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Evaluation of Toll-Like Receptor Expression in Blood Cells of Iraqi Patients with Irritable Bowel Syndrome

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ABSTRACT

low-grade inflammation has been observed in patients with irritable bowel syndrome (IBS), but the underlying mechanisms are not clear. Toll-like receptors (TLRs) may be involved in the IBS. Therefore, the present study aimed to assess the expression of TLR2, TLR4, and TLR8 in the blood of IBS patients from Iraq. mRNA levels were measured using real-time quantitative PCR (RT-qPCR), with β-Actin as the reference gene.

Results showed a significant reduction in TLR2 mRNA expression ($p < 0.01$), a significant elevation in TLR4 mRNA expression ($p < 0.05$), and no significant change in TLR8 ($p > 0.05$) mRNA levels compared to healthy controls. Our findings indicate that changes in TLR expression may contribute to the immune dysregulation linked to IBS. Further research with larger sample sizes and subgroup analyses is necessary to fully understand the mechanisms behind these changes and their clinical significance.

Keywords: *Irritable Bowel Syndrome, IBS, Toll-Like Receptor, Iraq, Immunity*

1. INTRODUCTION

Irritable bowel syndrome (IBS) is a condition disorder marked by abdominal discomfort, affecting 9% to 16% of the global population (1). Four subtypes of IBS are categorized according to stool consistency and shape: Diarrhea-predominant (IBS-D), Constipation-predominant (IBS-C), Mixed bowel habits (IBS-M), and Unclassified (IBS-U) (2). The exact causes of IBS are still unclear; however, it is found that factors such as stresses, changes in gut microbiota, physical injury, and food intolerances are all capable of causing IBS (3). Additionally, recent studies have found evidence of low-grade inflammation in patients with IBS (1). Low-grade inflammation is a multifactorial process (4) and is characterized by an elevated presence of mast cells, T lymphocytes, and enteroendocrine cells within the colonic mucosa (5). Understanding the mechanisms of low-grade inflammation in IBS patients further deepens the understanding of the disease.

Toll-like receptors (TLRs), as immune sensors in the gut, detect pathogen-associated molecular patterns (PAMPs) from microbes and lead to the secretion of inflammatory cytokines (4). Therefore, TLRs play a crucial role in triggering the pro-inflammatory immune responses necessary for eliminating infections (6). Nucleic acids (DNA or RNA), lipoproteins, glycoproteins, peptidoglycans, lipoteichoic acid, and lipopolysaccharide (LPS) are some of PAMPs (7). In humans, ten functional TLRs have been detected, which are primarily expressed by various immune cells (6).

Dysbiosis linked to intestinal infections and bacterial overgrowth can affect potentially epithelial permeability, causing contact between TLRs and PAMPs. This causes host immune response, which may contribute to the development of IBS and worsen its symptoms (5). However, TLRs are also involved in the development of other immune and inflammatory disorders that are not directly associated with microbial infections (8).

Little is understood about the role of TLRs in IBS patients. Studies have shown the abnormal levels of TLRs in patients with IBS. For example, Shukla et al. reported an elevated level of TLR-4 and TLR-5 in IBS patients and described that intestinal

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inflammation may be exacerbated by dysbiotic microbiota in a subgroup of IBS patients (9). The current study aims to explore the levels of expression of TLR-4, TLR-2, and TLR-8 in IBS patients compared to healthy controls in Iraq.

2. METHODS

Sample collection

In the present study, whole blood was collected from 40 individuals, comprising 20 healthy participants and 20 IBS patients. All participants were from Baghdad Hospital in Iraq and were referred by a specialist physician. The blood samples (5 milliliters) were drawn into tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant agent and promptly transferred to the laboratory.

The study received approval from the Ethics Committee of the University of Sciences, Faculty of Veterinary Medicine, and all ethical guidelines, including obtaining informed consent from the participants, were strictly adhered to throughout the research process.

RNA Isolation

For total RNA extraction from whole blood, the Blood RNA Isolation Kit (DNAZist, Iran; Cat. No.: S-1021-1) was used. In accordance with the manufacturer's protocol, two milliliters of blood were used for RNA isolation per sample. The RNA concentration was subsequently measured using a NanoDrop spectrophotometer. The extracted RNA from blood samples was stored at -20°C.

Complementary DNA (cDNA) synthesis

cDNA was reverse transcribed from an RNA template with the cDNA synthesis kit (DNAZist, Iran; Cat. No. S-1074-50) according to manufacturer guidelines. The amplification program was as follows: 25 ° C in 10 min, 55 ° C in half an hour, and 80 ° C in 5 min. The synthesized cDNA was then stored at −80 °C for future use.

Real-time quantitative PCR (RT-qPCR)

The expression of receptors was assessed with a real-time PCR machine (ABI step one, America). Furthermore, β-Actin was used as the reference gene to normalize the samples. The PCR reaction mixture was composed of 0.5 µL each of primer, 10 µL of SYBR Green Master Mix (Denazist, Iran), and 0.5 µL of template cDNA. The final volume was then adjusted to 20 µL by nuclease-free water. The specific primers of RT-qPCR, synthesized by SinaClone, Iran, are listed in Table 1. The PCR program included one cycle of initial denaturation at 95 ℃ in 10 minutes, followed by 40 cycles of denaturation at 95 ℃ in 15 seconds, annealing at 60 ℃ in 60 seconds, and a final melting step at 60 ℃ to 95 ℃. The $2^{-\Delta\Delta CT}$ method was applied to evaluate RT-qPCR data and determine relative alteration in gene expression.

Table 1. Primer Sequences

Gene name	Sequences	Reference
TLR-2	FP-GGAGGCTGCATATTCCAAGG	(10)
	RP-GCCAGGCATCCTCACAGG	
TLR-4	FP-AGTTTCCTGCAATGGATCAAGG	
	RP-CTGCTTATCTGAAGGTGTTGCA	
TLR-8	FP-AGCGGATCTGTAAGAGCTCCATC	
	RP-CCGTGAATCATTTTCAGTCAAGAC	
β-Actin	FP-CACCATTGGCAATGAGCGGTT	(11)
	RP-AGGTCTTTGCGGATGTCCACGT	

Statistical analysis

The expression levels of TLRs are displayed as mean ± standard deviation (SD). GraphPad Prism version 10 (GraphPad Software, USA) was used to perform the statistical analysis. Each experiment was repeated three times. The normality of data was checked using the Kolmogorov-Smirnov test, and independent t-test was used for group comparisons. If the p-value was less than 0.05, it was considered statistically significant.

3. RESULTS

Figure 1 shows the expression levels of TLR expression in IBS patients and healthy controls. Our investigation revealed distinct patterns of TLR mRNA expression among Iraqi IBS patients. Specifically, TLR2 mRNA was significantly downregulated ($p < 0.01$), whereas TLR4 mRNA expression was significantly upregulated ($p < 0.05$). In contrast, there was no significant difference ($p > 0.05$) in the TLR8 mRNA expression between the two groups.

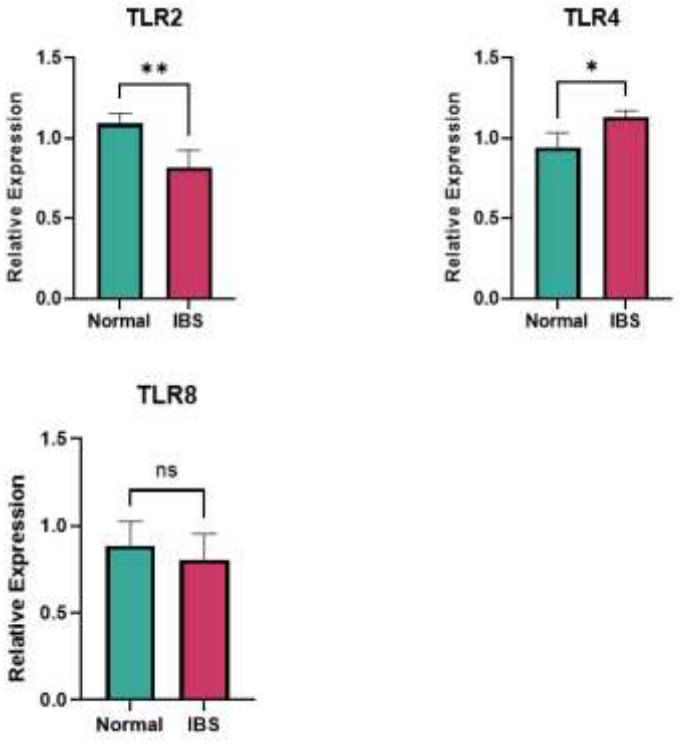

Figure 1. Expression levels of Toll-like receptors (TLRs) in blood samples from patients with irritable bowel syndrome (IBS) compared to healthy individuals. Statistical significance: **p < 0.01, *p < 0.05, and ns (not significant, p > 0.05).

4. DISCUSSION

The underlying causes of IBS, a functional gastrointestinal disease, remain unclear. Low-grade, chronic inflammation has been linked to the progression of IBS (12). This study investigated the mRNA expression levels of TLRs (2, 4, and 8) in IBS patients within an Iraqi population. Our findings reveal a significant reduction in TLR2 mRNA, a significant elevation in TLR4 mRNA, and no significant difference in TLR8 mRNA levels compared to healthy controls (Figure 1).

TLR2 has the capability to recognize a diverse range of ligands from different pathogens. It can also diagnose endogenous danger-associated molecular patterns (DAMPs). In the case of TLR2, ligand binding leads to heterodimerization with TLR6 or TLR1, leading to nuclear factor kappa B stimulation, inflammasome activation, and inflammatory cytokine release. This is crucial for establishing downstream immunity, which ultimately affects disease progression and host outcomes. Consequently, TLR2 serves a dual function during infection (13). In other studies, this receptor has also been studied in individuals with IBS. Most of the studies stated that there was no difference in the mRNA expression levels of TLR2 in the patients and healthy controls (9, 14-16). However, in the study by Koçak et al., although receptor levels were slightly elevated in IBS patients, the increase did not reach statistical significance (17). Unlike our research, which analyzed blood samples, these studies assessed TLR2 levels in intestinal samples.

Whereas earlier research noted no significant difference in the amount of TLR2 expression in IBS patients compared with controls, other reports note notable differences between different IBS subgroups. For instance, Koçak et al. reported that colonic TLR2 levels were upper in the IBS-D subgroup in comparison to both controls and IBS-C patients (17). Moreover, Belmonte et al. established that levels of TLR2 were higher in patients of IBS-M than in IBS-D or IBS-C patients (14). The decreased expression of TLR2 in our study may suggest impaired immunity, as research indicates that deficiencies in genes encoding TLRs or their downstream signaling proteins can increase susceptibility to infections (18).

TLR4 is stimulated by the LPS, initiating a series of signaling cascades (19). The activation of TLR4 by bacterial endotoxins

takes part in the development of both acute and chronic inflammatory diseases (20). On the other hand, overstimulation of TLR4 disturbs immune homeostasis by continuously releasing pro-inflammatory cytokines and chemokines, which play a role in the occurrence and progression of various diseases, including osteoarthritis, cancer, Alzheimer's disease, and sepsis (21). As previously mentioned, our study observed a significant increase in the level of this receptor in the IBS group versus the healthy group. This finding is consistent with the results of studies (9), (17), and (15). However, Belmonte et al. found no significant difference in TLR4 mRNA levels between IBS patients and healthy controls (14). Notably, a study revealed TLR4 variations in levels between various tissue samples. Dlugosz et al. reported that TLR4 expression was higher in the small intestinal mucosa of patients with IBS compared to healthy individuals. However, no such elevation was observed in colonic mucosa (16).

TLR8 primarily detects nucleic acids and is localized on intracellular vesicular membranes. Furthermore, it can detect bacterial RNA released inside the vacuoles of phagosomes (22). Limited research is available on the levels of TLR8 in IBS patients. Although the levels of TLR8 were reduced in this study, the decrease was not statistically significant. However, Brint et al. reported a significant decrease in this receptor (15).

5. CONCLUSION

In the present study, it was observed that TLR expression was changed in Iraqi IBS patients. TLR-2 mRNA expression was significantly decreased, while TLR-4 mRNA expression was significantly higher in IBS patients than in healthy controls. On the other hand, there were no significant changes in TLR-8 mRNA levels. The findings are consistent with previous research indicating that dysregulated TLR signaling could contribute to IBS. However, differences in TLR levels found in various studies illustrate that more research should be done on how the differences vary across various tissues and populations.

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