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Additional Information

Title Page

Genetic analyses of celiac disease in a Spanish population confirm association with CELIAC3 but not with CELIAC4

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Short title: Association studies in a celiac Spanish population

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Abstract

Genetic predisposition to celiac disease (CD) is determined primarily by the *HLA* genes (CELIAC1 region; 6p21), although many loci are involved in disease susceptibility. First we have analysed a large series of CD patients from the Spanish Mediterranean region who had previously been characterised for the HLA complex. We have investigated how relevant regions contribute to CD susceptibility: CELIAC3 (CD28/CTLA4/ICOS region on 2q33) and CELIAC4 (19p13) as well as the TNF-α and the LT-α loci by case-control and association analyses. We highlight the association with the +49*A allele of CTLA4 locus (p-value= 0.01), and the -308*A of TNF-α locus (p-value=0.0008) in DQ2 individuals, although an independent role for TNF-α as risk factor has not been proven. Moreover, we do not confirm the association with the CELIAC4 region polymorphisms described in other populations.

Keywords: Celiac disease; Spanish population; association analysis; case-control study; transmission disequilibrium test.

Celiac Disease (CD; MIM 212750) is an enteropathy of immunologic nature accompanied by small bowel mucosal atrophy due to a permanent intolerance to ingested wheat gluten or related proteins from wheat, rye and barley in genetically predisposed individuals (1). The association between CD and the *HLA* (Human Leukocyte Antigens) genes of the major histocompatibility complex on chromosome 6p21 (CELIAC1 region) is indisputable, although other non-HLA loci must also play a role in CD. Among them, the CELIAC3 region on chromosome 2q33 is highly remarkable. This contains the *CD28*, *CTLA4* (Cytotoxic T-Lymphocyte-associated Antigen 4) and *ICOS* (Inducible CO-Stimulator) genes (2, 3) that are regulators of T-cell activity. Two other chromosomal regions may be named: 5q31-33 and 19p13. The 5q31-33 region, also known as CELIAC2, contains genes encoding molecules involved in T-cell activation, which unleash the immune response and cellular differentiation, as well as the production of interleukines and other pro-inflammatory molecules (4). Finally, some notable genes lie within the 19p13 region, namely CELIAC4, such as the myosin IXB (*MYO9B*) gene that encodes an unconventional myosin involved in intracellular movements, in remodelling actin cytoeskeleton, besides being able to act as a GTPase activating protein on Rho (5).

Our study, carried out in a series of families and unrelated cases of CD from Eastern Spain (Mediterranean basin), aims to investigate the main genetic regions associated with CD (CELIAC1, CELIAC3 and CELIAC4), as well as *TNF-α* (Tumor Necrosis Factor α) and *LT-α* (Linfotoxin or *TNF-β*), which have been considered promising genes. We have not performed genetic analyses for the CELIAC2 region because to date studies focussing on the search for genes associated with CD have been unsuccessful (4 , 6, 7) and therefore, the initial expectations triggered by the CELIAC2 region have lost strength.

Our series comprised 168 paediatric patients: 13 familial cases and 155 isolated cases. Patients were diagnosed according to the ESPGHAN criteria (8). To begin, small bowel biopsy was performed in all cases, and a Marsh III lesion was required to establish the diagnosis, overall, to check diagnosis withdrawal and rechallenge with gluten, according to the ESPGHAN criteria, mainly in patients under 2 years old. These patients, together with their first degree relatives, were supervised at the Gastroenterology, the Paediatric Nutrition Section and/or the Digestive Medicine Unit of several hospitals in the Valencian region: the University Hospital La Fe and the University Hospital General both in Valencia, Hospital Lluís Alcanyís in Xàtiva and Hospital Francisco de Borja in Gandia. Patient's parents were aware of the investigative nature of the study and signed consent was obtained in all cases. As a control population, a total of 378 healthy individuals were included. Of this group, 186 were random bone marrow and organ donors from the Transfusion Centre of Valencia and the remaining 64 healthy trios (father, mother and child) were recruited in the University Hospital La Fe (Valencia).

First of all, we performed a power analysis for both association and case-control approaches, using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc). Disease prevalence was assumed to be 0.02 and D', disequilibrium linkage, 0.99. Considering (i) that the marker frequency was 0.4 and (ii) that the relative risk for disease allele heterozygote was 2 and for homozygote 4 (9), when the frequency of the disease allele was 0.5, 0.25 and 0.1, the power in the association analysis was estimated to be 76, 68 and 20%, respectively, and in the case-control study was found to be 94, 89 and 31% respectively.

All patients and their first degree relatives had previously been characterised for the HLA complex (CELIAC1). One hundred and thirteen (67.2%) out of 168 patients had the DRB1*03_DQB1*0201_DQA1*0501 haplotype (DQ2.5 haplotype): 14

individuals were homozygous and 99 were heterozygous. Among these heterozygous patients, 44 were DR3/DR7. In the remaining 55 heterozygous affected subjects, the DR3 haplotype was identified in other genotypes (DR3/DRX). The more relevant combinations were: DR3/DR11 (15 individuals), DR3/DR1 (8 individuals), DR3/DR5 (11 individuals) and DR3/DR13 (7 individuals). Giving consideration to the 55 non DR3 patients, 42 (25%) had the DR7/DR11 genotype and 6 individuals (3.57%) had the DR4-haplotype (expressing the DQ8 heterodimer); only one of them in homozygosis. Finally, 7 individuals (4.16%) expressed neither the DQ2 nor the DQ8 heterodimer. So, we confirmed that most of our patients bear the highest risk alleles: 92.26% of our cases are able to express the DQ2 molecule and 3.57% are DQ8.

Several studies have indicated that the HLA region could harbour peculiar genetic factors affecting disease susceptibility besides the *DQ* genes, such as TNF-α and LT-α loci that are pro-inflammatory molecules with a broad range of immunomodulatory activities. We have analyzed two polymorphisms, -308*A/G TNF-α and *Nco*I*A/G LT-α, that have previously been reported to be related to CD predisposition (10, 11). Neither marker showed deviation from Hardy-Weinberg equilibrium in the healthy group (data not shown). The case-control study pointed towards a possible involvement of both polymorphisms in CD (Table 1 and 2). Since the *TNF* genes are in linkage disequilibrium with the HLA region, we decided to investigate whether the DRB1*03_DQB1*0201_DQA1*0501 (DQ2.5) haplotype took part in the association with both TNF-α and LT-α loci. We performed an S-TDT (Sib Transmission Disequilibrium Test) analysis (12) for both -308*A/G TNF-α and *Nco*I*A/G LT-α polymorphisms in three familial groups: first of all we considered the population as a whole and secondly, we divided it into two groups based on the presence or absence of DQ2.5 haplotype population. Results showed that the association of disease with -308*A TNF-α was significant (Z'= 2.135, p-value= 0.01)

when we considered the whole population (63 families) without stratification. This association was clearly increased if we only took into account the 36 DQ2.5 positive families (Z'= 0.487, p-value= 0.0008), and it disappeared in the 27 DQ2.5 negative families (Z'= 0.762, n.s.). Our data lead us to believe that the possible contribution of the TNF-α locus to CD susceptibility is closely related to DQ2.5 haplotype. The -308*A TNF-α polymorphism is part of an ancestral haplotype named AH 8.1 [HLA –A*01, B*08, -308*A TNF-α, DRB1*03, DQB1*02] and the conservation of this AH 8.1 haplotype seems to be more evident in CD chromosomes (13). However, this observation is controversial. Van Belzen et al (14) analysed 18 loci located in the HLA region in unrelated DQ2 positive celiac patients and no evidence for association was found between CD and any of the studied loci, including -308*A TNF-α. By contrast, in other studies, genes for CD susceptibility located in the HLA region like *MICA* and *MICB* genes (MHC class I region) have been associated with CD (15, 16), and even the MICA-A5.1 allele has been demonstrated to be independently associated with CD, since association was also significant in DQ2 and DQ8 negative patients (17). Thus, collaborative studies are necessary to confirm these previous findings because we find once again that researches do not always yield convergent results.

Involvement of the CD28/CTLA4/ICOS region (CELIAC3) in CD has been broadly studied. Much is known regarding its immunological function: engagement of CD28 on naïve T cells by CD80/CD86 (B7) ligands on antigen presenting cells provides a potent co-stimulatory signal to T cells activated through their T-cell receptor; engagement of ICOS on T cells by ICOS ligand (B7-H2) also provides a positive proliferative and cytokine secretion signal, although cell surface ICOS is expressed on activated rather than naïve T cells suggesting a later regulatory function; cell surface CTLA4 is also up regulated on T-cell activation, has a higher affinity for its CD80/86 ligands than CD28, and provides a negative signal to regulate T-cell activation. Hence all three genes

control different aspects of the T-cell response, and their close genetic proximity probably enables integrated control of expression. Among these three genes, *CTLA4* is the most likely one to be associated (3). We have studied four polymorphisms on CTLA4 locus, -1147*C/T, +49*A/G, CT60*A/G and CT61*A/G, and for all of them, allele frequencies were in Hardy-Weinberg equilibrium in the control group (data not shown). Case-control analyses showed a uniform distribution for both allele and genotype frequencies (Table 1 and 2). However, when we performed an S-TDT analysis with 70 informative families, no significant deviation of the transmission alleles was observed, except for the +49*A allele that was statistically associated with CD susceptibility (Z'= 2.28, p-value= 0.01). Similar results (χ^2= 6.37, p-value= 0.016) were obtained when a TDT analysis was performed using the Haploview v3.2 software (18). Association with the +49*A allele has been found in some populations (19, 20) whereas results have been negative in others (21). The contradictory results could be indirect evidence that the studied polymorphisms are not the aetiological mutation. In fact, in other autoimmune diseases, the *CTLA4* gene has proved to be associated, but with the +49*G allele (20).

To study the putative association between +49*A allele and CD in depth, we performed haplotype analyses using the Haploview v3.2 software (18) in 84 families (informative and non-informative). On the basis of 1147*C/T:+49*A/G:CT60*A/G:CT61*A/G region, three haplotypes were notable (frequency >5%): CAAG (45.5%), CGGG (25.6%) and TAGA (16.3%). However, none of them were associated with CD. Amundsen et al (22) described that the haplotype TAGA displays an increased risk for CD, whereas other authors have concluded that on the basis of +49*A/G:CT60*A/G, CD is associated with the haplotype GG (21) or with the haplotype AA (3). Taking into account that making direct comparisons are complicated because study designs are not identical, on the whole the most

representative haplotypes in our population have also been associated with CD in others. We have also evaluated the linkage disequilibrium (LD) between each pair of analyzed polymorphisms. For this, we estimated the disequilibrium value D' by means of two approaches Haploview v3.2 and PyPopWin32-0.6.0 softwares (23), and in all cases the obtained value was near 1, except for the assessment made between the -1147*C/T and CT60*A/G markers, which were slightly lower (D'= 0.59 with Haploview and 0.81 with PyPop). In fact, three separate blocks of LD, each including one of the genes *CD28*, *CTL4* and *ICOS*, in the CELIA3 region has been reported (24, 25).

To analyse the CELIAC3 region in our series further, we decided to analyse a tag SNP within the *ICOS* gene, the c.602*A/C polymorphism. This marker is not in linkage disequilibrium with the CTLA4 polymorphisms and in this way, we could generate an extended haplotype that provide us more information of the CELIAC3 region. For the c.602*A/C marker, allele frequencies were in Hardy-Weinberg equilibrium in the control group (data not shown). Table 1 and 2 show the results of case-control analyses for both allele and genotype frequencies. A significant p-value was obtained for the distribution of the genotype frequencies (Table 2). By contrast, TDT and S-TDT analyses for the c.602*A/C SNP performed with 66 informative families did not yield association between this SNP and CD: χ^2= 0.72, p-value=0.39 for the TDT analysis, and Z'= 0.139, p-value= 0.44 for the S-TDT analysis. On the basis of 1147*C/T:+49*A/G:CT60*A/G:CT61*A/G:c.602*A/C region, the most representative haplotype was CAAGA (29.3%), followed by CAAGC (15.7%), CGGGA (14.4%), TAGAA (13.0%), CGGGC (11.5%) and TAGAC (8.8%). These more frequent haplotypes are those observed for the CTLA4 markers with the addition of the c.602 alleles, A or C. The remaining haplotypes constructed presented a frequency <5%. In any case, association analyses with CD were negative for all of them. In other populations, the c.602*A/C SNP has been associated with CD together with other three ICOS' SNPs and

specifically, the c.602*C allele forms part of a haplotype that show transmission preference to affected children (9). We have not found any evidence that emphasizes any of the c.602 alleles in our CD series. The so diverging results in the different studies make difficult to achieve definitive conclusions and highlight the need of functional analyses to characterise the true disease-causing variation.

The CELIAC4 region has been found to be a convincing region for CD susceptibility in some studies: an association between CD and the SNP rs2305764*A, located on *MYO9B* gene, have been reported (5). A 2.3-times higher risk factor for CD has been attributed to individuals who are homozygous for the variant rs2305764*A. In the CELIAC4 region we studied the STR marker *D19S899* and two SNPs, rs2305764 and rs2305767. Allele frequencies were in Hardy-Weinberg equilibrium in the control group except for rs2305764 (data not shown), so we decided to exclude this SNP. *D19S899* was studied in 328 and 197 chromosomes from celiac and healthy individuals, respectively. We found eight allelic variants that ranged from $(CA)_{15}$ to $(CA)_{25}$. The most common alleles were the $(GT)_{18}$ allele and the $(GT)_{19}$ allele with frequencies of respectively 33.23% and 27.43% in the celiac population an of 32.48% and 25.38% in the control group, respectively. Neither case-control study nor S-TDT approach revealed any significant p-value. With respect to rs2305767, allelic and genotype analyses gave similar allelic frequencies among patients and controls, with no statistical differences (Table 1 and 2). Moreover, the S-TDT analysis revealed no significant deviation of the transmission pattern for rs2305767 in 54 informative families (Z'= 1.155; p-value= 0.125). In the last months, several reports have concluded that contribution of MYO9B to the genetic predisposition to CD in distinct populations could be brought into question (26-29). Bearing in mind the aforementioned results in different populations and in spite of the particular aspects of study design that could affect non-replication of results, there seems to be more

evidence indicating that genetic variation in MYO9B does not have a major effect on CD susceptibility. Nevertheless, other genes located on the CELIAC4 region cannot be ruled out (7).

In summary, this is the first time that the most promising CD loci have been analyzed in a series of patients from Eastern Spain (Mediterranean area). We have concluded that both the CELIAC1 and CELIAC3 regions, besides the TNF-α locus, confer susceptibility for CD. By contrast, we have not confirmed the association with CELIAC4 region.

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Allele frequencies and case-control studies

		CD (%)	*Controls (%)*	*P-value (χ^2)**	*OR (95%CI)*
*-308*A/G TNF-α and NcoI*A/G LT-α polymorphisms*					
-308*A/G TNF-α	G	224 (77.77)	442 (86.32)	0.0026 (9.06)	0.55 (0.38-0.80)
	A	64 (22.22)	70 (13.67)		1.80 (1.24-2.62)
*Nco*I*A/G LT-α	G	115 (37.82)	155 (26.54)	0.0006 (11.51)	1.68 (1.25-2.26)
	A	189 (62.17)	429 (73.45)		0.59 (0.44-0.79)
CELIAC3 region (CTLA4 and ICOS polymorphisms)					
-1147	C	259 (79.93)	171 (79.16)	0.91 (0.011)	0.95 (0.62-1.46)
	T	65 (20.06)	45 (20.83)		1.05 (0.69-1.61)
+49	A	253 (75.29)	275 (73.92)	0.73 (0.11)	1.08 (0.77-1.52)
	G	83 (24.70)	97 (26.07)		0.93 (0.66-1.31)
CT60	A	172 (50.88)	131 (55.50)	0.25 (1.32)	0.81 (0.58-1.13)
	G	170 (49.11)	105 (44.49)		1.23 (0.88-1.72)
CT61	A	45 (19.73)	75 (22.18)	0.55 (0.35)	0.86 (0.57-1.30)
	G	183 (80.26)	263 (77.81)		1.16 (0.77-1.76)
c.602	A	155 (59.61)	168 (68.02)	0.070 (3.62)	0.69 (0.48-0.99)
	C	105 (40.38)	80 (32.25)		1.44 (1-2.07)
CELIAC4 region					
rs2305767	T	138 (58.9)	160 (58.8)	0.95 (0.003)	1.01 (0.71-1.44)
	C	96 (41.02)	112 (41.17)		0.99 (0.69-1.41)

*P-values were corrected using the Yates correction.

-308*A/G TNF-α (rs1800629) and *Nco*I*A/G LT-α (rs909253) polymorphisms were typed as in previous studies (30, 31). The -1147*C/T polymorphism was analyzed by SSCP (Single Strand Conformation Polymorphism) using the following primers: forward (F), 5′-ATGAGGCCTGAAAGAGGC-3′, and reverse (R), 5′-AAACAGACAGTTGTAACAGGG-3′. The +49*A/G (F, 5′-GCTCTACTTCCTGAAGACCT-3′, and R, 5′-CCAGCCAAGCCAGATTGGAG-3′) and CT60*A/G (F, 5′-CAGTATCTGGTGGAGTCTCC-3′, and R, 5′-GCAGGCGGTAAGAAAGGGG-3′) polymorphisms were genotyped by PCR-RFLP method using the *Bbv*I and *Mae*II enzymes, respectively, that identify the nucleotide change in both cases. The CT61*A/G dimorphism (F, 5′-TGGGTTAACACAGAC-3′, and R, 5′-AGCAACATAGGACCACAGG-3′) was analyzed using the denaturing high performance liquid chromatography (DHPLC) based on the WAVE technology procedure (32). The c.602*A/C (rs10183087) polymorphism on the *ICOS* gene was studied as reported elsewhere (33). With respect to the CELIAC4 region, primers for *D19S899* were obtained from the public online database (www.gdb.org; (34)) and was analyzed by PAGE (PolyAcrylamide Gel Electrophoresis); rs2305764 (F, 5′- ATGAGAGTCCGAGCAGGC-3′, and R, 5′- GGATGACGACCCATGGGAT-3′) was typed by PCR-RFLP using the *Bcn*I enzyme that recognises the change; and finally, rs2305767 (F, 5′- ATCCTGCTGTGATCTGGG-3′, and R, 5′- AAGGAACACAGTGTCGGG-3′) was typed by DHPLC, checking the homozygous samples by SSCP. Both SNPs, rs2305764 and rs2305767, were selected from Monssur et al (2005) (5).

Table 1

Genotype frequencies and case-control studies

		CD (%)	Controls (%)	P-value (χ^2)
*-308*A/G TNF-α and NcoI*A/G LT-α polymorphisms*				
-308*A/G TNF-α	GG	90 (62.50)	191 (74.60)	0.007 (9.84)
	AG	44 (30.55)	60 (23.43)	
	AA	10 (6.94)	5 (1.95)	
*Nco*I*A/G LT-α	GG	23 (15.13)	27 (9.24)	0.003 (11.64)
	AG	69 (45.39)	101 (34.43)	
	AA	60 (39.47)	164 (56.16)	
CELIAC3 region (CTLA4 and ICOS polymorphisms)				
-1147	CC	103 (63.58)	63 (60.0)	0.73 (0.63)
	CT	53 (32.71)	39 (37.14)	
	TT	6 (3.70)	3 (2.85)	
+49	AA	95 (56.54)	97 (52.15)	0.45 (1.58)
	AG	63 (37.5)	81 (43.54)	
	GG	10 (5.95)	8 (4.30)	
CT60	AA	40 (23.66)	27 (22.88)	0.07 (5.33)
	AG	92 (54.43)	77 (65.25)	
	GG	37 (21.89)	14 (11.86)	
CT61	AA	6 (5.55)	5 (2.95)	0.27 (2.59)
	AG	33 (30.55)	65 (38.46)	
	GG	69 (63.88)	99 (58.57)	
c.602	AA	38 (29.23)	56 (45.16)	0.03 (7.26)
	AC	79 (60.77)	56 (45.16)	
	CC	13 (10)	12 (9.68)	
CELIAC4 region				
rs2305767	CC	15 (12.8)	21 (15.44)	0.69 (0.70)
	CT	66 (56.41)	70 (51.47)	
	TT	36 (30.76)	45 (33.08)	

*P-values were corrected using the Yates correction

Table 2