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Increased Frequency of the Immunoglobulin Enhancer HS1,2 Allele 2 in Coeliac Disease

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Background: Coeliac disease (CD) is characterized by increased immunological responsiveness to ingested gliadin in genetically predisposed individuals. This genetic predisposition is not completely defined. A dysregulation of immunoglobulins (Ig) is present in CD: since antiendomysium antibodies (anti-EMA) are of the IgA class. One polymorphic enhancer within the locus control region (LCR) of the immunoglobulin heavy chain cluster at the 3′ of the C alpha-1 gene was investigated. The correlation of the penetrance of the four different alleles of the HS1,2-A enhancer of the LCR-1 3′ to C alpha-1 in CD patients compared to a control population was analysed. **Methods:** A total of 115 consecutive CD outpatients, on a gluten-free diet, and 248 healthy donors, age- and sex-matched, from the same geographical area were enrolled in the study. HS1,2-A allele frequencies were investigated by nested polymerase chain reaction (PCR). **Results:** The frequency of allele 2 of the enhancer HS1,2-A gene was increased by 30.8% as compared to the control frequency. The frequency of homozygosity for allele 2 was significantly increased in CD patients. Crude odds ratio (OR) showed that those with 2/2 and 2/4 (OR 2.63, $P < 0.001$ and OR 2.01, $P = 0.03$) have a significantly higher risk of developing the disease. In contrast, allele 1/2 may represent a protective genetic factor against CD (OR 0.52, $P = 0.01$). **Conclusions:** These data provide further evidence of a genetic predisposition in CD. Because of the Ig dysregulation in CD, the enhancer HS1,2-A may be involved in the pathogenesis.

Key words: Coeliac disease; genetic predisposition; immunoglobulin dysregulation; immunoglobulin enhancer HS1,2-A; non-HLA factors; polymorphism

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Coeliac disease (CD) is a multifactorial disorder of the small intestine influenced by both environmental and genetic factors and characterized by increased immunological responsiveness to ingested wheat gliadin or similar prolamins from rye, barley (1). Although, the genetic predisposition to CD has been associated with HLA DQ2 (2), it is still not fully understood. Several studies have suggested the involvement of at least one non-HLA gene (3–5), but the nature of this gene has remained elusive so far. The non-HLA DR;DQ factors may be linked to the different clinical manifestations of gluten sensitivity (6).

A dysregulation of Ig is present in CD as suggested by the fact that antiendomysium antibodies (anti-EMA) are of the IgA class although CD patients have a higher incidence of IgA defect (7).

An important role in the regulation of Ig production is ascribed to the locus control regions (LCRs) located within the duplicated cluster of the human Ig heavy chain genes coding for the constant regions (8). Both regions are involved in class switch and consequently in B lymphocytes maturation (9). These duplicated LCRs at the 3′ of both C alpha-1 and C alpha-2 genes, shown in Fig. 1A, contain a copy of the enhancers HS3, HS1,2 and HS4 (10). The enhancer HS1,2 internal to the locus control region of the Ig heavy chain cluster is the most active in transcription studies in vitro. HS1,2 contains specific sites for the binding of at least 4 transcription factors with 4 alleles of different lengths for a DNA sequence duplicated from 1 to 4 times (11, 12).

The recent demonstration that these alleles have a role in the progression of IgA nephropathy, another disease with IgA dysregulation (13), prompted us to evaluate the influence of the Ig polymorphic enhancer HS1,2 in the development of CD.

The alleles of the two duplicated loci have been named

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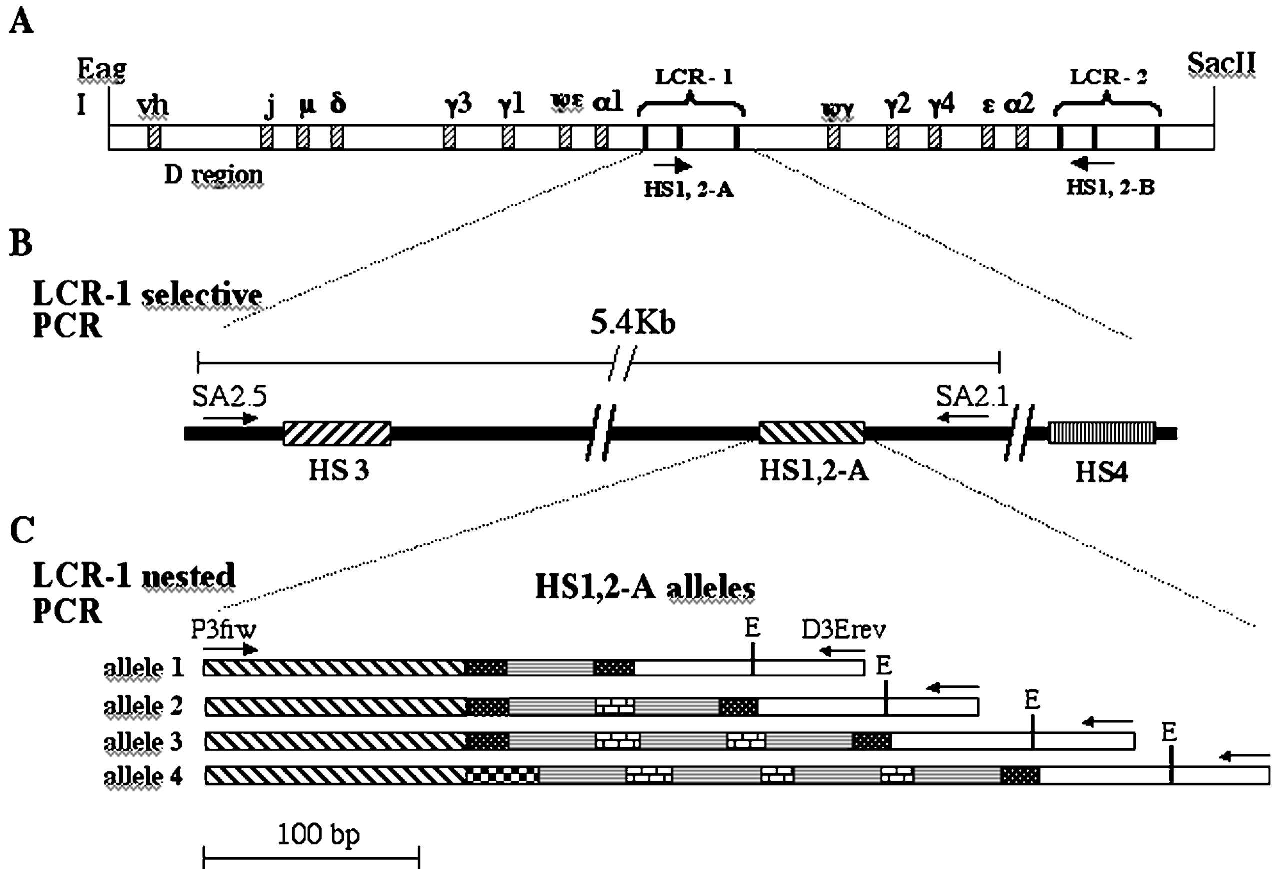

Fig. 1. A. Gene map of the Ig heavy region on chromosome 14 with the LCR-1 and LCR-2 3′ to the constant heavy genes alpha-1 and alpha-2, respectively. The enhancers HS1,2 in the two loci are inverted as shown by the two arrows. B. Enlargement of the LCR-1, the two primers (convergent arrows) SA2.5 and SA2.1 designed for the selective PCR of the alpha-1 region are shown. C. Enlargement of the HS1,2-A (3′ C alpha-1) enhancer, the two primers for the nested PCR amplifications P3frw and D3Erev (convergent arrows) are shown together with the four alleles. In the lower part, the four alleles are depicted with the different spacers (boxed with white dots, bricks or squared for allele 4) which separate the repeat of 38 bp (boxed with horizontal bars). The four alleles of HS1,2-A are reported in GeneBank (acc.n. AJ544218; AJ544219; AJ544220; AJ544221).

allele 1, 2, 3 and 4 and the enhancer mapping 3′ to C alpha-1 HS1,2-A and that mapping 3′ to C alpha-2 HS1,2-B.

In an attempt to find new genetic markers and to improve our understanding of the molecular basis of CD, we evaluated the allelic frequencies of Ig polymorphic enhancer HS1,2 in CD patients as compared to a healthy control population.

Methods

Patients

We enrolled 115 consecutive CD outpatients (29 M, 86 F, mean age 28.7 $\pm$ 16.5), diagnosed by histological analysis performed on bioptic specimens of intestinal mucosa and by the presence of anti-EMA, all of whom were on a gluten-free diet. Routine blood tests were also performed to assess the IgA levels. Furthermore, we collected data on the different clinical manifestations and the associated pathologies: patients were grouped according to Farrell & Kelly (14) (Table I). The control group was represented by 248 Italian healthy blood donors, age- and sex-matched, from the same geographical areas, thus taking into account the possibility of variations in the allelic frequencies (15). Genomic DNA was extracted from total blood according to standard procedures.

Polymerase chain reaction (PCR) assay

To estimate the frequencies of the 4 alleles of HS1,2-A, it was necessary to distinguish among the isoforms of the two separate loci of the LCR-1 and LCR-2 (Fig. 1A). In fact, the two loci have the same structural sequence, although inverted. The four alleles differ for 1–4 copies of a tandem repeat of 72 bp in allele 1, 124 bp in allele 2, 178 bp in allele 3 and 250 bp in allele 4, as shown in Fig. 1C (GeneBank acc.n. AJ544218, AJ544219, AJ544220, AJ544221). The first selective PCR encompassing the HS1,2-A region, shown in Fig. 1B, was performed on genomic DNA (100 ng) from peripheral blood nucleated cells with the primers described previously (8). The cycle conditions were changed to 94 °C 2′ for a first step, then 94 °C 30″, 61 °C 30″, 68 °C 5′ for 10

Table I. Different clinical manifestations of 115 CD patients studied

Gastro-intestinal features Diarrhoea, steatorrhea, recurrent abdominal pain, recurrent aphthous stomatitis	30 (26.1 %)
Extraintestinal features Iron deficiency anaemia, osteopenia, dental-enamel hypoplasia, vitamin K deficiency, hypertransaminasemia, thrombocytosis, arthropathy, polyneuropathy, ataxia, epilepsy, infertility, recurrent abortions, anxiety and depression, alopecia	59 (51.3 %)
Associated conditions Dermatitis herpetiformis, IgA deficiency, type 1 diabetes, autoimmune thyroid disease, vasculitis	26 (22.6)

cycles and 94 °C 30″, 59 °C 30″, 68 °C 5′ for 20 cicles, finally 72 °C 10′. PCRs were carried out in 50 µL reaction volume containing: 5 µL extracted DNA (100 ng), 1.5 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, Calif., USA), 15 pmole of each primer, 1.5 mM $MgSO_4$, 50 µM each of dNTP and 1X buffer High Fidelity (600 mM Tris-SO_4 pH 8.9, 180 mM ($(NH_4)_2SO_4$) (Invitrogen), by using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif., USA).

To prevent carry-over contamination, pre-PCR and post-PCR procedures were performed in separate rooms. PCR preparations were carried out with dedicated equipment in a laminar flow hood, by using aerosol-resistant plugged pipette tips (ART, Molecular Bio-Product, San Diego, Calif., USA); non-disposable devices were sterilized with ultraviolet irradiation after each use. Negative and positive controls, without a DNA template or with a control DNA of a heterozygote, were always included.

The nested second PCR to amplify the polymorphic core of the enhancer HS1,2 (Fig. 1C) was performed with 1/25 of the volume of the first PCR, avoiding the carry-over of the genomic DNA of the first reaction; control reactions were performed with 4 and 10 ng total genomic DNA, giving no visible amplification on gel agarose electrophoresis. The primers for this second PCR were P3frw (GACTCATTC-TGGGCAGACTTG) and D3Erev (GTCCTGGTCCCAAAG-ATGG), the cycle conditions were: first denaturation step 94 °C 5′ then 94 °C 30″, 56 °C 30″, 68 °C 60″, for 30 cycles; after the last cycle 72 °C 2′ (final extension step). This second PCR was carried on with the same volumes of the first PCR reaction and the same concentrations, except for the enzyme that was 1U of Platinum Taq DNA polymerase (Invitrogen). PCR products were analysed on 3.0% agarose gel stained with etydium bromide. Eight gel electrophoretic polymorphic patterns of PCR amplifications observed on agarose gel showed the typical difference of selected genomic alleles of HS1,2-A compared to unselected amplifications of HS1,2-A together with HS1.2-B (Fig. 2).

Statistical analysis

The frequencies of the variables considered are described in percentages. Comparisons between frequencies of categorical variables in different groups were performed by chi-squared test and the Fisher exact test, when required. To quantify the risk of developing CD associated with a specific allele, crude odds ratios (ORs) were calculated.

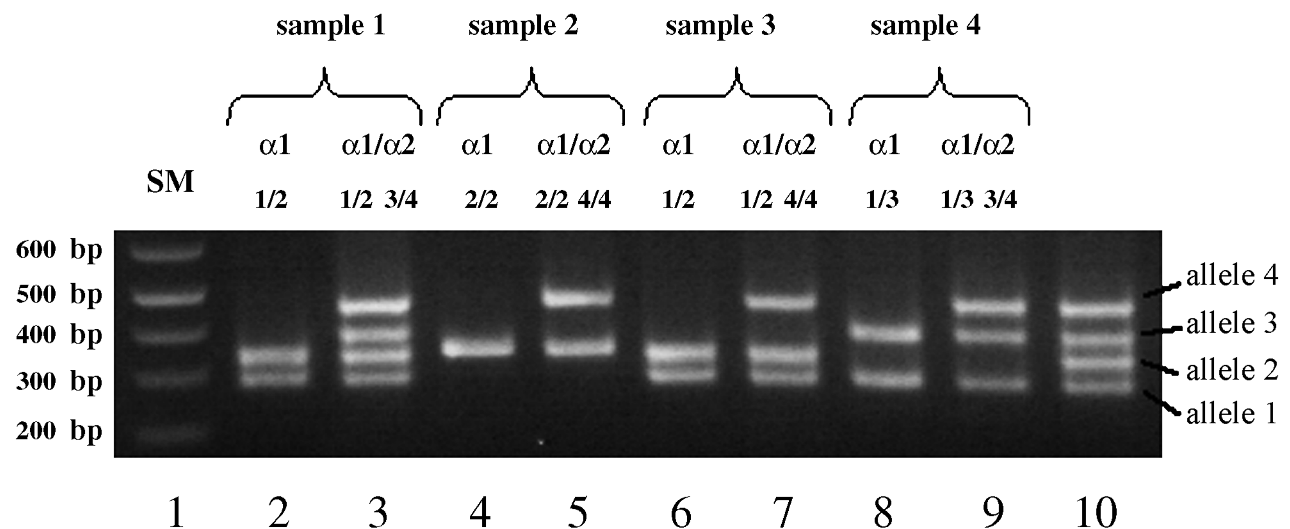

Fig. 2. Gel electrophoresis of 8 independent amplifications from 4 unrelated DNA genomic samples: lanes 3, 5, 7, 9 direct amplification of both HS1,2-A and HS1,2-B enhancers with genomic DNA template; lanes 2, 4, 6, 8 selective amplification of HS1,2-A enhancer with selective preamplified DNA (see text). Lane 1 contains '1Kb-plus' DNA ladder (Invitrogen, Carlsbad, Calif., USA); lane 10 the marker of the standard of the four alleles of HS1,2.

Table IIA. Number of observed genotypes and (expected) of control and CD samples

Genotype	Control (expected)	CD patients (expected)
1/1	52 (47.79)	23 (12.91)
2/2	37 (38.69)	37 (30.74)
3/3	2 (0.89)	0 (0.01)
4/4	4 (2.73)	3 (2.22)
1/2	84 (86.01)	24 (39.83)
1/3	12 (13.06)	2 (0.69)
1/4	18 (22.86)	5 (10.71)
2/3	13 (11.75)	0 (1.07)
2/4	25 (20.57)	21 (16.52)
3/4	1 (3.12)	0 (0.29)
	Tot. 248	Tot. 115

A logistic regression analysis was performed to calculate the adjusted OR for developing CD due to a specific allele, controlling for other covariates (geographical patterns and different alleles). The P value was set at 0.05. Stata 6.0 software (College Station, Texas) was used for statistical analysis.

Results

Genotype frequency distributions and estimates of allele frequencies for samples from both the Italian control population and CD patients are reported in Table II (A and B).

In the control group, the observed genotype distributions were in agreement with the Hardy-Weinberg expectation values, while for the CD sample, the P value of allele 2 frequency increase was highly significant ($P < 0.001$).

In the control and the CD groups, the frequency of allele 1 was 0.439 and 0.335, respectively, for allele 2, 0.395 and 0.517, for allele 3, 0.060 and 0.009, and for allele 4, 0.105 and 0.139, respectively. In summary, the frequency of allele 2 was increased by 30.8% as compared with the control frequency. In particular, in Table III we show the specific differences among the healthy group and the CD patients for the frequencies of the four alleles. In the control group and the CD patients, the frequency of the homozygosis for allele 2 was 15% and 32%, respectively. Crude OR showed that people with 2/2 and 2/4 (OR 2.63, $P < 0.001$ and 2.01, $P = 0.03$, respectively) have a significantly higher risk of developing CD, as shown in Table IV. In contrast, the allele 1/2 condition may represent a relatively protective genetic factor for CD development (OR 0.52; $P = 0.01$).

Table IIB. Frequencies of alleles ± relative standard error. Significance was calculated for Hardy-Weinberg equilibrium

Allele	Controls	CD patients
1	0.439 ± 0.022	0.335 ± 0.031
2	0.395 ± 0.022	0.517 ± 0.033
3	0.060 ± 0.011	0.009 ± 0.006
4	0.105 ± 0.014	0.139 ± 0.023
	Degrees of freedom = 5	Degrees of freedom = 3
	$\chi^2 = 3.54$	$\chi^2 = 19.99$
	$0.750 < P < 0.500$	$P < 0.001$

CD = coeliac disease.

Table III. Differences (delta) of the allele frequencies in coeliac disease (CD) patients subtracted by frequencies of healthy control blood donors

Allele	Delta frequencies
1	−0.104
2	+0.122
3	−0.051
4	+0.034

The recent report (13) of an excess of HS1,2 3′ to Ig alpha-1 2/2 homozygotes in patients with IgA nephropathy suggested analysing the single comparison concerning only the 2/2 genotype into a one-tail test, thus bypassing the Bonferroni correction resulting in a P value <0.001. No significant differences were observed when patients were stratified by age at diagnosis.

The multivariate analysis taking into consideration the OR due to a specific allele controlling for geographical pattern and other alleles showed a significant risk effect of the 2/2 genotype (adj. OR 3.6, 95% CI 1.1–10.6), a borderline risk effect of 2/4 genotype (adj. OR 3.1, 95% CI 0.9–9.8) and no significant effects of the other covariates. In fact, when the patients were grouped according to the presence of gastro-intestinal or extra-intestinal features (Table I), no significant differences between different genotypes were observed ($P = 0.257$). This suggests that the CD genetic marker described here may not be associated with those subgroups of the disease. Further analysis of associated conditions indicated that, when all such patients were considered together, they showed no significant differences between the three groups. However, individual subgroups of these patients were small. For instance, only 5 patients with dermatitis herpetiformis (DH) were included in our study and all had the 2/2 genotype.

Discussion

Our data show that the homozygosity of allele 2 of the HS1,2-A enhancer in CD was increased by 30.8%, as compared to controls.

Genotype 2/2 confers a statistically significant risk of

Table IV. Crude OR of the values of genotypes 1/1, 1/2, 2/2 and 2/4 calculated as reported in materials and methods. CI and P values are reported

Genotype	OR	95 % CI	P
1/1	0.95	0.55–1.64	ns
1/2	0.52	0.32–0.87	= 0.01
2/2	2.63	1.55–4.44	<0.001
2/4	2.01	1.08–3.75	= 0.03

OR = odds ration; CI = confidence interval; ns = not significant.

developing CD while genotype 2/4 has a borderline risk in the multivariate analysis. In the first logistic model the analysis included all the possible combinations of alleles and in the second model all the combinations and the geographical origin. Because of IgA dysregulation in CD, it is relevant that involvement of the enhancer HS1,2-A has been proposed to play a role in Ig class switching (16).

CD has a wide spectrum of clinical manifestations and our data may also prompt studies aimed at correlating the presence of allele 2 homozygosity with a defined clinical picture or with severity of the disease. As mentioned above, the association between genotypes of the HS1,2-A enhancer in IgA nephropathy constitute a risk factor for the prognosis of the disease. In our series of CD patients no significant differences were shown at the multivariate analysis when patients were grouped according to different clinical manifestations. However, further studies on larger samples of patients with associated conditions may detect new significant associations. For instance, five of our patients who also showed DH all presented with allele 2 homozygosity, but this sample size is too small to detect the difference with an adequate statistical power. In this regard, it is interesting that in a recent report it is suggested that the development of DH, a disorder also characterized by IgA dysregulation and the presence of IgA deposits in the skin, is linked to non-HLA factors (6).

The mechanisms leading to IgA dysregulation are not known, although new relevance has recently proposed a role for regulatory models of the NF-kB transcription factor in lymphocyte maturation, proliferation and death (17). In fact, lymphocyte proliferation and Ig production, or hyper-production, could be related to cytokines inducing NF-kB recruitment (18, 19). In our case the polymorphic DNA sequence of the alleles with 1 to 4 repetitions includes the consensus for NF-kB binding. It could be speculated that the different numbers of copies of the consensus where this transcription factor can bind might change the possibility of NF-kB to modulate the function of HS1,2 in the LCR, thus leading to different clinical features of the disease.

In conclusion, our data provide novel evidence of a genetic predisposition in CD and a possible candidate for a non-HLA locus. This marker may also be relevant in determining some associated conditions, such as DH.

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