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The use of indirect immuneofluorescent test in the diagnosis of coeliac disease

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SUMMARY

Objectives: Study the indirect immunofluorescent (IIF) profiles of the coeliac disease (CD)-specific auto antibodies and asses their role in the establishment of the diagnosis of this disease.

Material and Methods: Fifty consecutive children and adults patients suspected to have CD were studied. They were recruited from Ibn-Sina Teaching Hospital and Al-Wafaa Center for Endocrine Diseases from Mosul City during the period from January 2013 to April 2014. From each patient ten milliliters (ml) of blood was collected. The relevant laboratory tests including liver function test, renal function tests, and urinalysis were conducted. The remaining of the separated serum was kept in clean tubes and stored at -20^{o}C until use for IIF tests. The tested auto antibodies IIF technique included anti-deaminatedgliadin peptide (DGP) and anti-endomysial (EM) auto antibodies of IgA class.

Results: The classical symptoms were found to be the most common complaints reported. Failure to gain weight was common in the infant-preschool age group. Twenty out of fifty (30%) patients had growth retardation and/or short stature and found mostly among school and adolescent age groups. The DGP and anti-EM auto antibodies of IgA class were detected in 29/50 (58%) and 31/50 (62%) patients with CD respectively. Strong IIF intensities of anti-EM autoantibodies were found to be correlated with advanced mucosal architecture affection.

Conclusions: Simultaneous determination of the auto antibodies against endomysium and deaminated gliadin peptide helps in the establishment of the diagnosis CD. Dual positivity for the two antibodies and/or the degree of fluorescent intensity of anti-EMA have an association with the degree villous atrophy.

Key words: Coeliac disease, Indirect immunofluorescent, Endomysial antibodies.

INTRODUCTION

The European Society for Pediatric Gastroenterology, Hepatology and Nutrition proposed that coeliac disease (CD) couldbe defined as follows, "…an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals and characterized by a variable combination of gluten-dependent manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy" [1]. Gluten is a protein that appears in wheat, barley, rye and oat, compositing of prolamin and glutelin. The majorityof the proteins in food responsible for the immunereaction in CD are the prolamins. Because of their high glutamine contentand specific sequence patterns, prolamins are resistant to gastrointestinal proteolytic enzymes and are excellent substrates for deamidation by tissue transglutaminase [2].

Typical indications for serologic testing includeun explained bloating or abdominal distress; chronicdiarrhea, with or without mal absorption or the irritable bowel syndrome; abnormalities on laboratorytests that might be caused by malabsorption (e.g., folate deficiency and iron-deficiencyanemia); first-degree relatives with CD; and autoimmune diseases and other conditions known to be associated with CD [3,4].The most sensitive antibody tests for the diagnosis of CD are of the IgA class. The available tests include those for antigliadin antibodies (AGA), connective-tissue antibodies including anti-reticulin (ARA) and anti-endomysial antibodies, and antibodies directed against tissue transglutaminase (tTG) [3]. Since Serological tests are fundamental and the first step in the diagnosis of CD [5,6]. The current work is aimed to study the IIF profiles of the CD-specific autoantibodies and asses their role in the establishment of the diagnosis of CD.

SUBJECT, MATERIALS AND METHODS

Fifty patients suspected to have CD were studied. They were recruited from Ibn-Sina Teaching Hospital Al-Wafaa Center for Endocrine Diseases, Mosul, Iraq during the period from January 2013 to April 2014. All patients were first interviewed and examined clinically according to a special Questionnaire Form. Furthermore, all patients underwent small bowel biopsies and blood sample collection. Histopathological evaluation was carried out in the hospital's pathology unit of Al-jomhori Teaching Hospital. At the time of the study, the results of the previous screening tests by anti-tissue transglutaminase antibody assay were neglected to avoid selection bias. Patients with IgA deficiency and patients on gluten-free-diet were excluded.

Materials

All laboratory kits and reagents used throughout this study were purchased from Euroimmun company, Medizinische, Labordiagnostika AG, Lubeck, Germany. The used slides were consisted of 2 Biochips per field including liver monkey and gliadin (GAF-3X) BIOCHIPs.

Sample collection and processing

A sample of 10 ml blood was obtained from anti-cubital vein by sterile disposable syringes. Two milliliters were collected in tubes containing ethylenediamine tetraacitic acid (EDTA) to be used for performing complete blood count and ESR. The remaining blood was poured into plane tubes without anticoagulant and left for about half an hour, then centrifuged. The serum was separated by micropipette and kept in clean tubes. They stored at -20^{o}C until use for IIF tests. In addition, relevant laboratory tests including liver function test, renal function tests, and urinalysis were also conducted. The sample to be investigated have been diluted 1:10 in PBS-tween. Therefore, 11.1 µl of the sample were diluted in 100µl of PBS-tween in a polystyrene tube and mixed thoroughly using vortex for 4 seconds. The controls were ready to use after dilution with PBS-tween (1:10).

Titer plane Technique

Samples have been applied to the reaction tray. The Biochip slides were then placed into the recesses of the titer plane, where all Biochip's of the slides come into contact with the samples, andthe individual reactions commence simultaneously. If a positive reaction is obtained, specific antibodies of classes IgA, IgG and IgM attach to the antigens. In a second step, attached antibodies are stained with fluoresce in-labeled anti-human antibodies and made visible with fluorescence microscope. The fluorescence was read with the microscope initially in objective 20X and then focused using 40X(excitation filter: 488 nm, color separator: 510 nm, blocking filter: 520 nm, blue light).

Interpretation of the results

In primate liver: in case of positive sample, filamentous lining of the interlobular sinusoids reacted. Green circular areas were fluoresced against a dark background in case of gliadin (GAF-3X)-specific autoantibodies positive samples. In case of negative reaction, the entire gliadin (GAF-3X) biochip remained dark, the green circular areas described were not or hardly recognizable.

Data analysis

Basic descriptive statistics, including means, SDs, range, and percentages were used to characterize the study cases[7].

RESULTS

The demographic data, clinical characteristics and antibody profiles of patients with CD are shown in Table 1. Fifty patients (23 males and 27 females) suspected to have CD were studied. The age of patients was ranged from 2.5 to 38 (12.87 ± 7.53) years. The patients were classified into four age groups including, 7(14%) patients from infant-preschool age group (0-5), 15(30%) patients from school-age group (6-11), 20 (40%) patients from adolescent-age group (12-18) and 8 (16%) patients from adult-age group (> 18).

The classical symptoms (abdominal pain, distension, diarrhea and change in the stool pattern) were found to be the most common complaints reported. These complaints were more pronounced in the 1st two groups (infant-preschool age and school age groups). In addition, failure to gain weight were common in the infant-preschool age group. Twenty out of fifty (30%) patients had growth retardation and/or short stature (< to the third percentile for age) and found mostly among school and adolescent age groups. Fifteen out of these twenty patients had no gastrointestinal symptoms (i.e., short stature is the only presenting feature). Isolated iron deficiency anemia resistant to treatment was reported in three adult cases (referred by hematologists). Three out of fifty (6%) patients were found to have insulin dependent diabetes mellitus(IDDM). In addition, two female (17-years and 20-years old) patients were found to have

cryptogenic hepatitis. Elevated liver enzyme (alanine transaminases) was evident in the two cases. Moreover, jaundice was found in one patient who was also positive for anti-liver kidney microsomal (anti-LKM) antibodies. Liver biopsy was not available for the two cases.

All patients were referred for small intestinal biopsy at the hospital's Gastroenterology Unite. Histological evaluation was performed according to the Marsh classification system [8]. The following histological findings were obtained: (a) ten (20%) patients had normal mucosa (Marsh type 0); (b) ten (20%) patients had normal mucosal architecture with epithelial lymphocyte infiltration (Marsh type I); (c) seven (14%) patients had Inflammation, villous blunting and increased crypt:villous height ratio (hyperplastic) (Marsh type II); (d) twenty three (46%) patients with Marsh III lesions had severe inflammation, flat villi and hyperplastic crypts (destructive). Mixed Marsh II and Marsh III lesions were documented in one patient. Marsh type I, II and III were found to be celiac-related pathology, whereas Marsh type II and III lesions were considered to be diagnostic for CD.

The primate liver and gliadin (GAF-3x) Biochips were used for the detection of anti-endomysial (anti-EM) and anti-deaminated gliadin peptide (anti-DGP) antibodies (IgA class) respectively (Figure 2 and 3). The anti-EM antibodies were detected in 31/50 (62%) patients. Moreover, the anti-DGP antibodies were detected in 29/50 (58%) patients (i.e., dual positivity was detected in 29 cases). Twenty seven patients with biopsy-proven CD (Marsh II and Marsh III) and four patients with minimal pathology (Marsh I) were found to have anti-DGP and/or anti-EM antibodies. Nineteen (38%) patients were found to have negative serology for both antibodies. Six out of these nineteen patients had a Marsh I lesion, two patients had a Marsh II lesion and one patient had a Marsh IIIlesion, while the diagnosis of CD could be excluded in the remaining ten patients with normal mucosal architecture and negative serology. The patients with Marsh type I lesion including the six patients with negative serology (because of strong clinical suspicion of CD) were put on a trial of gluten free diet for further evaluation.

Three fluorescent intensities of anti-EM autoantibodies in relation to the autoantibody titers were identified including, weak, moderate and strong (Figure 1). The strong fluorescent intensities were found to be correlated with advanced mucosal architecture affection.

Table 1: Demographic data, clinical characteristics and antibody profiles of patients with CD.

Demographic data	Variable
Number of patients	50
Sex (M/F)	23/27
Age, range (mean) years	2.5–38 (12.87 ± 7.53)
0-5 years	7 (14)
6-11 years	15 (30)
12-18 years	20 (40)
> 18 years	8 (16)
Clinical characteristics	**No. (%)**
Classical (typical) symptoms	
Abdominal cramps/distention	23 (46)
Diarrhea/change in stool pattern	21 (42)
Vomiting	5 (10)
A typical symptoms (secondary to malabsorption)	
Growth retardation/short stature*	20 (40)
Unexplained anaemia§	3 (6)
Recurrent aphthous stomatitis¥	1 (2)
Associated conditions	
IDDM	3 (6)
Cryptogenic hepatitis	2 (4)
Linear IgA dermatosis	1 (2)
Biopsy results	
Marsh 0	10 (20)
Marsh I	10 (20)
Marsh II	7 (14)
Marsh III	23 (46)
Autoantibodies profile	
Anti-gliadin **(**GAF-3X) (IgA class)	29 (58)
Anti-endomysial (primate liver) (IgA class)	31 (62)

*:< to the third percentile for age, §: Iron deficiency, ¥:referred by a pediatrician. IDDM : insulin dependent diabetes mellitus.

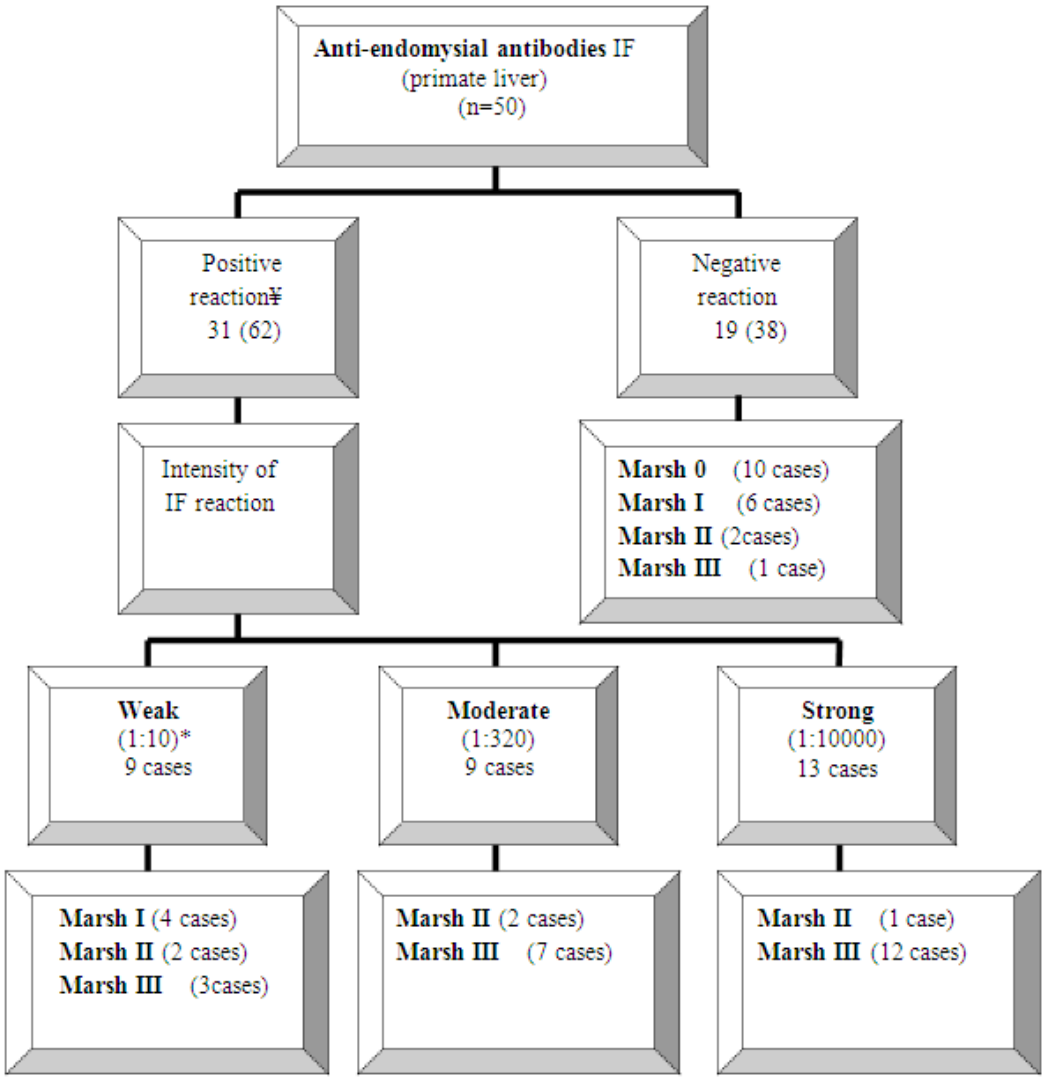
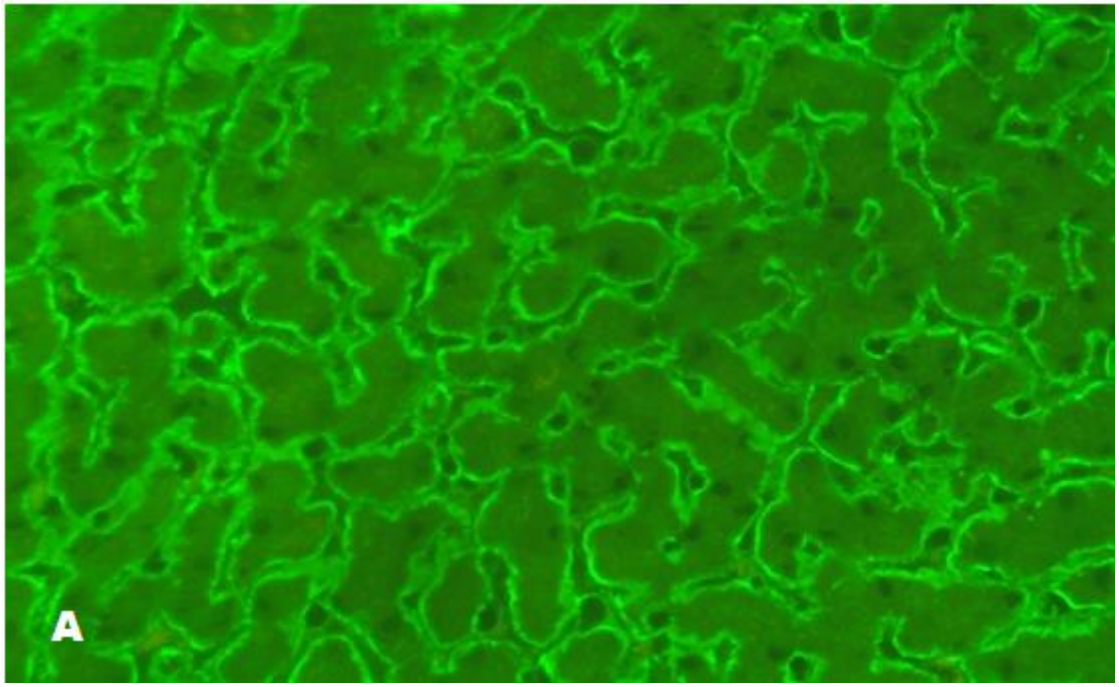


Figure 1: The different profiles of anti-endomysial antibodies and their relations to the results of small intestinal biopsy in patients with CD. ¥: all patients had anti-DGP antibodies except two patients (one had a Marsh I lesion and other had Marsh II lesion).*: titer.



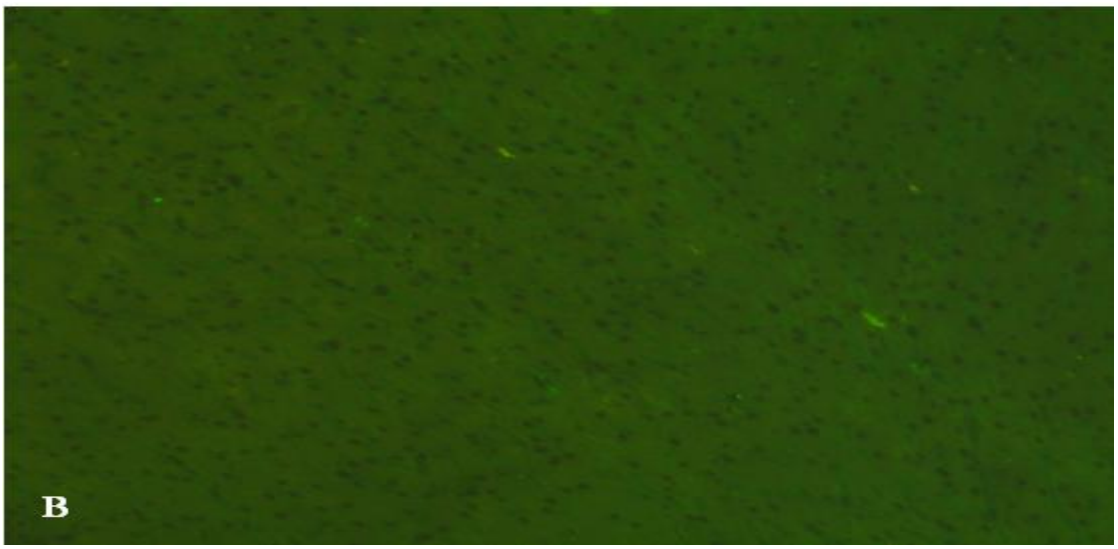


Figure 2: Primate liver: antibodies against endomysium. In case of positive reaction, the filamentous linings of the intralobular sinusoids react (A). In case of negative reaction, no fluorescent staining can be detected (B).

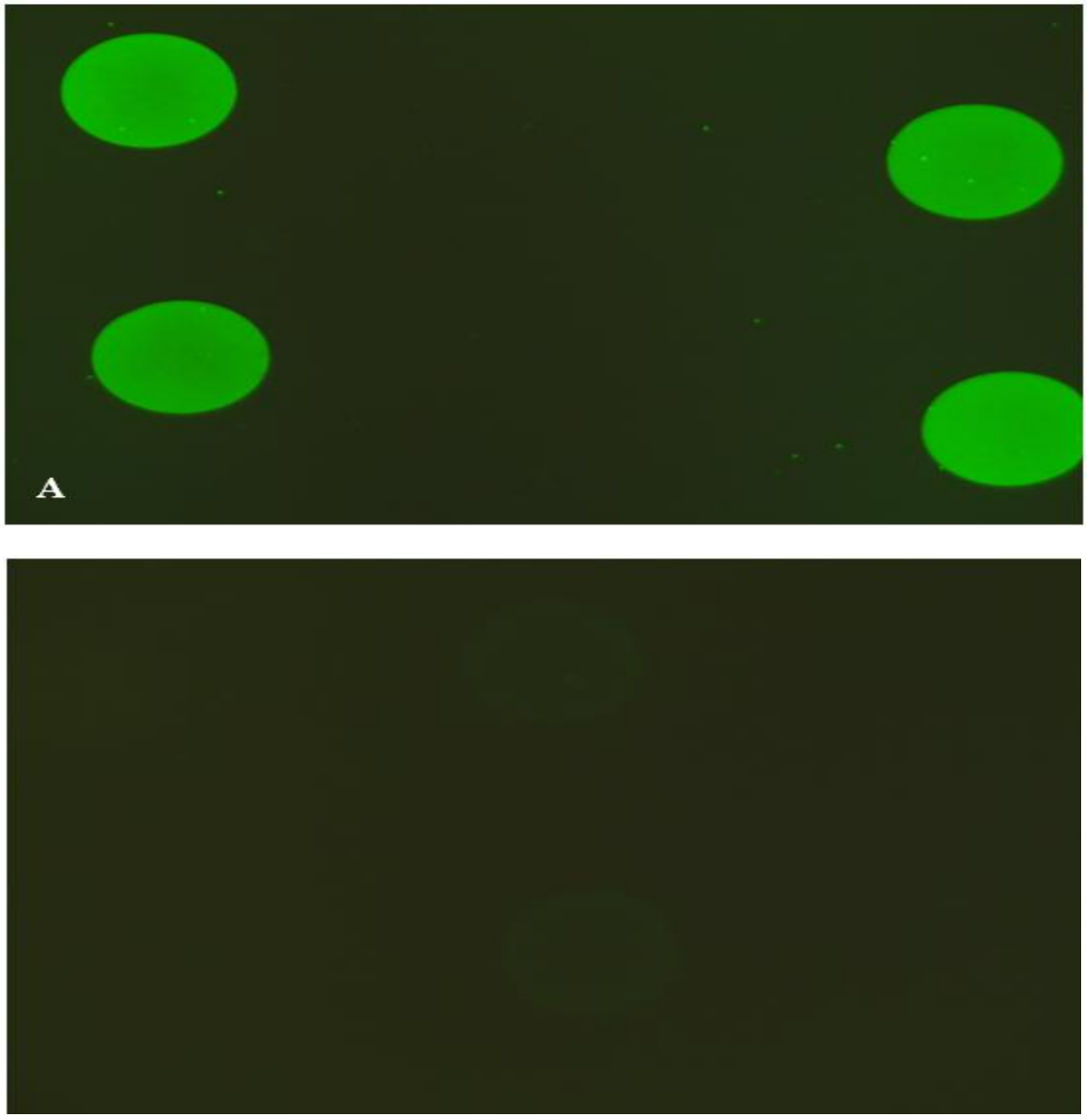


Figure 3: Gliadin (GAF-3X): fine droplets gliadin. In case of positive reaction, the circular areas show a green fluorescence in front of a dark background (A). The entire intrinsic Biochip is dark, the circular areas can hardly be detected or not at all in case of negative reaction (B).

DISCUSSION

Gluten sensitive enteropathy is an autoimmune disease which occurs in genetically predisposed individuals by the ingestion of gluten and related proteins. It is associated with different serious complications including for e.g., malabsorption, short stature and anaemia. Furthermore, the anti-EMA and more recently the anti-DGP antibody assays permits the efficient screening of symptomatic and non-symptomatic patients at risk for CD [9,10]. Therefore, a Biochip combination technique of IIF wasused in the current study as screening serological method for clinically suspicious CD cases.

More than two thirds (84%) of patients included in the present study were children and adolescent (12.87 ± 7.53). This may indicate that CD is more commonly found in young age groups. This is in agreement with Marine´ and Coworkers (2011) who found that the prevalence of CD in childhood was five times higher than in adults suggesting either environmental factors influencing infancy, or latency of CD in adulthood [11].

In the current work twenty three out fifty (46%) patients were found to Marsh type III of intestinal mucosal lesion [8]. Dual positivity for both antibodies was detected in 22/23 (95.6%) patients with Marsh type III. Moreover, the strong fluorescent intensities of anti-EMA were documented to be correlated with advanced mucosal architecture affection (13 patients with villous atrophy or Marsh III lesion had strong fluorescent intensity (1:10000)). These findings suggest that dual positivity for the two antibodies and/or the degree of fluorescent intensity of anti-EMA (in relation to the antibody titer) have an association with the degree mucosal villous atrophy (i.e., the disease is in the advanced stages). Furthermore, it may also indicate that the anti-EMA may have a pathogenic effect leading for further mucosal damage and complications. Dieterichet and Coworkers (1997) had identified epitope of anti-EMA as tissue transglutaminase2. Interestingly, gliadinis a preferred substrate for this enzyme, giving rise to novel antigenic epitopes [12]. Moreover, the anti-EMA have been reported in some, but not all studies, to decline or disappear in association with a clinical and/or histological response to a gluten free diet [13].

These findings are in line with Ozgenc and Coworkers (2003) who had detected the anti-EMA in 35/37 (95%) cases with atrophy and 4/9 (44%) without atrophy. They found a significant association between total villous atrophy and anti-EMA positivity [14]. In addition, Kurppa and Colleagues (2012) had reported that small-bowel mucosal villous atrophy and crypt hyperplasia (Marsh III) 85% of the anti-EMA-positive subjects. There was a significant association between high antibody values and more severe small-bowel mucosal deterioration; in total 94% of those with high anti-EMA titers evinced villous atrophy. The percentage of subjects evincing severe small-bowel mucosal damage increased progressively with higher anti-EMA titers [15]. Conversely, Salmi and Coworkers (2006) had reported 22 cases with IgA-competent CD and were negative for serum anti-EM antibodies. Patients with EMA-negative CD were older, had abdominal symptoms more often. They found that negative serum endomysial antibodies might be associated with advanced CD [16].

Six out of ten patients with minimal changes in the mucosal architecture (Marsh I), two patients with Marsh II and one patient with Marsh III were found to have negative serology for both anti-EM and anti-DGP antibodies. Negative serology for anti-EMantibody is commonly associated with mild mucosal architecture changes [17]. Moreover, this may indicate that the disease is in the early stage and may need further follow up to reevaluate both small intestinal biopsies and serum antibodies. It is estimated that 10 to 20% of the untreated CD patients remain serologically negative for anti-EM antibodies. Furthermore, seronegativity for the antibody testing could be also attributed to the deficiency in the IgA antibodies [18]. The IgA deficiency is more common in subjects with CD disease (1 in 40) than the general population (1 in 400) [19]. However, this is not applied to the patients studied in the present work because all of them were IgA competent.

The diagnosis of CD could be excluded in the ten patients with normal mucosal histology (Marsh 0) and negative serology for both antibodies (anti-EM and anti-DGP). Conversely, the diagnosis of CD could not be excluded in the patients with minimal or borderline mucosal affection (Marsh type I) including the six patients with negative serology (due to strong clinical suspicion of CD) because they have CD-related pathology (evident by small intestinal biopsy). They were put on a trial of gluten free diet for further follow up by small intestinal biopsy and serological tests because HLA typing is not clinically available for the patients included in the current study. It has been found that genetic testing (i.e., HLA typing for HLA-DQ2 and HLA-DQ8) can help to exclude CD when used in combination with other serological tests. However, HLA typing is costly and not always readily available. In addition, genotyping is still not recommended for routine testing because these are reasonably prevalent associations in the general population (i.e., HLA-DQ2 in approximately 30%) [20].

IN CONCLUSION

Simultaneous determination of the antibodies against endomysium and deaminatedgliadin peptide using a Biochip combination of tissue sections (primate liver) and single antigens (repetitive gliadin-analogue fusion peptide (GAF-3X)) respectively help in the establishment of the diagnosis of patients with CD. Dual positivity for the two antibodies

(anti-EM and anti-DGP) and/or the degree of fluorescent intensity of anti-EMA (in relation to the antibody titer) have an association with the degree of intestinal mucosal villous atrophy. In addition, this technique provides a rapid and accurate identification of both anti-DGP and anti-EMA. Therefore, further research studies are needed to study the ability of this screening method (Biochip combination technique) to replace the current screening assays of tissue transglutaminase.

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