Original Article

Toll-Like Receptor 7 a Novel Non-Invasive Inflammatory Genetic Sensor for Ulcerative Colitis Remission Monitoring

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Abstract

Background: Ulcerative colitis (UC) and Crohn's disease (CD) are two major types of inflammatory bowel diseases (IBDs). Toll-like receptors (TLRs) are expressed in the innate immune system compartments, in charge of identifying a wide range of microorganisms. The aim of the present study was to evaluate the expression of *TLR-2*, *-7*, and *-8* in peripheral blood mononuclear cells (PBMC) of UC patients as a novel non-invasive primary inflammation sensor for monitoring the clinical course of UC candidates.

Materials and Methods: In this cross-sectional study, total RNA was extracted from the PBMC of 42 UC patients along with 20 healthy donors. The mRNA levels of *TLR-2*, *-7*, and *-8* were assessed using the quantitative real-time polymerase chain (qRT-PCR) reaction.

Results: The present research study demonstrated no significant changes in *TLR-2* mRNA expression in UC patients in comparison with the control group ($P = 0.1264$), whereas significant elevation ($P = 0.0008$) was distinguished in the *TLR-7* expression of UC participants specifically during the remission course compared with healthy donors and flareup patients ($P = 0.0004$ and $P = 0.0063$, respectively). The last selected TLR, *TLR-8* was not shown remarkable changes either between UC patients and the control group or between clinical courses of the disease.

Conclusion: Here, among three nominated TLRs for predicting UC patients, *TLR*-7 was potentially selected according to the significant difference in mRNA expression in flareup UC patients and control donors. *TLR*-7 could be used as a novel non-invasive biomarker for monitoring UC patients in the active course of the disease.

Keywords: Biomarkers, colitis, inflammatory bowel disease, remission, toll-like receptor 7

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Introduction

Inflammatory bowel diseases (IBDs) are multifactorial disorders, which are classified into two major subgroups, named Crohn's disease (CD) and ulcerative colitis (UC).[1,2] The incidence and prevalence of IBDs have increased in recent decades; especially in developed countries, IBDs are more common than in developing countries.[3,4] More than two and a half million Europeans and over one million in the USA suffer from IBDs.[5,6] Although the exact figures for IBD patients in Iran are not clear, it seems that the number of patients suffering from IBD in Iran has increased significantly in recent years.[7,8] The disease course of UC is characterized by exacerbations and remissions, which may occur spontaneously

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or in response to treatment changes, superimposed infection. In untreated disease, UC usually exhibits chronic active colitis with the presence of active inflammation accompanied by features of chronic mucosal injury. With spontaneous healing or medical treatment, UC may become inactive or quiescent. Histologically, inactive (quiescent) colitis is characterized by marked architectural abnormalities in the absence of active inflammation.[9] Most cases of infectious colitis with the active colitis pattern have no specific diagnostic features on histological examination; hence, serologic studies or stool cultures are required for diagnosis.[10] The exact etiology of IBDs is unknown, but suggestions about the immune system dysfunction, genetic background, changes in microbiota, particular viruses, and environmental factors are the most possible theories of initiation and pathogenesis of IBDs.[11-13] The role of different genes and proteins that contribute to immune responses has been investigated in various studies.[14-19] Toll-like receptors (TLRs) are a class of pattern recognition receptors (PRRs) that initiate the innate immune response by distinguishing conserved molecular patterns for early immune recognition of a pathogen, composed of 10 different types.[20] TLRs activation not only leads to the inflammatory induction of responses but also antigen-specific adaptive immunity development. The inflammatory response through innate immunity in mammalians is induced by TLR, which is dependent on a common signaling pathway mediated by the pathogen-related molecular patterns and initiating NF-κB activation and other signaling pathways through the adapter protein[21,22] [Figure 1].

There is some evidence of an increment in the mRNA expression of *TLR-2* and *-4* in IBDs patients.[23-25] TLR-2 functionality is in the recognition of bacterial components such as peptidoglycan and fungi.[26] Based on further investigations, the expression of *TLR-7* and *-8* is down-regulated in inflammatory diseases such as irritable bowel syndrome.[27,28] TLR-7 and -8 are essential for the identification of some viral components such as nucleic acid-homologs structures in viruses.[29] Recent studies have indicated that the TLR expression profile in PBMC of IBD patients could be served as a reliable, non-invasive biomarker for IBD diagnosis or monitoring of the disease status.[30] Healthcare professionals apply several types of pharmaceuticals (anti-inflammatory drugs, immune system suppressors, and biosimilar therapies) for induction and maintenance of remission to improve the quality of life for ulcerative colitis-affected patients. The aim of the present study was to investigate the mRNA expression of *TLR-2*, *-7*, and *-8* in PBMC of participants to distinguish the flareup and remission course of colitis. Furthermore, the mRNA expression of recommended TLRs was compared in patients who had a history of taking different medications *5-ASA* [5-aminosalicylic acid] (*sulfasalazin/mesalazin, asacol, lialda, pantasa, lsalazin*, *prednizolon/azaram*), immunomodulation (*remicade, azotioprin, mercaptoperin, methoteroxcat, cyclosporin),* corticosteroids (*bedozonid, golimomab*), and biological drugs (*infliximab)* and supplements with non-started treatment patients. All patients' medications were produced by Tehran Chemie (Tehran, Iran).

Materials and Methods

Bioinformatics analysis

GeneMANIA (http://www.genemania.org) an informative, user-friendly web interface with a large set of functional association data was utilized for proving protein and genes interaction networks and visualizing pathways, gene co-expression, gene enrichment, and prediction of gene functions.

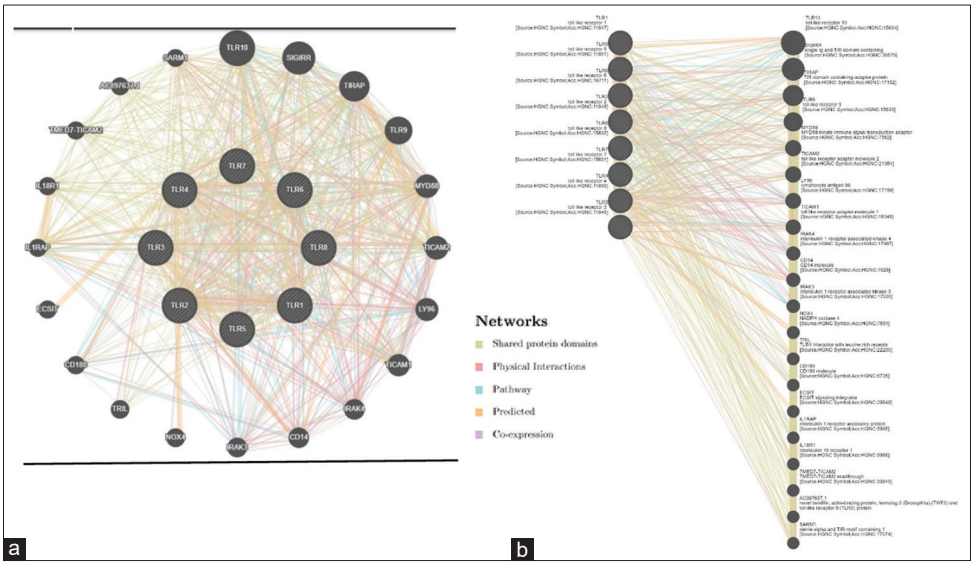

Figure 1: A Gene MANIA graph designed to visualize the gene–gene interaction network. Colon's main TLR network automatically was laid out. Different line colors in the network display the bioinformatics methods applied (mentioned in the style table). The edge thickness demonstrates the interaction strength and the node size shows the gene scores. From left (a) to right (b): Concentric bipartite, linear bipartite

Study population

The present study enrolled 62 UC candidates and 20 control donors (21 were in the active colitis course and 21 were in the inactive category. Twenty volunteers were assigned as healthy controls). Active or flare-up course of the disease was reported by two senior gastroenterologists and the support of experienced pathologists, according to patients' symptoms and inflammation detection accompanied by features of chronic mucosal injury. The inactive or remission course of colitis is characterized by marked architectural abnormalities in the absence of active inflammation. The most frequently remarked architectural abnormalities consist of atrophy, irregularity, and shortening of crypts, thickening of the muscularis mucosae, and metaplasia.[10] Patients were classified into five distinctive categories according to medication treatment protocol: 1) no treatment, 2) 5-ASA, 3) 5-ASA + corticosteroid, 4) 5-ASA + corticosteroid + immunomodulator, 5) 5-ASA + corticosteroid + immunomodulator + anti TNF. All dietary supplement consumption was recorded by questionnaire. The Ethics Committee at the research institute approved the informed consent form, case report form, and study protocol (IR. SBMU.IRGLD.1393.815).

RNA Isolation and quantitative real-time PCR

Five mL of whole blood samples were collected from all participants in the study. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Histopaque-1077 (Sigma-Aldrich, Merck Millipore, Japan) density gradient centrifugation and the RNA was extracted by Qiagen RNA Extraction (QIAamp, RNA Blood Mini Kit, Germany) in accordance with the manufacturer's protocol. The quality and quantity of RNA were resolved by spectrophotometric optical density measurement (260 and 280 nm) (NanoDrop spectrophotometer Technologies, Inc., Wilmington, DE, USA). The cDNA was synthesized by the Revert aid RT Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts, US). To evaluate the mRNA expression of *TLR-2*, *-7*, and *-8*, qRT-PCR was conducted using the Prime Script RT-PCR Takara kit (Shiga, Japan) and ABI 7500 real-time (2.3 version) PCR system (Applied Biosystems, Foster City, CA, USA). The primers were designed using the online tool (Primer 3) and checked by an offline application (Gene Runner 6.0). They are listed in Table 1.

Statistical analysis

Gene expression fold changes were calculated by the $2^{-\Delta\Delta CT}$ method. Student's *t*-test and one-way ANOVA analysis of variance test, with Tukey's multiple comparisons post-hoc tests in which $P < 0.05$ was considered as statistically significant were performed. GraphPad Prism 8 software (Graph Pad Software, Inc. La Jolla, CA, USA (https://www.graphpad.com/scientific-software/prism/) was utilized to draw the statistical graphs. The receiver operating characteristics (ROC) curve was constructed to describe the diagnostic specificity and sensitivity of biomarker selection. All statistical analyses were performed using SPSS v. 20 (SPSS Inc., Chicago, IL, USA).

Results

In silico colorectal TLR genes interaction

The PPI network analysis of the TLR genes interacting was performed using GeneMANIA (http://genemania.org/) to distinguish the deserved genes interaction and the main pathways. Figure 1 is shown, the colorectal TLR genes interacting with: *TLR10, SIGIRR, TIRAP, TLR9, MYD88, TICAM2, LY96, TICAM1, CD14, IRAK3, NOX4, TRIL, CD180, ECSIT, IL1RAP, IL18R1, TMED7,* and *SARM1.*

Demographic characteristics

In this case–control study, 21 patients with flareup UC and 21 in remission course were enrolled. The mean age of patients was evaluated at 36.8 ± 12.8. Among UC participants, 27 (64.3%) were females and 15 (35.7%) were males. The control group consisted of 20 healthy donors with a mean age of 52.2 ± 17.4. The gender distribution of normal cases was as follows: 11 (55%) were females and 9 (45%) were males. UC patients' drug consumption demonstrated over 80% use of 5-ASA and under 15% use of infliximab (Tehran Chemie, Tehran, Iran) expenditure. Information collected from patients demonstrated that below 30% of participants were using dietary supplements [Table 2].

Quantification of the expression of TLR-2,4,7 in PBMCs

The mRNA expression of *TLR-2, TLR-7,* and *TLR-8* in the PBMC of UC patients and normal donors were assessed by the qRT-PCR technique. Released data from expression analysis illustrated that the *TLR-2, -8* mRNA expression was not significantly different in UC cases and normal controls ($P = 0.1264$, $P = 0.25$, respectively) [Figure 2a, 2c].

Table 1: Primers were used in this study

Gene ID: (HGNC)	Gene Symbol	Primer Sequence 5' 3'	Product length (bp)
11848	TLR-2	F: 5'-GCTTTCCTGGGCTTCCTTTT-3' R: 5'-GGCATGTGCTGTGCTCTGTT-3	125 bp
15631	TLR-7	F: 5'-TTACCTGGATGGAAACCAGCTACT-3' R: 5'-TCAAGGCTGAGAAGCTGTAAGCTA-3'	72 bp
15632	TLR-8	F: 5'-CAGAATAGCAGGCGTAACACATCA-3' R: 5'-TGTCAAGGCGATTGCCACTGA-3'	161 bp
914	B2M	F: 5'-TGCTGTCTCCATGTTTGATGTATCT-3' R: 5'-TCTCTGCTCCCCACCTCTAAGT-3'	86 bp

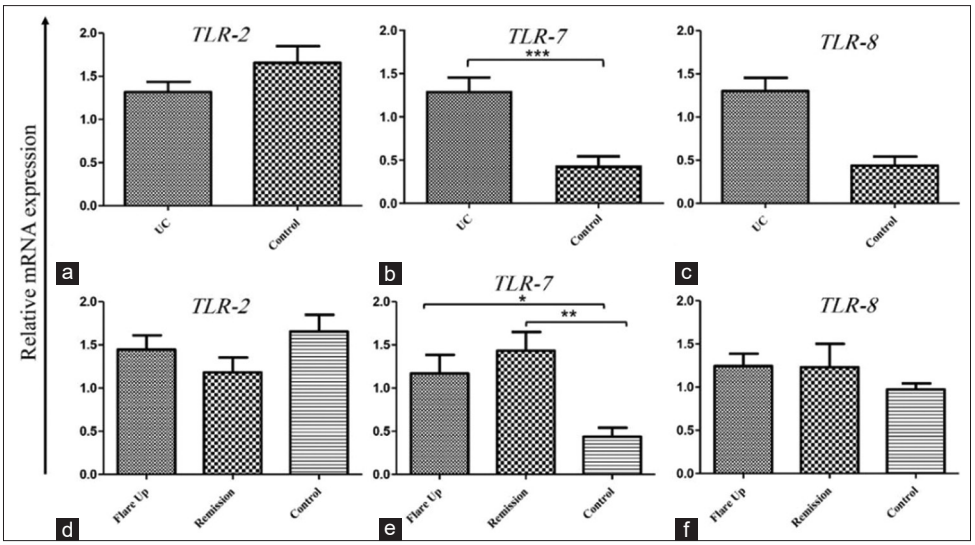

Figure 2: Quantitative real-time PCR analysis of expression of TLR-2, 7, 8 in human PBMC. (a) Relative mRNA expression of *TLR-2* in UC patients compared with healthy individuals. (b) Significant upregulation of *TLR-7* in UC patients compared with healthy individuals. (c) Relative mRNA expression of *TLR-8* in UC cases compared with healthy groups. (d) Relative mRNA expression of *TLR-2* at flareup and remission clinical course of ulcerative colitis. (e) A significant increment in relative mRNA expression of *TLR-7* at remission course of the disease. (f) Relative mRNA expression of *TLR-8* at the active and inactive course of UC. Student *t*-test and One-way ANNOVA analysis were applied. TLR-2, Toll-like receptor-2; TLR-7, Toll-like receptor-7; TLR-8, Toll-like receptor-8; UC, ulcerative colitis; (*$P < 0.05$) (**$P < 0.01$) (***$P < 0.001$)

In return, the results indicated significant upregulation of *TLR-7*in UC patients in comparison with healthy donors ($P = 0.0008$) [Figure 2b].

In advance, UC patients' determination of *TLR-2* and *TLR-8* mRNA expression in blood samples was not significantly different in flareup and remission course of disease [Figure 2d, 2f], but in particular, *TLR-7* was upregulated in patients who achieved remission in comparison with healthy individuals and fulminant cases ($P = 0.0004$, $P = 0.0063$, respectively) [Figure 2e].

History of medication and expression of toll-like receptors in UC patients

Pharmacological interventions in UC patients were subdivided into five groups as mentioned in the study population section. It is noteworthy that no statistically significant differences were observed among patients receiving different treatment procedures and mRNA expression levels of *TLR-2, TLR-7,* and *TLR-8* [Figure 3a–c]. Although the expression of *TLR-2* among UC candidates who consumed dietary supplements (Vit. D3 + calcium + folic acid) was decreased significantly in comparison with non-users ($P = 0.036$) [Figure 3d], no significant changes were observed in the expression of *TLR-7* and *-8* between UC supplement users and non-consumers [Figure 3e–f].

Characteristics of TLR-7 as predictive UC-related biomarkers

For evaluating the validity of *TLR-7 mRNA* expression as the potential biomarker for early diagnosis of UC, the receiver operating characteristics (ROC) curve was designed and the area under the ROC curve (AUC) was measured. The under the curve area estimated 76% (95% confidence interval [CI]: 0.55–0.97; $P < 0.038$) with specificity of 73% and sensitivity of (82%) [Figure 4].

Discussion

Although the exact causation and mechanisms underlying IBDs with respect to UC and Crohn's syndrome are not a clear but better understanding of the pathogenesis of inflammation, especially the clinical course of the disease can play an important role in the development of new and non-invasive approaches for molecular diagnosis and novel biological-based therapeutic protocols.[23,31] Recent studies have identified the essential role of TLRs in the recognition of the PAMPs and molecules associated with cellular and tissue damage (DAMPs).[32,33] In the laid-out gene–gene interaction network, immune factors were presented. The interaction between gut microflora and TLRs affects immune responses, and homeostasis was previously reported.[34] Different host responses appear using immune adjuvants by targeting distinguishable TLRs and their relevant adaptors. IFN-α secretion, further beneficial antigen presentation, cytotoxic T-lymphocyte activation, and Th1 responses by activation of TLR7, 8, and 9.[35] Cario reported that unwanted irritation of the mucosal immune system can be caused by intestinal microflora and environmental factors.[36] Any imbalance in microflora, host genetic background, and environmental factors could lead to abnormal TLR signaling in IBDs. According to our knowledge, evaluating the mRNA expression of *TLR-2*, -7, and -8 in PBMC of UC patients was performed for the first time at the Research Institute of Gastroenterology and Liver disease of Shahid Beheshti

Table 2: Demographic characteristics of the UC patients enrolled in the study

Variable	Patients ($n=42$)	Controls ($n=20$)
*Age (mean±SD)	36.8±12.8	52.2±17.4
*BMI (mean±SD)	26.4±5.9	26.3±4.2
*Gender (%)		
Male	15 (35.7%)	9 (45%)
Female	27 (64.3%)	11 (55%)
*Disease Clinical Course		
Active (Flare up)	21 (50%)	NA
Inactive (Quiescent)	21 (50%)	NA
*Drug History		
**5-ASA		
No	6 (14.3%)	NA
Yes	36 (85.7%)	NA
**Immunomodulation		
No	28 (66.7%)	NA
Yes	14 (33.3%)	NA
**Corticosteroids		
No	21 (50%)	NA
Yes	21 (50%)	NA
**Infliximab		
No	36 (85.7%)	NA
Yes	6 (14.3%)	NA
*Supplement use History		
**Vitamin D		
No	30 (71.4%)	NA
Yes	12 (28.6%)	NA
**Calcium		
No	30 (71.4%)	NA
Yes	12 (28.6%)	NA
**Folic acid		
No	27 (64.3%)	NA
Yes	(35.7%)	NA

SD; Standard deviation, BMI: Body mass index, 5-ASA: 5-aminosalicylic acid

University of Medical Sciences. The *TLR2* expression in intestinal epithelial cells is extensively upregulated in IBD patients compared to healthy individuals.[37] *TLR7* and *TLR8* are generally localized in intracellular sections of homo sapience, whereas *TLR-2* is expressed on cell surfaces.[38] *TLR2/1*, *TLR2/6*, and *TLR-4* recognized a wide variety of exogenous and endogenous stimuli including foreign lipids and lipopeptides.[23] Although researchers reported increased expression of *TLR-2* in the PBMC during inflammatory diseases, for example, irritable bowel syndrome (IBS), we observed no significant expression of *TLR-2* in the PBMC samples of UC patients compared with the control group.[39] Based on previous studies, significant upregulation of *TLR-2* was observed in the colonic mucosa of UC patients as well.[40,41] A research study by Cario *et al*.[42] showed that *TLR-2* plays an important role in signaling pathways and the protein expression of TLR2 in the active clinical course of IBD patients, also *TLR2* expression was significantly increased in the inflammatory cells of the lamina propria. Interestingly, UC patients who consumed dietary supplements such as Vit. D3 + calcium + folic acid demonstrated significant alteration in *TLR-2* mRNA expression, which might be related to some mechanisms of *TLRs* regulations and activation in the gastrointestinal tract.[43] Moreover, in our study, *TLR-7* expression evaluation showed a significant difference among UC participating patients with a different flareup and remission clinical courses of disease compared with the control group. According to the revealed data, it can be deduced that *TLR-7* could be used as a potential diagnostic biomarker in blood samples of UC patients to define the clinical course of colitis. Although *TLR-8* mRNA expression was not shown as a statistically significant difference between groups, the role of TLR-7 and -8 has been confirmed previously in inflammatory diseases. Contrary to the above information, Brint *et al.* reported that *TLR-7* and *-8* were decreased in patients with IBS.[44] Several studies have shown that the expression of *TLR-8* in the mucosa of UC and CD patients significantly changed between patients and the control group.[44] With all updated knowledge, there is still insufficient data for determining the trend of expression in *TLR-2, 7,* and *-8* in PBMC samples of IBD patients compared with healthy control donors. Finding novel biomarkers could facilitate early detection, therapeutic protocol, and monitoring of IBD patients. Several types of treatment protocols were prescribed for affected UC patients to induce and maintain an inactive course for ameliorating the standard of living; however, there is no difference recorded among prescribed medicaments.

Conclusion

The existing study investigated the mRNA expression of *TLR-2*, *-7,* and *-8* by a non-invasive sampling of participants to distinguish flareup and remission course of colitis. *TLR-7* can be used in the routine sampling of UC for remission monitoring.

Acknowledgement

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Abbreviations

(UC): Ulcerative colitis

(CD): Crohn's disease

(IBDs): Inflammatory bowel diseases

(TLR): Toll-like receptor

(PBMC): Peripheral blood mononuclear cells

(qRT-PCR): quantitative real-time polymerase chain reaction

(5-ASA): 5-aminosalicylic acid

(ROC): Receiver operating curve

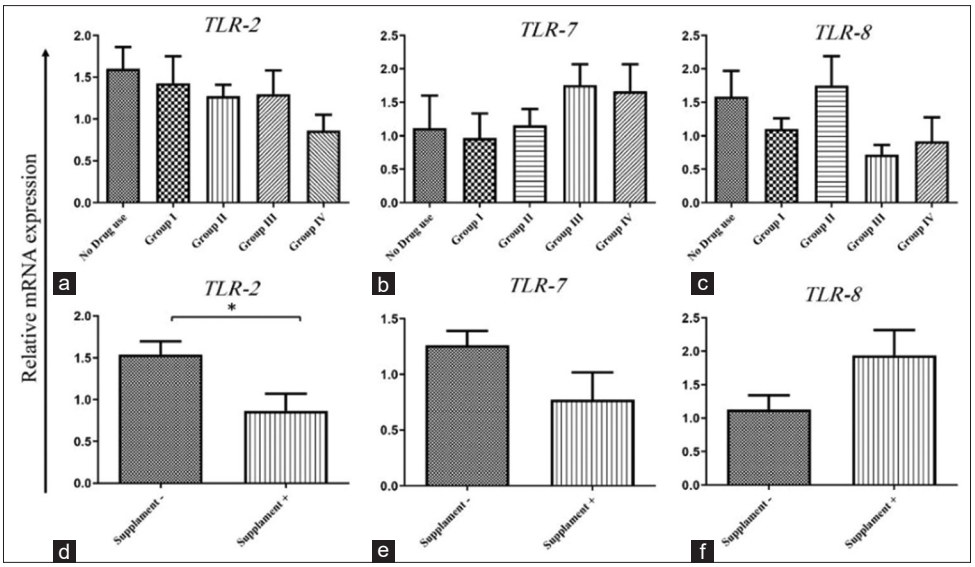

Figure 3: Real-time quantitative PCR analysis of *TLR-2, 7, 8* in human PBMC samples depending on drug consumption and dietary supplements. (a, b, c) Non-significant relative mRNA expression of *TLR-2, -7, -8* in accordance with the drug history groups described above. (d) Significant relative mRNA expression of *TLR-2* in dietary supplements users compared with non-users (e, f) Non-significant relative mRNA expression of *TLR-7, -8* in different supplement user's groups compared with non-users. Student *t*-test and One-way ANNOVA analysis were applied. TLR-2, Toll-like receptor-2; TLR-7, Toll-like receptor-7; TLR-8, Toll-like receptor-8; UC, ulcerative colitis; (*$P < 0.05$)

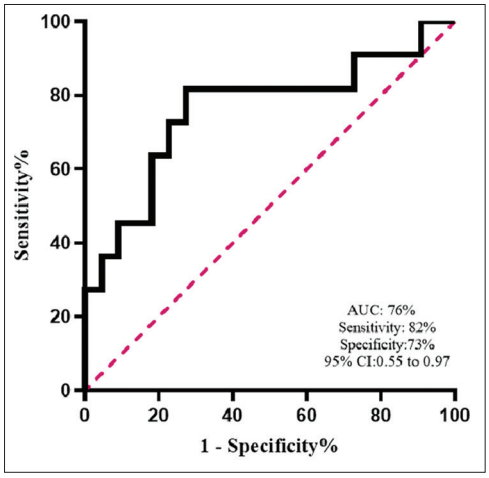

Figure 4: Receiver-operating characteristic (ROC) curves of normalized *TLR-7* expression to distinguish UC patients PBMC from normal individuals. The area under the curve (AUC) was determined for *TLR-7*

Ethical approval

The Ethics Committee at the Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences approved the informed consent form, case report form, and the study protocol (**IR.SBMU.IRGLD.1393.815**).

Consent of publication

The corresponding author (Ehsan Nazemalhosseini-Mojarad) claims that all manuscript contents and images in the present study can be published.

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Conflicts of interest

There are no conflicts of interest.

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