Abstract

Objective: Celiac disease (CD) is an immune-mediated chronic inflammatory disease associated with HLA-DQ2 and DQ8 molecules. We evaluated the role of HLA in the CD diagnostic algorithm in order to contribute to the development of practical indications for the use of HLA typing.

Material and methods: We selected 317 subjects typed for DR-DQ genes. CD was present in 123 patients, and 89 were included in the study; a control sample of 70 healthy individuals was recruited.

Results: 64% of patients with CD carried DQ2 heterodimer (α5β2), 13.5% carried DQ8 heterodimer without DQ2, 21.4% only showed β2 chain and 1.1% were positive for DQ2 α5 chain. The only presence of α5 chain did not predispose to CD, while DQB1*02 allele resulted more frequent than in other reports, pointing out the intrinsic correlation between β2 chain and CD. In the case-control study we observed a progression of increased risk, ranging from 1:7 for HLA-DQ2 homozygous to 1:85 for DQ8 heterozygous subjects. Overall, 8.6% of first degree family members were affected, exclusively in presence of HLA-DQ2, -DQ8 or DQB1*02, and CD was significantly more frequent among siblings than parents. Finally, considering the different patterns of clinical presentation among the HLA-DQ risk classes identified we found no relationship between CD clinical presentation and HLA-DQ risk categories.

Conclusions: Our results strengthen the evidence that HLA-DQ status strongly influences the development of CD and demonstrate that knowledge of a patient’s HLA-DQ genotype allows to establish clinically relevant genetic risk profiles.

Key words: Celiac disease. MHC class II. HLA heterodimer. Relative risk. Children.

Introduction

Celiac disease (CD) is an immune-mediated chronic inflammatory disease triggered by gluten ingestion that occurs in genetically predisposed individuals. The dietary ingestion of wheat gluten and similar proteins in barley and rye induces a T cell CD4+ immune response resulting in chronic inflammation and villous atrophy of the small intestine (1). There is a very strong genetic contribution to the pathogenesis of CD and most patients (95%) express HLA-DQ2 heterodimer (DQA1*05 DQB1*02), either in cis in DR3 individuals or in trans in DR5/DR7 patients, and the others express HLA-DQ8 (DQA1*03 DQB1*0302). HLA related genes are responsible for 40% of the genetic contribution in CD (2) and about 30% of the general population shows the HLA-DQ2 and/or DQ8 haplotype although only 1% develops CD; this means that HLA-DQ2 and HLA-DQ8 are necessary but not sufficient factors to cause CD. The absence of these alleles is significant for high negative predictive value; in fact, virtually all CD patients carry HLA-DQ2 and/or DQ8 molecules or one chain of the DQ2 heterodimer (3-5). Although CD is one of the most common chronic disease in western countries, most affected individuals go undiagnosed because of atypical symptoms or absence of symptoms (6,7). Serologic screening for the presence of anti-tTG is the first step in detecting new cases but the definitive diagnosis needs small bowel biopsy showing the typical histological abnormalities (8,9). We report our experience with HLA genotyping in a cohort of Italian subjects with the aim of contributing to the development...
of practical indications for the use of the HLA typing. We evaluated with a retrospective study the role of HLA in the CD diagnostic algorithm in individuals afferent to Siena University pediatrics department. Our work consists of three parts: a) dividing patients in different classes on the basis of the arrangement of HLA heterodimers in order to be able to expand HLA utility in establishing clinically relevant genetic risk profiles; b) evaluating the prevalence of HLA-DQ alleles associated with CD and of the disease itself in affected subjects’ first degree relatives; and c) analyzing the possibility of connecting different CD clinical presentation and HLA-DQ haplotypes.

MATERIAL AND METHODS

We selected 317 blood samples from subjects observed at the outpatient clinic for CD diagnosis and follow-up by the Department of Pediatrics of the University of Siena between 1985 and 2009, analyzed to discover HLA-DQ2 or HLA-DQ8 presence predisposing to the disease. All subjects gave informed consent. CD diagnosis was reached in 123 subjects, according to the revised criteria of the European Society for Pediatric Gastroenterology H epatology and Nutrition (ESPGHAN) and North American Society for Pediatric Gastroenterology, H epatology and Nutrition criteria (NASPGHAN), based on the characteristic histological lesions (8,9). Clinical, laboratory and instrumental data were retrieved from the medical files. We made all efforts to obtain missing data from patients lost during the follow-up or from those who were followed in other hospitals, by telephone interviews and contacting their physicians. Thirty-four patients were excluded because the HLA genotyping was performed with a different method, not comparable with the method subsequently adopted. Thus, a population of 89 CD patients (27 males and 62 females) was analyzed for this study, together with a control sample including 70 healthy individuals (19 male and 51 females) randomly selected among those who were negative at the CD serological screening.

Genetic testing

40/317 samples were tested with sero-immunological method and therefore excluded from the study to prevent bias in the outcome of the results. The 277 samples left were typed for DRB1, DQA1 and DQB1 genes at the Laboratory of the Pediatric Department by Sequence-Specific Primer-Polymerase Chain Reaction (SSP-PCR) using commercial Kit (Eurospital, Trieste, Italy).

HLA nomenclature

We adopted the HLA nomenclature introduced by Meziorni et al. (10). Briefly we indicated as DQ2 positive subjects carrying both the alleles DQA1*05 and DQB1*02. A single dose of DQ2 was identified as hetero-DQ2. Individuals DQA1*05 negative/DQB1*02 positive, in which β2 chain forms dimmers with an α chain different from DQA1*05, are named B2 positive. A single or double dose of DQB1*02 is labeled as B1*02/X or B1*02/*02 respectively. Subjects carrying only allele DQA1*05, without DQB1*02 are designated as α5. Individuals with an haplotype DQA1*0301/DQB1*0302 are indicated as D8 positive.

Statistical analysis

Statistical significance was calculated using 2 x 2 contingency tables by χ² test, corrected with Fisher exact test when sample sizes were small. Student’s t-test was used to compare means of different samples. Values of p < 0.05 were considered significant. Disease risk was expressed as 1:N, where N is the number of subjects among which one patient is present (10). We considered a disease prevalence of 1:100 in the general population and for each HLA-DQ haplotype, N was calculated as a percentage of controls with that particular haplotype multiplied by 100 and divided by percentage of patients with the same HLA-DQ status.

RESULTS

As a first step, we did a case-control study of 89 celiac patients and 70 healthy controls. 77.5% (n = 69) patients had DQ2 and/or DQ8 heterodimers. Among the DQ2/DQ8 negative patients 5% had DQA1*05 (α5 positive) and 95% had the DQB1*02 allele (B2 positive). No patient was negative for all the alleles predisposing to CD (Fig. 1A). The frequency of controls carrying DQ2 and/or DQ8 heterodimers was 30% (n = 21), consistent with literature data (2,9,10). Among the DQ2/DQ8 negative controls, the DQA1*05 allele was found in 28.6% (α5 positive), showing a negative association between CD and α5 phenotype (p = 0.02) and the DQB1*02 allele was found in 20.4% (B2 positive), showing a strong association between CD and B2 phenotype (p < 0.00001). 51% of controls DQ2/DQ8 negative lacked all the alleles predisposing to CD (Fig. 1B). Sample distribution of the HLA-DQ alleles in patients and controls is presented in table I. Two alleles coding for DQ β chains (*02/*02, *02/*0302 or *0302/*0302) were found respectively in 33.7% of patients and in 5.7% of controls, showing a high significance value (p = 0.000009). In particular the DQB1*02/*02 combination was found in 21/89 patients (23.6%) and in 1/70 controls (1.4%) (p = 0.000001). The DQB1*02/*0302 and DQB1*0302/*0302 combinations were observed in 9/89 patients and 3/70 controls. We estimated a gradient of CD risk comprised between 1:7 and 1:3580 based on the HLA haplotype. The HLA-DQ risk categories distribution in cases and in controls is reported in table II. The highest risk (1:7) was observed in DQ2
homzygous individuals or who carry DQ2 heterodimer associated with a double dose of the DQB1*02 allele. Subsequently, we observed subjects carrying both DQ2 and DQ8 (1:41), followed by two groups characterized by the presence of two β chains: B1*0302/*02 or B1*0302/*0302 (1:43), B1*02/*02 (1:45). We included the category carrying B1*0302 homozygous (DQ8DR4/DQ2DR4) together with individuals DQ8, B1*02 positive (DQ8DR4/DQ2DR7), because of the small numbers (1 case and no control). The disease risk for DQ2 subjects, showing a second allele DQB1 different from *02 (DQ2, B1*02/X ) resulted 1:47. We analyzed together in this category individuals carrying alleles in cis or in trans position, because no statistical difference emerged from other studies (11). The sole presence of DQB1*02 allele (B2, B1*02/X ) was associated with a CD risk of 1:75. Individuals carrying DQ8, without DQB1*02 allele have a disease risk of 1:85. In the last two groups the risk of developing CD is lower than in the general population, respectively 1: 1818 for individuals with α5 phenotype (DQ7DR5) and 1: 3580 for individuals without any predisposing allele. We did not consider whether DQA1*05 allele was homozygous or heterozygous because it was not relevant to the calculation of the CD risk gradient (12).

In the second part of the study, 105 first degree family members (63 parents, 29 siblings, 13 offsprings) were screened with tTG IgA and HLA-typed. 45/63 parents, 23/29 siblings and 11/13 offsprings carried HLA-DQ alleles predisposing to CD. Biopsies were performed in subjects having a positive serology result for diagnostic confirmation. 5/29 siblings, 3/63 parents and 1/13 children were affected, with a prevalence of CD in siblings significantly higher than in parents (17.2 % vs. 4.8 %) (p = 0.04). Figures 2A and 2B show the distribution of at risk DQ molecules among parents and siblings of the index cases and the affected subject percentage, all carrying HLA-DQ alleles predisposing to celiac disease. Overall 75.2% of first degree family members carried susceptibility factors and 8.6% (HLA-DQ +) were affected (Fig. 2C).

In the third part of the study we tried to establish if it is possible to correlate the different clinical presentation of CD and the genetic background (HLA-DQ), assuming that a gene-dose effect exists and may influence the clinical phenotype, modifying the immune response intensity. We included a population of 89 CD patients, 62 females and 27 males with a male to female ratio of 1:2.3 and 31 (34.8%) were adults. The mean age at diagnosis was 16.6 years (SD 16.7), higher in females (mean: 17.6; SD 16.1), than in males (mean: 14.5; SD 18.4), but not significantly

Table I. HLA-DQ2/DQ8 haplotype in cases and controls: the whole sample distribution of the HLA-DQ alleles in patients and controls

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Cases n = 89%</th>
<th>Controls n = 70%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other</td>
<td>0</td>
<td>35.8%</td>
</tr>
<tr>
<td>DQ2 e DQ8</td>
<td>3.4%</td>
<td>1.4%</td>
</tr>
<tr>
<td>DQ8/X</td>
<td>5.6%</td>
<td>4.3%</td>
</tr>
<tr>
<td>DQ8/DQ8</td>
<td>1.1%</td>
<td>0</td>
</tr>
<tr>
<td>DQ8 + β5</td>
<td>1.1%</td>
<td>1.4%</td>
</tr>
<tr>
<td>DQ8 + β2</td>
<td>5.7%</td>
<td>2.9%</td>
</tr>
<tr>
<td>α5</td>
<td>1.1%</td>
<td>20%</td>
</tr>
<tr>
<td>β2/X</td>
<td>19.2%</td>
<td>14.3%</td>
</tr>
<tr>
<td>β2/β2</td>
<td>2.2%</td>
<td>0</td>
</tr>
<tr>
<td>DQ2/DQ2</td>
<td>30.3%</td>
<td>12.9%</td>
</tr>
<tr>
<td>DQ2+ β2</td>
<td>5.6%</td>
<td>1.4%</td>
</tr>
<tr>
<td>DQ2+ β5</td>
<td>15.7%</td>
<td>0</td>
</tr>
<tr>
<td>DQ2 trans</td>
<td>0</td>
<td>4.2%</td>
</tr>
<tr>
<td>DQ2 trans</td>
<td>9%</td>
<td>1.4%</td>
</tr>
</tbody>
</table>

DQ2 = DQA1*05 and DQB1*02; DQ8 = DQA1*0301 and DQB1*0302; α2 = DQB1*02 in the absence of DQA1*05; α5 = DQA1*05 in the absence of DQA1*02; DQ2 trans = DQA1*05 and DQB1*02 in trans position in heterozygous subjects.
The mean age at presentation of symptoms, known in 47 subjects, was 12.3 years (SD 14.4), without any significant difference between males and females. We had sufficient information to divide 80/89 patients (90%) into 4 groups, based on the different clinical presentation: typical CD, atypical, silent, latent (Fig. 3):

1. **Typical celiac disease**, characterized by gastrointestinal symptoms and signs (failure to thrive, diarrhea, vomit, abdominal distension, abdominal pain, constipation, edema): 38 patients, 47.5%. Just 1/4 patients presented with diarrhea (20 patients, 25%) (13).

2. **Atypical celiac disease**, characterized by minimal or absent gastrointestinal symptoms and signs and by other clinical manifestations like iron deficiency anemia, isolated short stature, anorexia, osteoporosis, enamel alterations, dermatitis herpetiformis, recurrent aphthous stomatitis, alopecia, epilepsy in patients with occipital calcifications (CEC syndrome), increased transaminase levels, pubertal delay (18 patients, 22.5%).

3. **Silent celiac disease**, in absence of any symptoms (14 patients, 17.5%).

4. **Latent celiac disease**, when a subject, symptomatic or asymptomatic, presents with genetic markers for CD susceptibility, positive serology, but normal duodenal biopsy or just an increased number of IEL (type 1 according to Marsh; grade A according to Corazza and Villanacci) (14,15) (10 patients, 12.5%).

We divided patients into three categories based on HLA typing and risk gradient found in results 2 (Fig. 4):

- High risk subjects: DQ2, B1*02/02 (19 patients).
- Intermediate risk subjects: DQ2 and DQ8; DQ8, B1*02 positive or B1*0302 positive; β2, B1*02/02; DQ2, B1*02/X (39 patients).
- Low risk subjects: β2, B1*02/X; DQ8, B1*02 negative (22 patients).

Subjects carrying high risk genotype show in 53% of cases typical symptoms and in 26% of cases atypical symp-
tomts without any statistically significant difference (p = 0.09), but in a significantly lower percentage of cases (21%) silent or latent CD (p = 0.04), which we considered together (Fig. 4). We found among intermediate risk subjects a significantly greater frequency of typical CD (49%) than atypical CD (18%; p = 0.003), but not compared to silent or latent CD (33%; p = 0.1). We didn’t note in low risk group any statistically significant difference in distribution of typical CD (41%), atypical (27%; p = 0.2) and latent or silent (32%; p = 0.3).

DISCUSSION

CD is a multigenic, chronic inflammatory disease occurring in genetically susceptible individuals after gluten ingestion, characterized by chronic small bowel inflammation that results in villous atrophy. The presence of HLA-DQ2 and HLA-DQ8 molecules is necessary but not sufficient for the development of the disease and comprises more than 40% of the genetic risk (11,12,16). The European Genetic Cluster on Celiac Disease established that fewer than 0.5% of affected subjects are negative both for DQ2 and DQ8 (12). HLA typing is used as a genetic test for CD with a negative predictive value near to 100%. We present in this study the experience of the University of Siena Pediatrics Department with HLA typing, with the aim of contributing to the development of practical indications for the use of the HLA typing to stratify CD genetic risk. In the case-control study we confirmed the strong association between DQ2/DQ8 molecules and CD, even though the proportion of DQ2 positive patients in our cohort of Italian subjects was lower than those described in Northern Europe. The European Genetics Cluster on Celiac Disease investigated the presence of DQ2 and DQ8 molecules in 1008 patients from France, Italy, Finland, Norway, Sweden, UK and detected more frequent DQ2 negative patients in the South of Europe (France, Italy), than in the North (Finland, Norway, Sweden, UK) (15.5 vs. 9.8%), (2,17). We found that 64% of patients with CD carried DQ2 heterodimer (αβ2), 13.5% carried DQ8 heterodimer without DQ2, 21.4% only showed β2 chain and 1.1% of the remaining patients were positive for DQ2 α5 chain. The sole presence of α5 chain was found in 20% of controls, thus we can affirm that the sole α5 chain presence doesn’t predispose to CD. None of our patients was negative for all the alleles HLA-DQ associated with CD. Subjects carrying only DQB1*02 allele are more numerous than in other reports pointing out the intrinsic correlation between β2 chain and CD. Megiorni et al. (10) found that 5.9% of individuals with CD had only the β2 chain. Karrell et al. (17) were the first to show that CD could develop in patients coding for a heterodimer formed by the β2 chain together with β2 (DQ2DR7) instead of an α5 chain (DQ2DR3). In our study among the DQ2/DQ8 negative individuals the frequency of subjects carrying DQB1*02 allele was 95 versus 20.4% of the controls, leading to a very high p value (p < 0.00001) and showing a disease risk higher than in other European countries (11,12). In the case-control we provided a detailed estimation of the CD risk associated with each particular HLA-DQ status, considering a CD prevalence in the general population of 1:100. We observed a progression of increased risk, ranging from 1:7 for HLA-DQ2 homozygous to 1:85 for DQ8 heterozygous subjects. The same risk progression has been demonstrated in other groups of Italian subjects and in the United States population (18). As expected the HLA-DQ2 homozygous status, or anyway associated with a β2 chain double dose (DQ2,B1*02/*02), leads to the highest risk to develop CD which in our study was 1:7 vs. 1:47 in DQ2 heterozygous subjects. These data are concordant with what is affirmed by many authors (3,12,19-21), while Megiorni et al. (10) found the highest risk value for DQ2 and DQ8 positive subjects. In our experience these individuals are comprised in an intermediate risk category (1:41). Steppniak et al. (22) hypothesized that in patients carrying DQ2 and DQ8 heterodimers the higher CD risk could be due to a broadening repertoire of gluten peptides presented to T cells in the intestine. Homozygous subjects DQB1*02/*02 in absence of the DQA1*05 allele have an intermediate risk (1:45). We showed how the presence of DQB1*02 allele (β2 chain) in DQ8 positive individuals increases the risk of developing CD twofold (from 1:85 to 1:43). DQB1*02 heterozygous subjects (β2, B1*02/X) are comprised in a low risk category (1:75), together with subjects DQ8 positive when DQB1*02 and DQB1*0302 negative (1:85), in line with what was observed by other authors (3,19) and in contrast with Megiorni et al. (10) who found a risk lower for β2 heterozygous subjects than in the general population. Values lower than the disease prevalence in the general population were demonstrated for α5 subjects (1:1818) or DQ2, DQ8, β2, α5 negative.
subjects (1:3580). The differences in outcomes between the studies can be explained either by different HLA-risk group classifications or by variable sample size. Based on HLA-DQ genotype related risk gradient we identified three different classes of risk (see above). This classification could be employed to select patients who might require to undergo further tests and could determine the timing of follow-up programs, not yet encoded. Time interval between repeat serologic testing in literature varies from 0.5 to 20 years (19,23,24) and the data are hardly comparable for population heterogeneity. As recommended by NASPGHAN (8), HLA typing is particularly useful in first degree family members. We showed that, overall, 8.6% of first degree family members are affected and that CD is significantly more frequent among siblings (1/6) than parents (1/21). Moreover, CD developed exclusively in the presence of HLA-DQ2, -DQ8 or DQB1*02. Pre-disposing haplotype was present in 75.2% of first degree family members, consistent with incidence rates reported in the literature estimated from 63.5 to 83.1% (25). 24.8% of first degree relatives were negative for any at risk allele according to Chang et al. (23) who found 20% of first degree family members could be excluded from follow-up by genetic testing. HLA typing could be used to exclude from follow-up programs individuals who lack the necessary genotype to develop CD. We finally examined whether it was possible to establish a correlation between HLA-DQ haplotypes and the different clinical presentation of CD. Starting from the assumption that a gene-dose effect exists, which can modulate the immunological response, we expected to observe severe clinical CD presentation (typical CD) more often among DQ2DR3 homozygous patients, or anyway among subjects with a double dose of β2 chain (DQ2,B1*02/*02). We also expected to find more patients with latent or silent CD in the low risk category. We found no relationship between CD clinical presentation and HLA-DQ risk categories according to Vermeulen et al. (3) and Mustalathi et al. (26). In contrast, Nenna et al. (5) observed a percentage of CD typical form higher in DQB1*02 homozygous patients, as well as higher levels of tTG Ab titers. Karinen et al. reported that the DQB1*0201 allele homozygosity is associated with a more severe form of CD, younger age at clinical presentation and slower recovery of villous atrophy after gluten free diet (27). The differences in studies results can be explained either by the different classifications of at risk HLA-DQ haplotypes or by the different classification of CD clinical presentation. Differences in outcomes can also result from environmental factors such as breastfeeding, amount of gluten and timing of introduction in the diet, gastrointestinal infections (28,29).

In conclusion, our results strengthen the evidence that HLA-DQ status strongly influences the development of CD and demonstrate that knowledge of a patient’s HLA-DQ genotype allows to establish clinically relevant genetic risk profiles. An exact individual genetic risk assessment could improve current diagnostic strategies and thus prognosis. Identifying and quantifying the risk of developing CD in newborns from affected family members could be useful for programming individualized follow-up timing. An early diagnosis and a prompt treatment following a gluten free diet could prevent the development of a clinically severe CD and/or comorbidity and an early identification of children at risk leads to primary prevention. On the contrary subjects with ζ5 phenotype or without predisposing alleles could be ruled out from clinical and serological follow-up. In the future the goal will be to combine the HLA and the non-HLA risk genotype discovered by Genome Wide Association Study (GWAS) in a two step model to predict the CD genetic risk and to improve the identification of at risk individuals (30).

REFERENCES

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