



# Epigenetic Mechanisms of Genes Influencing Immune Response in Patients with Celiac Disease

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## ABSTRACT

**Aim:** The goal of this study was to investigate the expression levels of microRNAs (miRNAs) (miR-196b, miR-10a, miR-31-5p, and miR-338-3p) which regulate the genes involved in the proliferation and function of cells functioning in the inflammatory processes in Celiac patients' blood and tissue samples. Celiac disease (CD) is an inflammatory disease which affects people who are genetically predisposed to gluten consumption. The only treatment for this disease is a gluten-free diet.

**Materials and Methods:** The miRNA expressions were determined in blood and tissue samples from 12 pediatric patients with CD and from 8 healthy children using quantitative real-time PCR (qRT-PCR) and SybrGreen dye. The gene expression levels of miRNAs such as miR-196b, miR-10a, miR-338-3p, and miR-31-5p were compared between the two groups.

**Results:** There was a significant difference only in *miR-10a* gene expression levels between the control and patient blood samples. The greatest difference between the tissue and blood samples within the CD group were found in the expressions of miR-31-5p and miR-338-3p. It was seen that the patients' human leukocyte antigen tissue type was not associated with their miRNA expression profiles. In addition, there was no significant correlation between their Marsh classification and gene expression levels.

**Conclusion:** The significantly low level of miR-10a may be related to CD due to its effect on the immune response. Additionally, miR-10a may have potential as a non-invasive biomarker in the diagnosis of CD.

**Keywords:** miRNA, Celiac disease, qRT-PCR, gene regulation

## Introduction

Celiac disease (CD) is an autoimmune enteropathy which develops in the intestine because of gluten digestion in genetically susceptible people. Human leukocyte antigen

(HLA)-DQ2.5, DQ8, and DQ2.2 haplotypes are found in genetically susceptible individuals. When these people consume gluten-containing foods, their immune systems react. This response causes chronic inflammation, which

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harms the duodenum mucosa. The molecular processes which may aid in diagnosis are not completely understood (1).

The histopathological determination of villous atrophy and an increase in intra-epithelial lymphocytes in duodenal biopsies was previously accepted as the gold standard method for CD diagnosis. It will be extremely valuable to discover a new biomarker which can be detected in a patient's blood and can provide information about CD, particularly before intestinal damage occurs in the early stages (2).

Mature microRNAs (miRNAs) are small non-coding single-stranded RNAs which are 18-25 bp in length. They primarily prevent translation from the target mRNAs. These small molecules could be used as diagnostic biomarkers, particularly in CD. Several studies have been conducted to determine the role of several proteins which are targets for miRNAs (miR-192, miR-195-5p, miR-449a, and miR-638) in the pathogenesis of CD (3).

The goal of this study was to compare the gene expression levels of miRNAs such as miR-196b, miR-10a, miR-338-3p, and miR-31-5p, which may play a role in the pathogenesis of CD, in pediatric patients with a healthy control group. Our experiment was unique in that there had not been a study linking miR-196b and miR-10a to the etiology of CD, and miR-338-3p and miR-31-5p had not been explored in pediatric celiac patients to date.

## Materials and Methods

### Sampling

This study was a prospective case-control study which included 12 duodenal biopsy and blood samples from pediatric celiac patients and 8 blood samples from healthy children between 2019 and 2021. CD was excluded with negative anti-tissue transglutaminase (tTG) IgA-IgG and negative endomysial antibody (EMA) IgA-IgG in the healthy children group. CD diagnosis was made by duodenal biopsy in those who had positive tTG IgA- IgG and EMA IgA-IgG (4). All patients were screened for IgA deficiency. The HLA types of all participants were determined by the molecular method. Tissue samples were collected in an RNA saver tube (A.B.T, Turkey) and then lysed with liquid nitrogen. For the control tissue, a tissue sample was taken from a patient who was biopsied with the suspicion of CD but was found not to have CD. The association between the miRNA expression profiles and the clinical features of the patients was investigated.

Signed informed consent forms were obtained from the participants before this study took place. This study was conducted according to the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (approval no.: 01, date: 29.03.2019).

### miRNA Isolation from Sera and Tissue Samples

Sera samples were separated from whole blood samples by centrifugation at 4,000 rpm for 5 minutes. miRNAs were isolated from fresh sera samples according to the manufacturer's instructions using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany).

Firstly, tissue samples were lysed with liquid nitrogen using a sterile mortar and pestle. Then, miRNA isolation from these lysed tissue samples was performed according to the manufacturer's instructions using NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany).

The purity and concentration of miRNAs were measured with the NanoDrop 2000 Instrument (ThermoScientific, Wilmington, Delaware USA). Those samples with a ratio of 1.9-2.2 were accepted as pure (5).

### cDNA Synthesis

cDNA synthesis was performed according to the manufacturer's instructions using the MiScript II RT Kit (Qiagen, Germany). Before usage, the cDNA samples were diluted 1/100 according to the kit's instructions (6).

### The Analysis of miRNA Gene Expressions by Quantitative Real-Time PCR (qRT-PCR)

The quality and concentration of the cDNA samples were tested by qRT-PCR using RNU6 reference primer pairs (Qiagen, ABD). Threshold cycle (Ct) values between 15 and 35 were accepted as positive. In cases of low expression of RNU-6 control in blood, miR-16 primer was designed as the second control (F: CCGGAGTAGCAGCAGCGTAAAT R: ATCCAGTGCAGGTCCGA) (7). miR-196b, miR-10a, miR-31-5p, and miR-338-3p gene expression levels were determined by using gene-specific primer assays (Qiagen, USA) and reference primers in all cDNA samples. qRT-PCR was carried out with SYBR Green in a Rotorgene system. qRT-PCR conditions were optimized as 15 minutes at 95 °C, 40 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C, and 30 seconds at 70 °C. Each experiment was repeated three times (7).

### Statistical Analysis

Ct values above 35 were excluded from this study. The fold changes in gene expression values of all individuals

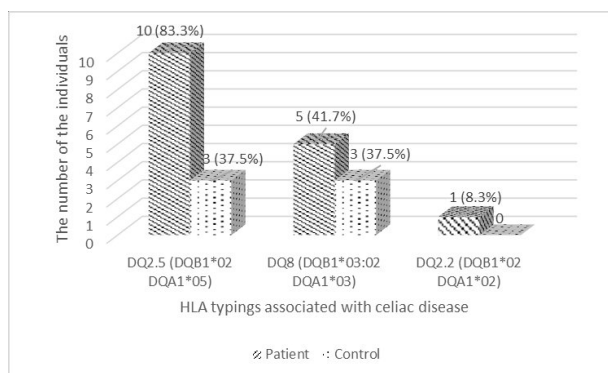
were calculated by the  $\Delta\Delta C_t$  method and RT<sup>2</sup> Profiler PCR sequence data analysis platform on the Qiagen website (<https://dataanalysis2.qiagen.com/pcr>). The relationships between the variables were determined by the chi-square test using the IBM SPSS Statistics 25 Software Program. The correlations between the variables were identified using the Pearson correlation test. All p-values <0.05 were accepted as statistically significant. The GraphPad Prism 9 Software Program was used to perform unpaired and paired Student's t-tests to compare mean Ct values between groups and within groups. The fold changes in the gene expression levels of the miRNAs in the patient group's biopsy and blood samples were compared with the control group of healthy children. Furthermore, the difference in the expression profiles in the miRNA values between the blood and tissue samples within the groups was determined.

## Results

The demographic and clinical features of the patient and control groups are given in Table I. One patient had type 1 diabetes mellitus (T1DM), and 11 patients were free from T1DM.

We found that 66.7% (n=8) of the patients and 37.5% (n=3) of the controls had the DRB1\*03DQB1\*02DQA1\*05 haplotype. The DRB1\*07DQB1\*02DQA1\*02 haplotype was found in 25% of the patients (n=3) and 12.5% of the controls (n=1), while the DRB1\*04DQB1\*03DQA1\*03 haplotype was found in 41.7% of patients (n=5) and 25% (n=2) of controls. Figure 1 gives the frequencies of the HLA types which are clinically associated with CD. Accordingly, 83.3% (n=10) of the patients had DQ2.5 (DQB1\*02 DQA1\*05) and 8.3% (n=1) had DQ2.2 (DQB1\*02 DQA1\*02) alleles, while 41.7% had the DQ8 allele.

There were no significant differences in the *miR-196b* and *miR-338-3p* gene expression levels between the



**Figure 1.** HLA-type frequencies in the patient and control groups  
HLA: Human leukocyte antigen

control and patient blood samples ( $p>0.05$  and  $p>0.05$ , respectively). In the CD group, there was a significant decrease in the *miR-196b* gene expression levels in the blood in comparison to the tissue samples ( $p<0.05$ ). Furthermore, there were significant differences in the *miR-338-3p* gene expression levels between the tissue and blood samples in the CD group ( $p<0.05$ ). The *miR-31-5p* gene expression level was significantly different in the patient blood samples compared to their tissue samples ( $p<0.05$ ). There was a significant difference between the control and patient blood samples for *miR-10a* ( $p<0.05$ ). In addition, there was no significant difference between the tissue and blood samples in the CD group ( $p>0.05$ ).

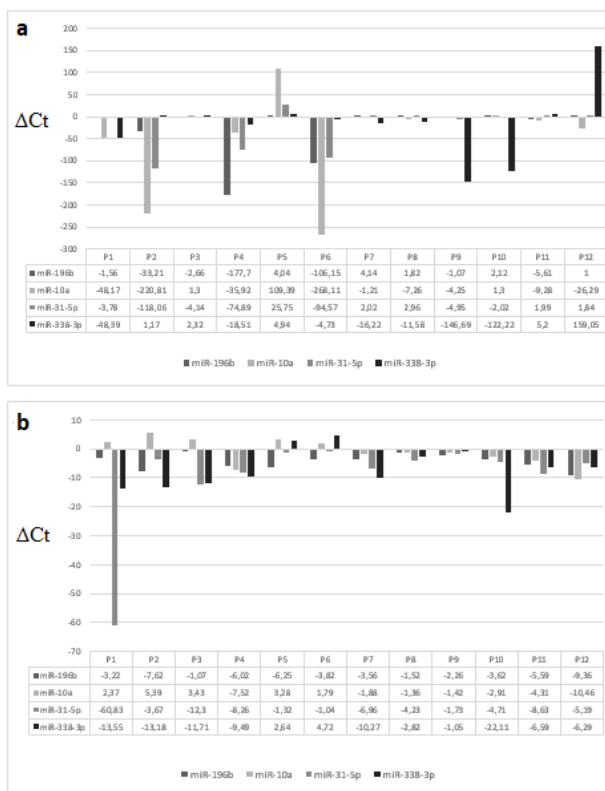
The highest number of changes in gene expression in the patient blood and tissue samples were observed in the *miR-338-3p* expression. This expression decreased in seven patients (58.3%), while it increased in four patients (33.3%) in their blood samples. However, nine patients (75%) showed a decrease, while two (25%) showed an increase in their tissue samples.

Other modifications were not statistically significant. *miR-10a* had the greatest decrease in expression in patient blood (>2,000 fold) (Figure 2). The greatest decrease in patient tissues was observed in the expression of *miR-31-5p* (>500 fold). There was no statistically significant relationship between the patient's HLA tissue type and miRNA expression profiles ( $p>0.05$ ). Additionally, there was no significant correlation between Marsh classification and gene expression levels ( $p>0.05$ ). None of the patients had IgA deficiency and there was no significant correlation between the relative Ct values and the IgA levels of the patients ( $p>0.05$ ).

**Table I.** Demographic and clinical information of the patient and control groups

	Patient group	Control group
Age (years) mean $\pm$ SD	7.67 $\pm$ 3.52	6.62 $\pm$ 3.58
Gender (% female)	11 (91.7)	5 (62.5)
T1DM n (%)	1 (8.3)	-
Marsh classification n (%)	3a	1 (8.3)
	3b	8 (66.7)
	3c	3 (25)

SD: Standard deviation, T1DM: Type 1 diabetes mellitus



**Figure 2. a)** The gene expression changes of miRNAs in the blood samples of patients with celiac disease. **b)** The gene expression changes of miRNAs in the tissue samples of the patients with celiac disease  
*miRNAs: microRNAs*

## Discussion

GATA-binding factor 6 (GATA6) is a zinc finger transcription factor which is involved in epithelial cell differentiation in the intestines (8). The GATA6 protein from the GATA transcription factor family is expressed in almost all gastrointestinal epithelial cells. It is important in intestinal epithelial cell proliferation and the development of colorectal cancer. GATA6 protein is suppressed by miR-196b (9). Although no studies have been conducted to directly link miR-196b to CD, it is thought that the GATA6 protein might play a role in the pathogenesis of this disease because it is involved in the transcription of some cytokine genes involved in the pro-inflammatory response. When we compared our patient group's blood samples to the control group, miR-196b expression was similar in the control and patient blood samples. In each group, the expression level was much lower in the tissue samples than in the blood samples. Due to its low expression, miR-196b may not have suppressed GATA6 protein synthesis in the patients, which may have resulted in the initiation of the inflammatory

processes. In celiac patients, this miRNA has never been studied in blood samples. Felli et al. (10) found that miR-196a was downregulated in biopsy samples taken from celiac patients.

Bone morphogenetic protein 2 expression is suppressed by miR-10a. *In vitro*, this protein inhibited intestinal epithelial cell growth and induced apoptosis. It also promoted differentiation while suppressing proliferation (10). It has also been reported that this miRNA inhibits the release of the cytokine IL-12/IL-23p40, which is released by active macrophages and dendritic cells during innate and adaptive immune responses, and that it plays a role in the activation of NK cells and helper T-cell differentiation (11). In addition, it inhibits dendritic cell response, nucleotide-binding oligomerization domain 2 expression, and Th1/Th17 cell activations (12). Wu et al. (12) reported that miR-10a expression decreased in their patient group with inflamed bowel disease. Similarly, miR-10a expression was lower in the blood samples of the patients than in the control group in this study. These findings suggested that miR-10a could be used as a blood marker to predict CD.

It has been proposed that miR-31-5p is involved in the mitogen-activated protein kinase pathway, Wnt signaling, and cytoskeletal remodeling. It has been reported that miR-31-5p was downregulated in the serum of celiac patients compared to controls (13). Similarly, in our study, miR-31-5p expression was found to be lower in the blood samples of the patients when compared to the controls. Also, it was significantly downregulated in the patient's biopsy samples. By determining the levels of expression in biopsy samples, this miRNA may help in the diagnosis of this disease.

It has been demonstrated that in celiac patients, low expression of miR-338-3p increases the expression of innate and adaptive immune response proteins (14). According to Felli et al. (10), celiac patients have low expression, particularly in biopsy samples. The expression was similar in the control and patient blood samples. The expression levels in the tissue samples were lower than in the blood samples within the CD group.

The pathogenesis of CD is influenced by specific HLA alleles. The *HLA-DQA1* and *HLA-DQB1* genes are the main determinants which cause genetic susceptibility to this disease. While nearly all celiac patients have the HLA-DQ2 type, the remaining patients have the HLA-DQ8 heterodimer (15). HLA-DQ2 alleles were found in 91.7% (n=11) of our patients. However, the patients' HLA types were not associated with miRNA expression in our study.

### Study Limitations

Our study's limitations include that it was performed as a single-center analysis and involved a limited number of participants. In addition, the effects of epigenetic mechanisms on the clinical findings could not be evaluated due to the small number of patients.

### Conclusion

The expression levels of the miRNAs investigated in this study were found to be consistent with their molecular pathways and other published findings. When compared to the control group, all of the miRNAs had lower levels of expression in the patient blood samples than the control group. Among these, the significantly low level of miR-10a may be related to the autoimmune mechanism in CD due to its effect on the immune response. However, because of its similar expression levels in the blood and tissue samples from the CD group, particularly, miR-10a should be investigated further as a possible blood biomarker for the diagnosis of CD.

### Ethics

**Ethics Committee Approval:** This study was conducted according to the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (approval no.: 01, date: 29.03.2019).

**Informed Consent:** Signed informed consent forms were obtained from the participants before this study took place

### Authorship Contributions

Surgical and Medical Practices: T.K.A., A.E., İ.P., Concept: M.B., M.S., Design: M.P., Y.Ç.A., B.N.D., İ.P., Data Collections or Processing: B.A., B.N.D., Analysis or Interpretation: M.P., A.E., M.S., Literature Search: A.Ö.K., Y.Ç.A., Writing: T.K.A., M.P., A.Ö.K.

**Conflict of Interest:** No potential conflict of interest was reported by the authors.

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