COELIAC DISEASE

MHC class I chain related gene A (MICA) modulates the development of coeliac disease in patients with the high risk heterodimer DQA1*0501/DQB1*0201

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Background and aims: Coeliac disease (CD) is an enteropathic disorder characterised by a strong association with major histocompatibility complex (MHC) heterodimer HLA-DQ2. It has been suggested that other HLA class I genes in combination with DQ may also contribute to CD susceptibility. The aim of this study was to investigate whether other candidate genes modify the risk of developing different clinical forms of CD.

Patients and methods: We studied 133 Spanish coeliac patients, divided according to their clinical presentation into typical and atypical groups, and 116 healthy controls. All were typed by polymerase chain reaction-sequence specific primers (PCR-SSP) at HLA-B, DRB1, DQA1, and DQB1 loci and for exon 5 of the MHC class I chain related gene A (MICA).

Results: No differences were found in the frequency of the DQA1*0501/DQB1*0201 heterodimer in either group. The risk of typical CD was significantly associated with the DR7/DQ2 haplotype (p_c=0.02, odds ratio (OR)=3.4, ethiological fraction (EF)=0.4). Extended haplotype (EH) 8.1 (B8/DR3/DQ2) was found to be overrepresented in the atypical form compared with the typical form (p_c=0.001, OR=4.19, EF=0.56). The trinucleotide repeat polymorphism MICA-A5.1 was found to be increased in the atypical group of patients compared with the typical group (p_c=0.00006, OR=8.63, EF=0.81). This association was independent of linkage disequilibrium with EH8.1 as this was also found to be increased in EH8.1 negative atypical patients compared with the typical group (p_c=0.004, OR=6.66, EF=0.56).

Conclusions: Our results showed that the risk of developing typical forms of CD was associated with DR7/DQ2 haplotype, and the presence of B8/DR3/DQ2 was significantly increased in atypical patients. In these, the MICA-A5.1 allele confers an additive effect to the DR3/DQ2 haplotype that may modulate the development of CD.

C oeliac disease (CD) is an enteropathy induced by wheat gliadins and related prolamins of other cereals in genetically susceptible individuals.[1] In these patients, gluten induces an immune response in the small bowel mucosa which causes villous atrophy and crypt hyperplasia. Recent serological screening studies performed in the general population of different countries have clearly shown that the real prevalence of CD is higher than classical reports.[2–5]

Pathological and clinical presentation of gluten sensitivity is variable.[6] From a clinical point of view, coeliac patients may present a clear digestive symptomatology and the so-called "*typical form*" of the disease. However, in some cases CD may be subclinical or oligosymptomatic and in other cases it can be completely asymptomatic.[7] These latter types of clinical presentation may be grouped together as "*atypical forms*" of CD and are more common among subjects included in several risk groups for CD, such as those represented by autoimmune diseases and first degree relatives of coeliac patients.[8] Better knowledge of the extradigestive manifestations of the disease together with the performance of screening in risk groups have largely increased the number of patients diagnosed with CD in recent years.[9]

There is a strong association between CD and the major histocompatibility complex (MHC). Previous studies have shown that CD is associated with HLA class I molecule B8. This association has been found to be secondary to the stronger association to the DR3 bearing haplotype. In Caucasians, DR3/DQ2 haplotypes carry the allele encoding B8 in linkage disequilibrium (extended haplotype (EH) 8.1). It has

been established that CD is strongly associated with the DQ2 heterodimer (DQA1*0501/DQB1*0201) that is encoded by more than 90% of coeliac patients.[10] The DQA1*0501 and DQB1*0201 genes are carried in the *cis* position in the DR3 bearing haplotype and in the *trans* position in the DR5/DR7 genotype. It has been suggested that the DR5/DR7 genotype confers a higher risk for developing the disease than the DR3/X genotype.[11 12] These haplotypes are different outside the HLA class II region. Another gene(s) in addition to class II haplotypes encoding DQA1*0501/DQB1*0201 may also contribute to the genetic predisposition to CD and can explain the observed associations.[11–13] Additionally, other genes, in different chromosomes, may play a role in the development of the disease.[14]

The pathogenesis of CD is relatively well known but some important questions remain unanswered. The role of intraepithelial TCRγδ T cells, notably increased in coeliac patients, is one of these unanswered questions.[11] Recently, Groh and colleagues proved that a group of these T cells, expressing diverse Vδγδ T cell receptors (TCR), recognise MHC class I chain related molecules MICA and MICB,[15] two non-classic HLA

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Table 1 Distribution of the HLA-DQ2 heterodimer in patients with typical and atypical coeliac diseas (CD) and healthy controls

	Controls (n=116)	Atypical CD (n=54)	Typical CD (n=79)
DQA1*0501/DQB1*0201	25 (21.55%)	47 (87%)*	68 (86%)†

*$p_c<10^{-6}$, odds ratio=24.44, 95% confidence interval (9.87–59.73), ethiological fraction=0.83.
†$p_c<10^{-6}$, odds ratio=22.50, 95% confidence interval (10.48–47.94), ethiological fraction=0.82.

proteins mainly expressed by enterocytes under stressful conditions.[16] MICA is a polymorphic gene located 47 kb centromeric to HLA-B.[17] The high degree of linkage disequilibrium between MICA and HLA-B, and the particular tissue expression pattern of this molecule, are important in considering MICA as an additional gene in the development of CD.[11][13] In fact, recent studies show a clear relation between MICA TM polymorphism and type I diabetes,[18] autoimmune Addison's disease,[19] and psoriatic arthritis.[20]

The aim of this study was to examine whether MICA confers additional susceptibility to the classical DR/DQ haplotypes in patients with typical and atypical CD.

METHODS
Subjects
A total of 133 coeliac patients were included in the study (females 82, males 51; mean age 23.7 (18.2) years). All were ambulatory patients, recruited consecutively over a two year period (from January 1998 to December 1999) from two Spanish hospitals. These patients were followed up yearly at their respective adult and paediatric gastroenterology outpatient clinics and represented 100% of the total number of ambulatory coeliac patients followed up during this period. The ethics committees of our hospitals approved the protocol and all patients gave writen informed consent before enrolling in the study.

Diagnosis of CD was made according to the revised European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) criteria.[21] All patients presented villous atrophy of variable degrees, with crypt hyperplasia of the small bowel mucosa, prior to commencement on a gluten free diet. Mean time of the gluten suppression period was 5.8 (4.6) years.

Coeliac patients were classified into typical and atypical, according to their clinical manifestations.[2][6–8] Seventy nine of the 133 patients (59.4%) had a clear digestive symptomatology, consisting of diarrhoea, flatulence, weight loss, and fatigue, and these patients were included in the typical form. Fifty four patients (40.6%) had extraintestinal, atypical, single predominant symptoms, or were symptomless, and were classified as atypical forms. The initial reason for consultation of these patients was anaemia (17), herpetiformis dermatitis (14), familial studies of coeliac patients (12), iron deficiency alone (6), and hypertransaminasaemia of unknown origin (5).

Mean age of the typical group was younger (18.2 (16.2) years) compared with the atypical group (31.6 (18.2) years) (p<0.001). Sex distribution in the typical group was 43 females and 34 males compared with 39 females and 17 males in the atypical group, with a greater proportion of females in the atypical group (NS). Mean age at the onset of symptoms was lower in the typical group (8.9 (13.9) years), compared with the atypical group (25.1 (18.1) years) (p<0.001). Age at diagnosis in the typical group was lower (11.5 (16.1) years) compared with the atypical group (29.4 (19.4) years) (p<0.001). Mean time on a gluten free diet was longer in the typical group (7.6 (6.7) years) than in the atypical group (3.2 (2.7) years) (p<0.001). Body mass index (BMI) was lower in the typical group (19.8 (3.7)) compared with the atypical group (22.4 (7.9)) (p<0.01).

No significant differences were found between the two groups of patients for concentration of haemoglobin, iron levels, serum ferritin, serum folic acid, aspartate aminotransferase and alanine aminotransferase, serum IgA, or positivity of IgA antigliadin or antiendomysium antibodies, or in the degree of villous atrophy found in duodenal biopsies.

A total of 116 random healthy blood donors from the Spanish population, not related to the patients and with no history of intolerance to gluten or its derivatives, iron deficiency, anaemia, or any abnormality in biochemical studies and who were negative for antigliadin and antiendomysium antibodies, were included in the study for genetic comparison (50 males and 67 females; mean age 35.3 (12.4) years (range 18–62)).

Laboratory methods
HLA typing
Analysis for typing class I antigens was performed using standard methods, and DNA polymerase chain reaction (PCR) amplification with sequence specific primers (SSP) was used to genotype the HLA-B allele[22] described as in linkage disequilibrium with DR3 and DR7. HLA-DRB1, -DQA1, and -DQB1 class II alleles were typed by PCR/SSP[23][24] and by sequence specific oligonucleotide probes (PCR/SSOP; INNO-LiPA, Innogenetics NV Ghent, Belgium).

MICA-TM allele typing
For analysis of microsatellite repeat polymorphism in the MICA gene, PCR was carried out by the same procedure as described by Ota and colleagues[25] except for the use of the primers flanking the TM region: sense MICA 5'-ACATTCCATGTTTCTGCTGTTG (MICA located 33 bp 3'- of exon 5) and the antisense primer 5'-TCACCTGG ACCCTCTGCAG (MICA exon/intron 5 boundary region).[20] The antisense primer was marked with Cy5'-amidite. Allele designation was based on the number of repeat units present in the PCR products and was detected using an automatic sequencer ALFexpress II (Amersham Pharmacia Biotech). Four distinct alleles consisting of CGT repetitions were designated as A4 (104 bp), A5 (107 bp), A6 (110 bp), and A9 (119). One additional A5 (A5.1) with one nucleotide insertion (G) was also detected (108 bp).

Statistics
Descriptive analyses were used to characterise the study population. The Fisher exact test was used to compare dichotomous variables, and an unpaired t test was used to compare differences in the means of continuous variables.

Allelic and haplotypic frequencies were calculated by direct counting and the significance of the association was determined using the χ^2 test (with Yates' correction). Haplotypic distribution was derived from molecular typing of HLA-B, HLA-DR, and the MICA gene. EHs were deduced according to the previously reported linkage disequilibrium.[26] The odds ratio (OR) was calculated by the cross product ratio. Exact confidence intervals (CI) of 95% were obtained. The p values were corrected (p_c) by multiplying by the number of comparisons: five for MICA TM alleles, 15 for MICA genotypes, 26 for HLA-DR/DQ genotypes, 16 for HLA-DQA/DQB haplotypes, and 26 for HLA-DQA/DQB/DRB haplotypes. A p_c value <0.05 was considered significant. The potential

Table 2 Frequency of the diverse HLA haplotypes in patients with coeliac disease (CD) and in healthy controls

	Healthy controls (n=116)	Typical CD (n=79)	Atypical CD (n=54)	Typical versus atypical		
				p_c	OR (95% CI)	EF
DR7/DQB1*0201	28 (24.13%)	41 (51.89%)*	13 (24%)	0.02	3.4 (1.58–7.29)	0.40
B8/DR3/DQB1*0201	12 (10.34%)	32 (40,5%)†	40 (74%)‡	0.001	4.19 (1.97–8.84)	0.56

OR, odds ratio; 95% CI, 95% confidence interval; EF, ethiological fraction.
*p_c=0.0026, OR=3.38, 95% CI (1.84–6.11), EF=0.36 (typical CD v healthy controls).
†p_c=0.00003, OR=5.78, 95% CI (2.74–12.06), EF=0.33 (typical CD v healthy controls).
‡p_c<10^{-6}, OR=24.28, 95% CI (10.27–56.26), EF=0.71 (atypical CD v healthy controls).

Table 3 Major histocompatibility complex class I chain related gene A (MICA) TM alleles in Spanish coeliac disease (CD) patients and controls

Allele genotype	Atypical (n=54)	Typical (n=79)	Controls (n=116)	Atypical versus typical		
				p_c	OR (95% CI)	EF
5.1	49 (90.74%)*	42 (53%)	51 (44%)	0.00006	8.63 (3.11–23.94)	0.81
4	9 (16.66%)	19 (24%)	30 (25.86%)	—	—	—
5	7 (13%)	20 (25%)	33 (28.44%)	—	—	—
6	21 (39%)	42 (53%)	50 (43.1%)	—	—	—
9	6 (11.11%)	13 (16%)	35 (30.17%)	—	—	—

OR, odds ratio; 95% CI, 95% confidence interval; EF, ethiological fraction.
*p_c<10^{-6}, OR=12.5, 95% CI (4.66–33.11), EF=0.84 (atypical v healthy controls).

impact for each marker was estimated by the ethiological fraction (EF) which indicates the proportion of disease cases among the total population that are attributable to one allele when OR >1.

RESULTS

Distribution of DRB1/DQA1/DQB1 haplotypes in typical and atypical forms of coeliac disease

The DQA1*0501/DQB1*0201 heterodimer was carried by 86% of patients with the typical form and 87% with the atypical form of CD (table 1). No differences were found between the groups for distribution of the HLA-DR genotypes (data not show). However, the distribution of haplotypes carrying the heterodimer was different between the two groups (table 2). EH8.1 (B8/DR3/DQB1*0201) encodes the DQA1*0501/DQB1*0201 heterodimer in the *cis* position. A strong association of EH8.1 was found in atypical patients compared with healthy controls (p_c<10^{-6}, OR=24.28, EF=0.71). This haplotype was also significantly increased in typical patients (p_c=0.00003, OR=5.78, EF=0.33). However, when we compared the frequencies of EH8.1 in both groups of patients, we found that this haplotype was significantly increased in atypical forms compared with typical CD (p_c=0.001, OR=4.19, EF=0.56). The incidence of the DR5/DR7 genotype carrying DQA1*0501/DQB1*0201 in the *trans* position was greater in the typical than in the atypical form of CD (17% v 1.85%; NS). The DR7/DQB1*0201 haplotype (encoding a β chain of the heterodimer) was found in 51.8% of typical patients (p_c=0.0026, OR=3.38, EF=0.36) whereas it was carried by only 24% of the atypical group (p_c=0.02, OR=3.4, EF=0.4, atypical v typical).

MICA-A5.1 allele is associated with atypical coeliac disease

We also investigated whether the differences observed with EH8.1 could be secondary to HLA class I genes, particularly at the MICA locus which has been suggested to be a candidate in the development of CD. Analysis of microsatellite polymorphism in the TM region of MICA showed a strong association between atypical forms and MICA-A5.1 (table 3). This allele was found in 91% of atypical patients and was carried by 44% of controls (p_c<10^{-6}, OR=12.5, EF=0.84) and 53% of typical

patients (this latter finding was not significantly different from controls). Furthermore, significant differences were found in the distribution of the MICA-A5.1 allele among atypical and typical coeliac patients (p_c=0.00006, OR=8.63, EF=0.81). Analysis of the MICA genotypes showed that A5.1/5.1 was present in 29% of atypical and in 13% of typical patients whereas it was carried by only 7% of controls (data not shown). Differences between atypical patients and controls for the A5.1/5.1 genotype reached statistical significance (p_c=0.002, OR=5.68, EF=0.23).

Increase in MICA-A5.1 in atypical coeliac disease is independent of B/DR/DQ linkage disequilibrium

Because MICA-A5.1 is associated with the EH8.1 haplotype, stratification was necessary to estimate the contribution of this allele independently of this linkage to susceptibility to the atypical CD form. All subjects, both patients and controls, who carried EH8.1 also carried the MICA-5.1 allele thus confirming the strong disequilibrium described between HLA-B and MICA. We investigated the MICA-A5.1 allele in a group of patients positive and negative for EH8.1 to establish whether MICA-A5.1, which predisposes to atypical CD, is due to linkage disequilibrium with EH8.1 (table 4). We examined 47 typical and 17 atypical patients not carrying the EH8.1 haplotype. The MICA-A5.1 allele was significantly increased in the atypical form (64.28% v 21.27%; p=0.004, OR=6.66,

Table 4 Distribution of major histocompatibility complex class I chain related gene A (MICA)-A5.1 allele in extended haplotype (EH) 8.1 positive and negative coeliac disease (CD) patients

	n	MICA-A5.1
EH 8.1 positive		
Typical	32	32 (100%)
Atypical	40	40 (100%)
EH 8.1 negative		
Typical	47	10 (21.2%)
Atypical	14	9 (64.3%)*

*p=0.004, odds ratio=6.66, 95% confidence interval (1.82–24.36), ethiological fraction=0.56.

EF=0.56). This confirms that the increased frequency of A5.1 in atypical forms is independent of EH8.1 association.

DISCUSSION

It is well known that the clinical presentation of gluten sensitive enteropathy is highly variable and this variability can be attributed to diverse factors including genetic and environmental influences. The aim of this study was to investigate the involvement of the MHC region in the development of typical and atypical forms of CD.

The abovementioned typical form is often encountered in infants between 9 and 18 months of age. However, when CD appears in childhood or in adults, other digestive or extradigestive symptoms are more common. These patients may be symptomless and diagnosed only because of a systematic search through family studies or other risk groups. All of these late and frequently mild clinical forms of the disease must be included in the so-called "atypical" forms of the disease. In our study, the characteristics of both coeliac patient groups were in agreement with those previously described. Patients with a typical clinical presentation were younger, had a longer mean follow up time, and were on a gluten free diet for longer than those patients in the atypical group. We observed a more pronounced female predominance and a higher body mass index among atypical patients.

Our analysis of HLA confirms the association between DQA1*0501/DQB1*0201 and CD, a finding consistent with previously data.[10] [12] No differences were found between groups for the distribution of the HLA-DR genotypes, as has been reported previously in other studies.[27] However, differences were found when we compared DR/DQ haplotype distribution. DR7/DQB1*0201 was increased in typical patients and EH8.1 was strongly associated with atypical forms. Previous studies have suggested that these haplotypes confer an increased risk of developing CD.[12] Our results suggest that in addition to DQA1*0501/DQB1*0201, another possible gene present in EH8.1 and/or DR7/DQB1*0201 haplotypes may modulate the development of CD. It has been suggested that an additional susceptibility gene(s) telomeric to class II contributes to CD susceptibility.[11] MICA is located 47 kb centromeric to HLA-B, is expressed on intestinal cells, and can be tested as a candidate gene in CD susceptibility. It has been reported that expression of this molecule is likely to be stress induced by promoter elements similar to heat shock protein (HSP70).[17] Our study showed that MICA-A5.1 transmembrane polymorphism was associated with a risk of atypical CD. This association could be the result of linkage disequilibrium with EH8.1. However, CD atypical patients carrying MICA-A5.1, but lacking EH8.1, were also significantly overrepresented compared with the group of typical patients. Thus the association found between MICA-A5.1 and atypical CD cannot be attributed exclusively to linkage disequilibrium with EH8.1.

Atypical CD is frequently associated with other autoimmune and endocrine diseases such as dermatitis herpetiformis, type 1 diabetes mellitus, and autoimmune Addison's disease. It has recently been shown that an unidentified gene in the class I region modifies the risk of developing both type I diabetes and CD.[28] It is notable that in APS-II Addison's disease and type I diabetes, a combination of MICA polymorphism and at risk class II haplotypes were associated with the highest genetic risks.[18] [19] Furthermore, it has been described that autoimmunity to transglutaminase is prevalent among patients with type 1 diabetes expressing DQ2/DQ2 homozygous genotypes.[29] This result suggests that this prevalence probably corresponds with the predominance of DR3,3/DQ2,2 genotypes found in the atypical form. In addition, a significant number of the relatives of patients with type 1 diabetes have the silent form of CD associated with DRB1*03/DQA1*0501/DQB1*0201.[30] Considering all of these observations, we hypothesise that the genetic factors determining type 1 diabetes and oligosymptomatic CD are closely linked with MICA-A5.1 in linkage disequilibrium with DR3/DQ2, and that this combination may play a role in the pathogenesis of both diseases.

In accordance with previous descriptions, the almost exclusive expression of MICA in gastrointestinal epithelium suggests that it may function as a ligand recognised by a subset of Tδγδ cells in the intestinal intraepithelial lymphocyte compartment.[15] Several studies have provided direct support for a functional role of the NKG2D receptor for MICA recognition on NK, Tγδ, and recently, CD8+ T cells.[31] The pattern of MICA tissue expression and the distribution of these cells correlate with the stage of mucosal transformation. In fact, intraepithelial recruitment of CD8+ and Tγδ cells is a characteristic feature of CD[11] which could participate in the destruction of the mucosa. This could be related to induced expression of MICA on intestinal epithelial cells that may be triggered by cellular stress mechanisms, infection, or inflammation. Recently, histological studies have demonstrated high expression of MICA molecule in biopsies of patients with active CD (S Caillat-Zucman, personal communication). The MICA-A5.1 allele carries a nucleotide insertion resulting in a premature stop codon which may encode a secreted form of the MICA molecule.[32] The MICA-A5.1 soluble form may protect against the development of typical CD by blocking or inhibiting enterocyte recognition and destruction by Tδγδ or CD8+ activated cells. This may be particularly important in patients DR3/DQ2 homozygous carrying the MICA-A5.1/5.1 genotype. In addition to DQ2 heterodimers, the presence of the MICA-A5.1 allele could explain the finding that DR3/DQ2 was found to be associated with the atypical form, and that DR5/DR7 was found to be prevalent in typical CD. These results provide a basis for further functional investigation of the roles that induced expression of MICA-A5.1 could play in the development of CD.

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