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Allelic frequencies of the hs1.2 enhancer within the immunoglobulin heavy chain region in Dayton, Ohio patients screened for celiac disease with duodenal biopsy

Jing Liu¹, Rebecca A. Law¹, Paul G. Koles², Jonathan C. Saxe^{3,4}, Michael Bottomley⁵, and Courtney E.W. Sulentic¹

¹Departments of Pharmacology & Toxicology, Wright State University, Dayton, OH

²Departments of Pathology, Wright State University, Dayton, OH

³Departments of Medicine, Wright State University, Dayton, OH

⁴Departments of Dayton Gastroenterology, Dayton, OH

⁵Departments of Boonshoft School of Medicine and the Statistical Consulting Center, Wright State University, Dayton, OH

Abstract

Background—Genetic and environmental factors contribute to the development of celiac disease (CD), but specific genetic predisposing factors remain poorly understood. One candidate is allele 2 of the hs1.2 enhancer within the immunoglobulin heavy chain region. In humans, there are four possible alleles and a previous study of an Italian cohort demonstrated a significantly increased frequency of allele 2 in patients with CD.

Aims—The purpose of the current study was to determine if a similar association between allele 2 and CD exists in an American population from Dayton, OH.

Methods—Subjects were screened for CD via esophagogastroduodenoscopy with duodenal biopsy. All biopsies were microscopically scored using a modified Marsh-Oberhuber classification. DNA was isolated from patients' buccal cells for hs1.2 genotype analysis using PCR.

Results—Unlike the Italian cohort, allele 2 frequency was not significantly different in patients with histopathologic evidence of CD compared to patients without such evidence. However, our patient population as a whole demonstrated a significantly increased allele 2 frequency when compared to that previously reported within diverse ethnic populations.

Conclusions—Since our comparative control patients do not necessarily reflect a healthy control population, an overall increase in allele 2 may reflect an association between allele 2 of the hs1.2 enhancer and a spectrum of gastrointestinal disorders.

Corresponding author: Courtney Sulentic, Ph.D., Phone: 937-775-3583, Fax: 937-775-7221, courtney.sulentic@wright.edu.

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Keywords

Immunoglobulin heavy chain region (*IGH*); 3' *IGH* regulatory region; gene regulation; autoimmune disease; celiac disease; Ig dysregulation

1. Introduction

Celiac disease (CD) is an autoimmune disease that occurs in about 1% of the general population, but this prevalence is highly variable according to geographic area [1]. Symptoms arise after the ingestion of gluten, a component of wheat, barley, and rye (reviewed by [2]). Multiple genetic and environmental factors may influence the development of CD. Through the well-studied connection between CD and HLA-DQ8 and HLA-DQ2.5, it has been established that these HLA alleles are present in almost all individuals with CD (reviewed by [2]). However, approximately 40% of individuals in Western countries possess these HLA molecules, highlighting the multifactorial nature of CD (reviewed by [2]). In familial studies, the contribution of HLA in CD heritability was quantified at less than 40% [3,4]. With the addition of 57 SNPs and 5 variants within the MHC region that are independent of the HLA-DQ alleles, the known genetic variations associated with CD amount to approximately 48% of the heritability of CD [5]. To clarify genetically unexplained heritability, genome-wide association studies have been conducted, identifying several non-MHC candidate genes (e.g. IL-2 and IL-21) that may influence the risk of developing CD [6-8].

Besides those identified via genome-wide association analysis, an additional genetic factor within the immunoglobulin heavy chain (*IGH*) region has been implicated in CD and in a number of autoimmune diseases [9-12]. This potential additional factor is allele 2 of the hs1.2 enhancer within the 3' *IGH* regulatory region (3' *IGHRR*). In animal models and cell culture, this region is crucial for Ig expression and class switch recombination, which impacts antibody concentrations [13-16]. In humans, the hs1.2 enhancer is polymorphic but the polymorphism is not defined by a single nucleotide polymorphism (SNP). Instead, it is defined by an approximately 53 bp sequence called the invariant sequence that can be tandemly repeated up to four times (Fig. 1) [13,17]. The four alleles of the hs1.2 enhancer are named for the number of invariant sequences they have, i.e. alleles 1, 2, 3, and 4 have 1, 2, 3, and 4 invariant sequences, respectively. In European populations, allele 1 and 2 are the most prevalent [18]. As the name implies, each repeat of the invariant sequence has high sequence identity and the invariant sequence contains binding sites for several transcription factors known to be involved in B-cell activation and differentiation into antibody-secreting cells [13,15,17]. Additionally, secretion of tissue transglutaminase antibodies, endomysial antibodies, and deaminated gliadin peptide antibodies is a characteristic of CD [19]. However, the relationship of these antibodies with clinical manifestations of CD is poorly understood. Two existing hypotheses are that the antibodies 1) promote damage of small intestinal mucosa or 2) might simply be an epiphenomenon occurring alongside CD pathology (reviewed by [20,21]).

In a previous study by Frezza et al., the authors reported an increased frequency of allele 2 in patients with CD and an increased risk of developing CD in patients with allele 2 [10]. Their patient population included individuals diagnosed with CD using an intestinal biopsy and the presence of anti-endomysial antibodies. The control population was comprised of Italian blood donors; a negative upper gastrointestinal duodenal biopsy was not a criterion for control individuals [10]. According to the American College of Gastroenterology Clinical Guideline for the diagnosis and management of CD, a small bowel biopsy should be performed when the suspicion of CD is high, regardless of serum antibody results, and is considered critical in patient evaluation [19]. In our study, all subjects had duodenal biopsies to support or exclude a diagnosis of CD.

Overall, our aim was to analyze the association between CD and allele 2 of the *hsl.2* enhancer. To do so, we enrolled 154 patients from two groups: 1) those undergoing esophagogastroduodenoscopy with duodenal biopsy (prospective group); 2) those with a previously established diagnosis of celiac disease confirmed by duodenal biopsy (retrospective group). We analyzed their genotypes for the *hsl.2* enhancer and categorized the patients as CD or non-CD based on microscopic findings. In keeping with the diagnostic guidelines, a patient was classified as having CD when histopathologic changes met criteria for categories 3A, 3B, or 3C in the modified Marsh-Oberhuber classification [19,22]. Our results did not confirm an association between histopathologic changes of mucosal atrophy and the presence of allele 2 of the *hsl.2* enhancer. We also did not find a significantly increased risk of developing CD in patients with allele 2. However, there is strong evidence that the allele 2 frequency of our patient population was significantly different from the allele 2 frequency in supposedly healthy populations from various countries, as previously reported [18]. This was an unexpected observation and may point to an association between allele 2 and gastrointestinal disorders in general.

2. Patients and methods

2.1. Patient data

Patient information in this double-blind study was obtained through review of patients' medical records by JCS. All patients came from the clinical practice of JCS and Dr. Donald Lutter. We recruited 154 patients from two groups: 1) those undergoing esophagogastroduodenoscopy with duodenal biopsy (prospective group); 2) those with a previously established diagnosis of celiac disease confirmed by duodenal biopsy (retrospective group). Written consent was obtained from all patients agreeing to participate in this study, which was reviewed and approved by the Wright State Institutional Review Board (SC#3584). Clinical and diagnostic information collected was limited to chart numbers, date of consent, histopathologic classification of duodenal biopsies, and final CD diagnosis. Although a double-blind study, the subjects were primarily white based on JCS's knowledge of the patients within his clinical practice. In keeping with the diagnostic guidelines, a patient was classified as having CD when histopathologic changes met criteria for categories 3A, 3B, or 3C (i.e. increased intraepithelial lymphocytes, crypt hyperplasia and increasing degrees of villous atrophy) in the modified Marsh-Oberhuber classification [19,22]. A patient was diagnosed as non-CD when their biopsy was classified as 0 (i.e. no

pathological evidence). Based on these criteria, 123 patients were classified as non-CD and 26 patients were classified as CD. Patients with a classification of 1 (i.e. increased intraepithelial lymphocytes) or 2 (i.e. increased intraepithelial lymphocytes and crypt hyperplasia) were not included since they did not meet the classification criteria for CD or non-CD. Only five patients were classified as 1 and no patients were classified as 2. Two pathologists (i.e. PGK and Dr. Lisa Farinella Anacker) independently evaluated the duodenal biopsies for modified Marsh-Oberhuber classification.

2.2. Human buccal cell samples, DNA isolation, and nested PCR analysis

Retrospective samples were collected from Dayton Gastroenterology patients by mailing a patient consent form, a mouth swab, and a stamped return envelope or by meeting at Dayton Gastroenterology and collecting a mouthwash sample. Mouthwash samples were collected from prospective patients prior to esophagogastroduodenoscopy. Informed consent was obtained from all subjects as approved by the Wright State University Institutional Review Board (#SC3584). DNA was extracted from buccal cells using the GenElute Mammalian Genomic DNA Kit following the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Nested PCR was completed as previously outlined [17,23]. Briefly, for the first round of PCR, 1 µg of genomic DNA was used as a template to selectively amplify a 5.4 kb fragment of the 3' *IGHRR*-1 (Fig. 1). For the second PCR round, 10 µl of the product from the first round of PCR served as the template DNA and a 297 to 456 bp product containing the *hs1.2* polymorphic region was amplified (Fig. 1). Primers and PCR conditions were as described previously [23]. The nested PCR products were stained for 40 minutes in SYBR Green I Nucleic Acid and visualized using an 8% polyacrylamide gel and the LAS3000 Fuji imager (Fujifilm Medical Systems USA, Stamford, CT) [23]. Alleles 1, 2, 3, and 4 were associated with the following sizes: 297, 350, 403, and 456 bp, respectively. Six members of the research team independently analyzed all genotyping gels for allele assignments. Additionally, allele assignments were confirmed by sequencing (Retrogen, San Diego, CA) a random sampling of nested PCR products as described previously [23].

2.3. Statistical analysis

Chi-square tests were run on SAS, version 9.4. When at least one cell in a contingency table had an expected cell count of less than 5, Fisher's exact test was used instead. To correct for potentially inflated Type I error from conducting multiple tests, a Bonferroni correction was applied. The significance level yielded was $\alpha = 0.0167$, which ensured that the experiment-wise Type I error rate was at most $\alpha = 0.05$. Using a series of chi-square tests, pairwise comparisons were made between the allele frequencies of the Dayton cohort and each of the eleven countries reported in the Giambra and coworkers study [18]. A sequentially rejective Bonferroni procedure was employed to keep the experiment-wise type I error rate at most $\alpha = 0.05$. Power analysis simulations were performed with R version 3.2.5.

3. Results

3.1. There is insufficient evidence to suggest an association between CD and allele 2

Several statistical analyses were conducted to determine if an association between CD and the presence of allele 2 exists within our patient cohort. The observed and expected *hs1.2*

genotype frequencies for CD and non-CD patients (Table 1) were used for chi-square statistics. Expected values were calculated by dividing the product of the row total and column total by the total number of subjects (Table 1). Patients with positive biopsies, as determined by a modified Marsh-Oberhuber classification of 3A, 3B, or 3C, were considered to have CD (i.e. 26 subjects of which 7 were classified as 3A, 15 as 3B, and 4 as 3C) and only those with a classification of 0 were classified as non-CD (i.e. 123 subjects). We first analyzed the three most common genotypes in this population (i.e. 143 subjects were either homozygous or heterozygous for the *hs1.2* alleles 1 and 2). Genotype analysis of subjects homozygous for allele 1 and the combination of subjects heterozygous for alleles 1 and 2 or homozygous for allele 2 or the analysis of each of these genotypes separately demonstrated a lack of significant associations between these genotypes and CD diagnosis (Table 2). Analysis using Fisher's Exact Test of all the genotypes identified in 149 subjects, including those with less common genotypes, also demonstrated no significant association between a particular *hs1.2* genotype and CD (Table 2). Additionally, no significant association was identified between a particular *hs1.2* genotype and histopathologic classification (Table 3). Furthermore, frequency analysis of the individual *hs1.2* alleles demonstrated no significant association with CD (Table 4) and a lack of association between the three most common *hs1.2* genotypes and CD was confirmed by odds ratio (OR) and 95% confidence interval (CI) analysis. The OR was not significantly greater than or less than 1 for CD patients exhibiting a 1,1 (OR 0.98, 95% CI:0.36-2.69) or 1,2 (OR 1.02, 95% CI: 0.43-2.43) or 2,2 (OR 1.00, 95% CI: 0.40-2.52) genotype. To check if the lack of significance in our study might be due to the sample size, extensive power analysis simulations were run to determine if we could reasonably increase the sample size to detect a significant association between CD and allele 2 within our patient cohort (data not shown). Even increasing the sample size to 10,000 only resulted in an average test power of less than 10%, supporting our conclusion that allele 2 and CD are not associated within our cohort regardless of the sample size.

3.2. The frequency of allele 2 differs significantly between the current population and previous population analyses

Since our results contrast with a previous analysis that associated *hs1.2* allele 2 with CD in an Italian patient cohort [10], using previously reported allele 2 frequencies we determined the observed and expected allele 2 frequency within our patient population compared to supposedly healthy, unrelated donors from Benin, Cameroun, Burkina, Mongolia, Siberia, Iceland, Bulgaria, Turkey, Spain, Italy, and Sicily [18] (Table 5). The frequency of allele 2 in our entire study population (i.e. no exclusion based on Marsh-Oberhuber classification) significantly differed from the combined data that was reported by Giambra and coworkers [18] ($p < 0.0001$). Additionally, separate chi square tests were run to compare the allele 2 frequencies from each of the eleven countries with the allele 2 frequency in our study population. A sequentially rejective Bonferroni procedure was used for this portion of the analysis, which keeps the experiment-wise type I error rate at most $\alpha = 0.05$. Other than Bulgaria, the allele 2 frequency in the Dayton cohort who underwent esophagogastroduodenoscopy was significantly higher than the frequency in healthy individuals from each of the various European, African, and Asian countries as reported by Giambra and coworkers [18] (Table 5).

4. Discussion

The diagnosis of CD can be challenging due to the absence of overt symptoms or the presence of atypical symptoms. Atypical extraintestinal manifestations may include disorders of the liver, blood, skin, endocrine system, musculoskeletal, or nervous system as well as other autoimmune disorders (reviewed by [24]). Current diagnostic limitations are highlighted by the fact that for every patient diagnosed with CD, an estimated five patients remain undiagnosed ([25], reviewed by [2]). Therefore, a better understanding of the causes of CD, specifically the genetic factors, may lead to improved diagnostic measures or treatments. Current understanding of the immune mechanisms involved in CD underscore a role of CD4⁺ T-cell activation in response to gluten peptide presented by HLA-DQ2.5, HLA-DQ2.2, and HLA-DQ8 (reviewed by [26]). These cells produce IFN- γ and IL-12, which can lead to intestinal damage via activation of intraepithelial CD8⁺ cells (reviewed by [26]). Furthermore, these gluten-reactive T cells can contribute to B-cell production of CD-specific antibodies such as anti-transglutaminase, anti-deaminated gliadin peptide, and anti-endomysial antibodies (reviewed by [20]). The role of these antibodies in the intestinal or extraintestinal manifestations of CD is not fully understood. However, they may promote damage of the small bowel by increased epithelial cell proliferation and increased passage of gliadin, but evidence relies heavily on *in vitro* studies (reviewed by [20]). Of note, another hypothesis is that the presence of these antibodies is simply an epiphenomenon, unrelated to the pathophysiology of CD, as supported by the occurrence of CD in a large subset of IgA-deficient individuals ([21] [27], reviewed by [28]). However, the presence of these antibodies, which also include IgG isotypes, in most patients with CD suggests a role larger than epiphenomenal, which is further supported by an association between the polymorphic hs1.2 enhancer and CD, as well as other autoimmune diseases [9-12]. The hs1.2 enhancer is part of a large transcriptional regulatory region (3' *IGHRR*) that is located 3' of the heavy chain constant regions in the *IGH* gene. In animal models and cell culture, this region is crucial for Ig expression and class switch recombination, which impacts overall antibody levels [13-16]. Therefore, the hs1.2 enhancer may influence the expression of CD-associated antibodies and be an additional contributing factor to the currently known genetic risk factors for CD [21].

A previous study by Frezza and coworkers reported a significant association of allele 2 of the hs1.2 enhancer with CD [10]. Their study was conducted with an Italian cohort and CD diagnosis was confirmed via small bowel biopsy and the presence of anti-endomysial (EMA) antibodies. The control group consisted of Italian blood donors that were matched with the CD patients by age, sex, and geographic area of origin [10]. The inclusion criteria for CD positive vs. control groups differ between the Frezza et al. study and the current study. In our study, only a positive biopsy was used for inclusion into the CD patient group and the control group consisted of patients with a negative biopsy. This is more in-line with the diagnostic criteria recommended by the American College of Gastroenterology [19]. Under the American College of Gastroenterology guideline, a small bowel biopsy should be performed, even in the case of negative serology tests, if CD is in the differential diagnosis. Under this guideline, an anti-EMA test is not necessary for the diagnosis of CD and the anti-tissue transglutaminase antibody is preferred to the anti-EMA for single serologic testing

[19]. In our study, biopsies were graded as Type 0, 1, 2, 3A, 3B, or 3C based on combinations of intraepithelial lymphocytes, crypt hyperplasia, and villous atrophy [19,22]. Type 3A, 3B, and 3C reflect increased intraepithelial lymphocytes, crypt hyperplasia and increasing degrees of villous atrophy; these findings are consistent with a diagnosis of CD in the appropriate clinical setting [19,22]. However, we do recognize that CD is not the single cause of villous atrophy; other causes include infection, drug-related enteropathy (especially non-steroidal anti-inflammatory drugs), protein-calorie malnutrition, lymphocytic enterocolitis, microvillous inclusion disease, Crohn disease, immunodeficiency syndromes, and tropical sprue [19,29].

Our analysis comparing the frequency of allele 2 between the CD and control groups differed from the findings of Frezza and coworkers [10]. We did not find an association between allele 2 and CD diagnosis. However, when the pairwise comparisons were made, the allele 2 frequency in our entire patient cohort (i.e. no exclusion based on Marsh-Oberhuber classification) was significantly different from the hs1.2 enhancer allele frequencies reported in a population study of subjects from various European, Asian, and African countries [18]. Specifically, the frequency of allele 2 within our Dayton, Ohio cohort was significantly greater than the frequency reported for each country except Bulgaria. Bulgaria had the highest frequency of allele 2 (0.558 +/- 0.036) in the Giambra et al. study [18], which was comparable to the frequency of allele 2 in our patient population (i.e. 0.5192 +/- 0.0693 for CD and 0.5244 +/- 0.0318 for non-CD, Table 5). Considering that the Dayton cohort was receiving care for gastrointestinal symptoms, these results may indicate an association between allele 2 of the hs1.2 enhancer and gut-related disorders.

Another consideration is the structure of the hs1.2 polymorphism, which is defined by an approximately 53 bp invariant sequence that can be tandemly repeated up to four times resulting in four possible alleles [13,17]. In European populations, allele 1 and 2 are the most prevalent and allele 2 has been implicated in CD and a number of autoimmune diseases [9-12,18]. The invariant sequence contains binding sites for several transcription factors known to be involved in B-cell activation and differentiation into antibody-secreting cells (Fig. 1) [13,15,17]. Furthermore, there is a binding site (i.e. dioxin response element, DRE) for the aryl hydrocarbon receptor (AhR), which can be activated by a variety of structurally diverse ligands, including dietary and microbiota-derived ligands [23,30]. Indeed, it is becoming increasingly acknowledged that the AhR is an important environmental sensor and regulator of mucosal immunity and AhR knockout mouse models have demonstrated an essential role of the AhR in the structural integrity of the gut (reviewed by [31]). AhR ligands have been found to induce either inflammatory or protective responses in the gut and have been implicated in inflammatory disease conditions (reviewed by [31]). Our previous work with human B-cell lines has demonstrated increased activity of the hs1.2 enhancer following AhR activation that increased with an increase in the number of the invariant sequence repeats ([23] and data not shown). Therefore, allele 2 of the hs1.2 enhancer may be more sensitive to modulation by dietary and microbiota-derived AhR ligands, perhaps influencing early stages of inflammatory disease states and interacting with other inflammatory mediators that can be modulated by AhR ligands. This is particularly intriguing in light of the increased frequency of allele 2 in our patient population receiving care for gastrointestinal symptoms.

A limitation of this study is the absence of healthy controls. All biopsy data were taken from patients who had esophagogastroduodenoscopy for gastrointestinal complaints, not from truly healthy individuals. However, this approach was taken based on the diagnostic criteria recommended by the American College of Gastroenterology as described above [19]. Additionally, due to the double-blind nature of this study, we did not match for sex, age, and ethnicity, as was done in the study by Frezza and coworkers [10]. This lack of matching may have impacted our results due to differences in allele frequencies by ethnicity or geographic area. However, our patient population was primarily white and because of being a Midwestern United States population, the ethnicity would likely be fairly heterogeneous. An additional consideration is the small number of patients diagnosed with CD in this study and the potential under-diagnosis of CD by basing diagnosis on a single biopsy [19]. It would be difficult to justify additional biopsies on our patient cohort, but we did conduct an extensive power analysis as discussed above, which supported our conclusion that allele 2 and CD are not associated within our cohort. The significant increase in allele 2 frequency in our patient cohort compared to that previously reported in control subjects [18] is an unexpected result. Although we cannot rule out a potential under-diagnosis of CD based on a single biopsy, this result supports further studies to fully elucidate the frequency in our geographic region of the hs1.2 enhancer alleles and the potential role of allele 2 in CD or other gastrointestinal disorders.

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Figure 1. The human *IGH* locus and polymorphic *hs1.2* alleles

(A) Schematic of the rearranged human *IGH* gene, including the variable (VDJ) and constant regions (μ , δ , γ_{1-4} , ϵ , and $\alpha_{1,2}$) and the known transcriptional regulators, i.e. V_H promoter, intonic enhancer (E_μ), germline promoters upstream of each constant region (open rectangles), and the two 3' *IGHRR*s (3' *IGHRR*-1 and 3' *IGHRR*-2) downstream of each α constant region. In the UCSC Genome Browser Database GRCh38/hg38, the 3' *IGHRR*-1 and 3' *IGHRR*-2 approximately span nucleotides 105,688,954 to 105,704,102 and 105,569,086 to 105,585,123 in Chr14 q32.33, respectively. The *hs1.2* enhancer (underlined) within the 3' *IGHRR*-1 has previously been shown to be polymorphic and allele 2 has been associated with CD and several autoimmune diseases [9-12]. (B) Representative sequence for allele 1 and allele 2 based on random sampling of nested PCR products generated from patient samples (buccal cells). Transcription factor binding sites - Sp1, DRE (AhR binding site), AP-1, NF1, and NF- κ B - are represented by increased font size with italics and/or bold type and agree with previous sequence analysis (EMBL AJ298015 and AJ298016 for allele 1 and allele 2 respectively) [13,17]. The invariant sequence that can be repeated within the *hs1.2* enhancer is underlined. (C) Representative genotyping gel of nested PCR products generated from four patients (P). Patients 1 and 2 are homozygous for allele 2 (350 bp); patients 3 and 4 are heterozygous for alleles 1 (297 bp) and 2 (350 bp). M denotes the 100 bp ladder.

Table 1
Observed and expected hsl.2 genotype frequency of CD and non-CD patients

Genotype	Diagnosis ^a	
	CD Observed (Expected)	Non-CD Observed (Expected)
<i>1,1</i>	6 ^b (6.1 ^c)	29 (28.9)
<i>1,2</i>	11 (8.4)	51 (51.2)
<i>1,3</i>	1 (0.5)	2 (2.5)
<i>1,4</i>	0 (0.2)	1 (0.8)
<i>2,2</i>	8 (8.0)	38 (38.0)
<i>2,3</i>	0 (0.2)	1 (0.8)
<i>2,4</i>	0 (0.2)	1 (0.8)

^a A positive CD diagnosis was indicated by a modified Marsh-Oberhuber classification of 3A, 3B, or 3C. Based on these criteria, 123 patients were classified as non-CD and 26 patients were classified as CD. The frequency is represented as the number of patients

^b observed vs.

^c expected to have a particular genotype.

Table 2
Observed frequency of the hs1.2 genotypes with CD or non-CD diagnosis

Genotype	Diagnosis ^a		Total
	CD	Non-CD	
1,1	6 ^b 17.14 ^c	29 82.86	35
1,2 & 2,2	19 17.59	89 82.41	108
Total	25	118	143
1,1	6 ^b 17.14 ^c	29 82.86	35
1,2	11 17.74	51 82.26	62
1,3	1 33.33	2 66.67	3
1,4	0 0.00	1 100.00	1
2,2	8 17.39	38 82.61	46
2,3	0 0.00	1 100.00	1
2,4	0 0.00	1 100.00	1
Total	26	123	149

^a A positive CD diagnosis was indicated by a modified Marsh-Oberhuber classification of 3A, 3B, or 3C. Observed frequency is represented as the

^b number and

^c percentage of CD vs. non-CD patients with a particular genotype. There was no significant association between genotype and diagnosis for all possible hs1.2 genotypes ($P=0.8903$) or for the most commonly observed hs1.2 genotypes (i.e. alleles 1 and 2) whether analyzed separately ($P=0.9970$) or with the combination of 1,2 and 2,2 ($P=0.9515$).

Table 3
Observed frequency of the hs1.2 genotypes for each Marsh-Oberhuber classification

Genotype	Marsh-Oberhuber Classification ^d				
	0	3A	3B	3C	Total
1,1	29 ^b 82.86 ^c	3 8.57	2 5.71	1 2.86	35
1,2 & 2,2	89 82.40	4 3.70	12 11.11	3 2.78	108
Total	118	7	14	4	143
1,1	29 ^b 82.86 ^c	3 8.57	2 5.71	1 2.86	35
1,2	51 82.26	3 4.84	8 12.90	0 0.00	62
1,3	2 66.67	0 0.00	1 33.33	0 0.00	3
1,4	1 100.00	0 0.00	0 0.00	0 0.00	1
2,2	38 82.61	1 2.17	4 8.70	3 6.52	46
2,3	1 100.00	0 0.00	0 0.00	0 0.00	1
2,4	1 100.00	0 0.00	0 0.00	0 0.00	1
Total	123	7	15	4	149

^a A positive CD diagnosis was indicated by a modified Marsh-Oberhuber classification of 3A, 3B, or 3C. Observed frequency is represented as the

^b number and

^c percentage of each specific Marsh-Oberhuber classification with a particular genotype. There was no significant association between genotype and diagnosis for all possible hs1.2 genotypes ($P=0.5679$) or for the most commonly observed hs1.2 genotypes (i.e. alleles 1 and 2) whether analyzed separately ($P=0.3291$) or with the combination of 1,2 and 2,2 ($P=0.5294$).

Table 4
Observed hs1.2 allele frequency of CD and non-CD patients

Allele	CD ^a	Non-CD	Frequency Difference ^c
1	0.4615 +/- 0.0691 ^b	0.4553 +/- 0.0318	0.0062
2	0.5192 +/- 0.0693	0.5244 +/- 0.0318	-0.0052
3	0.0192 +/- 0.0190	0.0122 +/- 0.0070	0.0070
4	0	0.0081 +/- 0.0057	-0.0081

^a A positive CD diagnosis was indicated by a modified Marsh-Oberhuber classification of 3A, 3B, or 3C. Observed frequency is represented as the

^b mean +/- standard error and the

^c difference in allele frequency of CD vs. non-CD patients.

Table 5
Observed and expected hs1.2 allele 2 frequency of all study subjects compared to the anthropogenic study by Giambra and coworkers [18]

Country	Allele 2		Total	P-value
	Yes Observed (Expected)	No Observed (Expected)		
<i>Dayton, USA^c</i>	160 ^a (97 ^b)	148 (211)	308	N/A
<i>Benin^d</i>	0 (63)	200 (137)	200	< 0.0001
<i>Cameroun</i>	15 (54)	157 (118)	172	< 0.0001
<i>Burkina</i>	10 (63)	192 (139)	202	< 0.0001
<i>Mongolia</i>	50 (60)	140 (130)	190	< 0.0001
<i>Siberia</i>	39 (44)	101 (96)	140	< 0.0001
<i>Iceland</i>	59 (60)	133 (132)	192	< 0.0001
<i>Bulgaria</i>	106 (60)	84 (130)	190	0.4038
<i>Turkey</i>	79 (65)	127 (141)	206	0.0025
<i>Spain</i>	72 (65)	136 (143)	208	0.0001
<i>Italy</i>	123 (93)	173 (203)	296	0.0105
<i>Sicily</i>	73 (63)	127 (137)	200	0.0006
Total	786	1718	2504	

The frequency of allele 2 represented as the

^a observed and

^b expected number. The

^c frequency of allele 2 out of 308 possible hs1.2 alleles in the 154 subjects recruited for this study (Dayton, OH) is significantly higher than the

^d frequencies previously reported by Giambra and coworkers [18], with the exception of Bulgaria. *P*-values pertain to pairwise comparisons of allele 2 frequency in the Dayton, USA population to each of the other countries, respectively.