First evidence for an association of a functional variant in the microRNA-510 target site of the serotonin receptor-type 3E gene with diarrhea predominant irritable bowel syndrome

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Diarrhea predominant irritable bowel syndrome (IBS-D) is a complex disorder related to dysfunctions in the serotonergic system. As cis-regulatory variants can play a role in the etiology of complex conditions, we investigated the untranslated regions (UTRs) of the serotonin receptor type 3 subunit genes HTR3A and HTR3E. Mutation analysis was carried out in a pilot sample of 200 IBS patients and 100 healthy controls from the UK. The novel HTR3E 3'-UTR variant c.76G>A (rs62625044) was associated with female IBS-D (P = 0.033, OR = 8.53). This association was confirmed in a replication study, including 119 IBS-D patients and 195 controls from Germany (P = 0.0046, OR = 4.92). Pooled analysis resulted in a highly significant association of c.76G>A with female IBS-D (P = 0.0002, OR = 5.39). In a reporter assay, c.76G>A affected binding of miR-510 to the HTR3E 3'-UTR and caused elevated luciferase expression. HTR3E and miR-510 co-localize in enterocytes of the gut epithelium as shown by in situ hybridization and RT–PCR. This is the first example indicating micro RNA-related expression regulation of a serotonin receptor gene with a cis-regulatory variant affecting this regulation and appearing to be associated with female IBS-D.

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal (GI) disorder affecting up to 20% of the population (1), in which patients report abdominal pain or discomfort associated with disordered defaecation or change in bowel habit. Patients can present with either constipation (IBS-C) and diarrhea (IBS-D) or a mixture of both (IBS-M).

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Notably, women are twice as likely to be affected as men (2) and a high proportion of IBS patients are reported to show comorbidity with psychiatric conditions like anxiety or depression (3,4). The disorder accounts for nearly half of gastroenterology clinic referrals, markedly reduces quality of life and treatment remains far from satisfactory. The lack of adequate treatment and the unsuccessful development of new drugs stem from poor understanding of its pathophysiology. Nevertheless, there is evidence of an altered GI motility, with some studies reporting slow GI transit, reduced motility and incidence of high amplitude propagating contractions in IBS-C (5). In IBS-D, accelerated transit, increased motility and high amplitude propagating contractions were described (6). Other studies have suggested that approximately two-thirds of IBS patients may also be more viscerally sensitive to intra-luminal events such as distension than healthy subjects, with slightly more patients with IBS-D being affected than IBS-C (7). Why some but not all patients exhibit this phenomenon remains unknown, but evidence is emerging of a possible genetic link to these phenotypic variations. Family studies provide strong evidence for a clustering of functional bowel disorders in families. Furthermore, twin studies clearly demonstrate an increased concordance rate in monoyzotic compared to dizyoztic twins. Thus, hereditary factors are likely to be involved in the etiology of IBS (8–10), with recent studies estimating this to be ~50% in females (11).

The 5-HT3 receptor is a Cys-loop ligand-gated ion channel composed of five subunits (12). It is an important mediator of the action of 5-HT, and has been shown to play a key role in the motor-sensory function of the gut (13). In the GI tract, 5-HT3 receptors are located on peripheral nerve terminals of both vagal and spinal primary afferent neurons innervating the gut, as well as on myenteric and submucosal neurons. They have also been described in the spinal cord and throughout the brain, mostly in the limbic and cortical regions. Importantly, the 5-HT3 receptor antagonist alosetron is effective in reducing pain by maybe its interaction with receptors in the spinal cord and/or the brain. However, not all patients respond to this agent, particularly males, which might be explained by the genetic variability of 5-HT3 receptor genes.

To date, five human 5-HT3 receptor subunit genes have been isolated: HTR3A, HTR3B, HTR3C, HTR3D and HTR3E (14–16). The 5-HT3A subunit seems to have a key function in the formation of 5-HT3 receptors since it is the only subunit that can form functional homopentamers. In contrast, all other subunits form functional heteromers when co-expressed with the 5-HT3A subunit (15,17). Expression analyses of all five subunit genes have revealed that the 5-HT3E subunit is exclusively expressed in GI tissues-like colon, small intestine and stomach, whereas the other subunits are more ubiquitously expressed (16,18). This indicates that the 5-HT3E subunit may play a distinct and specific role in the formation and function of 5-HT3 receptors in the human GI tract.

Genetic variations that alter gene expression cause phenotypic diversity and play an important role in disease susceptibility especially with regard to complex conditions (19). As evidence accumulates that cis-regulatory variants in the untranslated regions (UTRs) of genes may have a significant impact on gene expression (20,21), the aim of this study was to investigate whether variants in the UTRs of the 5-HT3 receptor genes HTR3A and HTR3E may be predisposing or contributing to the clinical signs of IBS with diarrhea.

RESULTS

Sequence variants HTR3E c.*76G>A and HTR3A c.-42C>T are associated with IBS-D

We analyzed the 5'- and 3'-UTR of HTR3A and HTR3E in DNA samples of 100 IBS-D, 100 IBS-C patients and 100 healthy controls in a pilot cohort from the UK. Using dHPLC and direct sequencing of the generated PCR products, four sequence variants for HTR3A were identified. Two of these were located in the 5'-UTR (c.-42C>T, c.-25C>T) and two in the 3'-UTR (c.*70C>T, c.*503C>T). For HTR3E, one variant located in the 5'-UTR (c.-189G>A) and four in the 3'-UTR (c.*76G>A, c.115T>G, c.*138C>T, c.*191T>C) of the gene were found (Supplementary Material, Table S1). Statistical analyses on the genotype frequencies obtained for the identified variants of both genes were performed to determine whether there are significant differences between the two IBS subgroups and healthy controls.

We found the novel HTR3E variant c.*76G>A (rs62625044) and the HTR3A variant c.-42C>T (rs10626213) associated with the IBS-D phenotype of the disease.

The heterozygous genotype of the HTR3E c.*76G>A variant was more frequent in female IBS-D patients compared to female healthy controls [P = 0.033, odds ratio (OR)=8.53, 95% CI = 1.04–70.28] or female IBS-C patients [P = 0.125, OR = 3.03, 95% CI = 0.88–10.52] or compared to the pooled group of non IBS-D females (P = 0.010, OR = 4.13, 95% CI = 1.30–13.14), whereas there were no genotype frequency differences between female IBS-C patients and female healthy controls (Table 1). There were no significant differences between male IBS patients and healthy males or between patients of both sexes compared to controls of both sexes (Supplementary Material, Table S3). For the HTR3E c.*76G>A variant, no deviation from the Hardy–Weinberg equilibrium (HWE) was detected in the IBS patients or the healthy controls. In a replication study, we genotyped c.*76G>A in an independent cohort of 119 German IBS-D patients and 195 healthy controls and confirmed the association of this variant in female IBS-D patients in the German cohort (P = 0.0046, OR = 4.92, 95% CI = 1.49–16.30; Table 1). The association remained significant after Bonferroni correction for testing two variants in the German sample (P = 0.0092). There were no significant c.*76G>A frequency differences in German male patients compared to healthy males and the comparison of c.*76G>A in German patients and controls of both sexes (Supplementary Material, Table S3) resulted in less significant results than the comparison of just females. Pooled analysis of c.*76G>A in female IBS-D patients from both countries resulted in a highly significant association (P = 0.0002, OR = 5.39, 95% CI = 1.90–15.28, adjusted for cohort;Table 1). We performed retrospective power calculation for the pooled cohort of IBS-D and control females using a
miR-510 interaction, we co-transfected the 3′-UTR c.*76G allele constructs (100%; $P = 0.0002$, OR = 8.53, CI = 1.04–70.28) in a minor allele dominant model. Frequencies (in%) were rounded up or down to the nearest whole number. UK, United Kingdom.

The HTR3E c.*76G>A variant disrupts the binding site for miR-510 and significantly increases protein expression level in HEK293 and Colo320 cells

To investigate putative functional consequences of the HTR3E c.*76G>A variant, we cloned the full-length 3′-UTR of HTR3E carrying the c.*76G or c.*76A allele downstream of a luciferase reporter gene. Both constructs were transfected into colon carcinoma cells Colo320, but no difference in luciferase activity was detected (data not shown). By in silico analysis of miRNA binding sites using miRBase, we were able to identify a putative binding domain for hsa-miR-510 (miR-510; MI0003197) covering bases −80 downstream of the stop codon of HTR3E (Fig. 1A). The calculated score of 19.12 (22) for putative binding of miR-510 was the highest of 41 predicted miRNAs with calculated scores in the range of 13.94–19.12. To confirm a putative binding of miR-510 to HTR3E, we co-transfected the 3′-UTR c.*76G construct with different concentrations of miR-510 precursor molecules or same amounts of negative control miRNA (Ambion). Higher concentrations of miR-510 (40 or 100 nM, but not 4 nM) led to a significant reduction of luciferase activity to 55–60% compared to negative control miRNA (100%; $P < 0.001$) (Fig. 1B). These findings confirm the predicted binding of miR-510 to the 3′-UTR of HTR3E and demonstrate a dose-dependent reduction of reporter gene expression. Since the binding site for miR-510 includes the sequence variant c.*76G>A, we next investigated if the presence of the c.*76A variant allele interferes with the ability of miR-510 to interact with the 3′-UTR of HTR3E. We co-transfected the HTR3E 3′-UTR c.*76G and c.*76A luciferase constructs with 40 nM of miR-510, negative control miRNA or anti-miR-510 precursor molecules into Colo320 and HEK293 cells. The anti-miR-510 precursor molecules are single-stranded RNA molecules which specifically knock-down endogenous miR-510. As predicted, the c.*76A variant constructs co-transfected with miR-510 showed significantly higher (~180%) luciferase expression compared to c.*76G allele constructs (100%; $P < 0.001$) in HEK293 cells, which do not endogenously express the 5-HT3E receptor subunit (Fig. 2B). We confirmed these findings in 5-HT3E expressing Colo320 cells (Fig. 2C). In both cell lines, no significant luciferase activity differences exist when co-transfecting negative control or anti-miR-510 precursor molecules.

<table>
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<tr>
<th>Genotype</th>
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<th>c.*76G/c.*76G</th>
<th>c.*76G/c.*76A</th>
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<td>3 (3%)</td>
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<td>0 (0%)</td>
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<td>(IBS-C+controls; n = 160)</td>
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<td>10 (13%)</td>
<td>0 (0%)</td>
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<td>Controls Germany (n = 132)</td>
<td>128 (97%)</td>
<td>3 (2%)</td>
<td>1 (1%)</td>
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<td>IBS-D pooled (n = 143)</td>
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<td>18 (13%)</td>
<td>0 (0%)</td>
<td></td>
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<tr>
<td>Controls (n = 197)</td>
<td>192 (97%)</td>
<td>4 (2%)</td>
<td>1 (1%)</td>
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</table>

Values indicate number of patients and healthy controls with the respective genotype. Odds ratios (OR), 95% confidence intervals (CI) and $P$-values calculated using the Fisher’s exact test ($P_{F}$) or the $\chi^2$ test ($P_x$) in a minor allele dominant model. Frequencies (in%) were rounded up or down to the nearest whole number.
The HTR3E c."76G>A variant does not affect HTR3E mRNA levels in Colo320 cells

We performed quantitative real-time PCR to assess mRNA levels of HTR3E in Colo320 cells transfected with the pcDNA3 HTR3E-Myc-3'-UTR c."76G or c."76A construct in combination with miR-510 or negative control miRNA precursor molecules. No differences in the HTR3E mRNA levels (normalized to neomycin transferase mRNA levels) were detectable for any combination of transfected constructs (Supplementary Material, Fig. S1). This indicates that the binding of miR-510 to the HTR3E mRNA does not seem to affect mRNA transcription levels or mRNA stability, but decreases gene expression at the translational level.

HTR3A, HTR3E and miR-510 are co-expressed in enterocytes and myenteric plexuses of the human colonic mucosa

To further investigate the interaction of miR-510 and HTR3E seen by in vitro analyses, we performed in situ hybridization on human colon tissue sections to check for overlapping expression of these two genes and, in addition, also for HTR3A expression. We found all three genes to be co-expressed specifically in enterocytes of the colonic mucosa (Fig. 3) as well as in myenteric plexuses (not shown). The co-expression of 5-HT3A and 5-HT3E in the respective cells was confirmed by immunofluorescence experiments using specific antibodies (Fig. 3). These results indicate that the expression of the 5-HT3E subunit in vivo can be controlled by the co-expressed miR-510 and that 5-HT3 receptors located in the investigated region comprise 5-HT3A and 5-HT3E subunits.

Confirmation of HTR3E and miR-510 co-expression in enterocytes of the colonic mucosa by RT–PCR

Microdissected tissue of the epithelial layer of human colonic mucosa was analyzed by RT–PCR. We found HTR3E and miR-510 both expressed in enterocytes, whereas no expression of HTR3E and only low expression of miR-510 were detectable within the lamina propria (Fig. 4).

The HTR3A c.-42C>T variant is associated with IBS-D in UK patients and causes elevated 5-HT3A receptor density in membranes of transfected HEK293 cells

Applying a minor allele dominant model, the HTR3A c.-42C>T variant was significantly more frequent in UK
IBS-D patients compared with both the healthy control \( (P = 0.020, \ OR = 2.01, \ 95\% \ CI = 1.11–3.63) \) and IBS-C subgroups \( (P = 0.034, \ OR = 1.89, \ 95\% \ CI = 1.05–3.40) \) or compared with a pooled group of non-IBS-D individuals (IBS-C and controls; \( P = 0.009, \ OR = 1.95, \ 95\% \ CI = 1.18–3.22) \) (Table 2). After Bonferroni correction for testing nine variants in this cohort, none of the findings remained formally significant. There were no genotype frequency differences in IBS-C patients compared to healthy controls. The results of the analysis of c.-42C>T in IBS patients and controls categorized by gender are given in Supplementary Material, Table S4. The power calculation for the sample of UK IBS-D patients and controls resulted in a value of 0.63 using an allele frequency of 0.15 and an odds ratio of 2.01. The association of c.-42C>T with IBS-D could not be confirmed in a replication study with German IBS-D patients and controls. No deviation from the HWE was present in the IBS-subgroups or in the control group for the c.-42C>T genotypes. With regard to statistics, the association of the c.-42C>T variant with IBS-D appears weaker compared to the HTR3E c.*76G>A variant, nevertheless it seems to have the same functional consequences with regard to expression regulation. In a previous study, we demonstrated that the presence of the c.-42T allele in the 5'-UTR of HTR3A causes higher luciferase reporter

**Figure 3.** 5-HT3A, 5-HT3E and miR-510 are co-expressed in enterocytes of human colon sections. Expression of HTR3A, HTR3E and miR-510 as detected by *in situ* hybridization. Neg. contr., scramble miRNA hybridization (Exiqon). 5-HT3A and 5-HT3E (both in green) subunit expression as detected by immunofluorescence analysis.
gene expression levels compared to the c.-42C allele (23). Here, we performed radioligand binding assays using HEK293 cells transiently transfected with the pcDNA3 HTR3A 5'-UTR c.-42C or c.-42T constructs (Fig. 5A) to investigate differences in binding of the 5-HT3 receptor radioligand [3H]GR65630. Maximum binding capacity (Bmax) for cells expressing the homomorphic c.-42T variant was significantly higher (142 ± 17%, n = 5) than for cells expressing the homomorphic c.-42C allele receptor (Fig. 5B), whereas radioligand affinity remained unchanged (data not shown). These findings confirm our previous results and furthermore implicate that the c.-42C>T variant results in elevated expression levels of 5-HT3A subunits, which can be interpreted as a higher homomeric receptor density on the cell surface.

**DISCUSSION**

This study has identified the functional HTR3E c.*76G>A variant to be associated with the diarrhea phenotype of IBS in females and delivered first evidence for a microRNA regulated expression of 5-HT3 receptor genes. The variant showed highly significant association with female IBS-D in two independent cohorts from the UK and Germany. This underlines the possible impact of c.*76G>A in the etiology of female IBS-D while the relevance of the HTR3A c.-42C>T variant remains questionable as it failed replication in the German patients. In vitro studies showed that both variants lead to an increased expression of the 5-HT3A and 5-HT3E subunits at the translational level by affecting cis-regulatory mechanisms. Luciferase assays revealed that the presence of a miR-510 binding site in the 3'-UTR of HTR3E significantly reduces expression of the luciferase carrying the c.*76G allele 3'-UTR. This confirmed the predicted functional interaction of miR-510 with the HTR3E mRNA and indicates regulation of 5-HT3E subunit expression by miR-510 in vivo. MiRNAs typically bind to the 3'-UTR of their targets and repress translation. It is hypothesized that regulation by miRNAs is responsible for adjustment of gene dosage within the cell. It is currently estimated that miRNAs account for ~1% of predicted genes in higher eukaryotic genomes and that up to 30% of genes might be regulated by miRNAs. However, only very few miRNAs have been functionally characterized in detail (24,25). Recently, SNPs residing in miRNA-binding sites were shown to affect the expression of miRNA targets and contribute to the susceptibility to complex disorders such as cancer, asthma, cardiovascular disease and Tourette syndrome (26–30). In the present study, the c.*76G>A variant diminishes the ability of miR-510 to bind to the 3'-UTR of HTR3E, thereby protecting the mRNA from translational repression. As HTR3E is exclusively expressed in the GI tract, the upregulation of gene expression caused by c.*76G>A may specifically affect 5-HT3 receptors in the digestive system of variant carriers. Increased 5-HT3E subunit expression might cause changes in receptor composition or lead to higher receptor density in the cell membrane as seen in vitro for homomorphic 5-HT3A receptors carrying the c.-42C>T variant.

Interestingly, we found the strongest association of the HTR3E variant c.*76G>A with IBS-D in female patients. Gender-related differences in IBS prevalence (two thirds of patients are female) and in response to pharmacological treatment are described, yet poorly understood. Our findings indicate that female c.*76G>A variant carriers have a much higher risk for developing IBS-D than male carriers. Further studies are needed to clarify why the c.*76G>A variant seems to have this gender specific effect. This could be related to the fact that gonadal hormones influence GI motility and sensory afferent pathways and central processing of visceral stimuli (31).

By in situ hybridization and immunofluorescence experiments, we found HTR3A, HTR3E and miR-510 co-expressed in enterocytes and myenteric plexuses of human colon sections. The co-expression of HTR3E and miR-510 in enterocytes was confirmed by RT–PCR of microdissected tissue. These findings suggest that native 5-HT3 receptors in colonic enterocytes are comprised of 5-HT3A and 5-HT3E subunits and that the expression of the latter is regulated by the co-expressed miR-510. This is, to our knowledge, the first study describing the presence of 5-HT3 receptors in colonic enterocytes, suggesting that 5-HT3 receptors are involved in human mucosal secretion stimulated by serotonin. This remains controversial, as a recent study suggested no involvement of 5-HT3 receptors in secretion, at least in human duodenum (32). Further analyses of human GI tract tissue samples using different 5-HT3 agonists and antagonists are needed to elucidate a potential involvement of 5-HT3 receptors in the regulation of mucosal secretion.

In a previous study, the c.-42C>T variant was shown to be located in an upstream open reading frame (uORF) of HTR3A resulting in an amino acid exchange (Pro16Ser) in the predicted upstream peptide (23). Peptides encoded by uORFs are thought to participate in the regulation of gene expression by blocking the scanning ribosome during the elongation phase and by diminishing translation of the downstream gene (33). We have now demonstrated that the c.-42C>T variant leads to higher 5-HT3A receptor density in the membranes of transfected HEK293 cells. The 5-HT3A subunit
seems to play a crucial role in receptor formation, as it is the only 5-HT₃ subunit that can form functional homopentameric receptors in vitro. In addition, heteromeric assemblies of 5-HT₃ₐ, 5-HT₃ₐ, 5-HT₃ₐ, and 5-HT₃ₐ form functional receptors (17). It is, therefore, possible that the presence of the c.-42C>T variant might affect composition and density of native 5-HT₃ receptors in various human tissues. Patients suffering from IBS frequently show comorbidity with anxiety and depression (3,4). Previous studies have found c.-42C>T to also be associated with bipolar affective disorder, harm avoidance in women and modulation of amygdaloid activity (23,34,35). Therefore, elevated expression levels of the 5-HT₃ₐ subunit caused by the c.-42C>T variant may contribute to both, dysfunction of brain and gut-related serotonin signaling mediated by 5-HT₃ receptors. As the association of the c.-42C>T variant failed replication in the German IBS-D and controls cohort, its impact in the etiology of IBS-D remains unclear yet associations to psychiatric traits cannot be excluded and further studies are necessary to evaluate the role of this variant in IBS-D.

Depending on the localization and receptor composition, the functions of 5-HT₃ receptors within the GI tract and CNS are many and diverse. 5-HT₃ receptors are located on the intrinsic and extrinsic neural pathways, spinal and CNS neurons and, as shown in the present study, on mucosal enterocytes. In the gut, they are thought to be involved in motor-sensory function and secretion (36,37). Thus, it is possible that the increased 5-HT₃ₐ and 5-HT₃ₐ subunit expression might lead to an increased density of 5-HT₃ receptors or alter the receptor composition and cause hypercontractility and secretion along with visceral hypersensitivity, features typical of IBS-D. Our hypothesis is supported by the fact that 5-HT₃ receptor antagonists-like alosetron and ondansetron seem to directly antagonize mediated by 5-HT₃ receptors. As the association of the c.-42C>T variant failed replication in the German IBS-D and controls cohort, its impact in the etiology of IBS-D remains unclear yet associations to psychiatric traits cannot be excluded and further studies are necessary to evaluate the role of this variant in IBS-D.

### Table 2. HTR3A c.-42C>T (rs1062613) genotypes (and frequencies) in IBS patients and healthy controls

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>c.-42C/c.-42T</th>
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<tbody>
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<td>Sample group</td>
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<td>55 (56%)</td>
<td>35 (36%)</td>
<td>8 (8%)</td>
</tr>
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<td>70 (71%)</td>
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<td>49 (25%)</td>
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</tr>
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<td>IBS-D Germany (n = 119)</td>
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<tr>
<td>Controls Germany (n = 195)</td>
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</tr>
<tr>
<td>IBS-D pooled (n = 217)</td>
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<td>Controls pooled (n = 295)</td>
<td>193 (65%)</td>
<td>89 (30%)</td>
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Values indicate number of patients and healthy controls with the respective genotype. Odds ratios (OR), 95% confidence intervals (CI) and P-values calculated using the χ² test in a minor allele dominant model. Frequencies (%) were rounded up or down to the nearest whole number. UK, United Kingdom.

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<td>13 (4%)</td>
</tr>
</tbody>
</table>

Values indicate number of patients and healthy controls with the respective genotype. Odds ratios (OR), 95% confidence intervals (CI) and P-values calculated using the χ² test in a minor allele dominant model. Frequencies (%) were rounded up or down to the nearest whole number. UK, United Kingdom.

**A** pcDNA3 HTR3A-5'UTR c.-42C

pcDNA3 HTR3A-5'UTR c.-42T

<table>
<thead>
<tr>
<th>B</th>
<th>Bmax (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.-42C</td>
<td>5.0</td>
</tr>
<tr>
<td>c.-42T</td>
<td>10.0</td>
</tr>
</tbody>
</table>

**B** Maximum [3H]GR65630 binding (Bmax) to membranes of HEK293 cells transfected with pcDNA3 HTR3A-5'-UTR c.-42C or c.-42T constructs. Values are means ± SEM of five experiments. *P < 0.05.

In conclusion, our data represent an important step in understanding the complex mechanisms of 5-HT₃ receptor expression regulation. We found cis-regulatory mechanisms to fine-tune expression of the receptor subunits 5-HT₃ₐ and 5-HT₃ₐ by translational repression. Two functional variants residing within these crucial regulatory elements have been identified with both resulting in increased expression of the respective subunit. The HTR3E c."76G>A variant seems to...
affect microRNA regulated gene expression and appears to be strongly associated with female IBS-D. The HTR3A c.-42C>T variant counteracts the translational repression caused by a uORF, yet its role in the etiology of IBS-D remains unclear as it was associated in only one of the two investigated IBS-D patient groups. We hypothesize that the increased expression of the 5-HT3E and presumably the 5-HT1A subunit may affect neural signaling either predisposing or causing at least some of the symptoms associated with female IBS-D. Our findings contribute to the understanding of IBS-D pathomechanism and may open novel therapeutic options in the treatment of this disease.

MATERIALS AND METHODS

IBS patients and healthy controls from the UK

The HTR3E mutation analysis was carried out on a pilot cohort of 100 patients with IBS-D (aged 18–66 years; mean age 41.5 years; 32 male), 100 IBS-C patients (aged 18–65 years; mean age 40.5 years; 5 male) and 100 healthy controls (aged 18–63 years; mean age 35.3 years; 35 male). For the HTR3A mutational analysis, 98 IBS-D patients (aged 18–66; mean age 41.7 years; 31 male) and 99 IBS-C patients (aged 18–65 years; mean age 40.6 years; 5 male) of the same patient pools and the 100 healthy controls were screened. IBS patients with a mixed bowel habit (IBS-M) were excluded from the study. IBS patients were recruited from the Out Patients Departments of the University Hospitals of South Manchester (tertiary patients excluded), local general practices, advertisement in regional news papers and an existing departmental volunteer pool of patients. All satisfied the Rome II criteria for IBS and predominant bowel habit subtype (42). All patients underwent appropriate investigations to exclude organic disease (1) and did not show any functional disorder of the upper GI tract that was more prominent than their IBS. In addition, no subject had a history of major psychiatric disorder or history of alcohol or substance abuse. Healthy controls were recruited by advertisement. All subjects were Caucasian and drank below the recommended safe alcohol limit (<21 units/week), smoked <5 cigarettes per day and had not participated in a clinical trial of any drug within the previous 30 days. Written consent was obtained from all subjects and the study was approved by the South Manchester Medical Research Ethics Committee.

German IBS-D patients and healthy controls

For the replication study, we investigated 119 unrelated IBS-D patients (aged 19–79; mean age 44.6 years; 44 male) recruited from the outpatient clinic of the medical department at Charité University Medical Center, Campus Virchow-Klinikum, Berlin. The patients satisfied the Rome III criteria for IBS-D and the presence of other diseases that could explain symptoms was excluded by appropriate investigations. Ethical approval was given by the ethical committee of the Charité Campus Mitte (CCM). The German control series consisted of 195 healthy, unrelated blood donors (36–55 years of age; mean age 43.4 years; 63 male). They were recruited in 2004 and 2005 by the Institute of Transfusion Medicine and Immunology (Mannheim, Germany). According to the German guidelines for blood donation, all blood donors were examined by a standard questionnaire. All blood donors consented to the use of their samples for research studies. The study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany).

Preparation of genomic DNA

Genomic DNA was prepared from blood samples taken from both the patients and healthy controls using standard protocols (43).

Polymerase chain reaction

PCRs were performed in 25 µl volumes containing 50 ng of genomic DNA as template, 10 pmol of each primer, 200 µM dNTPs (MBI Fermentas), 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris–HCl, 0.1% Triton X-100 and 1.25 U of Taq DNA Polymerase (NEB). Thermal cycling was performed in a PTC-200 (MJ Research) or Mastercycler gradient thermal cycler (Eppendorf). Annealing temperatures (T_a) and sequences of the UTR-specific HTR3A and HTR3E primers are shown in Supplementary Material, Table S2. Cycling conditions were: Initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, T_a for 30 s and 72°C for 30 s. The final extension step was at 72°C for 5 min. A 3 µl aliquot of each PCR product was analyzed on a 1.5% agarose gel.

dHPLC analysis

Prior to direct sequencing, the WAVE DNA fragment analysis system was used as a tool for fast and sensitive detection of unknown sequence variants according to conditions recommended by the manufacturer (Transgenomic). The formation of heteroduplexes was achieved by denaturing the PCR products at 95°C for 5 min and gradually cooling them down to 4°C in 45 cycles (–2°C per cycle). A 5 µl aliquot of PCR product was loaded on the DNASep column (Transgenomic). Gradient parameters and column temperatures for each amplicon were calculated using the software supplied with the WAVE system. Each amplicon was analyzed at two different column temperatures (Supplementary Material, Table S2). In case of detection of a putative sequence variant within an amplicon, all samples were subject to direct sequencing of the respective amplicon to assure detection of homozygous variants which are not detectable by using dHPLC.

Purification and direct sequencing of PCR products

A 5 µl aliquot of PCR product was treated with 2 U shrimp alkaline phosphatase (SAP) and 5 U exonuclease I (Exol; MBI Fermentas) for 15 min at 37°C followed by inactivation at 80°C for 15 min. Two microliter of the Exol/SAP-treated PCR product was used for direct sequencing using the DYENamic ET Terminator Cycle Sequencing Kit according to the manufacturer’s protocol (GE Healthcare). The MegaBACE 1000 sequencer and the software provided by the manufacturer.
Expression and luciferase reporter constructs

The pcDNA3 HTR3A-5'-UTR c.-42C and c.-42T constructs (Fig. 5A) were constructed by cloning the respective 5'-UTR upstream of an existing pcDNA3 HTR3A cDNA construct. To create the pRL-TK HTR3E-3'-UTR c.-76G and c.-76A renilla luciferase reporter constructs (Fig. 2A), the respective full-length HTR3E 3'-UTR fragments were amplified from genomic DNA using forward primer 5'-ATTATCTAGAGCA GGTGCTCACCTGCCAAC-3' and reverse primer 5'-ATTAT CTAGACTGCAGAATTATTTATT GG-3' (both with an XbaI tail). The XbaI-digested PCR products were ligated into the XbaI site of the pRL-TK renilla luciferase vector (Promega). Constructs were purified using the PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen) and integrity of insert sequence and orientation was verified by sequencing using the MegaBACE system (GE Healthcare).

Cell culture and transfection

HEK293 and Colo320 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. For luciferase assays, the cells were split into 24-well plates at approximately 5.0 × 10⁵ cells per well prior to transfection. Cells were transiently transfected using 4 μg of polyethylenimine (Sigma-Aldrich) per 1 μg of construct DNA and cells were harvested 24 h after transfection. For radioligand binding assays, HEK293 cells were split into 75 cm² cell culture flasks and transfected by TransIT®-293 (Mobitec). The assay was performed 48 h after transfection.

Luciferase assay

Four hundred nanograms (per well) of renilla luciferase reporter construct (pRL-TK HTR3E 3'-UTR c.-76G/c.-76A) and 100 ng (per well) of reference construct pGL3-Control (firefly luciferase; Promega) were co-transfected in the presence of 4, 40 or 100 nM of hsa-miR-510 pre-miR precursor molecules or pre-miR negative control no. 1 or hsa-miR-510 anti-miR miRNA inhibitor (Ambion). The luciferase assay was performed using the dual-luciferase reporter assay system (Promega) and a Luciferase assay was performed 48 h after transfection.

Membrane preparation and radioligand binding assay

Radioligand binding with the 5-HT₃ receptor ligand [³H]GR65630 (86 Ci/mmol; PerkinElmer) was carried out on membranes of HEK293 cells transfected with either the pcDNA3 HTR3A-5'-UTR c.-42C or the c.-42T construct as described previously.

Preparation of tissue sections and in situ hybridization

Six unaffected, normal colon tissue samples from four female and one male patient (55–78 years old; three colonic cancer patients and two patients with diverticulitis) were used for cryosections. Frozen tissue sections (12 μm) were fixed in 1 × PBS containing 4% paraformaldehyde for 20 min and then washed twice in 1 × PBS for 10 min each. The sections were dehydrated and stored at −80°C. Prior to hybridization, the sections were thawed and rehydrated. The HTR3A- and HTR3E-specific hybridization probes were synthesized from 3'-UTR cDNA fragments, subcloned into the pSTBlue-1 vector (Novagen), using the MAXIscript in vitro transcription kit (Ambion). Sense and antisense probes were generated using T7 or Sp6 polymerase. The probes were labeled with digoxigenin (DIG) by adding DIG RNA Labeling Mix (Roche) and purified using NucAway spin columns (Ambion) according to the manufacturer’s protocols. For detection of miRNA-510 expression, a specific 5'-DIG-labeled antisense-labeled nuclear acid (LNA) oligonucleotide (Exiqon) was used. The 5' DIG-labeled scramble-miRNA (negative control) was purchased from the same company. MiRNA in situ hybridizations were performed according to a protocol recommended by Exiqon at a hybridization temperature of 56°C. The HTR3A- and HTR3E-specific probes were hybridized at 68°C using a modification of a previously published protocol (44).

Immunofluorescence

For localization of 5-HT₃A and 5-HT₃E subunits in human colon tissue sections, immunofluorescence experiments were carried out as follows: tissue sections (8 μm) were fixed by incubation in 4% paraformaldehyde for 20 min. Afterwards, they were washed three times for 10 min in 1 × PBS at room temperature. Then slides were blocked in 4% goat serum/0.25% Triton-X-100/PBS. The primary antibodies rabbit anti-5-HT₃A or anti-5-HT₃E were diluted 1:100 in blocking solution and applied overnight at room temperature. Afterwards, tissue sections were washed 3 × 10 min in 1 × PBS at room temperature and incubated in blocking solution containing the Alexa Fluor 488-labelled (Invitrogen) secondary antibody goat anti-rabbit for 3 h. From then on, every step was carried out light protected. After washing for three times for 5 min in 1 × PBS, a nuclear counterstain with 4',6-Diamidino-2-phenylindol (DAPI, 1:10.000 in 1 × PBS) was carried over following by two washes in 1 × PBS. Sections were mounted in Vectashield (Vector) and stored at 4°C until microscopical investigation by a Zeiss Axiophot.

Laser microdissection and pressure catapulting

Fresh frozen colon mucosa samples were cut into 18 μm thick sections using a cryostat (Leica CM1850, Leica Microsystems) and processed as following: the sections were mounted on membrane slides (PEN-membrane, 1 mm glass, Carl Zeiss MicroImaging GmbH) and incubated for 10 min at −20°C in RNAlater®-ICE (Ambion). For further preservation, samples were fixed in ethanol and stained in cresyl violet acetate (1% (w/v) in ACS-grade ethanol, Sigma-Aldrich) for 15 s. Subsequently, the slides were washed in ethanol and incubated...
for 5 min in xylene. After air-drying, the slides were mounted on the stage of an inverse microscope which is a component of a Microbeam LMPC System (Carl Zeiss MicroImaging GmbH). We employed the RoboLPC method to microdissect and capture the appropriate tissue fragments (approx. 10 mm² epithelium, ~100 000–250 000 cells). For sample collection, we applied 0.5 ml AdhesiveCaps® opaque (Carl Zeiss MicroImaging GmbH).

RNA isolation and RT–PCR of microdissected samples
Total RNA was isolated from each sample using the Chomczynski/Sacchi (45) method according to the manufacturer’s instructions (peqGOLD TriFast™ kit, PeqLab Biotechnologie GmbH). For precipitation 2 μl of Pellet Paint® Co-Precipitant (Novagen) per sample was used. After DNase treatment (DNA-free™, Ambion), 200 ng of RNA was reverse transcribed using the Accuscript® High Fidelity first strand cDNA synthesis kit (Stratagene). PCR was performed using HotStarTaq DNA Polymerase (Qiagen) according to the manufacturer’s protocol. Primers are given in Supplementary Material, Table S5.

Statistics
Comparison of genotype frequencies, association analyses and test for deviation from the HWE were performed using an online tool provided by the Institute of Human Genetics in Munich (http://ihg.gsdf.de/cgi-bin/hw/hwa1.pl) and the SAS v9.1 software (SAS Institute Inc.). For the association analyses, the frequencies of genotypes were compared in a 2 × 2 contingency table. We calculated P-values using the χ² test. In those cases where the expected value of at least one cell of the contingency table was below 5, we used the Fisher’s exact test. Corrections for multiple testing were performed as indicated in the results. For sample power calculations, we used Quanto 1.2 (http://hydra.usc.edu/gxe). For the luciferase and radioligand binding assay results, the independent samples t-test was performed using the MedCalc software (http://www.medcalc.be).

In silico analysis of microRNA binding sites
We performed in silico analysis of micro-RNA binding sites, using the miRBase Target database, release 8.2 (http://microrna.sanger.ac.uk) (22).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. L.A.H. has received remuneration for advice and speaking (Novartis, Solvay, Clasado, Sapphire Therapeutics) together with financial support for the conduct of physiological research from Novartis Pharmaceuticals, GlaxoSmithKline, Pfizer, Solvay Pharmaceuticals and Danone Research. P.J.W. has received remuneration for advice and the department has also received financial support from Novartis Pharmaceuticals, GlaxoSmithKline, Pfizer, Solvay Pharmaceuticals, Rotta Research, Procter and Gamble, Danone Research, Astellas Pharma and Tillotts Pharma. All other authors declare no conflicts of interest.

REFERENCES