Immunohistochemical Expression of Antitissue Transglutaminase 2 in Tissue Injuries: An Interpretation Beyond Celiac Disease

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Abstract: Tissue transglutaminase 2 enzyme plays a diverse role in intracellular and extracellular functioning. Aberrant expression of anti-TG2 antibody has recently been proposed for extraintestinal identification of celiac disease (CeD), but its utility is questionable. To examine whether anti-TG2 immunohistochemical (IHC) staining can be of diagnostic value in identifying extraintestinal involvement in CeD, tissue blocks of patients with IgA nephropathies (IgAN), minimal change disease, membranous glomerulonephritis, membrano-proliferative glomerulonephritis, normal kidney, intestinal biopsies from CeD, tropical sprue, nonspecific duodenitis, and inflammatory bowel disease; liver biopsies from patients with chronic hepatitis B and C, acute liver failure (ALF), and CeD-associated liver diseases were retrieved and subjected to IHC staining for anti-TG2. H-score was calculated by multiplying the area of positivity and stain intensity. Anti-TG2 stain H-scores were almost similar in IgAN and non-IgANs (H-score 6.31 ± 3 vs. 7.03 ± 2.7); however, H-scores in both of these groups were significantly higher than in normal renal parenchyma (1.6 ± 1.5). Only 6.2% patients with IgAN with anti-TG2 immunostain positivity showed a positive anti-TG2 antibody serology and villous abnormalities, suggestive of CeD. Intestinal biopsies from patients with CeD, tropical sprue, nonspecific duodenitis, and inflammatory bowel disease also showed high anti-TG2 H-scores, with no statistically significant differences. Liver biopsies from patients with both ALF, as well as chronic liver diseases showed high anti-TG2 H-scores; with highest stain expression in ALF. In conclusion, IHC expression of anti-TG2 stain correlates with both acute and chronic tissue injuries, irrespective of etiology and organ involvement. It is not a reliable marker for diagnosis of CeD.

Key Words: antitissue transglutaminase 2, immunohistochemical stain, marker of tissue injury, celiac disease, extraintestinal celiac disease, IgA nephropathy

The diagnosis of celiac disease (CeD) is based on a combination of clinical manifestations, a positive celiac serology [antitransglutaminase antibody (anti-TG Ab), antiendomysial Ab, or antideamidated peptide Ab], and presence of villous abnormalities on histology.1,2 The hypersensitivity to immunogenic gluten peptides in CeD, once thought to be limited to only intestine, is now known to involve many other extraintestinal organs including skin, liver, and brain; hence, CeD is now considered as a multisystem disorder altogether. As the anti-transglutaminase 2 enzyme (TG2) Ab crosslinks with tissue TG enzymes present in various organs, demonstration of anti-TG2 in extraintestinal organs using immunohistochemical (IHC) technique was conceived as a promising technique for establishing a diagnosis of extraintestinal organ involvement in CeD. In a recent meta-analysis including 23 studies, Gatti et al5 reported anti-TG2 deposits in symptomatic CeD, both in adults (100%) and children (73.2% to 100%). Similar deposits were also noted in patients with potential CeD (64.7% to 100%) and dermatitis herpetiformis (63% to 79%). However, utility of anti-TG2 IHC stain in diagnosis of CeD is debated,6 and it has been accepted that, to establish the diagnosis of an extraintestinal CeD, demonstration of colocalization of IgG anti-TG2 and human IgA, using dual immunofluorescence technique is required.7 However, availability of colocalization study is sparse, needs the supervision of expert pathologists, not easy to perform, and interpret. Hence, the diagnosis of extraintestinal CeD is still difficult and the controversial results found in literature needs to be sorted out.

Hence, to explore, if anti-TG2 staining of biopsies can predict association of CeD with other diseases, which have a known association with CeD, such as IgA nephropathy (IgAN), we performed immuno-staining of...
tissue with anti-TG2. For this purpose, tissue specimens from renal, hepatic, and intestinal diseases, with known relation to CeD as well as unrelated conditions, were subjected to anti-TG2 IHC staining.

**PATIENTS AND METHODS**

**Recruitment of Archived Biopsies From Various Organs**

**Recruitment of Patients With IgAN and Retrieval of Their Kidney Biopsies**

From the registry of patients with chronic kidney diseases, 151 patients with IgAN were identified and paraffin-embedded tissue blocks were retrieved from 105 of them. The diagnosis of IgAN was made on the basis of a combination of clinical features such as proteinuria and hematuria, focal or diffuse mesangial expansion and mesangial deposit of IgA and/or C3 antibodies, as seen in immunofluorescence study. Immunofluorescence stain intensity grade ≥2, was taken as positive for all the antibodies used. All the renal biopsies had been reported by expert renal pathologists.

**Recruitment of Kidney Biopsies From Patients With Other Nephropathies (as Disease Controls) (n = 29)**

Renal biopsies from patients with minimal change disease (MCD) (n = 15), membranous glomerulonephritis (n = 8), and membro-proliferative glomerulonephritis (n = 6) were retrieved and included as disease controls. Renal biopsies from patients with MCD showed no definite pathology on light microscopic or immunofluorescence examination; they, however, showed podocytopathies on electron microscopic examination. In membranous glomerulonephritis, the renal glomerular capillaries showed thickening, with a granular capillary wall deposit of IgG and C3 and subepithelial immune complex deposits on electron microscopy. Membrano-proliferative glomerulonephritis was diagnosed based on the presence of diffuse proliferative pattern of the glomeruli, with subendothelial, mesangial, or subepithelial deposit of immune complex and C3 on immunofluorescence.

**Recruitment of Kidney Biopsies as Normal Controls (n-5)**

Normal renal parenchyma adjacent to renal tumors, were sampled from nephrectomy specimens and included as normal renal controls. Presence of any preexisting renal disease was excluded. Kidney biopsies having fibrosis or significant inflammation were excluded after histological examination. From the database, a random selection of biopsies were done to avoid selection bias.

**Recruitment of Liver Biopsies**

The paraffin-embedded blocks of liver biopsies from patients with chronic hepatitis B (CHB), chronic hepatitis C (CHC) (n = 8), idiopathic chronic liver disease with associated CeD (n = 4), and postmortem liver biopsies from patients with acute liver failure (ALF) (n = 10) were retrieved and included in this study. In the liver biopsies of patients having ALF, the presence of the chronicity was ruled out based on clinical and histologic criteria.

**Intestinal Mucosal Biopsies**

Duodenal mucosal biopsies from patients with CeD (all having villous abnormalities of modified Marsh grade 3c, along with serum anti-tTG > 10 times the cutoff value for a positive test; n = 22), tropical sprue (TS, based on typical clinical features, exclusion of other causes and prolonged response to antibiotic treatment; n = 5), and nonspecific duodenitis (negative celiac serology, HLA DQ2 and DQ8 negative, and histology not suggestive of CeD; n = 10) were retrieved from the records. Colonic mucosal biopsies from patients with inflammatory bowel disease (IBD) (n = 10) were also retrieved.

**Recruitment of Prospective Cases**

We invited patients with anti-tissue transglutaminase 2 enzyme (tTG2) stain positive IgAN, included in this study, to undergo screening for CeD, and 32 of them agreed to get screened. Informed and written consent were obtained from all of them. All these patients aged above 18 years underwent screening by using anti-tTG Ab ELISA test (AESKU Diagnostics, Wendelshelm, Germany) and duodenal biopsies thereafter. Positive and negative controls were used. An anti-tTG titer of > 18 U/mL was considered positive. Grading of villous abnormalities was done using the modified Marsh criteria. CeD diagnosis was made based on the modified European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria.

**IHC for Anti-tTG 2 Ab and Reporting**

From the paraffin-embedded blocks, thin slides of 4 to 5 μm were cut and processed as per the standard protocol of our laboratory. All these slides were subjected to IHC staining for IgG anti-TG2 rabbit polyclonal Ab (1:200, Lot no GR118477-1; AbCam) directed against the tissue TG2 enzyme. Standard overnight IHC staining method was followed. Universal secondary Ab (CRF antipolyvalent HRP polymer; Scy Tek Laboratories, Logan) was used and the reaction was developed by using 3,3'-diaminobenzidine. The reaction was developed under the visual supervision of a pathologist to avoid any overstaining. The stain was standardized on duodenal biopsy showing histologic modified Marsh grade 3c changes, having serum anti-tTG titer > 200 U/mL.

Interpretation of IHC stain was done in respect to stain distribution (area of positivity grade 0: negative, <1% positive; grade 1: 1% to 10% positivity; grade 2: 11% to 33% positivity; grade 3: 34% to 66% positivity; and grade 4: >66% positivity) and stain intensity (grade 1: faint expression; grade 2: moderate expression; grade 3: strong expression) in different tissue parts separately, such as epithelial cells, endothelial cells, and stroma etc. This scoring system was adopted from a previous published IHC study. The H-score was calculated by multiplying...
the grade for area positivity × grade of stain intensities. The composite H-score was calculated by multiplying the H-scores in different compartments of an organ, as for example, H-score of glomeruli × H-scores of tubules × H-scores of endothelial cells × H-score of the interstitium. The latter was calculated for the purpose of comparing the anti-tTG2 staining pattern across different organs included here.

The Ethics Committee of our Institution approved the study.

**Statistical Tests**

Pearson χ² test was used for the comparison of the distribution and intensities of expression of anti-tTG2 in different compartments of the organs and Wilcoxon Rank-Sum test was used for the comparison of the H-scores among the groups. $P < 0.05$ was considered as statistically significant.

**RESULTS**

**Expression of Anti-TG2 Stain in the Renal Biopsies**

Only focal discontinuous expression of anti-TG2 stain was noted in renal tubular epithelial cells in control renal biopsies (Figs. 1A, B). No expression of this stain was identified in normal renal glomeruli and interstitial fibroblasts. Therefore, a composite H-score of anti-TG2 immunostaining was very low in control renal biopsies. In MCD, anti-TG2 staining was comparatively more diffuse in renal tubular cells; neither the glomeruli, nor the interstitial cells showed significant stain expression (Fig. 1C). In other renal diseases, strong staining was noted in the areas of fibrosis and sclerosis (Figs. 1D, E). The vascular endothelial cells, in all renal biopsies showed uniform and strong expression of anti-TG2 immunostain (Table 1).

The H-score of anti-TG2 immunostain in renal glomeruli was significantly higher both in IgAN (8.10 ± 3.47, $P = 0.04$) and in other non-IgAN (9.08 ± 2.7, $P = 0.004$); in comparison with control renal biopsies (4.80 ± 1.79). Among the IgA and non-IgAN, no statistically significant difference of H-score was noted in renal glomeruli ($P = 0.29$). Similar overexpression of anti-TG2 immunostain was also observed in renal tubules of both IgA and non-IgAN, in comparison with controls (Table 1).

**The Pattern of Expression of Anti-TG2 Ab in the Liver Biopsies From Patients With Liver Diseases**

Anti-TG2 staining in the liver biopsies was comparatively low in CHB and CHC (composite H-score 5 ± 3.16); moderate in patients with chronic liver disease associated with CeD (composite H-score 8 ± 0); and was maximum in the postmortem liver biopsies from patients with ALF (composite H-score 10 ± 3.53) (Table 2). In all the liver biopsies included, expression of anti-TG2 immunostaining was uniform and strong in the vascular endothelial cells of portal tracts and in the endothelialized sinusoids. Although the anti-TG2 expression was identified both in the hepatocytes and in the bile ducts, the expression was maximum in the periportal hepatocytes (Fig. 1F). The liver biopsies from patients with chronic liver disease associated with CeD (n = 4) included in this study, showed: nonspecific histologic changes in one (macronodular steatosis involving <5% area of cores, not suggestive of nonalcoholic steatohepatitis); features of NASH (with NASH-clinical research network grade 4/8 and stage 3-4/6) in one; and features compatible with chronic cryptogenic hepatitis with fibrosis stage 4/6 in the other. Anti-TG2 staining was strong in the ballooned and steatotic hepatocytes (arrows), in addition to the periportal hepatocytes (Figs. 1G, H).

The liver biopsies from patients with CHB and CHC had ≥ stage 3 fibrosis in 5 of them, whereas the other 3 patients had fibrosis stage < 3/6 (according to the modified Ishak histologic grading and staging system). In fibrotic livers, anti-TG2 staining was diffuse both in hepatocytes, bile ducts, and in fibroblasts (Fig. 1I).

**Pattern of Anti-TG2 Stain Expression in the Intestinal Biopsies**

There was no significant difference of anti-TG2 composite H-scores in the intestinal biopsies of patients with CeD (10.4 ± 2.3), TS (9.6 ± 2.2), and nonspecific duodenitis (8.4 ± 2.8) (Figs. 1J–L). In colonic biopsies from IBD, also showed high anti-TG2 H-scores (Table 3). In CeD, the staining was more prominent near the mucosal surface epithelium, than that in the crypts (Fig. 1K). The vascular endothelial cells also showed strong anti-TG2 staining in all the cases.

**Prevalence of Anti-TG2 Seropositivity in Patients With Anti-TG2 IHC Stain Positive IgAN**

Of 32 patients who agreed to undergo screening for CeD, 5 (15.6%) had high serum IgA anti-tTG2 Ab titer. Of these 5 patients, 2 showed very high anti-tTG Ab titer (4-fold and 9-folds, upper limit of cutoff value) and another 3 had 2-fold higher Ab titer. Out of these 5 patients, 2 showed villous abnormalities of modified Marsh grade 3b.

**DISCUSSION**

In this study, we observed overexpression of anti-tTG2 staining not only in various chronic diseases of kidney, liver, small intestine, and colon (in comparison with corresponding normal controls); but also in the acute liver disease. There was no difference in anti-TG2 staining in between renal biopsies of patients with IgAN and non-IgAN. Also, no difference of staining was noted in intestinal biopsies from patients with CeD, TS, non-specific duodenitis, and IBD. The expression of anti-TG2 immunostain, while was limited predominantly in the zone 1 hepatocytes (periportal) in normal liver, staining was noted at the zone of hepatocyte damage and fibrosis, in diseases as in NASH, CeD-associated liver diseases, and in chronic hepatitis. In ALF anti-TG2 H-score was maximum in the viable hepatocytes. Johnson et al. have also reported diffuse anti-TG2 staining pattern in the
tubular epithelial cells, renal glomeruli, macrophages, interstitial fibroblasts, and in endothelial cells of both IgAN and non-IgAN.

Tissue TG, which is the target of both anti-TG2 IHC stain and circulating serum anti-TG2 serum Ab in patients with CeD, is a calcium-dependent enzyme.

**FIGURE 1.** In normal kidney anti-TG2 stain was noted only in vascular endothelial cells and focally in tubular epithelial cells (arrow) (A, × 40; B, × 100). In minimal change disease, more numbers of tubules showed anti-TG2 positivity (C, × 40). In renal biopsies from membranoproliferative glomerulonephritis, staining was noted in the atrophied, as well as viable tubules, interstitial fibroblasts (D, × 40), and in sclerosed glomeruli of IgA nephropathy (arrows) (E, × 100). In normal liver biopsies, anti-TG2 staining was seen around the periportal hepatocytes (F, × 40). However, in the NASH, staining was also noted near the steatohepatic change (arrows) (G, × 40; H, × 100) and diffusely in hepatocytes, bile ducts, and fibroblasts in chronic viral hepatitis with cirrhosis (arrows) (I, × 100). Anti-tTG2 staining was also noted both in the villous and crypt enterocytes in the duodenal biopsies from patients with tropical sprue (J, × 40); whereas predominantly a mucosal surface expression (arrow) in celiac disease (K, × 40) and diffuse positivity in nonspecific duodenitis were noted (L, × 40).

**TABLE 1.** Comparative Composite Scores of Anti-TG2 Antibody Staining in Renal Biopsies From Patients With IgA Nephropathy, Non-IgA Nephropathies, and Control Normal Biopsies

<table>
<thead>
<tr>
<th>Pattern and Intensity of Staining</th>
<th>Control Renal Parenchyma (n = 5)</th>
<th>Renal Biopsies From IgAN (n = 105)</th>
<th>Renal Biopsies From Other Nephropathies (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-score in tubules</td>
<td>1.60 ± 1.52</td>
<td>6.31 ± 3.00</td>
<td>7.03 ± 2.71</td>
</tr>
<tr>
<td></td>
<td>P-value for IgAN vs. control, 0.001</td>
<td>P-value for IgAN vs. other nephropathy, 0.27</td>
<td>P-value other nephropathies vs. control, 0.001</td>
</tr>
<tr>
<td>H-score in glomeruli</td>
<td>4.80 ± 1.79</td>
<td>8.10 ± 3.47</td>
<td>9.08 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>P-value vs. control, 0.04</td>
<td>P-value vs. other nephropathies, 0.29</td>
<td>P-value vs. control, 0.004</td>
</tr>
<tr>
<td>H-score in blood vessels</td>
<td>12 ± 0</td>
<td>11.84 ± 1.00</td>
<td>10.82 ± 1.49</td>
</tr>
<tr>
<td></td>
<td>P-value vs. control, 0.65</td>
<td>P-value vs. other nephropathies, 0.001</td>
<td>P-value vs. control, 0.09</td>
</tr>
<tr>
<td>Total composite H-scores</td>
<td>136 ± 84.28</td>
<td>673.23 ± 482.41</td>
<td>710.77 ± 435.92</td>
</tr>
<tr>
<td></td>
<td>P-value vs. control, 0.02</td>
<td>P-value for IgAN vs. other nephropathies, 0.5</td>
<td>P-value vs. control, 0.008</td>
</tr>
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</table>

IgAN indicates IgA nephropathy.
tightly regulated and expressed in various organs in vertebrates, both within the cells and also in the extracellular space. Although the intracellular TG2 regulates the cell proliferation, differentiation, and apoptosis; the extracellular component helps in stabilizing the extracellular matrix. TG2 has also been implicated in the fibrogenesis of liver through the release of TG-mediated IL-13 and transforming growth factor β, which was further validated by demonstration of reduction in the expression of IL-13 and amelioration of the liver fibrosis, with application of cystamine, an inhibitor of TG2. It has been hypothesized that the TG2 leaks from the damaged or ruptured cells into the extracellular matrix and crosslinks with the extracellular fibrillar matrix proteins to form irreversible (g-glutamyl) lysine bonds, which stabilizes the matrix. Hence, anti-TG2 IHC staining alone, which detects this tissue enzyme, both inside and outside the cells, has no link with the occurrence of CeD. In this regard, identification of colocalization of IgA Ab and IgG anti-TG2, has been shown as the correct way of identification of extraintestinal CeD. However, as said previously, colocalization study is set-up dependent and needs technical and pathologic skills for proper interpretation. Hence, we wanted to see, if anti-TG2 IHC stain if at all can be used as a marker of CeD.

Patients with IgAN were evaluated prospectively for presence of CeD, as the possible etiological association between the CeD and IgAN have been documented in various studies. Hence, in the cases of IgAN, which showed anti-tTG2 IHC positivity, we tried to pursue them further to see if this positive staining is indicative of CeD or not? Finally, out of these IHC-positive cases, only 15.6% cases showed high anti-tTG2 serum titer, and 6.2% of them showed histologic villous abnormality (both showing modified Marsh grade 3b); indicating a poor utility of anti-tTG2 IHC stain alone, as a diagnostic method for detection of extraintestinal CeD.

Dрастич et al have also reported nonselective expression of anti-TG2 immunostain in the liver biopsies from patients with autoimmune hepatitis, toxic hepatitis, liver diseases associated with CeD, and in metastatic carcinoma. We also noted nonspecific staining pattern in liver biopsies.

Biagi et al had identified anti-TG2 IHC staining as a specific marker for CeD. Furthermore, Sakly et al demonstrated anti-TG2 immunohistochemical staining in proportion to the different grades of villous atrophy in CeD. However, subsequently, Villanacci et al and Gorgun et al had identified that, anti-TG2 IHC staining is not specific for CeD. In the index study also, the expression of this stain was identified almost uniformly in intestinal mucosal biopsies not only in patients with CeD but also from patients with TS, nonspecific duodenitis, and even IBD. Almarzooqi and colleagues have also reported higher expression of anti-TG2 immunostain in the upper part of the duodenal mucosal epithelium, compared with that in the crypts, in patients with CeD. Such a differential expression pattern may be due to cross linking of the tissue TG with the absorbed gliadin peptide in the epithelium and subepithelium. Although higher expression of anti-TG2 in the upper part of mucosa in patients with CeD is indicative, it cannot be considered to be a reliable marker of CeD.

In conclusion, the expression of anti-TG2 IHC stain is a nonspecific marker of both chronic and acute tissue injuries, and it is not a reliable marker to suggest the presence of CeD. Hence, anti-TG2 IHC stain alone does not play any significant role in defining intestinal and extraintestinal involvement in CeD.

<table>
<thead>
<tr>
<th>Pattern and Intensity of Staining</th>
<th>Liver Diseases Associated With CeD</th>
<th>Postmortem Liver Biopsies From Patients With ALF</th>
<th>Liver Biopsies From Patients With CHB and CHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. samples</td>
<td>4</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>H-score (mean ± SD)</td>
<td>8 ± 0</td>
<td>10 ± 3.53</td>
<td>5 ± 3.16</td>
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</table>

ALF indicates acute liver failure; CeD, Celiac disease; CHB, chronic hepatitis B; CHC, chronic hepatitis C.

<table>
<thead>
<tr>
<th>Pattern and Intensity of Staining</th>
<th>Duodenal Biopsies in CeD</th>
<th>Duodenal Biopsies of Tissue vs. CeD</th>
<th>Duodenal Biopsies of Nonselective Duodenitis vs. CeD</th>
<th>Colonic Biopsies in IBD vs. CeD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. samples</td>
<td>22</td>
<td>5</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>H-score (mean ± SD)</td>
<td>10.4 ± 2.3</td>
<td>9.6 ± 2.2</td>
<td>8.4 ± 2.8</td>
<td>7.1 ± 4.3</td>
</tr>
</tbody>
</table>

CeD indicates celiac disease; IBD, inflammatory bowel disease; TS, tropical sprue.
REFERENCES