Introducing New Potential Biomarkers for Celiac Disease among the Genes Extracted from General Databases

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ABSTRACT

BACKGROUND:
Inflammatory cytokines play roles in the pathogenesis of celiac disease. To introduce new diagnostic markers in patients with celiac disease for easy, fast, low cost, and non-invasive diagnosis, we evaluated the peripheral blood expression levels of interleukin-15 (IL-15), interleukin-17A (IL-17A), interleukin23A (IL-23A), granzyme B (GzmB), T-box transcription factor 21 (TBX21), and tumor necrosis factor alpha-induced protein 3 (TNFAIP3) of patients compared with the healthy controls, which were extracted from public databases organized in a protein-protein interaction network, in this group.

METHODS:
Peripheral blood mononuclear cells were collected from 30 patients with celiac disease and 30 healthy subjects. Total RNA was extracted, and mRNA expression levels of targeted genes were investigated by the quantitative real-time polymerase chain reaction (PCR) method. SPSS software was used for statistical analysis. Receiver operating characteristic (ROC) curve analysis was performed to characterize the diagnostic ability of the studied genes.

RESULTS:
The expression of IL-15, IL-17A, IL-23A, GzmB, TBX21, and TNFAIP3 genes in peripheral blood mononuclear cells of patients with celiac disease showed a significant increase compared with the control group. Among them, TNFAIP3, IL23A, and GzmB have better resolution and diagnostic value in differentiating patients with celiac disease from healthy controls.

CONCLUSION:
Our results suggest that TNFAIP3, IL23A, and GzmB could be useful and sensible markers in differentiating patients with celiac disease from healthy controls. However, the diagnostic relevance of other genes recognized by pathway analysis needs to be further investigated.

KEYWORDS:
Autoimmune diseases, Blood, Celiac disease, Inflammation, Polymerase chain reaction

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INTRODUCTION

Celiac disease (CD) is an autoimmune disorder triggered by gluten ingestion and characterized by small intestinal mucosal abnormality, chronic diarrhea, weight loss, and malabsorption in genetically predisposed individuals who carry HLA DQ2/DQ8 heterodimers.[1-3] Immune system

revolution in patients with CD has been investigated in various studies. The chronic inflammatory response in CD is maintained by cytokines and chemokines that induce differentiation and proliferation of immune cell types and activate T cells and B cells in the lamina propria during the active phase of the disease.[4,5] As a proinflammatory cytokine, interleukin (IL)-15 upregulation in CD induced by gluten peptides leads to Th17 and Th1 responses activation, natural killer (NK)-like phenotype promotion in CD8+ T cells, disruption of intestinal immune homeostasis, and a range of inflammatory consequences.[6,7] T-box 21 (TBX21/T-bet) is known as a transcription factor that is induced in the mucosa of patients with CD by IL-15 and causes Th1 cell development and interferon gamma (IFN-γ) production, the important cytokine in Th1 responses.[8] IL-15 also promotes upregulation of granzyme B (GzmB), a serine protease that is mainly found in the granules of cytotoxic lymphocytes and natural killer cells and plays a key role in lymphocyte mediated cytotoxicity in intraepithelial lymphocytes (IELs) of the small intestine of patients with CD.[9,10] IL-17A, which is known as IL-17, is another critical proinflammatory cytokine secreted from Th17 cells. The gluten-specific IL-17A production by Th17 cells has been observed in CD. In fact, Th17 cells have the ability to stimulate the production of other inflammatory cytokines and chemokines and initiate potent inflammatory responses.[11-15] Studies showed a relation between increased mucosal IL-17A response and villous atrophy in CD.[16]

The heterodimeric cytokine interleukin-23 (IL-23 or IL23A/IL12B) is a member of the IL-12 family produced by dendritic cells and macrophages, which has a role in the pathogenesis of several tissue-specific autoimmune diseases. According to the result of studies greater peripheral blood mononuclear cell (PBMC) production of IL-23 caused by gliadin can contribute to the pathogenesis of CD and is critical for Th17-mediated immune response activation.[17-19]

Moreover, tumor necrosis factor alpha-induced protein 3 (TNFAIP3), also known as A20, is involved in negatively regulation and termination of the nuclear factor kappa B (NF-κB) signaling pathway and controlling of inflammation in the pathology of CD. TNFAIP3 inhibited the expression of different proinflammatory cytokines like IL-17.[20-22]

Recent screening studies have shown that the prevalence of CD has doubled from 0.01%-0.05% to 1-2% over the past few decades.[23-25] Due to the absence of accurate diagnostic tools, 75%-90% of patients with CD remain undiagnosed.[24,26] The combination of intestinal mucosal changes evaluation by small-bowel biopsy and positive serum anti-transglutaminase-2 antibodies (anti-tTG), anti-endomysium antibodies, and gliadin peptide antibodies are used to diagnose CD. Despite advances in serology, there are currently no antibody tests with 100% sensitivity and specificity for CD.[27,28] Therefore, diagnosis of CD requires the development of accurate and facilitated screening tests that allow rapid and non-invasive screening of asymptomatic populations and the identification of such patients in high-risk groups (e.g, first-degree relatives and patients with various autoimmune disorders).[29] These non-invasive diagnostic methods for CD are under review these days.[30] In this study, we aimed to determine new diagnostic factors in CD to help gastroenterologists and clinicians in their clinical practice.

MATERIALS AND METHODS

Bioinformatics Analysis

CD-related genes were extracted from general databases (technical documents based on proteomics and microarrays) and organized in the protein-protein interaction network using the STRING database as a plugin of Cytoscape software version 3.6. For finding related biochemical pathways, the main genes were introduced and enriched through Gene Ontology (GO). After network analysis, 20 CD-related genes were introduced as hub-bottleneck nodes. According to the bioinformatics analysis in our previous study, GzmB, IL15, IL17A, IL23A, TBX21, and TNFAIP3 genes were selected as commonly expressed genes in blood and small intestine tissue.[22]

Study Population and Sample Collection

Thirty adult patients with CD, who did not start a gluten-free diet, (15 women and 15 men) with a mean ± SD age of 33 ± 10 years as case and 30 healthy subjects (15 women and 15 men) with a mean± SD age of 35 ± 12 years were recruited from Gastroenterology and Liver Diseases Research Center, Shahid Beheshti

University of Medical Sciences during February 2020-2021. The diagnosis of CD was based on positive serology (anti-tTG IgA and EMA IgA) confirmed by modified Marsh grade ≥2 villous abnormalities. Healthy subjects had negative serology tests for CD. They were matched with CD subjects in terms of sex and age. Patients with Marsh 0-I, pregnant and lactating women, patients with any other autoimmune/gastrointestinal diseases, and those who had a history of non-steroidal anti-inflammatory and immune suppressors drug intake were excluded from the study. Non-use of immune suppressors or anti-inflammatory agents were considered as inclusion criteria. Patients with CD and healthy people did not have a significant medical history in the past, such as hypertension and diabetes. Whole blood samples (5 mL) were collected in EDTA tubes. The tubes were placed at 4°C until RNA extraction.

RNA Extraction, Complementary DNA Preparation, and Real-Time PCR

Total RNA was extracted from PBMCs using YTA RNA Extraction (YEKTA TAJHIZ AZMA) kit and quantified by spectrophotometric optical density measurement (260 and 280 nm). Seven micrograms of RNA were reverse transcribed to cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, catalog number: K1691). The expression level of IL15, IL17A, IL23A, GzmB, TBX21, and TNFAIP3 mRNA were evaluated by quantitative real-time polymerase chain reaction (PCR). Real-time PCR using ABI 7500 real-time (2.3 version) PCR system (Applied Biosystems, Foster City, CA, USA), with Takara SYBR Master Mix instructions (Shiga, Japan).

Primers for IL15, IL17A, IL23A, GzmB, TBX21, TNFAIP3, and beta-2-microglobulin (B2M) (as a housekeeping gene) were designed by Gene Runner and Primer3 online programs (Table 1) and were confirmed via nucleotide BLAST searches (NCBI).

Statistical Analysis

Data are presented as means ± standard deviations. Student *t* test was used to analyze the difference in IL15, IL17A, IL23A, GzmB, TBX21, and TNFAIP3 mRNA expression levels between subject groups. To draw the graphs, GraphPad Prism software version 5 (GraphPad Software, Inc. La Jolla, CA, USA; https://www.graphpad.com/scientific-software/prism/) were utilized. The receiver operating characteristic (ROC) curve was used to characterize the diagnostic value of studied genes. *P* values ≤ 0.05 were considered significant.

RESULTS

mRNA Expression

We examined the expression level of IL15, IL17A, IL23A, GzmB, TBX21, and TNFAIP3 mRNA in PBMCs from 30 patients with CD compared with 30 healthy control using real-time RT-PCR and determined the changes in the expression level of these genes between the case and the control groups through the relative quantification method. In this method, the rate of gene expression changes is measured as fold change.

Figure 1 shows the mRNA expression level of IL15, IL17A, IL23A, GzmB, TBX21, and TNFAIP3 mRNA in PBMC of patients with CD compared with the controls. We found that IL15 (P =0.0085; Figure 1A), IL17A (P = 0.0008; Fig. 1B), IL23A (P < 0.0001; Figure 1C), GzmB (P < 0.0001; Figure 1D), TBX21 (P =0.0197; Figure 1E), and TNFAIP3 (P <0.0001; Figure 1F) mRNA levels in the PBMC of patients with CD was significantly increased compared with the controls.

Evaluation of the Diagnostic Value of These Genes in CD

To evaluate the diagnostic value of IL-15, IL-17A, IL-23A, GzmB, TBX21, and TNFAIP3 genes in peripheral blood samples of patients with CD, the ROC curve was plotted using SPSS software (v. 21), and the area under the curve (AUC) was obtained for each of the variables. According to the ROC curve, the peripheral blood mRNA level of IL-15, IL-17A, IL-23A, GzmB, and TNFAIP3 genes was able to distinguish patients with CD from healthy controls (Table 2).

IL-17A and IL-15 with AUC=0.7-0.8 had relatively good diagnostic power. GzmB with AUC=0.8-0.9 had good diagnostic power, and IL-23A and TNFAIP3 with AUC=0.9-10 had excellent diagnostic power in differentiating between patients with CD and healthy controls (Figure 2).

Table 1. Primers used for quantification

Gene symbol	Primer sequence	Product length
IL15 F	5′-TTGTGGATGGATGGCTGCT-3′	87 bp
IL15 R	5′-TCTCATTACTCAAAGCCACGGT-3′	
IL17A F	5′-AGGCCCCTCAGAGATCAAC-3′	91 bp
IL17AR	5′-TTGTGTGGTGCCTTGATCAG-3′	
IL23A F	5′-ACAACAGTCAGTTCTGCTTGC-3′	204 bp
IL23A R	5′-GACTGAGGCTTGGAATCTGC-3′	
GzmB F	5′-CCTGGGAAAACACTCACACACA-3′	91 bp
GzmB R	5′-GTCGTAATAATGGCGTAAGTCAGAT-3′	
TBX21 F	5′-CCTTGGGGGAGATCACTACTC-3′	166 bp
TBX21 R	5′-CATGCTGACTGCTCGAAACT-3′	
TNFAIP3 F	5′-CATCCACAAAGCCCTCATCGAC-3′	118 bp
TNFAIP3 R	5′-ATTGCCGTCACCGTTCGTTT-3′	
β2MG F	5′-CCAGCGTACTCCAAAGATTC-3′	102 bp
β2MG R	5′-ATGTCGGATGGATGAAACCC-3′	

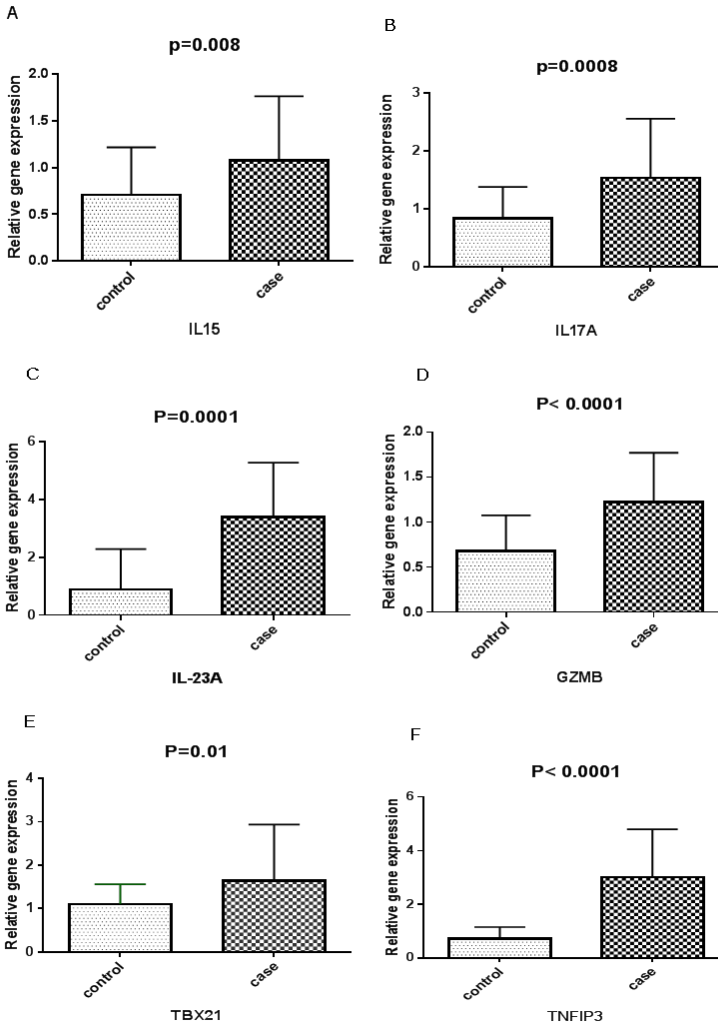

Fig. 1: The mRNA expression level of IL15, IL17A, IL23A, GzmB, TBX21, and TNFAIP3 mRNA in PBMC of patients with CD compared with the controls. (A) IL15 ($P = 0.0085$); (B) IL17A ($P = 0.0008$); (C) IL23A ($P < 0.0001$); (D) GzmB($P < 0.0001$); (E) TBX21($P = 0.0197$); (F) TNFAIP3 ($P < 0.0001$).

Table 2. The sensitivity and the specificity of IL15, IL17A, IL23A, GzmB, TBX21, and TNFAIP3 genes

Gene symbol	Sensitivity%	Specificity%	AUC	Cutoff	*P* value
IL15	57	93	0.7379	0.50	0.0003
IL17A	37	50	0.7160	0.34	0.0026
IL23A	91	85	0.9076	0.75	< 0.0001
GzmB	95	69	0.8363	0.64	< 0.0001
TBX21	42	92	0.5693	0.33	0.3015
TNFAIP3	80	93	0.9099	0.72	< 0.0001

Abbreviation: AUC, area under curve.

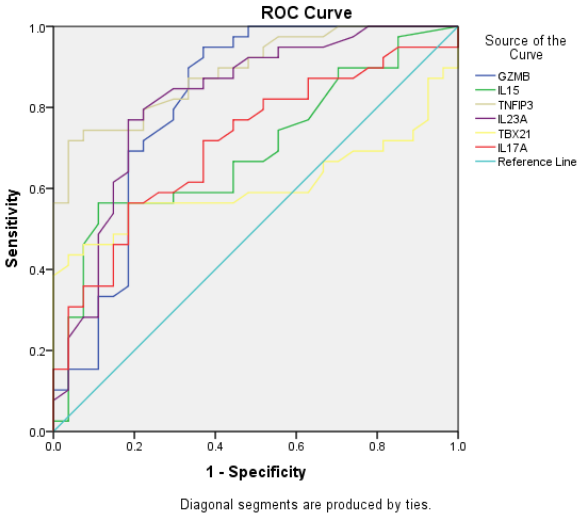

Fig. 2: ROC curve of GzmB, IL15, IL17A, IL23A, TBX21, and TNFAIP3 genes in differentiating patients with CD from healthy controls.

DISCUSSION

The aim of this study was to evaluate the PBMC mRNA expression level of those CD-related genes extracted by bioinformatic analysis in the peripheral blood and intestinal tissue samples of patients with CD compared with healthy subjects (IL-15, IL-17A, IL-23A, GzmB, TBX21, and TNFAIP3). Available diagnostic tests for CD include a combination of serologic tests and histologic evaluation of the small intestine. Finding new diagnostic markers that can be evaluated in patients' blood samples can lead to a faster, easier, more cost-effective, and non-invasive diagnosis of CD. The results of this study and other similar studies can be helpful in this regard, although further additional research is needed.

Like many autoimmune diseases, several cytokines and signaling proteins play roles in CD pathogenesis.[31] According to our results, the expression level of IL-15, IL-17A, IL-23A, GzmB, TBX21, and TNFAIP3 genes in the peripheral blood sample of the patients with CD was significantly increased compared with the control group.

IL-15 is a proinflammatory cytokine that exerts different biological functions in CD pathogenesis and is required for the development of villous atrophy.[5] Aghamohamadi and colleagues demonstrated that IL-15 gene expression is increased in biopsy specimens of CD patients with Marsh II compared with the control group.[31] Mention and co-workers also found the overexpression of IL-15 in lamina propria and intestinal epithelium of patients with active CD.[9]

TBX21/T-bet is a transcription factor induced by IL-15 in the intestine of patients with CD that causes IFN-γ production. Frisullo and others reported that T-bet expression was higher in peripheral blood mononuclear cells of untreated patients with CD than treated subjects and controls.[32] Increases in T-bet expression have also been reported in mucosal samples of patients with CD than controls by Monteleone and colleagues.[33]

GzmB is a cytotoxic protease promoted by IL-15 and has a key role in lymphocyte-mediated cytotoxicity in IELs of the small intestine of patients with CD. Mention and colleagues reported that GzmB expression was significantly up-regulated in biopsy specimens of patients with active CD and refractory celiac sprue and attributed its induction to IL-15.[9] Moreover, Pohjanen and co-workers decreased the expression of protease inhibitor 9 expression, a GzmB inhibitor that is crucial for duodenal homeostasis, in duodenal biopsies of patients with CD together with increased GzmB expression, apoptosis rate, and severity of villous atrophy.[10]

IL-17A is also a critical proinflammatory cytokine, which affects villous atrophy in CD. Faghih and others, in their study on duodenal biopsies of treated and untreated patients with CD observed a high level of IL17A gene expression than controls.[34] Lahdenperä

et al also found that the mucosal expression of IL-17 was elevated in children with untreated CD, and they stated that Th17 immunity occurs at the late stage of disease and adherence to a gluten-free diet can downregulate it.[35] IL-23 has a role in the pathogenesis of several tissue-specific autoimmune diseases like CD and contributes to Th17-mediated immune response activation. Harris and others found that wheat gliadin induces IL-1 signaling pathway that led to increased IL-23 expression in PBMC from patients with CD related to HLA-DQ2(+) healthy controls.[18]

TNFAIP3 (A20) is involved in controlling inflammation in the pathology of CD. Trynka and colleagues in 2009 for the first time, reported that single nucleotide polymorphisms in the A20 region on 6q23.3 was a disease risk factor in CD.[20]

As it turns out, the results of previous studies are in line with our findings. Among the studied variables, TNFAIP3, IL-23A, and GzmB, which have a higher AUC than others, have better resolution in differentiating patients with CD from healthy controls.

CONCLUSION

Several serum/plasma biomarkers have previously been suggested for fast and non-invasive diagnosis of CD. Our results suggest that TNFAIP3, IL-23A, and GzmB could be useful and sensible markers in differentiating patients with CD from healthy controls. However, the diagnostic relevance of other genes recognized by pathway analysis needs to be further investigated.

Figure 3 schematically shows the role of GzmB, IL-15, IL-17A, IL-23A, TBX21, and TNFAIP3 genes in immune responses and inflammatory processes.

Induction of IL-15 and 23 gene expression by genetic and environmental factors leads to CD4+ T cells proliferation (GzmB acts as an intermediate) and IL-17, IFNγ, IL-21, IL-4, IL-2 mediated inflammation. Infiltration of macrophages and B and T cells to the inflamed site increases the expression of mediators such as TBX21 and NFKB, which have a role in inflammation control (eg. through TNFAIP3 expression induction).

AUTHORS' CONTRIBUTION

MRN and MRT: Substantial contributions to conception and design; EKh, NA: acquisition and analysis and interpretation of data; EKh, BF. N. M. Gh: Drafting the manuscript, MRN, NA, SJS, MHH: revising the manuscript critically for important intellectual content. All authors revised the

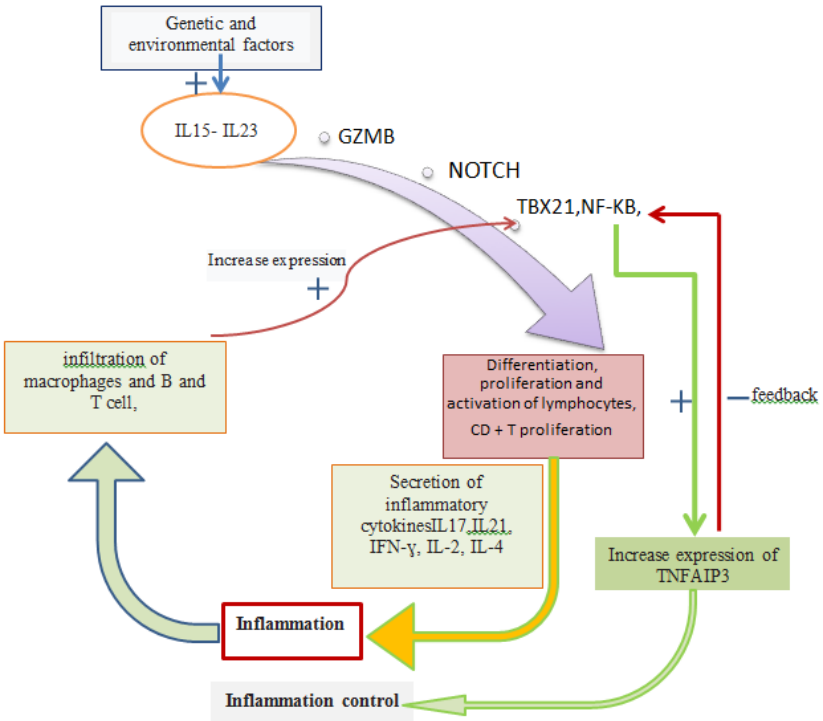

Fig. 3: The roles of GzmB, IL15, IL17A, IL23A, TBX21, and TNFAIP3 genes in immune responses and inflammatory prosses (directly or indirectly) are schematically shown.

manuscript and approved the final version for submission.

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ETHICAL APPROVAL

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1399.1146). Participants were informed of the content of the study, and written consent was signed by all participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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