Original
ISSN 2413-0516

Evaluation of T-Bet immunostaining in the counting of intraepithelial lymphocytes in celiac disease

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(Submitted: 10 July 2020 – Revised version received: 19 July 2020 – Accepted: 21 August 2020 – Published online: 30 October 2020)

Abstract
Objective: In this study, we aimed to evaluate CD3, T-bet, and GATA3 staining of intraepithelial lymphocytes (IELs) in comparison to the routine H&E stains in celiac disease.
Methods: A total of 50 patients, in whom celiac disease was diagnosed based on a combination of clinicopathological features, were enrolled in the study. Duodenal biopsied tissues were processed routinely into formalin fixed paraffin embedded (FFPE) blocks. Sections were prepared and stained with H&E and each of CD3, T-bet and GATA3. A number of histological criteria were measured to calculate the Marsh score. The results were analyzed using the IBM SPSS analytic software.
Results: A positive correlation was found between the count of T-bet stained cells and the increase of IELs (*P*-value = 0.001). In addition, low count of GATA3 stained cells was seen in almost all cases. The count of GATA3 stained cells was not affected by the increase in IELs count.
Conclusions: The majority of increased IELs were stained with T-bet. Whereas in normal IELs count is less than half the IELs were stained with T-bet. This would indicate that T-bet immunostaining is a potential alternative to H&E and/or CD3-based counting of IELs.
Key words: T-bet; Immunostaining; Gluten sensitive enteropathy; Distribution pattern; Marsh grading; Immunohistochemistry.

Introduction

Celiac disease (CD) is an immune-mediated enteropathy.[1] It is characterized by an immune-mediated response to gluten present in foods.[2] CD-associated symptoms are often triggered by production of autoantibodies and in many cases flattening of the intestinal villi.[3]

Its prevalence is currently estimated at 1:100 of the Western countries,[4] and is rising in the Middle Eastern populations to 1.5:100.[5-7] CD may occur in adults and children,[8] with a female/male ratio of 3:1.[9] About 90% of the CD patients carry a major histocompatibility complex class II molecule, human leukocyte antigen (HLA) variant DQ2 and most of the remainder of patients carry an HLA variant named DQ8.[10]

The clinical presentation of CD ranges from asymptomatic form to a symptomatic which could represent a general malabsorption (typical or classical) or extra intestinal symptoms (Atypical or symptomatic).[11, 12] The long-term consequences of undiagnosed and/or untreated CD can lead to a form of unresponsiveness (refractory celiac) to gluten-free diet (GFD) and malignancies.[11] Because of its atypical presentation, a large portion of CD patients remain undetectable. This atypical form include the presentation of patients with extra intestinal symptoms, a silent form which can present with positive serology and histological changes of intestinal mucosa with no symptoms[13]; a potential CD form which can present with a positive serology with no architectural changes in mucosa histology; and a latent form which can be defined as normal histological mucosa with no serological abnormalities.[14, 15]

The diagnosis of CD requires a joint clinicopathological approach; the recommended first-line test is serology with immunoglobulin A (IgA) tissue transglutaminase, deaminated gliadin IgA and/or IgA endomysial antibodies. These serological tests show high levels of sensitivity and specificity, but biopsy is the gold-standard to confirm the diagnosis.[16] The latter being based on the modified Marsh grading system and is the most conclusive current test for CD.[17] The modified Marsh grading criteria includes an architectural changes of the mucosa (atrophy of villi and crypts hyperplasia) with a rise in the number of intraepithelial lymphocytes (IELs).[18]

The classical histopathology changes of CD are partial or total villous atrophy and increase of IELs. However, the pathology classification of CD is changing, with recognition that CD may show minimal pathology (normal architecture and an IEL count ≥25⁄100 enterocytes). This entity is also described as lymphocytic duodenitis, and recommendation of follow-up serology testing is paramount in this condition.[19, 20] An increase in the number of IEL is the hallmark of early stage CD as it is the first and most sensitive index of the effects of gluten on the mucosa.[21] Yet, the threshold of what is considered normal is still debatable. Studies have suggested a count of 20-25/100 EC to be borderline lymphocytosis with a value equal or above 25 as pathologic[22] other authors suggests even a lower number to be as an increase in IELs.[23, 24] The distribution of the IELs is also being considered as a morphological feature of CD.[21, 25]

The IELs comprise of a significant lymphocyte population residing in close immediacy to the intestinal lumen between enterocytes in the intestinal epithelium.[21, 26] Phenotypically, in CD patients IELs are T lymphocytes, of which the majority 70% are CD8+ T cells and less than 20% are CD4+ T-cells. B cells are not present.[21, 26-28] Several studies have suggested CD to be of a Th1-mediated immune response.[29, 30] The predominant cytokine produced by T-cell isolated from CD patients mucosa which have a significant role in the lesion formation is IFN gamma.[31] This view is supported by other studies on

the intestinal lamina propria isolated T cells, underlined by the increase of cytokines IFN gamma and IL-18.[32, 33] T-bet is a specific T box transcription factor which correlates with IFN gamma expression in Th1 and natural killer cells.[34] Reports suggest that the T-bet transcription factor is upregulated in active CD then down regulated to normal levels in treated (on GFD) CD patients.[32] However, the significance of counting T-bet expressing cells was not evaluated thoroughly.

This study was aimed to investigate the value of the counting and distribution of types of IELs in relation to other parameters to improve the diagnosis of CD.

Materials and Methods

This is a cross-sectional study of which the samples collected were formalin-fixed paraffin embedded (FFPE) proximal duodenal tissue of 50 consecutive CD patients. They were collected from Al-hussainy teaching hospital, Al-kafeel super-specialty hospital and Al-sajjad medical laboratory in the city of kerbala from August 2019 to February 2020. CD was diagnosed based on a combination of suggestive clinical presentation, positive serology, and/or indicative histology based on the H&E staining results.

Processing and staining of the FFPE samples was performed. H&E staining of the sections was done and Marsh grading was judged according to the modified Marsh criteria.[35] The Marsh grading system was chosen in this research as it provides a wide more detailed view of the histopathological changes (lesions) that appears in the duodenal mucosa of CD patients.[36] These changes include the count of IELs and the villus architecture, being preserved or atrophied in addition to the degree of atrophy and the hyperplasia of the crypts. Immunohistochemistry was preformed, on 3–5 µm sections, using monoclonal antibodies directed against CD3 (clone PC3/188A, mouse monoclonal IgG1, Dako A/S Denmark, GATA3 (clone HG3-31, mouse monoclonal antibodies, Santa Cruz Biotechnology, Inc.) and T-bet (clone 4B10, mouse monoclonal IgG1, Santa Cruz Biotechnology, Inc.). Prior to incubation with the primary antibodies, the de-waxed 3–5 µm thick sections were subjected to an antigen retrieval procedure consisting of a high-temperature heating of the sections immersed in EnVision™ FLEX Target Retrieval Solution, high pH for 30 min. Bound antibodies were made visible using a detection kit purchased from Leica biosystems Newcastle Ltd. UK, NovolinkTM Polymer Detection System. The resulting image was a nuclear staining for the T-bet and GATA3, while the CD3 showed a membranous staining of brown color due to the DAB chromogen. Data were analyzed using IBM SPSS analytic software.

Results

Of the 50 samples, 38 (76%) were females and 12 (24%) were males, female to male ratio was 3.2:1. Their ages range 3–65 years. Of those patients, only 34 (68%) of them had a positive serology (Table 1).

On the H&E stain, 40 (80%) of the samples showed an increase of the IELs (30 IELs/100 EC) in the duodenal villi while the other 10 samples showed only a borderline increase (20–29 IELs/100 EC). On staining with the CD3 monoclonal antibody, of the 40 (80%) samples only 32 (64%) cases showed an increase of IELs of 30/100 EC. While the remaining of the 40 (80%) had either a borderline or a normal count of IELs, as did the other 10 (20%) cases.

Table 1. **Patient's characteristics.**

Characteristics		Frequency	Percent%
Gender	Female	38	76.0
	Male	12	24.0
Age Groups	0-10	1	2.3
	11-20	12	27.3
	21-30	14	31.8
	31-40	9	20.5
	41-50	7	15.9
	>51	1	2.3
Serology	Negative	14	28.0
	Positive	34	68.0
	Borderline	2	4.0
Marsh grade	0*	17	34.0
	1	10	20.0
	2	6	12.0
	3*	17	34.0

* All grades of Marsh (IIIa, IIIb, IIIc) are expressed in this table as grade III.
* Grade 0 are patients with positive serology and a borderline increase of IELs which is counted as (20-29/100EC) in this study.

The T-bet monoclonal antibodies were used to label and track the Th1 lineage of IELs in CD. The IELs in the 32 (64%) samples counted by CD3, were counted by T-bet, with the T-bet most of the cases had 30 IELs/100 EC, the remaining few showed a borderline count 20–29 IELs/100EC. In general, the staining with T-bet showed a lower count of IELs. This decrease of the IELs count could be attributed to the fact that T-bet can only stain the Th1 lymphocyte cell, while the CD3 stains all of the CD3 barring T-lymphocytes including Th1 and Th2, making the T-bet to be a more specific marker for CD than do CD3.

On staining with GATA3, a markedly low percentages of 5% of the IELs in CD samples were stained with GATA3. Deducing that the Th2 has no significant role in the pathogenesis of CD. The weak presence of Th2 cells in the duodenal biopsy of CD-diagnosed patients further supports the previous finding, which is that CD is of a Th1-mediated immune response.

Table 2. **Means and slandered deviations of the IELs count with different stains in celiac disease diagnosed patients.**

Stain	IELs Count (mean SD)	P value compared to H&E
H&E	41.25±6.2	-
CD3	34±5.8	0.001
T-bet	30.2±6.8	0.001
GATA 3	3.2±3.49	0.001

A highly significant correlation was found between the number of IELs stained by T-bet and the Marsh grades (P = 0.001). It was observed that with the increase of IELs, counted by H&E and/or by CD3 staining, there was also an increase in the T-bet stained IELs (Th1 lineage), showing that most of the increased IELs were T-bet positive. These results suggest the possibility of using the T-bet as an important tool for the counting of IELs and possibly the diagnosis of CD.

Nineteen cases of the diagnosed CD (positive serology), Marsh stages I, II, IIIa, IIIb, showed a high staining percentage of the IELs with T-bet (70–90%), and CD3 (80–100%), while the GATA3 showed a markedly low staining percentage (1–5%) (Fig. 1, Table 3).

In a comparison with the diagnosed celiac cases (which either had a positive serology or an indicative histology), of the negative serology cases which showed an indicative histology (Marsh I-IIIc), all showed a staining percentage of no less than 70% with T-bet while the GATA3 showed less than 5% staining positivity. As for the cases with positive serology, some with borderline increase in IEL showed increased T-bet positivity with also no GATA3 staining. Interestingly, of the positive serology cases, two of them had a normal count of IELs (18) yet they had a high staining positivity for the T-bet of no less than 80%. While another case with negative serology and a borderline increase IEL count (20–29/100EC) with CD3 showed a high positive staining of the IELs with the T-bet monoclonal antibodies.

Regarding the distribution patterns of the T-bet stained IELs in the villi of the duodenum mucosa, Table 4 shows the majority 68% of the cases showed regular distribution of the T-bet stained IELs throughout the villi body, while only 2% of the cases showed an aggregation of the IELs in the base of villi. The relation between Marsh stages and the distribution pattern of T-bet stained IELs is highly significant.

Table 3. **Percentage of stained cells in different counts of IELs.**

Stain	Normal IELs count	Increases IELs count
CD3	<40	80-100 %
T-bet	0-40 %	70-90 %
GATA3	1-5 %	0-7 %

* A different expression level of T-bet stained cells were observed when there was a borderline increase of IELs.

Discussion

Histopathological assessment of biopsied duodenal tissue is of vital role for the diagnosis of CD. However, a subjective grading system that is currently being used tends to misdiagnose

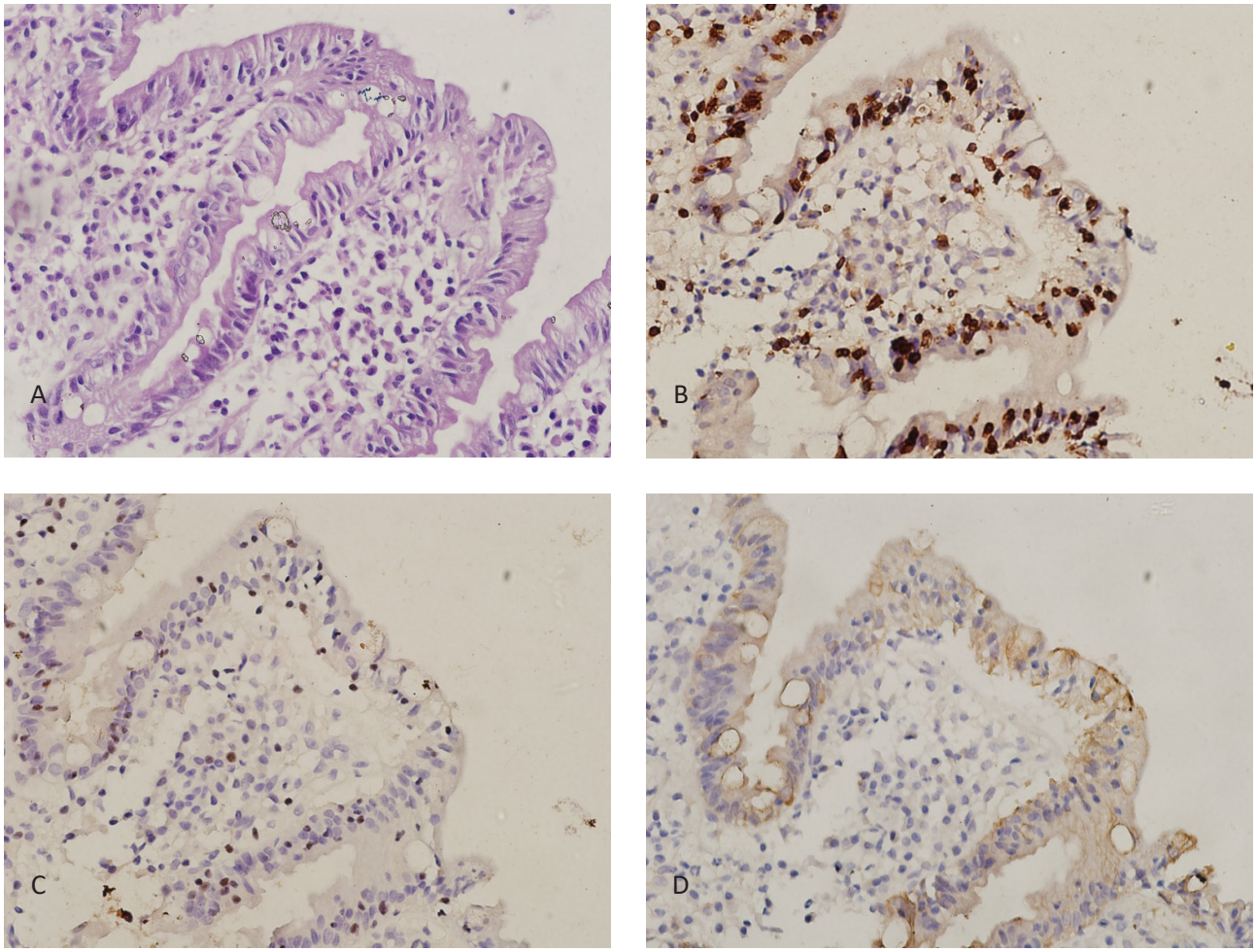

Fig 1. **High power view of CD diagnosed patient Marsh IIIa, the same tissue sample compared in different stains. Black arrows: shows the stained intraepithelial lymphocytes. A: H&E, B: CD3 cytoplasmic staining pattern, C: T-bet staining of the Th1 type IELs (the responsible in CD pathogenesis) P-value < 0.001, D: GATA-3 staining of Th2 type IELs shows minimal count of IELs. X40.**

Table 4. **Distribution pattern of T- bet stained IELs in correlation with Marsh grades in the duodenal villi of celiac disease diagnosed patients.**

		Marsh stages				Total	Percent %	*p* value
		0	1	2	3*			
Distribution pattern of T-bet stained IELs in villi	Flat villi	0	0	0	3	3	6 %	p<0.01
	Aggregated in the tip	0	2	0	10	12	24 %	
	Aggregated in the base	0	0	1	0	1	2 %	
	Regular distribution	17	8	3	6	34	68 %	

Marsh grade 3 includes (IIIa, IIIb, IIIc).

some cases, especially in the absence of an architectural changes.[37] Counting the IELs in H&E stained sections is not a straightforward process. It is not always possible to distinguish the IELs nuclei from the enterocytes as they may be present in a multitude of shapes or due to the overlapping and lack of contrast between cells. The usage of immunohistochemistry is very helpful in the counting of IELs.[38] Owing to the fact that it is cell type specific staining, IHC offers the ability to distinguish the type of IELs, and the nuclei form the enterocytes.

Studies have suggested that the IHC staining of IELs by CD3 can improve the diagnosis in CD patients of the Marsh I lesions,[38, 39] as the T-lymphocytes are CD3+ cells. The guidelines of British society of gastroenterology stipulate the that use of IHC staining in borderline cases is vital.[37, 40] Many other studies used the CD3 mononuclear antibody to improve the count of IELs in an attempt to either detect the early stages of CD or improve contrast to count IELs in seropositive cases or simply to study the celiac-related IELs.[19, 41]

In the current study, counting of IELs based on CD3 was compared to H&E based counting. We found that the count of the CD3 stained cells was lower than that stained with H&E. T-bet staining of duodenal IELs in celiac diagnosed cases was investigated in this study. A highly significant correlation was found between the number of IELs and the percentage of T-bet staining.

CD3 eliminated the issue of overcounting the IELs and their overlapping with other cell nucleus that was faced with the H&E. However, it is not specific for CD. Recent study proposed that the CD3 was of no more use in the diagnosis of the normal histology cases of CD than is the staining with H&E (20). Therefore, the search of a more specific marker for CD would serve as a more reliable method for diagnosing CD, especially in early and potential cases.

Very few studies examined T-bet expression in limited number of CD patients.[42, 43] They found the T-bet expression in IELs of duodenal biopsy was increased, therefore a role for T-bet was suggested in CD. However, the use of T-bet expression in the diagnosis of CD was not investigated. The current study aimed to investigate T-bet role in CD. These results shows that most of the increased IELs were T-bet positive, suggesting that T-bet expression in IELs of CD patient is of significance. It also is supportive of the studies which suggests that CD is a Th1-mediated inflammatory reaction. The T-bet staining was found to be superior to the CD3 IHC staining in settings of the contrast, intensity, and background staining probably due to the fact that T-bet selectivity of only Th1 cells rather than all the CD3+ cells like with the CD3 monoclonal antibodies.

These findings suggests the possibility of using the T-bet monoclonal antibodies for counting IELs in CD as a useful diagnostic tool, while the GATA3 was found to be of low expression. This was similar to what was found in the literature.[43, 44] Of the 50 cases, 2 cases with positive serology had a high positivity for T-bet even with a normal IEL counts (0–19). This result could be interpreted with a potential or a latent CD of the 2 cases indicating the usefulness of using T-bet in detecting latent or potential CD cases.

In 19 cases of serology positive (including anti-tTG IgA) and histology indicative of CD, increased IELs in CD3 and H&E staining with crypt hyperplasia and some cases showed atrophy in the villi in addition to the clinical indications, the T- bet expression in IELs was of no less than 70% of the cells.

In 10 cases of serology positive (but anti-tTG IgA negative) and a negative histology, meaning no increase in IELs, no crypt hyperplasia nor villus atrophy, they showed less than 40% expression of T-bet by IELs. While in 10 cases of seronegative with an architectural changes including villus atrophy (IIIa, IIb, IIIc), the T-bet staining positivity was also high (70–10%).

Another criteria that have been suggested in the literature by some authors is to distinguish early stages of CD via the distribution pattern of the IELs. A study of over 400 biopsies suggested that IELs in CD were not normally distributed.[41] Dickson et al. suggested that the loss of the normal "decrescendo" pattern of normal IELs distribution of duodenal villi of patients is suggestive of CD (Dickson et al., 2006). Mino *et al.* found that an aggregation of IELs in the tip of villi observed in CD3 staining is suggestive of CD (Mino and Lauwers, 2003). Goldstein *et al.* found that a regular distribution pattern along the villi was most sensitive of an association of celiac Marsh I lesion (Goldstein and Underhill, 2001), it was also suggested that the increase of mean IEL in the villus tip is associated with CD (Goldstein and Underhill, 2001). In other words, they suggested that the regular distribution of IELs ≥25 in architecturally normal villus is indicative of CD, they also note that a Gestalt approach should be applied in assessing the biopsy by the pathologists. These findings are similar to what we found in the duodenal biopsy of CD patient, regular distribution of increased IELs in the duodenal villus. These results were highly significant in correlation with the Marsh stages.

All of the mentioned cases have studied the IELs distribution with either the CD3 staining or with the H&E stain. In the current study, the IELs distribution was examined with the T-bet monoclonal antibodies which showed 10 cases of Marsh III with an aggregation of cells in the tip. Another 6 cases had a regular distribution. On the other hand, 17 cases of Grade 0 Marsh with seropositive and ≤19 IELs had also a regular distribution of IELs along the villi, which showed a statistical significance in comparison with Marsh grading system ($P <$

0.001) and ($P < 0.017$) in comparison with the T-bet stained IELs. This could be suggestive that the distribution pattern of IELs can be indicative of early stage CD.

Conclusion

The majority of increased IELs were stained with T-bet, whereas in normal IELs count, there was less than half the IELs stained with T-bet. This would indicate that T-bet immunostaining is a potential alternative to H&E and/or CD3-based counting of IELs.

Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

The author declare that research involved human participants and consent was obtained.

This research was funded by the authors.

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