Yes/no	169/149	0/248*
Family history of myocardial infarction		
Yes/no	179/137	71/177*
Myocardial infarction in case history		
Yes/no	158/162	0/248*
Stenosis		
In one artery/more than one artery	81/237	0/0*
Body mass index, kg/m^2	28.2 (3.8)	28.0 (2.7)
Serum cholesterol, mmol/l†	6.25 (0.83)†	6.29 (0.78)
HDL cholesterol, mmol/l†	1.27 (0.17)†	1.31 (0.21)
LDL cholesterol, mmol/l†	4.04 (0.93)†	4.11 (0.89)
Triglycerides, mmol/l†	2.04 (0.7)†	1.96 (0.81)

*p<0.00001 cases *v* controls.
†Six months after the surgery, cases were treated by lipid lowering drugs.

ANOVA test was used to estimate the impact of the polymorphisms on the quantitative traits.

RESULTS

Frequencies of the genotypes and alleles

The frequencies of the genotypes and alleles in question in CAD patients and controls are presented in table 2. Significant differences were observed in the frequencies of C4B*Q0 between CAD patients and controls. The results were overall in Hardy-Weinberg equilibrium, with the exception of the C4B*Q0 allele distribution in CAD patients, where there was deviation (χ^2=12.41, p=0.0004) from the equilibrium. Analysis of the genotype distribution of this polymorphism showed that the deviation from the HWE was exclusively because of an increased frequency of the C4B*Q0/C4B*Q0 homozygotes among patients (p=0.02, odds ratio (OR)=3.8, 95% confidence interval 1.2-13.2), suggesting a recessive mode of action of this polymorphism on CAD.

Study of association between alleles tested and known risk factors for CAD

The mean values of several quantitative variables were compared between the different genotypes in both groups. No significant association with the lipid parameters (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) and blood pressure was observed (data not shown). The mean body mass index (BMI) in carriers of TNFα −308A was also compared with that of non-carriers, as was done by Herrmann *et al*.[5] In contrast to their results, we have not found significant differences between carriers and non-carriers and no association was found between obesity and the TNFα −308A allele (data not shown).

The mean blood glucose levels of carriers of TNFα −238A were lower than that of non-carriers, 5.52 (SD 0.57) mmol/l *v* 6.38 (SD 2.01) mmol/l (p=0.05), but the TNFα −238A allele frequency in subjects with diabetes did not differ significantly from that of subjects without diabetes (4.3% and 4.5% in subjects with and without diabetes, respectively).

Investigation of allelic combinations

We investigated with the exact p test whether the distribution of the different combinations of alleles corresponded to the expected values. Deviation from the equilibrium was found in patients with CAD in TNFα −308A and C4A*Q0 alleles (exact p<0.001, linkage disequilibrium (LD) coefficient (±|D'|) was 0.61, that is, the observed disequilibrium is 61% of the

Table 2 Genotype and allele frequencies in patients with CAD undergoing CABG (n=318) and in controls (n=248)

	Status	Genotype frequency 11/12/22* (%)	Allele 2 frequency (% (SE))
TNFα -238A	Cases	90.2/9.8/0.0	4.9 (1.7)
	Controls	90.7/9.3/0.0	4.6 (1.8)
TNFα -308A	Cases	72.0/25.2/2.8	15.4 (2.9)
	Controls	72.9/26.2/0.9	13.9 (3.1)
Bf*F	Cases	64.2/31.4/4.4	20.4 (3.2)
	Control	69.5/27.3/3.2	17.0 (3.3)
C4A*Q0	Cases	73.9/24.5/1.6	13.8 (2.7)
	Controls	70.9/27.4/1.7	15.6 (3.3)
C4A*6	Cases	91.5/7.9/0.6	4.6 (1.7)
	Controls	95.6/4.4/0.0	2.2 (1.3)
C4B*Q0	Cases	76.1/19.5/4.4†	14.2 (2.8)†
	Controls	81.5/17.3/1.2	9.9 (2.7)

*2 represents the allele under consideration, 1 codes for all other alleles.
†Deviation from Hardy-Weinberg equilibrium (χ^2=12.41, p=0.0004). The frequency of the homozygous carriers of the C4B*Q0 in CAD patients differs significantly from that of controls (p=0.02, odds ratio=3.8 (95% confidence interval 1.2–13.2). The frequency of the C4B*Q0 allele is different in cases and in controls (p=0.02, OR=1.5 (1.0–2.1)).

Table 3 Maximum likelihood frequencies of allelic combinations. Comparisons of ratios between the frequency of TNFα -308A+C4A*Q0 allelic combinations with that of TNFα -308A without C4A*Q0 alleles, and with that of TNFα -308G + C4A*Q0 alleles in cases and controls. The same comparisons with the TNFα-238A, C4A*6, and TNFα-238G allelic combinations

	Frequency in cases	Ratio in cases	Frequency in controls	Ratio in controls	p	Odds ratio (95%CI)
(A) TNFα-308A + C4A*Q0	8%		3.8%		0.005	2.2 (1.3–3.8)
(B) TNFα-308A + no C4A*Q0	6.8%		10.5%		0.04	0.6 (0.4–0.9)
(C) TNFα-308G + C4A*Q0	7.1%		8.5%		0.5	0.8 (0.5–1.3)
(A/B)		1.18		0.36	0.0005	3.2 (1.7–6.3)
(A/C)		1.13		0.45	0.008	2.5 (1.3–4.9)
(D) TNFα-238A + C4A*6	3.3%		0.8%		0.009	4.2 (1.4–12.3)
(E) TNFα-238A + no C4A*6	1.6%		3.2%		0.1	0.5 (0.2–1.1)
(F) TNFα-238G+ C4A*6	1.3%		1.4%		1.0	0.9 (0.3–2.5)
(D/E)		2.1		0.25	0.001	8.4 (2.2–31.7)
(D/F)		2.5		0.6	0.06	4.6 (1.0–20.1)

theoretical maximum disequilibrium value) and in TNFα −238A and C4A*6 alleles ($p<0.001$, $\pm|D'|=0.71$) and in both patients and controls in C4B*Q0 and Bf*F alleles ($p<0.001$, $\pm|D'|=0.3$ in cases; $p<0.05$, $\pm|D'|=0.2$ in controls). The linkage between TNFα −308A and C4A*Q0 alleles was supported by the findings that five patients were homozygous for the C4A*Q0 allele and three of them had AA (60%) and one GA genotype at the TNFα −308 position. Complete negative LD ($\pm|D'|=-1$; the two alleles are never present on the same haplotype) was found in patients between TNFα −308A and C4A*6 and between TNFα −238A and C4AQ*0 and in both patients and controls between C4A*Q0 and C4B*Q0.

We examined the impact of these allelic combinations on the susceptibility to CAD. We computed the maximum likelihood frequencies of the different allelic combinations with an expectation maximisation algorithm and compared them between cases and controls. The estimated frequency of the TNFα −308A + C4A*Q0 allelic combinations and that of the TNFα −238A + C4A*6 (table 3) were significantly higher in CAD patients than in controls (8% *v* 3.8%, and 3.3% *v* 0.8%, respectively). In contrast, the frequency of the TNFα −308A without C4A*Q0 (pooled all C4A alleles, which is not C4A*Q0) was higher in controls (6.8% *v* 10.5%). The prevalence of other allelic combinations did not differ between the two groups (data not shown). If the ratios between the frequency of patients with TNFα −308A + C4A*Q0 alleles and with TNFα −308A without C4A*Q0 were compared in cases and in controls, the p value was 0.0005 and the OR 3.2 (95%CI 1.7-6.3), indicating that the risk of having CAD was 3.2 times higher in patients with TNFα −308A+C4A*Q0 alleles than with TNFα −308A without C4A*Q0. When the same ratio between patients with TNFα −308A + C4A*Q0 alleles and with TNFα −308G + C4A*Q0 were compared in cases and in controls, the p value was 0.008 and the odds ratio 2.5 (95%CI 1.3-4.9), indicating that the risk of having CAD was 2.5 times higher in patients with TNFα −308A + C4A*Q0 than in patients with TNFα −308G + C4A*Q0 allelic combination. Similar trends were observed in respect of TNFα −238A+C4A*6 (table 3), although because of the low prevalence of C4A*6, the comparison of the ratio between patients with TNFα −238A + C4A*Q0 alleles and with TNFα −238G + C4A*6 did not show a significant difference ($p=0.06$). These results suggest that the two allelic combinations (TNFα −308A + C4A*Q0 and TNFα −238A+C4A*6) give higher susceptibility to CAD than either allele alone.

Differences in the frequencies of alleles and allelic combinations between CAD patients with or without myocardial infarction in their case history

To investigate the role of these alleles and allelic association in CAD, we analysed the clinical status and case histories of the patients. The CAD patients were divided into two groups based on the occurrence of myocardial infarction in their case history before the bypass surgery. The frequencies of TNFα −308A and the C4A*Q0 alleles were significantly higher in patients with MI than without MI (table 4). The TNFα −308A and the C4A*Q0 alleles occurred together significantly more frequently in patients with preoperative MI than without preoperative MI, while there was no such association in the case of TNFα −238A + C4A*6 allelic combination.

There was no association between other symptoms (extent of stenosis, pre- and postoperative angina pectoris, thrombosis, left or right heart failure, hypertension, embolism, syncope, diabetes) and these alleles or allelic combinations (not shown).

DISCUSSION

In this study we investigated the distribution and association of six alleles in the MHC in CAD patients undergoing CABG and in controls. There is some evidence that complement plays

Table 4 Frequencies of alleles and allelic combinations in CAD patients undergoing CABG with or without myocardial infarction in their preoperative case history

	Preoperative MI	No preoperative MI	p	Odds ratio (95%CI)
No	158	162	0.8	—
TNFα -308A	19.9 (4.5)	12.0 (3.6)	0.004	1.8 (1.2–2.8)
C4A*Q0	15.7 (4.1)	9.0 (3.1)	0.005	1.9 (1.2–3.1)
TNFα-308A + C4A*Q0	10.8%	4.9%	0.02	2.2 (1.2–4.1)
TNFα -238A + C4A*6	3.5%	3.1%	1.0	1.1 (0.5–2.7)

MI=myocardial infarction.

an important role in the establishment of atherosclerosis. Complement C4 is a precursor of a subunit of the enzyme complex C3 convertase and is encoded by two closely related genes. The protein products of these loci are called C4A and C4B. Both genes are highly polymorphic and there is a relatively high frequency of the non-expressed variants, termed C4A*Q0 and C4B*Q0.[11] These alleles are associated with several autoimmune diseases.[12 13] The rare, haemolytically inactive C4A*6 allotype was reported to be associated with rheumatic heart disease.[14]

Our present findings indicate that the susceptibility of homozygous carriers of the C4B*Q0 allele to severe CAD is higher than that of non-carriers. Previously we found the prevalence of C4B*Q0 to be markedly lower in healthy, elderly, Hungarian people, particularly in men, as compared to healthy, young subjects.[22] We explained this observation by an increased morbidity and mortality from some diseases in middle aged carriers of the C4B*Q0 allele. This assumption was supported by our more recent observation indicating an increased frequency of the C4B*Q0 allele in 60-79 year old myocardial infarction patients as compared with age matched, healthy controls.[23]

Little is known about the mechanism of this greater susceptibility to cardiovascular disease associated with the C4B*Q0 allele. Here we looked for a possible link between the C4B*Q0 allele and some characteristics and symptoms of CAD patients undergoing CABG. We found, however, no differences in these factors between carriers and non-carriers of this allele.

Several studies have shown association between atherosclerosis and certain bacterial and viral pathogens. The most compelling evidence for an infectious factor in atherosclerosis is related to *Chlamydia pneumoniae*.[24] The complement system plays a principal role in the defence against bacterial infection. Therefore, it can be assumed that homozygous carriers of the silent C4B*Q0 allele have an impaired capacity to eliminate or mitigate *Chlamydia pneumoniae* infection. Finally, it cannot be ruled out that C4B*QO is a marker of a known or unknown gene in the MHC with linkage disequilibrium, or there are still unknown interactions with products of genes at other linked loci, which increase the susceptibility to the disease.

TNFα is an inducible cytokine with a wide range of proinflammatory and immunoregulatory actions. Through its effect on lipid metabolism,[25] obesity,[5] insulin resistance,[16] and endothelial function,[26] and stimulation of growth factors and adhesion molecules,[27] it could be involved in cardiovascular pathophysiology. The large and stable interpersonal differences in TNFα production indicate a genetic background. Wilson *et al*[28] raised considerable interest with their report that the −308A allele in the promoter region is transcribed in vitro at seven times the rate of the −308G allele. Moreover, the −308A allele has also been found to correlate with enhanced spontaneous and stimulated TNFα production in vivo.[29] Several studies have investigated TNFα polymorphisms in diseases in which dysregulation of TNFα production might have played a role and several of them found association between TNFα −238 and −308 promoter polymorphisms and some diseases.[5 6 16] Herrmann *et al*[5] investigated patients with coronary heart disease and found no association between polymorphisms in TNFα and susceptibility to the disease. The frequencies of TNFα −308A and −238A in patients with CAD undergoing CABG did not differ significantly from those of in controls in our present study either.

Investigation of the distributions of the different combinations of alleles showed some deviation from the calculated values, which was not totally unexpected, since all alleles are in the MHC within a stretch of a few hundred kilobases and linkage disequilibrium is one of the characteristic features of the MHC. However, it must be noted that the C4A genotypes were obtained by protein analysis, and it is possible that the quantitative null alleles were caused by multiple nucleotide changes resulting in the pooling of several different alleles as a single null allele. Therefore, the finding that there is linkage disequilibrium in CAD patients between TNFα −308A and C4AQ*0 does not necessarily mean that the two variants are in one haplotype, but together they are over-represented in these patients, suggesting that there might be a connection between the coincidence of the simultaneous occurrence of these variants and the development of CAD. Haplotype analysis would be needed to clarify whether there is functional importance that the alleles are in *cis* or *trans* positions.

At present it is not possible to explain the higher simultaneous occurrence of these two allelic combinations in patients with severe CAD, since apart from the higher occurrence of preoperative MI in patients with TNFα −308A + C4A*Q0 alleles, there were no clinical or laboratory parameters that differed in patients with or without these haplotypes. However, it can be hypothesised that since each allele was found to be involved in immunological disturbances, which play important roles in CAD, the simultaneous occurrence of the alleles increases the susceptibility to the development of the disease. It is also possible that genes linked with these alleles are also involved, since there are other candidate genes in the close vicinity,[8 30] including lymphotoxin α and β (related to TNFα), heat shock protein 70 (putative role in autoimmune inflammation), allograft inflammatory factor (allograft rejection), leucocyte specific transcript-1 (involved in macrophage activation), and several other genes with uncertain or unknown functions.

The TNFα −308A and C4A*Q0 alleles separately and together occurred at higher frequency in CAD patients with preoperative MI in their case history than in patients without MI. It is well known that the TNFα and the complements are involved in local inflammatory reactions in myocardial infarction.[10 31] Several studies reported raised TNFα levels in patients after MI, expressed by cardiac myocytes and macrophages migrated into the myocardium.[31] Complement activation is a key event mediating the deleterious effects of the local inflammatory response occurring in the infarcted myocardium. A partial C4 deficiency, which may include defective handling of immune complexes, can also be an additional risk factor in MI. In addition, inflammation is known to increase the probability of rupture of the vulnerable plaques.[32] Therefore it is reasonable to assume that the inflammation associated TNFα −308A and C4A*Q0 alleles may facilitate plaque rupture in MI. Besides, the connection between these alleles and high relative risk of CAD and MI correlates well with the recent findings that the imbalance of inflammatory processes (CRP, IL6, and IL1β) increases the risk of future cardiovascular disease significantly.[33]

Our study shows the importance of investigation of allelic association in the search for disease susceptibility genes. Several studies have investigated the TNFα polymorphisms in diseases in which dysregulation of TNFα production might have played a role and several of them found no association between TNFα −308A and −238A alleles and the suspected diseases.[5 34] It is possible that these negative results would change drastically if the C4A alleles were also considered.

Retrospective case-control studies may suffer from several biases, which may lead to false positive and false negative results. We have matched our patient and control groups for age, sex, and ethnicity to reduce this possibility. On the other hand, a survival bias cannot be avoided in a disease association study and prospective studies will be necessary to confirm the role of these alleles in CAD. Moreover, in this study only those CAD patients were analysed who were sent for CABG. Because only the most severe CAD patients undergo CABG, this is a selected population; thus it is possible that the conclusions of this study may not be extended to CAD patients in general, but only to the most severe cases. Furthermore, it is also possible that patients referred for CABG with MI in their case histories differ from patients with MI in general. This could be an

Key points

- There is an increased susceptibility to CAD in homozygous carriers of the C4B*Q0 allele.
- Subjects simultaneously carrying the TNFα –308A and C4A*Q0 or the TNFα –238A and the C4A*6 alleles have an increased risk for developing severe CAD.
- Among CAD patients, carriers of the TNFα –308A and the C4A*Q0 alleles have a higher risk of myocardial infarction.

explanation of the differences between the results of this study and those of a recent report by Nityanand *et al*,[35] where no association of C4A*Q0 and MI was found.

Another important concern might be that the gene pool of the cases and the controls differs, which could account for the associations observed. In the quest of alleles contributing to the susceptibility to the disease, several other polymorphisms were investigated in these populations on other chromosomes as well[36–38] (Szalai *et al*, unpublished data). The distributions of the vast majority of these alleles did not differ between cases and controls, for example, ACE D, 53.1%, 54.2%; apoE4, 10.1%, 9.2%; factor V Leiden mutation of the blood coagulation system, 4.8%, 4.1%; PlA2 allele of the platelet glycoprotein IIb/IIIa receptor, 11.3%, 13.5%; F allele of the C3 component of complement, 16.5%, 16.7%; chemokine receptor 5 CCR5Δ32, 10.5%, 11.7%; CCR264I, 12.3%, 11.3%; stromal derived factor 1-3'A, 19.1%, 20.4%; RANTES-28G, 4.2%, 3.3%; RANTES-403A, 20.8%, 17.8%; methylenetetrahydrofolate reductase 677T, 34.8%, 37.2%; apo(a) $(TTTTA)_n$ repeat polymorphism, mean n= 8.6 (SD 1.1) *v* 8.5 (SD 0.8), in cases and controls, respectively. Altogether, 32 polymorphisms were investigated and only two differences have been found between the two populations (MCP-1 –2518[36] and C4B*Q0 (this report)), both of which could contribute to the susceptibility to CAD. The careful selection of our patients and these results ensure that the differences in the gene pool between cases and controls are as minimal as possible.

In summary, according to this study, homozygous carriers of the C4B*Q0 allele have an increased risk of developing severe CAD. The simultaneous occurrences of the TNFα –308A + C4A*Q0 and the TNFα –238A + C4A*6 alleles are higher in CAD patients undergoing CABG than in healthy controls. Among CAD patients, carriers of the TNFα –308A + C4A*Q0 allelic combination have a higher risk of myocardial infarction.

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CTLA-4/CD28 gene region is associated with genetic susceptibility to coeliac disease in UK families

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Coeliac disease (CD) is a malabsorption disorder characterised by a small intestinal enteropathy that reverts to normal on removal of dietary gluten. Susceptibility to disease has a strong genetic component. Ninety percent of patients in northern Europe have the HLA class II alleles DQA1*0501 and DQB1*0201, which encode the cell surface molecule HLA-DQ2.[1] However, haplotype sharing probabilities across the HLA region in affected sib pairs suggest that genes within the MHC complex contribute no more than 40% of the sib familial risk of CD, making the non-HLA linked gene (or genes) the stronger determinant.[2]

Attempts have been made to identify these loci using genome wide linkage studies. Zhong *et al*[3] performed an autosomal screen in 45 affected sib pairs from the west coast of Ireland, using 328 microsatellite markers. They found evidence of linkage with lod scores of greater than 2.0 in five areas: 6p23 (separate from HLA), 7q31.3, 11p11, 15q26, and 22cen. A larger genome wide search involving 110 affected Italian sib pairs using 281 markers found no evidence of linkage in these five areas.[4] It did, however, propose a novel susceptibility locus at 5qter, important in both symptomatic and silent CD, and another at 11qter, which appeared to differentiate the two forms. In UK families an initial genome wide search,[5] followed by a study of 17 candidate regions[6] identified five areas with lod scores of greater than 2.0: 6p12, 11p11, 17q12, 18q23, and 22q13. Of these, 11p11 replicates one of the loci identified by Zhong *et al*[3] and it is likely that this area contains an important non-HLA susceptibility locus. However, in general the results of these studies are disappointingly inconsistent.

A number of candidate genes have been investigated in linkage and association studies. Of these, the only region with repeatedly positive results is the locus on chromosome 2q33 containing the cytotoxic T lymphocyte associated (*CTLA-4*) gene and the *CD28* gene. CD28 and CTLA-4 molecules are expressed by T lymphocytes and interact with their ligands B7-1 (CD80) and B7-2 (CD86) during antigenic stimulation of T cells via the T cell receptor. CD28 provides a co-stimulatory signal to T cell activation, while CTLA-4 provides a negative signal and thus is thought to be an important regulator of autoimmunity.[7] *CTLA-4* was investigated in a French study of coeliac patients versus controls and showed allelic association of disease with the A allele of the position +49 A/G dimorphism (+49*A/G).[8] The association has recently been replicated in a study of Swedish families,[9] which also showed some evidence of linkage and association with neighbouring microsatellite markers. A study of Finnish families also showed linkage and association in this region[10]; however, this was maximal at the marker locus D2S116, and association was not detected at +49*A/G. A study of *CTLA-4/CD28* in Italian and Tunisian families,[11] however, showed no evidence of linkage or association.

The *CTLA-4/CD28* gene region has shown linkage and/or association with a number of chronic inflammatory disorders, including type 1 diabetes.[12 13] These studies show linkage and association of *CTLA-4* polymorphisms with type 1 diabetes in Italian, Spanish, and French populations, but not in UK, Sardinian, or Chinese data sets.[13] We have studied this region in our own sample of UK coeliac families using the transmission/disequilibrium test (TDT). In contrast to conventional case-control studies, the TDT is not liable to produce false positive results owing to unrecognised population stratifications.

MATERIALS AND METHODS

Family sample

Affected subjects with both parents available for genotyping were selected from our established collection of multiply affected pedigrees.[6] In pedigrees where more than one subject fulfilled this criterion, only one was selected on a random basis. This was to ensure that the study would test for association rather than just linkage. Additional trios of affected subjects with their parents were recruited with the help of an article written for the UK Coeliac Society newsletter. A total of 166 families were studied; however, in 24 of these families only one parent was available for genotyping. All affected subjects were diagnosed according to the revised ESPGAN criteria.[14]

The study was the approved by the St Thomas's Hospital Ethics Committee and all subjects provided informed consent.

Microsatellite and SNP genotyping

Six finely spaced microsatellite markers and two single nucleotide polymorphisms (SNPs), within and surrounding the *CTLA-4* gene were genotyped (fig 1). Markers were chosen in

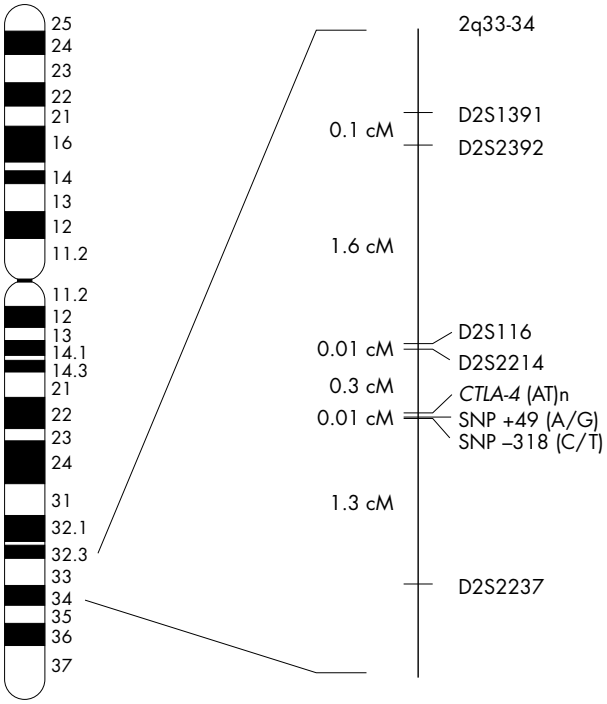

Figure 1 Map of chromosome 2q33-2q34 showing the position of microsatellite markers and SNPs used in the study.

Table 1 Results of allele-wise TDT analysis using ETDT

Marker	χ^2 df	p value
D2S1391	13.8, 6	0.032
D2S2392	14.4, 16	NS
D2S116	3.7, 10	NS
D2S2214	19.4, 7	0.007
CTLA-4	12.6, 15	NS
D2S2237	6.7, 10	NS
SNP+49	0.19, 1	NS
SNP-318	0.02, 1	NS

order to allow a direct comparison with previous studies, and at a density sufficient to realistically detect any locus across the region using association methods. Microsatellites were genotyped in all 166 families, but SNPs were genotyped only in the 142 families in which both parents were available for genotyping. Microsatellite markers were selected from the Genethon and CHLC maps: D2S116, D2S2392, D2S2214, D2S2237, D2S1391, and CTLA4(AT) (fig 1). Fluorescently labelled polymerase chain reaction (PCR) products were analysed using the ABI377XL Genetic Analyser (Applied Biosystems). Products were sized using the Genescan version 3.1 program, and scored using the Genotyper version 2.5 program (Applied Biosystems, Foster City, CA USA; http:www.appliedbiosystems.com).

The two known dimorphisms within the *CTLA-4* gene, +49*A/G and –318*C/T, were genotyped using the PCR-restriction fragment length polymorphism (PCR-RFLP) method. PCR reactions for +49*A/G were performed using forward and reverse primers previously described by Marron *et al*.[13] The 152 bp product was cleaved for 16 hours at 60°C using 10 units of *Bst*EII per reaction. The digested A allele yields a fragment of 130 bp and the G allele yields an intact 152 bp fragment. The T allele of –318*C/T also creates a restriction site,[15] allowing a similar technique to be used for genotyping. Primers were therefore designed around this site: forward primer 5′-TGGACTGGATGGTTAAGGATG-3′ and reverse primer 5′-AGAAGGCACTTGAATAGAAAGC-3′. The 275 bp PCR product was cleaved for 16 hours at 37°C using 4 U *Mse*I per reaction. The C allele produces a 262 bp fragment, whereas the T allele produces a 169 bp and a 95 bp fragment. All fragments were separated on a 2% agarose gel and visualised with ethidium bromide staining under UV fluorescence. Microsatellite and SNP data were checked for genotyping errors using the PEDCHECK program.[16]

Data analysis

Data for biallelic markers were analysed using the transmission disequilibrium test (TDT).[17] In order to avoid biases, only subjects with data available from both parents were included in the analysis. For multiallelic markers such biases do not arise, as long as the affected subject is heterozygous for the marker,[18] so all 166 families were analysed. Multiallelic data were analysed using the ETDT program,[19] which carries out a logistic regression analysis to determine whether different marker alleles vary in terms of their probability of being transmitted from a heterozygous parent to an affected offspring. When this overall allele-wise test was significantly positive for a marker, we examined the transmission counts for individual alleles, to see which one(s) made the major contribution to the observed effect.

RESULTS

The results of the allele-wise TDT analysis as performed by the ETDT program are shown in table 1. D2S2214 provides evidence for unequal transmission of different alleles to affected offspring (χ^2 =19.4, 7 df, p=0.007). Examining the individual allele transmissions (table 2), it seems that most of this effect is the result of preferential transmission of allele *278, which is transmitted from 92 heterozygous parents and not transmitted from 49 (χ^2=13.1, 1 df, p=0.0003). A lesser contribution (χ^2=9.333, 1 df, p=0.002) to the effect is made by allele *276. The only other somewhat positive result is with D2S1391 (χ^2 =13.8, 6 df, p=0.03), but given that eight markers were tested this result could easily have occurred by chance. For both *CTLA-4* single nucleotide polymorphisms, alleles were transmitted approximately equally, providing no evidence at all for the direct involvement of these polymorphisms in the susceptibility to CD.

Table 2 Transmission of individual alleles of D2S2214 to affected offspring from heterozygous parents

Allele	*274	*276	*278	*280	*282	*284	*286	*288
Passed	25	28	92	4	2	1	15	9
Not passed	33	56	49	7	6	2	18	5
Chi-squared (1 df)	1.103	9.333	13.113	0.818			0.273	1.143
p value	NS	0.002	0.0003	NS			NS	NS

DISCUSSION

This study of UK families provides further evidence that the *CTLA-4/CD28* gene region on chromosome 2q33 contains an important non-HLA susceptibility locus for coeliac disease. This evidence comes from a positive TDT result with the D2S2214 microsatellite (p=0.007), indicating that allelic association with the disease is present with this locus. The use of the TDT method rather than a case-control sample means that this positive result is not the result of unrecognised population stratifications. Although association has not previously been shown with this marker, it has been shown with two nearby markers, D2S116 and +49*A/G. D2S116 lies 0.01 cM centromeric of D2S2214 and showed association significant at p=0.0001 in one previous study.[10] The +49*A/G SNP lies 0.3 cM telomeric of D2S2214 and has been found to be associated with CD in two previous studies with significance p=0.0001[8] and p=0.007.[9] We have found no evidence for association of +49*A/G or D2S116 in the current study. The –318*C/T SNP lies within the *CTLA-4* gene promoter region and is thus a candidate to be an aetiological polymorphism, but it did not show any evidence of association in the current study. The third known polymorphism of the *CTLA-4* gene is the *CTLA-4* (AT) microsatellite positioned in the 3′ untranslated region of exon 3. Theoretically, this variation could affect gene expression by affecting mRNA stability; however, in keeping with two previous studies,[9 10] we failed to detect any evidence for association.

The finding that different polymorphisms within the same region are positive in different samples is consistent with the hypothesis that none of them influences susceptibility to CD directly but that there is another, as yet untested, susceptibility locus within the region. Different patterns of linkage disequilibrium between markers in different populations, along with chance variations, would then account for the different results obtained. Association of the *CTLA-4/CD28* region with genetic susceptibility to coeliac disease has now been reported in UK, French, Finnish, and Scandinavian populations. No association has yet been found in Italian, Tunisian, or Dutch[20] populations. These differences might result from chance factors, from different frequencies of the susceptibility locus among cases in different populations, or because of differences in the pattern of linkage disequilibrium across the region. It may be of some interest that the pattern observed across Europe seems different from that found in the type 1 diabetes studies, in particular in relation to UK families, in that association is found for coeliac disease but not for diabetes.

We have used a TDT design which provides a robust test for association and which may be more powerful at detecting loci in complex diseases than linkage analysis,[21 22] at least when attention can be restricted to a small region. Association based studies can also provide a more precise localisation than linkage studies since they are based on the presence of linkage disequilibrium, which in outbred human populations only exists between markers within a few hundred kb of DNA sequence (probably <300 kb).[23–25] *CTLA-4* and *CD28* are both plausible candidate genes and are separated by only 25-150 kb. However, a polymorphism within these genes that predisposes to CD has not yet been identified. Disease predisposition may lie in undiscovered polymorphisms of *CTLA-4* or *CD28*, or alternatively within another gene in very close proximity. A database search of the region using Genemap 99 (http://www.ncbi.nlm.nih.gov/genemap99/) and Ensembl (http://www.ensembl.org/) did not show any genes with known immunological or gut related functions. Future work will therefore involve detailed mapping of the region with identification of further SNPs within *CTLA-4/CD28* and in the surrounding region in order to identify the aetiological polymorphism that confers susceptibility to coeliac disease.

Key points

- Genetic susceptibility to coeliac disease is not entirely explained by known HLA associations.
- Association of coeliac disease with the *CTLA-4/CD28* gene region on chromosome 2q33 has been reported in some European populations but not in others.
- This study of UK families did not show significant association with any of the three known polymorphisms of the *CTLA-4* gene.
- The study did, however, show significant association with D2S2214 (p=0.007), a microsatellite marker 0.3 cM centromeric of the *CTLA-4* gene.

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A common ancestral haplotype in carrier chromosomes from different ethnic backgrounds in vacuolating megalencephalic leucoencephalopathy with subcortical cysts

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Vacuolating megalencephalic leucoencephalopathy with subcortical cysts (VL) is a newly described, inherited leucodystrophy (MIM 604004). Clinically, the disease is characterised by accelerated head growth beginning in the first year of life and resulting in extreme macrocephaly and mild delays in gross motor milestones. In most cases, these early manifestations are followed by evolution of pyramidal symptoms and signs, cerebellar ataxia, epilepsy, and in older patients dystonia and athetosis.[1] Cognitive function is relatively spared in most patients. Brain imaging with computed tomography (CT) or magnetic resonance imaging (MRI) shows diffuse cerebral white matter swelling with progressive cystic-like changes, prominent in the frontotemporal regions, with preservation of grey matter structures.[1 2] Pathological specimens from VL patients showed splitting of the myelin sheaths between the lamellae consistent with an oedematous process, with sparing of the exons.[3] Although progressive in nature, VL is characterised by a relatively mild clinical course compared to the severity of the neuroradiological findings.[4 5] About 70 cases of the disease have been described in different ethnic groups. The molecular basis of this disorder remains unknown. The inheritance is autosomal recessive and the disease gene was recently mapped to a 3 cM interval between D22S1161 and the telomere of chromosome 22q.[6] Linkage was established in a group of 13 Turkish families all originating from rural areas of central and south eastern Anatolia. No shared alleles or shared haplotypes were detected between the Turkish families.

METHODS

Six of the seven families included in this study (fig 1) have been described in detail by Ben Zeev *et al*.[7] Families 1, 2, 4, and 6 are of Libyan Jewish origin and family 3 is of Turkish Jewish origin. Family 7 is of non-Jewish Indian origin and family 5 is of mixed ancestry. The father is of Libyan Jewish origin and the mother is Ashkenazi. The parents in families 2, 3, and 4 are first cousins, while the parents in the other families are unrelated. The study was approved by the Helsinki Committee at the Sheba Medical Centre and participants gave informed consent. Computed screening of the full chromosome 22 sequence, telomeric to D22S922, showed two new CA repeats located in clones WI14811 and STS28616. These polymorphic repeats were amplified with the primers 5′-GGAGAAT CACTTAAACTCAG-3′ and 5′-TTCAGCAGTTTTTCTGTCCC-3′, 5′-TGGAAGAAAGAAATCTCAAA-3′ and 5′-TGAACTCAAGGT TTGCTAAG-3′, respectively. The markers N66C4 and *ARSA*[6] were amplified with primers 5′-TGTACATCCTTACTGCTCG-3′ and 5′-ACGGCAGTGGGGAAACACAA-3′, 5′-CCGGCCAAA AATGACTTTTA-3′ and 5′-CTGGAAAGAGCAAGACCCTG-3′, respectively. Amplification was carried out as described elsewhere.[8] Lod scores were calculated with the LINKAGE (version 5.1) package of programs, assuming recessive inheritance and a disease allele frequency of 0.004. Haplotypes were