The Possible Role of Viruses in the Development of Irritable Bowel Syndrome

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Original Article

ABSTRACT

Background

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder characterized by abdominal pain and changes in intestinal movements in the absence of structural or biochemical abnormalities. In spite of the high prevalence, its etiology is unknown, and there are no specific diagnostic laboratory tests.

Material and Methods:

In a case/control study, 36 biopsy samples taken from patients with IBS and 30 biopsy samples as control were obtained. Expression of Toll-like receptor 3 (TLR 3), Toll-like receptor 9 (TLR 9), and Retinoic acid-inducible gene I (RIG 1) in macrophages, lymphocytes, and plasma cells in terms of percentage and color intensity were evaluated by immunohistochemistry. The obtained data were statistically analyzed using Mann Whitney U and Chi-square tests.

Results:

The differences between percentage and intensity in the sample and control groups were significant for all three receptors in the epithelial cells. In plasma cells, the difference in TLR 3 and TLR 9 percentage was significant but not significant for RIG. However, in terms of intensity, it was not significant for any of them. In lymphocytes and macrophages, there was no significant difference for any of the receptors.

Conclusion:

Since the expression of some virus sensing receptors on the different cell types of the intestine increases following infection, we concluded that IBS might be related to viral infections of the intestine. Future studies are needed to reveal the exact nature of the suspected viral agents. Some viruses producing gastroenteritis in humans, such as coronavirus, can be suspected.

Keywords: Irritable bowel syndrome, Toll-like receptor, Retinoic acid-inducible gene, Viral infection, Coronavirus, COVID-19

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INTRODUCTION

Irritable bowel syndrome (IBS) is the most well-known functional gastrointestinal disorder in which abdominal pain and/or discomfort is related to changes in bowel habits and with characteristics of disordered defecation without structural, inflammatory, or biochemical defects (1, 2). IBS influences up to 21% of the general population (3) and is more common in women than men (4).

It is a symptom-based state that is recognized by the ROME IV criteria (5). Based on the main stool pattern, the IBS is subtyped into four groups: IBS-C (constipation dominant), IBS-D (diarrhea dominant), IBS-M (mixed), and IBS-U (un-subtyped) (6). IBS does not predispose patients to a serious disease, but it profoundly influences personal satisfaction and causes a considerable economic burden in both direct and indirect consumptions around the world (7, 8).

The etiology and conditions related to the triggering and development of IBS have not been well understood (8), but it seems that some factors, for example, stress (9), autonomous nervous system dysfunctions (10), irregular gut motility (11), changes in intestinal microbiota (12), and genetic susceptibility (13) have roles. Increasing studies point to the role of innate immunity in the pathophysiology of IBS (14).

Pattern recognition receptors (PRRs) are responsible for detecting the attendance of microorganisms. They detect structures conserved amongst microbial species that are called pathogen-associated molecular patterns (PAMPs) (15). Based on protein domain homology, PRRs arrange into one of five families: Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and the Absent in melanoma 2 (AIM2)-like receptors (ALRs) (16). Toll-like receptors (TLRs) are type I transmembrane glycoproteins, which play a key part in the immune reaction against microorganisms. Ten human TLRs have been recognized to date. Among them, TLR3 detects the double-stranded RNA (dsRNA), and TLR9 identifies CPG DNA (17). Among the three RIG-Like Receptor family members, RIG-I and melanoma differentiation-associated gene 5 (MDA5) detect distinct viral RNA species (18).

Activation of PRRs triggers signaling events that cause immune system activation and expression of inflammatory cytokines and chemokines (19). In patients with IBS, there is some evidence of low-grade intestinal inflammation with activated lymphocytes and mast cells and enhancement of pro-inflammatory cytokines expression (20,21).

The association between TLRs and colonic inflammation has previously been reported in patients with IBS (22). Also, it was shown in some studies that the level of PRRs changed in colons of patients with IBS, which can be indicative of a change in intestinal microbiota in these patients (23,24). The possible role of viruses in the development of some non-digestive diseases of unknown etiology, including Multiple sclerosis (MS), Guillain-Barré syndrome, and type 1 diabetes, has already been suggested (25-27).

Some viruses, such as rotavirus and adenovirus, have a prominent role in the development of gastrointestinal problems, and they can be considered as an alarm for intestinal cells and cause inflammation and irritability (28,29). There have also been reports of an increase in the incidence of IBS and some other diseases of unknown etiology in patients recovering from the COVID-19 pandemic, which supports our hypothesis (30,31). So, it seems logical that viral infections can contribute to the etiology of IBS.

Thus, we decided to evaluate the expression of viral recognition receptors including TLR3, TLR9, and RLR1 in biopsy samples of patients with IBS by Immunohistochemistry (IHC) technique for the first time and compare them with the healthy group.

MATERIAL AND METHODS

Samples:

Human mucosal biopsy samples were obtained during April 2014-December 2015 from the colons of 36 patients with IBS undergoing colonoscopy at Afzalipour Hospital (Kerman, Iran). IBS diagnosis was made using the Rome III criteria and clinical assessment by a gastroenterologist.

Control biopsy samples were taken from 30 patients undergoing colon cancer screening. None of the patients (control or IBS) had Inflammatory bowel disease (IBD), celiac disease, intestinal infection, autoimmune diseases, immunodeficiencies, and colon cancer. All subjects provided written consent before participation in the study.

Participants' information was collected, and age matching was performed. Sampling was carried out with patients' collaboration.

Biopsy samples were placed in 10% formalin containers and transferred to Pathology Laboratory, School of Medicine, Kerman University of Medical Sciences.

Immunohistochemistry:

Paraffin-embedded samples were cut in 5-mm sections and mounted on clean glass slides. The paraffin was removed with xylene, and the samples were rehydrated. The process of sample fixation can lead to protein crosslinking, which masks epitopes and can restrict antigen-antibody binding. We used an antigen retrieval technique to prevent this problem. The slides were rinsed 1×5 min in tween 0.5% and 2×5 min in Tris-buffered saline (TBS). Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase solution for 5 minutes. After immersing in distilled water for 5 mins, for blocking of non-specific labeling, the samples were incubated in serum blocking solution for 30 mins. Then the sections were incubated with 50 microliters of primary rabbit polyclonal antibodies (Abcam, USA) against TLR3 (diluted at 1:5000), TLR9 (diluted at 1:2500), and RIG1(diluted at 1:200) for overnight at 4°C. To obtain suitable dilution, the tonsil tissue and nasal polyps were used as positive control and adipose tissue and skeletal muscle as the negative control. The sections were then washed 3×5 min with TBST (a mixture of TBS and Tween 0.5%). The surface of each slide was coated with 50 microliter Horseradish peroxidase (HRP) solution containing some peroxidase molecules and secondary goat anti-mouse-rabbit antibodies (1:200; Dako, Glostrup, Denmark) that are connected by dextran bridges. Incubation was done for 30 minutes in a wet place at room temperature, and slides were washed three times with TBST for 5 minutes each one. 50 microliters of substrate/chromogen mixture were added and incubated for 10 minutes in a dark place. Slides were placed in hematoxylin dye for a minute, and the nuclear staining was performed for differentiation between background and specific color. The slides were washed with TBST for 5 minutes again. The sections were subsequently dehydrated through an ascending ethanol series and were transparent with xylene, and were covered with a coverslip. After drying slides glue, the samples were evaluated under the light microscope, and cells were counted.

Slides evaluation:

All histological sections were evaluated by an expert pathologist. Four groups of mucous cells (epithelial, lymphocyte, macrophage, and plasma cell) were separated and evaluated. Results including percentage (P) and color intensity (I) of each group of cells were estimated, and immunoreactive score (IS) was calculated for each group (IS =P cell × I cell or $\sqrt{P cell \times \mathrm{I} Cell}$).

For each tissue section, the percentage of positive cells was scored on a scale of 0 to 3 for the percentage of tissue stained: 0 (0% positive cells), 1 (1% to 25%), 2 (26% to 50%) or 3 (51% to 100%). Staining intensity was scored on a scale of 0 to 3: 0, negative staining; 1, weak staining; 2, moderate staining; or 3, strong staining.

Data expression and statistical Analysis:

Data were analyzed using Mann–Whitney U and Chi-square tests.

Analyses were done by running the SPSS software version 22. Two-tailed P values less than 0.05 were considered statistically significant.

RESULTS

There are normally about 60±10 immune cells in each mm^2 of the slide, but this number was increased to 250±10 immune cells in our sample group. In our control group, about 60% lymphocyte, 30% plasma cell, and 10% macrophage were observable that changed into around 75% lymphocyte, 15% plasma cell, and 10% macrophage in the sample group. For the comparison of the percentage and color intensity of the cells with TLR3, TLR9, and RIG1 in the two control and sample groups, the Chi-square test was used. The significance level of the test was considered at P value ≤ 0.050.

Based on this, the difference between P (percentage) in the sample and control groups was significant for all three receptors in the epithelial cells ($P < 0.001$ for all three). In plasma cells, this difference was significant for TLR3 with $P = 0.040$ and for TLR9 with $P = 0.010$ but, it was not significant for RIG1. In lymphocytes and macrophages, it

was not significant for any of the receptors.

The difference between I (intensity) in the sample and control groups was significant for all three receptors in the epithelial cells ($P < 0.001$ for all three), but the difference was not significant for any of the receptors in plasma cells, lymphocytes, and macrophages.

The staining index (SI) was calculated based on the multiplying square of the color intensity by the percentage of the colored cells, and the Mann-Whitney test was used for the results. The significance level of the test was considered at P value ≤ 0.050.

All three proteins TLR3, TLR9, and RIG1 were observed in the four-cell groups (epithelial cells, plasma cells, lymphocytes, and macrophages), and they were at a medium level in the sample group (2 of 4) and at low in the control group (1 of 4). The difference between SI in the sample group and the control group was significant for all three receptors in the epithelial cells ($P < 0.001$ for all three). This difference was significant in plasma cells for TLR3 ($P = 0.033$), for TLR9 ($P = 0.020$), and for RIG1 ($P = 0.026$). In lymphocytes, the difference between the sample and control group was significant for TLR3 ($P = 0.030$). It should be noted that these differences are only mathematically significant and are not considered at the clinical level.

In this study, there were 14 subjects in the IBS-C group, 12 in the IBS-D group, 10 in the IBS-M group, and there were no subjects in the IBS-U group. For the comparison of the SI of the TLR3, TLR9, and RIG1markers in the IBS subgroups, the Chi-square test was used. Based on this, there were no significant differences between the subgroups of disease and the expression of the mentioned markers in the epithelial cells. The significance level of the test was considered at P value ≤ 0.050.

DISCUSSION

The examination results of the number of immune cells existing in the intestinal mucus, including plasma cells, lymphocytes, and macrophages in the sample and control groups, show the increase of these cells in the intestinal mucus in the sample group compared with the control group. This increase in the number of immune cells in the patient group compared with the control group can show a microscopic inflammation, while no microscopic inflammation is observed in patients with IBS. The increase of immune cells can show the formation of immunologic response in the intestinal mucus of these patients. Also, the results show an increase in the TLR3, TLR9, and RIG1 expression on the surface of epithelial cells and plasma cells in the sample group compared with the control group. This study is the first of its kind that has evaluated these three receptors in the protein level in the mucus of patients suffering from IBS.

Based on the results, the increase of the TLR3 expression in the epithelial cells compared with the plasma cells and lymphocytes in the sample group was more significant compared with the control group. Since the epithelial cells are at the front line of fighting viruses and can be essentially considered as a part of the immune system, it is not unexpected that among the cells under study, the epithelial cells had the highest amount of TLR3 expression. In several studies, TLR3 has been examined in the inflammatory diseases of the intestine. Cario and Podolsky in 2000 showed that the expression level of TLR3 protein in patients infected with Crohn's disease was lower compared with the healthy people, and the level in patients suffering from ulcerative colitis was not different from healthy people (32). Based on the study of Fan and Lit in 2015, it was observed that the expression level of gene and protein TLR3 in the intestinal mucus of the patients infected with ulcerative colitis was not different from the control group (33). Also, the study of Ostvik and colleagues in 2013 showed that TLR3 increased in patients suffering from intestine inflammation (34). Since the etiology of IBS is yet unknown and that TLR3 is responsible for realizing virus dsRNA, it can be said that the increase in the expression of TLR3 is possibly caused by a viral infection. However, the proof of this issue needs more study and research. Since TLR3 recognizes the dsRNA ligand, it may be possible to relate this viral infection to the intestinal viruses having dsRNA, such as adenovirus. Of course, this hypothesis cannot be totally rejected that maybe viruses with dsRNA that do not have an intestinal role, for example, respiratory viruses can also be responsible for IBS creation, and in this case, it is necessary to examine stool samples of patients with IBS for the existence of the antigens of these viruses or the antibody of these viruses in their serum samples and

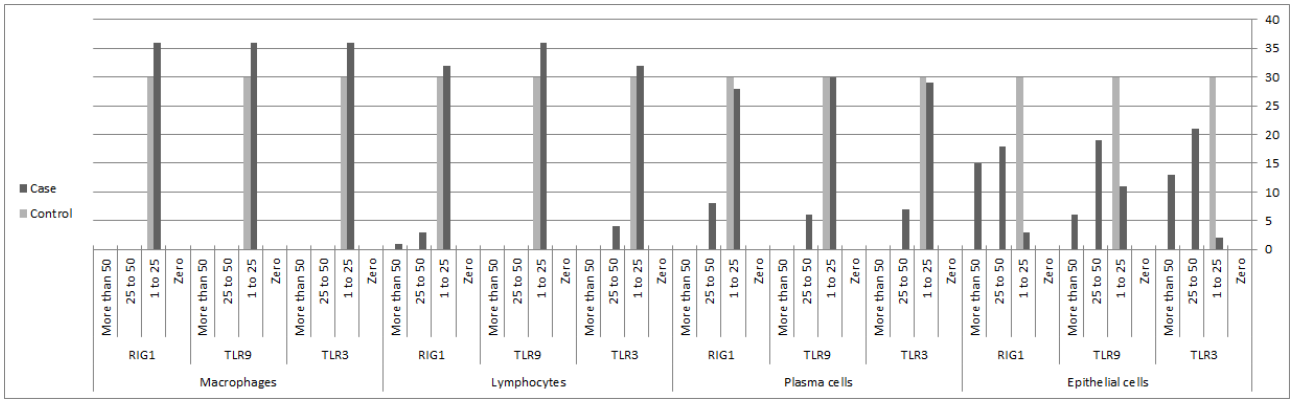

Fig. 1: Comparing the percentage of epithelial cells, plasma cell, lymphocyte, and macrophage with RIG1, TLR9, and TLR3 markers in the sample and control groups

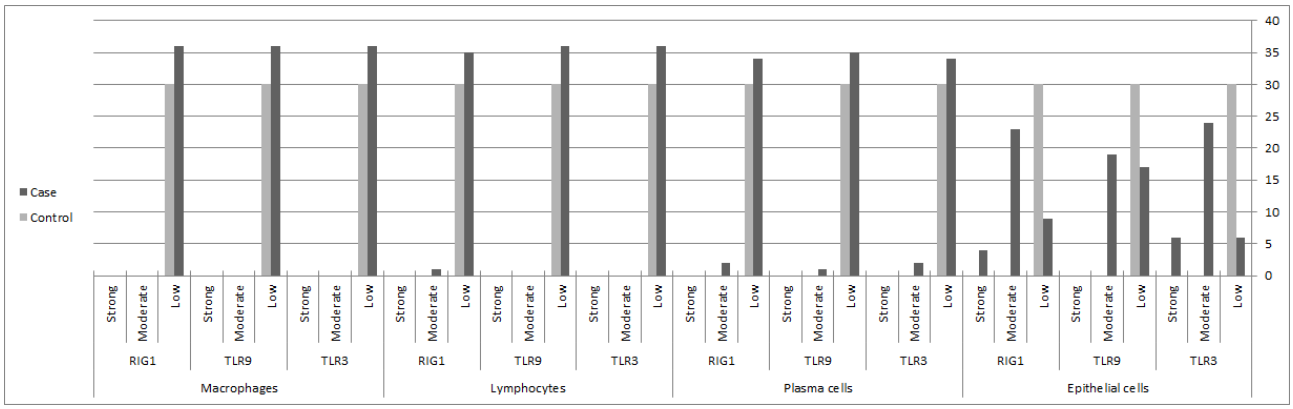

Fig. 2: The comparison of color intensity of the epithelial cells, plasma cells, lymphocytes, and macrophages with RIG1, TLR9, and TLR3 markers in the control and sample groups

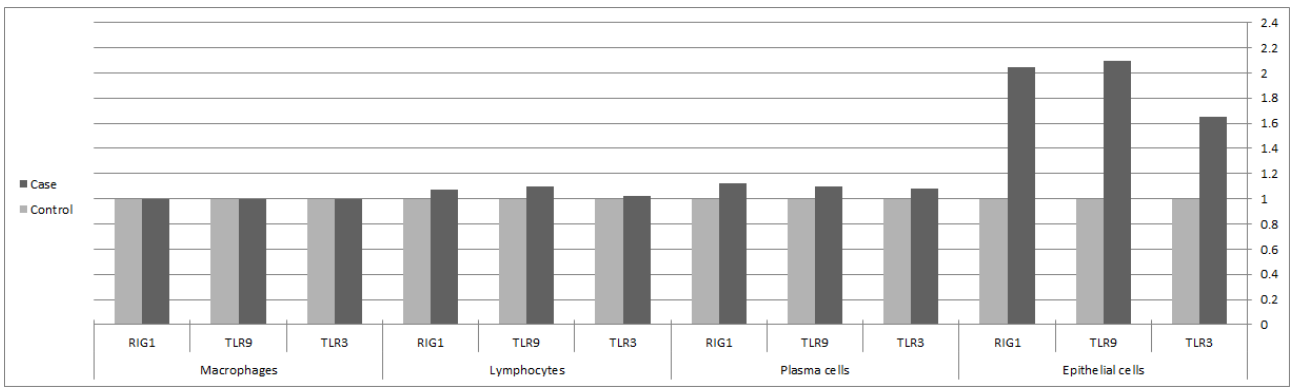

Fig. 3: Comparing the mean of the staining index for the epithelial cells, plasma cells, lymphocytes, and macrophages with TLR3, TLR9, and RIG1 markers in the sample and control groups

compare it with the control group. The role of unknown viruses in the environment cannot be ignored as well.

In this study, we observed the increase of the protein expression of TLR9 (as the receptor recognizing viral CpG DNA) in the epithelial and plasma cells of the sample group compared with the control group. Based on the previous notions about the increase of TLR3 expression (as the receptor recognizing viral CpG DNA) in the

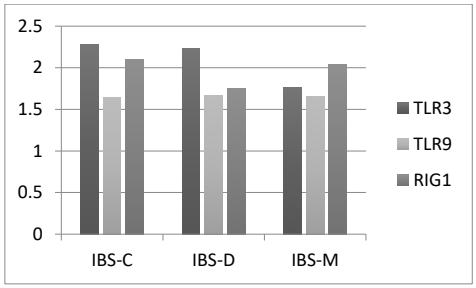

Fig. 4: The comparison of staining intensity of TLR3, TLR9, and RIG1 markers in the subgroups of IBS

epithelial cells, plasma cells, and the lymphocytes of the sample group, this increase strengthens the possibility of a viral infection. Types of DNA viruses are recognized by TLR9 that are not viruses with a proven role in intestinal diseases (35). Of course, it is possible to imagine a new role with the examination of their antigens in the stool sample or antibody of these viruses in the serum sample of the patients with IBS compared with the control group. It should also be noted that in addition to viral infections, bacterial infections can also be involved in the formation of this disease. The study of Belmonte and colleagues in 2012 with the use of q-PCR technique and immunofluorescence showed that the expression amount of gene and protein TLR2 (ligand for peptidoglycan and lipoprotein), and TLR4 (ligand for LPS) did not have a significant difference in patients suffering from IBS compared with the control group (36). Based on this study, it could be said that to some extent, the possibility of a bacterial factor is weaker than a viral infection. This is while the study of Brint and co-workers in 2011 shows the increase of the expression of TLR4 gene in patients with IBS compared with the control group that contradicts with the results of the present study and the study of Belmonte (23). Nevertheless, the prove of the role of bacteria in the creation of IBS needs more study and research. Also, in an unpublished study by Mohammadi and colleagues, TLR3 and TLR9 were studied for the possible role of viruses in the hydatidiform mole disease that has an unknown etiology like IBS. They showed that the level of TLR3 and TLR9 increased in the sample group compared with the control group (37). Also, the study of Fan and Liu in 2015 showed that the level of expression of gene and protein TLR2, TLR4, and TLR9 in the intestinal mucus of the patients infected with ulcerative colitis increased compared with the control group, while the expression levels of gene and protein TLR1 and TLR3 were not different in the sample and control groups (33). The study of Munoz and others also shows the increase in the expression of TLR9 gene in patients with ulcerative colitis compared with the control group (38). This could show the possible effect of TLR changes in the creation of ulcerative colitis.

The findings of our study also show the increase of the protein RIG1 expression (as the receptor recognizing dsRNA and viral ssRNA) in the epithelial cells and plasma cells of the sample group compared with the control group. This increase in the expression in addition to the increase in the expression of TLR3 (as the receptor recognizing viral dsRNA) and TLR9 (as the receptor recognizing viral CPG DNA) strengthens the hypothesis for the existence of a viral infection. This can guide future studies about the function of the innate immune system fighting viruses.

On the other hand, regarding the possible relation between RNA viruses and IBS and also increase in RIG1 expression (as the recognizing receptor of viral dsRNA and ssRNA), the possible infection with any of the RNA digestive viruses, including coronavirus, rotaviruses, adenoviruses, astroviruses, and noroviruses can be noted that all of them can cause gastroenteritis (39-42). In the end, there was no significant difference between the staining index of TLR3, TLR9, and RIG1 markers in the subgroups of IBS. It seems that the disease subgroups do not affect the expression of viral detecting TLRs.

Based on the above discussion and the possible role of viruses in the IBS, maybe with the examination of digestive viruses and non-digestive viruses, the etiology of this disease can be better understood.

CONCLUSION

Based on the increase in the expression of TLR3, TLR9, and RLR1 in patients with IBS in comparison with the control group, the hypothesis of the possible relation between viral infections and the emergence of IBS are strengthened, which needs more studies and examination with more precise methods in the gene level. Based on the results, it is possible that this possible viral infection can be the result of ssRNA, dsRNA, and CpG DNA viruses.

So, some viruses that produce gastroenteritis in humans, such as coronavirus, can be suspected.

Since TLR9 recognizes the ligand of CpG DNA virus and bacteria, the possibility should be considered that besides viral infections, bacterial infections can also be involved in the creation of this disease.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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