Association between single nucleotide polymorphisms of the transient receptor potential vanilloid 1 (TRPV-1) gene and patients with irritable bowel syndrome in Korean populations

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Abstract

Background and study aims : **Transient receptor potential vanilloid type 1 (TRPV1) plays a crucial role in pain perception and its expression is up-regulated in patients with irritable bowel syndrome (IBS). The aim of this study was to investigate the potential association between Single nucleotide polymorphism (SNPs) of the TRPV-1 gene and patients with IBS.**

Patients and methods : **We chose to focus on three SNPs in the human TRPV1 coding region (rs222749, rs9894618 and rs222747) in 80 healthy controls and 103 IBS patients. We developed the high resolution melting (HRM) method to determine the genotyping of rs222747 and rs9894618 and the genotyping of rs222749 was also determined by direct sequencing method.**

Results : **The CG genotype of rs222747 was 58.8% in controls and 45.6% in the IBS group. The GG genotype of rs222747 was 15.0% in controls and 20.4% in the IBS group. The CT genotype of rs222749 was 31.3% in controls and 32.0% in the IBS group. The CC genotype of rs9894618 was 98.8% in controls and 100.0% in the IBS group. There was no significant difference in allele frequency of these three SNPs of the TRPV1 gene between controls and the IBS group. Also, no significant difference was observed between the IBS subtypes.**

Conclusions : **These results suggest that the SNPs of the TRPV1 gene may not be associated with IBS in Korean populations. Further studies with large cases are needed to validate the results of the present study.** (Acta gastroenterol. belg., **2012**, 75, **222-227**).

Key words : TRPV1, Single nucleotide polymorphism, Irritable bowel syndrome.

Introduction

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder characterized by recurrent abdominal pain, altered bowel habits that may be either diarrhea or constipation or an erratic bowel habit that has features of both, and relief of abdominal pain with defecation. IBS has been known to affect up to 20% of a given population. Severe IBS can reduce health-related quality of life of all age groups and is seen to be more common in women (1-3). IBS can be classified as either diarrhea predominant (IBS-D), constipation predominant (IBS-C), and alternate diarrhea and constipation (IBS-A) (4).

The pathophysiology of IBS remains unknown. Many new hypotheses have been proposed to explain IBS including alterations in intestinal motility and visceral hypersensitivity, alterations in brain-gut regulatory pathways, postinfectious or postinflammatory changes in digestive neuroimmune function, and alterations in intestinal microflora as possible mechanisms (5-8). Among them, mounting evidence suggests that visceral hypersensitivity plays an important role in the pathogenesis of IBS.

The transient receptor potential vanilloid type 1 (TRPV1) is a non-selective ligand-gated cation channel that may be activated by a wide variety of exogenous and endogenous physical and chemical stimuli, leading to painful burning sensation (9-11). TRPV1 is found mainly in the nociceptive neurons of the peripheral nervous system and is activated by capsaicin, noxious temperature, acidosis, and high concentration of endogenous cannabinoid (9-11). Previously, TRPV1 was shown to be involved in mechanosensation (12,13) and visceral hypersensitivity of the colon in animal experiments (14, 15). A recent study showed that increased TRPV1 expression in nerve fibers in recto-sigmoid biopsies of IBS patients correlate with the degree of abdominal pain (16-18). These accumulating data suggest that the modulation of TRPV1 is one of the important steps in the development of pain and may be a novel drug target for treatment of chronic pain and hyperalgesia in IBS.

Pain sensitivity varies significantly in the human population. This variation in the sensitivity to pain could be associated with polymorphism of genes that participate in pain perception such as TRPV1 (19). HapMap analysis reveals that there are at least six SNPs that affect structural domains of *TRPV1*. $TRPV1^{K2N}$ (rs9894618) and $TRPV1^{P91S}$ (rs222749) affect the intracellular amino terminus of *TRPV1*. $TRPV1^{I315M}$ (rs222747) is localized in the ankyrin repeat-containing domain which plays a role in mediating protein-protein interactions and homotetramerization of the channel. $TRPV1^{T469I}$ (rs224534) is predicted to be located in the extracellular loop between membrane-spanning helices 1 and 2 and $TRPV1^{I585V}$ (rs8065080) is predicted to reside within

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membrane spanning helix 5 (20). Until now, it remains unclear whether genetic variation of the TRPV-1 has functional roles in the pathogenesis of IBS. The aim of this study was to investigate the potential association between three SNPs of the TRPV-1 gene and patients with IBS in the Korean population.

Patients and Methods

Patients characteristics and samples

We enrolled 103 patients diagnosed with IBS, who visited the outpatient clinic of our department and 80 healthy controls, who visited the health promotion center for a routine checkup in Chonnam National University Hospital, Gwangju, Korea between March 2007 and February 2008. IBS was diagnosed according to the Rome III criteria. None of the patients had other gastrointestinal disease, severe co-morbidity or history of substance abuse within the previous two years. Controls did not have any gastrointestinal disease or other systemic conditions. This study was approved by the institutional review board of the Chonnam National University Hospital, Gwangju, Korea. All subjects were provided with written information on the project, and fully informed consent was obtained as well.

SNP search in database and Primers

The SNPs in the human TRPV1 gene were obtained from the NCBI Genebank database (http://www.ncbi.nlm.nih.gov/projects/SNP/). We chose to focus on three non-synonymous SNPs in the human TRPV1 coding region. Two SNPs in exon 1, rs9894618 and rs222749, were located in the NH2 terminus of the proteins. rs222747 affected exon5 and localized to the region of the ankyrin repeat domains, which are postulated to mediate protein-protein interactions. We developed the high resolution melting (HRM) method to determine the genotyping of the rs222747 and rs9894618, and the genotyping of the rs222749 was also determined by direct sequencing method. The primers for each genotyping were as follows : rs222747, 5'-ACGAAGTTTGTGACGAGCA-3'/5'-ATTCCCTTCTTGTTGGTGAG-3' ; rs9894618, 5'-CACAGAGGATCCAGCAAGG-3'/5'-GTTAGGGTCTCCATCCAGG-3' ; and rs222749, 5'-TTGGGAAGGGTGACTCGGA-3'/5'-TGAGGAAGGACGCTGGACC-3'.

DNA extraction

Genomic DNA was extracted from the diagnostic peripheral blood sample by QIAamp DNA blood minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Briefly, blood samples with lysis buffer were incubated at 56°C for 10 min and added 100% ethanol of equal volume of the sample. Sample mixture was transferred to QIAamp Mini spin column and centrifuged at 6000g for 1 min. Flow-through containing lysis buffer were discarded and DNA bound to QIAamp column membrane was washed in centrifugation with two different provided wash buffers. Purified DNA was eluted from QIAamp column membrane using elution buffer. The extracted DNA was stored at 4°C until analyzed.

High Resolution Melting (HRM) analysis

DNA samples were diluted to the same concentration (50 ng/μL) and used as templates in a nested real-time PCR-HRM assay using a pair of internal polymerase chain reaction (PCR) primers. We designed pairs of primers flanking each SNP to amplify DNA fragments shorter than 200 bp. PCR mixes were prepared using a Platinum PCR Supermix (Invitrogen, Carlsbad, CA, USA) and SYTO 9 intercalating dye (Invitrogen) and run in a Rotor-Gene 6000 (Corbett Research, Mortlake, NSW, Australia) PCR machine. The cycling profile was as follows : hold at 95°C for 3 min ; 45 cycles of 95°C for 20 s and 56°C for 30 s ; hold at 72°C for 3 min. A premelt hold at 50°C for 30 s was allowed prior to HRM at 75-85°C with a 1°C increment per step. The melt profile generated for each amplicon following real-time PCR was analyzed using Rotor-Gene 6000 Series Software (Version 1.7).

Directly Sequencing

PCR was carried out using 10 pmol of specific primer, 250 μM dNTPs, 50 ng of genomic DNA, and 1 U Go taq. Polymerase (Promega, Madison, WI, USA) in the buffer provided by the manufacturer. The cycling conditions were as follows ; 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s. PCR products were separated by agarose gel electrophoresis and purified using a PCR product purification kit. For sequencing, 1.0 μg of purified PCR products were incubated with the use of a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). After ethanol purification, the reaction products were analyzed using a sequencer (ABI 3100).

Statistical analysis

The statistical software program used was Statistical Package for the Social Science (Version 15.0 ; SPSS, Chicago, USA). The statistical significance of the difference between healthy controls and the IBS group was estimated by one way analysis of variance (ANOVA) and logistic regression analysis. A value of $p < 0.05$ was considered statistically significant.

Results

Clinical characteristics of the study population

One hundred –three IBS patients that met the Rome III criteria for IBS and 80 healthy controls were analyzed in this study. The IBS patients had a mean age of 39.2 years (range 22-74), and 57.3% of the patients were

Table 1. — **Clinical characteristics of the healthy controls and IBS patients**

	Control (n = 80)	IBS (n = 103)	IBS-D (n = 61)	IBS-C (n = 20)	IBS-A (n = 22)	p-value
Age (yr)						
Mean ± SD	39.4 ± 12.5	39.2 ± 12.8	39.7 ± 12.4	42.0 ± 11.9	41.6 ± 14.8	0.47
Range, median	(22~69, 38)	(22~74, 35)	(22~73, 35)	(23~65, 42)	(23~74, 36)	
Sex						
Female, n (%)	44 (55.0%)	59 (57.3%)	32 (53.3%)	16 (80.0%)	11 (50.0%)	0.12

IBS, irritable bowel syndrome ; IBS-D, IBS with diarrhea predominant pattern ; IBS-C, IBS with constipation predominant pattern ; IBS-A, IBS with alternate diarrhea and constipation pattern ; SD, standard deviation.

female. In the control group, the mean age was 39.4 years (range 22-69), and 55.0% were female. Sixty-one patients of the IBS group had IBS-D, 20 had IBS-C, and 22 had IBS-A. There was no statistical difference between controls and the IBS group in age or sex (p >0.05), but the IBS-C subtype had a higher proportion of females (80%, 16/20) than other subtypes (Table 1).

TRPV1 receptor exon 5 (rs222747) polymorphism in controls and IBS group

The rs222747 was detected by HRM analysis, and the genotypes with different alleles were distinguished by distinct melting profiles. However it was not possible to directly discriminate the three groups corresponding to each genotype (Fig. 1A, B). The comparison of genotype distributions and allele frequencies of TRPV1 receptor exon 5 between controls and the IBS group is shown in Table 2. The frequency of the CG genotype was 58.8% (47/80) in controls and 45.6% (47/103) in the IBS group. The frequency of the GG genotype was 15.0% (12/80) in controls and 20.4% (21/103) in the IBS group. The frequency of the G-carrier genotype was 73.8% (59/80) in controls and 66.0% (68/103) in the IBS group. There was no significant difference in allele frequency between the two groups. In IBS subtype analysis, the frequencies of the CG and GG genotypes were 43.3% (26/61) and 20.0% (12/61), respectively, in IBS-D subtype, 35.0% (7/20) and 35.0% (7/20), recpectively, in IBS-C, and 63.6% (14/22) and 9.1% (2/22), respectively, in IBD-A. There was no statistically significant difference between IBS subtypes.

TRPV1 receptor exon 1 (rs9894618) polymorphism in controls and IBS group

rs9894618 was detected by HRM analysis, and the genotypes with different alleles were distinguished by distinct melting profiles. However, it was not possible to directly discriminate the three groups corresponding to each genotype (Fig. 2A, B). The comparison of genotype distributions and allele frequencies of TRPV1 receptor exon 1 between controls and IBS is shown in Table 3. The frequency of the CC genotype was 98.8% (79/80) in controls and 100.0% (103/103) in the IBS group. Only 1 patient (1.2%) had CA type in controls. There was no patient with CA or AA genotypes in the IBS group. The data did not show any statistical significance.

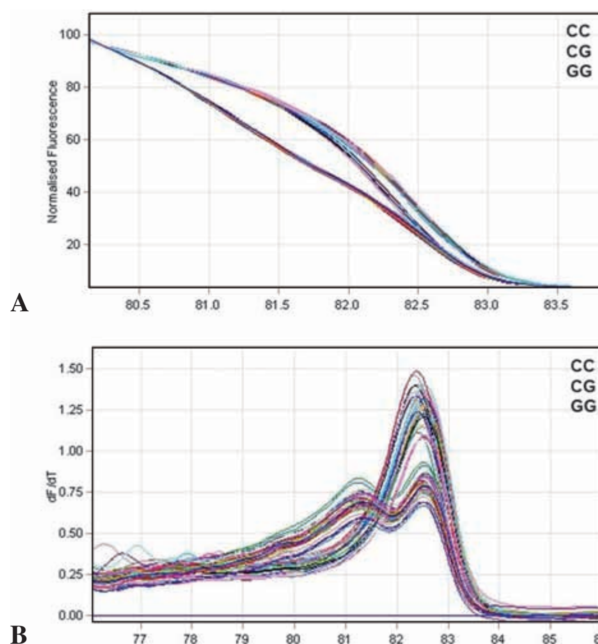

Fig. 1. — High resolution melting curve profiles of rs222747. (A) The graph displays the temperature normalized melting curve for tested samples. The three genotypes of rs222747 are shown in the normalized melting plot. (B) The graph shows a difference curve displaying the difference in fluorescence of the respective sample relative to the wild type. It was not possible to directly discriminate the three groups corresponding to each genotype.

TRPV1 receptor exon 1 (rs222749) polymorphism in controls and IBS group

The three genotypes of rs222749 were detected by direct sequencing (Fig. 3A, B, C). As shown in Table 4, the frequency of CT genotype was 31.3% (25/80) in controls and 32.0% (33/103) in the IBS group. The frequency of the TT genotype was 5.0% (4/80) in controls, but no patients in the IBS group had the TT genotype. The frequency of the T-carrier genotype was 36.3% (29/80)

Table 2. — **Genotype distribution of rs222747 between healthy controls and IBS patients**

Genotype /Allele	Control n (%)	IBS, n (%) OR (95% CI) p-value	IBS-D, n (%) OR (95% CI) p-value	IBS-C, n (%) OR (95% CI) p-value	IBS-A, n (%) OR (95%CI) p-value
CC	21 (26.2%)	35 (34.0%) 1 (reference)	22 (36.7%) 1 (reference)	6 (30.0%) 1 (reference)	6 (27.3%) 1 (reference)
CG	47 (58.8%)	47 (45.6%) 0.60 (0.31-1.18) 0.138	26 (43.3%) 0.53 (0.25-1.14) 0.102	7 (35.0%) 0.52 (0.16-1.74) 0.290	14 (63.6%) 1.04 (0.35-3.09) 0.940
GG	12 (15.0%)	21 (20.4%) 1.05 (0.43-2.56) 0.915	12 (20.0%) 0.96 (0.35-2.59) 0.927	7 (35.0%) 2.04 (0.56-7.50) 0.282	2 (9.1%) 0.58 (0.10-3.36) 0.546
G-Carrier	59 (73.8%)	68 (66.0%) 0.69 (0.36-1.32) 0.261	38 (63.3%) 0.62 (0.30-1.27) 0.188	14 (70.0%) 0.83 (0.28-2.44) 0.736	16 (72.7%) 0.95 (0.33-2.75) 0.923

IBS, irritable bowel syndrome ; IBS-D, IBS with diarrhea predominant pattern ; IBS-C, IBS with constipation predominant pattern ; IBS-A, IBS with alternate diarrhea and constipation pattern ; CI, confidence interval.

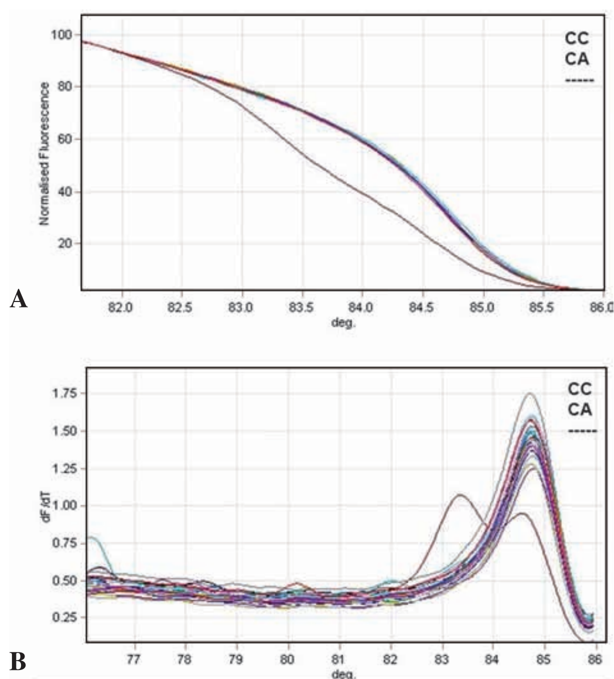

Fig. 2. — High resolution melting curve profiles of rs9894618 (A). The graph displays the temperature normalized melting curve for tested samples. The three genotypes of rs9894618 are shown in the normalized melting plot. (B) The graph shows a difference curve displaying the difference in fluorescence of the respective sample relative to the wild type. It was not possible to directly discriminate the three groups corresponding to each genotype.

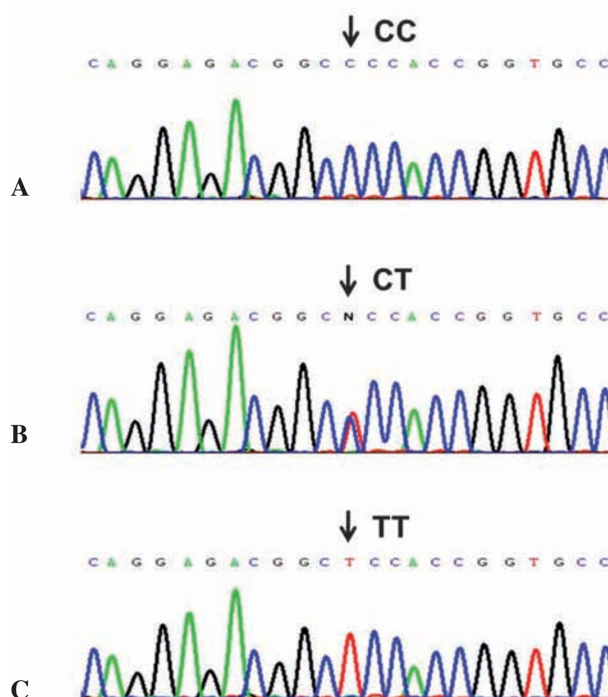

Fig. 3. — DNA sequencing of rs222749. (A) Sample of a case that was homozygous for CC. (B) Sample of a case that was heterozygous for CT. (C) Sample of a case that was mutated for TT.

in controls and 32.0% (33/103) in the IBS group. There was no significant difference in allele frequency between the two groups. In IBS subtype analysis, the frequency of the CT genotype was 31.1% (19/61) in the IBS-D subtype, 25.0% (5/20) in IBS-C, and 40.9% (9/22) in IBS-A. No significant difference was observed between the IBS subtypes.

Discussion

The TRPV1 is a nonselective cation channel with high permeability for Ca (2+) and may play a role in the development and maintenance of visceral pain and hypersensitivity states. It is activated not only by noxious heat but also by ligands containing such as capsaicin, hydrogen ion, ethanol, and a variety of arachidonic acid-derived lipid mediators (9-11). TRPV1 is associated with primary afferent neurons and TRPV1-positive nerve

Table 3. — **Genotype distribution of rs9894618 between healthy controls and IBS patients**

Genotype /Allele	Control n (%)	IBS, n (%) OR (95% CI) p-value	IBS-D, n (%) OR (95% CI) p-value	IBS-C, n (%) OR (95% CI) p-value	IBS-A, n (%) OR (95%CI) p-value
CC	79 (98.8%)	103 (100%) 1 (reference)	61 (100%) 1 (reference)	20 (100%) 1 (reference)	22 (100%) 1 (reference)
CA	1 (1.2%)	0	0	0	0
AA	0	0	0	0	0
A-Carrier	1 (1.2%)	0	0	0	0

IBS, irritable bowel syndrome ; IBS-D, IBS with diarrhea predominant pattern ; IBS-C, IBS with constipation predominant pattern ; IBS-A, IBS with alternate diarrhea and constipation pattern ; CI, confidence interval.

Table 4. — **Genotype distribution of rs222749 between healthy controls and IBS patients**

Genotype /Allele	Control n (%)	IBS, n (%) OR (95% CI) p-value	IBS-D, n (%) OR (95% CI) p-value	IBS-C, n (%) OR (95% CI) p-value	IBS-A, n (%) OR (95%CI) p-value
CC	51 (63.7%)	70 (68.0%) 1 (reference)	42 (68.9%) 1 (reference)	15 (75.0%) 1 (reference)	13 (59.1%) 1 (reference)
CT	25 (31.3%)	33 (32.0%) 1.02 (0.54-1.92) 0.951	19 (31.1%) 0.97 (0.47-2.00) 0.932	5 (25.0%) 0.79 (0.25-2.45) 0.676	9 (40.9%) 1.53 (0.57-4.11) 0.399
TT	4 (5.0%)	0	0	0	0
T-Carrier	29 (36.3%)	33 (32.0%) 0.88 (0.47-1.63) 0.684	19 (31.1%) 0.84 (0.41-1.70) 0.620	5 (25.0%) 0.68 (0.22-2.10) 0.497	9 (40.9%) 1.32 (0.50-3.50) 0.579

IBS, irritable bowel syndrome ; IBS-D, IBS with diarrhea predominant pattern ; IBS-C, IBS with constipation predominant pattern ; IBS-A, IBS with alternate diarrhea and constipation pattern ; CI, confidence interval.

fibers are located in the muscle layer, enteric nerve plexuses and mucosa of the gastrointestinal tract, as shown by immunohistochemical studies in animal experiments (23,24). Also, its expression is up-regulated in painful disorders including functional dyspepsia, inflammatory bowel disease and IBS (16-18,21,22). In particular, Akabar *et al*. demonstrated increased mucosal nerve fibers immunoreactive to TRPV1 in colonic biopsy specimens from IBS patients and a correlation between the abdominal pain score and TRPV1 levels in an IBS group (16). These findings suggest that TRPV1 represents a promising target with therapeutic potential for the treatment of gastrointestinal hyperalgesia, including IBS.

Pain sensitivity varies substantially among humans. This inter-individual variability in pain sensitivity could be associated with genetic polymorphism (19). Genetic polymorphism might impede on the generation, transmission, and processing of nociceptive information.

At least six non-synonymous polymorphisms of the human TRPV1 gene are have been found through a variety of approaches (20). Among the known SNPs, two SNPs in exon 1, $TRPV1^{P91S}$ (rs222749) and $TRPV1^{K2N}$ (rs9894618), are located in the intracellular amino terminus of the protein. $TRPV1^{I315M}$ (rs222747) in exon 5 is localized in the ankyrin repeat-containing domain and is postulated to mediate protein-protein interactions (20). Apart from structural changes, at least two SNPs, $TRPV1^{P91S}$ (rs222749) and $TRPV1^{I315M}$ (rs222747), modify the functional properties of the channel and induce increased TRPV1 protein expression due to an increased copy number, and we included those two SNPs in our study (25). Because $TRPV1^{K2N}$ (rs9894618) is located in the NH2 terminus of the TRPV1 protein such as $TRPV1^{P91S}$ (rs222749), we also considered the SNP as a candidate gene for functional properties. Despite $TRPV1^{I585V}$ (rs8065080) affects the transmembrane domain which confers responsiveness to capsaicin, it is identified as a conservative substitution and not included in the subject of initial interest. Until now, there are no data about the functional consequences of TRPV1 gene polymorphism in IBS. Therefore, we first investigated whether the three SNPs in the human TRPV1 gene are associated with IBS patients.

In our study, the GG genotype of the rs222747 was more common in IBS patients (20.4%) than in controls (15.0%). The TT genotype of the rs222749 was only observed in controls (5.0%), and none found in the IBS group. However, there was no significant difference in allele frequencies of these three SNPs of the TRPV1 gene between controls and the IBS group. In IBS subtype

analysis, we did not find any association between the TRPV1 polymorphism and IBS patients. These results suggest that these three SNPs of the TRPV1 gene may not be associated with IBS in the Korean population.

There are limitations regarding our study. First, our study was performed with small groups of patients (80 controls, 103 IBS patients) and an uneven distribution of IBS subtypes. It is the major concern of our study. Second, we did not evaluate the association between three SNPs and the degree of abdominal pain because a smaller sample size.

Despite of these limitations, our study is the first report to identify the association between SNPs in the TRPV1 gene and IBS patients. Further studies with a large number of cases are needed to validate the results of this study and to clarify the functional properties of TRPV1 gene polymorphisms in IBS patients.

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