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HLA-DQ2 homozygosis increases tTGA levels at diagnosis but does not influence the clinical phenotype of coeliac disease: A multicentre study

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Abstract

Background and purpose: Magnitude of gluten-specific T-cell responses in coeliac disease (CD) might be dependent on HLA-DQ2 gene dose. We aimed to investigate the effects of HLA-DQB1*02 allele dose on clinical outcomes.

Methods: We reviewed the charts of all coeliac patients attending to three Hungarian university clinics after 1997 and included those patients, who (a) were diagnosed with CD, (b) underwent high-resolution HLA typing and (c) were ≥18 years at the time of data collection. HLA typing was performed to determine DQB1*02 allele dose. Patients were divided into risk groups by DQB1*02 allele dose, as follows: high-, intermediate- and low-risk groups corresponded to a double, single and zero doses, respectively. We used ANOVA and Pearson's chi-squared test to explore association between HLA risk and clinical variables.

Results: A total of 727 coeliac patients attended the clinics but only 105 (14.4%) patients were eligible for inclusion. High, intermediate and low HLA risk patients comprised 35.3%, 52.3% and 12.3% of the study population, respectively. Double dose of HLA-DQB1*02 was more frequent in patient with high tTGA level (>10 times the upper limit of normal; $p = 0.045$). Gene dose was not associated with younger age at diagnosis ($p = 0.549$), gender ($p = 0.739$), more severe diagnostic histology ($p = 0.318$), more frequent classical presentation ($p = 0.846$), anaemia ($p = 0.611$), metabolic bone disease ($p = 0.374$), dermatitis herpetiformis ($p = 0.381$) and autoimmune diseases ($p = 0.837$).

Conclusions: Our study shows a significant gene dose effect in terms of tTGA level at diagnosis, but no significant association between HLA-DQB1*02 allele dose and the clinical outcomes in CD.

KEYWORDS
coeliac disease, gene dose, HLA-DQ2, Homozygosis, Phenotype

1 | INTRODUCTION

Coeliac disease (CD) is a highly heritable, immune-mediated systemic disorder. The consumption of gluten-containing cereals triggers a T-cell-mediated immune cascade resulting in impairment of small intestinal villous architecture and systemic manifestations. Nearly all (>99%) CD patients are positive for HLA-DQ2 and/or DQ8 (Abraham & Inouye, 2015; Korponay-Szabo, Troncone, & Discepolo, 2015).

DQ molecules are MHC class II proteins serving critical functions in the immune response to foreign antigens. Gliadin peptides are presented (with rare known exceptions) on DQ2 or DQ8 molecules. Tissue transglutaminase generates negatively charged peptide residues in the deamidation of gliadin. Acting as high-affinity ligands, the peptide residues bind to HLA-DQ2 and DQ8 molecules of antigen-presenting cells' (APCs). Then, CD4+ T cells recognize this complex, initiate cellular activation and trigger T-cell immune responses. Sequelae include severe tissue destruction accompanied by remodelling of small intestinal villous architecture (Kupfer & Jabri, 2012; Tjon, Van Bergen, & Koning, 2010).

The diagnosis of CD can be excluded by HLA typing because of its outstanding negative predictive value approaching 100% (Diaz-Redondo, Miranda-Bautista, Garcia-Lledo, Gisbert, & Menchen, 2015; Rubio-Tapia et al., 2013). The current paediatric guideline recommends HLA typing if patients are diagnosed without intestinal biopsy (Husby et al., 2012). Although the diagnostic applicability of HLA typing is clear, its role in risk stratification is still under debate. Homozygotes have the potential to synthesize the highest possible number of identical DQ2 molecules with effective antigen-presentation properties. Unlike heterozygotes, who have multiple potential allele combinations, only some of them can present gliadin peptides (van Belzen et al., 2004). Accordingly, DQ2.5 homozygotes have a fivefold risk of CD, as compared to heterozygotes (Abraham & Inouye, 2015; Koning, 2012; Pietzak, Schofield, McGinniss, & Nakamura, 2009; Vader et al., 2003; van Belzen et al., 2004).

The way how HLA-DQ2 gene dose modifies clinical phenotype is unclear. High-risk DQ2.5 homozygous patients might have earlier disease onset with a more severe disease course as compared to those with other haplotypes (Agardh et al., 2015; Biagi et al., 2012; Congia et al., 1994; Demarchi et al., 1983; Jores et al., 2007; Karinen et al., 2006; Mubarak, Spierings, Wolters, Otten et al., 2013; Nenna et al., 2008; Zamani, Modares-Sadegi, Shirvani, Zamani, & Emami, 2014; Zubillaga et al., 2002). However, many studies did not confirm this thesis (Akar, Yildiz, Sevinc, & Sokucu, 2015; Greco et al., 1998; Maŕia Jesús et al., 2012; Murray et al., 2007; Mustalahti, Holopainen, Karell, Maki, & Partanen, 2002; Piccini et al., 2012; Ploski, Ek, Thorsby, & Sollid, 1993; Ros, Ros, Sanchez-Valverde, & Gimeno, 2010; Thomas et al., 2009; Vermeulen et al., 2009).

Early identification of high-risk patients would be of utmost importance. A closer follow-up and a stricter gluten-free diet might help them to avoid the development of life-threatening complications (e.g., malignancies) (Megiorni & Pizzuti, 2012; Romanos et al., 2009). We aimed to investigate the association between HLA-DQB1*02 allele dose with clinical parameters thereby attempting to define the role of HLA status in clinical risk stratification of CD patients.

2 | METHODS

2.1 | Patients

We included patients who were (a) diagnosed with CD by the current guidelines in one of three Hungarian university clinics (1st Department of Internal Medicine, Division of Gastroenterology, University of Pécs, Pécs; 2nd Department of Internal Medicine, Semmelweis University, Budapest; 2nd Department of Internal Medicine, University of Debrecen, Debrecen), (b) underwent HLA typing after 2010 (when the current PCR-based method was installed) and (c) were ≥18 years at the time of data collection.

Clinical data were retrieved from medical files retrospectively by independent investigators, blinded to the HLA status of the patients. Since HLA genotyping is not mandatory in CD patients, it is not incorporated in our routine diagnostic management.

Scientific and Research Ethics Committee of the Medical Research Council has granted ethical approval of this research project (45098–2/2016/EKU).

2.2 | HLA genotyping

Genomic DNA was isolated from peripheral venous blood (QIAamp DNA Blood Mini Kit). We used polymerase chain reaction with sequence-specific primers (PCR-SSP) and sequence-specific oligonucleotide probes (PCR-SSO) on commercial kits (Inno-Train HLA Ready Gene PCR-SSP kit, Olerup PCR-SSO kit and SSO One Lambda Luminex kit) at Hungarian National Blood Transfusion Service (Budapest and Pécs) and at Department of Laboratory Medicine, Clinical Center, University of Debrecen (Debrecen). Both DQA1 and DQB1 alleles, including allele dose, were typed. Genotypes were determined by haplotypes.

The HLA risk categories corresponded to HLA-DQ2 gene dose, as follows: (a) high-risk HLA-DQ2.5 homozygotes (DQ2.5/DQ2.5) and compound heterozygotes (DQ2.5/DQ2.2) with a double dose of DQB1*02 alleles; (b) intermediate-risk HLA-DQ2.5 heterozygotes (DQ2.5/DQX) and HLA-DQ2 in trans (DQ2.2/DQ7) with a single dose of DQB1*02 allele; and (c) low-risk HLA groups (HLA-DQ8/DQX, HLA-DQ2.2/DQX, X corresponded to any alleles except for DQ2.5) with zero doses of DQB1*02 allele.

2.3 | Clinical features

Age at diagnosis corresponded to the date of definite diagnosis when gluten-free diet was introduced. We defined the clinical presentation as per Oslo criteria as classical CD (with malabsorptive syndrome, e.g., diarrhoea and weight loss, irrespective of extraintestinal manifestations) and non-classical CD (without malabsorptive syndrome e.g., atypical gastrointestinal symptoms, extraintestinal manifestations) (Ludvigsson et al., 2013). We classified diagnostic small intestinal histology according to Corazza-Villanacci (Corazza &

Villanacci, 2005). Levels of tissue transglutaminase antibody (tTGA) were measured at diagnosis with ELISA. Positive tTG serology was further divided into two groups: patients with high and low titre levels were defined as >10 times or <10 times of the upper limit of normal (ULN), respectively. Haemoglobin levels <130 and <120 g/L indicated anaemia in men and women, respectively. Metabolic bone disease (including osteopenia and osteoporosis) was defined as measuring a *T*-score <−1.0 standard deviation by dual-energy X-ray absorptiometry (DEXA). Concurrent autoimmune diseases, malignancies and dermatitis herpetiformis were assessed, as well.

2.4 | Statistical analysis

We performed Pearson's chi-squared test to analyse the association between HLA risk and categorical variables and one-way ANOVA to compare age at diagnosis across HLA risk groups. $p < 0.05$ indicated the rejection of the null hypothesis. Statistical analysis was carried out by IBM SPSS Statistics v 20.0 (IBM's Corporate, New York, USA).

3 | RESULTS

3.1 | Demography

A total of 727 coeliac patients attended the clinics between November 1997 and May 2016, of them 105 (14.4%) were eligible for inclusion in the study. High, intermediate and low HLA risk patients comprised 35.3%, 52.3% and 12.3% of the study population, respectively. Fifteen patients (14.3%) were diagnosed in childhood (<18 years), and another 90 (85.7%) in adulthood (≥18 years). We observed female predominance in our cohort (73 female vs. 32 male patients) without significant gender differences between the HLA risk groups ($p = 0.739$).

3.2 | Age, clinical presentation, serology and histology at diagnosis

Mean age at diagnosis was 31.2 years (*SD*: 15.747, range: 0.5–78 years). Age at diagnosis did not differ significantly between the risk groups ($p = 0.549$).

Forty-five of the 105 patients (42.9%) had classical CD. We failed to prove a significant association between HLA risk and clinical presentation ($p = 0.846$): 15 of 37, 25 of 55 and 5 of 13 patients had classical CD in the high, intermediate and low HLA risk groups, respectively (Table 1).

tTGA was measured in 70 of 105 patients at diagnosis, nine of them (12.9%) were seronegative (3, 2 and 4 cases with zero, single and double dose of HLA-DQB1, respectively). In the other 35 cases, the serological diagnosis was based on endomysium antibody positivity. Of the tTGA-positive patients, 34 had a low level of tTGA (2, 17 and 15 cases with zero, single and double dose of HLA-DQB1, respectively) and another 27

TABLE 1 Risk stratification and clinical phenotype

Locus B1 PCR1	Locus B1 PCR2	B1*02 allele dose	HLA genotype	Classical phenotype (*n*)	Non-classical phenotype (*n*)
High risk				15	22
B1*0201	B1*0201	Double	DQ2.5/DQ2.5	8	16
B1*0201	B1*0202	Double	DQ2.5/DQ2.2	7	6
Intermediate risk				25	30
B1*0201	B1*05	Single	DQ2.5/DQ5	5	9
B1*0201	B1*06	Single	DQ2.5/DQ6	3	7
B1*0201	B1*0301	Single	DQ2.5/DQ7	9	6
B1*0201	B1*0302	Single	DQ2.5/DQ8	2	4
B1*0201	B1*0303	Single	DQ2.5/DQ9	1	1
B1*0202	B1*0301	Single	DQ2.2/DQ7	5	3
Low risk				5	8
B1*0202	B1*0202	Zero	DQ2.2/DQ2.2	0	0
B1*0202	B1*04	Zero	DQ2.2/DQ4	0	1
B1*0202	B1*06	Zero	DQ2.2/DQ5	1	0
B1*0202	B1*0302	Zero	DQ2.2/DQ8	2	1
B1*0302	B1*0302	Zero	DQ8/DQ8	0	1
B1*0302	B1*04	Zero	DQ8/DQ4	0	1
B1*0302	B1*06	Zero	DQ8/DQ6	0	1
B1*0302	B1*0301	Zero	DQ8/DQ7	2	3

In the left foremost column, 3 groups are highlighted with bold (High risk, Intermediate risk, and Low risk), rows below indicate those alleles belonging to these groups. Bold numbers indicate the total number of patients within these groups dichotomized into classical and non-classical phenotypes.
HLA: human leucocyte antigen; PCR: polymerase chain reaction.

TABLE 2 Gene dose and clinical parameters in CD patients with autoimmune disease(s)

Patients	Gender	Age at diagnosis (years)	HLA genotype	B1*02 allele dose	Clinical phenotype	Autoimmune disease
1	Female	41	DQ2.5/DQ2.5	Double	Classical	Autoimmune thyroid disease
2	Male	18	DQ2.5/DQ2.5	Double	Classical	Ulcerative colitis
3	Female	4	DQ2.5/DQ7	single	classical	Crohn's disease
4	Male	25	DQ2.5/DQ5	Single	Classical	Crohn's disease
5	Male	28	DQ2.5/DQ7	Single	Non-classical	Autoimmune thyroid disease
6	Female	20	DQ2.5/DQ2.5	Double	Classical	Autoimmune thyroid disease
7	Female	36	DQ2.2/DQ7	Single	Classical	Ulcerative colitis
8	Female	26	DQ2.5/DQ8	Single	Non-classical	Alopecia areata
9	Female	56	DQ2.5/DQ5	Single	Classical	Autoimmune thyroid disease
10	Female	59	DQ2.5/DQ5	Single	Classical	Sarcoidosis
11	Female	2	DQ2.5/DQ7	Single	Classical	Autoimmune thyroid disease
12	Female	27	DQ2.5/DQ7	Single	Non-classical	Autoimmune thyroid disease
13	Female	24	DQ2.5/DQ2.5	Double	Classical	Autoimmune thyroid disease
14	Female	17	DQ2.5/DQ2.5	Double	Non-classical	Autoimmune thyroid disease
15	Female	31	DQ2.5/DQ5	Single	Non-classical	Autoimmune thyroid disease
16	Female	51	DQ2.5/DQ5	Single	Non-classical	Autoimmune thyroid disease
17	Female	35	DQ2.5/DQ5	Single	Non-classical	Lichen ruber planus
18	Male	10	DQ8/DQ7	Zero	Non-classical	Alopecia areata
19	Female	41	DQ2.2/DQ5	Zero	Classical	Autoimmune liver disease
20	Female	36	DQ2.5/DQ2.2	Double	Non-classical	Rheumatoid arthritis, myasthenia gravis
21	Male	43	DQ2.5/DQ7	Single	Classical	Autoimmune thyroid disease
22	Female	23	DQ2.5/DQ2.5	Double	Non-classical	Autoimmune thyroid disease
23	Female	46	DQ2.5/DQ2.2	Double	Non-classical	Autoimmune thyroid disease
24	Female	62	DQ2.5/DQ5	Single	Classical	Autoimmune thyroid disease, ulcerative colitis
25	Male	37	DQ8/DQ7	Zero	Non-classical	Autoimmune thyroid disease
26	Female	29	DQ2.5/DQ2.5	Double	Non-classical	Psoriasis
27	Female	14	DQ2.5/DQ6	Single	Classical	Ulcerative colitis
28	Male	30	DQ2.5/DQ6	Single	Classical	Ulcerative colitis, sacroileitis

HLA: human leucocyte antigen.

patients had high level of tTGA. In the comparison of seropositive patients with the double dose to those with the single dose, higher levels of tTGA were more frequent as in the former group ($p = 0.045$).

We detected no significant association between diagnostic histological severity and HLA risk ($p = 0.318$), even if we included only patients diagnosed in adulthood.

3.3 | Anaemia and metabolic bone disease

Gene dose effect seems to be unimportant in the development of anaemia ($p = 0.611$) and metabolic bone disease ($p = 0.374$) either in the whole study population or in the subgroup of patients diagnosed in adulthood. However, 14, and 43 of 105 cases did not have available baseline haemoglobin levels and DEXA scores. Forty of 91 patients (44.0%) had anaemia while 38 of 62 patients (61.3%) had metabolic bone disease.

3.4 | Concomitant autoimmune diseases

Twelve patients (11.4%) had dermatitis herpetiformis, all of them were in the high or intermediate HLA risk groups, and nearly half of them had classical CD. The frequency of dermatitis herpetiformis was independent of HLA status ($p = 0.381$). Interestingly, DH was diagnosed exclusively in high-risk DQ2.5 homozygotes (five patients) or intermediate-risk patients (seven patients).

Twenty-eight patients (26.7%), with a prominent female predominance of 3:1, had a concurrent autoimmune disease. The most frequent autoimmune disease was autoimmune thyroid disease (15 patients). Seven patients had inflammatory bowel disease (five cases

of ulcerative colitis and two cases of Crohn's disease), two patients had alopecia areata, and there were isolated cases of rheumatoid arthritis, myasthenia gravis, lichen ruber planus, sarcoidosis, psoriasis, sacroileitis and autoimmune liver disease. Type 1 diabetes mellitus was not observed in the study population. Autoimmunity was independent of HLA status ($p = 0.837$), as shown in Table 2.

3.5 | Malignant tumours

Malignant tumours were diagnosed in three patients: a female patient (DQ2.2/DQ7 heterozygote) developed malignant melanoma at the age of 38 years (CD was diagnosed at the age of 36 years), another female patient (DQ2.5 homozygote) died of pancreatic adenocarcinoma at the age of 75 years (CD was diagnosed at the age of 59 years), and a male patient (DQ2.5 homozygote) had lung adenocarcinoma at the age of 55 years (CD was diagnosed at the age of 46 years). Here, the low case number did not allow us to perform statistical analysis.

Refractory CD did not occur in the study population.

4 | DISCUSSION

Although the theoretical background suggests a significant gene dose effect in CD, we proved only association between HLA-DQ2 gene dose and tTGA level but not in connection with the clinical features.

The influence of DQ2 gene dose on clinical phenotype is rather vague, lack of consensus on risk stratification systems might contribute to the contradictory results. It is pervasive that DQ2.5 or DQ2 homozygosis confer the highest risk of adverse outcomes, but authors are divided in terms of low-risk categories (Abraham & Inouye, 2015; Delgado et al., 2014; Gudjonsdottir et al., 2009; Koning, 2012; Margaritte-Jeannin et al., 2004; Medrano et al., 2012; Megiorni & Pizzuti, 2012; Mubarak, Spierings, Wolters, Otten et al., 2013; Nenna et al., 2008; Piccini et al., 2012; Romanos et al., 2009; Ros et al., 2010; Rostami-Nejad et al., 2014; Vader et al., 2003; van Belzen et al., 2004; Vermeulen et al., 2009). In accordance, we stratified risk by the number of DQB1*02 alleles (i.e., gene dose) so that patients with DQ2.5/2.5 and DQ2.5/2.2 represented the high-risk category (Table 1). As expected, all of our patients were positive for HLA DQ2 and/or DQ8. Although double-dose DQB1*02 is more frequent in this population than that of reported earlier (35.2% vs. 19.7% and 21.3%) (Piccini et al., 2012; Thomas et al., 2009), DQ2.5/2.5 frequency proved as high as in a previous work (22.9% vs. 19.2%) (Stankovic et al., 2014).

Lack of gene dose effect was reported frequently, for example in terms of age at presentation of first symptoms (Greco et al., 1998; Vermeulen et al., 2009); age (Akar et al., 2015; Greco et al., 1998; Murray et al., 2007; Mustalahti et al., 2002; Thomas et al., 2009), histological damage (Murray et al., 2007; Thomas et al., 2009; Vermeulen et al., 2009) and clinical symptoms (Akar et al., 2015; Greco et al., 1998; Murray et al., 2007; Vermeulen et al., 2009) at diagnosis; anaemia or haemoglobin levels (Akar et al., 2015; Thomas et al., 2009); coeliac-specific serology (Murray et al., 2007); iron, mineral and vitamin deficiencies (Akar et al., 2015; Thomas et al., 2009); metabolic bone disease (Thomas et al., 2009) and autoimmunity (Malamut et al., 2012). On the contrary, studies reported significant gene dose effect in terms of, for example age (Liu, Lee, & Agardh, 2014; Nenna et al., 2008; Ploski et al., 1993; Zubillaga et al., 2002), clinical presentation (Congia et al., 1994; Demarchi et al., 1983; Karinen et al., 2006; Nenna et al., 2008; Zubillaga et al., 2002) and histological damage (Jores et al., 2007; Karinen et al., 2006) at diagnosis; coeliac-specific serology (Agardh et al., 2015; Nenna et al., 2008; Thomas et al., 2009), anaemia (Karinen et al., 2006), autoimmune diseases (Lionetti et al., 2014; Liu et al., 2014) and malignant tumours (Al-Toma et al., 2006; Biagi et al., 2014; Malamut et al., 2012).

Gene dose effect might determine magnitude of T-cell responses. APCs extracted from DQ2.5 homozygotes induced more prominent T-cell proliferation and interferon γ production, as compared to HLA-DQ2.5/DQX heterozygotes (X corresponded to any haplotypes except for DQ2.5). Taken together, the number of HLA-DQ2.5 molecules on APCs determines the magnitude of T-cell responses (Vader et al., 2003), in other words "quantity matters" (Koning, 2012). Enhanced antibody response, observed in our study in homozygotes as well, seems to support this theory.

Among alleles encoding DQ2.5, the number of B1*02 is decisive; the presence of double-dose A1*05 is not essential to trigger full-strength immune responses (Karinen et al., 2006; Medrano et al., 2012; Megiorni et al., 2009; Piccini et al., 2012; Pisapia et al., 2016; van Belzen et al., 2004). The most prominent response is expected in DQ2 homozygotes (including DQ2.5/DQ2.5) and in compound heterozygotes (DQ2.5/DQ2.2), who have a double dose of B1*0201 (Abraham & Inouye, 2015; Demarchi et al., 1983; Kupfer & Jabri, 2012; Margaritte-Jeannin et al., 2004; Pietzak et al., 2009; Rostami-Nejad et al., 2014). DQ2.5/DQX heterozygotes and those with DQ2.2/DQ7.5 genotype (so-called DQ2.5 *trans*) have a single dose of B1*02 allele (Korponay-Szabo et al., 2015; Mearin et al., 1983; Mubarak, Spierings, Wolters, van Hoogstraten et al., 2013). Here, the chance of synthesizing (high affinity) DQ2.5 molecules is only 25%; therefore, this haplotype is deemed to be an intermediate-risk one (van Belzen et al., 2004; Vader et al., 2003). Other HLA haplotypes, not producing functioning DQ2 molecules, are accompanied by low risk of CD.

On the contrary, a few findings oppose gene dose effect. Equal magnitude of specific T-cell responses characterizes homo- and heterozygotes. In addition to heterozygotes, the amount of DQA1*05 and DQB1*02 mRNS exceeded the expected 50% of those measured in homozygotes (Pisapia et al., 2016).

Coeliac disease is strongly HLA-linked but the effect of non-HLA loci (not taken into account in this study) can outweigh that of HLA. In a study, HLA risk stratification was complemented with 10 non-HLA loci which resulted in the allocation of 10% of study population from the moderate- to the high-risk group (Romanos et al., 2009). Besides, nongenetic (environmental) factors contribute to

CD phenotype (Greco et al., 1998; Gudjonsdottir et al., 2009; Piccini et al., 2012; Vermeulen et al., 2009).

Taken together, it is possible that genetic (non-HLA) and environmental factors masked a significant gene dose effect. However, the retrospective design (missing data) restricts the validity of our findings. The low case numbers and event rates raise concerns about the occurrence of β-type error. Only a small minority (14.4%) of patients underwent HLA typing because it is not mandatory at diagnosis (Husby et al., 2012). The low case number did not allow us to perform separate analysis on patients with childhood diagnosis. Some clinical features, especially symptoms, are difficult to be categorized without bias; therefore, we used the classical/non-classical distinction (as per the Oslo classification) to decrease risk of bias (Ludvigsson et al., 2013).

The HLA status is an unalterable, lifelong persistent marker, not influenced by age, gluten intake or environment. The diagnostic yield of HLA typing is clear, whereas the usefulness of HLA-based risk stratification has remained questionable. Our study showed that patients with a double dose of HLA-DQB1*02 have higher tTGA titre at diagnosis but we failed to prove an association between HLA-DQ genotypes and the clinical features, namely age at diagnosis, clinical presentation (classical vs. non-classical CD), frequency of anaemia, dermatitis herpetiformis, metabolic bone disease, autoimmune diseases and malignancies. Large number, prospective studies are needed to clarify the role of HLA risk stratification in clinical practice.

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