Medical Research Archives

ESMED European Society of Medicine

OPEN ACCESS

Published: January 31, 2023

Citation: Lomash A, Singh R, et al., 2023. Utility of human leukocyte antigen DQ2 and DQ8 genotypes in Celiac disease: Two sides of the coin, Medical Research Archives, [online] 11(1). https://doi.org/10.18103/mra.v11i1.2864

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DOI
https://doi.org/10.18103/mra.v11i1.2864

ISSN: 2375-1924

RESEARCH ARTICLE

Utility of human leukocyte antigen DQ2 and DQ8 genotypes in Celiac disease: Two sides of the coin

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ABSTRACT

Human Leukocyte Antigen (HLA) DQ2 and DQ8 are the known risk genotypes for Celiac Disease. Diagnosis of celiac disease requires serology testing and intestinal biopsy however, genetic testing could also be implemented because of its high negative predictive value. Our study, initiated prior to the publication of 2020 guidelines, evaluated the utility of HLA DQ2 and DQ8 genotyping in the cohort of 537 children with celiac disease and their 1420 first-degree relatives. We attempted to evaluate its applicability in low-middle-income countries like India. Prevalence of celiac disease was observed at 18.52% and 15.68% based on serological and histopathological diagnosis in first-degree relatives. HLA DQA1*0501 and DQB1*0201 alleles were the most frequently observed alleles in index cases (84.4% versus 86.4%), biopsy proven first-degree relatives (77.9% vs 72.15) and serology negative first-degree relatives (67.7% vs 58.3%) ($p<0.001$). A strong association of DQA1*0501 and DQA1*0301 alleles was observed with high serology positivity ($p<0.05$). The majority of the subjects in our cohort had histopathologic scores of 3c (54.80%), 3b (22.13%), 3a (22%) and grade 2 (1.05%) ($p<0.001$). HLA DQB1*0201 was observed as 100% in cases with Marsh grade 2, 72.5% in grade 3a, 82.7% in grade 3b and 85.6% in grade 3c ($p=0.017$) mucosal lesions. HLA DQA1*0501 and DQB1*0201 alleles of the DQ2 genotype also predicted the severity of intestinal mucosal damage assessed by Marsh grading when used in conjunction with anti-tTG-IgA. When performed in-house using polymerase chain reaction-sequence specific primers method, the assay was economical in identification of mucosal severity in index cases and the first-degree relatives. Its value as a model for additive predictive value in the diagnostic algorithm of cases with tTG-IgA less than 10 times of the upper limit of normal needs further evaluation.

1 Introduction

Celiac disease (CeD) is an autoimmune, gluten-related enteropathy associated with the presence of human leukocyte antigen (HLA) genotype DQ2 and DQ8 [1]. It results from immune dysregulation arising from gluten and related cereal proteins [2]. CeD is a common health problem with a varied global pooled prevalence based on serology (1.4%) and histopathology (0.7%) [3]. Its frequency in India seems to be higher in the Northern parts of the country as wheat is the predominant staple food in the North, a finding partly explained by the wheat-rice shift separating North from South India [4]. Although CeD is one of the most common lifelong autoimmune diseases, most affected individuals remain undiagnosed. This is probably due to the fact that many patients have atypical symptoms or are oligosymptomatic/asymptomatic [5], detected as part of 'at risk' population screening of first-degree relatives (FDRs) of a patient with CeD, patients with Type 1 diabetes Mellitus and other autoimmune diseases [6,7]. The established biomarker for serological screening of CeD with high sensitivity and specificity is anti-tissue transglutaminase-IgA (tTG-IgA) [1,8]. Other sensitive biomarkers for CeD are anti-endomysial-IgA antibodies (EMA-IgA), anti-deamidated gliadin peptide-IgA (DGP-IgA) and amino acid citrulline [9–11]. Diagnostic confirmation for CeD is the histopathology analysis of duodenal biopsy as per modified Marsh classification; however, upper gastrointestinal endoscopy is an invasive procedure. The biopsy sparing approach recommended by ESPGHAN since 2012 has its limitations and advantages [1]. The ESPGHAN 2020 guidelines has recommended that the additive value as susceptibility gene testing from the diagnostic algorithm does not hold promise.

Genome Wide Association Studies (GWAS) on CeD have implicated the role of multiple genes, but the most commonly evaluated complex is the HLA class II, specifically the DQ2 and DQ8 region [12]. Though the population frequency of the HLA DQ2 and DQ8 genotypes is close to 30%, only 1% of these ultimately develop CeD [13]. The clustering of CeD in families provided the first conceptual understanding of the role of HLA in CeD. Concordance in monozygotic and dizygotic twins is high at approximately 50%-80% and 10% respectively, whereas prevalence among FDRs varies from 5% to 22.5% [14,15]. CeD has a strong genetic association with the HLA gene that is estimated to account for up to 40% of the genetic susceptibility [16]. About 90% of patients with CeD are positive for the HLA DQ2 genotype. Trans (DQA1*0201-DQB1*0202) and cis (DQA1*0501-DQB1*0201) forms of the DQ2 heterodimer are listed as DQ2.2 and DQ2.5, respectively [17]. The negative predictive value (NPV) is one of the cardinal characteristics of the HLA DQ2 and DQ8 CeD susceptibility, though it comes at the cost of poor specificity [18,19].

In the Indian context, studies related to the distribution of HLA DQ2 and DQ8 genotype frequency in the 'at risk' cohort of FDRs is sparse. This may be partially explained by the non-availability of the testing facilities and the high cost per test. In addition, there is a paucity of data regarding the applicability of ESPGHAN 2020 guidelines [8] in the Indian setting. Therefore, we intended to address the role of HLA genotyping by an in-house assay to evaluate the additive predictive value of HLA in the diagnostic algorithm of CeD and to discuss the feasibility and applicability of new guidelines for the early detection of at-risk FDRs.

2 Materials & Methods

2.1 Study design

This prospective cohort study was conducted in the Northern Indian population between February 2014 and July 2019. Institutional ethical approval was taken as per guidelines approved by ICMR (Indian Council of Medical Research) vide letter no F.1/IEC/MAMC/(31)/3/2012/No:236. Written informed consent was obtained from all study subjects/parents as applicable. The CeD and FDRs (parents and siblings) were diagnosed according to the ESPGHAN 2012 guidelines [1]. Written informed consent of the parent/s of all the study subjects was obtained after explaining the aims and the freedom of choice for participation in the study. The detailed clinical history and relevant clinical findings were entered in a predesigned proforma.

2.2 Serology, gastrointestinal endoscopy and histopathology

Serum tTG-IgA levels were assessed by Enzyme Linked Immuno Sorbent Assay (ELISA) method using a commercial kit, HYCOR, (CA, USA) on a Microplate reader, BioRad (Model 680, CA). The cut-off for tTG-IgA positivity was taken as >7U/L per kit specification. All patients with elevated tTG-IgA titre (>7 U/L) underwent at least four punched intestinal biopsies. Two mucosal fragments were obtained from the duodenal bulb and three to four samples from the distal duodenum. Serology positive FDRs also underwent endoscopic procedures. Endoscopic procedures in cases with

age up to 12 years were performed at Maulana Azad Medical College (MAMC) and associated LN Hospital, and those above 12 years of age at the Department of Gastroenterology, Govind Ballabh Pant Institute of Post Graduate Medical Education and Research (GIPMER). Histopathologic reporting was done by the Department of Pathology, GIPMER. All biopsy specimens were evaluated by an experienced pathologist blinded to the serology results and classified according to modified Marsh-Oberhuber classification [20]. Subjects classified with grades 2 or more were diagnosed as being affected by CeD as per ESPGHAN 2012 guidelines [1]. Figure 1 depicts the histomorphology of biopsy tissue graded as per ESPGHAN 2012 criteria.

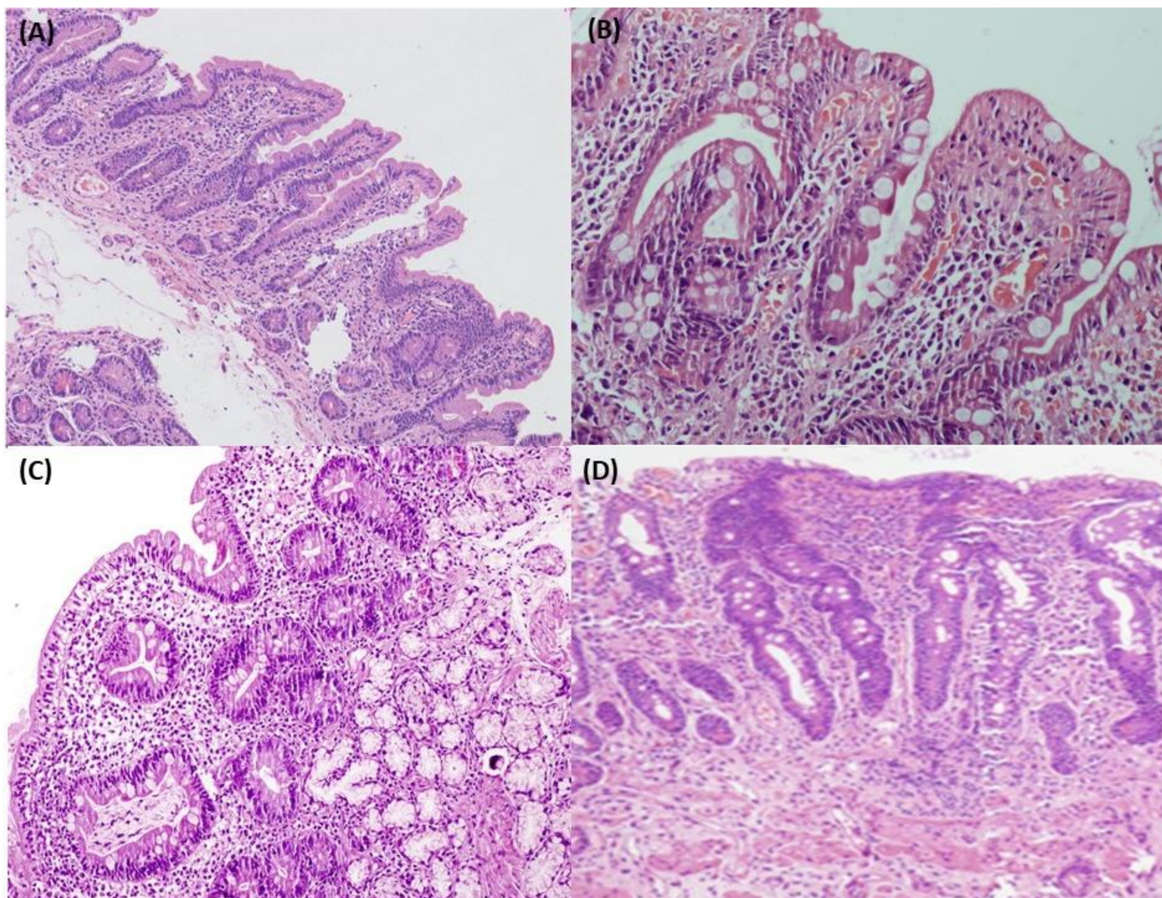

Figure 1: Image depicts the histomorphology of duodenal biopsy tissue in healthy subjects (A) and in CeD patients. Images (B), (C) & (D) depict 3a (mild), 3b (moderate) and 3c (severe) villous atrophy as per modified Marsh grading.

2.3 HLA-Genotyping

One ml venous blood was collected in EDTA vial and DNA extraction was performed by phenol chloroform method with minor procedural modifications and stored at -20°C until analysis [21]. All individuals were evaluated for the presence of HLA DQ2 (DQA1*0501, DQB1*0201, DQA1*0201 & DQB1*0202) and DQ8 (DQA1*0301 & DQB1*0302) genotypes by a rapid PCR-SSP (Polymerase Chain Reaction-Sequence Specific Primer) based identification method. A control fragment of Human Signaling Lymphocyte Activation Molecule Associated protein (HSAP) gene was amplified to check the fidelity of the reaction. Primer sequences for HLA genotyping were adopted from Olerup O et al. 1993, Doolan A et al. 2005 and Scola L et al. 2008 [22–24] (Table 1). Reactions were performed in 25 µl of volume and run over Veriti (Applied Biosystems, CA, USA) thermal cycler. Amplified products were run over 3% agarose gel electrophoresis and visualized on Gel Doc EZ system using Image Lab software, version 5.0 (BioRad, CA, USA).

Gene/Allele	Direction	Sequences	Product size
HSAP	Forward	5'-GTGGTTGGAGGCAGATACAATATGG-3'	435 bp
	Reverse	5'-GCTAAACAGGACTGGGACCAAAA-3'	
*DQA1*0501*	Forward	5'-AGCAGTTCTACGTGGACCTGGGG-3'	149 bp
	Reverse	5'-GGTAGAGTTGIGAGAGCGTTTAATCAGA-3'	
*DQB1*0201*	Forward	5'-CGCGTGCGTCTTGGATGAGCAGAAG- 3'	108 bp
	Reverse	5'-GGCGGCAGGCAGCCCCAGCA-3'	
*DQA1*0201*	Forward	5'-ACGGTCCCTCTGGCCAGTT-3'	170 bp
	Reverse	5'-CAGGATGTTCAAGTTATGTTTTAG-3'	
*DQB1*0202*	Forward	5'-TCCGGTGGTTTCGGAATGG-3'	146 bp
	Reverse	5'-GTGCTCCACGTGGCAGGT-3'	
*DQA1*0301*	Forward	5'-TTCACTCGTCAGCTGACCAT-3'	183 bp
	Reverse	5'-CAAATTGGACGGGTCAAATCTTCT-3'	
*DQB1*0302*	Forward	5'-GACGGAGCGCGTGCGTTA-3'	122 bp
	Reverse	5'-AGTACTCGGCGTCAGGCG-3'	

Table 1 depicts the primer sequences and amplified product size for HSAP gene and HLA alleles

2.4 Statistical analysis and costing

Statistical analysis was performed using SPSS software, version 23. Data distribution was checked by the Shapiro-Wilk test. Qualitative variables were depicted as percentages and calculated using the standard formulae. The difference among the groups were calculated by Fisher Exact Test and Chi square test. Quantitative variables were expressed in Median (First and Third quartile, Q1-Q3). The difference among the groups was calculated by Mann-Whitney U test. A significance level was interpreted at p value <0.05 with a confidence interval of 95 %. We also assessed the cost for HLA genotyping per haplotype using an in-house PCR-SSP approach. We evaluated the consumption of reagents and genotyping cost per sample was calculated. Instrument and labor cost was excluded from the calculation.

3 Results

A total of 4719 cases with a strong clinical suspicion of CeD were screened, of which 558 (11.82%) index cases were confirmed. Figure 2 depicts the graphical representation of the increasing trends in CeD diagnosis from 2014 to 2019. The group was comprised of 297 (53.2%) females and 261 (46.8%) males (mean age at diagnosis, 86.08 ± 45.41 months; age range 13.00 - 216.00 months). The characteristic histopathologic grading confirmed the disease state, but the HLA genotypes of 21 cases were not available; hence these were excluded, and the remaining 537 cases were evaluated in the study and their FDRs were screened. Further, 1565 FDRs were recruited in the study and 145 observations were not included (58 lost to follow up cases, no serology data of 62 subjects at baseline and 25 FDRs who transformed to overt CeD positive on serial follow up). Off the remaining 1420 evaluated FDRs, 236 (18.52%) were serology positive at initial contact and 222 (15.63%) were also confirmed by histopathology analysis. 1157 serology negative FDRs were used for comparative analysis in the study as the incidence of CeD development in this subset was considered low. Figure 3 depicts the study flow.

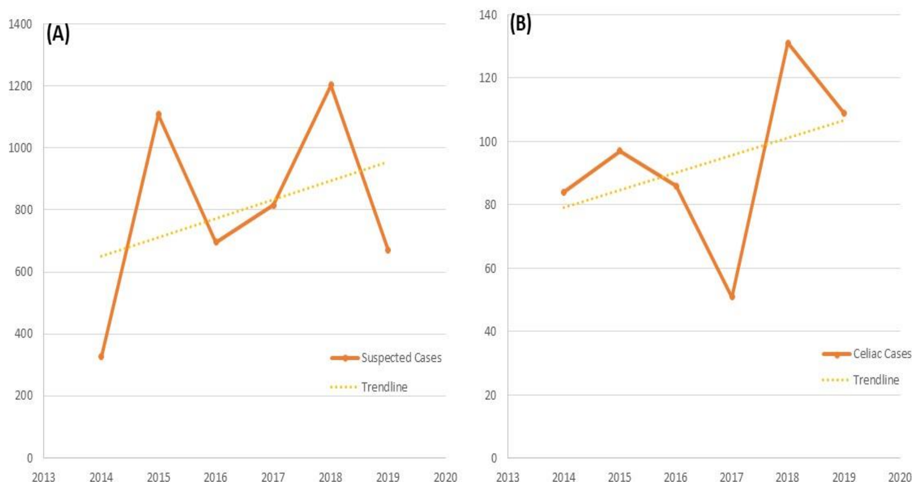

Figure 2: The line diagram depicts the year wise distribution of suspected and confirmed cases with CeD. (A) represent the number of suspected cases per year; 327 cases in 214, 1109 in 2015, 696 in 2016, 815 in 2017, 1203 in 2018 and 669 cases till July 2019. (B) represent the number of biopsy confirmed CeD cases; 84 in 2014, 97 in 2015, 86 in 2016, 51 in 2017, 131 in 2018 and 109 in 2019. Trendline represents the increasing trend of suspected and confirmed CeD cases in corresponding charts

3.1 Frequency distribution and association of HLA genotype in index cases and FDRs

The comparative analysis of the frequency of HLA genotypes in patients with CeD and total FDRs revealed HLA DQ2 genotypes being frequently present in 515 (95.95%) index cases and 1250 (82.9%) FDRs ($p<0.001$) followed by HLA DQ8 genotype were observed in 59 (11.0%) index cases and 65 (4.3%) FDRs ($p<0.001$). A small fraction of the index cases (6.95%) carried both HLA DQ2 and DQ8 genotype. The HLA DQ2 genotype, further subclassified into HLA DQ 2.5 and HLA DQ 2.2 subtypes were observed in 508 (94.6%) vs 1220 (81.02%), ($p<0.001$) and 162 (30.02%) vs 376 (25.0%), ($p=0.051$) in index cases and FDRs respectively. The frequency of individual alleles of HLA DQ2.2 (DQA1*0501 and DQB1*0201), DQ2.5 (DQA1*0201 and DQB1*0202) and DQ8 (DQA1*0301 and DQB1*0302) were observed as 453 (84.4%) vs 1036 (68.7%), 464 (86.4%) vs 911 (60.5%), 121 (22.5%) vs 327 (21.7%), ($p=0.967$) and 59 (11.0%) vs 87 (5.8%), 54 (10.1%) vs 63 (4.2%), ($p=<0.001$) and 9 (1.7%) vs 5 (0.3%), ($p=0.004$) in index cases and FDRs respectively. The detailed CeD predisposing HLA-DQ genotypic distribution among CeD patients and comparison with the frequency in the serology negative subjects group is tabulated in Table 2. Figure 4 depicts the frequencies of HLA genotypes among index cases, total FDRs, biopsy proven and serology negative FDRs.

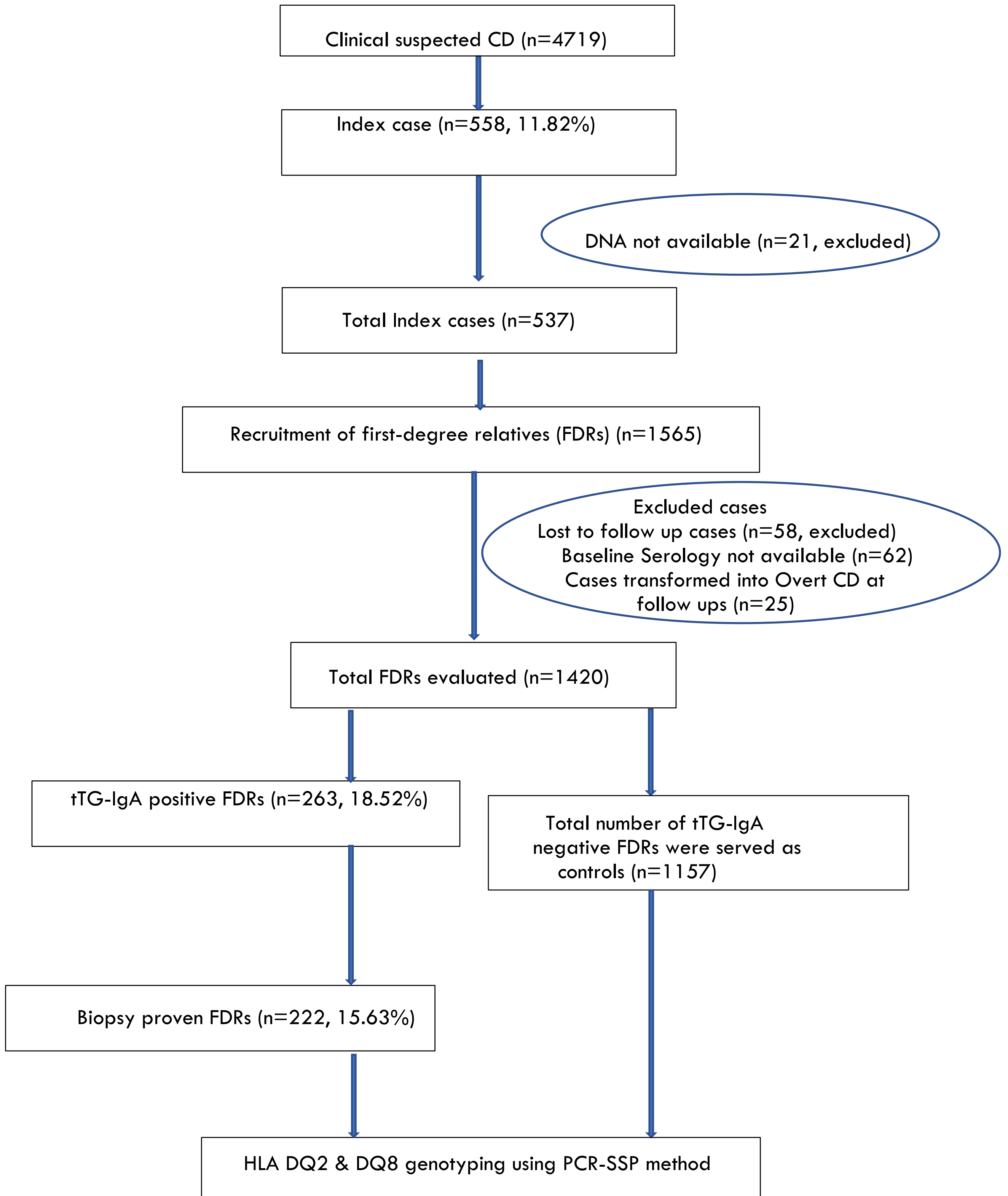

Figure 3: Algorithm depicts the study flow; enrolled cases, inclusion and exclusion of the numbers of cases and their corresponding FDRs

HLA genotype	Index case (n=537)	tTG-IgA negative FDRs (n=1157)	Biopsy proven FDRs (n=222)	p-value
DQ2	515 (95.9%)	955 (82.5%)	193 (86.9%)	<0.001
DQ2.5	508 (94.6%)	929 (80.3%)	192 (86.5%)	<0.001
DQ2.2	162 (30.2%)	289 (25.0%)	48 (21.6%)	0.039
DQ8	59 (11.0%)	50 (4.3%)	10 (4.5%)	<0.001
*DQA1*0501*	453 (84.4%)	783 (67.7%)	173 (77.9%)	<0.001
*DQB1*0201*	464 (86.4%)	675 (58.3%)	160 (72.1%)	<0.001
*DQA1*0201*	121 (22.5%)	252 (21.8%)	40 (18.0%)	0.348
*DQB1*0202*	59 (11.0%)	65 (5.6%)	15 (6.8%)	<0.001
*DQA1*0301*	54 (10.1%)	49 (4.2%)	9 (4.1%)	<0.001
*DQB1*0302*	9 (1.7%)	1 (0.1%)	3 (1.4%)	<0.001

Table 2 depicts the distribution of HLA genotype in index cases, tTG-IgA negative and positive FDRs

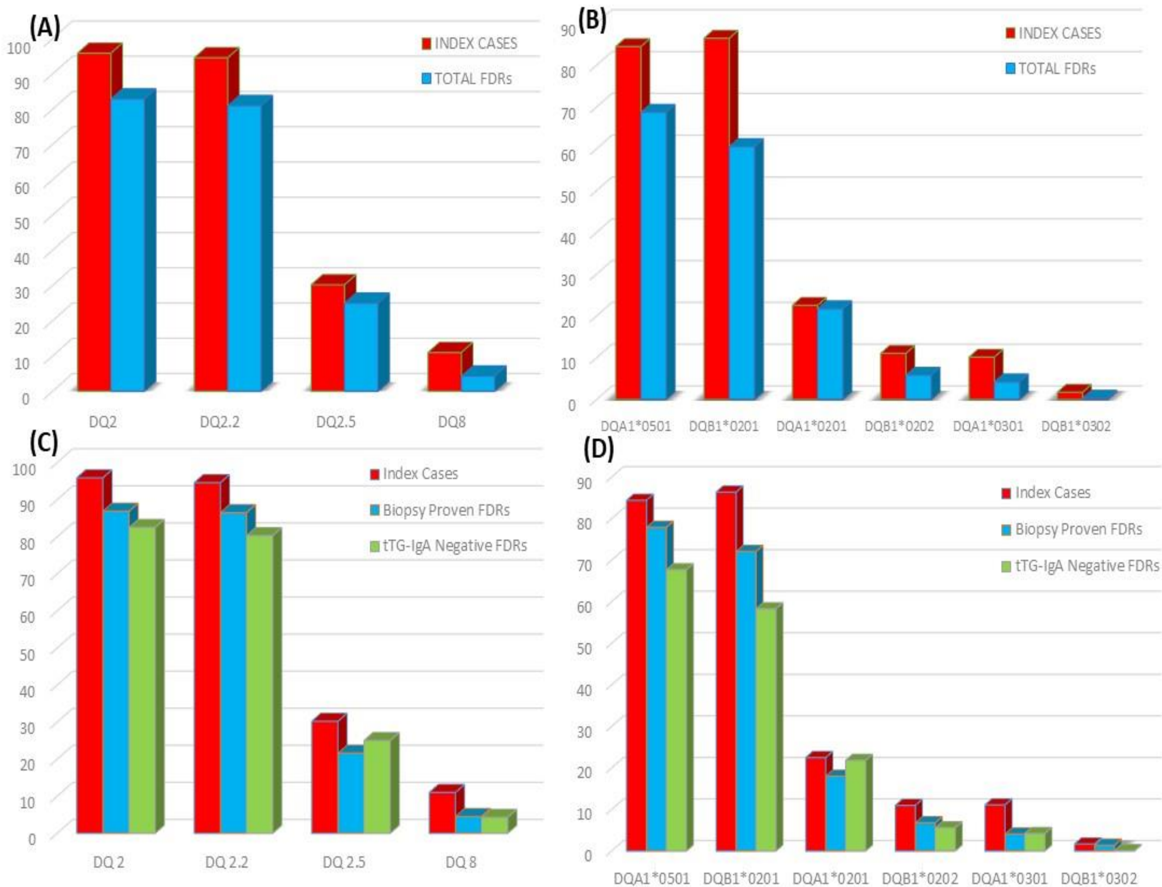

Figure 4: Bar diagram depicts the frequency of HLA genotypes in CeD cases and FDRs. (A) and (B) depicts the comparison in HLA frequencies between index cases and total FDRs whereas (C) and (D) depicts the comparison between index cases, biopsy proven FDRs and tTG-IgA negative FDRs. Charts represent a decreasing trend in frequencies from DQ2 to DQ8 in every study groups

3.2 Association between HLA genotype and tTG-IgA serology

a) Frequency distribution of HLA genotype among serology positive and negative cases

The analysis was performed between 753 serology positive subjects and 1189 serology negative subjects. Most frequent genotype was HLA DQ2, observed in 703 (93.4%) serology positive subjects and in 984 (82.8%) serology negative subjects (p<0.001). Presence of HLA DQ2.2 and DQ2.5 haplotype of HLA DQ2 genotype were observed in 208 (27.6%) vs 305 (25.7%), (p=0.563) and 695 (92.3%) vs 956 (80.4%), (p<0.001) in serology positive and negative subjects, respectively. Presence of individual alleles DQA1*0501, DQB1*0201, DQA1*0201, DQB1*0202, DQA1*0301 and DQB1*0302 in serology positive and negative subjects were observed in 621

(82.5%) vs 806 (67.8%) subjects (p<0.001), 619 (82.2%) vs 699 (58.8%) subjects (p<0.001), 160 (21.2%) vs 266 (22.4%) subjects (p=0.356), 73 (9.7%) vs 67 (5.6%) subjects (p=0.002), 64 (8.5%) vs 50 (4.2%) subjects (p<0.001) and in 12 (1.6%) vs 2 (0.2%) subjects (p<0.001) respectively.

b) Comparison of HLA genotype with tTG-IgA levels

The Median (IQR) overall tTG-IgA levels in study subjects, index cases, biopsy proven FDRs and in serology negative subjects with positive HLA DQ2 genotype were 4.13 U/ml (0.0-821), 119.0 U/ml (0.3-821), 48.06 U/ml (7.01-227.2) and 1.29 U/ml, (0.0-7.0), respectively. In subjects with negative DQ2 genotype similar trends were observed i.e., 2.08 U/ml (0.08-604.6), 123.25 U/ml (23.58-250), 12.18 U/ml (7.41-604.6) and 1.27 U/ml (0.08-6.98), respectively. A statistically significant difference was observed in overall cases (<0.001) and in biopsy proven FDRs (p=0.043), while there was no difference between the index cases (p=0.898) and tTG-IgA negative FDRs (p=0.614).

Median tTG-IgA levels in overall study cases with presence and absence of HLA DQ8 genotype (52.03 U/ml, 0.04-801 vs 3.32 U/ml, 0-821, p<0.00A1), index cases (138.0 U/ml 33.5-801, vs 109.0 U/ml, 0.3-821, p=0.004), biopsy proven FDRs (81.73 U/ml, 8.9-151.74 vs 42.55 U/ml, 7.01-604.6, p=0.518) and also in serology negative subjects were (1.23 U/ml, 0.04-691 vs 1.29 U/ml, 0-7, p=0.331) respectively (Table 3). In figure 5, box and whiskers plot depict the tTG-IgA levels in presence or absence of HLA haplotypes.

HLA genotype	Anti tTG-IgA levels Median (IQR), U/ml;			
Parameters	Overall cases (1886)	Index cases (n=537)	Biopsy proven FDRs (n=222)	tTG-IgA negative FDRs (n=1157)
DQ 2.2				
Present	3.21 (0-812.3)	101.89 (0.72-812.3)	36.91 (7.12-227.2)	1.24 (0-6.86)
Absent	3.8 (0.02-821)	123.08 (0.3-821)	44.59 (7.01-604.6)	1.3 (0.02-7)
(p-value)	0.959	0.299	0.585	0.198
DQ 2.5				
Present	4.2 (0-821)	115.1 (0.3-821)	48.16 (7.01-227.2)	1.29 (0-7)
Absent	1.8 (0.02-604.6)	132.5 (23.58-450.6)	12.04 (7.41-604.6)	1.21 (0.02-6.98)
(p-value)	<0.001	0.285	0.024	0.836
*DQA1*0501*				
Present	4.46 (8-821)	122 (0.3-821)	47.13 (7.01-227.2)	1.27 (0-7)
Absent	2.31 (0.01-604.6)	100 (3.33-450.6)	20.54 (7.12-607.16)	1.29 (0.01-6.98)
(p-value)	<0.001	0.018	0.083	0.264
*DQB1*0201*				
Present	5.77 (0.01-821)	109.44 (0.3-821)	47.7 (7.08-222.7)	1.38 (0.01-7)
Absent	1.71 (0-812.3)	135.11 (85.56)	23.13 (7.01-604.6)	1.1(0-6.98)
(p-value)	<0.001	0.009	0.133	0.005
*DQA1*0201*				
Present	2.7 (0-812.3)	101 (3.65-812.3)	44.2 (7.12-202.9)	1.23 (0-6.86)
Absent	4.07 (0.02-821)	123.25 (0.3-821)	43.19 (7.01-604.6)	1.3 (0.02-7)
(p-value)	0.176	0.312	0.620	0.217
*DQB1*0202*				
Present	11.3 (0.04-312.5)	119.72 (0.72-312.5)	76.7 (7.48-227.2)	1.19 (0.04-6.63)
Absent	3.5 (0-821)	119.72 (0.3-821)	42.5 (7.01-604.6)	1.29 (0-7)
(p-value)	0.008	0.712	0.282	0.556
*DQA1*0301*				
Present	48.75 (0.04-801)	135 (33.5-801)	71.21 (8.9-142.35)	1.05 (0.04-6.91)
Absent	3.34 (0-821)	110.52 (0.3-821)	42.6 (7.01-604.6)	1.29 (0-7)
(p-value)	<0.001	0.015	0.806	0.373
*DQB1*0302*				
Present	122.33 (2.1-229)	135 (49.51-229)	18.12 (8.9-151.74)	2.1 (2.1-2.1)
Absent	3.63 (0-881)	119.72 (0.3-821)	43.77 (7.01-604.6)	1.29 (0-7)
(p-value)	<0.001	0.369	0.765	0.554

Table 3 depicts the association between HLA genotype and tTG-IgA levels among overall cases, index Case, biopsy proven FDRs and tTG-IgA negative FDRs

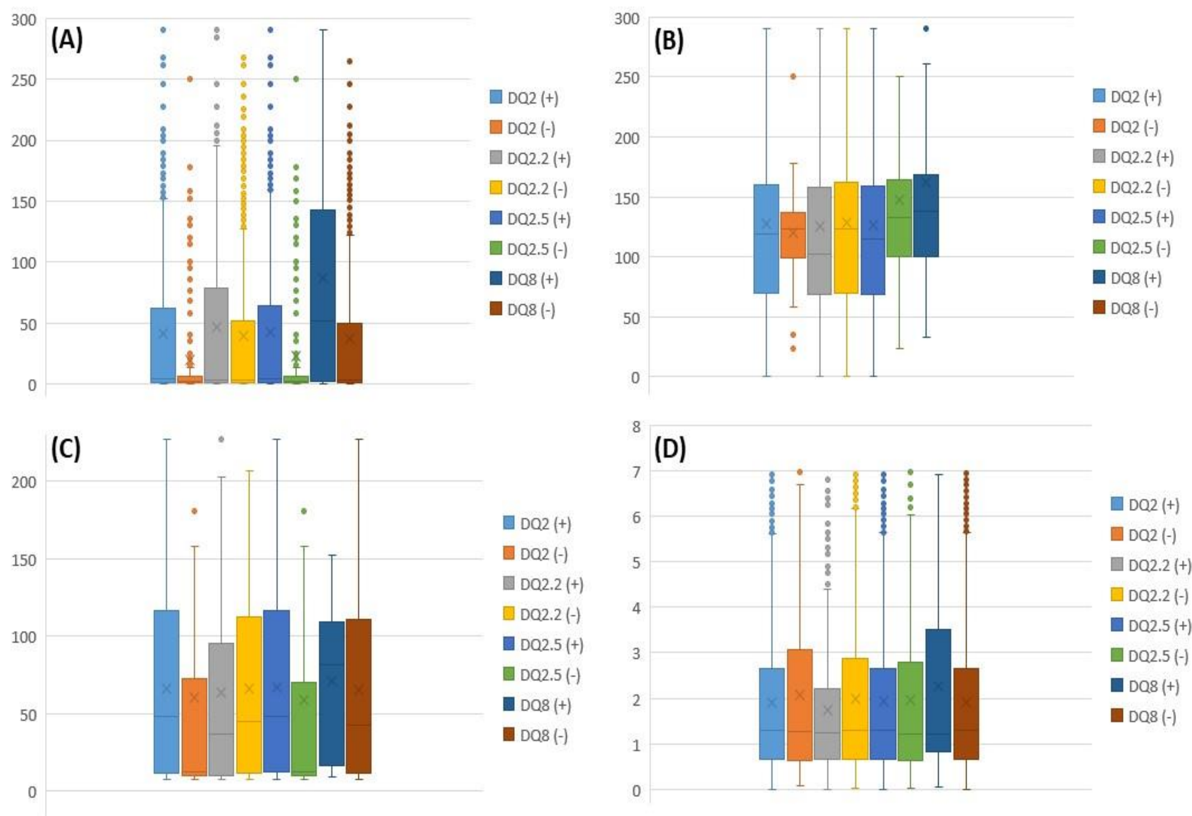

Figure 5: Box and whiskers plot depicts the median (IQR) tTG-IgA levels in cases with the presence of HLA genotype and in those without HLA genotypes. (A) represent the tTG-IgA levels in the presence/absence of HLA genotypes in overall cases, (B) represents the levels in index cases, (C) represents the levels in biopsy proven FDRs and (D) represents the levels in tTG-IgA negative FDRs

3.3 Distribution and association of HLA genotype with histopathology

Subjects with the presence of HLA DQ2 and DQ8 genotypes and their individual alleles were segregated based on their frequency in different Marsh grades.Table 4 shows the distribution of HLA haplotypes according to the classification provided by Rostami et al [25]. Figure 6 depicts the graphical distribution of HLA genotypes among the Marsh grades.

HLA genotype	Grade 2 (n=8)	Grade 3a (n=167)	Grade 3b (n=168)	Grade 3c (n=417)	p-value
*DQA1*0201/DQB1*0202*	1 (12.5%)	47 (28.1%)	40 (23.8%)	122 (29.3%)	0.667
*DQA1*050/ DQB1*0201*	8 (100%)	140 (8.0%)	157 (93.5%)	396 (95.0%)	0.005
*DQA1*0501*	5 (62.5%)	122 (73.1%)	140 (83.3%)	359 (86.1%)	0.008
*DQB1*0201*	8 (100.0%)	121 (72.5%)	139 (82.7%)	357 (85.6%)	0.017
*DQA1*0201*	1 (12.5%)	36 (21.6%)	33 (19.6%)	91 (21.8%)	0.952
*DQB1*0202*	0 (0.0%)	15 (9.0%)	14 (8.3%)	45 (10.8%)	0.817
*DQA1*0301*	1 (12.5%)	7 (4.2%)	11 (6.5%)	45 (10.8%)	0.069
*DQB1*0302*	0 (0.0%)	3 (1.8%)	2 (1.2%)	7 (1.7%)	0.938

Table 4 depicts frequency distribution and association of HLA genotype with Marsh grading in collective biopsy proven subjects.

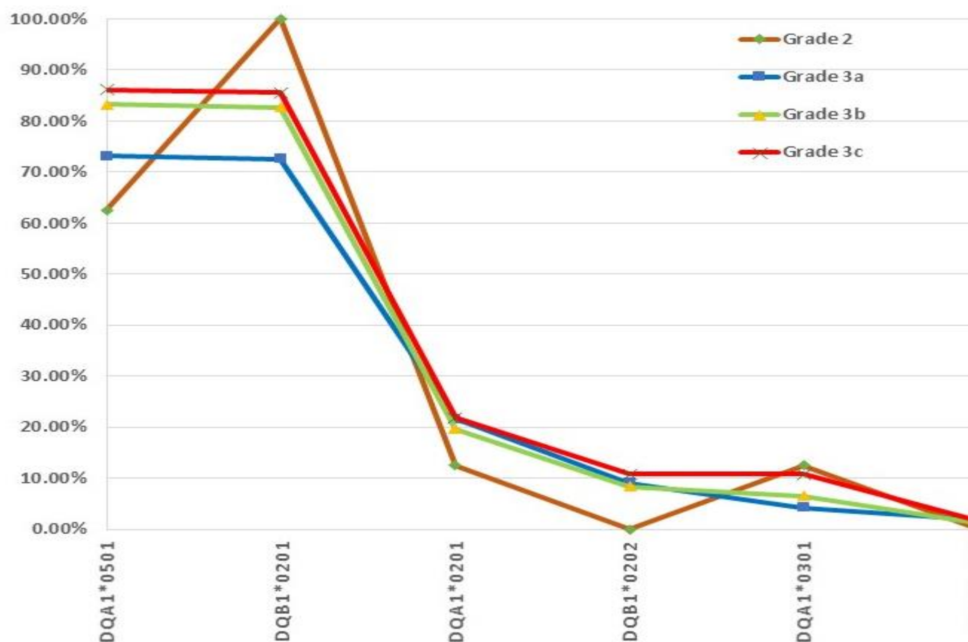

Figure 6: Line diagram depicts the percentage of HLA genotypes in cases with Marsh 2, 3a, 3b and 3c grade villous atrophy. Chart depicts that HLA DQ2 (DQA1*0501 and/or DQB1*0201) genotype is most frequently observed in CeD cases with every Marsh grade

3.4 Analysis of serology positive cases with tTG-IgA levels less 10 times of upper limit of normal range (<10 X ULN)

Median tTG-IgA levels in cases <10 X ULN was 20.51 u/ml (7.01-69.74). Upon histopathology analysis, 5 cases (1.83%) had Marsh grade 2 score, 155 (56.77%) had 3a, 106 (38.82%) had 3b and 6 (2.19%) had grade 3c type mucosal lesions respectively. HLA DQA1* 0501 and DQB1*0201 were the most frequent genotypes observed being 212 (77.65%) and 210 (76.93%). Proportion of other genotypes including DQA1*0201, DQB1*0202, DQA1*0301 and DQB1*0302 was 56 (20.51%), 21 (7.69%), 13 (4.76%), and (3, 1.09%) respectively.

4 Discussion

In our cohort, the point prevalence of CeD among those with a suspicion of CeD was 11.82% in a hospital-based setting. CeD autoimmunity was estimated to be 18.52% in FDRs. However, the prevalence of biopsy confirmed CeD in FDRs was 15.63%. Studies on the prevalence of CeD among FDRs of CeD patients have shown significant variability [26,27]. These are presumably secondary to differences in population biotypic characteristics, methods of diagnosis (biopsy vs serologic testing) and dietary variations. The differing time periods of studies in context of spatial differences due to increased recognition of atypical CeD may also be responsible for the variations. The development of CeD is a dynamic process and the risk of development after the diagnosis of an index case is variable. Considering the genetic susceptibility, it is expected that FDRs may demonstrate an increased prevalence against the baseline and increased propensity to manifest CeD when followed up on a longitudinal time scale [28,29]. Wessels et al reported that the initial seronegative siblings age >10 years were unlikely to develop overt CeD [30]. However, in our study, 60 siblings (4.22%) were initially seropositive in this age bracket and of those initially seronegative (n=201, 14.15%), 7 (3.5%) developed CeD over next 2 years of follow up. The findings from our study need a longer follow up period to evaluate the possibility of overt CeD in FDRs, as reported by Wessels et al that the chances to develop CeD in initially evaluated serology negative siblings with age >10 years are very low. Thus, the longitudinal follow up is of vital importance in the age bracket of children less than 10 years.

HLA DQ2 alleles were most frequently observed in 95% of index cases and 86.9% in histologically confirmed FDRs. HLA DQ8 genotype was observed in 11% of index cases and in 4.5% histologically confirmed FDRs. There was a statistical difference

in the proportion of these 2 genotypes compared to their presence in FDRs ($p<0.05$).

It is pertinent to note that HLA DQ2 and DQ8 genotypes depicted by its frequency has an important role in those with manifest CeD and this highlights the utility of HLA DQ2 and DQ8 genotyping for understanding the severity of the lesion where the tTG-IgA assay is less than 10 times of the upper limit of normal (<10 X ULN). Testing for HLA DQ2/DQ8 genotypes would still be a very useful modality in those CeD cases in whom gluten free diet (GFD) was initiated without histopathological confirmation and complete recovery of the enteropathy happened subsequently. The HLA DQ2 and DQ8 testing would suggest a susceptibility locus with a high degree of sensitivity [28]. The frequency of HLA DQ2 and DQ8 genotypes in our study is in concordance with other studies by various authors [18,23,31–39]. A study from North Indian population reported HLA DQ2 positivity of 85.7% in FDRs however, HLA DQ8 genotype was not present even in a single subject [40]. Mishra et al genotyped 127 biopsy confirmed FDRs and reported that 73% showed the presence of HLA DQ2 and 10.2% had HLA DQ8 genotype [41]. Singla et al reported the presence of HLA DQ2 as 16.7% in homozygous state in and 50% in heterozygous state in biopsy proven 18 FDRs. Similarly, the presence of HLA DQ8 as 5.6% each in homozygous and heterozygous state. The presence of HLA DQ2/DQ8 was reported in 22.2% cases. Collectively, the presence of HLA DQ2 and DQ8 genotypes was observed in 100% of the FDRs [42].

Overall, HLA DQB1*0201 ($p=0.08$), DQA1* 0301($p<0.001$) and DQB1*0302 ($p<0.001$) were observed to be associated with elevated tTG-IgA levels. It was also observed that tTG-IgA levels were highly elevated in the presence of DQB1*0302 followed by DQA1*0301 and DQB1*0201. Moreover, in the absence of these alleles, the tTG-IgA levels were less than the reference range. Similarly, in biopsy proven FDRs it was observed that the tTG-IgA levels were 4 times more with the presence of HLA DQ2.5 genotype ($p=0.024$). This denotes that HLA DQB1*0201, DQA1*0301, DQB1*0302 alleles and DQ2.5 genotype were good predictors of increased serology levels. Mills et al conducted a study to observe the relationship between HLA DQA1/DQB1 genotype, IgA deficiency and celiac serology on 13,358 adult patients and reported that homozygous genotype, HLA DQ2.5 or HLA-DQ2.5/DQ2.2 were the strongest predictor of positive tTG-IgA serology ($p<.0001$). The study also concluded that screening with tTG-IgG has low sensitivity and specificity in those cases carrying single HLA DQ2.5 allele [43]. However, the significance of individual alleles in the cohort was not reported.

The comparative analysis between the enteropathy severity as illustrated by Marsh grading and HLA genotypes revealed higher proportion of subjects with Marsh grade 3c were DQ2 positive (413, 96.4%). Decreasing trends in number of subjects were noted in the descending order from 3c to grade 2 ($p<0.001$). Similar trends were observed with HLA DQ2.5 ($p=0.005$), DQ8 ($p=0.042$), DQA1*0501 ($p=0.008$) and DQB1*0201 (0.017) as depicted in table 4. In all the described genotypes, highest number of subjects were noted with Marsh 3c grade. Our findings are in synchrony with other studies. Murad H et al reported the frequency of DQ2 and DQ8 were highest in Marsh III type lesion in patients with CeD. The frequency of HLA DQ2 and DQ8 in Marsh I, II, II lesion was 9.5% and 0%, 23.8% and 4.8%, 52.4% and 9.5% in subjects with CeD respectively [35]. Our results may have greater translational value as we genotyped a robust sample size of 537 index cases with CeD and their 1420 FDRs in comparison to other studies. Amarapurkar DN et al reported the presence of HLA genotype in 13 (38%) subjects with Marsh grade I lesions, 9 (29%) with Marsh grade II lesions and 10 (32%) in grade III lesions. The authors concluded that HLA genotype is predominantly associated with CeD patients in India with the lack of the availability of HLA DQ2/DQ8 genotype testing facility [18].

Ceylan et al reported that the most common Marsh classification was type 4 hypoplastic and 3b destructive type in HLA DQA1*0501 and DQB1*0201 positive patients [44]. Our results suggest that HLA genotype DQ2, DQ8 and DQ2.5 subtypes with individual alleles DQA1*0501 and DQB1*0201 are strong predictors of severe mucosal lesion in patients with CeD. This of great importance as HLA is a non-invasive approach to ascertain the degree of mucosal injury in FDRs with a high risk of CeD development. HLA status can also delineate degree of compliance with tTG-IgA serology at specific time interval may thus be good in conjunction with genotyping.

We have also performed the analysis between the tTG-IgA positive cases who had levels <10 X ULN to seek the utility of HLA DQ2 and DQ8 genotype testing in view of sparing invasive approach. HLA DQA1*0501 and DQB1*0201 alleles were more frequent in cases with tTG-IgA levels <10 X ULN when compared to tTG-IgA negative FDRs. There

were no differences observed in frequencies of HLA DQA1*0201, DQB1*0202, DQA1*0301 and DQB1*0302 between the cases with tTG-IgA <10 X ULN and seronegative FDRs.

The main limitation of HLA typing for DQ2 and DQ8 genotypes in CeD patients is the cost involved in the testing. Kumar P et al estimated the approximate cost of HLA testing at US$350 and this was substantially less than the combinations of serology, endoscopy and duodenal biopsies ($2000) [19]. Shrivastava et al reported a single test for HLA DQ2 and DQ8 was worth of $104 using a commercially available kit [40]. In the present study, we also assessed the cost for HLA genotyping using an in-house PCR-SSP approach. We evaluated per sample cost of genotyping was approx. $11.77 which is lower than endoscopy and biopsy procedure (approx. $75) in the Indian scenario. The total time spent in genotyping by using PCR-SSP method is 3 hours/ haplotype (excluding DNA extraction). PCR-SSP method also facilitates large scale genotyping in a single run and thus is a promising approach for HLA typing. PCR-SSP method is time saving and cost effective.

Our study represents one of the largest cohorts from North Indian population with HLA DQ2 and DQ8 genotyping. The strength of our study is robust sample size and sequential follow up of the subjects. The potential limitations include false positivity, requirements for more DNA sample and HLA DQ2 and DQ8 genotyping by PCR-SSP method is not a confirmatory test. The confirmatory approach is sequencing which is labor intensive, tedious and costs almost 2 times more (US$20) per test. In low resource settings with high disease prevalence, PCR-SSP approach is therefore a feasible method of genotyping. Because of the referral pattern of our tertiary care center, we enrolled predominantly Northern Indian population with a high index of suspicion of CeD. However, in community settings, the applicability of the HLA DQ2/DQ8 genotypic testing needs further exploration. Another limitation is that we enrolled FDRs as controls in our study, who were serology negative at initial evaluation however, we evaluated only those FDRs who remained persistently negative for 24 months in the final analysis. We also did not rule out IgA deficiency which could have affected our correlation with tTG assay which is predominantly an IgA based assay.

5 Conclusions

Our findings suggest that HLA DQA1*0501 and DQB1*0201 alleles were the most frequently observed in patients with CeD and in their FDRs. These alleles were the strongest predictors of tTG-IgA seropositivity and severity of mucosal lesions. Our study also supports the use of in-house PCR-SSP method for screening of HLA DQA1*0501 and DQB1*0201 alleles in high-risk groups for early detection. When performed in-house using PCR-SSP approach, the assay was economical in identification of mucosal severity in index cases and the FDRs. Its value as a model for additive predictive value in the diagnostic algorithm needs further evaluation. Though its role is both for prediction of severity in index cases and FDRs, the other side of coin needs further studies to evaluate the biopsy sparing approach in cases with tTG-IgA <10 X ULN as evidenced by cascade screening.

Conflicts of Interest Statement: The authors have no conflicts of interest to declare.

Acknowledgment: We acknowledge the staff of the Genome Sequencing Lab, Genetic division, Department of Pediatrics for their constant support during the study especially Mrs. Jyotsana Gour, Himani Vashistha and Vinita Bansal for the technical help in the study. We are also thankful to DBT, Delhi for the initial funding of 1 year as a proof-of-concept study (vide grant number BT/PR6635/PFN/20/767/2013).

Author contributions: Concept: SK, AL; Design: SK, AL; Supervision: RS, PK, VVB, APD, ASP, SKP, SK; Resources: RS, PK, VVB, APD, ASP, SKP, SK, BD; Materials: AL; Data Collection and/or Processing: AL; Analysis and/or Interpretation: AL; Statistical analysis; RG; Literature Search: AL; Writing Manuscript: AL, SK, BD; Critical Review: SK, RS, BD

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