The Diagnostic Utility of Flow Cytometry in Celiac Disease Presented Isolated Iron Deficiency Anemia

Rafet Mete[1], *Mustafa Oran*[2], *Burcu Altındağ Avcı*[2], *Burhan Turgut*[3]

[1]Department of Gastroenterology, Faculty of Medicine, Namık Kemal University, Tekirdag, Turkey
[2]Department of Internal Medicine, Faculty of Medicine, Namık Kemal University, Tekirdag, Turkey
[3]Department of Hematology, Faculty of Medicine, Namık Kemal University, Tekirdag, Turkey

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ABSTRACT

Background: *Flow cytometric analysis of intestinal intraepithelial lymphocytes contributes to the diagnosis of celiac disease. Celiac disease may present with iron deficiency anemia alone which is considered as one of the forms of atypical celiac disease. In this study, we have aimed to investigate the diagnostic utility of flow cytometric analysis of intraepithelial lymphocytes in this atypical form.*

Methods: *Three groups were formed: the patients with unexplained iron deficiency (group 1), the patients with celiac disease (group 2), and the patients who underwent gastroduodenoscopy for other reasons (group 0). Duodenal biopsy samples were used for flow cytometric analysis of intraepithelial lymphocytes. T cell receptor gammadelta intraepithelial lymphocytes and CD3−/CD103+ intraepithelial lymphocytes were determined with relevant monoclonal antibodies. Sensitivity–specificity calculation was performed to evaluate the usability of flow cytometric variables as diagnostic tests.*

Results: *Group 1 had 22 patients, group 2 had 14 patients, and group 0 had 56 patients. In the comparison of the 3 groups, CD3+/TCRγδ+ intraepithelial lymphocytes were found to be higher in celiac patients than other cases. CD3+/TCRγδ+ intraepithelial lymphocyte was evaluated for its usability as a diagnostic test. The cut-off value of CD3+/TCRγδ+ intraepithelial lymphocyte as 16.39% according to receiver operating characteristics curve analysis determined celiac disease in 14 of 22 patients in group 1 with 91.7% sensitivity and 80.4% specificity.*

Conclusions: *Although celiac disease is diagnosed with serologic tests and histologic examination, successively, the increase in intestinal CD3+/TCRγδ+ intraepithelial lymphocytes may be used as a diagnostic test, and it may assist in revealing atypical forms of celiac disease.*

Keywords: *Atypical celiac disease, iron deficiency anemia, flow cytometry, IELs*

INTRODUCTION

Celiac disease is a small bowel disease caused by dietary gluten, characterized by mucosal inflammation, villous atrophy, and crypt hyperplasia.[1,2] The most widely accepted pathogenetic mechanism is that antigen presenting cells carrying human leukocyte antigen (HLA)-DQ2 and DQ8 antigens present modified gluten peptides to T lymphocytes in the intestinal mucosa.[3] The classical type of celiac disease is characterized by diarrhea, weight loss, or malabsorption, as well as the presence of antibodies to gliadine, and in particular tissue transglutaminase.[1] However, the use of serological, genetic, and histological data in diagnosis has led to the identification of other types of celiac disease such as atypical and subclinical types.[4]

In the atypical form of celiac disease, patients have only minor gastrointestinal complaints, but anemia, osteoporosis, arthritis, increased transaminases, neurological symptoms, and infertility can be observed.[4] In the subclinical form of celiac disease, there are no symptoms or very mild symptoms, but celiac disease may occur during follow-up.[4]

There are 2 separate lymphoid components in the intestinal mucosa; lamina propria T lymphocytes (LPLs) and intraepithelial lymphocytes (IELs). Both components contribute to the pathogenesis of celiac disease. While IELs provide natural immunity, LPLs are effective in providing adaptive immune response.[5] Since IELs are easier to obtain from the intestinal mucosa and have more characteristic changes than LPLs in celiac disease, they are more suitable for flow cytometric analysis (FCA).[1,3,6]

The first detectable immune abnormality in celiac disease is the increase in absolute and relative numbers of αβ and γδ IELs.[5] Both αβ and γδ IELs proliferate in situ in

Corresponding author: ***Rafet Mete****, e-mail:* ***rafetmete@yahoo.com***
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celiac disease. The increase in αβ IELs is associated with disease activity and returns to normal with gluten-free diet.[7] In spite of that, the increase in the γδ IELs is relatively less affected by the gluten-free diet. While γδ IELs in the intestinal mucosa account for 8% of all IELs in healthy controls, this rate is around 28% in celiac disease and does not alter much throughout the phases of the disease.[7]

In atypical and subclinical celiac disease, the demonstration of the increase in γδ IELs and the decrease in CD3–/CD103+ IELs by flow cytometry might contribute to the diagnosis.[3,7] Iron deficiency anemia is a common symptom of celiac disease and may sometimes be its only clinical manifestation.[1,4] Therefore, in this study, we have aimed to determine whether the IELs isolated from intestinal mucosa and studied by flow cytometry could contribute to the diagnosis of this atypical form of celiac disease.

PATIENTS AND METHODS

Patients

The study was planned prospectively, and 3 groups were formed; group 1 included the patients with unexplained iron deficiency anemia despite detailed diagnostic research and without clinical finding for classical celiac disease therefore were thought that they could have atypical celiac disease (research group). Group 2 comprises the patients with celiac disease or patients diagnosed with celiac disease in the course of the study (positive control group). Celiac disease diagnosis was based on autoantibody positivity such as anti-endomysial and/or anti-transglutaminase antibodies and histopathological evaluation of duodenal biopsy material as recommended.[8] Group 0 included the cases who underwent gastroduodenoscopy for other reasons such as dyspepsia and with no signs of celiac disease and no histopathological findings of celiac disease (negative control group). Patients under 18 years of age, who have known neoplastic disease, who are pregnant, and with renal or liver insufficiency were excluded. Upper gastrointestinal endoscopy was performed for all cases, and at least, 4 biopsy samples were obtained in all cases from second part of duodenum. One biopsy sample was sent for flow cytometric study, and other samples were sent to pathology department for histological assessment. Routine blood analyses of all cases were recorded.

Isolation of Intraepithelial Lymphocytes

Duodenal biopsy material was transferred to Roswell Park Memorial Institute tissue culture medium supplemented with 10% fetal calf serum without calcium. It was carefully crushed with a scalpel and shaken for 60 minutes. The obtained cells were washed with isotonic NaCl and made ready for flow cytometric study.

Immunophenotyping

In order to determine the ratio of TCRγδ IELs and CD3–/CD103+ IELs in total IETs, we employed monoclonal antibodies developed against CD3, CD103, and TCRγδ and labeled with different fluorescent agents. Monoclonal antibodies (CD3-phycoerythrin cyanin 5.1, CD103-flourescein isothiocyanate, and TCRγδ-phycoerythrin) were obtained from Navios Flow Cytometer (Beckman Coulter, Miami, FL, USA). Cell suspensions (100 µL) containing IELs were incubated with monoclonal antibody combinations for the appropriate time, with optimal concentrations determined previously, followed by fixation, and run on a Beckman Coulter Navios (USA) flow cytometry instrument. The data obtained were analyzed on 2 parameter cytograms (dot plot) using Kaluza program. CD3+/TCRγδ+ and CD3–/CD103+ IEL ratios in total lymphocytes were determined. Figure 1 shows a sample histogram of a patient.

Statistical Analysis

Variables between the 3 groups were compared by Kruskal–Wallis test. Chi-square test was used in the analysis of categorical data. Sensitivity–specificity calculation was performed to evaluate the usability of flow cytometric variables as diagnostic tests. The receiver operating characteristics (ROC) curve was drawn, and cut-off values were determined for continuous variables. Cases with celiac disease were identified in the research group (group 1) with the sensitivity–specificity determined by the cut-off values.

RESULTS

Demographic and laboratory characteristics of the patients are given in Table 1. Group 1 had 22 patients, group 2 had 14 patients, and group 0 had 56 patients. Ten of the patients with celiac disease were diagnosed within the year preceding the study. Four patients were diagnosed previously and 2 of them had been on a gluten-free diet. There was no statistically significant difference between the groups in terms of age and gender. There was no statistically significant difference between the groups in terms of hemoglobin value and ferritin, albumin, and C reactive protein serum levels. No difference in hemoglobin value and ferritin serum level between the groups was thought to have occurred due to the fact that

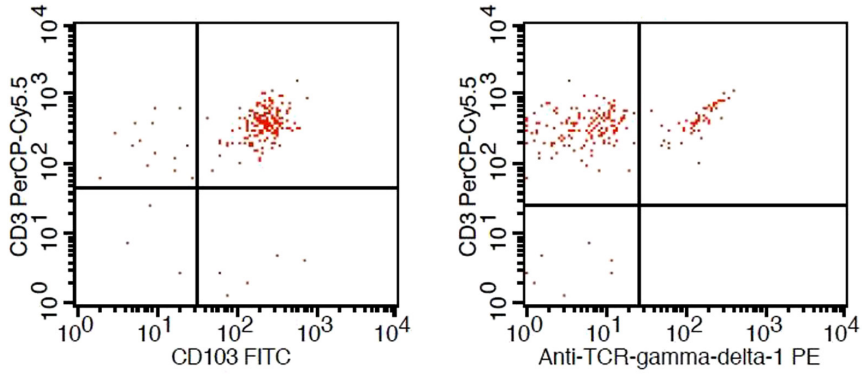

Figure 1. A representative histogram of a celiac patient showing the percentages of CD3+/TCRγδ+ IELs and CD3–/CD103+ IELs. IELs, intraepithelial lymphocytes; TCRγδ, T cell receptor gammadelta.

some of the patients received iron replacement therapy during the study.

CD3+/TCRγδ+ IEL and CD3–/CD103+ IEL percentages in intraepithelial total lymphocyte population were determined by flow cytometry. The distribution of IELs between the groups is given in Table 2. CD3+/TCRγδ+ IEL percentage was statistically different between the groups; the patients diagnosed with celiac disease (group 2 patients) had higher percentages than both groups. CD3–/CD103+ lymphocyte percentages were lower in the patients with celiac disease than other patients; however, the difference was not statistically significant.

CD3+/TCRγδ+ IELs are statistically significantly different between positive and negative control groups; therefore, we did a ROC analysis and sensitivity–specificity calculation for this variable to evaluate the usability of it as a diagnostic test. Two patients in the positive control (group 2) were not included in the ROC analysis because they were under the gluten-free diet, and the gluten-free diet would affect CD3+/TCRγδ+ IEL percentages.

Table 1. Demographic and Clinical Characteristics of the Patients

Variables	Group 1, n = 22	Group 2, n = 14	Group 0, n = 56	*P*
Age, years	38.09 ± 14.47	38.86 ± 11.19	45.05 ± 12.20	.052
Gender, F/M, n	13/9	10/4	45/11	.153
Hemoglobin, g/dL	11.99 ± 2.11	12.39 ± 2.51	12.22 ± 2.09	.87
Ferritin, ng/mL	24.13 ± 39.63	13.23 ± 11.23	28.34 ± 35.87	.42
Albumin, g/dL	4.54 ± 0.32	4.30 ± 0.38	4.46 ± 0.40	.26
CRP, mg/dL	1.85 ± 1.72	8.04 ± 15.89	4.88 ± 6.40	.10

All the parameters, except gender, are given mean ± SD. CRP, C reactive protein; SD, standard deviation.

Table 2. The Distribution of Intraepithelial Lymphocytes Between the Groups

Variables	Group 1, n = 22	Group 2, n = 14	Group 0, n = 56	*P*
CD3+/TCRγδ+ lym %	24.14 ± 18.43	37.08 ± 24.34	11.40 ± 7.88	<.0001
CD3–/CD103+ lym %	9.89 ± 13.26	3.72 ± 2.82	12.37 ± 14.61	.094

In the ROC analysis, the area under the curve was calculated as 0.936 ± 0.032 and the CI as 0.873-0.999. Accordingly, the diagnostic value of CD3+/TCRγδ+ was statistically significant ($P < .0001$). According to ROC analysis, when the cut-off value of CD3+/TCRγδ+ lymphocytes was taken as 16.39%, it was determined that this parameter could diagnose celiac disease with 91.7% sensitivity and 80.4% specificity. When this cut-off value was applied, 14 patients (63.7%) were compatible with celiac disease in the research group (group 1). Only 4 of these 14 patients had celiac antibody positivity. There were only 2 patients with CD3+/TCRγδ+ negative (under the cut-off), although the antibody was positive. There was no correlation between antibody test and CD3+/TCRγδ+ variable in the research group. Table 3 shows the correlation analysis between CD3+/TCRγδ+ and antibody test results.

DISCUSSION

Celiac disease is diagnosed by serological tests and intestinal biopsy.[8] Although serologic tests are used first line,

Table 3. The Correlation Analysis Between CD3+/TCRγδ+ and Antibody Test Results

	Antibody Positive, n (%)	Antibody Negative, n (%)	*P*
CD3+/TCRγδ+ positive	4 (18.2%)	10 (45.5%)	.856
CD3+/TCRγδ+ negative	2 (9.1%)	6 (27.3%)	

Positivity means the percentage of CD3+/TCRγδ+ is higher than cut-off value.

it is the gold standard to show the characteristic histopathological findings in the biopsy material.[8] The most sensitive and specific serological tests are tissue transglutaminase (tTG) IgA and antiendomysium IgA (EMA).[8,9] Sensitivity and specificity of tTG IgA and EMA ranged from 85% to 97%.[8-11]

Although anti-tTG and EMA antibodies are good indicators of the active phase of the disease, the definitive diagnosis requires intestinal biopsy to show typical histological abnormalities (villus disruption, crypt hyperplasia, and lymphocyte infiltration).[8] For cases lacking concordance between serological tests and intestinal biopsy, additional evaluations may be needed. In such cases, genotyping of HLA DQ2/DQ8 may be helpful, in spite of that, there are still some cases with difficulties in diagnosis.[8,12,13]

Flow cytometric study of IELs obtained from intestinal mucosa has been shown to contribute to the diagnosis of celiac disease.[1,3] The first detectable immune abnormality in celiac disease was shown to be the increase in absolute and relative numbers of αβ and γδ IETs.[5] Therefore, it is thought that atypical or subclinical celiac patients can be diagnosed by FCA before autoantibodies appear.

The proportions of CD3+/TCRγδ+ IELs and CD3−/CD103+ IELs in total IEL population (the so-called ''IEL immunophenotype'') have been used with success in the diagnosis of celiac disease.[14] Similarly, the evaluation of TCRγδ count and CD3-IELs in duodenal mucosal samples using flow cytometry can provide a high level of diagnostic accuracy for celiac disease.[15] Therefore, the proportions of these IELs in total IELs were measured in our study.

In our study, CD3+/TCRγδ+ and CD3−/CD103+ IELs were compared between the groups. In the comparison of the 3 groups, CD3+/TCRγδ+ IELs were found to be higher in celiac patients than other cases, in spite of that, probably due to low numbers in the groups, CD3−/CD103+ IEL did not differ statistically significant between the 3 groups. Therefore, we evaluated the variable of CD3+/TCRγδ+ IEL for its usability as a diagnostic test.

In our study, the contribution of immunophenotyping of IELs obtained in the intestinal mucosa by flow cytometry was investigated in unexplained iron deficiency patients who could be evaluated as an atypical form of celiac disease. We took the cut-off value of CD3+/TCRγδ+ IEL as 16.39% according to ROC curve analysis, and it was determined that this parameter could diagnose celiac disease with 91.7% sensitivity and 80.4% specificity. Fourteen of the unexplained iron deficiency patients (63.7%) checked against the cut-off value came out as compatible with celiac disease while only 4 of them had antibody positivity.

Immunophenotyping of IELs has been used with success in the diagnosis of pediatric celiac disease; the presence of a "celiac IEL immunophenotype" has been shown to have 94% sensitivity and specificity in the diagnosis of pediatric celiac disease.[16] Sanchez-Castanon et al.[17] studied celiac IEL immunophenotype in 60 celiac patients (40 active and 20 silent) and compared their results with 161 normal patients. Active and silent celiac patients had significantly higher levels of TCRγδ+IEL than non-celiac patients, and also the number of CD3-IEL cells was lower in celiac patients than in non-celiac patients. In this study, some cut-off values according to ROC curve analysis are proposed for CD diagnosis of IEL lymphogram. In a previous study, Valle et al. evaluated 312 patients with suspicion of celiac disease, 42 of whom were designated as difficult cases. Flow cytometric analysis of IELs in those cases allowed correct diagnoses in 39 out of these 42 cases (92.8%) with a sensitivity of 91.7% (95% CI: 61.5% to 99.8%) and a specificity of 93.3% (95% CI: 77.9% to 99.2%) for the diagnosis of celiac disease in this setting.[18] In the study of Valle et al., difficult cases were defined as the cases in which celiac disease could neither be confirmed nor denied through serology and histology. In our study, a group of patients with probable atypical celiac disease were selected. Intraepithelial lymphocyte immunophenotype was found to be highly (63.7%) compatible with celiac disease in our iron deficiency cases, of which the underlying cause could not be determined despite extensive research (gastroscopy, colonoscopy, celiac serology, extraintestinal system cancer screening, urinary systems examination, and gynecological examination in women).

It has been shown that both αβ and γδ IELs proliferate in situ in celiac disease and the increase in αβ IELs was

associated with disease activity and disappeared with the gluten-free diet, whereas the increase in γδ IELs was relatively less affected by the gluten-free diet.[7] Therefore, FCA of intestinal IEL can be more effective in the diagnosis of celiac disease and may be useful in the analysis of other atypical forms. In our study, in accordance with the literature, CD3+/TCRγδ+ IELs were found to be as high in the intestinal mucosa as in patients who were previously diagnosed with celiac disease and started a gluten-free diet. In spite of this, we did not include these cases in the ROC analysis.

It is important in clinical practice to differentiate celiac disease from other conditions with villous atrophy. Flow cytometry can be effective in distinguishing celiac disease from other diseases presenting with villous atrophy such as intolerance to cow's milk or soy protein, infectious diarrhea, tropical sprue, intestinal stasis syndrome, protein energy malnutrition, autoimmune enteropathy.[3] We did not evaluate this issue in our study.

In conclusion, although the most sensitive and specific tests for the diagnosis of celiac disease have been shown to be tTG, IgA, EMA, and typical histological abnormalities (villus atrophy, crypt hyperplasia and lymphocyte infiltration), the increase in intestinal CD3+/TCRγδ+ IELs can be used as a diagnostic test, and it may assist in revealing atypical forms of celiac disease.

Ethics Committee Approval: Ethics committee approval was received for this study from Ethics committee of Tekirdağ Namık Kemal University School of Medicine in 01.03.2012 with approval number 2012.01.01.07.

Informed Consent: Written informed consent has been obtained from all the participants involved in this research.

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Conflict of Interest: The authors have declared that no conflicts of interest exist.

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