Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women


Background and aims: Serotonin (5-hydroxtryptamine, 5-HT) is an important factor in gut function, playing key roles in intestinal peristalsis and secretion, and in sensory signalling in the brain-gut axis. Removal from its sites of action is mediated by a specific protein called the serotonin reuptake transporter (SERT or 5-HTT). Polymorphisms in the promoter region of the SERT gene have effects on transcriptional activity, resulting in altered 5-HT reuptake efficiency. It has been speculated that such functional polymorphisms may underlie disturbance in gut function in individuals suffering with disorders such as irritable bowel syndrome (IBS). The aim of this study was to assess the potential association between SERT polymorphisms and the diarrhoea predominant IBS (dIBS) phenotype.

Subjects: A total of 194 North American Caucasian female dIBS patients and 448 female Caucasian controls were subjected to genotyping.

Methods: Leucocyte DNA of all subjects was analysed by polymerase chain reaction based technologies for nine SERT polymorphisms, including the insertion/deletion polymorphism in the promoter (SERT-P) and the variable tandem repeat in intron 2. Statistical analysis was performed to assess association of any SERT polymorphism allele with the dIBS phenotype.

Results: A strong genotypic association was observed between the SERT-P deletion/deletion genotype and the dIBS phenotype (p = 3.07 \times 10^{-5}; n = 194). None of the other polymorphisms analysed was significantly associated with the presence of disease.

Conclusions: Significant association was observed between dIBS and the SERT-P deletion/deletion genotype, suggesting that the serotonin transporter is a potential candidate gene for dIBS in women.
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Elegant genetic manipulation studies in mice have investigated the latter possibility. The authors found that mice in which SERT was deleted demonstrated a gut function phenotype which was markedly different from wild-type littermates. The transgenic animals displayed altered bowel habit such that either diarrhoea (the majority) or constipation was observed. Interestingly, recent reports have described SERT polymorphisms in the SERT gene which lead to either up- or downregulation of its expression. Such altered expression has thus far been reported in lymphocytes, platelets, and brain tissue, and although similar changes have not yet been investigated in the gut, it is possible that they would be found here also. The possibility exists that such polymorphisms may be present in subtypes of IBS patients, and the predicted resultant changes in 5-HT content (recently reported by Moses and colleagues) or function in these patients may underlie some of their symptomology and indeed response to drugs. To date, two studies have addressed these possibilities: Pata reported no association between SERT polymorphisms and the presence of IBS, regardless of subtype, but identified the short/short (s/s) homozygous polymorphism as a risk factor for a constipation predominant form of the disease (cIBS) and the long/short (l/s) heterozygous polymorphism similarly for dIBS. Camilleri and colleagues demonstrated greater inhibition of colonic transit in response to treatment with the 5-HT3 antagonist alosetron in dIBS patients with the SERT polymorphism genotype which produces increased expression of the transporter and thus presumed enhanced uptake of 5-HT. Although this study was not aimed at investigating disease association, another study from the same group, which has been reported in abstract form, suggested a trend towards significance for the odds ratio for the s/s polymorphism (versus the heterozygous genotype) to be associated with the presence of cIBS.

The SERT gene encoding the SERT protein is located on chromosome 17q11.2-q12. The main gene transcript of 2.5 kilo base pairs (kb) (Ensembl Accession No L05568) contains 14 exons which span approximately 31 kb of genomic DNA transcript. Some evidence of alternative transcript splicing has been reported in the literature. One of the first SERT polymorphisms characterised was the variable number of tandem repeats (VNTR) in intron 2 of the gene and consists of 17 base pair (bp) repeats. The SERT gene linked polymorphic region (SERT-P), originally known as 5HTT-LPR, was first reported in 1996 by Heils and colleagues. It consists of a 44 bp insertion/deletion in the gene creating long (l) and short (s) allelic variants, respectively. The polymorphism is located within a GC rich region composed of 20–23 bp repeat units with the s and l alleles having 14 and 16 repeats, respectively. The same study also reported evidence for allele dependent differential SERT promoter activity. Additional work has shown that cells homozygous for the insertion allele (l/l) exhibit a higher uptake of serotonin compared with those having one or two copies of the deletion allele (l/s or s/s). Therefore, this suggests that the presence of one or more copies of the s allele reduces not only the level of transcription but also levels of protein expression and reuptake of serotonin. Both the VNTR and SERT-P polymorphisms have been extensively investigated in genetic association studies for various complex behavioural traits and disorders such as bipolar disorder, obsessive-compulsive disorder, autism, schizophrenia, substance abuse, and eating disorders, and perhaps more interestingly with respect to the present study, in fibromyalgia, anxiety, and depression, which are thought to be comorbid with IBS.

There are few literature reports to date showing genetic evidence of a genetic risk factor in IBS (see above), although several twin studies based on questionnaires have shown that there is such a factor for the development of IBS. In the present study, we therefore aimed to investigate the association of the IBS phenotype with nine SERT polymorphisms in a large population of IBS patients and controls.

MATERIALS AND METHODS

Patient and control population

A total of 194 samples from North American Caucasian IBS patients were used in this study. These patients were a subset of subjects who participated in the GlaxoWellcome phase III clinical trials (S3BA3001 and S3BA3002) for the ala
troehydrochloride compound (Lotronex) and were willing to participate in the genetic substudy after providing fully informed consent. All patients were females (age range 18–83 years) with a history of IBS (diarrhoea as the major bowel habitue). These subjects had at least six months of recurrent symptoms that fulfilled the Rome I criteria for IBS, had normal sigmoidoscopic/colonoscopic results within five years and who, during a two week baseline period, had daily mild to severe abdominal pain/discomfort, and an average stool frequency of greater than 2.5 normal to watery stools/day. Patients were excluded if they had an unstable medical disorder, another gastrointestinal disorder, a major psychiatric disorder, or substance abuse within the previous two years. Further details of the study population and results of these two trials are published elsewhere. No samples from patients with cIBS were collected in these clinical trials.

A panel of 448 North American Caucasian random female control samples was collected from various sources (table 1). As the IBS status for these samples was largely unknown, the number of control samples collected was higher than the patient collection, allowing for an incidence of IBS of up to 20% in the general population. As the population was not selected on the basis of their IBS status, age of onset matching was not considered necessary.

Single nucleotide polymorphism (SNP) identification

All available genomic sequence (both exonic and intronic) surrounding the SERT exons (including 10 kb 5’ and 3’ flanking sequence) was amplified by polymerase chain reaction (PCR) in overlapping fragments on 30 random female Caucasian DNA samples (healthy volunteers with full consent) followed by direct sequencing of the product. The resulting sequence for these samples were aligned to allow for the detection of polymorphisms in each amplified fragment of the gene. Approximately 18 kb of genomic sequence was screened in total and a total of 17 SNPs were identified. Seven of these were selected for genotyping on the...
basis of their minor allele frequency (>5%) in the 30 samples analysed (table 2).

**Assay development and genotyping**

TaqMan allelic discrimination (5’ nuclease extension) assays were designed for the seven selected SNPs using the Primer Express program from Applied Biosystems. In AT nucleotide rich regions of the gene, TaqMan Turbo probes were utilised to increase the effective annealing temperature. Details of probes (Applied Biosystems, Foster City, California, USA) and primers used can be found in table 3. All assays were performed in an 8 µl reaction volume, except for the 5HTT-G674A assay where the reaction volume was 25 µl. The standard reaction mixture contained 8 ng of genomic DNA (25 ng for assay 5HTT-G674A), 0.6× universal PCR mastermix (Applied Biosystems), 900 nmol/l of each primer, and 100 nmol/l of both VIC- and FAM-fluorescently labelled probes (200 nmol/l of each Turbo probe used for assays 5HTT-G160A and G769T). All assays were processed using the following standard cycling conditions: 50 °C for two minutes, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds plus 62 °C for 60 seconds. When cycling was finished, reactions were kept at 15 °C until plates were finished, reactions were kept at 15 °C until plates were analysed on an ABI7700 TaqMan machine using the SDS1.7 software. The 17 bp VNTR in intron 2 was analysed by PCR using the primers detailed in table 3. Amplification was performed in a 25 µl reaction volume containing 50 ng of genomic DNA,
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1 x Perkin-Elmer PCR buffer (no MgCl2), 1.5 mmol/l MgCl2, 0.2 mmol/l deoxynucleotide triphosphates, 10 pmol of each primer, and 1 unit of AmpliTaq Polymerase (Perkin-Elmer, Boston, Massachusetts, USA). DNA was denatured at 94°C for four minutes and subjected to 35 cycles of 94°C for one minute, 60°C for one minute, and 72°C for one minute, followed by a final extension at 72°C for 10 minutes. Amplification products (10 μl) were resolved by electrophoresis on 4% Nusieve 3:1 agarose gels next to a 1 kb DNA molecular weight standard (Life Technologies, Carlsbad, California, USA) and visualised with ethidium bromide staining. The expected product sizes for alleles 9, 10, 11, and 12 are 253 bp, 270 bp, 287 bp, and 304 bp, respectively.

The 44 bp insertion/deletion polymorphism in the promoter region (SERT-P) was analysed by PCR using the primers detailed in table 3. Amplification was performed in a 25 μl reaction volume containing 200 ng of genomic DNA, 1 x Perkin-Elmer PCR buffer (no MgCl2), 1.5 mmol/l MgCl2, 0.2 mmol/l deoxyadenosine triphosphate, 0.2 mmol/l deoxycytidine triphosphate, 0.2 mmol/l deoxythymidine triphosphate, 0.1 mmol/l deoxyguanosine triphosphate, 0.1 mmol/l 7-deaza-deoxyguanosine triphosphate, 20 pmol of each primer, 5% DMSO, and 0.5 unit of AmpliTaq Polymerase (Perkin-Elmer). DNA was denatured at 95°C for four minutes and subjected to 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for one minute followed by a final extension at 72°C for seven minutes. Amplification products (25 μl) were resolved by electrophoresis on 3.5% Nusieve 3:1 agarose gels next to a 1 kb DNA molecular weight standard (Life Technologies) and visualised with ethidium bromide staining. The expected product sizes for the deletion (del or s) and insertion (ins or L) alleles are 484 bp and 528 bp, respectively.

Statistical analysis
Testing for Hardy-Weinberg equilibrium (HWE)
The genotype distribution for all SNPs for both cases and controls were tested for HWE using a χ² test (comment: the test was done for the number of expected genotypes, not the frequencies).

Association test
χ² tests were carried out to test for association between allele and phenotype (allelic association) and also between genotype and phenotype (genotypic association).

Odds ratio
Odds ratio and confidence intervals were estimated using SAS software.6 We applied the methods described in chapter 2 of Stokes and colleagues,25 specifically we used the “Proc Freq” procedure using the “Measures” option. Data for the del/ins and ins/ins genotypes were collapsed into one group to form a 2×2 table of “del/del” and “non del/del” individuals against disease status.

Linkage disequilibrium (LD)
LD is a measure of the association between alleles at different loci. LD tends to decline with distance between polymorphisms and generally exists for polymorphisms that are less than 100 kb apart. LD was measured using r² and D’ statistics.26

RESULTS
Genotype analysis of SERT polymorphisms
As the availability of SNPs in the public domain was limited when this study was performed, SNPs were isolated by re-sequencing all available genomic (exonic, intronic, untranslated region plus 10 kb flanking sequence) in 30 random female Caucasian DNA samples. A total of 17 SNPs were identified and a total of seven SNPs (available in the dbSNP database http://www.ncbi.nlm.nih.gov/SNP/index.html; see table 2 for SNP reference ID) were selected for genotyping on the basis of their minor allele frequency (˃5%). These seven SNPs were screened using the TaqMan 5’ nuclease extension assay. PCR based assays were used to screen SERT-P and VNTR polymorphisms, which were performed in duplicate to control for genotyping error. A total of 194 IBS patient samples and 448 control samples were analysed for the nine polymorphisms (table 2).

Association between IBS disease phenotype and SERT genotypes
Statistical analyses were performed to investigate the association between genotypes and the dIBS phenotype.

Genotype distribution for all polymorphisms was checked for deviation from HWE. Deviation was observed for the 5HTT-VNTR assay in the control study population (p = 0.0084). As this could suggest a problem with the assay, data related to this assay were excluded from further analysis.

Association analyses were performed for the eight remaining polymorphisms. The strongest association was observed between the s/s genotype of the SERT-P polymorphism and the dIBS phenotype (p = 3.07×10⁻⁴). Table 4 shows the genotype distribution between dIBS cases and controls for this polymorphism. The percentage of s/s genotypes was nearly double in dIBS cases compared with controls at the expense of the heterozygote genotypes. The odds ratio for these data was 2.23 for s/s individuals versus non–s/s individuals with 95% confidence limits of 1.51–3.31, respectively, providing further statistical support for an association between the s/s genotype and the dIBS phenotype. Although deviation from HWE was also observed for the SERT-P polymorphism, this was seen for dIBS cases only (p = 3.1×10⁻⁴) and could be an effect of the genotypic disease association observed or the functional nature of this polymorphism. No association was observed with any of the other SERT polymorphisms.

LD analysis was performed to identify how many of the polymorphisms analysed in this study share the same block of LD (data not shown). The D’ measurement of LD indicates that there may be breakdown of LD between the SERT-P and 5HTT-G674A polymorphisms. Also, none of the other polymorphisms are in perfect LD (r >0.9) with the SERT-P polymorphisms. In fact, the only high r measurement (>0.8) was observed between polymorphisms 5HTT-G160A and G769T.

Although association between the dIBS phenotype and a combination of two or more SERT polymorphism alleles (also called haplotypes) was also studied, none of the haplotypes showed a more significant association compared with the single polymorphism p values reported here (data not shown).

Table 4: Genotype distribution and associated odds ratio for the SERT-P polymorphism in diarrhoea predominant irritable bowel syndrome (dIBS) cases and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>dIBS cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>s/s</td>
<td>77 (17.2%)</td>
<td>61 (31.4%)</td>
</tr>
<tr>
<td>l/s</td>
<td>214 (47.8%)</td>
<td>62 (31.9%)</td>
</tr>
<tr>
<td>l/l</td>
<td>138 (30.8%)</td>
<td>63 (32.8%)</td>
</tr>
<tr>
<td>No genotype obtained</td>
<td>19 (4.2%)</td>
<td>8 (4.1%)</td>
</tr>
</tbody>
</table>

Odds ratio (95% CI) for s/s v non-s/s genotypes: 2.23 (1.51–3.31)
DISCUSSION

To date there has been little evidence for the involvement of genetic factors in the development of IBS. Although there have been indications from non-genetic studies that there may be familial associations in adults with IBS, including some twin studies,\(^44\) no candidate gene has been associated with any IBS phenotype. Recently, Pata et al\(^44\) reported no association between SERT-P polymorphisms and the presence of IBS, but did suggest the short/short \((s/s)\) homozygous polymorphism as a risk factor for the constipation predominant form of the disease and the long/short \((l/s)\) heterozygous polymorphism similarly for dIBS. Interestingly, the \(s/s\) and \(l/s\) variants have both been shown to lead to less transporter protein expression and less serotonin reuptake.\(^20\) 21 and thus the apparently opposing associations with cIBS and dIBS reported are difficult to interpret based on predicted functional consequences of the polymorphisms. The authors noted that these conclusions were drawn from very small samples \((n = 26\) for cIBS; \(n = 18\) for dIBS) and as such interpretation of the results should be limited. However, in their pilot study involving 173 patients with IBS (mixed subgroup population) or chronic abdominal pain, Kim and colleagues\(^26\) also determined a trend towards an association between the \(s/s\) polymorphism and the constipated (IBS) phenotype. It is not immediately obvious how a polymorphism that produces a reduced level of functional reuptake transporter, and elevated 5-HT levels, results in a constipated phenotype but plausible mechanisms do exist (see below).

In stark contrast with these earlier studies, the present data from a North American study population provide evidence of an association between the \(s/s\) genotype of the SERT-P polymorphism and dIBS, which is perhaps somewhat more intuitive based on the predicted functional consequence of raised levels of 5-HT in the bowel. This study represents the first evidence for a statistically significant association between the dIBS phenotype and a polymorphism in the SERT gene. It is not known at this time whether the commonly used diagnostic distinction of IBS sufferers into constipation predominant, diarrhoea predominant, or alternators, has any true physiological or genetic basis, but further studies of this type will surely help to define this more precisely.

In our study, the distribution of the genotypes in the dIBS cases clearly reflects an increase of the \(s/s\) genotype when compared with the control population. From the allelic analyses, no such association with the phenotype was found, suggesting that two alleles are required to see the effect of association. In this study and others,\(^24\) the distribution of the genotypes in the general population is biased towards the heterozygous \(l/s\) form, with the \(s/s\) genotype being least frequent, accounting for only approximately 18%. The increased risk of carrying this genotype in the dIBS disease population appears to be at the expense of the more common heterozygous form as \(l/s\) frequency is reduced and that of the \(l/l\) form is unchanged. This may explain in part the lack of allelic association described above.

The few studies that have been performed in vitro to date suggest that the \(s\) allele is dominant, such that both the heterozygous \((l/s)\) and homozygous \((s/s)\) genotypes result in reduced transporter expression and thus function, compared with the \(l/l\) genotype.\(^20\) \(^21\) Therefore, if we hypothesise that such reduced SERT expression results in dIBS symptoms, then both genotypes containing the \(s\) allele should be associated with the presence of the disease. This was not what we found; indeed, analysis of the differences between the frequency of combined \(s/s+l/s\) relative to the \(l/l\) genotype was not performed as the genotype frequencies of these two groups were virtually identical and therefore quite obviously not significantly different. The important point to stress from our analysis, as mentioned above, is the greater proportion of \(s/s\) genotypes in the disease population compared with controls, apparently resulting from a shift from the \(l/l\) form. The overall risk of bearing a copy of the \(s\) allele is unchanged in the disease but rather the shift from heterozygous to homozygous forms appears to be the more relevant factor. Perhaps the small trend for a greater functional consequence of the homozygous genotype in vitro (Lesch et al described a greater significance reported for the \(s/s\) to \(l/l\) comparison than for the \(l/s\) to \(l/l\) comparison) could help us understand these disease phenotype findings. However, direct correlation between in vitro findings in isolated cells and disease symptom expression is a huge leap and probably not a sensible link to try to claim.

Genotyping for the SERT-P polymorphism was performed in duplicate for all samples and the genotypes were scored in duplicate by different individuals, and thus it is unlikely that these results are due to genotyping error. This study has not determined how far the block of LD containing the SERT-P polymorphism extends upstream of the SERT gene and no LD map is available for the area surrounding the gene. Therefore, we cannot rule out the possibility that another polymorphism upstream of the SERT gene, which is in high LD with the SERT-P polymorphism, may also be associated with the dIBS phenotype. The genotypic association is also reflected in the HWE deviation observed for dIBS cases, and this method of detecting disease association in a heterogeneous disease such as IBS has been reported in the literature.\(^56\)

There can be no doubt that genetic studies on populations collected during clinical trials are unfortunately far from perfectly structured due to the strict limitation on the individuals that can be collected and their willingness to be involved in a genetic analysis. In the case of this IBS study, it was not possible to collect data from cIBS patients and so the analysis and conclusions are restricted solely to the dIBS population. Collection of matched controls was also problematic but this problem was addressed by increasing the number of controls used to over twice the number of cases. We have demonstrated (unpublished data) that when matched controls are not available, an increased number of controls from a general ethnic match increases study power and suppresses confounding effects such as population stratification and admixture. However, as the SERT-P polymorphism is known to show frequency variability between ethnic groups,\(^37\) \(^38\) additional studies based around the genetic hypothesis formulated in this work should be undertaken to replicate the association demonstrated.

It is interesting to speculate how the evidence of association between the \(s/s\) genotype of the SERT-P polymorphism with diarrhoea predominant IBS might provide some insight into the underlying pathophysiology of the disease, at least in this subgroup of female patients. As described above, reduced transcription of the 5-HT transporter would be expected to lead to enhanced levels of 5-HT in the bowel of these patients, which in turn might be predicted to result in increased bowel motility and secretion, and subsequent diarrhoea; a situation not dissimilar to that described for SERT knockout mice.\(^19\) Moreover, as observed in the SERT\(^−/−\) mice, it is intriguing to consider the possibility that such increased 5-HT may also lead to a constipated phenotype, as suggested by earlier studies.\(^24\) \(^26\) This may occur following 5-HT receptor desensitisation in the presence of high levels of agonist for long periods, or indeed could be produced by excessive stimulation of the 5-HT receptor bearing enteric neurones such that the normal motility pattern is stimulated to such an extent that coordination of enteric reflexes breaks down and contractile and secretory function fail. At a cellular level, such excessive neuronal depolarisation may result in “depolarisation block”. In this condition, the neuronal membrane
potential remains depolarised above the threshold for sodium channel activation, and further action potential generation is not possible. In this way, varying degrees of excitation of enteric neurones might contribute to both hyper- and hypomotility and secretion.

The potential effects of reduced uptake of 5-HT are not however restricted to the neurones of the enteric nervous system. Excessive or prolonged stimulation of extrinsic afferents may also result in the development of neuronal sensitisation, at peripheral, spinal, or higher CNS levels, such that perception of sensations from the bowel is heightened, resulting in symptoms of urgency, bloating, and pain. Such neuronal hypersensitivity is thought to be a key factor in the generation of sensory IBBS symptoms, and can be mimicked in animals by repetitive physical stimulation of the gastrointestinal tract, which may be inappropriately enhanced. This procedure by which sensory perception of events in the gut may not be based on genuine absorptive or secretory function, but rather on the perception of movement. However, in this way, varying degrees of excitation of extrinsic afferent nerves might contribute to either intrinsic afferent nerves by excessive or prolonged stimulation of extrinsic afferents which may then further develop into a state of hypomotility and secretion.

This study shows that the SERT polymorphism, or a polymorphism in linkage disequilibrium with the SERT polymorphism, may play a role in the development of IBS. We do not believe that the reduction in SERT is the sole cause of dIBS in our population but we do believe it might be a contributing factor. Perhaps a combination of this polymorphism with many other factors, such as raised ECG cell number, could explain the variety of symptom patterns observed across the disease population. The recent finding of reduced SERT expression in IBS patient biopsies, coupled with our own findings, does encourage speculation that there is a role for altered transporter function in the disease. While research continues into the variety of possible factors which initiate, maintain, and exacerbate the symptoms suffered in IBS, the data reported in the present study clearly provide further support for the key role of the serotonin system in IBS pathophysiology and will hopefully stimulate further effort towards identifying and understanding further potential genetic and other factors that might contribute to the disease.

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Conflict of interest: All authors are or were employees of GlaxoSmithKline.

REFERENCES


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