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Elevated interleukin-17A levels despite reduced microRNA-326 gene expression in celiac disease patients under gluten-free diet

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Introduction: The microRNA-326 (miR-326) gene, by targeting ETS Proto-Oncogene 1 (ETS1), regulates the differentiation and interleukin-17A production of T helper 17 (Th17) cells. Celiac disease (CD) is an intestinal autoimmune disorder, in which the cascade of Th17 cells plays an important role in its pathogenicity. The aim of this study was to evaluate the expression changes of miR-326 and its two target genes ETS1 and IL-17A in celiac disease patients under a gluten-free diet (GFD). We expected the expression of miR-326 and IL-17A gene to decrease, and the expression of the ETS1 gene to increase, following the adherence to GFD.

Methods: Peripheral blood samples of 40 CD patients under GFD (for more than 1 year) and 40 healthy individuals were collected. RNA was extracted, cDNA was synthesized and the miR-326, ETS1 and IL-17A gene expressions were evaluated by the quantitative polymerase real-time qPCR method. P-value < 0.05 was considered statistically significant.

Results: Although miR-326 mRNA expression was significantly lower in CD patients (P = 0.001), no significant difference was observed in ETS1 mRNA level between the two groups (P = 0.54), but IL-17A was significantly overexpressed in CD patients (P=0.002). No significant correlation was observed between the expression of the studied genes and the patients' symptoms and Marsh classification.

Conclusion: Adherence to the GFD for one to two years did not have the expected effect on the expression of genes in this panel. The most important finding that contradicted our hypothesis was the observation of high IL-17A levels in CD patients despite dieting, which may be related to the protective effect of this cytokine on intestinal tight junctions, which needs to be confirmed in further studies.

Key words: autoimmune diseases, celiac disease, gene expression, microRNAs, reverse transcriptase polymerase chain reaction.

- We studied the expression profiles of miR-326 and its two target genes (ETS1 and IL-17A) in peripheral blood samples of gluten-free diet treated (1 to 2 years) celiac patients in comparison to the healthy controls.
- We expected the expression of miR-326 to decrease, the expression of the ETS1 gene to increase, and the expression of the IL-17A gene to decrease following the adherence to GFD. At the end of the study, the results did not fully support this hypothesis.
- Elevation of IL-17A may be effective in maintaining patients' intestinal tight junctions, which need to be evaluated in further studies.

INTRODUCTION

Celiac disease (CD) is a chronic autoimmune disorder characterized by small intestinal enteropathy induced by gluten ingestion in genetically predisposed subjects [1-3]. Gluten proteins, which are highly resistant to gastrointestinal digestion, are found in different cereals like wheat, rye, and barley [1, 4]. CD prevalence is estimated to be about 0.5-1% in the

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general population [5, 6]. Innate and adaptive immune responses play an important role in CD pathogenesis [7]. CD pathogenesis is associated with a dominant activation of Th1 lymphocytes that express high levels of Th1-related proinflammatory cytokines [8]. Th17 cells are also involved in CD pathogenesis and produce proinflammatory cytokines like interleukin (IL)-17A [9]. According to the reports, the inflammatory properties of Th17 cells contribute to promoting the production of chemokines and other inflammatory cytokines and elevating the synthesis of tissue-damaging proteases. It has been reported that IL-17A is highly produced in the inflamed intestine of patients with CD [1].

MicroRNAs (miRNAs) are short non-coding ribonucleic acids with 19–23 nucleotides in length, which play essential roles in the regulation of the host genome expression at the post-transcriptional stage [10]. miR-326 has 20 nucleotides and is encoded through intron 1 of chromosome 11 [11]. Over-expression of miR-326 is directly or indirectly related to the pathogenesis of autoimmune disorders [12]. Mir-326 regulates T cell differentiation and promotes IL-17A production. In fact, miR-326 improves the differentiation of Th17 cells by targeting the ETS Proto-Oncogene 1 (ETS-1) transcription factor, which is the negative regulator of the Th17 differentiation and participates in regulating the homeostasis of immune cells [13, 14]. Recent studies reported the miR-326 regulation of Th17 differentiation by affecting ETS-1 expression in the pathogenesis of several diseases [14]. Moreover, it has been reported that ETS-1 is one of the genes that has an association with CD in the 5' untranslated region (UTR) region [15]. Many genetic studies also reported the association between rs11221332 of ETS1 and celiac disease [16].

Due to the importance of a more detailed study on the pathogenesis of celiac disease, the necessity of studying the effect of gluten free diet (GFD) on the pathological conditions caused by the disease and the emerging role of microRNAs in the diagnosis and treatment of various disorders, this study aimed to investigate the effect of GFD on the expression of miR-326, ETS1 and IL-17A, as a panel that has not been studied so far in celiac disease.

MATERIAL AND METHODS

Study population and sample collection

In the present study, 40 confirmed CD patients by serological tests (serum IgA tTG antibody [tTGA] and/or anti-endomysial antibody [EMA IgA]) and histopathology evaluations, who referred for routine celiac disease checkups (diet, symptoms check and serological tests) to the celiac department at the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran during September–March of 2019–2020, have been recruited. Celiac disease patients were on a gluten-free diet for one to two years. Forty healthy individuals with no history of celiac disease and other autoimmune disorders up to their first-degree relatives, who had undergone annual checkup without any complicating risk factors, and were willing to participate in the study were considered as a control group. Subjects with other autoimmune disorders and inflammatory conditions, colorectal cancer, inflammatory bowel disease, gastrointestinal infections, pregnancy, or lactation were excluded from the study.

The research become authorized through the Ethical Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Ethics code: IR.SBMU.RIGLD.REC.1399.036). All participants have been asked to cautiously study and sign an informed consent. Peripheral blood samples were collected from patients and controls into EDTA tubes. Demographic data, as well as clinical symptoms (at the time of diagnosis), have been registered earlier than blood sampling. None of the cases have a complication (symptomatology or serologic) at the time of sampling.

RNA extraction and cDNA synthesis

YTA RNA Extraction Kit (YEKTA TAJHIZ AZMA, Taiwan) was used to extract total RNA from peripheral blood samples. The concentration and purity of the extracted RNA were determined using NanoDrop spectrophotometry (optical density =260/280) and samples were stored at −80°C until the next use. Complementary DNA (cDNA) synthesis was done by the Biofact cDNA Synthesis Kit (2 steps 2x RT-PCR Pre-mix Taq, Korea).

Primer designing and gene expression analysis

Gene sequences were obtained from the National Biotechnology Information Center (NCBI) Web site at https://www.ncbi.nlm.nih.gov, and primers were designed using Gene Runner

software. The sequence of designed primers and their properties are shown in Table 1. The specificity of the primers was checked by performing PCR experiments and products were separated by electrophoresis on a 1.5% agarose gel, and bands were visualized using UV-fluorescence.

mRNA expression of IL-17A, ETS1 and Beta2 micro-globulin (B2M), as house-keeping gene, were assessed using quantitative polymerase real-time qPCR with SYBR Premix Ex Taq (RealQ Plus 2x Master Mix Green-Amplicon, Japan). Stem-loop RT-PCR was done to determine the level of miR-326 and U6 (for normalization) using SYBR Premix Ex Taq (Tli RNase H Plus;Takara, Kusatsu, Japan). The $2^{-\Delta\Delta Ct}$ was used to analyze the relative changes in genes expression ($\Delta Ct=Ct^{gene}-Ct^{control}$).

Table 1
Primers used in qPCR

Gene Symbol	Primer sequence	Length
miR-326 (Forward)	5´CCTCTGGGCCCTTCCTCCAGGTCGT 3´	25
miR-326 (Reverse transcription)	5´GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACCTGGAG 3´	50
Universal reverse	5´CCAGTGCAGGGTCCGAGGTA 3´	20

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) (V.21) and GraphPad, Prism (V.6). The student's t-test was used for the analysis of the significance of differences in averaged gene expressions. The correlation between variables was assessed by Pearson's correlation test. A P-value < 0.05 considered statistically significant.

RESULTS

Demographic and clinical characteristics

Forty CD patients who had been on a gluten-free diet (for one to two years) consisting of 28 (70%) females and 12 (30%) males were included in this study. Moreover, 40 healthy individuals consisting of 23 (57.5%) females and 17 (42.5%) males were considered as the control group. The mean age of patients and controls were 36.75± 15.26 and 34.32 ± 4.9, respectively. There was no significant difference between age (p= 0.8) and gender (p= 0.75) among CD patients and controls.

The most common gastrointestinal symptoms of CD patients (at the time of diagnosis) were bloating (65%) and weight loss (52.5%), and the most common extra-intestinal symptom were anemia (67.5%) and fatigue (60%) (Fig. 1).

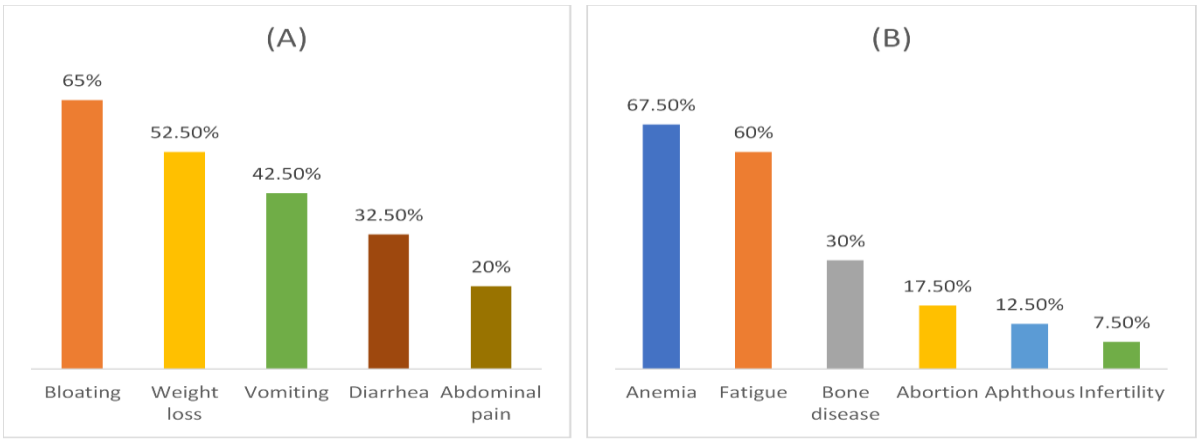


Fig. 1. Symptoms reported by CD patients: most patients complained of two or more symptoms. A: Gastrointestinal symptoms, B: extraintestinal symptoms. CD: Celiac disease.

mRNA expression of IL-17A, ETS1 and miR-326

We investigated the mRNA expression of IL-17A, ETS1, and miR-326 in peripheral blood samples of CD patients and healthy control subjects using B2M and U6 as housekeeping genes. A significant increase in IL-17A mRNA level was shown in CD patients' samples (Mean±SEM:11.9 ± 1.1) in comparison to controls (Mean±SEM:6.79 ± 1.07), (p=0.002), (Fig. 2a). The mRNA expression of ETS1 did not show a significant difference between patients (Mean±SEM:0.72 ± 0.08) and controls (Mean±SEM:0.79 ± 0.07), (p=0.54). (Fig. 2b). Moreover, the expression level of miR-326 exhibited a significant decrease in CD patients' specimens (Mean±SEM:0.08 ± 0.01) compared to the control group (Mean±SEM:0.9 ± 0.15), (p=0.001) (Fig. 2c).

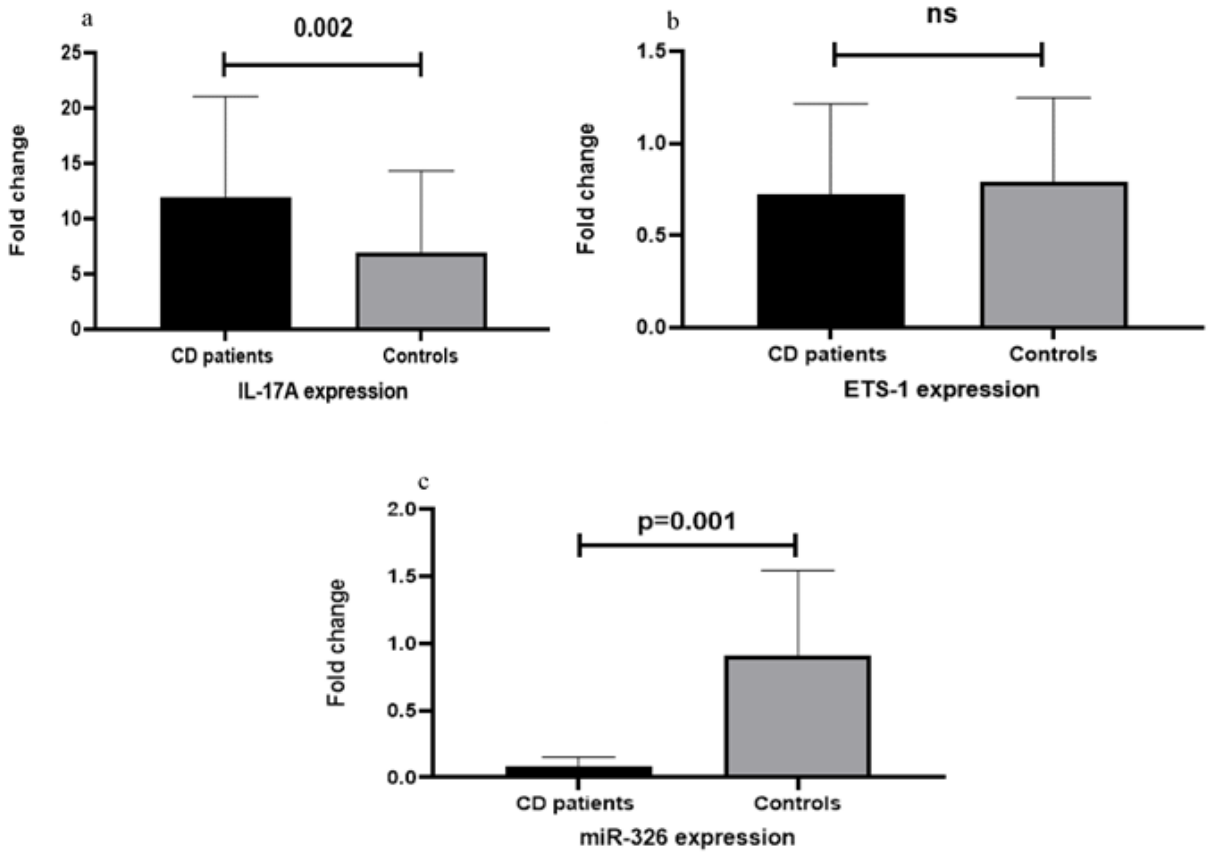


Fig. 2. Analysis of relative expression levels in CD patients compared to normal controls using real-time PCR assay. The analysed genes were as follows: (A) IL-17A, (B) ETS1and (C) miR-326. CD: Celiac Diseas; ETS1: ETS Proto-Oncogene; IL-17A: Interleukin 17a; miR-326: MicroRNA 326.

Correlations

Bivariate Pearson's correlation showed that there is not any significant correlation between IL-17A, ETS1 and miR-326 genes expression and CD patients' symptoms (Table 2).

Moreover, there was not any significant correlation between the expression of the studied genes and the patients' Marsh classification (Table 3).

DISCUSSION

The expression of ETS1 and miR-326 genes in autoimmune diseases such as autoimmune thyroiditis, multiple sclerosis, type 1 diabetes, etc. has been evaluated in previous studies but no similar study has been performed on patients with celiac disease [17–19]. Gene expression studies which have assessed the contribution of genes to the pathophysiology of

CD and the effect of GFD on this contribution are crucial in providing a deeper insight into CD development and management [20, 21].

The relationship between miR-326, ETS1 and IL-17-producing T-helper cells have been reported in previous studies [22–24]. According to the present study, while miR326 mRNA levels were significantly decreased in GFD-treated CD patients (P = 0.001), ETS1 levels were unchanged (P = 0.54) and IL-17A levels were significantly increased (P =0.002) compared to controls. Farisulla *et al.* conducted a study on 15 untreated, 15 GFD-treated CD patients (for at least 1 year), and 30 healthy controls and reported that the expression level of IL-17 in the peripheral blood samples of untreated and treated CD patients did not show a significant change relative to healthy controls [25]. Khalkhal *et al.* conducted a bioinformatics study on CD intestinal tissues and peripheral blood mononuclear cell (PBMC) or whole blood samples using a microarray-based technique and reported that the IL-17A gene is one of the genes that has increased expression in blood and small intestinal tissue samples of patients with celiac disease [26]. Faghih *et al*. by examining small intestinal tissue samples of untreated celiac disease patients and treated subjects in comparison with the control group showed that IL-17A gene expression in both groups of patients has a significant increase relative to the control group [20]. It is noteworthy that La Scaleia *et al.* reported that the size and dynamics of circulating tissue T IL-17A+ cells are defective in celiac disease, and this defect has a negative effect on the efficiency of the epithelial barrier and is associated with mucosal damage in patients with CD. They concluded that the IL-17 cytokine family can also be effective in protecting tight junctions [17]. Therefore, it can be considered that if the increase in the level of IL-17A in GFD treated CD patients is not due to inadvertent consumption of gluten by them, it may be related to the protective effect of this cytokine on intestinal tight junctions, which needs to be confirmed in further studies.

Table 2
The correlation between clinical symptoms and IL-17A, ETS1 and miR-326 genes expression in celiac disease group

Symptoms	IL-17A mRNA expression		ETS1 mRNA expression		miR-326 mRNA expression	
	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient
Diarrhea	0.647	0.101	0.514	-0.131	0.638	0.183
Nausea and vomiting	0.222	0.259	0.179	-0.266	0.244	0.433
Bloating	0.422	-0.164	0.445	0.147	0.476	0.274
Anemia	0.103	-0.320	0.766	0.058	0.143	0.497
Weight loss	0.701	-0.081	0.310	-0.195	0.346	0.361
Aphthous	0.347	-0.196	0.464	-0.144	0.4	-0.212
Infertility	0.471	0.166	0.543	-0.141	0.32	-0.76
Abortion	0.408	-0.186	0.318	-0.208	0.555	-0.247
Fatigue	0.534	0.128	0.829	0.042	0.825	0.087
Bone disease	0.731	-0.076	0.353	-0.190	0.4	0.12
Abdominal pain	0.725	0.137	0.182	-0.707	0.2	0.19

Table 3
The correlation between Marsh classification and IL-17A, ETS1 and miR-326 genes expression in celiac disease group

Variables	P-value	Correlation coefficient
IL-17A mRNA expression	0.245	0.273
Ets1 mRNA expression	0.722	0.096
miR-326 mRNA expression	0.209	0.498

CONCLUSION

In the present study, the expression profiles of miR-326 and its two target genes (ETS1 and IL-17A) were evaluated in GFD-treated CD patients compared to the control group.

In fact, we expected the expression of miR-326 to decrease, the expression of the ETS1 gene to increase, and the expression of the IL-17A gene to decrease following the adherence to GFD, but the results did not fully support this hypothesis. Apart from the effect of gluten cross-contamination of their diet and/ or the duration of GFD adherence by patients on the expression of genes in this panel, there is a need to investigate the effect of interfering factors on their expression. It is also possible that elevation of IL-17A can be effective in maintaining patients' intestinal tight junctions, which need to be evaluated in further studies.

Introducere: *miR-326 prin legarea de protooncogena ETS reglează diferențierea limfocitelor Th17. Boala celiacă (CD) este o boală autoimună caracterizată și de modificări ale Th17. Scopul studiului a fost de a evalua expresia miR-326 la pacienții cu boală celiacă aflați pe dietă fără gluten (GFD).*

Metode: *40 pacienți cu CD cu durata tratamentului GFD mai veche de 1 an și 40 de martori sănătoși au fost incluși în studiu. Din sângele periferic a fost extras ARN-ul, iar expresia miR-326, ETS1 și IL-17A a fost evaluată prin PCR.*

Rezultate: *Deși nivelurile miR-326 au fost mai mici la pacienții cu CD (p=0.001) nu s-au observat diferențe între expresia ETS1, iar IL-17A a fost supraexprimată la CD (p=0.002). Nu s-au observat asocieri semnificative între genele studiate și caracteristicile clinice ale pacienților.*

Concluzii: *Aderența GFD nu a avut efectul scontat pe expresia genelor. Nivelurile crescute ale IL-17A ar putea fi legate de efectul protector al acestei citokine pe joncțiunile intestinale, date ce trebuie confirmate în studii viitoare.*

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