ORIGINAL ARTICLE

Association of single nucleotide polymorphisms (rs1799883) and gene expression of I-FABP with celiac disease

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ABSTRACT

Objectives: Fatty acid-binding protein 2 (FABP2) is released into the systemic circulation following enterocyte damage, and its rs1799883 polymorphism is associated with several chronic diseases. This study aimed to investigate the rs1799883 and FABP2 intestinal gene expression in Iranian celiac disease (CD) patients in comparison to controls.

Methods: We collected peripheral blood samples of 103 celiac disease patients and 103 healthy controls randomly in 2019 and genotyped for rs1799883 polymorphism by PCR-RFLP method. FABP2 mRNA expression was also measured by RT-qPCR in duodenal biopsy samples of celiac disease patients and healthy individuals.

Results: Statistical analysis of the mean allele frequencies and genotypes showed no significant difference between the two groups. Intestinal mRNA expression of FABP-2 was significantly higher in celiac disease patients than controls (p=0.01) and decreased from Marsh I to Marsh III in patients' group (p=0.004).

Conclusion: Taken together, rs1799883 could not be considered as a risk factor in the development of celiac disease in the Iranian population.

Keywords: celiac disease, fatty acid-binding proteins, polymerase chain reaction, FABP2 protein; polymorphism

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INTRODUCTION

Celiac disease (CD) is a common chronic inflammatory disorder that occurs in genetically susceptible individuals, those who express HLA-DQ2 and/or DQ8 haplotypes (1-5). The main triggering factor for CD is gluten protein, which is found in cereals such as wheat, barley, and rye and causes immune-mediated enteropathy in CD patients (6-8). CD prevalence is 1-2% in the general population and 0.3–2.9% in the paediatric population (5, 6, 9, 10). Currently, the diagnosis of celiac disease is based on intestinal biopsy examination, serological and genetic tests (2, 6).

Intestinal fatty acid-binding protein 2 (FABP2) or I-FABP, a subset of nine different types of FABP that expressed especially in the small intestine, is one of the important and effective genes in the transport and metabolism of long-chain fatty acids (11, 12). FABP2 constitutes about 2% of the enterocyte total proteins and is released into the circulation by enterocyte damage (what happens in CD) (12). Moreover, change in FABP expression by affecting the PPARγ signalling pathway has a role in the regulation of inflammatory reactions in the small intestine (13). Previous studies have shown that serum FABP2 levels increased in celiac disease, acute intestinal ischemia, necrotizing enterocolitis, sepsis, and Crohn's disease (14-17). Oldenburger et al. (18) proposed that serum *I-FABP* level evaluation in patients with a tTG ≥ 50 could be useful in non-invasive diagnosis of CD patients.

The G to A single nucleotide transition at codon 54 of FABP2 results in alanine (Ala) to threonine (Thr) substitution in exon 2 (Ala54Thr (rs1799883) polymorphism) (19, 20). The association of this polymorphism with several chronic diseases has been extensively investigated so far. It has been reported that rs1799883 is associated with obesity, insulin resistance, type 2 diabetes mellitus (T2DM) and metabolic syndrome (21, 22). None of the previous studies investigated the association between rs1799883 polymorphism and celiac disease; therefore, the aim of this study was to investigate the Ala54Thr polymorphism and FABP-2 intestinal gene expression in patients with celiac disease compared with healthy individuals in an Iranian population.

MATERIALS AND METHODS

Subjects

This case-control study included 103 celiac disease patients (their disease confirmed by clinical presentation, histological examination and serological tests) and 103 healthy controls (with no history of CD and other immune-related diseases) who were referred to GI clinics of Taleghani Hospital (Tehran, Iran) during 2019. Subjects who used any prohibited medications, females who were pregnant, patients who had another autoimmune/intestinal disorder, obese subjects, and those who failed to cooperate with researchers were excluded from the study. Fresh whole blood (5uL) was collected from all study volunteers using standardised venepuncture in EDTA anticoagulant tubes. Duodenal biopsy samples were also collected from CD patients and healthy individuals.

This study was approved by the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases (RIGLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.745). All participants signed an informed written consent form.

DNA isolation

Total genomic DNA was extracted from the peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The quality and quantity of extracted DNA were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, US).

Genotyping

Ala54Thr polymorphism was identified by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primer sequence 5′-CAAAGGCAAT-GCTACCGAG-3′ (forward) and 5′-AGTTAGTGAA-GGAGACCTGC-3′ (reverse) were used to amplify a 410bp fragment. By the following conditions, the polymerase chain reaction (PCR) in a 25μL total volume was performed. PCR reaction contained 2.5μL of 10x PCR Buffer, 0.5μL of 10mmol/L dNTP Mix, 1μL of each forward/reverse primers, 0.5μL of Taq DNA polymerase, 1μL of Template DNA, and 18.5μL of sterilised distilled water. The reaction was immediately started by a denaturation step for 5 minutes at 95°C, followed by 40 cycles of 40 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C, and a final extension step for 10 minutes at 72°C. The PCR products were visualized using 1.5% agarose gel electrophoresis and then 5μL of above PCR products digested by 0.3μL HhaI restriction enzyme (Fermentas, Burlington, Canada). The enzyme cutting reaction was performed by incubating the samples at 37°C for 6 hours in a bain-marie. Finally, the products were visualized using 3% agarose gel electrophoresis with 100bp marker.

DNA sequencing
Approximately 20% of the PCR products (22 cases and 22 controls matched according to the gender) were randomly selected for DNA Sanger sequencing and sent to Gene Fanavaran. DNA sequences were determined by Bioedit software.

RNA extraction, cDNA synthesis and quantitative real time PCR (RT-qPCR)
Total RNA was extracted from duodenal biopsy specimens using EZ1 RNA Tissue Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription of RNA to cDNA was also performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc, USA), according to the manufacturer protocol. Specific primer sequences were designed using gene runner software (V.3.05) as follow: (FABP2 Forward) 5′-ACGGACAGACAATGGAAACG-3′ and (FABP2 Reverse) 5′-GCCAAGAATAATGCTCAATCC-3′. Real-time quantitative–PCR (RT–qPCR) was performed on a Rotor-Gene Q real-time PCR system (QIAGEN) using RealQ Plus 2x Master Mix Green (AMPLIQON, Denmark). Amplification was performed under the subsequent conditions: 95°C for 15 min, followed by 40 cycles of 95°C for 10 seconds, 58°C for 30 seconds, and 60°C for 30 seconds. β-2 microglobulin was used as a housekeeping gene. The relative expression was calculated by performing the $2^{-\Delta\Delta CT}$ method.

Statistical analysis
Statistical analysis was performed using SPSS statistics for Windows (Version 21.0, SPSS Inc., Chicago, IL, USA). Comparisons were carried out by Chi-square test, logistic regression, Multinomial Logistic Regression, Student's t-test, and ANOVA. Data are presented with a 95% confidence interval and a p-value < 0.05 was considered to be statistically significant.

RESULTS

Study groups characteristics
Of the 103 CD patients (average age: 28.55 years; average body mass index (BMI): 21.55 kg/m^2) 31 (30.1%) were male and 72 (69.9%) were female. Control group (average age: 29.91 years; average body mass index (BMI): 23.53 kg/m^2) also consists of 34 (33%) male and 69 (67%) female. More than half of the participants (59.2%) were married and the mean weight and BMI values were significantly different between the CD and control groups (*p*=0.006 and 0.002, respectively) (Table1).

PCR- RFLP results
The RFLP-generated profile comprised two fragments at 192 and 218bp for wild-type homozygotes (G/G: Ala/Ala); three fragments with lengths of 410, 192 and 218bp for heterozygotes (A/G: Thr/Ala) and one fragment with a length of 410bp for the mutant homozygotes (A/A: Thr/Thr) (Figure1).

Genotypes and alleles frequencies
Allelic frequency and genotypic distribution were compatible with the Hardy-Weinberg equilibrium. As summarized in Table 2, in both patient and control groups, the GG genotype had the most frequency (50.5% and 53.4%, respectively) and the AA (24.2%) and AG (22.3%) genotype had the lowest frequency in CD patients and control subjects, respectively. There was no statistically significant difference in this regard between the two studied groups (p>0.05). There was no statistically significant difference in allele frequencies between CD patients and controls too (Table 2).

Direct sequencing
In order to confirm the results of PCR-RFLP, 20% of the PCR products (randomly selected) were sequenced (the golden standard method for genotyping). The sequencing data matched the result from PCR–RFLP. Figure 2 shows the AG heterozygote genotype of rs1799883.

Results of gene expression
FABP2 mRNA expression was measured by RT-qPCR in duodenal biopsy samples of CD patients and healthy individuals. A significant increase in FABP2 mRNA level was shown in the CD patients' intestinal specimens in comparison with healthy controls (p=0.01). (Figure 3)

Association of FABP2 mRNA level with the severity of tissue damage in CD patients
Pathologic reports of those patients selected for FABP2 mRNA expression analysis were Marsh I in 30%, Marsh II in 40%, and Marsh III in 30%. According to our results, levels of FABP2 mRNA expression decreased significantly with increasing severity of tissue damage (Marsh I to III) (p=0.004) (Table 3).

Table 1. Anthropometric parameters of the case and control groups

Variable	Case (n=103)	Control (n=103)	p-value
Age	28.5±13.9	29.9±14.4	0.492
Weight (kg)	54.1±19.2	59.8±20.6	**0.006**
BMI (kg/m^2)	21.55±4.71	23.54±4.45	**0.002**

Independent sample t-Test statistical tool used to compare difference between the means of two groups. Data were expressed as mean ± standard deviation (SD). Significant differences in bold

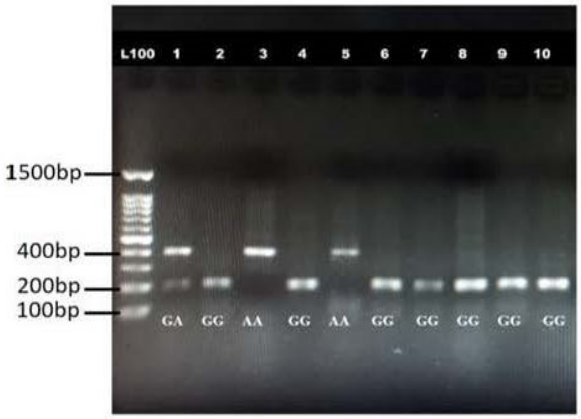

Figure 1. PCR-RFLP results after digestion with the restriction enzyme Hha I on samples. Lanes 1 – 10, pattern of digestion of PCR products. Lane L100, DNA ladder (Roche, 100bp).

Table 2. Comparing Allelic and Genotypic frequencies in celiac disease patients and control subjects using logistic regression analysis.

Variable	Genotype and allele	Celiac patient (n=103)	Healthy control (n=87)	CI	OR	P-value
Genotype	AA	25 (24.2%)	25 (24.3%)	Ref	Ref	Ref
	AG	26 (25.3%)	23 (22.3%)	0.483-1.851	0.942	0.870
	GG	52 (50.5%)	55(53.4%)	0.425-1.646	0.836	0.605
Allele	A	76 (36.9%)	73 (35.5%)	Ref	Ref	Ref
	G	130(63.1%)	133(64.5%)	0.845-1.130	0.977	0.758

CI = 95% Confidence Interval; OR = Odds Ratio

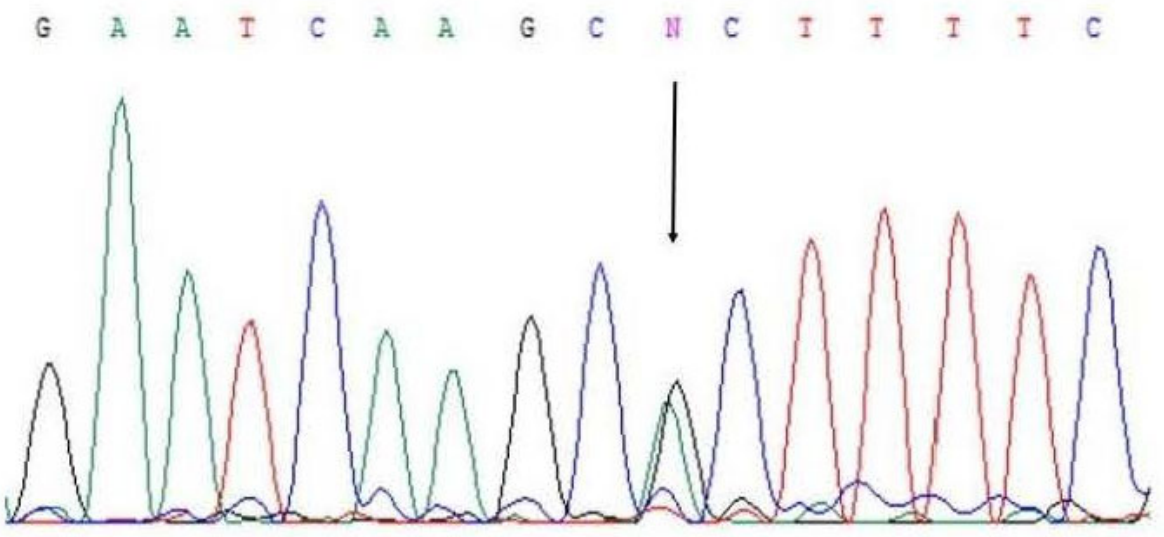

Figure 2. Sequence of rs1799883

Table 3. Analysis of variance (ANOVA) statistical analysis tool used to compare the FABP2 gene expression between Marsh I, II and III.

Marsh Classification	Mean	Std. Deviation	P-value between groups
1	2.5067	0.45446	0.004
2	1.2775	0.48815	
3	0.6967	0.27934	
Total	1.4720	0.84659	

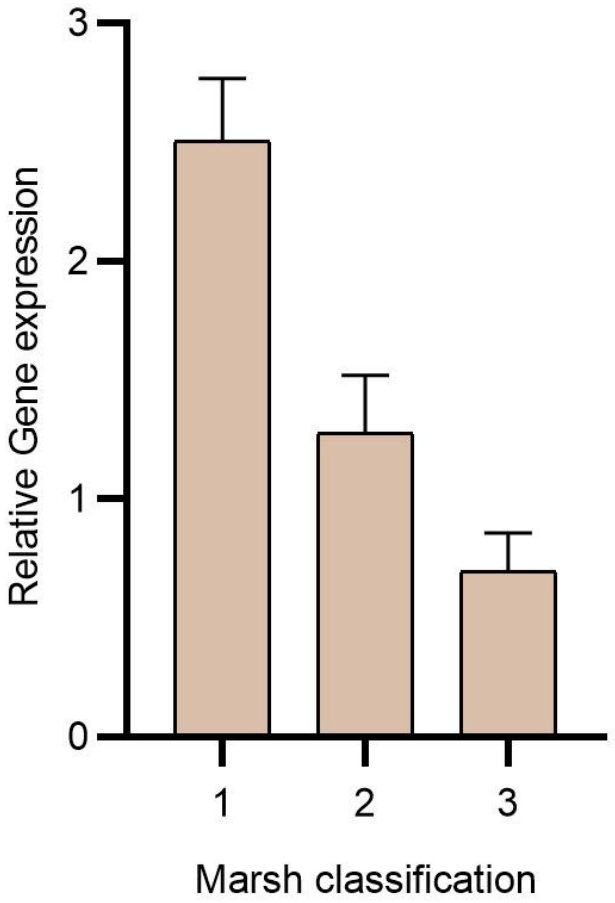

Figure 3. The FABP2 gene expression comparison between groups (P=0.01).

DISCUSSION

Celiac disease is an immune-mediated enteropathy accompanied by intestinal enterocyte damage and villous atrophy (2, 6, 23, 24). FABP2, which is an intestinal epithelial cell specific protein, is released into the systemic circulation following epithelial cell loss and enterocytes damage. Hence, its circulating concentration is useful for evaluating intestinal injury (25). Adriaanse et al. (26) in their study reported the association between increased serum FABP2 levels and the degree of histological abnormalities in CD patients. Similarly, Uhde and co -workers (25) reported increased circulating FABP2 levels and its association with intestinal epithelial cell damage in non-celiac gluten sensitive patients (25). Oldenburger et al. (18) also considered serum I-FABP level as a diagnostic marker for CD patients. Moreover, it has been proposed that, IFABP can be used as a differentiation marker to assess preterm neonate's intestinal maturation (27).

In the present study, we observed a significant overexpression of FABP2 in CD patient's intestinal biopsies compared to normal subjects ($p < 0.01$). In this regard, Bottasso Arias et al. (6), found that IFABP expression is increased within immature enterocytes isolated from the crypts in active CD patients compared to healthy control. They considered this increased expression of IFAB as a sign of accelerated developmental program of enterocytes. Meanwhile, they observed a significant reduction in the mRNA levels of IFABP in untreated CD patients with severe enteropathy compared to non-CD controls and considered it as a consequence of histological changes. Simula et al. (28), also reported reduced IFABP level in duodenal samples from active CD patients in comparison to healthy subjects. Our results also showed that, the level of FABP2 mRNA expression was significantly decreased from Marsh I to Marsh III and our results confirmed Bottasso Arias and Simula et al. findings. It can also be explained by considering the fact that, IFABP is highly expressed in the villi tip and with increasing severity of the disease, the number of villi decreases dramatically. Accordingly, Vreugdenhil et al. (29) reported that IFABP could be considered as an early marker of intestinal damage in CD patients.

FABP2 has two types (A54 or T54) which are formed by Ala54Thr (rs1799883) polymorphism at codon 54 in exon 2 that results in alanine substitution by threonine (19). It has been reported that rs1799883 polymorphism is associated with several chronic diseases like insulin resistance, obesity, type 2 diabetes mellitus, etc. (21, 22). For instance, Albala et al. (21) demonstrated that the carriers of the Thr54 allele of Ala54Thr polymorphism of FABP2 are at increased risk of obesity and insulin resistance. Vimaleswaran et al. (30) also showed that there was a significant relationship between the Thr54 allele with metabolic syndrome and hypertriglyceridemia in the urban South Indian population. To our knowledge, we investigated the association analysis of rs1799883 polymorphism in CD patients for the first time and our results did not show any significant difference in the genotype distribution/allele frequency of this polymorphism between CD patients and controls. According to our findings, there is not any relationship between rs1799883 polymorphism and CD and this polymorphism does not have a role in susceptibility to CD in the Iranian population.

CONCLUSION

In conclusion, although various studies reported an association between I-FABP polymorphism and different disorders, our data showed that this polymorphism could not be considered as a risk factor in the development of CD in the Iranian population. However, further studies with higher patient numbers and in other societies are needed to confirm/reject the result of our study.

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