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Tight junction protein changes in irritable bowel syndrome: the relation of age and disease severity
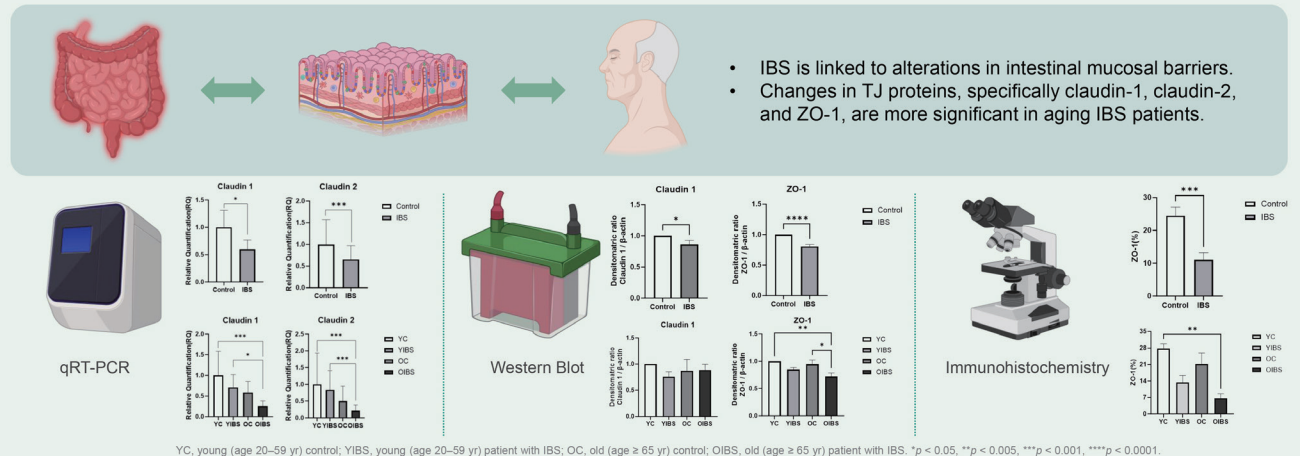
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Tight junction protein changes in irritable bowel syndrome: the relation of age and disease severity

This study aims to evaluate changes in tight junction (TJ) proteins in irritable bowel syndrome (IBS) patients and explore how these changes relate to aging and disease severity.

- IBS is linked to alterations in intestinal mucosal barriers.
- Changes in TJ proteins, specifically claudin-1, claudin-2, and ZO-1, are more significant in aging IBS patients.

YC, young (age 20–59 yr) control; YIBS, young (age 20–59 yr) patient with IBS; OC, old (age ≥ 65 yr) control; OIBS, old (age ≥ 65 yr) patient with IBS. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$.

Conclusion — ZO-1 expression was especially decreased in the aged IBS group. These results suggest that ZO-1 might be the prominent tight junction protein causing IBS in the aging population.

Background/Aims: The etiology of irritable bowel syndrome (IBS) is associated with intestinal mucosal barrier damage. However, changes in the tight junction (TJ) proteins in IBS have not been fully elucidated. This study aimed to evaluate TJ protein changes in IBS patients and the relationship between aging and disease severity.

Methods: Thirty-six patients with IBS fulfilling the Rome IV criteria and twenty-four controls were included. To evaluate the change of TJ in the colonic mucosa, quantitative reverse transcription polymerase chain reaction, western blot, and immunohistochemistry (IHC) were performed, respectively.

Results: The entire IBS group (n = 36) exhibited decreased levels of claudin-1 and -2 mRNA compared to the control group (n = 24), with statistical significance ($p < 0.05$). Additionally, in western blot analyses, both claudin-1 and ZO-1 levels were significantly reduced in the IBS group compared to the control group (n = 24) ($p < 0.05$). IHC analysis further revealed that ZO-1 expression was significantly lower in the IBS group than in the control group ($p < 0.001$). This trend of reduced ZO-1

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expression was also observed in the moderate-to-severe IBS subgroup ($p < 0.001$). Significantly, ZO-1 expression was notably lower in both the young- ($p = 0.036$) and old-aged ($p = 0.039$) IBS groups compared to their respective age-matched control groups. Subtype analysis indicated a more pronounced decrease in ZO-1 expression with advancing age.

Conclusions: ZO-1 expression was especially decreased in the aged IBS group. These results suggest that ZO-1 might be the prominent TJ protein causing IBS in the aging population.

Keywords: Irritable bowel syndrome; Tight junction; Aging; Colon

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal (GI) disorder characterized by chronic abdominal pain and altered bowel habits. This reduces the quality of life and places a significant economic burden on the medical system and society [1-4]. The pathophysiology of IBS is multifactorial, involving alterations in visceral hypersensitivity, GI motility, mucosa-associated immune alterations, microbiome, and intestinal permeability [5]. Recent studies have shown the role of intestinal barrier dysfunction in IBS. Several previous studies have suggested that intestinal permeability is increased in patients with IBS with predominant diarrhea (IBS-D) or in those with post-infection IBS [6].

The intestinal epithelium forms a crucial barrier that regulates the selective passage of nutrients and prevents the infiltration of harmful pathogens and toxins into the underlying tissues [7]. Tight junction (TJ) proteins, which serve as molecular gatekeepers that control paracellular permeability, are central to the integrity of this barrier. TJs consist of various proteins, including claudins, occludins, and zonula occludens, all of which play pivotal roles in maintaining the structural and functional integrity of the intestinal epithelial barrier [8-10].

Alterations in TJ proteins may be a key factor that contributes to the disrupted gut barrier observed in patients with IBS. The dysregulation of these proteins can lead to increased intestinal permeability, thereby allowing luminal antigens to translocate into the underlying mucosa. This, in turn, can trigger immune responses, low-grade inflammation, and an array of GI symptoms characteristic of IBS [11,12].

Among the proteins composing TJs, the following three proteins have been mainly studied: the transmembrane proteins, occludin and claudin-1, and the cytosolic protein, zonula occludens-1 (ZO-1) [8,13-15]. However, in patients with IBS, the role of TJ proteins has been poorly investigated. Previous studies have recently shown an increase in the colonic paracellular permeability in patients with IBS associated with a decrease in ZO-1 mRNA level [16]. Other studies have reported that occludin mRNA expression remained not modified [17]. However, a recent study has reported a decrease in the occludin expression in the colonic mucosa of patients with IBS [18].

While several studies have investigated the association between TJ protein levels and IBS, the exact nature of these changes, their mechanisms, and their clinical implications remain a subject of ongoing research and debate. Although studies have identified changes in TJ proteins in patients with IBS, the relationship between these changes and the aging process has not been well elucidated.

Therefore, we aimed to investigate the changes in TJ proteins associated with aging and disease severity in patients with IBS, thereby contributing to a deeper understanding of the pathophysiological mechanisms in IBS.

METHODS

Participants

This study was conducted prospectively at a tertiary care center from 2018 to 2022. All participants were recruited from Korea, with the control group comprising individuals who underwent colonoscopies as part of a health checkup. Under the guidance of a well-trained interviewer, participants received colonoscopies and completed questionnaires regarding IBS symptoms. We excluded patients with any macroscopic or histologic abnormalities of the colonic mucosa, a history of GI surgery, neuromuscular diseases, GI malignancies, or any other organic bowel diseases. Participants were categorized into control or IBS groups, with only IBS-D patients enrolled according to the Rome IV criteria,

as shown in previous studies [19,20]. IBS-D is commonly known to be more prevalent, and it was expected that the expression of TJ proteins would be more closely associated with IBS-D. The control group consisted of individuals with normal colonic mucosa and no evidence of organic or functional bowel disease.

Colonoscopy biopsy

All participants underwent a comprehensive colonoscopy, during which biopsies were taken from the sigmoid colon. Using standard biopsy forceps, we collected six tissue samples of the colon mucosa. These samples were then analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR), western blotting, and immunohistochemistry (IHC). Specifically, two colon mucosal biopsy samples were allocated for each analysis method: qRT-PCR, western blot analysis, and IHC.

Questionnaire

Thirty-six patients with IBS fulfilling the Rome IV criteria and twenty-four controls were included in this study. IBS symptom severity was assessed using the IBS Severity Scoring System [19]. The overall IBS score ranged from 0 to 500, and patients were divided into 3 groups according to score, as follows: mild IBS, < 175; moderate IBS, 175–300; and severe IBS, > 300 (Supplementary Fig. 1) [21]. The IBS and control groups were classified according to age. Further, both groups were categorized into the old- and young-aged groups. To clarify differences between ages, participants aged 65 years or older were categorized into the old-aged group, whereas those aged 20–59 years were categorized into the young-aged group.

qRT-PCR

Total RNA was extracted from human sigmoid colon tissues using QIAzol reagent (Qiagen, Venlo, The Netherlands), according to the manufaturer's instructions. Using 2 μg of the total RNA as a template, the first strand cDNA was synthesized using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The ABI ViiA7 Real-time PCR system (Applied Biosystems, Waltham, MA, USA) was used for quantitative real-time PCR (qPCR) amplification and detection. qPCR was run in triplicates, each of 10-μg reaction volume using Power SYBR® Green PCR Master mix (ABI), following the manufacturer's instructions. Thermal cycling was performed under the following conditions: 50°C, 2 minutes; 95°C, 10 minute; 40 cycles of 95°C, 15 seconds; and 60°C, 1 minute. Used primers are described in Supplementary Table 1. All expression levels were normalized to *GAPDH* levels of the same sample. The relative quantity of target gene expression to housekeeping genes was measured using the comparative Ct method (Supplementary Table 1).

Western blot analysis

Based on the qRT-PCR results, we selected TJ proteins for western blot analysis. Claudin-1, -2, occludin, and ZO-1 were selected for analysis as they are likely to be related to disease severity and age based on PCR analysis. Total protein was extracted from sigmoid colon tissues using RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) and PMSF (Sigma Aldrich, St. Louis, MO, USA). Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Primary antibodies used claudin-1 (rabbit, 1:250, Invitrogen, Carlsbad, CA, USA, 51–9,000), claudin-2 (rabbit, 1:250, Invitrogen, 51–6,100), occludin (rabbit, 1:250, Invitrogen, 40–4,700), ZO-1 (rabbit, 1:500, Invitrogen, 40–2,200), and β-actin (rabbit, 1:200, Cell Signaling Technology, 4,970). Rabbit-HRP (1:1,000, 7074S, Cell Signaling Technology) was used as the secondary antibody (Supplementary Table 2). We performed densitometric quantification using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and removed irrelevant or blank lanes from blot images to present our data in a streamlined way.

Immunohistochemical analysis

Based on the qRT-PCR and western blot results, we selected ZO-1 as the TJ protein for IHC analysis. Two professionally trained pathologists performed the IHC analysis. The immunohistochemical experiment was commissioned by SuperBioChips (SuperBioChips Laboratories, Seoul, Korea). The primary antibody used was ZO-1 (rabbit, 1:20, ATLAS, Stockholm, Sweden). Samples were analyzed using the BX43F microscope (Olympus, Tokyo, Japan). Expression percentage was assessed as a ratio between the number of cells stained with ZO-1 divided by the total number of cells counted in a section. ZO-1 expression was quantified using the ImageJ software. (IHC score, diffuse + strong = 3, focal + strong or diffuse + weak = 2; focal + weak = 1; negative = 0) (Supplementary Table 2).

Statistical methods

All statistical analyses were performed using Statistical Package for the Social Sciences (version 18.0; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, LLC, Boston, MA, USA). Data were expressed as means ± standard error of the mean. The IBS and control groups were analyzed by age, sex, and body mass index (BMI). Each classification criterion was explained in the questionnaire. One-way analysis of variance test and unpaired t-test were used to compare data between different groups. When the number of samples was greater than 10 and less than 30, the normality test was used to analyze the data, and the Mann–Whitney test and Kruskal–Wallis test were used to analyze the data with less than 10 samples. p values of < 0.05 were considered statistically significant.

Ethics statement

The study's protocol received approval from the Institutional Review Board (IRB) of Kyungpook National University Hospital (IRB 2019-06-020), and written informed consent was secured from all participants.

RESULTS

This study included 36 participants with IBS and 24 controls. The baseline characteristics of the participants are presented in Table 1. Twenty-three and thirteen patients were included in the IBS group's young- and old-aged groups, respectively. The control group consisted of 24 participants, of whom 12 and 12 were included in the young- and old-aged groups, respectively. The mild and moderate-to-severe IBS groups comprised 16 and 20 participants, respectively. The average symptom duration in patients with IBS was 34.3 weeks. Moreover, all groups had more than 32 weeks of symptom duration. The average and standard deviation of symptom duration in the IBS subgroups are described in Table 1.

qRT-PCR analysis of TJ mRNA

Compared with the control group, claudin-1, -2, -4, junctional adhesion molecule A (JAM-A), occludin, and ZO-1 mRNA levels all showed a tendency to decrease the IBS group. However, only claudin-1 and -2 showed a statistically significant decrease ($p < 0.05$) (Fig. 1A). In the subgroup analysis, the mild IBS group tended to have lower mRNA levels than the control group; however, only claudin-1 showed

Table 1. Baseline characteristics of healthy controls and patients with IBS

Variable	Control (n = 24)			IBS (n = 36)					p value			
	Young-aged control (n = 12)	Old-aged control (n = 12)	(total)	Severity		Aging		(total)	Control vs. IBS	Among IBS severity	Control (M vs. F)	IBS (M vs. F)
				IBS1 (n = 16)	IBS2 (n = 20)	Young-aged IBS (n = 23)	Old-aged IBS (n = 13)					
Age (yr)	47.8 ± 5.4	70.7 ± 7.9	59.3 ± 13.4	46.6 ± 15.0	50.2 ± 17.8	37.8 ± 8.9	67.7 ± 5.6	48.6 ± 16.5	0.011	0.526	0.940	0.073
Sex (M/F)	8/4	6/6	14/10	5/11	12/8	13/10	4/9	17/19	0.399	0.086	NA	NA
BMI (kg/m²)	23.5 ± 1.5	23.2 ± 2.5	23.4 ± 2.0	22.3 ± 3.5	22.2 ± 2.8	23.1 ± 2.8	20.9 ± 2.9	22.3 ± 3.0	0.080	0.628	NA	NA
IBS symptom duration (wk)	NA			36.0 ± 22.6	33.7 ± 10.3	34.8 ± 14.3	32.5 ± 3.5	34.3 ± 12.2	NA	NA	NA	NA

Values are presented as mean ± standard error of the mean or number only.
IBS, irritable bowel syndrome; IBS1, mild IBS; IBS-SSS questionnaire < 176; IBS-2, moderate-to-severe IBS, IBS-SSS questionnaire ≥ 176; Young-aged IBS, aged 20–59 years; Old-aged IBS, age ≥ 65 years; M, male; F, female; BMI, body mass index; NA, not applicable.
$p < 0.05$ is considered statistically significant.

a statistically significantly decrease ($p < 0.05$), and no significant difference was noted in claudin-2, -4, JAM-A, occludin, and ZO-1 (Fig. 1B). Claudin-1, -2, and ZO-1 showed a tendency to decrease in the old-aged and IBS groups. However, claudin-4, JAM-A, and occludin did not show any tendency or statistical significance. Claudins-1 and -2 were statistically significantly reduced in the old IBS group compared to the young IBS group ($p < 0.05$) (Fig. 1C). In the subgroup analysis by sex, the decrease in Claudin-1, Claudin-2, and ZO-1 TJ mRNA levels in IBS patients was found to have a greater effect in women than in men, although this difference was not statistically significant (Supplementary Fig. 2). In the analysis by BMI, TJ mRNA levels tended to increase in cases of obesity, but this trend also did not reach statistical significance (Supplementary Fig. 3).

Western blot analysis of TJ proteins

Claudin-1, -2, occludin, and ZO-1 protein levels were measured by western blot. Compared with the control group, claudin-1, -2, occludin, and ZO-1 protein levels all showed a tendency to decrease in the IBS group. Furthermore, claudin-1 ($p = 0.040$) and ZO-1 ($p < 0.0001$) protein levels showed a statistically significant decrease in the IBS group (Fig. 2A). In the subgroup analysis, claudin-1 ($p = 0.304$), -2 ($p = 0.832$), and occludin ($p = 0.690$) protein levels showed no significant difference in IBS severity; however, only ZO-1 showed a statistically significant decreased level in both the mild ($p = 0.031$) and moderate-to-severe IBS groups ($p = 0.006$) (Fig. 2B). Regarding claudin-1, -2, occludin, and ZO-1 protein levels, a tendency to decrease was observed in the old-aged groups compared with those in the young-aged group. However, claudin-1 ($p = 0.452$), -2 ($p = 0.291$),
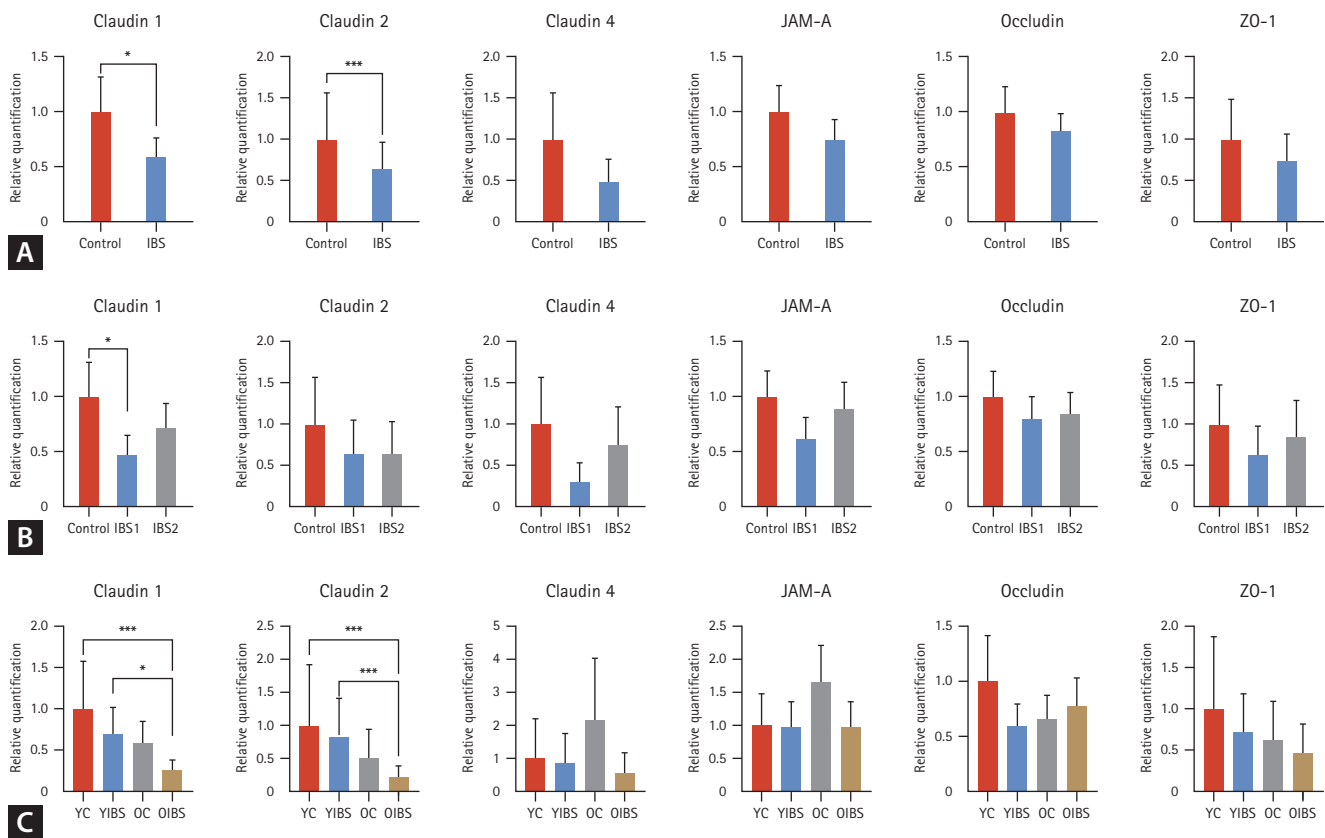

Figure 1. Claudin-1, -2, -4, JAM-A, occludin, and ZO-1 mRNA expression levels using reverse transcription polymerase chain reaction in the colonic mucosa of the controls and patients with IBS according to IBS severity and aging. (A) mRNA expression levels with or without IBS. (B) mRNA expression levels according to IBS severity. (C) mRNA expression levels according to IBS severity and aging. JAM-A, junctional adhesion molecule; ZO-1, zonula occludens-1; IBS, irritable bowel syndrome; IBS-1 (mild), IBS-SSS questionnaire < 176 (n = 14); IBS-2 (moderate-to-severe), IBS-SSS questionnaire ≥ 176 (n = 20); YC, young (age 20–59 yr) control (n = 12); YIBS, young (age 20–59 yr) patient with irritable bowel syndrome (n = 21); OC, old (age ≥ 65 yr) control (n = 12); OIBS, old (age ≥ 65 yr) patient with irritable bowel syndrome (n = 13). *$p < 0.05$; ***$p < 0.001$.

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and occludin (p = 0.883) showed no statistical significance. Compared with the young- and old-aged control group, the old-aged IBS group showed a significantly decreased ZO-1 level (p = 0.047) (Fig. 2C).

IHC analysis of ZO-1

In the IHC analysis, patients with IBS showed a significantly lower ZO-1 expression than controls (p < 0.001) (Fig. 3A). In the subgroup analysis, both mild and moderate-to-severe IBS groups tended to have lower ZO-1 expression levels than the control group. However, the mild IBS group did not show statistical significance (p = 0.229), and the moderate-to-severe IBS group had a significantly lower ZO-1 expression level than the control group (p < 0.001) (Fig. 3B). The young-aged group tended to have a higher ZO-1 expression level. Older age and moderate to severe symptoms tended to be associated with lower levels of ZO-1 expression. In the subtype analysis according to age, no dif-

ference was observed between the young- versus old-aged control groups (p = 0.506), and the young- versus old-aged IBS groups (p = 0.515). The old-aged IBS groups (p < 0.05) showed a statistically significantly lower ZO-1 expression than the young-aged control group (Fig. 3C).

DISCUSSION

Our study elucidates the complex interplay between TJ protein expression, aging, and disease severity in patients with IBS-D. The differential expression of TJ mRNA or proteins, notably claudin-1, -2, occludin, and ZO-1, underscores the multifaceted nature of IBS-D pathophysiology, particularly in the context of intestinal barrier function.

The statistically significant decrease in claudin-1 and -2 mRNA levels in the IBS group compared to controls (p < 0.05) highlights the potential role of these gene expres-
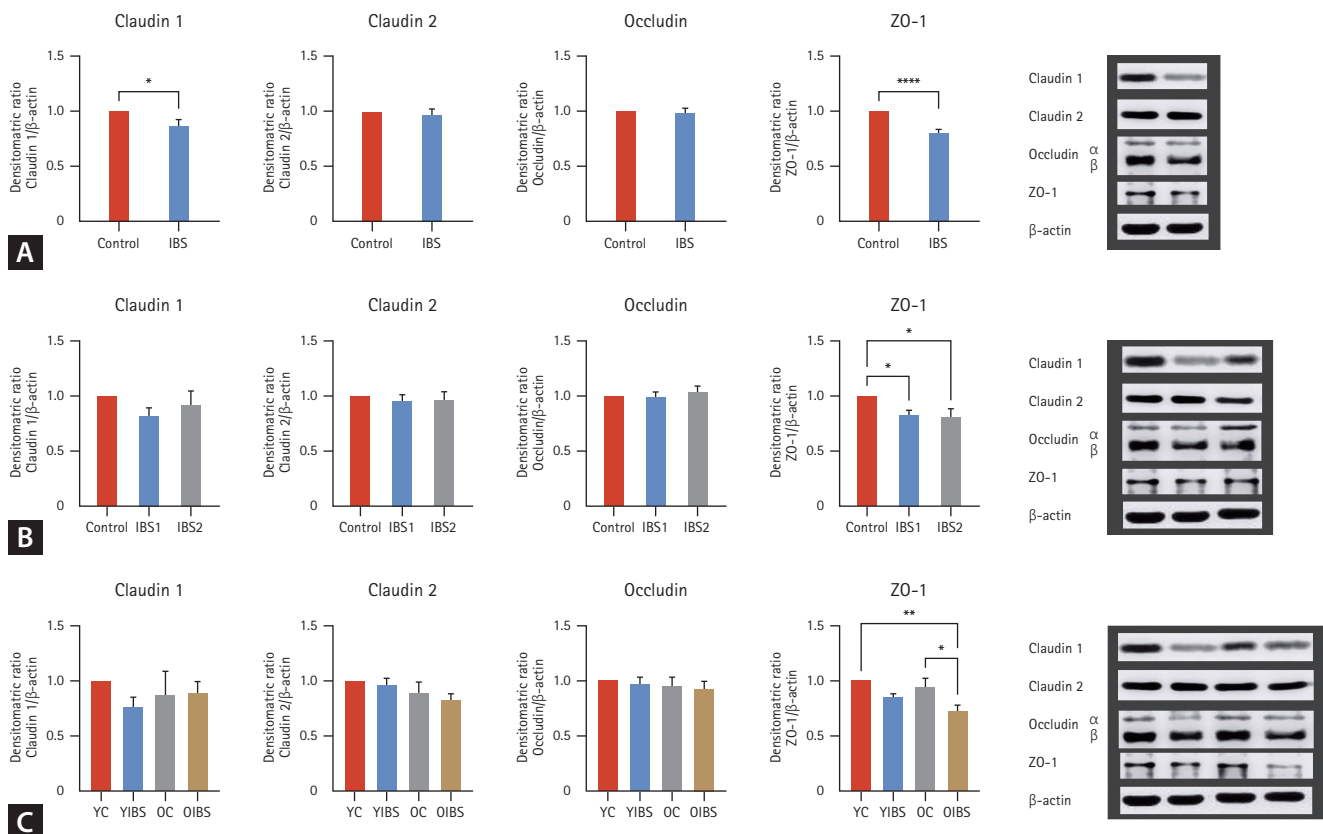

Figure 2. Claudin-1, -2, occludin, and ZO-1 in western blot analysis. (A) mRNA expression levels with or without IBS. (B) mRNA expression levels according to IBS severity. (C) mRNA expression levels according to IBS and aging. ZO-1, zonula occludens-1; IBS, irritable bowel syndrome; IBS-1 (mild), IBS-SSS questionnaire < 176 (n = 14); IBS-2 (moderate-to-severe), IBS-SSS questionnaire ≥ 176 (n = 20); YC, young (age 20–59 yr) control (n = 12); YIBS, young (age 20–59 yr) patient with irritable bowel syndrome (n = 21); OC, old (age ≥ 65 yr) control (n = 12); OIBS, old (age ≥ 65 yr) patient with irritable bowel syndrome (n = 13). *p < 0.05; **p < 0.005; ****p < 0.0001.

sions in contributing to the compromised intestinal barrier integrity observed in IBS-D. Claudin-1 and -2 are crucial for maintaining the selectivity and permeability of the TJ barrier, and their decreased expression may facilitate increased intestinal permeability, a hallmark feature observed in many IBS-D patients [22,23]. This phenomenon could exacerbate the translocation of luminal contents, thereby triggering inflammatory responses and symptomatology associated with IBS-D [20]. Notably, the specificity of ZO-1's significant decrease across different analyses (western blot and IHC) further supports its critical role in TJ integrity and the pathogenesis of IBS-D. The reduction in ZO-1 protein, particularly pronounced in the old-aged IBS group and the moderate-to-severe IBS subgroup, aligns with existing theories suggesting a degradation of intestinal barrier function with aging and increased disease severity.

The paracellular permeability of the intestinal barrier is regulated by a complex protein system that constitutes TJs. Transcellular or paracellular pathways can be described in three ways. The first is the pore pathway, which is a high-capacity size- and charge-selection pathway regulated by the claudin family [24]. The second is the leak pathway, which is a nonselective low-dose pathway primarily regulated by ZO-1, occludin, and myosin light chain kinase [25,26]. Finally, unrestricted pathways are opened owing to the loss of TJ complexes, usually as a result of cell death, apoptosis, or mucosal damage. This pathway can allow the passage of large macromolecules and even microorganisms across the epithelium [12]. A decreased membrane protein expression triggers an inflammatory response in the intestinal wall, thereby leading to changes in intestinal sensitivity and motility with intestinal barrier permeability disruption
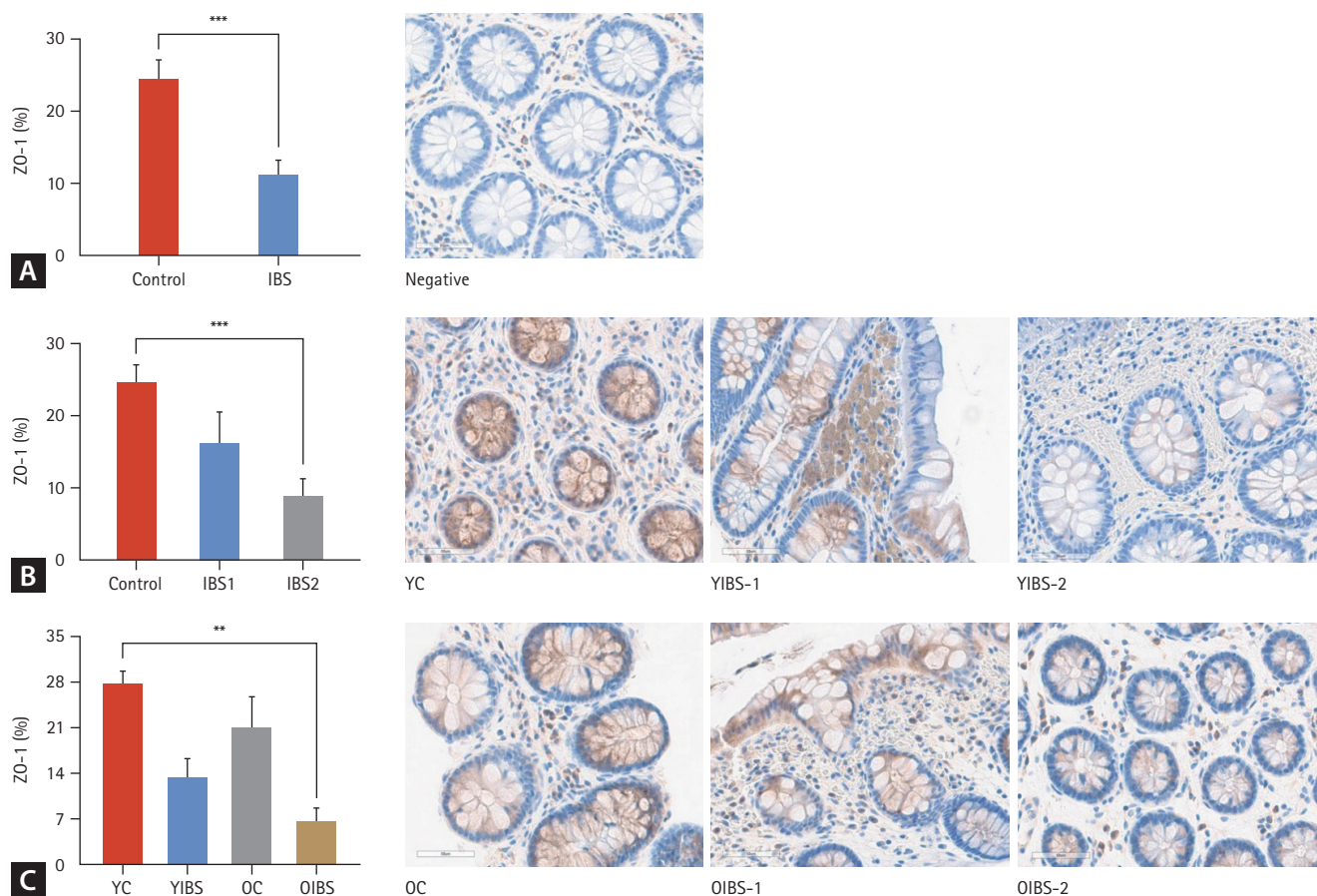

Figure 3. ZO-1 in the immunohistochemical analysis (×40). (A) mRNA expression levels with or without IBS. (B) mRNA expression levels according to IBS severity. (C) mRNA expression levels according to IBS and aging. ZO-1, zonula occludens-1; IBS, irritable bowel syndrome; IBS-1 (mild), IBS-SSS questionnaire < 176 (n = 14); IBS-2 (moderate-to-severe), IBS-SSS questionnaire ≥ 176 (n = 20); YC, young (age 20–59 yr) control (n = 12); YIBS, young (age 20–59 yr) patient with irritable bowel syndrome (n = 21); OC, old (age ≥ 65 yr) control (n = 12); OIBS, old (age ≥ 65 yr) patient with irritable bowel syndrome (n = 13). **$p < 0.005$; ***$p < 0.001$.

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[10,22,23]. Coëffier et al. [27] have linked epithelial barrier disruption and increased permeability to both the downregulation of the TJ scaffolding protein ZO-1 and proteasome-mediated occludin degradation in the colonic mucosa of patients with IBS. Several studies have shown increased intestinal barrier permeability and decreased TJ protein expression in patients with IBS-D. ZO-1 and occludin protein expressions were significantly lower in the colonic mucosa of patients with IBS compared with those of the controls. Zeng et al. [28] have reported that the mRNA expressions of ZO-1 and occludin were significantly reduced in IBS-D. Camilleri et al. [29] have reported that the RNA sequencing of claudin-1 was reduced in IBS-D. Zhen et al. [30] have stated that patients with IBS-D had significantly reduced occludin production compared with the controls. Moreover, Lee et al. [10] reported that ZO-1 gene expression was decreased in females, and no difference in claudin-1 and occluding was observed. Western blot showed a decrease in ZO-1 [10]. However, in some studies, no differences were noted. Ishimoto et al. [31] and Camilleri et al. [32] have shown that the mRNA levels of ZO-1, occludin, and claudin-1 were similar between each IBS subtype and controls. The expression of TJ proteins showed different results in each study, and no clear data were observed on the subgroup (Table 2). In our study, western blot analysis revealed that patients with IBS exhibited significantly decreased levels of claudin-1 and ZO-1 proteins ($p < 0.05$). Subgroup analysis showed that ZO-1 protein levels were significantly lower in both mild and moderate-to-severe IBS groups ($p < 0.05$). In the older IBS group, ZO-1 protein levels were significantly

lower than those in the younger IBS group ($p < 0.05$). The IHC analysis showed that patients with IBS had significantly lower ZO-1 expression compared to those in the control group ($p < 0.001$). The moderate-to-severe IBS group had significantly lower ZO-1 expression levels than the control group ($p < 0.001$). The older IBS groups ($p = 0.039$) showed statistically significant lower ZO-1 expression compared to the control group. The ZO-1 expression level decreased with age. Further, it was lower in individuals with more severe IBS symptoms. In a rat model, age-induced mRNA expression downregulation and decreased ZO-1 and occludin protein expressions were observed in the ileum [30]. Previous studies on the expression of TJ proteins related to age and severity lacked sufficient data in humans. Our study may provide evidence for changes in TJ proteins according to aging and disease severity. Our findings reveal a nuanced relationship between aging, disease severity, and TJ protein expression, especially ZO-1 protein. While the decrease in TJ proteins was more pronounced in the old-aged IBS group, it's particularly interesting that age itself did not significantly affect ZO-1 expression levels when comparing young and old control groups. This suggests that the observed alterations in TJ protein expression are more directly associated with the presence and severity of IBS-D rather than aging alone. The significant decrease in ZO-1 expression in the old-aged IBS group compared to young-aged controls, and its statistical significance across different degrees of IBS severity, underscores the potential exacerbating effect of aging on the disease's impact on intestinal barrier function. These findings align with the hypothesis that IBS-D's pathophysiology may

Table 2. Changes in colonic mucosal tight junctions in patients with IBS

Study (year)	IBS type (study population)	Tight junction type	Method (PCR, WB, or IHC)	Main results
Zeng et al. (2008) [28]	IBS-D (n = 30)	Occludin	PCR	↓Occludin
Camilleri et al. (2015) [29]	IBS-D (n = 47)	CLDN-1, ZO-1, Occludin	RNA sequencing	↓CLDN-1, = ZO-1, Occludin
Zhen et al. (2015) [30]	IBS-D (n = 42)	Occludin	WB, IHC	WB: ↓occludin IHC: occludin (faint and discontinuous)
Lee et al. (2015) [10]	IBS-D (n = 21)	ZO-1, CLDN-1, Occludin	PCR, WB	PCR: ↓ZO-1 in females, = CLDN-1, Occludin WB: ↓ZO-1
Camilleri et al. (2014) [32]	IBS-D (n = 9)	Occludin, ZO-1	PCR	= CLDN-1, Occludin, ZO-1
Ishimoto et al. (2017) [31]	IBS-D (n = 17)	CLDN-1, CLDN-2, CLDN-7, JAM-1, Occludin, ZO-1	PCR	PCR: = CLDN-1, CLDN-2, CLDN-7, JAM-1, Occludin, ZO-1

IBS, irritable bowel syndrome; PCR, polymerase chain reaction; WB, western blot; IHC, immunohistochemistry; IBS-D, IBS with predominant diarrhea; CLDN, claudin; ZO-1, zonula occludens-1; RNA, ribonucleic acid; JAM, junctional adhesion molecule.

be partly due to a compromised barrier function, which is further impacted by aging. In this study, the expression levels of mRNA and protein showed a significant decrease in the control group and IBS-D group. However, there was a tendency for these levels to decrease even more in the mild IBS group, contrary to the disease severity. Although the expression levels of mRNA and protein did not correlate with disease severity, particularly in IBS, disease severity is determined based on subjective symptoms felt by the patient using the IBS-SSS questionnaire. Since the method of measuring the severity of these symptoms is not based on pathology and biology, the decrease in TJ mRNA and protein may not align with the symptoms. These changes in TJ proteins may be related to symptoms due to alterations in mucosal permeability and the resulting increase in immune cells and inflammatory mediators caused by a compromised epithelial barrier [33]. Further studies are needed to confirm the association with symptoms.

This study had several limitations. First, the relatively small sample size and the different number of samples per group limited the ability to conduct appropriate analyses of generalizations and subtypes. Second, biopsy samples were taken from the sigmoid colon, and TJ protein expression may vary depending on the location in the GI tract, making identification of the entire intestine difficult. In addition, most previous studies have been conducted in the sigmoid colon, and there is a lack of research in other regions of GI tract. The expression of TJ proteins is not consistent across different sites [22]. Third, this study focused on patients with IBS-D; therefore, results may vary depending on different subtypes. Fourth, IBS is a multifactorial disorder wherein dietary habits, lifestyle, and other potential contributing factors may be present, and these factors may not be considered or controlled. Finally, to evaluate TJ proteins, we utilized various techniques (qRT-PCR, western blot, and IHC). Variability in methodologies and potential technical limitations may introduce bias or affect the consistency of results. Additionally, there may be limitations in terms of specific populations, cross-sectional studies, and clinical relevance.

Our study demonstrates that IBS-D is associated with significant alterations in the expression of specific TJ mRNA and proteins, notably claudin-1, -2, and ZO-1. These changes are further influenced by the severity of the disease and the age of the patients, suggesting that interventions aimed at restoring TJ protein expression could be beneficial, especially in older patients or those with moderate-to-severe IBS-D.

Future research should focus on elucidating the mechanisms underlying these alterations and exploring therapeutic strategies to enhance intestinal barrier function in IBS-D.

KEY MESSAGE

1. Age and disease severity are associated with alterations in tight junction proteins, which may contribute to increased intestinal permeability in IBS patients.
2. Tight junction protein ZO-1 expression is significantly decreased in patients with IBS, especially in older patients and those with more severe symptoms.
3. This study suggests that ZO-1 is the prominent tight junction protein involved in the pathogenesis of IBS, with potential implications for understanding the disease mechanisms in older populations.

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Supplementary Figure 1. The irritable bowel syndrome patient severity scoring system questionnaire (English and Korean version). Adapted from Francis et al. Aliment Pharmacol Ther 1997;11:395-402 [19].

IBS QUESTIONNAIRE

Name: _____ G.P. Name: _____

Address: _____ Address: _____

_____ _____

_____ _____

Telephone: _____ Telephone: _____

Date of birth: _____

Marital status: Single / Married / Divorced / Widowed / Co-Habit

Occupation: _____ **Sex:** M   F

Ethnic background: Caucasion (white) / Afro-Carribean / Asian / Oriental

Fathers Occupation (even if retired): _____

INSTRUCTIONS

This form is designed to enable us to record and monitor the severity of your IBS. It is to be expected that your symptoms might vary over time, so please try and answer the questions based on how you <u>currently</u> feel (ie over the last 10 days or so). All information will be kept in **strict** confidence.
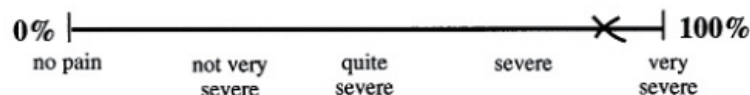
1. For questions where a number of different responses are a possibility please circle the response appropriate to you.

2. Some questions will require you to write in an appropriate response.

3. Some questions require you to put a cross on a line which enables us to judge the severity of a particular problem.

For example:

How severe was your pain?

Please place your cross (✗) anywhere on the line between 0-100%
in order to indicate as accurately as possible the severity of your symptom.
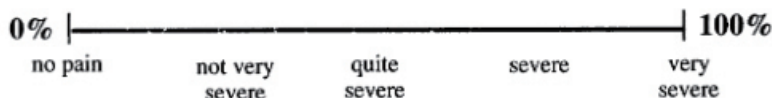
This example shows a severity of approximately 90%.

0% ├────────────────────────────✗─┤ 100%

  no pain   not very   quite   severe   very
               severe   severe   severe

1

PART 1 : SEVERITY SCORE

1. **a)** *Do you currently suffer from abdominal (tummy) pain?*   **YES**   **NO**

For office use only

Circle appropriate box

SCORE

   b) *If yes, how severe is your abdominal (tummy) pain?*

0% |————————————————————————| 100%

no pain   not very   quite   severe   very
                 severe   severe   severe

   c) *Please enter the number of days that you get the pain in every 10 days.*
   For example if you enter 4 it means that you get pain 4 out of 10 days. If you get pain every day enter 10
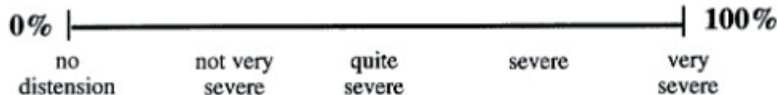
   Number of days with pain [   ]   x10

2. **a)** *Do you currently suffer from abdominal distension**   **YES**   **NO**
   (bloating, swollen or tight tummy)
   (*women, please ignore distension related to your periods)

Circle appropriate box

   b) *If yes, how severe is your abdominal distension/tightness*

0% |————————————————————————| 100%

no   not very   quite   severe   very
distension   severe   severe   severe

3. *How satisfied are you with your bowel habit?*

0% |————————————————————————| 100%

very   quite   unhappy   very
happy   happy   unhappy
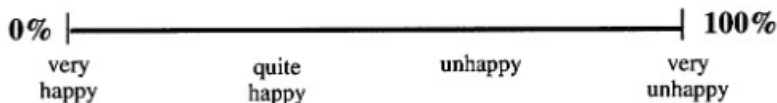
4. *Please indicate with a cross on the line below how much your Irritable Bowel Syndrome is affecting or interfering with your life in general*

0% |————————————————————————| 100%

not at all   not much   quite   completely
                                      a lot

IBS SEVERITY SCORE: [   ]

2

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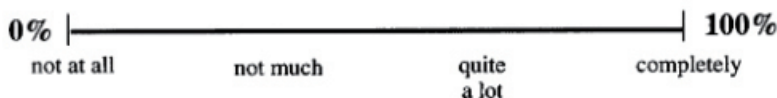
PART 2 : OTHER IBS DATA

BOWEL HABIT

5. a) *What is the most number of times you open your bowels per day/week/month?*

Number of times [   ] per day / week / month *(Circle appropriate)*

Note: For some people the answer to part a and b could be the same

b) *What is the least number of times you open your bowels per day/week/month?*

Number of times [   ] per day / week / month *(Circle appropriate)*

6. In the following questions you may circle more than one answer:

Are your motions ever:

a) *normal*   often / occasionally / never   *(Circle appropriate)*

b) *hard*   often / occasionally / never   *(Circle appropriate)*

c) *very thin (like string)*   often / occasionally / never   *(Circle appropriate)*

d) *in small pieces (like rabbit pellets)* often / occasionally / never   *(Circle appropriate)*

e) *mushy (like porridge)*   often / occasionally / never   *(Circle appropriate)*

f) *watery*   often / occasionally / never   *(Circle appropriate)*

7. In the following questions you may circle more than one answer:

Do you ever: *Circle appropriate box*

a) *pass mucus (or slime or jelly) with your motions*   YES   NO

b) *pass blood with your motions*   YES   NO

c) *have to hurry/rush to the toilet to open your bowels*   YES   NO

d) *strain to open your bowels*   YES   NO
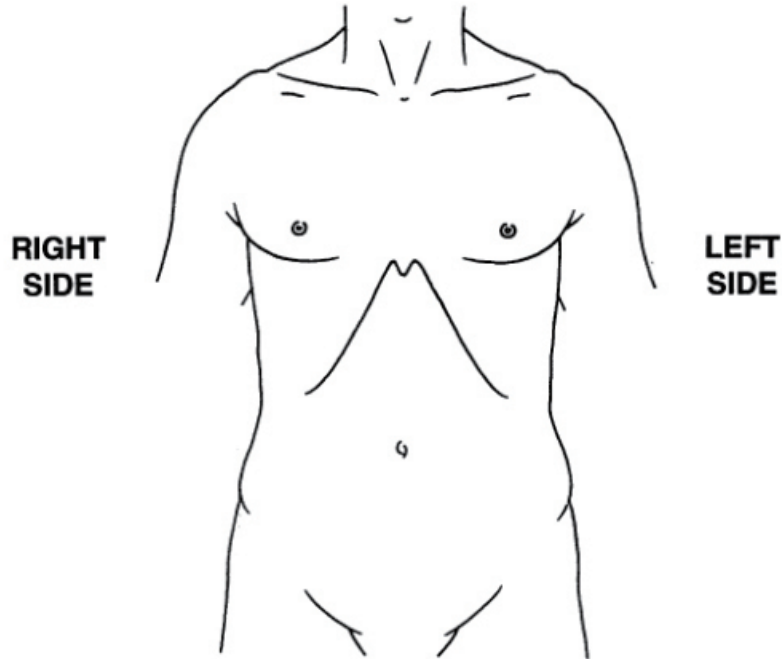
e) *feel you haven't emptied your bowel completely*   YES   NO
    after you have passed a motion

3

PART 2 : Continued

SITE OF PAIN

Please mark with a cross (x) on the diagram below where you get your pain
(use more than one x if necessary)

RIGHT SIDE **LEFT SIDE**

8. Do you ever:

a) notice your stools are more frequent or loose
    when you get pain

YES   NO
Circle appropriate box

b) notice whether the pain is frequently eased
    by opening your bowels

YES   NO
Circle appropriate box

9. In the last year on approximately how many weeks were you:

i) absent from work due to IBS
    (enter 52 if you have given up completely work because of IBS)   _ _ _ _ _ _ _

ii) at work suffering from IBS   _ _ _ _ _ _ _

4

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과민성대장증후군 설문지

이름 : _____

주소 : _____

전화번호 : _____

생년월일 : _____

결혼상태 : 미혼/기혼/이혼/동거중 : _____

직업 : _____   성별 : _____

시작하기 앞서...

이 양식은 과민성대장증후군의 증상의 심한정도를 기록하고 관찰할 수 있도록 고안되었습니다. 이 병의 증상은 시간이 지남에 따라 달라질 수 있으므로 현재 느끼는 지에 따라 질문에 답하세요. 모든 정보는 엄격한 기밀로 유지됩니다. .

1. 여러가지 다른 답변이 가능한 질문에 대해서는 해당 답변에 동그라미를 표시하십시오.

2. 어떤 질문은 적절한 답변을 생각해서 답변해야 합니다.

3. 어떤 질문들은 특정한 문제들의 심한 정도에 대해서 판단 후 선에 X표를 표시해야 합니다.

예를 들어,

[얼마나 통증이 있습니까?] 라는 질문에는

0~100% 사이에 당신의 증상을 가장 잘 표현할 수 있는 정도를 선위에 X 표를 표시하면 됩니다.

아래 예시는 증상의 정도가 90%정도에 해당할 때의 답변입니다.

0% ├─────────────────────✗─┤ **100%**
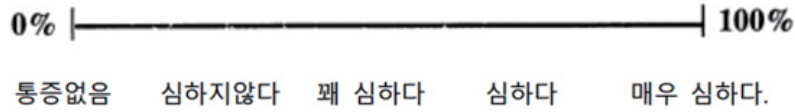
통증없음   심하지않다   꽤 심하다   심하다   매우 심하다.

첫번째 : 중증도 점수

네	아니오

해당하는 답에 동그라미를 그리세요

1. 가) 당신은 현재 복통이 있습니까?

   나) 만약 그렇다면 복통이 얼마나 심한가요?

   0% |————————————————————| 100%

   통증없음   심하지않다   꽤 심하다   심하다   매우 심하다.

   다) 매 10일마다 통증이 있는 날 수를 적으세요

   예를 들어 4를 적으셨다면 10일중에 4일을 통증을 느끼셨다는 뜻입니다. 10일 내내 통증을 느끼셨다면 10을 적으세요

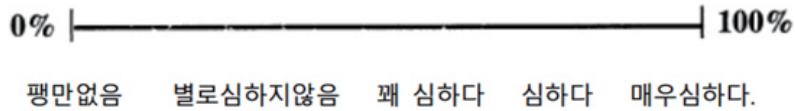   통증을 느낀 날 수   [   ]   x10
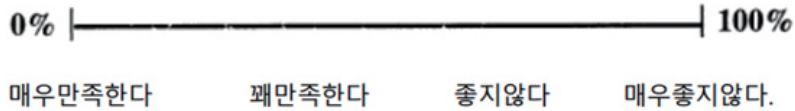
2. 가) 현재 복부 팽만으로 고통받고 있습니까?*

네	아니오

   해당하는 답에 동그라미를 그리세요

   (배가 부풀어 오른 느낌, 또는 꽉찬 느낌)

   (* 여성의 경우 생리기간중 생긴 팽만감은 무시하시기 바랍니다.)

   나) 만약 그렇다면 복부 팽만의 심한정도는 어느정도 인가요?

   0% |————————————————————| 100%

   팽만없음   별로심하지않음   꽤 심하다   심하다   매우심하다.

3. 당신의 배변에 대해서 얼마나 만족하고 계십니까?

   0% |————————————————————| 100%

   매우만족한다   꽤만족한다   좋지않다   매우좋지않다.

4. 과민성대장증후군이 얼마나 당신의 삶을 방해하거나 영향을 주는지를 선위에 X 표로 표시해주세요.

   0% |————————————————————| 100%

   전혀   별로   꽤 많이   전적으로
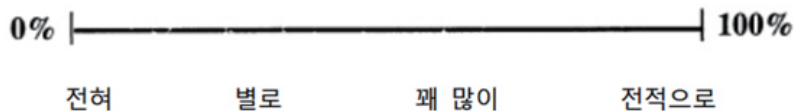
<center>두번째 : 배변습관</center>

5. **가) 하루/일주/한달 사이에 얼마나 변을 보나요?**

   하루/일주/한달에 [   ] 번 (해당하는 곳에 동그라미를 치세요)

(가)와 나) 답변이 같을 수도 있습니다.)

   나) 하루/일주/한달 사이에 얼마나 변을 보나요?

   하루/일주/한달에 [   ] 번 (해당하는 곳에 동그라미를 치세요)

6. **다음의 질문에는 한가지 이상의 답에 동그라미를 칠수 있습니다.**

    당신의 변은

가) **정상적이다**   가끔씩 그렇다./항상 그렇다/그렇지 않다. (동그라미 치세요)

나) **딱딱하다**   가끔씩 그렇다./항상 그렇다/그렇지 않다. (동그라미 치세요)

다) **매우 얇다 (줄처럼)**   가끔씩 그렇다./항상 그렇다/그렇지 않다. (동그라미 치세요)

라) **토끼똥 처럼 매우 작다.**   가끔씩 그렇다./항상 그렇다/그렇지 않다. (동그라미 치세요)

마) **진흙처럼 나온다.**   가끔씩 그렇다./항상 그렇다/그렇지 않다. (동그라미 치세요)

바) **물처럼 나온다.**   가끔씩 그렇다./항상 그렇다/그렇지 않다. (동그라미 치세요)

7. **해당하는 답에 동그라미를 치세요**

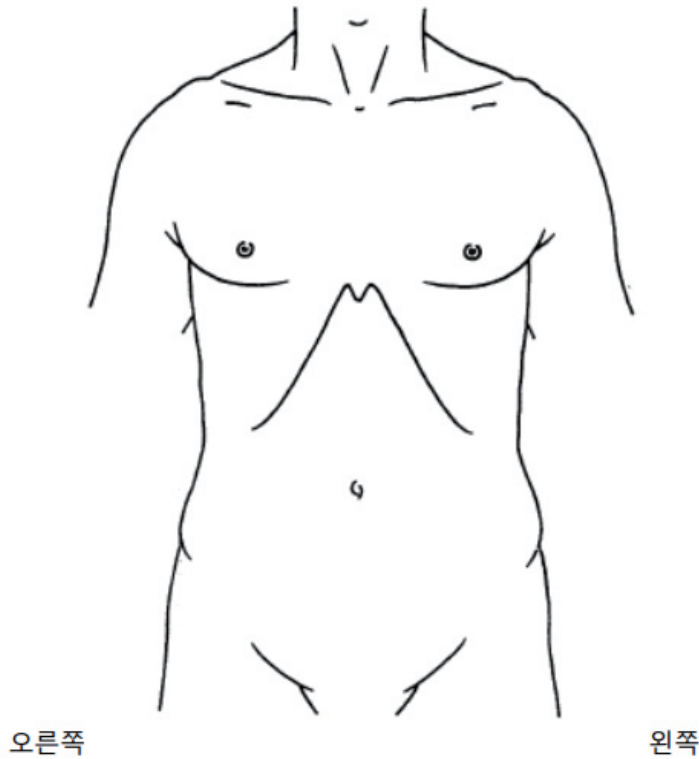   당신의 변은   해당하는 답에 동그라미를 그리세요

가) **점액처럼(또는 콧물이나 젤리) 나온다**   네   아니오

나) **피가 같이 묻어나온다.**   네   아니오

다) **변을 보기위해 급하게 가야한다.**   네   아니오

라) **변을 보기위해 긴장을 한다.**   네   아니오

마) **변을 보고 나서도 변을 다 본 것 같지 않다.** 네   아니오

세번째 : 통증의 부위

다음 그림에 통증이 있는 부위를 x 표로 표시하세요

(필요하면 하나 이상의 x 표를 표시해도 됩니다.)

오른쪽   왼쪽

해당하는 답에 동그라미를 그리세요

8. 가) 통증이 있을 때 변이 자주 나오거나 묽게 나오나요? | 네 | | 아니오 |

    나) 변을 보고 나서 통증이 자주 호전되나요? | 네 | | 아니오 |

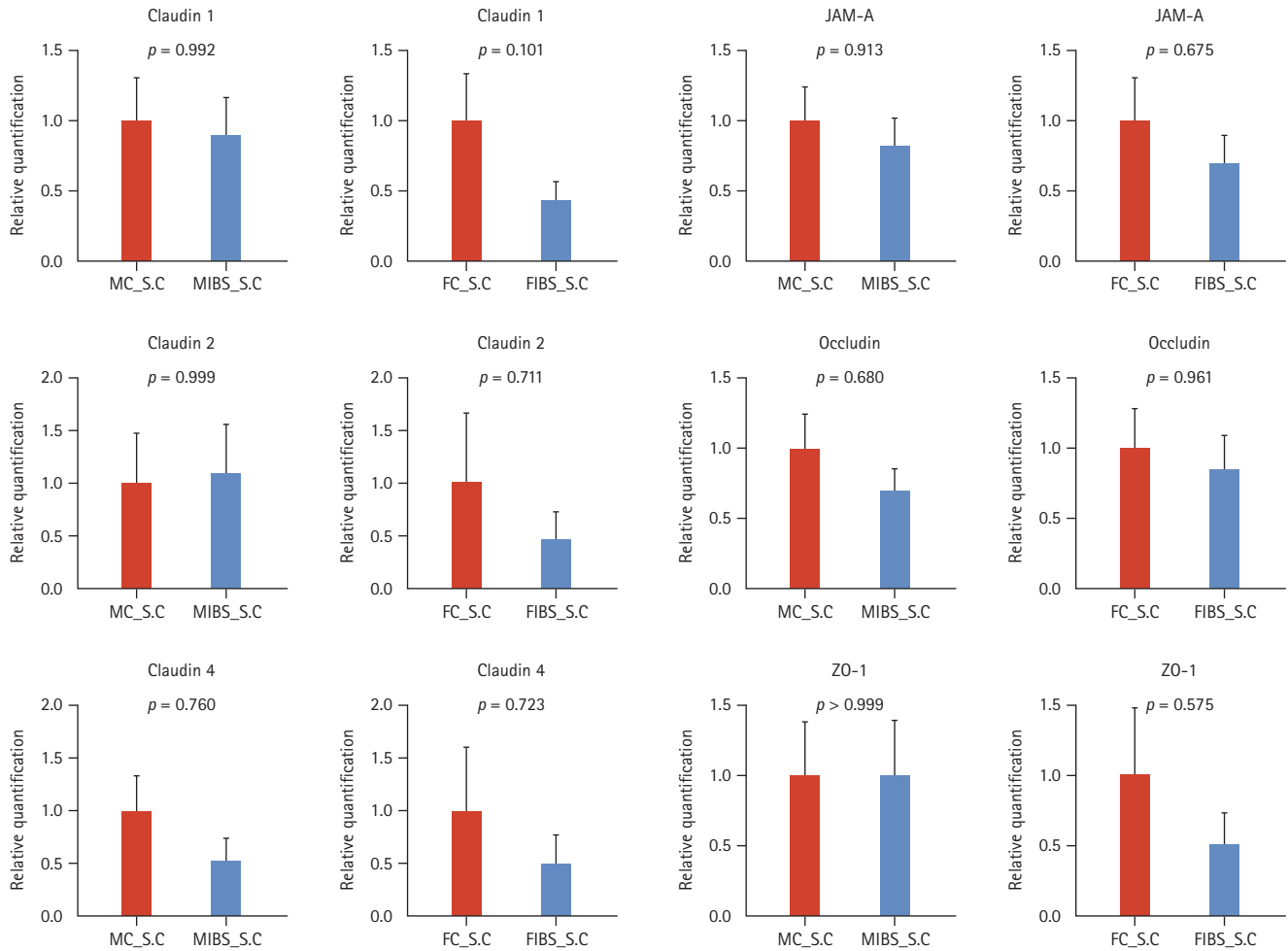9. i) 지난 한해동안 몇 주 동안 과민성대장증후군으로 인하여 일을 하지 못하였습니까?

    (만약 과민성대장증후군 때문에 일을 일년간 모두 쉬었다면 52 를 쓰세요)

                                              _____ 주
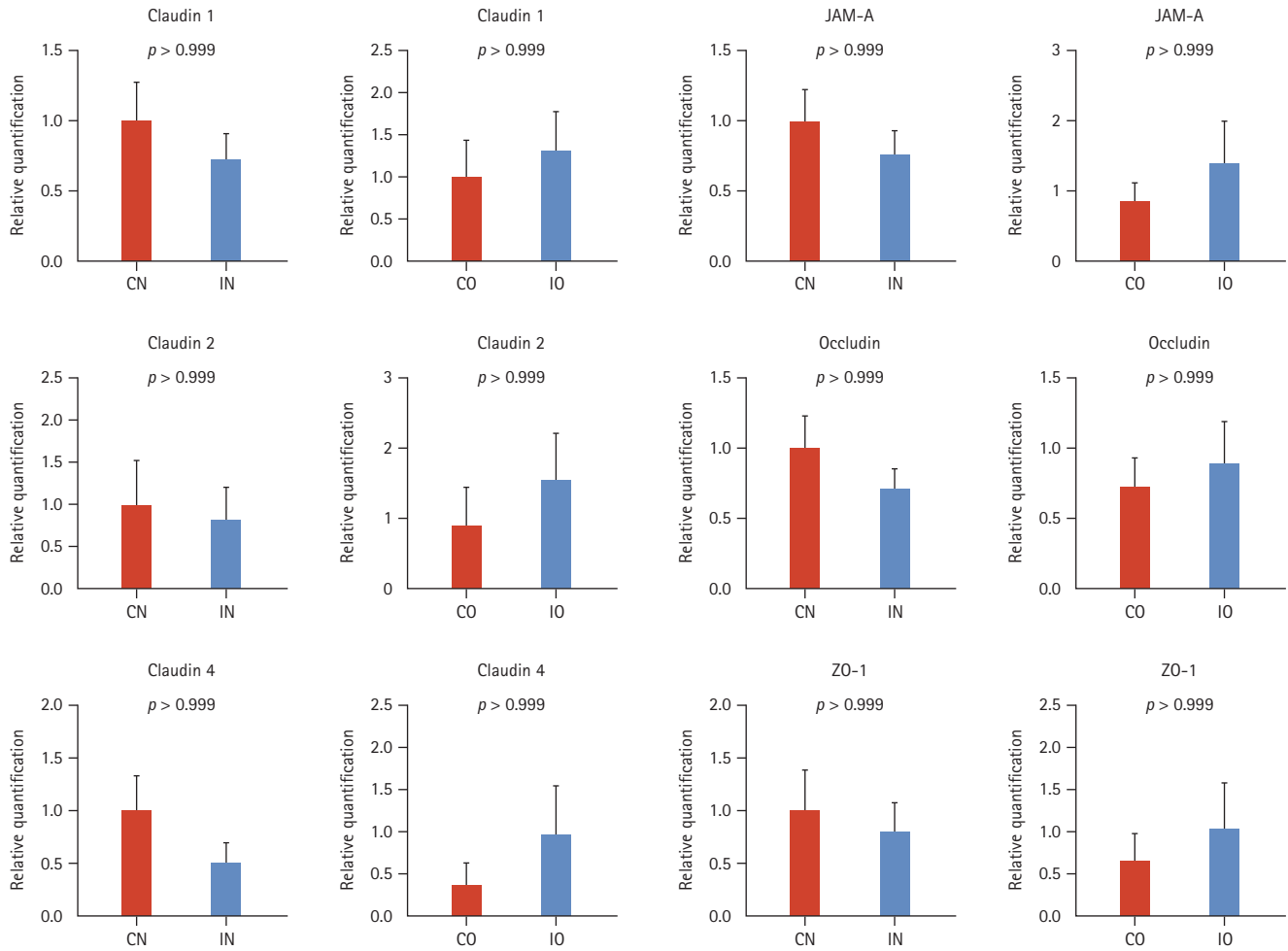
  ii) 지난 한해동안 몇 주 동안 과민성대장증후군으로 일을 하는데 불편감을 겪으셨습니까?

                                              _____ 주

Supplementary Figure 2. Claudin-1, -2, -4, JAM-A, occludin, and ZO-1 mRNA expression levels using reverse transcription polymerase chain reaction in the colonic mucosa of the controls and patients with IBS according to sex. JAM-A, junctional adhesion molecule; ZO-1, zonula occludens-1; IBS, irritable bowel syndrome; MC, male control (n = 14); S.C, sigmoid colon; MIBS, male irritable bowel syndrome patient (n = 15); FC, female control (n = 10); FIBS, female irritable bowel syndrome patient (n = 22).

Supplementary Figure 3. Claudin-1, -2, -4, JAM-A, occludin, and ZO-1 mRNA expression levels using reverse transcription polymerase chain reaction in the colonic mucosa of the controls and patients with IBS according to body mass index. JAM-A, junctional adhesion molecule; ZO-1, zonula occludens-1; IBS, irritable bowel syndrome; CN, control normal weight (n = 18); IN, IBS normal weight (n = 24); CO, control obesity (n = 6); IO, IBS obesity (n = 4).

Supplementary Table 1. Primers used for quantitative reverse transcription polymerase chain reaction

Name	Sequence	Tm
GAPDH F	GGCATCCTGGGCTACACTGA	60.6
GAPDH R	GGAGTGGGTGTCGCTGTTG	60
Claudin-1 F	GCGTTGCCAGTTCCTTTTACC	59.4
Claudin-1 R	TGCAAGCACAAAGTCCGATCAA	60.4
Claudin-2 F	ATTGTGACAGCAGTTGGCTT	57.3
Claudin-2 R	CTATAGATGTCACACTGGGTGATG	57.5
Claudin-4 F	GCCTATGGATGAACTGCGT	56.7
Claudin-4 R	CCACGATGATGCTGATGATGAC	58.6
Occludin F	GGAAGAGCAGGAAGGTCAAAGA	59
Occludin R	CGCCGCCAGTTGTGTAGTC	60.4
ZO-1 F	GAAGATCGCGTCCCCATGTAT	59
ZO-1 R	TGGGTAGGGCTGTTTGTCATC	59
JAMA F	GAGGCACGGAATGGGTATGG	59.6
JAMA R	CAGGGTTACAAGGACGGCTG	59.7

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; ZO-1, zonula occludens-1; JAMA, junctional adhesion molecule; F, forward primer sequence; R, reverse primer sequences; Tm, melting temperature.

Supplementary Table 2. Primary antibodies used for western blot and immunohistochemical analysis

	Company	Cat #	Host	Final dilution
Claudin-1	Invitrogen	51–9,000	Rabbit	1:250
Claudin-2	Invitrogen	51–6,100	Rabbit	1:250
Occludin	Invitrogen	40–4,700	Rabbit	1:250
ZO-1	Invitrogen	40–2,200	Rabbit	1:500
ZO-1	ATLAS	HPA001636	Rabbit	1:200
β-Actin	Cell Signaling	4970	Rabbit	1:10,000

ZO-1, zonula occludens-1; Cat#, Catalogue number.