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Investigating the expression of anti/pro-inflammatory cytokines in the pathogenesis and treatment of ulcerative colitis and its association with serum level of vitamin D

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Ulcerative colitis is an idiopathic gastrointestinal disease described by chronic inflammation of the digestive system. Cytokines may be responsible for immunopathogenesis, mucosal and tissue damage, and even treatment response. In addition to its role in calcium and phosphorus homeostasis and bone health, vitamin D is an immunomodulatory and anti-inflammatory agent. Understanding the role of cytokines may lead to improving the pathogenesis and treatment of this disease, therefore we aimed to investigate the relative gene expression of pro- and anti-inflammatory cytokines in biopsy samples taken from the affected area in the colon of ulcerative colitis patients and its association with serum vitamin D levels. A total of 47 ulcerative colitis patients were enrolled in this case-control study. The case group consisted of 23 patients with treatment-resistant ulcerative colitis, and the control group consisted of 24 ulcerative colitis patients responding to routine treatment. Serum vitamin D levels were measured by ELISA method. Real-time PCR was employed to quantify the relative expression of pro- and anti-inflammatory cytokines in colon biopsy samples from case and control groups. The pro-inflammatory cytokines included tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), interleukin-1β (IL-1β), IL-6, IL-8, IL-17 A, and IL-33, while the anti-inflammatory cytokines were IL-10, IL-35, and TGF-β. Data are showed as mean ± standard deviation (SD), and p values < 0.05 were considered statistically significant. The mean age of the control group was 45.88 ± 18.51 years, while that of the case group was 41.30 ± 13.01 years. The relative gene expression of TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-17 A, IL-33, TGF-β, IL-10, and IL-35, in the case and control groups did not exhibit statistically significant differences ($p > 0.05$). However, the gene expression levels of the principal pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, were elevated in treatment-resistant patients compared to patients who responded to treatments. No correlation was observed between serum vitamin D levels and the gene expression of pro- and anti-inflammatory cytokines ($p > 0.05$). The present study did not identify a statistically significant correlation between the expression of pro- or anti-inflammatory cytokines and treatment response. Therefore, routine treatments had no effect on the expression of these cytokines in treatment-resistant patients. Additionally, serum vitamin D levels were not related to the relative expression of pro- and anti-inflammatory cytokines in the affected area of the colon of these patients. Despite the need for further research on the protective and pathological roles of cytokines and vitamin D, regular screening and early and complementary treatment may be beneficial in reducing inflammatory symptoms in these patients.

Keywords Ulcerative colitis, Cytokine, Vitamin D, Inflammation, Pathogenesis, Treatment

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Inflammatory bowel disease (IBD), as a chronic illness, is associated with gastrointestinal tract inflammation[1,2]. Ulcerative colitis is an idiopathic chronic type of IBD, which is determined by continuous and diffuse mucosal inflammation of the colon and rectum and extends proximally. Another form of IBD is Crohn's disease[1,2]. The incidence of Ulcerative colitis in Western countries is high, while in developing countries is increasing. The disease was once a rare disorder and only elevated considerably in Europe and North America in the second half of the twentieth century. During the last two decades, its prevalence doubled every decade and spread to developing countries[3]. The age of high incidence of ulcerative colitis is between 30 and 40 years old, and there is no difference in the incidence between men and women[4,5]. Ulcerative colitis has enteric glial cells enteric neurons, and bowel necrosis pathophysiological processes. Typical presenting symptoms, like abdominal pain, bloody diarrhea, rectal urgency, hematochezia, and tenesmus are the main clinical manifestations. In some patients, extra-intestinal manifestations also may be present. The diagnosis of ulcerative colitis in the clinical setting is primarily based on evidence of continuous colonic acute and chronic inflammation, typically shown by endoscopy, with signs of chronic colitis revealed by confirmatory biopsy specimens. The main goals of treatment are inducing and maintaining remission, improving the quality of life, and decreasing the risk of complications. Treatment classically is determined based on the severity of symptoms. For mild to moderate disease, 5-aminosalicylates are the mainstays of treatment. Patients who show the moderate to severe form of disease or resist 5-aminosalicylate therapy are usually treated with corticosteroids followed by a steroid agent with a thiopurine, anti-tumor necrosis factor-α (TNF-α), or adhesion molecule inhibitors[6]. Almost half of the patients with ulcerative colitis with extensive and chronic symptoms undergo surgery in the first ten years of their disease. Despite additional treatment and improvements in patient care, approximately 15% of ulcerative colitis patients still require proctocolectomy. These patients have ulcerative colitis that is resistant to medical treatment or have developed colitis-associated colorectal dysplasia or cancer[7,8].

Despite significant advancements in research on IBD in recent years, the causative factors and precise mechanisms underlying the pathogenesis of ulcerative colitis remain incompletely understood. However, various factors, including genetic predisposition, environmental influences, and immune dysregulation in the mucosa, can cause the development of ulcerative colitis[8]. Abnormal immune responses to the commensal bacteria are one factor in initiating inflammation in the colon mucosa. Also, it is proposed that dysregulated expressions of anti/pro-inflammatory cytokines are involved in the pathogenesis of ulcerative colitis disease[9,10]. Cytokines certainly are linked to the initiation, amplification, and persistence of many chronic and acute diseases such as ulcerative colitis[11]. Cytokines themselves are directly responsible for mucosal and tissue damage, however, some of them induce a specific immune response in ulcerative colitis[11]. They play various roles in ulcerative colitis through the generation of platelet-activating factors, pro-inflammatory mediators, leukotrienes, nitric oxide, transcription factors activation, and apoptosis inhibition[11]. The most important and influential pro- and anti-inflammatory cytokines that play an essential role in the pathogenesis and treatment of various acute and chronic diseases include IFN-γ, TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-17 A, IL-21, IL-23, and also IL-10, IL-35, and TGF-β[11–13].

Vitamin D is a secosteroid whose classical function is calcium-phosphorous homeostasis and the maintenance of bone health. The non-classical function of vitamin D includes the regulation of cellular proliferation, differentiation, apoptosis, and modulation of innate and adaptive immune responses. Furthermore, during the last decades with the discovery of vitamin D receptors in different tissues, several other biological functions of vitamin D have increasingly become apparent and its protective effects in many human diseases such as hypertension, cardiovascular, diabetes, autoimmune, cancers, dermatological, allergic, and acute and chronic inflammatory disorders have recognized[14,15]. Understanding the role of cytokines can lead to important advances in the diagnosis and treatment of this disease. Considering that the increase in the level of cytokines can aggravate inflammation and damage in the intestine, therefore, the identification and detection of pro- and anti-inflammatory cytokines in ulcerative colitis can be very helpful in the diagnosis and treatment of this disease. Since some changes in biological factors occur locally and at the site of disorders and may not be reflected in the peripheral blood, we decided to investigate the expression of these cytokines in the involved inflammatory environment. For this reason, this research aims to investigate the expression of important and influential pro- and anti-inflammatory cytokines in treated and treatment-resistant biopsy samples of ulcerative colitis patients and its association with serum level of vitamin D.

Materials and methods

Subjects

This study was conducted as a two-group case-control study. The control group consisted of 24 people with ulcerative colitis who responded to routine treatment protocols and who had a colon biopsy sample taken at the time of initial diagnosis of the disease. The case group consisted of 23 people with ulcerative colitis who did not respond to treatment. Three stages of treatment were used to treat ulcerative colitis. The treatment steps of the patients were as follows: The first stage of treatment was 5-aminosalicylic acid (5-ASA) treatment. The second stage of treatment was treatment with glucocorticoids and azathioprine. The third stage of treatment was biological treatment with anti-TNF-α, which included infliximab and adalimumab. All selected patients with ulcerative colitis were clinically evaluated and underwent further evaluation with biochemical tests, colonoscopy and colon biopsy. The inclusion criteria for the study involved patients who responded to treatment (control group) and those who did not respond to treatment (case group). The exclusion criteria included individuals

with diabetes, rheumatism, irritable bowel syndrome, immune system deficiencies, use of antibiotics in the six months prior to sampling, and patients with kidney failure.

The present study was approved by the Ethics Review Board of the Hamadan University of Medical Sciences (IR.UMSHA.REC.1401.787) and the informed consent obtained from all subjects was also approved. All methods were conducted in accordance with the relevant guidelines and regulations.

Measurement of serum vitamin D concentration

From each participant, 5 mL of peripheral blood was collected in a serum separator tube for the measurement of serum levels of vitamin D. Serum samples were obtained by centrifugation (3000 rpm / 10 min) at room temperature and stored at – 20 °C up to use. Vitamin D concentrations were quantified using a commercially available ELISA kit (Pishtaz Teb, Iran) according to the manufacturer's instructions. Serum vitamin D levels < 20 ng/mL were considered deficient, levels ≥ 20 ng/mL and < 30 ng/mL were considered inadequate, and levels ≥ 30 ng/mL were considered sufficient[16].

Colon biopsy samples collection

After subjects were identified, two colon biopsy samples were collected from each subject, one sample for pathological examination to confirm ulcerative colitis and the other sample for RNA extraction. Microtubes containing 1000 µL RNAlater Tissue Collection Solution (QIAGEN, Germany) were used for RNA preservation and stored at – 70 °C until RNA extraction.

Total RNA extraction

This procedure was performed from biopsy samples using the FavorPrep™ Tissue Total RNA Extraction Kit (Favorgen Biotech Corp, Taiwan) according to the manufacturer's guidelines. The ratio and concentration of RNA samples were assessed by calculating the absorbance at 260 and 280 nm using a spectrophotometer (BioTek Synergy HTX Reader, USA). To assess the purity and integrity of the extracted RNA, samples were loaded onto a freshly prepared 1% agarose gel stained with a safe stain and subjected to electrophoresis in 1× TAE buffer at 100 V for 30 min. The bands associated with 18 S and 28 S rRNAs indicated that the RNA samples were intact, while the smaller band of 5 S rRNA appeared faint, indicating a low level of RNA degradation. Samples were then fixed at 50 ng/µL and stored at -70 °C until reverse transcription.

Reverse transcription and cDNA synthesis

RNA samples were reverse transcribed into cDNA using reverse transcription cDNA synthesis kits (Yekta Tajhiz Azma, Iran) according to the manufacturer's guidelines. The cDNA reaction was prepared to a final volume of 20.0 µL, including reverse transcriptase enzyme (1.0 µL), random hexamer primers (2.0 µL), H2O (4.2 µL), dNTP mix (100 mM; 0.8 µL), buffer (2.0 µL), and extracted RNA (10.0 µL). This solution was placed in a thermocycler (Mastercycler gradient, Eppendorf, Germany) and subjected to the following cycling conditions: 70 °C for 5 min, then 37 °C for 60 min, followed by heating to 70 °C for another 5 min. Generated cDNA products were either used immediately or stored at – 20 °C for up to 1 week or at –70 °C for long-term storage.

Designing of gene-specific primers for real-time PCR

These primers were designed using mRNA sequences provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using Beacon Designer 7.9 (PREMIER Biosoft; USA) according to standard criteria. To avoid unwanted genomic DNA amplification, the PCR primers extended exon-exon boundaries or targeted different exons. To confirm that the designed primer pairs could effectively amplify the target genes and to determine the optimal annealing temperature, conventional PCR was performed at different temperatures ranging from 55 to 65 °C. A Molecular Size Marker 50 bp (CinnaGen, Iran) was inserted into a freshly prepared 2% agarose gel stained with a safe stain and subjected to electrophoresis in 1× TAE buffer at 100 V for 30 min. The size of different amplified PCR products was determined and verified by agarose gel electrophoresis and visualization under a gel documentation system. The nucleotide sequences of the designed gene-specific primers are presented in Table 1.

Relative quantification using quantitative real-time PCR (qPCR)

qPCR was performed using cyber green reagents (Yekta Tajhiz Azma, Iran) on a Rotor Gen 6000 machine (QIAGEN, Germany). Each reaction included 2× cyber green master mix (5 µL), consisting of cDNA (3 µL), each primers (1.0 µL), and RNase-free water (1.0 µL). The following thermocycling conditions were used: 95 °C for 4 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was then performed to evaluate the quality of the amplified product and to identify any nonspecific amplification. To normalize cytokine expression levels, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was utilized as a reference for each sample. (relative expression). Finally, the normalized results were reported as differences between groups.

Statistical analysis

The statistical analyses were conducted using the statistical software package SPSS 20 and the graphing software GraphPad Prism 6.0. The Shapiro-Wilk test was employed to ascertain the normality of the data, thereby informing the selection between parametric and non-parametric tests. In instances where normality was confirmed, equal variance was subsequently tested, and a t-test was employed for the purpose of comparing the control and case groups. In instances where the requisite normality was not met, the Mann-Whitney test was employed. Furthermore, Fisher's exact and chi-squared tests were employed to evaluate the relationships between categorical variables, with p-values less than 0.05 indicating statistically significant differences.

Name	Primer sequence	Tm (°C)	Accession number	Amplicon size (bp)
TNF-α	Forward primer: 5′-AGCCTCTTCTCCTTCCTGATC-3′ Reverse primer: 5′-CCAGAGGGCTGATTAGAGAGA-3′	58.60 58.04	*NM_000594.4*	121
IL-1β	Forward primer: 5′-GTGTTGTCATCAGACTTTGACCGTA-3′ Reverse primer: 5′-GAGAGCTTTCAGTTCATATCGACCA-3′	58.39 58.34	*NM_000576.3*	146
IL-6	Forward primer: 5′-GCCACTCACCTCTTCAGAACGAAT-3′ Reverse primer: 5′-GCCTCTTTGCTGCTTTCACACAT-3′	61.92 61.69	*NM_000600.5*	117
IL-8	Forward primer: 5′-GCTCTCTTGGCAGCCTTCC-3′ Reverse primer: 5′-TGGTCCACTCTCAATCACTCTCAG-3′	60.75 61.59	*NM_000584.4*	156
IL-10	Forward primer: 5′-GGTTGCCAAGCCTTGTCTGA-3′ Reverse primer: 5′-CATTCTTCACCTGCTCCACGG-3′	60.82 61.28	*NM_000572.3*	199
IL-17 A	Forward primer: 5′-CCTCATTGGTGTCACTGCTACTG-3′ Reverse primer: 5′-GTCCTCATTGCGGTGGAGATTC-3′	61.17 61.32	*NM_002190.3*	227
IL-33	Forward primer: 5′-TAACCCTGAGTCCTACAAAAGAC-3′ Reverse primer: 5′-CACAGTTGGAGTGCATATTATGAAG-3′	57.52 58.28	*NM_033439.4*	132
IL-35	Forward primer: 5′-GATGTACCAGGTGGAGTTCAAGAC-3′ Reverse primer: 5′-CCGGTTCTTCAAGGGAGGATTT-3′	60.86 60.29	*NM_000882.4*	164
IFN-γ	Forward primer: 5′-GCAGGTCATTCAGATGTAGCGGATA-3′ Reverse primer: 5′-CTTCCTTGATGGTCTCCACACTCTT-3′	62.52 62.26	*NM_000619.3*	178
TGF-β	Forward primer: 5′-CAAGTGGACATCAACGGGTTCA-3′ Reverse primer: 5′-CGCACGCAGCAGTTCTTCT-3′	61.07 61.31	*NM_000660.7*	191
GAPDH	Forward primer: 5′-GGAGTCCACTGGCGTCTTC-3′ Reverse primer: 5′-TTGCTGATGATCTTGAGGCTGTT-3′	60.08 60.56	*NM_002046.7*	159

Table 1. The nucleotide sequences of primers used in quantitative RT-PCR for gene expression analysis.

Variables		Control group ($n=24$); frequency (%)	Case group ($n=23$); frequency (%)	*P*-Value
Age; Mean (Std. dev.)		45.88 (18.51)	41.30 (13.01)	0.331
Sex	Male	17 (70.83)	14 (60.87)	0.547
	Female	7 (29.17)	9 (39.13)	
Smoking	No	20 (83.33)	22 (95.65)	0.348
	Yes	4 (16.67)	1 (4.35)	
Alcohol use	No	20 (83.33)	22 (95.65)	0.348
	Yes	4 (16.67)	1 (4.35)	
History of *Helicobacter pylori*	No	70 (74.47)	24.70 (9.82)	0.589
	Yes	24 (25.53)	26 (10.99)	
Ulcerative colitis type	Distal colitis	18 (1.41)	20 (0.00)	0.221
	Extensive colitis	40 (7.07)	35.80 (16.13)	0.699
	Left-sided colitis	25.33 (10.31)	21 (0.00)	0.727
	Pancolitis	23.18 (8.12)	26.06 (10.96)	0.519

Table 2. Demographic information, risk factors, and other variables in two groups.

Results

Demographic outcomes and other variables

The mean age (std. dev.) in the control and case groups was 45.88 (18.51) and 41.30 (13.01), respectively. In both groups, the majority of the sexes were males. The frequency of smoking and alcohol consumption was 4 (16.67%) in the control group and 1 (4.35%) in the case group. There were no significant differences in demographic variables, including age and sex, between the study participants. Demographic information, risk factors, and other variables in two groups are shown in Table 2.

Gene expression analysis

The relative expression of IL-1 was 0.06 in the control group and 0.11 in the case group, and there was no significant relationship between this cytokine and ulcerative colitis. Also, the expression of inflammatory cytokine TNF-α was almost the same in both groups, so it was 0.037 in the controls and 0.038 in the case group, and this is probably due to the use of anti-inflammatory mediators of TNF-α in the case group. The lowest expression of inflammatory cytokines in both groups was related to IL-17 A and IL-35 (Table 3; Fig. 1).

The results of patient data analysis based on the type of involvement showed that based on the type of pancolitis involvement, the highest expression of cytokines was related to IL-8 with a rate of 0.34 for the control group and it was the same interleukin with a rate of 0.24 for the case group. Also, based on the type of left-sided involvement, the highest expression of cytokines related to IL-8 was 0.04 for the control group and the

Variable	Control group ($n=24$) Mean (Std. dev.)	Case group ($n=23$) Mean (Std. dev.)	*P*. Value
IL-1β	0.06 (0.07)	0.11 (0.18)	0.610
IL-6	0.01 (0.02)	0.02 (0.05)	0.949
IL-8	0.19 (0.49)	0.24 (0.43)	0.907
IL-33	0.006 (0.01)	0.005 (0.01)	0.718
TNF-α	0.037 (0.09)	0.038 (0.13)	0.278
IL-17 A	0.01 (0.02)	0.006 (0.01)	0.170
INF-γ	0.04 (0.11)	0.02 (0.05)	0.064
IL-35	0.01 (0.06)	0.004 (0.02)	0.131
IL-10	0.00074 (0.001)	0.00063 (0.001)	0.882
TGF-β	0.025 (0.03)	0.017 (0.01)	0.0898

Table 3. The results of the relative expression of inflammatory and anti-inflammatory cytokines. ‡: From Mann–Whitney test.

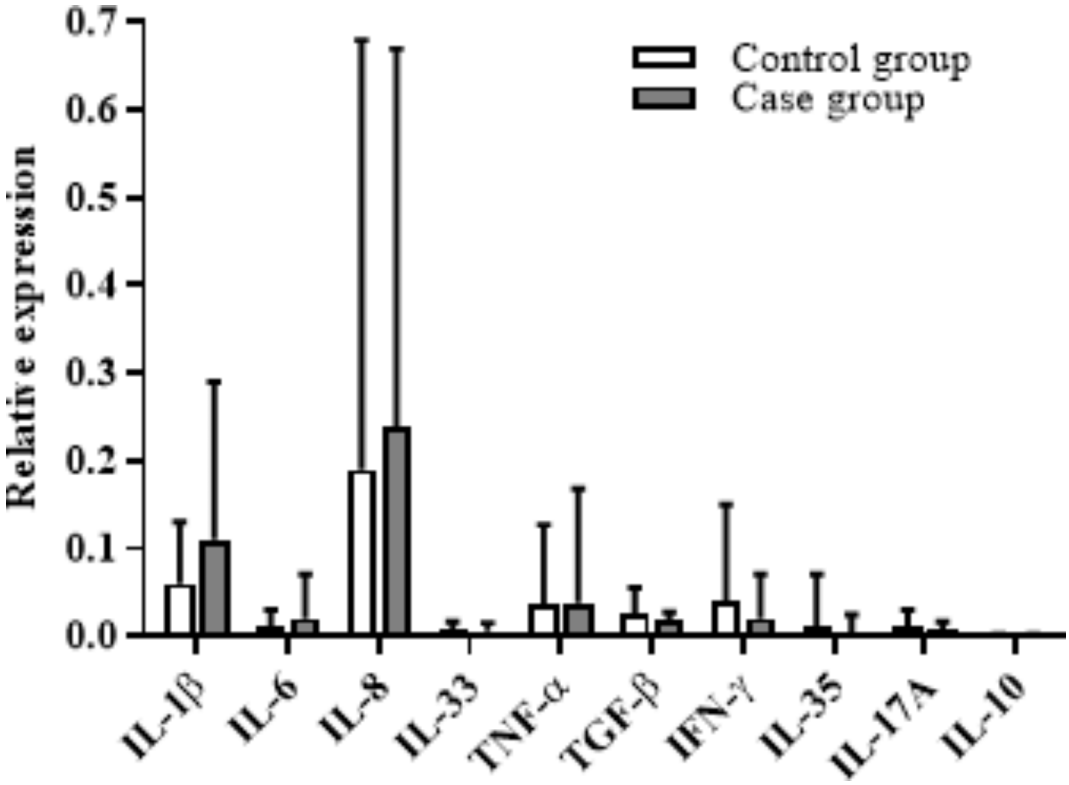

Fig. 1. Gene expression of pro- and anti-inflammatory mediators. The gene expression of anti/pro-inflammatory mediators in biopsy samples were assessed. IL-1β, TNF-α, IL-17 A, IL-10, IL-8, IL-6, IL-35, TGF-β IL-33, and IFN-γ gene expression levels in case group vs. the controls indicated no significant difference ($P>0.05$). The means ± STDs of the experiments are indicated in each bar graph.

same interleukin with a rate of 1.44 for the case group. The results of the expression of anti/pro-inflammatory cytokines based on the type of colon involvement are shown in Table 4.

Comparison of serum vitamin D levels with anti/pro-inflammatory factors in two groups

Vitamin D has anti-inflammatory and immunomodulatory properties and affects serum cytokine levels. Through unclear mechanisms, it is associated with the pathogenesis of ulcerative colitis in patients. Therefore, this study investigated the hypothesis that its efficacy in ulcerative colitis patients may be mediated by the expression of anti/pro-inflammatory cytokines in colon biopsy samples. According to linear regression analysis, serum vitamin D concentrations showed no correlation with gene expression levels of anti/pro-inflammatory cytokines in the colon biopsy samples of both treated and treatment-resistant ulcerative colitis patients (Table 5).

Variables	Ulcerative colitis type	Control group ($n=24$) Mean (Std. dev.)	Case group ($n=23$) Mean (Std. dev.)	*P*. Value
IL-1β	Pancolitis	0.09 (0.09)	0.12 (0.16)	0.921
	Leftsided	0.03 (0.04)	0.66 (0.00)	0.117
	Distalcolitis	0.04 (0.01)	0.01 (0.00)	0.221
	Extensive colitis	0.06 (0.08)	0.02 (0.02)	0.439
IL-6	Pancolitis	0.02 (0.03)	0.01 (0.02)	0.767
	Leftsided	0.002 (0.003)	0.25 (0.00)	0.116
	Distalcolitis	0.02 (0.01)	0.00 (0.00)	0.221
	Extensive colitis	0.00 (0.00)	0.00 (0.00)	0.439
IL-8	Pancolitis	0.34 (0.70)	0.24 (0.40)	0.505
	Leftsided	0.04 (0.05)	1.44 (0.00)	0.117
	Distalcolitis	0.14 (0.06)	0.01 (0.00)	0.221
	Extensive colitis	0.04 (0.05)	0.05 (0.02)	1.00
IL-33	Pancolitis	0.007 (0.01)	0.0066 (0.01)	0.677
	Leftsided	0.002 (0.001)	0.00 (0.00)	0.602
	Distalcolitis	0.02 (0.02)	0.001 (0.00)	0.221
	Extensive colitis	0.003 (0.00)	0.003 (0.003)	0.699
TNF-α	Pancolitis	0.044 (0.11)	0.050 (0.16)	0.300
	Leftsided	0.013 (0.027)	0.057 (0.00)	0.223
	Distalcolitis	0.15 (0.15)	0.005 (0.00)	0.221
	Extensive colitis	0.002 (0.002)	0.002 (0.00)	1.00
TGF-β	Pancolitis	0.04 (0.04)	0.02 (0.01)	0.374
	Leftsided	0.012 (0.02)	0.04 (0.00)	0.223
	Distalcolitis	0.01 (0.014)	0.005(0.00)	1.00
	Extensive colitis	0.02 (0.02)	0.006 (0.002)	0.699
IFN-γ	Pancolitis	0.03 (0.07)	0.02 (0.06)	0.114
	Leftsided	0.005 (0.00)	0.01 (0.00)	0.117
	Distalcolitis	0.32 (0.26)	0.001 (0.00)	0.221
	Extensive colitis	0.002 (0.002)	0.001 (0.00)	0.439
IL-35	Pancolitis	0.03 (0.09)	0.005 (0.02)	0.374
	Leftsided	0.00 (0.00)	0.00 (0.00)	0.384
	Distalcolitis	0.005 (0.005)	0.00 (0.00)	0.221
	Extensive colitis	0.00 (0.00)	0.00 (0.00)	0.699
IL-17 A	Pancolitis	0.008 (0.01)	0.007 (0.01)	0.361
	Leftsided	0.01 (0.02)	0.001 (0.00)	0.862
	Distalcolitis	0.04 (0.05)	0.00 (0.00)	0.221
	Extensive colitis	0.006 (0.008)	0.003 (0.002)	1.00
IL-10	Pancolitis	0.001 (0.002)	0.00 (0.00)	0.430
	Leftsided	0.00 (0.00)	0.005 (0.00)	0.117
	Distalcolitis	0.00 (0.00)	0.00 (0.00)	0.221
	Extensive colitis	0.00 (0.00)	0.00 (0.00)	1.00

Table 4. Relative expression of inflammatory/anti-inflammatory cytokines according to the type of colon involvement. ‡: From Mann–Whitney test.

Discussion

Cytokines are a type of protein that is produced by the immune system cells and are associated with the immune response and inflammatory processes of the body[17]. In ulcerative colitis, which is a type of IBD, the level and activity of cytokines increase. This increase may lead to the aggravation of inflammation and damage in the intestines[18–21]. Their unbalanced increase or decrease can lead to the aggravation of the disease and its side effects. Therefore, targeted therapies for ulcerative colitis may include controlling cytokine levels to help improve the patient's condition. For instance, anti-cytokine drugs such as anti-TNF-α drugs can be effective in decreasing inflammation and symptom improvement in IBD patients[18–21]. Therefore, the treatment of IBD includes the control and balance of cytokines and the body's inflammatory response. This includes the use of anti-cytokine drugs, anti-inflammatory drugs, and immune-modulating drugs[22–24]. Also, proper nutrition and stress reduction can be effective in controlling the level of cytokines and improving the condition of patients with IBD[25]. There was no significant relationship between the expression of anti/pro-inflammatory cytokines in treated and resistant ulcerative colitis cases. Even based on the type of colon involvement, there is no significant

Variables	Control group			Case group			Total sample		
	N = 24; Mean (Std.dev.)	Spearman's rho of Vitamin D & Variable	*P*.Value*	*N* = 23; Mean (Std.dev.)	Spearman's rho of Vitmin D & Variable	*P*.Value	*N* = 47; Mean (Std.dev.)	Spearman's rho of Vitamin D & Variable	*P*. Value
IL-1	0.06 (0.07)	0.01	0.966	0.11 (0.18)	− 0.14	0.523	0.09 (0.14)	− 0.08	0.612
IL-6	0.01 (0.02)	− 0.05	0.818	0.02 (0.05)	− 0.12	0.582	0.01 (0.04)	− 1.0	0.504
IL-8	0.19 (0.49)	− 0.21	0.316	0.24 (0.43)	− 0.17	0.428	0.21 (0.46)	− 0.20	0.176
IL-33	0.006 (0.01)	0.03	0.875	0.005 (0.01)	0.29	0.182	0.00 (0.01)	0.13	0.366
IL-35	0.037 (0.09)	0.12	0.569	0.038 (0.13)	− 0.07	0.758	0.04 (0.11)	− 0.11	0.450
TGF-β	0.025 (0.03)	− 0.16	0.469	0.017 (0.01)	0.05	0.809	0.02 (0.02)	− 0.07	0.636
IFN-γ	0.04 (0.11)	− 0.20	0.339	0.02 (0.05)	0.02	0.928	0.03 (0.09)	− 0.18	0.224
TNF-α	0.01 (0.06)	0.04	0.846	0.004 (0.02)	0.14	0.526	0.01 (0.04)	0.05	0.744
IL-17	0.01 (0.02)	− 0.37	0.072	0.006 (0.01)	0.05	0.813	0.01 (0.02)	− 0.21	0.153
IL-10	0.00 (0.001)	0.00	0.987	0.00 (0.001)	0.26	0.237	0.00 (0.00)	0.09	0.556

Table 5. The interaction between vitamin D concentration with anti/pro-inflammatory cytokines. *Test of H0: IL-1 (for example) and vitamin D are independent in the Treated group.

relationship between the two groups with the expression of cytokines. However, the expression of IFN-γ and TGF-β increased in the control group. The noteworthy point in this study is the lack of difference in the TNF-α expression in the two groups. This is due to the use of biological drugs such as anti-TNF-α to treat people with treatment-resistant colitis, and it shows the effect of this drug in reducing this inflammation. In a study that examined the expression of IL-17A in ulcerative colitis patients compared to healthy individuals using the real-time PCR, its expression was higher in unhealthy individuals than in healthy ones[26]. These findings were contrary to those of ours, where this cytokine expression was very low; this discrepancy is likely attributed to the selection criteria of our study participants. Another research assessed the serum levels of inflammatory cytokines in two groups: those with/without ulcerative colitis, using the ELISA method. Their results indicated that individuals with ulcerative colitis had higher concentrations of IL-8, while the IL-17A, IL-10, and IFN-γ levels were lower. Consequently, they concluded that IL-8 serves as a diagnostic marker for this disease[27]. Although our study found no statistically significant relationship between this inflammatory cytokine and the disease, clinically, the two groups were significantly different. In a separate study, it was demonstrated that the IL-1Ra' allele 2 was markedly overexpressed in ulcerative colitis patients than in those without the disease. These findings suggest that IL-1Ra can determine genetic susceptibility and the pathogenesis of ulcerative colitis[28]. Here, the IL-1β expression was found to be higher in the treatment-resistant cases in comparison to the treated group. We also examined the expression levels of cytokines based on the severity and extent of involvement. According to our findings and colonoscopy results, the severity of involvement was categorized into four levels, and cytokine expression results varied across these categories in both groups. This indicates that the severity of involvement influences the increase or reduction in the expression of anti/pro-inflammatory cytokines.

Vitamin D is a secosteroid that regulates calcium and phosphate metabolism, playing a crucial role in maintaining bone health. Additionally, vitamin D contributes to immune regulation. Immune cells, such as macrophages, regulate their local concentration of calcitriol by expressing 1-alpha-hydroxylase[29–31]. This effect is linked to the cytokine environment, where there is a link between serum vitamin D concentrations and lower IL-6, IL-1β, and TNF-α concentrations, and also higher IL-4 and IL-10 concentrations. Conversely, vitamin D deficiency is linked to a profile of pro-inflammatory cytokines and chronic inflammatory conditions[29–31]. Therefore, a deficiency in vitamin D leads causes an increase in the release of key IL-6, TNF-α, and IL-1β pro-inflammatory cytokines. Conversely, vitamin D supplementation alters the cytokine profile to an anti-inflammatory state through suppressing the IL-1β, TNF-α, and IL-6 generation. Furthermore, vitamin D can also inhibit the IL-17 A generation[29–31]. Since serum vitamin D levels are lower in ulcerative colitis patients than in controls, vitamin D may be linked to its pathogenesis[29–33]. One possible mechanistic link related to this association could involve the balance between anti/pro-inflammatory cytokines[29–33]. Therefore, we assessed the association between serum vitamin D and the gene expression of anti/pro-inflammatory cytokines in biopsy samples. Linear regression analyses showed no correlations between serum vitamin D concentrations and the gene expression of anti/pro-inflammatory cytokines in the biopsy samples. In this study, there was no significant relationship between the serum level of vitamin D and the expression of pro- and anti-inflammatory cytokines and responses to treatment. Therefore, it seems that the serum level of vitamin D did not affect the expression of pro- and anti-inflammatory cytokines in the affected area in the colon of treated and treatment-resistant ulcerative colitis patients and it has no role in the expression of these cytokines and the pathogenesis of the disease. Although in other studies, the relationship between serum level of vitamin D and gene expression or serum level of pro- and anti-inflammatory cytokines and disease pathogenesis has been observed[34–37] our study did not show this relationship. This may be because we investigated the local expression of pro- and anti-inflammatory cytokines and did not investigate their relationship in the peripheral blood. Another reason could be that they compared the healthy control group and the ulcerative colitis patients, while both of our study groups had ulcerative colitis.

Although the statistical analysis of the data between the treatment-resistant and treatment-responsive patient groups did not yield statistically significant results, the findings indicated that the gene expression levels of the principal inflammatory cytokines, including IL-6, IL-1β, and TNF-α, were elevated in the treatment-resistant patient group in comparison to the control group that demonstrated a response to treatment. This increase suggests the presence of a more robust immune response and chronic inflammation that is unresponsive to conventional therapeutic modalities. In these patients, the equilibrium between pro- and anti-inflammatory cytokines is disrupted, which can result in the exacerbation of symptoms and the recurrence of the disease.

In non-refractory patients, inflammatory cytokine levels are typically lower or more effectively controlled. This may be indicative of a favorable response to the administered treatments.

The distinction in inflammatory cytokine secretion between refractory and non-refractory ulcerative colitis patients serves as a crucial indicator for evaluating disease severity and gauging treatment efficacy and understanding of these differences can facilitate the development of more effective treatment strategies.

Given the limitations of our study, which include a small sample size and the lack of a healthy control group, we recommend that this research be replicated with a larger sample size. In addition, it is critical to include a well-defined healthy control group free of any disease to allow for more robust comparisons and increase the validity of the findings. This approach will not only provide a clearer understanding of the relationship between cytokine expression, vitamin D levels and inflammatory bowel disease (IBD), but will also strengthen the overall conclusions drawn from the data.

Conclusion

This study revealed no significant correlation between the expression of pro- and anti-inflammatory cytokines in the control and case groups. However, the gene expression levels of key pro-inflammatory cytokines were observed to be higher in patients with refractory ulcerative colitis than in patients who responded to treatment.

Furthermore, serum vitamin D levels were not correlated with the relative expression of cytokines in the affected colon of these patients. Despite the need for further research into the protective and pathology.

ical roles of cytokines and vitamin D, regular screening and early and adjunctive treatment may prove beneficial in reducing inflammatory symptoms in these patients.

Data availability

All data generated or analyzed during this study were included in this article but the raw data are available from the corresponding author on reasonable request.

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Author contributions

MYA, RYM designed and supervised the study. MYA, AA and FS performed data interpretation. AA, MYA, RYM, FS, LS, BN, HK and AM were responsible for data collection and doing experiments. MYA, AA and MKM analyzing the statistical results of the study. MYA, RYM and AA writing the original draft and edite the manuscrip. All authors approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1401.787). Written informed consent was obtained from all participants.

Additional information

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