Association of polymorphic variants in serotonin re-uptake transporter gene with Crohn’s disease: a retrospective case-control study

Aim  To analyze the distribution of SLC6A4 gene polymorphisms in Crohn’s disease (CD) patients and their association with the disease.

Methods  We evaluated the presence/absence of promoter (5-HTTLPR, rs25531) and intron 2 (STin2 VNTR) polymorphic variants of SLC6A4 gene in a retrospective case-control study including 192 CD patients and 157 healthy controls (HC). Genotyping was performed by polymerase chain reaction. The association of polymorphisms with CD and its clinical subtypes was analyzed using χ2 and Fisher exact test, binary logistic regression, and haplotype analysis.

Results  CD patients and healthy controls had similar sex (88 [45.8%] vs 84 [53.5%] women, respectively; P = 0.154) and age (41.3 ± 12.8 years vs 41.7 ± 8.8 years, respectively, P = 0.091) distribution. Significant differences were observed in the STin2 genotype and allele distribution between CD patients and healthy controls (P = 0.003 and P = 0.002, respectively) and between the corresponding female subgroups (P = 0.004 and P = 0.007, respectively), with a significant negative association of biallelic ss (STin2.9 and STin2.10) STin2 genotype with CD (P = 0.013, age- and sex-adjusted odds ratio [OR] 0.5, 95% confidence interval [CI] 0.29-0.86; women: P = 0.006, age-adjusted OR 0.32, 95% CI 0.14-0.72) and a significantly higher S-STin2.12 (S-HTTLPR/rs25531: S-STin2: STin2.12) haplotype distribution in CD patients (P = 0.004, OR 1.62, 95% CI 1.16-2.26). There was no significant association between S-HTTLPR and rs25531 genotype or allele frequencies and CD and between any SLC6A4 polymorphic loci with clinical CD subtypes.

Conclusion  STin2 VNTR polymorphism of SLC6A4 gene may contribute to CD pathogenesis.
Inflammatory bowel disease (IBD), with its constituent clinical phenotypes, Crohn’s disease (CD) and ulcerative colitis (UC), represents a major relapsing gastrointestinal (GI) disorder, with a combined incidence of 2-20 per 100 000 individuals in the developed countries (1-4). IBD susceptibility is influenced by a variety of factors, including genetic polymorphism, GI motility, stress response, visceral hypersensitivity, abnormal immune response, and its reaction to enteromicrobial pathogens (1-4).

Neuroimmunological interactions are also important because various pro- and anti-inflammatory cytokines may affect neuronal activity and the release of neurotransmitters influencing the activity of immuno-effector cells in the GI tract. The best characterized among them is serotonin (5-HT), 90% of which is produced and secreted by intestinal enterochromaffin cells (5-8). Alterations in 5-HT biosynthesis, quantity, release, or clearance are important for both sensory signal transduction in GI motility and the development of visceral hypersensitivity (5-8). 5-HT is also a chemotactic molecule and may promote lymphocyte activation and secretion of pro-inflammatory cytokines (5-8). Therefore, upon its release and binding to targeted receptors, 5-HT action must be rapidly terminated. This is maintained by the action of serotonin re-uptake transporter (SERT), expressed by serotonergic neurons and the mucosal enterocytes (5-8).

Animal models and studies on human cell lines and tissues indicate increased 5-HT availability and reduced SERT expression in the inflamed colon, accompanied with an increased expression of inflammatory genes, thus supporting the idea that the loss of SERT gene (SLC6A4) transcription can either cause intestinal inflammation or result from it (8,9). SLC6A4 gene polymorphisms have also been linked with its translation and expression (10,11).

The most extensively studied among them are 5-HTTLPR, rs25531 (A/G) single nucleotide (SNP), and STin2 VNTR (variable number of tandem repeats) polymorphic regions found in the promoter (5-HTTLPR and rs25531) and intron 2 region (STin2 VNTR) of SLC6A4 gene (Figure 1) (12-19).

Sikander et al (20) has reported a significant association of 5-HTTLPR polymorphism with UC and microscopic colitis (MC). However, the association between 5-HTTLPR and CD has never been determined. The same was true for other SLC6A4 polymorphic regions in any form of IBD (11,20). Therefore, the aim of this study was to compare genotype and allele frequencies of 5-HTTLPR, rs25531 and STin2 VNTR.
polymorphic regions in CD patients and healthy control participants and investigate their association with CD. Given the crucial role of SERT protein in the regulation of intestinal 5-HT availability, we hypothesized that the SLC6A4 polymorphism may contribute to the genetic predisposition for the development of CD.

PATIENTS AND METHODS

Patients

This retrospective case-control study included all 192 consecutive CD patients diagnosed between December 2012 and 2016 at the University Hospital Center Zagreb (Table 1). CD diagnosis was established according to standard endoscopic, radiological, and histopathological criteria (21). The healthy control group consisted of 157 participants with no family history of IBD or irritable bowel disease. They were selected from 217 completely healthy voluntary participants (hospital employees or their acquaintances) who were compatible according to age (outside the age range of CD group members) were excluded from further analysis. All participants signed the informed consent for study participation and data publication. The study protocol was approved by the Ethics Committee of the University Hospital Center Zagreb (Approval number 04/31-JG; contract number 108-1081874-1917, start date January 1, 2007).

DNA extraction and genotyping

Blood samples were collected by venipuncture in EDTA BD Vacutainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and genomic DNA was isolated from peripheral blood leukocytes using the salting out procedure (22). Study participants were genotyped for both promoter and intron 2 polymorphisms of the SLC6A4 as previously described (22-24). Biallelic 5-HTTLPR variants were discriminated with some modifications as previously described (22-24). The following primers were used: forward 5′-ATG CCA GCA CCT AAC CCC TAA TGT-3′, reverse 5′-GGA CCG CAA GGT GGG CGG GA-3′. The 5-HTTLPR polymorphic region was subdivided into functional variants (S, LA, LG) by rs25531 (A/G) SNP using a TaqMan qPCR procedure according to Hu et al (25). Amplification was performed in a 7500 qPCR System under the following conditions: at 50°C for 2 minutes, at 95°C for 15 seconds, and at 62.5°C for 1.5 minutes. Genotype sequencing/capillary electrophoresis (ABI PRISM 3100 Genetic Analyzer, ThermoFisher Scientific, Waltham, MA, USA) was performed to acquire controls. The STin2 VNTR polymorphism was discriminated using the method described by Ito et al (26). For the PCR reaction, the following primer pair was used: forward 5′-GTCAGTATCACAGGCTGCGAG-3′, reverse 5′-TGTTCCTAGTCTTACGCCAGTG-3′.

Statistical analysis

The normality of data distribution was analyzed by Shapiro-Wilk test. Data are presented as frequencies or means with standard deviations (±SD). The groups were compared using t test or Mann-Whitney test for parametric and non-parametric continuous variables, respectively, and Pearson χ² test for categorical variables. Conformity of genotype distributions to Hardy–Weinberg equilibrium was assessed using goodness-of-fit χ² test. The difference in allele frequency was determined by using either χ² or Fisher exact test as appropriate. Crude and adjust-
ed odds ratios (OR) (controlling for age and sex) and the corresponding 95% confidence intervals (CI) determined by binary logistic regression were also used to test the association between CD and genotype variants of both promoter and intron 2 SLC6A4 gene polymorphisms. Association analysis based on patient sex, or clinical CD subtypes such as age at diagnosis, localization, and disease behavior were also performed. Considering that LG allele has the same transcriptional activity as the S allele, the triallelic 5-HTTLPR-rs25531 genotypes were also coded in a clustered biallelic form (LL, LS, and SS; Figure 1). Likewise, the triallelic STin2 VNTR polymorphism was also coded in a biallelic (lL - ls - ss; Figure 1) genotype form. All biallelic genotype forms were analyzed under the codominant, dominant, and recessive model. Construction of haplotype, linkage disequilibrium (LD), and haplotype association analysis were calculated by permutation test using the online SHEsisPlus software (27).

The power analysis (to compute a priori sample size and the post-hoc power of the study) was performed using G*Power software (version 3.1.9.2 for Windows) (28). For the analysis of 5-HTTLPR genotype frequencies with a χ² goodness-of-fit test with the small effective size (w = 0.2, df = 2); power (1-β) = 0.80, and α = 0.017 (Bonferroni corrected P value), the required total sample size was 329, and the post-hoc achieved power (1-β) of analysis was 0.83-1.00 for genotype distribution and 0.99-1.00 (w = 0.2 - 0.5; df = 2) for allele frequency analysis. Furthermore, with a power of 0.80 we were able to detect an effect size of 0.223 for a significant difference in STin2 VNTR genotype frequencies. The two-tailed P < 0.05 was considered significant and corrected according to Bonferroni procedure (the corrected level of significance is: Pc = 0.05/N; N - number of independent tests). All reported P values were uncorrected unless stated otherwise. Statistical analysis was performed using SPSS Statistics software trial version (IBM Corp., Armonk, NY, USA), unless stated otherwise.

RESULTS

Population characteristics

There were 88 (45.8%) women and 104 (54.2%) men in the CD group, and 84 (53.5%) women and 73 (46.5%) men in the control group (Pearson χ² = 2.033, P = 0.154). The groups were also comparable according to age (mean±SD: 41.3 ± 12.8 years vs 41.7 ± 8.7 years, respectively; Mann-Whitney test, P = 0.091).

TABLE 2. 5-HTTLPR, STin2 VNTR and rs25531 SNP allele frequency distribution in patients with Crohn’s disease and healthy controls*

| Polymorphism              | No. (%) of participants | Pearson χ² | Individual comparisons |
|---------------------------|-------------------------|-------------|------------------------|
|                           | CD                      | controls    | χ²        | P        | χ²         | P        | OR (95% CI) |
| 5-HTTLPR                  |                         |             |           |          |           |          |            |
| L                         | 231 (60.2)              | 208 (66.2)  | 2.74      | 0.098    | 2.74      | 0.098    | 0.77 (0.56-1.05) |
| S                         | 153 (39.8)              | 106 (33.8)  | 2.74      | 0.098    | 1.30 (0.95-1.77) |
| rs25531                   |                         |             |           |          |           |          |            |
| L’                        | 212 (55.2)              | 185 (58.9)  | 3.76      | 0.152    | 0.97      | 0.325    | 0.86 (0.64-1.16) |
| L’’                       | 19 (5.0)                | 23 (7.3)    | 1.73      | 0.188    | 0.66      | 0.35-1.23 |
| L’’’                      | 153 (39.8)              | 106 (33.8)  | 2.74      | 0.097    | 1.30 (0.95-1.77) |
| STin2 VNTR                |                         |             |           |          |           |          |            |
| 12                        | 242 (63.0)              | 174 (55.4)  | 12.03     | 0.002†   | 4.15      | 0.042    | 1.37 (1.01-1.86) |
| 10                        | 141 (36.7)              | 130 (41.4)  | 1.59      | 0.207    | 0.82      | 0.301-1.12 |
| 9                         | 1 (0.3)                 | 10 (3.2)    | 0.002     | 0.98     | 0.08      | 0.01-0.62 |

*CD – Crohn’s disease; 5-HTTLPR – serotonin reuptake transporter (SLC6A4) length polymorphic region; STin2 VNTR – variable number of tandem repeats (VNTR) found in intron 2 of SLC6A4 gene; rs25531 – single nucleotide (SNP) polymorphism found in the background of longer 5-HTTLPR allele variant; L – long 5-HTTLPR allele; S – short 5-HTTLPR allele; L’ – long 5-HTTLPR allele associated with the higher transcriptional activity; L’’ – long 5-HTTLPR allele exhibiting lower serotonin uptake and transcriptional activity equivalent to the short (S) allele of the 5-HTTLPR polymorphic region; L’’’ – STin2 VNTR alleles; OR – odds ratio; CI – confidence interval.

†Bonferroni correction P = 0.05/3 = 0.017.

§Bonferroni non-adjusted P values.

‖Logistic regression adjusted for age and sex.
Association analysis

According to the Hardy-Weinberg equilibrium, there was no deviation in the 5-HTTLPR, STin2, and rs25531 genotype distribution in CD group (5-HTTLPR: $\chi^2 = 0.557$, df $= 1$, $P = 0.756$; STin2: $\chi^2 = 0.585$, df $= 3$, $P = 0.964$) and healthy control group (5-HTTLPR: $\chi^2 = 0.156$, df $= 1$, $P = 0.924$; rs25531: $\chi^2 = 2.358$, df $= 3$, $P = 0.124$). Furthermore, allele and genotype frequencies of all polymorphic loci in the control group corresponded well to the previously published data for Croatian and other European populations (18,29-31).

CD group and control group showed a different distribution of STin2 VNTR allele frequencies, which remained significant after Bonferroni correction (Table 2). This was also the case for women with CD ($\chi^2 = 9.850$, df $= 2$, $P = 0.007$) and their healthy female controls (data not shown). In addition, STin2.12 allele showed a positive and STin2.9 allele a negative association with CD both in the overall (Table 2) patient sample and in the subgroup of female patients (data not shown). However, the association remained significant only for the rare STin2.9 allele in the overall study sample and the subgroup of female patients after Bonferroni correction (Table 2). No significant association following Bonferroni correction was found between STin2 VNTR allele frequency and clinical CD subtypes (data not shown).

There was no significant association in 5-HTTLRP and rs25531 allele frequency between the CD and control group or between the corresponding sex subgroups (data not shown).

### Table 3. Logistic regression analysis of STIN2 VNTR genotype distribution in patients with Crohn’s disease and healthy controls*

| Polymorphism | No. (%) of participants | Logistic regression |
|--------------|-------------------------|---------------------|
|              | CD                      | controls            | crude OR (95% CI) | $P^*$ | OR (95% CI) $^†$ | $P^†$ |
| **5-HTTLPR** |                         |                     |                   |       |                   |       |
|              |                         | 0.038$^i$           |                   |       |                   |       |
|              |                         | 0.043$^i$           |                   |       |                   |       |
| **rs25531** |                         |                     |                   |       |                   |       |
|              |                         | 0.002$^i$           |                   |       |                   |       |
|              |                         | 0.009$^i$           |                   |       |                   |       |
| **5-HTTLPR** |                         |                     |                   |       |                   |       |
| **rs25531** |                         |                     |                   |       |                   |       |
|              |                         | 0.002$^i$           |                   |       |                   |       |
|              |                         | 0.009$^i$           |                   |       |                   |       |
| **5-HTTLPR** |                         |                     |                   |       |                   |       |
| **rs25531** |                         |                     |                   |       |                   |       |
|              |                         | 0.002$^i$           |                   |       |                   |       |
|              |                         | 0.009$^i$           |                   |       |                   |       |
| **5-HTTLPR** |                         |                     |                   |       |                   |       |
| **rs25531** |                         |                     |                   |       |                   |       |
|              |                         | 0.002$^i$           |                   |       |                   |       |
|              |                         | 0.009$^i$           |                   |       |                   |       |
| **5-HTTLPR** |                         |                     |                   |       |                   |       |
| **rs25531** |                         |                     |                   |       |                   |       |
|              |                         | 0.002$^i$           |                   |       |                   |       |
|              |                         | 0.009$^i$           |                   |       |                   |       |
| **5-HTTLPR** |                         |                     |                   |       |                   |       |
| **rs25531** |                         |                     |                   |       |                   |       |
|              |                         | 0.002$^i$           |                   |       |                   |       |
|              |                         | 0.009$^i$           |                   |       |                   |       |

*CD – Crohn’s disease; STN2 VNTR – variable number of tandem repeats (VNTR) found in intron 2 of serotonin reuptake transporter (SLC6A4) gene; OR – odds ratio; CI – confidence interval. $l$ – Long (12); STN2 alleles; $s$ – Short (10, 9); STN2 alleles. 12, 10, 9; STN2 VNTR alleles.

†Bonferroni non-adjusted $P$ values.

‡Logistic regression adjusted for age and sex.

§Overall STIN2 VNTR genotype distribution in CD patients and healthy controls: $\chi^2 = 15.857$, df $= 4$, $P = 0.003$.

||Global $P$ value for logistic regression analysis.

¶Rare genotype variants grouped together.

**Significant $P$ values ($<P$). Bonferroni correction for genotype distribution analysis $P_c^* = 0.008$ (0.05/6 – number of genotypes) and for logistic regression analysis $P_c^† = 0.017$ (0.05/3 – number of genetic model comparisons).
not shown). The results remained the same after the analysis for clinical CD subtypes (data not shown). Nevertheless, we did observe a positive, although non-significant, association of S allele of 5-HTTLRP and rs25531 polymorphism with CD (Table 2).

Pearson χ² test showed significant differences in STin2 genotype distribution (Table 3) between CD patients and healthy controls ($P = 0.003$) and between the corresponding female (χ² = 15.326, df = 4, $P = 0.004$) subgroups (data not shown). This was even more pronounced when carriers of the rare STin2 genotype forms (STin2 12/9 + STin2 10/9; Fisher exact test for CD vs controls; $P = 0.002$) were grouped together (overall CD: χ² = 15.720, df = 3, $P = 0.001$; female subgroup: χ² = 15.326, df = 3, $P = 0.002$). Furthermore, STin2 12/12 and STin2 12/10 genotypes were more common, while STin2 10/10 genotype was less common among CD patients and controls. The same was true when we analyzed the corresponding female subgroups only (data not shown). However, even at very liberal correction for multiple comparisons of $P_c = 0.008$ ($P_c = 0.05/N$ – number of genotypes analyzed), we did not find any significant difference in the distribution of individual STin2 VNTR genotypes between CD and controls (Table 3).

In logistic regression analysis, the co-dominant triallelic STin2 genotype model did not show a significant association of individual STin2 genotypes with CD (Table 3).

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**TABLE 4.** Logistic regression analysis of 5-HTTLRP and rs25531 genotype distribution in patients with Crohn’s disease and healthy controls*

| Polymorphism | No. (%) of participants | Logistic regression |  |  |
|--------------|-------------------------|---------------------|---|---|
|              | CD                      | controls            | crude OR (95% CI) | OR (95% CI) | P † | P ‡ |
| 5-HTTLRP     |                         |                     |                |              |     |     |
| biallelic    |                         |                     |                |              |     |     |
| codominant   |                         |                     |                |              |     |     |
| L/L          | 67 (34.9)               | 70 (44.6)           | reference 1.0   | reference 1.0 | 0.180 | 0.200 |
| L/S          | 97 (50.5)               | 68 (43.3)           | 1.50 (0.94-2.35) | 0.909 | 1.48 (0.94-2.35) | 0.090 |
| S/S          | 28 (14.6)               | 19 (12.1)           | 1.54 (0.79-3.02) | 0.210 | 1.52 (0.77-2.98) | 0.230 |
| dominant     |                         |                     |                |              |     |     |
| L/L          | 67 (34.9)               | 70 (44.6)           | reference 1.0   | reference 1.0 | 0.180 | 0.200 |
| L/S-S/S      | 125 (65.1)              | 87 (55.4)           | 1.50 (0.97-2.31) | 0.070 | 1.49 (0.97-2.30) | 0.070 |
| recessive    |                         |                     |                |              |     |     |
| L/L-L/S      | 164 (85.4)              | 138 (87.9)          | reference 1.0   | reference 1.0 | 0.180 | 0.200 |
| S/S          | 28 (14.6)               | 19 (12.1)           | 1.24 (0.66-2.32) | 0.500 | 1.22 (0.65-2.29) | 0.530 |
| 5-HTTLRP/rs25531 |                 |                     |                |              |     |     |
| biallelic    |                         |                     |                |              |     |     |
| codominant   |                         |                     |                |              |     |     |
| L'/L'        | 57 (29.7)               | 57 (36.3)           | reference 1.0   | reference 1.0 | 0.410 | 0.410 |
| L'/S'        | 98 (51.0)               | 71 (45.2)           | 1.38 (0.86-2.23) | 0.190 | 1.39 (0.86-2.24) | 0.180 |
| S'/S'        | 37 (19.3)               | 29 (18.5)           | 1.28 (0.69-2.35) | 0.430 | 1.25 (0.68-2.31) | 0.470 |
| dominant     |                         |                     |                |              |     |     |
| L'/L'        | 57 (29.7)               | 57 (36.3)           | reference 1.0   | reference 1.0 | 0.410 | 0.410 |
| L'/S'- S'/S' | 135 (70.3)              | 100 (63.7)          | 1.47 (0.90-2.40) | 0.130 | 1.47 (0.90-2.41) | 0.130 |
| recessive    |                         |                     |                |              |     |     |
| L'/L'- L'/S' | 155 (80.7)              | 128 (81.5)          | 1.47 (0.74-2.93) | 0.270 | 1.46 (0.73-2.90) | 0.290 |
| S'/S'        | 37 (19.3)               | 29 (18.5)           | 1.28 (0.69-2.35) | 0.430 | 1.25 (0.68-2.31) | 0.470 |

*CD – Crohn’s disease; 5-HTTLPR – serotonin reuptake transporter (SLC6A4) length polymorphic region; rs25531 – single nucleotide (SNP) polymorphism found in the background of longer 5-HTTLPR allele variant; L', S', L or S clustered biallelic 5-HTTLPR/rs25531 model based on transcriptional activity: L'/L'>L'/S'>S'/S' OR – odds ratio, CI – confidence interval.

†Bonferroni non-adjusted P values; Bonferroni correction for genotype distribution analysis $P_c = 0.0083$ ($P_c = 0.05/N$ – number of genotypes) and for logistic regression analysis $P_c = 0.0166$ ($P_c = 0.05/N$ – number of genetic model comparisons).

‡Logistic regression adjusted for age and sex.

§Overall 5-HTTLRP genotype distribution between CD and healthy control group: χ² = 3.410, df = 2, $P = 0.182$; iliglobal $P$ value of logistic regression analysis.

¶Overall triallelic 5-HTTLPR/rs25531 genotype distribution between CD and healthy control group: χ² = 5.674, df = 5, $P = 0.339$. 

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ever, when men and women were analyzed together under the recessive biallelic model (Table 3) or when women only were analyzed (data not shown), carriers of biallelic ss (s = STin2 10 or STin2 9, STin2 genotype form exhibited a significant negative association with CD compared with ll and ls carriers (female patients: Wald = 7.564, df = 1, P = 0.006, age-adjusted OR = 0.32, 95% CI = 0.14-0.72), which remained significant after sex and/or age adjustment (female patients: Wald = 7.564, df = 1, P = 0.006, OR adjusted by age = 0.32, 95% CI = 0.14-0.72). Nevertheless, only the variation of co-dominant STin2 model in which the rare genotypes (STin2 12/9 and 10/9) were grouped together showed significant global P values in crude and adjusted logistic regression analysis, and only when male and female patients were analyzed together (Table 3).

No significant difference was observed in 5-HTTLPR and rs25531 genotype distribution between CD group and controls (Table 4) or between sex subgroups and clinical CD subtypes (data not shown). The same was found when biallelic 5-HTTLPR and rs25531 genotype forms were analyzed under the codominant, dominant, or recessive genetic model. Nevertheless, we did observe more frequent, although non-significant, distribution of heterozygous (LS, LALG, L'5) and homozygous SS genotype forms in the CD group (Table 4).

**TABLE 5.** The frequency of SLC6A4 haplotypes in patients with Crohn’s disease and their healthy controls*

| SLC6A4 haplotypes | No. (%) of participants | CD | controls | χ² | P† | OR (95% CI)† |
|------------------|-------------------------|----|----------|----|----|-------------|
| 5-HTTLPR | rs25531 | STin2 VNTR | | | | |
| L | L | STin2 10 | 110 (28.6) | 97 (30.8) | 0.417 | 0.518 | 0.89 (0.65-1.24) |
| S | - | STin2 12 | 131 (34.1) | 76 (24.2) | 8.133 | 0.004‡ | 1.62 (1.16-2.26) |
| L | L | STin2 12 | 102 (26.5) | 88 (28.0) | 0.186 | 0.665 | 0.93 (0.66-1.30) |
| S | L | STin2 10 | 9 (2.3) | 6 (1.9) | 0.083 | 0.775 | 0.70 (0.29-1.82) |
| S | - | STin2 10 | 22 (5.7) | 27 (8.5) | 2.719 | 0.103 | 0.73 (0.29-1.82) |
| L | L | STin2 12 | 9 (2.3) | 10 (3.1) | 0.461 | 0.496 | 0.73 (0.29-1.82) |
| L | L | STin2 9 | 1 (0.2) | 7 (2.2) | 5.910 | 0.015 | 0.11 (0.01-0.94) |
| S | - | STin2 9 | 0 (0.0) | 3 (0.9) | 3.684 | 0.054 | NA |

* CD – Crohn’s disease; 5-HTTLPR – serotonin reuptake transporter (SERT/SLC6A4) length polymorphic region; STin2 VNTR – variable number of tandem repeats (VNTR) found in intron 2 of SLC6A4 gene; rs25531 – single nucleotide (SNP) polymorphism found in the background of longer 5-HTTLPR allele variant; L = Long 5-HTTLPR allele exhibiting lower serotonin uptake and transcriptional activity equivalent to the short (s) allele of the 5-HTTLPR polymorphic region; L = Long 5-HTTLPR allele exhibiting lower serotonin uptake and transcriptional activity equivalent to the short (s) allele of the 5-HTTLPR polymorphic region; Global result – Pearson χ² = 18.29, Pearson P = 0.011; OR – odds ratio; CI – confidence interval.

†Bonferroni non-adjusted P values and confidence intervals; Bonferroni correction for haplotype analysis: Pc = 0.006 (0.05/8 - number of haplotype comparisons).

‡Significant P value (<P).

FIGURE 2. Linkage disequilibrium (LD) in SLC6A4 gene with D* and r2 values.

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Haplotype analysis

S-HTTLPR and rs25531 polymorphic region were in strong linkage disequilibrium, while LDs between them and STin2 VNTR polymorphic region were relatively weak (Figure 2). After Bonferroni correction, only S-S-STin2.12 haplotype showed a significantly higher frequency in CD group (Table 5). The higher frequency in CD group was also revealed for L-LG-STin2.10 haplotype, but it was not significant (Table 5).

DISCUSSION

Our data revealed a significant difference in STin2 VNTR genotype and allele distribution between the overall CD group and healthy controls and between the female patient group and the corresponding control group. There was also a significant negative association of biallelic ss (STin2. 10/10 and STin2. 10/9 vs STin2. 12/12, 12/10 and 12/9 combined) genotype form and CD. We also found a significantly higher S-STin2.12 (S-HTTLPR/rs25531: S - STin2: STin2.12) haplotype distribution among CD patients. However, we did not find any association between S-HTTLRP and rs25531 genotype or allele frequencies and CD or between any SLC6A4 polymorphic loci and clinical subtypes of CD. Nevertheless, our results indicate that STin2 VNTR polymorphism of SLC6A4 gene may contribute to CD pathogenesis.

Altered function and down-regulation of SERT protein expression paralleled with abnormal 5-HT concentration, both locally in epithelial layer and in the circulation, has been documented in several human GI inflammatory conditions including CD, UC, and MC, as well as diverticulitis and active celiac disease (32-34). This was also documented in several animal models of intestinal inflammation (35-39). Pro-inflammatory mediators and growth factors released during IBD may down-regulate SERT transcription and decrease SERT protein expression and function (40-43).

Another possibility is that some individuals with IBD have a genetic predisposition leading to altered SERT expression with consequent changes in gastrointestinal 5-HT levels, which contributes indirectly to pro-inflammatory conditions in the affected intestinal mucosa.

However, despite the recognition that intestinal inflammatory diseases, such as CD, can have a strong genetic component, polymorphisms so far linked with SERT transporter have not been associated with CD.

Furthermore, in previous reports on GI diseases only STin2 VNTR was observed as an attractive candidate for a possible association of the SERT with IBS (10,44). Although IBD and IBS are usually viewed as dichotomous conditions, they exhibit similar alterations in serotonergic-signaling mechanisms (33,44-46). Due to the development of IBS symptoms in IBD patients in remission and the clinical overlap between IBD and IBS, some authors even argued that they may represent clinical manifestations of a pathophysiological spectrum of the same disease (47,48). Several population studies showed that IBS patients have an increased risk of becoming IBD patients than patients without prior IBS history, and this effect may be greater for CD (49,50).

Reports regarding the link between STin2 polymorphism and IBS were controversial and inconclusive, with most of them indicating no association with IBS or its clinical subtypes (47). However, Wang et al (51) found that IBS patients had a greater frequency of STin2.12/10 and a lower frequency of STin2.12/12 genotype compared to controls, but identified no significant difference in STin2 polymorphism among different clinical subtypes of IBS.

Current research in functional implications of the STin2 polymorphism is also inconclusive. It is known that STin2 acts as a transcriptional regulator and has allele-dependent enhancer-like properties that may influence tissue-specific regulation of SLC6A4 gene (18,52). However, it seems that individual SLC6A4 polymorphisms have a weak influence compared with the combined effect of S-HTTLPR and STin2 region (14,18,52,53). Therefore, their allelic combinations should be identified before concluding about functional and phenotypic associations. LD between these two loci, ranging from moderate (European) to very strong (Native Americans), was found in most of the studied populations (31). In addition, a partial linkage of STin2.12 allele with S allele of S-HTTLPR (S12 haplotype combination) has been reported and had stronger enhancer-like properties on SLC6A4 transcription than L10 or S10 haplotype (S3,54). We found a significantly higher distribution of S12 haplotype in CD group, showing its positive association with disease occurrence. However, since we did not measure the level of SERT expression or 5-HTT plasma or tissue levels, we were not able to indicate any functional consequences of this finding.

We did not confirm the association of S-HTTLRP and rs25531 with CD occurrence, but we did observe the tendency toward higher frequencies of heterozygote S-HTTLRP and rs25531 genotype forms in CD group.
This effect of molecular heterosis was also described in some other studies of 5-HTTLPR (55-57).

To the best of our knowledge, there are no reports analyzing the relationship between the promoter polymorphic regions of the SLC6A4 gene with CD. Sikander et al (20) demonstrated a potential association between 5-HTTLPR polymorphism and UC and MC by finding a significantly lower frequency of SS vs non-SS (LL and LS combined) 5-HTTLPR genotypes in MC patients or UC patients in remission and significantly higher serum 5-HT levels (SS>LS>LL) in these two patient groups. This was especially the case in MC LS and SS genotype carriers compared with controls (20). In addition, UC patients with active disease and SS genotype also had increased 5-HT levels compared with control subjects expressing the same genotype (20). Similar to our results in CD patients, they did not observe any significant differences in 5-HTTLPR genotype and allele distributions between UC patients with active disease and healthy controls or between male and female patient subgroups (20). Likewise, Shiotani et al (34) also observed no significant association of 5-HTTLRP variants and UC. Our results were contrary to the findings of Sikander et al (20), as we found a higher frequency of SS genotype in CD group.

The observed discrepancies are possibly due to heterogeneous background of CD and other forms of IBD and/or ethnic or regional differences between the studied groups. They are also consistent with the previously mentioned hypothesis that 5-HTTLPR might represent only one of the contributing polymorphic factors responsible for the disease occurrence.

Regarding the IBS and 5-HTTLPR polymorphism, different associations were reported, but the results were contradictory and inconclusive even for the same population under the study. The most recent meta-analysis found no significant association between 5-HTTLPR and IBS in overall population, while in the IBS subtype- and ethnic subgroup-based analysis the LL genotype was demonstrated as a risk factor for constipation predominant IBS (58).

With respect to rs25531 polymorphism, we found only two studies examining its association with IBS, and only one reported a positive association with disease occurrence with three times higher odds ratio for the LG allele distribution in IBS patients compared to controls (59,60).

There are several limitations to our study. The sample size was too small for a comprehensive genotype analysis, so the results cannot be generalized and should be interpreted cautiously. Second, we did not determine the 5-HT levels and were unable to evaluate the functional consequences of individual genotype or haplotype variants on SERT expression. Third, association studies of unrelated individuals, such as ours, warrant cautious interpretation as unknown sources of population stratification may affect the results.

In conclusion, we demonstrated significant differences in STin2 genotype and allele distribution between CD patients and healthy controls, and a negative association of biallelic ss STin2 genotype variant and a positive association of S12 haplotype with CD disease. Further large-scale studies in this and other populations aimed to confirm the obtained results and decipher the exact functional role of STin2 VNTR polymorphic region in intestinal inflammatory diseases are warranted.

Oxford Centre for Evidence-based Medicine level of evidence 3b/4a.

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Declaration of authorship All authors except BO conceived and designed the study, all authors acquired the data, analyzed and interpreted the data, drafted the manuscript, critically revised the manuscript for important intellectual content, gave approval of the version to be submitted, and agree to be accountable for all aspects of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

References
1. Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol. 2015;12:205-17. Medline:25732745 doi:10.1038/nrgastro.2015.34
2. Strober W, Fuss L, Mannon P. The fundamental basis of inflammatory bowel disease. J Clin Invest. 2007;117:514-21. Medline:17332878 doi:10.1172/JCI30587
3. Burisch J. Crohn’s disease and ulcerative colitis. Occurrence, course and prognosis during the first year of disease in a European population-based inception cohort. Dan Med J. 2014;61:B4778. Medline:24393595
4. Xia B, Cruijsius J, Meuwissen S, Pen a A. Inflammatory bowel disease: definition, epidemiology, etiologic aspects, and immunogenetic studies. World J Gastroenterol. 1998;4:446-58. Medline:11819343 doi:10.3748/wjg.v4.i5.s.446
5. Kraneveld AD, Rijne r A, Nijkamp FP, Gars sen J. Neuro-
immune interactions in inflammatory bowel disease and irritable bowel syndrome: future therapeutic targets. Eur J Pharmacol. 2008;585:361-74. Medline:18417115 doi:10.1016/j.ejphar.2008.02.095
6 El-Salhy M, Solomon T, Hausken T, Gilja OH, Hatlebakk JG. Gastrointestinal neuroendocrine peptides/amines in inflammatory bowel disease. World J Gastroenterol. 2017;23:5068-85. Medline:28811704 doi:10.3748/wjg.v23.i28.5068
7 Gershon MD. Serotonin is a sword and a shield of the bowel. Science. 1996;274:1527-31. Medline:8929413 doi:10.1126/science.274.5292.1527
8 Heils A, Teufel A, Petri S, Stöber G, Riederer P, Bengel D, et al. Allelic variants of the serotonin transporter gene regulatory region. Science. 1996;274:1527-31. Medline:8929413 doi:10.1126/science.274.5292.1527
9 Heils A, Teufel A, Petri S, Stöber G, Riederer P, Bengel D, et al. Allelic variation of human serotonin transporter gene expression. J Neurochem. 1996;66:2621-4. Medline:8632190 doi:10.1046/j.1471-4149.1996.66002621.x
10 Nakamura M, Ueno S, Sano A, Tanabe H. The human serotonin transporter linked polymorphism (5HTTLPR) shows ten novel allelic variants. Mol Psychiatry. 2000;5:32-8. Medline:10673766 doi:10.1038/sj.mp.4000698
11 Lesch KP, Wolozin BL, Murphy DL, Riederer P. Primary structure of human platelet serotonin (5HT) uptake site-identity with the brain 5-HT transporter. J Neurochem. 1993;60:2319-22. Medline:7684072 doi:10.1111/j.1471-4149.1993.tb03522.x
12 De Luca V, Tharmalingam S, King N, Straus J, Bulgin N, Kennedy JL. Association study of a novel functional polymorphism of the serotonin transporter gene in bipolar disorder and suicidal behaviour. Psychopharmacology (Berl). 2005;182:128-31. Medline:15986189 doi:10.1007/s00213-005-0046-z
13 Wendland JR, Martin BJ, Kruse MR, Lesch KP, Murphy DL. Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5-HTTLPR and rs25531. Mol Psychiatry. 2006;11:224-6. Medline:16402131 doi:10.1038/sj.mp.4001789
14 Hranilovic D, Stefuji F, Furac J, Kubat M, Balija M, Jeney B. Serotonin transporter gene promoter (5-HTTLPR) and intron 2(VNTR) polymorphisms in Croatian suicide victims. Biol Psychiatry. 2003;54:884-9. Medline:14573315 doi:10.1016/S0006-3223(03)00179-3
15 Batterbye S, Oglivie AD, Smith CA, Blackwood DH, Muir WJ, Quinn JP, et al. Structure of a variable number tandem repeat of the serotonin transporter gene and association with affective disorder. Psychiatr Genet. 1996;6:177-81. Medline:9149321 doi:10.1097/00041444-199624000-00001
16 Sikander A, Sinha SK, Prasad KK, Rana SV. Association of serotonin transporter promoter polymorphism (5-HTTLPR) with microscopic colitis and ulcerative colitis. Dig Dis Sci. 2015;60:887-94. Medline:25532499 doi:10.1007/s10620-014-3482-y
17 Feuerstein JD, Cheffetz AS. Crohn disease: epidemiology, diagnosis, and management. Mayo Clin Proc. 2017;92:1088-103. Medline:28601423 doi:10.1016/j.mayocp.2017.04.010
18 Markeljevic J, Sarac H, Bozina N, Hensigsberg N, Simic M, Cicin Sain L. Serotonin transporter gene polymorphisms: Relation with platelet serotonin level in patients with primary Sjogren's syndrome. J Neuroimmunol. 2015;282:104-9. Medline:25903736 doi:10.1016/j.jneuroim.2015.04.002
19 Rausch JL, Johnson ME, Fei YJ, Li QJ, Shendarkar M, Hoby HM, et al. Initial conditions of serotonin transporter kinetics and genotype: influence on SSRi treatment trial outcome. Biol Psychiatry. 2002;51:723-32. Medline:11983186 doi:10.1016/S0006-3223(01)01283-5
20 Bozina N, Medved V, Kuzman MR, Sain I, Sertic J. Association study of olanzapine-induced weight gain and therapeutic response with SERT gene polymorphisms in female schizophrenic patients. J Psychopharmacol. 2007;21:728-34. Medline:17092963 doi:10.1111/j.1471-4422.2007.01000.x
21 Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD, et al. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. Am J Hum Genet. 2006;78:815-26. Medline:16642437 doi:10.1086/503850
22 Itso K, Yoshida K, Sato K, Takahashi H, Kamata M, Higuchi H, et al. Association of anxiety related traits with a polymorphism in the serotonin transporter gene. J Neural Transm. 2006;113:301-6. Medline:17695343 doi:10.1007/s00702-005-1038-8
23 Liu Z, Shen H, Zhu QM, Dong ZM, Zhao JW, Liang YL, et al. Association of anxiety related traits with a polymorphism in the serotonin transporter gene. J Neural Transm. 2006;113:301-6. Medline:17695343 doi:10.1007/s00702-005-1038-8
24 Shi YY, He L. SHesis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res. 2005;15:97-8. Medline:15740637 doi:10.1038/sj.cr.7200272
25 Faul F, Erdfelder E, Lang AG, Buchner AG. *Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39:175-91. Medline:17693343 doi:10.3758/BF03193146
26 i to K, Yoshida K, Sato K, Takahashi H, Kamata M, Higuchi H, et al. Association study of a neutral polymorphism of the serotonin transporter gene and association with affective disorder. Psychiatr Genet. 1996;6:177-81. Medline:9149321 doi:10.1097/00041444-199624000-00001
27 Shi YY, He L. SHesis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res. 2005;15:97-8. Medline:15740637 doi:10.1038/sj.cr.7200272
28 Faul F, Erdfelder E, Lang AG, Buchner AG. *Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39:175-91. Medline:17693343 doi:10.3758/BF03193146
29. Bozina N, Mihaljević-Peles A, Sagud M, Jakovljević M, Sertić J. Serotonin transporter polymorphism in Croatian patients with major depressive disorder. Psychiatr Danub. 2006;18:83-9. Medline:16804504
30. Noskova T, Pivac N, Nedic G, Kazantseva A, Gaysina D, Fakhudhina G, et al. Ethnic differences in the serotonin transporter polymorphism (S-HTTLPR) in several European populations. Prog Neuropsychopharmacol Biol Psychiatry. 2008;32:1735-9. Medline:18700061 doi:10.1016/j.pnpbp.2008.07.012
31. Gelernter J, Cubells JF, Kidd JR, Pakstis AJ, Kidd KK. Population studies of polymorphisms of the serotonin transporter gene. Am J Med Genet. 1999;88:61-6. Medline:10050969 doi:10.1021/ol980918r1999020588:1-c6-1:AID-AMIG11-3.0.CO;2-K
32. Tada Y, Ishihara S, Kawashima K, Fukuba N, Sonoyama H, Kusunoki R, et al. Downregulation of serotonin reuptake transporter gene expression in healing colonic mucosa in presence of remaining low-grade inflammation in ulcerative colitis. J Gastroenterol Hepatol. 2016;31:1443-52. Medline:26676714 doi:10.1111/jgh.13268
33. Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blazyk H, et al. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology. 2004;126:1657-64. Medline:15188158 doi:10.1053/j.gastro.2004.03.013
34. Shiotani A, Kusunoki H, Kimura Y, Ishii M, Imamura H, Tarumi K, et al. S100A expression and interleukin-10 polymorphisms are associated with ulcerative colitis and diarrhoea predominant irritable bowel syndrome. Dig Dis Sci. 2013;58:2314-23. Medline:23595519 doi:10.1007/s10620-013-2677-y
35. Gill RK, Anbazhagan AN, Esmaili A, Kumar A, Nazir S, Malakooti J, et al. Epidermal growth factor upregulates serotonin transporter in human intestinal epithelial cells via transcriptional mechanisms. Am J Physiol Gastrointest Liver Physiol. 2011;300:G627-36. Medline:21727353 doi:10.1152/ajpgi.00563.2010
36. Porter CK, Cash BD, Pimentel M, Akinseye A, Riddle MS. Risk of inflammatory bowel disease following a diagnosis of irritable bowel syndrome. JAMA. 2015;313:111-2. Medline:25799172 doi:10.1001/jama.2014.14148
37. Bertrand PP, Barajas-Espinosa A, Neshat S, Bertrand RL, Lomax AE. Analysis of real-time serotonin (5-HT) availability during experimental colitis in mouse. Am J Physiol Gastrointest Liver Physiol. 2010;298:G466-S5. Medline:20019165 doi:10.1152/ajpgi.00318.2009
38. Oshima S, Fujimura M, Fukimiya M. Changes in number of serotonin-containing cells and serotonin levels in the intestinal mucosa of rats with colitis induced by dextran sodium sulfate. Histochem Cell Biol. 1999;112:257-63. Medline:10530609 doi:10.1007/s004180050445
51 Wang BM, Wang YM, Zhang WM, Zhang QY, Liu WT, Jiang K, et al. Serotonin transporter gene polymorphism in irritable bowel syndrome. Zhonghua Nei Ke Za Zhi. 2004;43:439-41. Medline:15312441
52 Fiskerstrand CE, Lovejoy EA, Quinn JP. An intronic polymorphic domain often associated with susceptibility to affective disorders has allele dependent differential enhancer activity in embryonic stem cells. FEBS Lett. 1999;458:171-4. Medline:10481059 doi:10.1016/S0014-5793(99)01150-3
53 Hranilovic D, Stefulj J, Schwab S, Bormann-Hassenbach M, Albus M, Jernej B, et al. Serotonin transporter promotor and intron 2 polymorphisms: relationship between allelic variants and gene expression. Biol Psychiatry. 2004;55:1090-4. Medline:15158428 doi:10.1016/j.biopsych.2004.01.029
54 Ali FR, Vasiliou SA, Haddley K, Paredes UM, Roberts JC, Miyajima F, et al. Combinatorial interaction between two human serotonin transporter gene variable number tandem repeats and their regulation by CTCF. J Neurochem. 2010;112:296-306. Medline:19860858 doi:10.1111/j.1471-4159.2009.06453.x
55 Yeo A, Boyd P, Lumsden S, Saunders T, Handley A, Stubbins M, et al. Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women. Gut. 2004;53:1452-8. Medline:15361494 doi:10.1136/gut.2003.035451
56 Sonuga-Barke EJ, Kumsta R, Schlotz W, Lasky-Su J, Marco R, Miranda A, et al. A functional variant of the serotonin transporter gene (SLC6A4) moderates impulsive choice in attention-deficit/hyperactivity disorder boys and siblings. Biol Psychiatry. 2011;70:230-6. Medline:21497794 doi:10.1016/j.biopsych.2011.01.040
57 Steffens DC, Taylor WD, McQuoid DR, Krishnan KR. Short/long heterozygotes at SHTTLPDR and white matter lesions in geriatric depression. Int J Geriatr Psychiatry. 2008;23:244-8. Medline:17702053 doi:10.1002/gps.1869
58 Zhang ZF, Duan ZJ, Wang LX, Yang D, Zhao G, Zhang L. The serotonin transporter gene polymorphism (5-HTTLPR) and irritable bowel syndrome: a meta-analysis of 25 studies. BMC Gastroenterol. 2014;14:23. Medline:24512255 doi:10.1186/1471-230X-14-23
59 Kohen R, Jarrett ME, Cain KC, Jun SE, Navaja GP, Symonds S, et al. The serotonin transporter polymorphism rs25331 is associated with irritable bowel syndrome. Dig Dis Sci. 2009;54:2663-70. Medline:19125330 doi:10.1007/s10620-008-0666-3
60 Farjadian S, Fakhrzadi B, Moenini M, Nasiri M, Fatollahi MR. Serotonin transporter gene polymorphisms in Southwestern Iranian patients with irritable bowel syndrome. Arab J Gastroenterol. 2013;14:59-62. Medline:23820502 doi:10.1016/j.ajg.2013.03.001