Corticotropin-Releasing Hormone Receptor 1 Gene Variants in Irritable Bowel Syndrome

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Abstract

Background: Corticotropin-releasing hormone (CRH) acts mainly via the CRH receptor 1 (CRH-R1) and plays a crucial role in the stress-induced pathophysiology of irritable bowel syndrome (IBS). Several studies have demonstrated that variants of the CRH-R1 gene carry a potential risk for depression, but evidence for an association between CRH-R1 genotypes and IBS is lacking. We tested the hypothesis that genetic polymorphisms and haplotypes of CRH-R1 moderate the IBS phenotype and negative emotion in IBS patients.

Methods: A total of 103 patients with IBS and 142 healthy controls participated in the study. Three single-nucleotide polymorphisms of the CRH-R1 gene (rs7209436, rs242924, and rs110402) were genotyped. Subjects’ emotional states were evaluated using the Perceived-Stress Scale, the State-Trait Anxiety Inventory, and the Self-rating Depression Scale.

Results: The TT genotype of rs7209436 (P = 0.01) and rs242924 (P = 0.02) was significantly more common in patients with IBS than in controls. Total sample analysis showed significant association between bowel pattern (normal, diarrhea, constipation, or mixed symptoms) and the T allele of rs7209436 (P = 0.008), T allele of rs242924 (P = 0.019), A allele of rs110402 (P = 0.047), and TAT haplotypes (P = 0.048). Negative emotion was not associated with the examined CRH-R1 SNPs.

Conclusion: These findings suggest that genetic polymorphisms and the CRH-R1 haplotypes moderate IBS and related bowel patterns. There was no clear association between CRH-R1 genotypes and negative emotion accompanying IBS. Further studies on the CRH system are therefore warranted.
stimulation evokes colonic motility and mediates visceral nociception. In contrast, CRH-R2 stimulation inhibits gastric emptying and may reduce visceral perception [4]. Activation of CRH-R1 causes a proinflammatory response, whereas stimulation of CRH-R2 provokes anti-inflammatory changes [2]. Treatment with a specific CRH-R1 antagonist attenuates anxiety and increases colonic motility under stressful conditions after colorectal distention in rats [18]. Another specific CRH-R1 antagonist also reduced the increased brain activation in response to expected threats in IBS patients compared with a placebo [19]. These studies suggest that CRH signals via CRH-R1 are likely to be a key determinant of brain-gut function in response to stress in IBS patients.

The gene encoding CRH-R1 is located on chromosome 17q21.31 and contains 14 exons spanning 51 kb [20,21]. Variation in the CRH-R1 gene has been found to be a risk for depression following childhood maltreatment [22–25]. The variability of genes that encode the proteins which play a pivotal role in regulating the HPA axis influence the inter-individual clinical response to antidepressants [26,27]. Previous study from our laboratory reported that less maternal care and maternal overprotection form risk for IBS-like symptoms in 7-year old children [28]. Earlier studies have reported moderation of the effects of maltreatment on depression and neuroticism by a three-allele haplotype of CRH-R1 involving the single-nucleotide polymorphisms (SNPs) rs7209436, rs110402, and rs242924 [22,23,29]. In these studies, the TAT haplotype protected against depression in individuals who had been severely maltreated. These findings led us to predict that the CRH-R1 SNPs and the TAT haplotype might be associated with IBS and/or negative emotion in IBS patients.

In the present study, we investigated the association between variation in three CRH-R1 SNPs and the presence of IBS or negative emotion in patients with IBS. We hypothesized that genetic polymorphisms and/or haplotypes of CRH-R1 may moderate the effects of IBS symptoms as well as depression or anxiety in IBS patients. Our findings suggest that genetic polymorphisms and the CRH-R1 haplotypes moderate IBS and related bowel patterns, although there was no clear association between CRH-R1 genotypes and negative emotion accompanying IBS.

**Materials and Methods**

**Subjects**

In total, 103 patients (43 males and 60 females) with IBS who were diagnosed at the Department of Psychosomatic Medicine, Tohoku University Hospital, were enrolled in the study (mean age 22.0±2.0 years; range 19–29). Patients with organic diseases were excluded. In addition, 142 healthy volunteers (78 males and 64 females) were recruited at Tohoku University as controls (mean age 22.0±2.3 years; range 19–32). Subjects without any symptoms or signs with medical interview and physical examination were identified as healthy controls. IBS patients were diagnosed according to Rome III criteria [30]. In brief, IBS was defined as recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months associated with two or more of the following symptoms: improvement with defecation, onset associated with a change in frequency of defecation, and/or onset associated with a change in form (appearance) of stools. These criteria were fulfilled for the previous 3 months with symptom onset at least 6 months prior to diagnosis. According to Rome III criteria, IBS was classified as IBS with diarrhea (D), constipation (C), or mixed symptoms of diarrhea and constipation (M). Unclassified IBS patients were classified as IBS-M. All subjects provided written informed consent and this study was approved by the Tohoku University Ethics Committee. Serial patients who agreed to participate in this study were enrolled.

**Evaluation of negative emotion**

Emotional state was rated using the Perceived Stress Scale (PSS) [31,32], the State–Trait Anxiety Inventory (STAI) [33,34], and the Self-rating Depression Scale (SDS) [35,36]. The Japanese versions of STAI, SDS, and PSS have been well validated and their reliability has been confirmed [32,34,36].

**Genotyping**

Peripheral blood was collected from the forearm vein of each subject with a heparinized syringe. DNA was then extracted from the lymphocytes using a standard protocol [37]. Three SNPs (rs110402, rs242924, rs7209436) in the regulatory region of the CRH-R1 gene were genotyped using direct sequencing and TaqMan real-time polymerase chain reaction (PCR) [Figure 1].

PCR amplification was carried out using the following primer pairs designed with primer3 version 4.0. [http://frodo.wi.mit.edu/primer3/]:

| Primer | Forward | Reverse |
|--------|---------|---------|
| rs110402 | 5’- AGA GGA AGT GTA GCT CTA CTT GTG AGC CTG-3’ | 5’- CTG GTC CCA CAT CTC ATG GTA GCT GC-3’ |
| rs242924 | 5’- GAA ACT GAG GCA TGG GAG AG-3’ | 5’- CCA CAT CTC ATG GTA GCT GC-3’ |
| rs7209436 | 5’- CCT TTG TTC TCA CCT CAT CC-3’ | 5’- GGA TTG TTG ACT CAA CGG CT-3’ |

PCR reactions were performed using a thermal cycler (ABgene Odyssey, Hampshire, UK) in a total volume of 50 μl solution consisting of 0.2 μM of each primer, 1.25 U Prime STAR HS DNA Polymerase, 200 μM deoxynucleotide triphosphate, 1× Prime STAR buffer, and recombinant Taq DNA Polymerase (TAKARA BIO INC., Shiga, Japan). After initial denaturation at 94°C for 4 min, amplification was performed using 35 cycles at 94°C for 1 min (denaturation),

![Figure 1. SNPs of the CRH-R1 gene examined in this study. CRH-R1 is located on chromosome 17q21.31. SNPs of rs7209436, rs242924, and rs110402 are covering the gene in first and second intron on 5’ end including promoter region, a total region of 51.55 kb that has links with haplotype block. The structure was determined using the confidence interval method in Haplovie such that we were able to estimate haplotypes for every participant with a posterior probability of >0.998. These SNPs had a minor allele frequency of >1.0% in the Japanese population.](image)
60°C for 1 min (annealing), and 72°C for 1 min (extension), followed by final elongation at 72°C for 7 min. Amplification products were separated on 2% agarose gel by electrophoresis. PCR products were purified from agarose gel using a QiA quick Gel Extraction Kit (Qiagen, Hilden, Germany). Amplimers were removed using CENTRI-SEP Columns (Princeton Separations, Adelphia, NJ), and excess dye terminators were sequenced directly using the ABI PRISM dRodamine TM Gel Extraction Kit (Qiagen, Hilden, Germany). Amplimers were PCR products were purified from agarose gel using a QIA quick. Statistical analysis

We used Haploview [38] to determine the linkage disequilibrium (LD) structure of the SNPs within the CRH-R1 gene and test for Hardy-Weinberg equilibrium. We also compared the LD structure of a subgroup of CRH-R1 SNPs. The genotypes, alleles, and TAT haplotypes of CRH-R1 SNPs were compared between IBS patients and controls, or between patients with different bowel patterns (normal, constipation, mixed, and diarrhea) using the chi-squared test. The effects of variation in CRH-R1 SNPs and TAT haplotypes on emotional states were examined with two-way analysis of variance (ANOVA). A post hoc test was performed to determine the significance of genotype effects. Statistical analyses were performed using SPSS PASW Statistic version 18.0 software (IBM Inc., New York, NY). Results are expressed as mean ± S.E., and P<0.05 was considered significant.

Results

Using Haploview we were able to estimate haplotypes for every participant with a posterior probability greater than 0.998, which allowed us to assign a score of 0, 1, or 2 copies of the TAT haplotype to every individual with a very high degree of certainty. The TAT haplotype accounted for 84% (410/245×2) of all haplotypes in the sample, with its complement CGG accounting for the remaining 16% (80/245×2).

Table 1 shows the genotype distribution and Table 2 shows the allelic frequency. Sex was not significantly associated with the SNPs and number of TAT haplotypes. The rs7209436 TT genotype was significantly more common in IBS patients than in controls (χ²(1) = 8.66, P = 0.01) (Figure 2A) and rs242924 (χ²(1) = 7.64, P = 0.02) (Figure 2B), but not on rs110402 (Figure 2C). TAT haplotype copies were tendentially but not significantly different between IBS and controls (χ²(1) = 5.88, P = 0.053) (Figure 2D).

There were no significant associations between IBS subtypes (D, C, and M) and genotypes of the three SNPs. However, bowel habit pattern (normal, D, C, and M) was significantly associated with T allele expression of rs7209436 (χ²(2) = 11.75, P = 0.008) (Figure 3A), T allele expression of rs242924 (χ²(3) = 9.97, P = 0.019) (Figure 3B), and TAT haplotypes (χ²(2) = 12.68, P = 0.048) (Figure 3D).

Table 3 shows that there are associations between TAT haplotype copy number and group (IBS patients vs. controls), gender (males vs. females), and bowel patterns (normal, D, C, and M). There was a significant gender effect in IBS patients with two copies of the TAT haplotype, as evidenced by more diarrhea in men and more constipation/mixed symptoms in women (χ²(1) = 17.17, P = 0.001). Of the genotypes, the TT of rs7209436 (P = 0.001, Fisher’s test) and rs242924 (P = 0.001, Fisher’s test), and the AA allele of rs110402 (P = 0.001, Fisher’s test) showed a

| Table 1. Genotypes and haplotype frequencies for three CRHR1 SNPs in IBS patients and controls. |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Controls n (%) n = 142                         | IBS Patients n (%) n = 103 |                  |                  |                  |
|                                               | Male (n = 78)   | Female (n = 64) | Male (n = 43)   | Female (n = 60) |
| rs7209436                                     |                 |                 |                 |                 |
| TT                                            | 53 (37.3)       | 46 (32.4)       | 34 (33.0)       | 45 (43.7)       |
| CT                                            | 25 (17.6)       | 18 (12.7)       | 9 (8.7)         | 11 (10.7)       |
| CC                                            | 0 (0.0)         | 0 (0.0)         | 0 (0.0)         | 4 (3.9)         |
| rs110402                                      |                 |                 |                 |                 |
| AA                                            | 51 (35.9)       | 45 (31.7)       | 34 (33.0)       | 42 (40.8)       |
| AG                                            | 26 (18.3)       | 19 (13.4)       | 9 (8.7)         | 14 (13.6)       |
| GG                                            | 1 (0.7)         | 0 (0.0)         | 0 (0.0)         | 4 (3.9)         |
| rs242924                                      |                 |                 |                 |                 |
| TT                                            | 59 (41.5)       | 46 (32.4)       | 37 (35.9)       | 47 (45.7)       |
| CT                                            | 19 (13.4)       | 18 (12.7)       | 6 (5.8)         | 10 (9.7)        |
| GG                                            | 0 (0.0)         | 0 (0.0)         | 0 (0.0)         | 3 (2.9)         |
| TAT haplotype                                 |                 |                 |                 |                 |
| 0 copies                                      | 1 (0.7)         | 0 (0.0)         | 0 (0.0)         | 4 (3.9)         |
| 1 copy                                        | 26 (18.3)       | 21 (14.8)       | 9 (8.7)         | 14 (13.6)       |
| 2 copies                                      | 51 (35.9)       | 43 (30.3)       | 34 (33.0)       | 42 (40.8)       |

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### Table 2. Allele expressions in controls and IBS patients and IBS subtypes for three SNPs of the CRH-R1 gene.

|        | Controls (n = 142) | IBS patients (n = 103) | Total (n = 245) |
|--------|--------------------|------------------------|-----------------|
|        | All                | C                      | M               | D               |                |
| rs7209436 | T allele           | 0                      | 4               | 0               | 4              |
|         | T allele<sup>+</sup> | 142                    | 99              | 33              | 241            |
|         | C allele           | 99                     | 79              | 26              | 178            |
|         | C allele<sup>+</sup> | 43                    | 24              | 10              | 67             |
| rs110402 | A allele           | 1                      | 4               | 2               | 0              | 5              |
|         | A allele<sup>+</sup> | 141                   | 99              | 30              | 240            |
|         | G allele           | 96                     | 76              | 21              | 172            |
|         | G allele<sup>+</sup> | 46                    | 27              | 11              | 73             |
| rs242924 | T allele           | 0                      | 3               | 2               | 0              | 3              |
|         | T allele<sup>+</sup> | 142                   | 100             | 30              | 242            |
|         | G allele           | 105                    | 84              | 23              | 189            |
|         | G allele<sup>+</sup> | 37                    | 19              | 9               | 56             |

IBS subtype: C, constipation; M, mixed; D, diarrhea.

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**Figure 2.** Difference in genotype of CRH-R1 SNPs between controls and IBS patients. The SNPs rs7209436 (A), rs242924 (B), and rs110402 (C), and TAT haplotype (D) were shown. The SNPs rs7209436 ($P = 0.013$) and rs242924 ($P = 0.022$, $\chi^2$-test) in IBS patients significantly differed from those in controls.

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significant predominance of diarrhea in men and female dominance of constipation/mixed symptoms in women.

The SDS score in IBS patients was significantly higher than in controls ($P = 0.02$) (Table 4). The PSS score in IBS patients was also significantly higher than in controls ($P = 0.03$). The STAI scores in IBS patients did not differ from those in controls. The two-way ANOVA indicated that the SDS scores in IBS patients were significantly higher than in controls despite the $rs242924$ genotypes (TT, GT, and GG) ($P = 0.013$) and the $rs7209436$ genotypes (TT, CT, and CC) ($P = 0.009$) (Figure 4). However,

**Figure 3. Difference in bowel patterns between allele of CRH-R1 SNPs.** Each panel indicates bowel patterns (normal, constipation, mixed, or diarrhea) in the SNPs of (A) $rs7029436$ (C+ vs C−, T+ vs T−), (B) $rs242924$ (G+ vs G−, T+ vs T−), and (C) $rs110402$ (G+ vs G−, A+ vs A−) and (D) TAT haplocopies (0, 1, or 2 copies). Significant differences in bowel patterns between the $T$ alleles of $rs7029436$ ($P = 0.008$), the $T$ alleles of $rs242924$ ($P = 0.02$), the $A$ alleles of $rs110402$ ($P = 0.047$), and among TAT haplocopies ($P = 0.048$, $\chi^2$-test) of CRH-R1 SNPs were observed.

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**Table 3. IBS subtype (bowel pattern) in relation to TAT haplotype copy number and sex.**

| TAT copies | Control (n = 142) | IBS patients (n = 103) | Association with TAT haplotype copies $P$ value |
|------------|------------------|------------------------|-----------------------------------------------|
|            | IBS subtype (bowel pattern) | Control vs. IBS | Male vs. female |
| 0 | Male | 1 | 0 | 0 | 0 | 0.05 | 0.048 | 0.082 |
| | Female | 0 | 2 | 2 | 0 | |
| 1 | Male | 26 | 3 | 2 | 4 | 0.58 |
| | Female | 21 | 6 | 2 | 6 | |
| 2 | Male | 51 | 4 | 8 | 22 | 0.001 |
| | Female | 43 | 17 | 17 | 8 | |

IBS subtype: C, constipation; M, mixed; D, diarrhea.

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among both IBS patients and controls, there were no significantly different interactions between rs7209436, rs110402, and rs242924 genotypes. Similarly, there were no significantly different allele distributions or psychological scores.

Discussion

To our knowledge, the present study is the first to show an association between IBS symptoms and SNPs of the CRH-R1 gene. Our finding of an increased frequency of the TT genotype in rs7209436 and rs242924 supports the main hypothesis that genetic polymorphisms and haplotypes of CRH-R1 control the IBS phenotype. The genotype and allele frequency of CRH-R1 SNPs in Japanese general population are presented in the database (HapMap Project: http://hapmap.ncbi.nlm.nih.gov/index.html.en). TT genotype of rs7209436 and rs242924 are around 70% of normal population. Therefore, increased frequency of TT genotype of rs7209436 and rs242924 alone cannot explain relationship between CRH-R1 SNPs and IBS. However, IBS individuals are characterized by more TT, less CT, and more CC of rs7209436 and more TT, less GT, and more GG of rs242924 than controls in our study. These findings suggest more homozygous preference on rs7209436 and rs242924 SNPs in parents of IBS individuals, resulting either 0 or 2 copies of TAT haplotype. It is of great interest to see whether these findings are replicated in another population or not. Moreover, decreased intermediate (heterozygous) genotypes of CRH-R1 SNPs may relate to fundamentals of pathophysiology of allostatic load [1] in IBS patients. Allostatic load is either repeated stress overtime, lack of adaptation, prolonged response, or inadequate response [1]. In other words, pathological response to stress is not only toward one direction with exaggerated and overactive response but also toward another direction with impaired and hypoactive response. Stress responsiveness and genotyping in IBS patients are promising issue in the near future.

SNPs of rs7209436, rs242924, and rs110402 are covering the gene in the first and second intron on 5’ end including promoter region of CRH-R1 gene [20,21]. They form a haplotype block [22,23,24]. Earlier studies indicated the influence of child abuse, the above SNPs [22], and haplotype copy numbers [22,23,24] on adult depression. CRH plays a major role in negative emotion formation through the 5-hydroxytryptamine (5-HT, serotonin)-2 receptor signaling pathway [39]. CRH-R1 activation leads to increased numbers and sensitivity of 5-HT2 receptors on the cell membrane of post-synaptic neurons [39]. We previously reported the effect of the 5-HT transporter gene-linked polymorphic region on colorectal distension-induced activation of the anterior cingulate cortex [40]. We showed that overt anxiety, which is recognized by lexical processing, was not different between the genotypes. Because in the present study we also found an association between CRH-R1 genotypes and IBS symptoms, but not negative emotion measured by psychometric tests, CRH-R1 genotypes may affect mainly physical (e.g., brain-gut) reactivity to stressors.

### Table 4. Perceived stress, depression, and anxiety in controls and IBS patients.

|                     | Controls (n = 142) | IBS patients (n = 103) | P value |
|---------------------|-------------------|------------------------|---------|
|                     | Mean   | SD     | Mean  | SD     |         |
| SDS                 | 39.1   | 8.5    | 41.6  | 4.2    | 0.02    |
| PSS                 | 26.3   | 9.2    | 28.8  | 8.2    | 0.03    |
| STAI (state)        | 44.2   | 9.7    | 45.7  | 9.2    | 0.22    |
| STAI (trait)        | 46.6   | 10.9   | 49.2  | 10.3   | 0.07    |

SDS: Self-rating Depression Scale, PSS: Perceived Stress Scale, STAI: StateTrait Anxiety Inventory.

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**Figure 4. Self-rating Depression Scale and CRH-R1 SNPs.** Two-way ANOVA showed that IBS patients with rs7029436 (P = 0.009) (right) and rs242924 (P = 0.013) (left) had significantly higher depression scale scores than controls with the same genotypes. However, there was no gene–group interaction.

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The association between CRH-R1 genotypes and bowel movement is also in line with a possible link between CRH-R1 genotypes and brain-gut reactivity to stressors. T alleles of rs7209456 and rs242294, and the A allele of rs110402 mediated diarrhea, while the lack of these alleles mediated constipation. Similarly, an increased number of TAT haplotypes were associated with an increased prevalence of diarrhea, while fewer copies were associated with constipation. There is a high degree of CRH immureactivity as well as an abundance of CRH-R1 receptors in the gut [41], especially in the myenteric plexus [42,43]. Systematic excitation of myentric neurons occurs after the application of CRH via CRH-R1 receptors [42,43]. Administration of CRH causes diarrhea in rats, which mimics stress-induced diarrhea in IBS patients [44].

Among IBS patients, men with two copies of the TAT haplotype had more diarrhea while women had more constipation/mixed symptoms. Individual allele analyses also supported this finding; the T alleles of rs7209456 and rs242294, and the A allele of rs110402 predisposed toward more diarrhea in men and more constipation/mixed symptoms in women. These findings are not surprising as an increased prevalence of diarrhea in men with IBS and more constipation/mixed symptoms among women has previously been reported [45]. Because 73.8% of individuals with IBS had two copies of the TAT haplotype, these findings may simply reflect the fact that two copies are present in the majority of IBS patients. However, sexual dimorphism in the CRH system was recently recognized: chronic variable mild stress induced more CRH mRNA in the paraventricular nucleus of male rats while the same stress decreased the level of CRH peptide in female rats [46]. In male rats subjected to perinatal stress, CRH-R1 mRNA expression was significantly greater in the central amygdala and basolateral amygdala [47]. In female rats subjected to stress during the perinatal period, CRH-R1 mRNA expression was greater than controls only in the medial amygdala [47]. By contrast, the effect of sex on CRH and CRH-R1 signaling in the myenteric plexus is largely unknown. The findings in this study of possible sexual dimorphism in the CRH system need further clarification.

Contrary to our hypothesis, no clear association between negative emotion and CRH-R1 SNPs was found. Our data indicate that depression and perceived stress, but not anxiety, increased IBS in patients regardless of CRH-R1 genotype. This is partially consistent with our previous report [13] and the results of others [45]. However, mean SDS score in IBS patients was below 49 and within normal range in this study. The findings of recent studies have provided behavioral and neuroendocrine evidence of stress vulnerability in GG homozygous individuals of SNP rs110402 in the CRH-R1 gene [48]. Among GG homozygotes, activation in the subgenual anterior cingulate cortex was greater in participants with major depressive disorder compared with controls [46]. However, only 0.7% of controls and 3.9% of IBS patients were GG homozygous for rs110402 in this study. Therefore, the lack of association between negative emotion and CRH-R1 gene may be explained by the small numbers of GG homozygotes.

This study has several limitations. First, the number of subjects is small. However, the study with even smaller subject populations (n = 99) have reported SNP-phenotype analyses [48]. Second, endophenotypes in our study were more global than biological endophenotypes. Although it is of great interest to identify the association between CRH-R1 polymorphisms and the presence of IBS or bowel patterns in IBS patients, biological endophenotypes should be identified in future studies. Finally, the function of CRH-R1 SNPs remains incompletely understood. The studied SNPs are located in introns and they do not influence sequence of the CRH-R1 protein directly. However, they may influence alternative mRNA splicing [49,50] and expression of protein [51]. CRH-R1 expression and activity is regulated at the gene level by mRNA alternative splicing that results in a number of CRH-R1 variants [52]. This process can generate putative CRH-R1 receptor variants with distinct structural and signaling properties [53,54]. Moreover, intron 1 of CRH-R1 contains 3 highly conserved regions that may have regulatory functions (according to the UCSC Genome Browser database, http://genome.ucsc.edu/)[22]. Because functional intronic regulatory elements have been reported for several genes, these CRH-R1 intronic regions could affect transcriptional modulation of gene function [22]. For instance, the GG genotype of rs110402 in CRH-R1 presumably causes increased expression of CRH-R1 [48]. More research is necessary to solve these limitations.

In conclusion, our findings support the hypothesis that genetic polymorphisms and haplotypes of CRH-R1 mediate IBS and related bowel patterns. However, we could not find a clear association between CRH-R1 genotypes and negative emotion. Further studies on IBS and the CRH system are therefore warranted.

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Author Contributions
Conceived and designed the experiments: N. Sato MA SF. Performed the experiments: N. Sato N. Suzuki AS. Analyzed the data: N. Sato TO M. Kanazawa M. Kano SF. Contributed reagents/materials/analysis tools: M. Kanazawa E. Tomita M. Kano TM MA SF. Wrote the paper: N. Sato N. Suzuki TK SF.

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