ORIGINAL ARTICLE

The genes expression of NF-κB inflammation pathway in treated celiac disease patients

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ABSTRACT

Objectives: Celiac disease (CD) is a heritable chronic inflammatory disease that generally leads to a wide spectrum of clinical symptoms. The present research aimed to investigate whether the expression of key genes (NF-kB, REL, and TNFAIP3) induces inflammatory meditated NF-kB signaling changes in patients treated with a gluten-free diet compared to the healthy group.

Methods: Biopsy specimens from the distal duodenum and blood sampling were collected from 50 patients with CD (37.06 ± 7.02 years old) under gluten-free diet for at least 1 year and 50 healthy individuals (34.12 ± 4.90 years old) served as a control group. RNA was extracted from samples, cDNA synthesised and primer pairs were designed for NF-kB, REL, and TNFAIP3 gene expression. Quantitative real-time PCR was used to analyze the relative gene expression.

Results: A total of 50 CD patients (72% men and 23 % women) were included in this study. The results showed that the expression of NF-κB1 and c-Rel in tissue sample and blood samples did not have a significant difference compared to the control group (P > 0.05), whereas the expression of TNFAIP3 was significantly lower than the control group (P< 0.05).

Conclusions: Generally, it seems that the disrupted gene pattern of the NF-kB pathway can affect the optimal immune response control, indicating some interactive inflammatory reactions in CD patients. These results light unknowns in interactive inflammatory reactions in CD patients and explain the common complex immune reaction in CD.

Keywords: Celiac disease, gluten-free diet, NF-kB, c-Rel, TNFAIP3.

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INTRODUCTION

The common autoimmune gluten-sensitive enteropathy is called celiac disease (CD) (1). CD is a heritable chronic inflammatory diseases that generally leads to a wide spectrum of clinical symptoms (2). The anti-tissue transglutaminase antibodies elevations and histopathological damage to the small intestine with villous shortening, chronic inflammation, intra-epithelial lymphocyte infiltration, and activation of lamina propria T cells are generally seen in CD patients (3,4). Nevertheless, when the patients are follow the gluten-free diet, the histopathological signal goes into remission (5). Some evidences indicates that the T-cell mediated immune response to gliadin plays a key role in starting events in the pathogenic cascade of CD (6,7). Also, the pathway of the gluten-induced immune response in CD is not yet completely understood to date (8). However, the recent discovery of candidate genes identified by genome-wide association studies has led to tremendous progress (9,10). Therefore, determining the pattern of these gene expressions may guide to finding reliable pieces of functional pathways that complete the puzzle of the gluten-induced immune response (11). A common paradigm in the pathogenesis of the CD is the genes whose expression is induced in the inflamed mucosa such as NF-kB pathway genes and important mediators such as REL and TNFAIP3 present CD-associated variants (9,12).

NF-kB is a transcription factor and a crucial regulator of lymphocyte activation and adaptive immune response. Additionally, recent studies showed that gliadin effects on enterocytes could be mediated through oxidative stress, followed by NF-kB activation and IL15 up-regulation (13). Some signaling mediators and regulatory mechanisms such as TCR/CD28 colligation can induce NF-kB activation (14). The NF-kB activation involves phosphorylation and degradation of small cytosolic IkB inhibitors, catalyzed by the IkB kinase complex leading to the transcriptional activity of NFkB1/RELA heterodimers (15,16). In this signaling pathway, a MALT1 inhibitor is an important procaspase modulator that can cleave TNFAIP3 leading to the loss of NF-kB inhibition downstream of IL1- and toll-like receptor signaling (17). Thus, the study of the gene expression of the most central functional components in the NFkB route may help to understand the inflammatory reaction in the CD patients. The present research aimed to investigate whether the expression of key genes (NF-kB, REL, and TNFAIP3) induces inflammatory meditated NF-kB signaling changes in the blood and biopsy specimens of celiac disease patients under gluten-free diet compared to the healthy group.

MATERIALS AND METHODS

Patients

Biopsy specimens were obtained from 50 patients with CD and 50 individuals without any history of autoimmune diseases served as the healthy control groups. Subjects were referred to the Research Institute for Gastroenterology and Liver Diseases, Taleghani Hospital,Tehran, Iran during 2018-2019. Four biopsy specimens were obtained, histological examination was performed on three of these, while one sample tissue was immediately frozen in liquid nitrogen beforentesting. Diagnosis of CD was based on positivity for anti-transglutaminase antibody (anti-tTG IgA), villous atrophy, typical mucosal lesions with crypt hyperplasia, and increased number of intraepithelial lymphocytes according to the modified Marsh classification (18). The control group was negative for anti-tTG IgA, and their duodenal histology was normal. Blood samples were collected from all CD patients and healthy controls. Informed consent was received from each individual who has been nominated for blood and tissue screening and gene expression analysis of Nf-κb1, c-Rel, and TNFAIP3. This study was approved by the local ethics committee of the University Islamic Azad, Iran (IR.SBMU.IRGLD.REC.1398.006).

RNA extraction and cDNA synthesis

RNA was extracted from blood buffy coat and biopsy specimens by using a commercial kit (YTA Total RNA Purification Mini Kit, Yektatajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. The quantity of the extracted RNA was determined by a NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, US) at wavelengths: 260 and 280 nm and the quality of the RNA was analysed by agarose gel electrophoresis. Then, total RNA (1 µg) was reverse transcribed into High Capacity cDNA using a Reverse Transcription Kit (Primer Script TMRT Reagent Kit, Takara Bio, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. cDNA samples were stored at -20°C until used as a template for Q-PCR.

Quantitative real-time PCR

The primers were designed using the GeneRunner software and Primer3 (ver.4) online program (19). The sequence of these genes is shown in Table 1. Quantitative real-time PCR was performed by using a Rotorgene Real-Time PCR System (Hilden, Germany) and SYBR Master Mix (BioFACT™ 2X Real-Time PCR Master Mix including SYBR® Green I (Daejeon, Korea) according to the manufacturer's instructions for the detection. The samples were amplified in 20 µL reaction mixtures containing 10 µL SYBR Green Master Mix, 1 µL of cDNA, and 0.5 µL of each primer and 8 µL water for three

genes, as well as 1μL of each primer and 7μL water for housekeeping gene (β2M). For each primer pair, NTC (no template control) was used, and cDNA sample was replaced by RNase-free water. Instrument settings for all amplification reactions were 95°C for 15 min; 40 cycles of 95°C for 5 sec; 59°C for 40 sec; and 72°C for 40 sec for NFkB1 gene; 95°C for 15 min; 40 cycles of 95°C for 5 sec; and 59°C for 40 sec for c-Rel gene. For the TNFAIP3 gene amplification reactions were 95°C for 15 min; 40 cycles of 95°C for 5 sec; and 59°C for 40 sec. The melting curve shows the absorption peak, and all samples were run in triplicate. The analysis of gene expression was performed based on the relative gene expression by analyzing the melting curve of the ΔΔCt method. The constitutive expression of target genes in the tissues was calculated as a ratio of the $2^{-\Delta Ct}$ target gene and endogenous reference gene, according to the 2 method (20), where the ΔΔCt is the number of PCR cycles at which the signal of fluorescence exceeds the threshold level. The relative gene expression in each group samples was shown as a fold change between the level of gene expression in control and patient samples and was calculated using the $2^{-\Delta\Delta Ct}$ method (20-22).

Data analysis and statistics

The exact ΔΔCt method was used to calculate the relative expression of each gene. SPSS (ver.20) software was used for statistical analysis of clinical symptoms and findings. Graph pad Prism (ver.7) software was also used to assess differences between groups using t-test and ANOVA, and Spearman correlation was used to assess differences between groups (23). Sstatistical significance was defined at $P<0.05$

RESULTS

Characteristics of patients

The average age of the patients and control groups were 37.06, ± 7.02 and 34.12 ± 4.90 years respectively and the majority of CD patients were male (72%). The mean of BMI in controls (27.11 ± 3.42) was higher than the patients' group (20.23 ± 5.78). The frequency of gastrointestinal symptoms and extra- intestinal symptoms in the CD patients are shown in Table 2. Based on this result diarrhea (60%) and abdominal cramps (58%) were the most common GI symptoms and weight loss (70%) and anaemia (62%) were the most frequent extra-GI symptoms. Based on the results of Fisher's exact test, no statistically significant difference was observed between extra-gastrointestinal symptoms and pathological findings in the CD group ($P<0.05$).

Gene expression in monocytes

The results of real-time PCR data showed that the mean expression of NFkB1 gene in the PBMC of patients group was 1.039 and the mean expression of NFkB1 gene in the healthy group was 0.987 and this difference was not statistically significant ($p=0.472$). In addition, the mean expression of c-Rel gene in the group of patients and control group were 1.087 and 0.895 respectively. Accordingly, there was no significant difference in the expression of c-Rel gene in the control group and CD patients ($p=0.439$). On the other hand, the results showed that the mean expression of TNFAIP3 gene in the group of patients was 0.8032 and 1.6882 in the control group. Therefore, the expression of TNFAIP3 from patients who underwent a gluten-free diet for six months to one year was significantly lower than the control group ($P<0.001$; Figure1).

Gene expression in tissues

As shown in figure 2, the analysis of gene expression of NF-κB1 and c-Rel in tissue of patients were not significantly different compared to the control group ($p=0.254$ and $p=0.341$ respectively). Our results demonstrated that the expression of TNFAIP3 of patients who were on a gluten-free diet for more than six months was significantly lower than the control group ($p=0.03$).

Table1. Sequences and specifications of primers.

Target gene	Oligonucleotide sequences (5'-3')	Product length (bp)	TM (°c)
NFkB1	AGAAGAAGTGCAGAGGAAACGT CCACCGCCACTACCAAACAT	111	58.39 59.35
c-Rel	TGAACAACCCAGGCAGAGG TGTTCGGTTGTTGTCTGTGC	100	58.83 57.30
TNFAIP3	TCATCCACAAAGCCCTCATC ATT GCCGTCACCGTTCGTTT	119	57.30 57.30
β2M	TGCTGTCTCCATGTTTGATGTATCT TCTCTGCTCCCCACCTCTAAGT	112	59.70 62.12

Table 2. Frequency distribution of people based on gastrointestinal symptoms and extra-intestinal symptoms in CD patients.

Symptoms		(%)
Gastrointestinal symptoms	Bloating	22
	Diarrhea	60
	Nausea and vomiting	32
	Abdominal cramps	58
Extraintestinal symptoms	Weight loss	70
	Anaemia	62
	Bone diseases	34
	Nervous problems	16
	Menstrual problems	12
	Infertility	6
	Abortion	16
	Skin problems and aphthous stomatitis	2

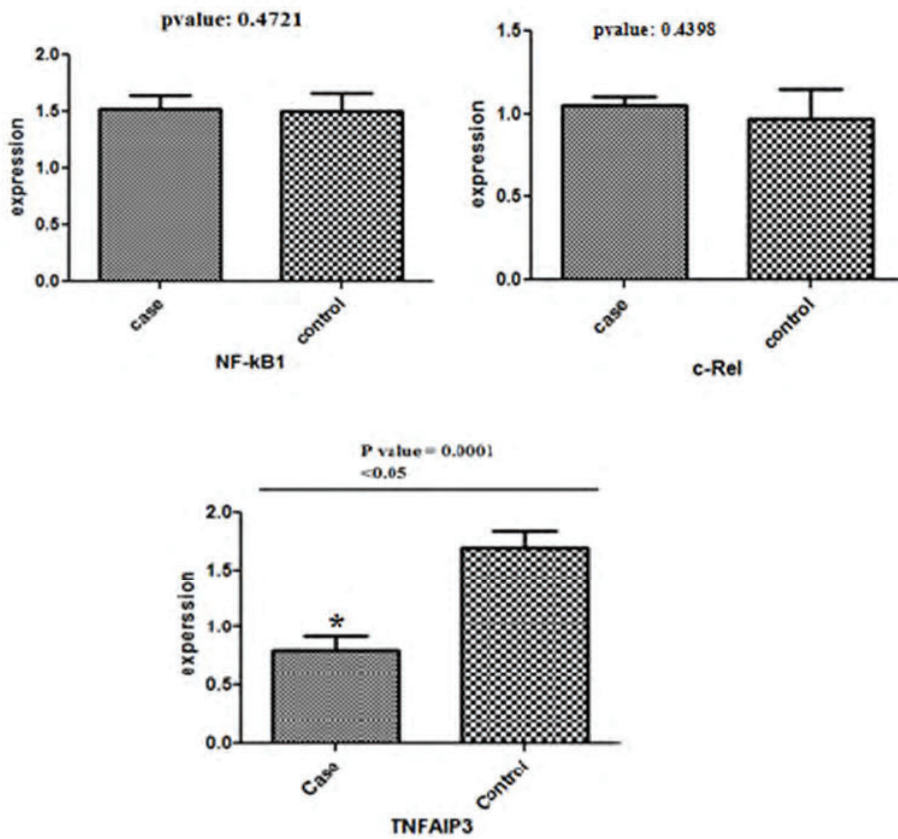

Figure 1. Constitutive expression of NF-kB1, c-Rel, and TNFAIP3 in monocytes from the control group (right bar) and CD patients who received the gluten-free diet for six months to one year (left bar). Gene expression was quantified by RT-qPCR and their expression level was expressed relative to the expression of the β2M gene (n=25). The star indicates significant differences between groups (p<0.05, mean± SD).

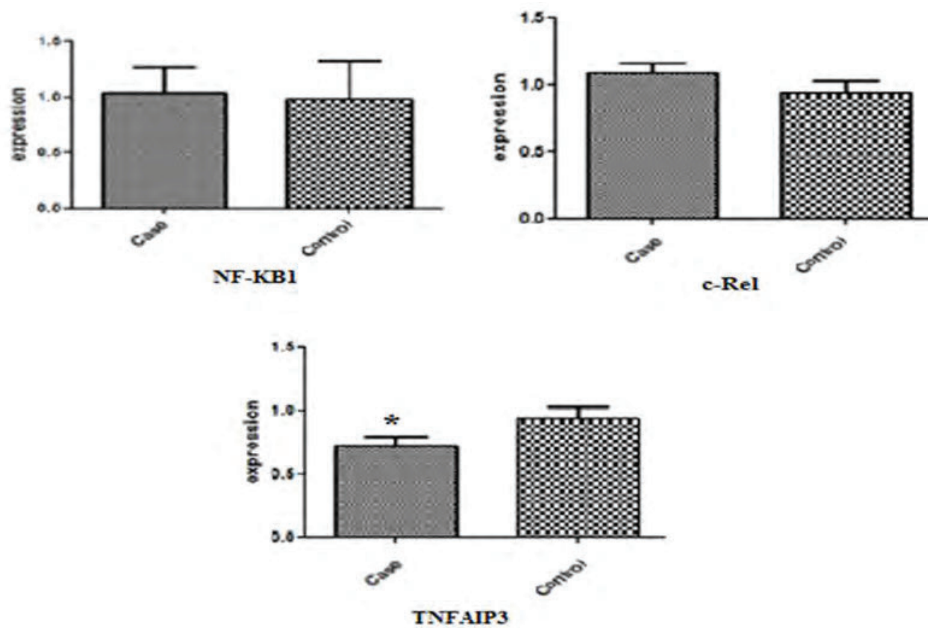

Figure 2. Constitutive expression of NF-kB1, c-Rel, and TNFAIP3 in the tissue of control group (right bar) and tissues of CD patients who received the gluten-free diet for six months to one year (left bar). Gene expression was quantified by RT-qPCR and their expression level was expressed relative to the expression of the β2M gene (n=25). The star indicates significant differences between groups P<0.05; mean ± SD.

DISCUSSION

Evidence show that the NF-kB pathway is constitutively upregulated in CD (12). Previous studies confirmed that NF-kB is a key regulator of inducible gene expression in both innate and adaptive immune responses (24). Besides, the NF-kB family transcription factors can control the development and maintenance of the cells and organs that comprise the immune system at multiple stages (25,26). Since the characteristics and functions of NF-kB and key NF-kB-mediators, such as REL and TNFAIP3, have been associated with susceptibility to CD (9), this pathway is an interesting candidate to have a prominent effective role in the development of the CD.

According to our results, the expression of NF-kB and Rel genes in monocytes and intestinal tissue of CD patients was not significantly different from the control group but the expression of the TNFAIP3 gene in both targets were significantly decreased in CD patients. The tumor necrosis factor-alpha-induced protein 3 (TNFAIP3) gene is a TNF-inducible gene, which is a negative feedback inhibitor of TNF signaling (27). Recent genome-wide association studies have revealed associations between TNFAIP3 and CD (9). The expression of TNFAIP3 in inflammatory responses can modulate the ubiquitination status of central components in NF-κB, IRF3, and apoptosis signaling cascades; therefore, it can restrict and terminate inflammatory responses (28).

Cielo *et al.* showed that the expression of NF-kB pathway genes, such as NF-kB and TNFAIP3, were upregulated in untreated CD patients and consequently the inflammatory processes genes were up-regulated, whereas the genes involved in the cell adhesion/integrity of the intestinal barrier were down-regulated (29). Thus, our study showed that strict gluten-free diet for six months to one year may modulate NF-kB pathway genes and cause downregulation of TNFAIP3 genes. It is hypothesised that, the abnormal response to dietary antigens may not be related to the regulation of molecular pathways but abnormalities of gene structure. Besides, disrupting the regulatory equilibrium of the NF-kB pathway and the effect of gene expression may be related to alteration in the methylation of NF-kB-related gene promoter (13). These hypotheses require further research.

A small sample size, the absence of CD patients who are not on a gluten-free diet (new cases), as well as the lack of protein base methods for validation of the results were the limitations of this study.

In conclusion, our study has revealed the different expression of the candidate genes in the NF-kB pathway between CD patients who received a gluten-free diet for six months to one year and the control group in monocytes and intestinal tissues. We also observed downregulation of the TNFAIP3 gene in CD. The disrupted gene pattern of the NF-kB pathway can affect different gene levels of this pathway and provoke the disruption of the tight regulatory equilibrium that ensures its optimal immune response control. These results highlight unknowns in interactive inflammatory reactions in CD patients and explain the common complex immune reaction in CD.

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