IRRITABLE BOWEL SYNDROME

Association of distinct α₂ adrenoceptor and serotonin transporter polymorphisms with constipation and somatic symptoms in functional gastrointestinal disorders

H J Kim, M Camilleri, P J Carlson, F Cremonini, I Ferber, D Stephens, S McKinzie, A R Zinsmeister, R Urrutia

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Correspondence to:
Professor M Camilleri,
Mayo Clinic, Charlton
8-110, 200 First St SW,
Rochester, MN 55905,
USA; camilleri.michael@
mayo.edu

Accepted for publication
10 December 2003
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Background: The role of genetics in the phenotypic manifestations of irritable bowel syndrome (IBS) is unclear. Our aims were: (1) to compare the prevalence of polymorphisms of alpha 2 (α_2) adrenoceptors, norepinephrine transporter, and serotonin transporter protein (soluble carrier protein member 4 (SLC6A4)) promoter in patients with lower functional gastrointestinal disorders (FGID) and in healthy controls; and (2) to test associations of these genetic variations with symptoms of IBS and high somatic symptom scores.
Methods: Validated bowel and somatic symptom questionnaires characterised the phenotype: 90 with IBS constipation (IBS-C), 128 IBS diarrhoea, 38 IBS alternating bowel function, and 20 chronic abdominal pain. Logistic regression analyses assessed associations of different polymorphisms for α_2 adrenoceptor and *SLC6A4* with IBS or chronic abdominal pain phenotypes and high somatic score.
Results: Two distinct polymorphisms independently appeared to be associated with the phenotype IBS-C: α_{2C} Del 322–325 (odds ratio (OR) 2.48 (95% confidence interval (CI) 0.98, 6.28); p = 0.05) and α_{2A} −1291 (C→G) (OR 1.66 (95% CI 0.94, 2.92); p = 0.08) relative to wild-type. Overall, the α_{2C} Del 322–325 polymorphism (alone or combined with other polymorphisms) was also significantly associated with a high somatic symptom score (OR 2.2 (95% CI 1.06, 4.64); p = 0.03). Combinations of polymorphisms were also associated with high somatic scores.
Conclusion: Functionally distinct α_{2A} and α_{2C} adrenoceptor and serotonin transporter polymorphisms are associated with constipation and high somatic symptoms in patients with lower functional gastrointestinal disorders, although the strength of the genetic contribution to the phenotype is unclear.

Irritable bowel syndrome (IBS) is a biopsychosocial disorder affecting 9–17% of patients of all ethnic groups.[1] It is associated with abnormal gastrointestinal motor function, visceral sensitivity, and psychosocial or autonomic dysfunction.[2] Psychosocial factors, particularly stress, can alter colonic motility, enhance colonic sensation,[2] and influence the timing of patients' presentation to physicians.[3 4]

There is evidence of sympathetic adrenergic dysfunction in a subgroup of patients with IBS.[5 6] Adrenergic agents alter the motor and sensory function of the human gastrointestinal tract.[7–9] The most prominent effects in the human colon were observed with α_2 agents.[7] Twin studies suggest a genetic component in IBS.[10–12] However, the influence of genetic factors that may modulate adrenergic and serotonergic functions in IBS is largely unknown.

Three human α_2 adrenoceptor subtypes have been cloned and characterised: 2$_A$, 2$_B$, and 2$_C$ subtypes.[13–16] Prejunctional α_{2A} and α_{2C} adrenoceptor subtypes regulate the release of norepinephrine from sympathetic nerves through negative feedback at presynaptic nerve endings. The potential of the α_2 adrenoceptor subtypes in the motor and sensory dysfunctions of disturbances of gastrointestinal function is shown in the model in fig 1. It is conceivable that polymorphisms of the genes encoding for these receptors may result in loss of normal synaptic autoinhibitory feedback and enhanced presynaptic release of norepinephrine. Synaptic levels of norepinephrine are modified by the norepinephrine transporter (*NET*); a mutation of *NET* in one family has been associated with autonomic dysfunction.[17] Genetic disorders of α_2 mechanisms could conceivably alter functions of relevance

to IBS: gut motility, pain sensation, autonomic imbalance, anxiety, and somatisation.

Serotonin (5-hydroxytryptamine (5-HT)) modulates sensorimotor functions in the digestive tract. There are seven subclasses of serotonergic receptors, differentiated on the basis of structure, molecular mechanism, and function.[18] The actions of enteric 5-HT are terminated by reuptake by the 5-HT transporter (SERT). SERT in the gut is similar to that in the brain of the same species[19]; gastrointestinal motility is abnormal in *SERT* knockout mice.[20] The approved gene symbol for *SERT* is SLC6A4 (solute carrier family 6 (neurotransmitter transporter, serotonin), member 4); this abbreviation will be used in the remainder of this manuscript. Adaptive changes occur in the subunit composition of enteric 5-HT₃ receptors in SLC6A4 knockout mice. Such changes are reflected in altered 5-HT₃ receptor affinity and desensitisation and hence in function in response of the

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Abbreviations: IBS, irritable bowel syndrome; IBS-C, irritable bowel syndrome with predominant constipation; IBS-D, irritable bowel syndrome with predominant diarrhoea; FGID, functional gastrointestinal disorders; FC, functional constipation; FD, functional diarrhoea; CAP, chronic abdominal pain; SoSc, somatic symptom score; NET, norepinephrine transporter; 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; SERT-P, serotonin transporter promoter; SLC6A4, solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; α_{2C} Del 322–325, deletion in third intracellular loop of α_{2C} adrenoceptor protein; α_{2A}Lys251, a cytosine to guanine transversion at position 753 that changes amino acid 251 of the third intracellular loop of the α_{2A} adrenoceptor from asparagine to lysine; α_{2A} −1291 (C→G), cytosine to guanine transversion in promoter of α_{2A} receptor protein; OR, odds ratio; PCR, polymerase chain reaction
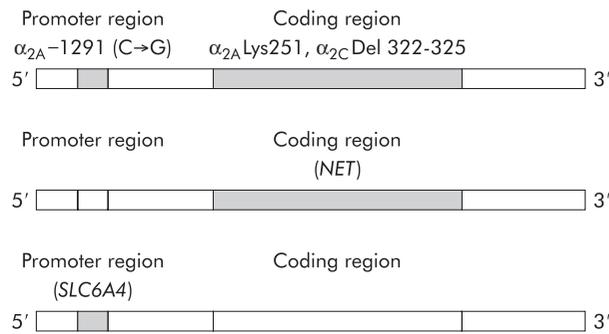
Figure 1 Candidate polymorphic or mutated genes involved in altered adrenergic and serotonergic functions. Top: α_{2A} and α_{2C} adrenoreceptor polymorphisms. α_{2A} −1291 (C→G), cytosine to guanine transversion in promoter of α_{2A} receptor protein; α_{2A}Lys251, a cytosine to guanine transversion at position 753 that changes amino acid 251 of the third intracellular loop of the α_{2A} adrenoceptor from asparagine to lysine; α_{2C} Del 322–325, deletion in third intracellular loop of α_{2C} adrenoceptor protein. Middle: norepinephrine transporter (*NET*). Bottom: solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (*SLC6A4*) (serotonin transporter promoter (SERT-P)).

receptor to 5-HT released from enteroendocrine cells.[21] Serotonin type 3 (5-HT$_3$) receptors are involved in the colonic motor response to feeding in health and IBS.[18] The colonic transit response to the 5-HT$_3$ antagonist, alosetron, in IBS patients with diarrhoea is influenced by the genotype controlling the promoter for SLC6A4.[22]

There is also evidence that the two biogenic amines, serotonin and norepinephrine, interact in modulating gastrointestinal functions. For example, norepinephrine causes 5-HT release from enterochromaffin cells in mouse ileal tissues via α_2 adrenoceptor subtypes coupled to a pertussis toxin sensitive G protein[23] or via β_2 adrenoceptors in rat duodenal mucosa.[24] Thus we were interested in exploring the potential combined effects of genetic mechanisms that influence serotonin and norepinephrine. Similarly, underactivation of serotonergic function and overactivation of noradrenergic function modulate the brain circuitry involved in euthymic and abnormal mood and anxiety states.[25] Thus altered control of noradrenergic and serotonergic systems may result in symptoms of depression and anxiety, which are frequently associated with IBS at the time of presentation to physicians.

Selection of candidate genes for association studies

α_{2A} and α_{2C} adrenoceptor polymorphisms can act synergistically to alter the feedback regulation of norepinephrine release through their effect on the prejunctional α_2 adrenoceptor.[16] Moreover, most of the presynaptic receptors inhibiting acetylcholine release are of the α_{2A} subtype.[26] α_{2A} Adrenoceptors are located on somatic and visceral afferents and may be associated with reporting of chronic abdominal pain and somatic symptoms.[27] α_{2B} Adrenoceptor gene polymorphisms were not included in our candidate gene approach as the biological action of α_{2B} adrenoceptors on vascular function (for example, hypertension) appears dependent on a sodium retention state, and renal medullary actions (for example, release of nitric oxide) also counteract the hypertensive effect of norepinephrine[27 28] mediated through the α_{2B} adrenoceptor.

Norepinephrine reuptake requires a transporter protein (NET). A mutation of the gene encoding NET[17] results in a non-functional NET, increased norepinephrine, and functional overstimulation of the sympathetic nervous system in response to physiological stimuli, such as orthostatic

hypotension syndrome, neurocirculatory asthenia, and chronic fatigue syndrome.

The actions of enteric 5-HT are terminated by 5-HT transporter mediated uptake. We elected to study a polymorphism in the functional promoter region. This has been associated with gastrointestinal and neurobiological dysfunctions,[20 21 29] neuropsychiatric disorders,[29] and with altered response of IBS patients to a 5-HT$_3$ antagonist.[22]

Homozygous wild-type or long alleles reflect normal function. For the association studies performed, we assumed that the gene confers a functional disadvantage if there was a homozygous (short) or heterozygous polymorphism. This was based on the literature that shows that the heterozygote state in knockout mice confers a change in biological function that mirrors that of the homozygous state. Thus, for example, marked increases in the stress hormone adrenocorticotropin were found in the plasma of homozygous −/− and heterozygous +/− knockout mice compared with their control littermates. These data suggest that homozygous short and heterozygous SLC6A4 genotypes in mice are both associated with an increased stress-responsive phenotype.[30] Moreover, the SLC6A4 heterozygous state was associated with reduced colonic transit response to alosetron compared with wild-type.[22] There is also evidence that heterozygous variation in α_2 receptors may impart a change in some α_2 mediated functions or responses to medications. α_2 Agonist treatment of mice heterozygous for the α_{2A} adrenoceptor ($\alpha2_A$ adrenoceptor +/−) lowers blood pressure without inducing sedation.[31] Thus there is compelling evidence to hypothesise that the heterozygous polymorphisms may be biologically relevant.

Study hypothesis and aims

Our hypothesis is that single or combined polymorphisms of α_2 adrenoceptors and serotonergic receptors are associated with IBS phenotypes and somatic symptom scores. To assess the potential role of genetic determinants in IBS, we considered five polymorphisms in four candidate genes or their promoters. The polymorphisms selected for study modify adrenergic or serotonergic functions, and have been previously demonstrated to affect smooth muscle function, visceral sensation, bioamine metabolism, sympathetic function (for example, cardiac or vascular tone), or psychological state.[32–35]

The aims of this study were: (1) to compare the distributions of polymorphisms of α_{2A} and α_{2C} adrenoceptors, norepinephrine transporter, and serotonin transporter protein promoter in patients with lower functional gastrointestinal disorders (FGID—that is, IBS or chronic functional abdominal pain (CAP)) and in healthy controls; and (2) to assess the association of these polymorphisms with phenotypes of IBS and CAP, and with high somatic symptom scores.

METHODS

Asymptomatic healthy controls and patients with IBS

All participants (18–75 years) completed a validated bowel disease questionnaire (including questions that corresponded to Rome II criteria[2]) and a somatic symptom checklist.[36] The latter has been used extensively in the literature to identify patients with a propensity to report somatic symptoms.[36] The somatic symptom score was summarised as a mean of the frequency and severity scores over the 16 items, each recorded on a scale of 0–4.

Symptoms surveyed were: headache, backache, wheezing, trouble breathing, difficulty sleeping, fatigue (tiredness), depression (feeling sad or blue), general stiffness, palpitations, joint pains, eye pain associated with reading, dizziness, weakness, nervousness (or shakiness), hot or cold spells, and

high blood pressure. Subjects were classified as having a high somatic score when a subject's mean score across the 16 domains was >0.75, which was the 90th percentile of mean scores in the healthy participants in this study. We have used these two questionnaires extensively in epidemiological studies (for example, in patients with diabetes).[37]

IBS participants were selected from an administrative database of 752 patients with IBS residing within a 150 mile radius of Rochester, Minnesota, USA, and were recruited by mailing. All IBS patients had already been evaluated by a staff gastroenterologist using clinically indicated tests, including endoscopy, biopsies, and tests of rectal evacuation. Healthy volunteers were recruited by public advertisement in Rochester, Minnesota. All participants gave informed consent for the study which was approved by the Mayo Foundation Institutional Review Board.

There is evidence of a significant likelihood of category transitions[38] between constipation predominant IBS (IBS-C) and functional constipation (FC), and similar transitions in the categories of diarrhoea predominant IBS (IBS-D) and functional diarrhoea (FD). Hence we have grouped patients whose symptoms at the time of the questionnaire suggested IBS-C and FC into one group (designated IBS-C) and those with IBS-D and FD into a second group (designated IBS-D).

DNA analysis by polymerase chain reaction amplification, identification, and sequencing

Venous blood drawn from a forearm vein was stored as de-identified samples. We isolated DNA from whole blood from 394 participants by the alkaline lysis method using the QIAamp DNA Blood Maxi Kit, (Qiagen Inc., Valencia, California, USA). Molecular assays were adapted from previously published papers to detect the candidate mutations or polymorphisms of interest.

We used the polymerase chain reaction (PCR) based fragment length assays to identify polymorphisms for the α_{2C} and α_{2A} adrenoceptor coding regions and in the α_{2A} adrenoceptor promoter. We confirmed polymorphisms by direct sequencing. Where there was no restriction site available, as for NET and the SLC6A4, polymorphisms were identified by direct sequence alone.

Sequences used in these studies were obtained from GenBank/EBI Data Bank, and accession numbers are given below. Briefly, we performed PCR amplification using TaKaRa LA Taq with GC buffers (TaKaRa Shuzo Co., Ltd, Japan) in a total volume of 50 µl solution containing 750 ng template DNA, 0.4 µM primers, 2.5 units TaKaRa LA Taq, 20 mM dNTPs, and GC buffer I containing 2.5 mM $MgCl_2$. After denaturing DNA samples at 94°C for one minute, we set up cycling conditions at 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for two minutes followed by an extension at 72°C for five minutes. The polymorphisms were amplified using a Perkin Elmer Gene Amp 9700 PCR thermal cycler.

α_{2C} and α_{2A} PCR products were digested for one hour and visualised by electrophoresis using 3% metaphor agarose (BioWhittaker Molecular Applications Inc., Rockland, Maine, USA). Polymorphisms were confirmed by direct sequencing performed at the Mayo Molecular Biology Core Facility using an ABI PRISM 377 DNA sequencer with XL Upgrade and 96 well Upgrade (Perkin-Elmer Applied Biosystems, Foster City, California, USA).

Detection of polymorphisms in the α_2 adrenoceptor subtypes

(A) Within the promoter region for the α_{2A} adrenoceptor, a C to G transversion results in a Msp1 restriction fragment length polymorphism located at −1291 base pairs upstream of the origin of transcription.[39] This polymorphism is designated by the abbreviation α2A −1291 (C→G). Genotype GG has been detected as an apolymorphic band of 174 bp, and genotype CC as two polymorphic bands of 121 and 53 bp. In a Japanese control group, 36% had C allele and 64% G allele, with GG homozygosity in 16%, CG heterozygosity in 40%, and CC homozygosity in 44%.[39] In contrast, a study of White Swedish men revealed allele frequencies of 23% allele C and 77% allele G, with no CC, 46% CG, and 54% GG genotypes.[40] Data from European American healthy controls have not been reported previously. Altered expression of the gene causes loss of receptor function.

GenBank accession number was #M23533. A 523 bp region containing the Msp1 polymorphic site was amplified by PCR using the following primers: sense position 661:

Table 1 Patient characteristics, prevalence of SLC6A4, α_{2C} Del 322–325, and α_{2A} −1291 (C→G) polymorphisms, and mean somatic score (SoSC) in 276 patients with lower functional gastrointestinal disorders (FGID) and 120 healthy controls

	Controls	Literature controls* (%)	All Lower FGID Patients	IBS-C	IBS-D	IBS-Alt	CAP
N	120		276	90	128	38	20
Age (mean, range)	36 (18–72)		49 (18–82)	52 (23–82)	47 (18–77)	47 (18–69)	47 (26–78)
Sex (n (% female))	95 (79)		226 (82)	83 (92)	98 (77)	30 (79)	15 (75)
SoSC (mean (SD))	0.31 (0.31)		0.9 (0.6)	0.89 (0.57)	0.83 (0.55)	0.93 (0.65)	0.84 (0.62)
Caucasian (n (%))	107 (89)		267 (97)	86 (96)	124 (97)	37 (97)	20 (100)
SLC6A4							
Wild-type (%)	31	36	36	40	32	42	35
Heterozygous (%)	49	47	46	43	48	45	45
Homozygous polymorphism (%)	20	16	18	17	20	13	20
Any polymorphism (%)	69	63	64	60	68	58	65
α_{2C} Del 322–325							
Wild-type (%)	92	94	89	85	91	86	95
Heterozygous (%)	6	4	9	11	7	11	5
Homozygous polymorphism (%)	2	2	2	4	2	3	0
Any polymorphism (%)	8	6	11	15	9	14	5
α_{2A} −1291 (C→G)							
Wild-type (%)	57	48	52	45	55	53	55
Heterozygous (%)	36	43	43	47	39	47	45
Homozygous polymorphism (%)	7	8	5	8	6	0	0
Any polymorphism (%)	43	51	48	55	45	47	45

SoSC, mean somatic symptom score (mean often and mean bothersome scores for 16 symptom "items"). Each "item" is on based on a scale of 0–4.
Lower FGID group comprises the four groups: irritable bowel syndrome with predominant constipation (IBS-C), irritable bowel syndrome with predominant diarrhoea (IBS-D), irritable bowel syndrome with alternating bowel function (IBS-Alt), and chronic abdominal pain (CAP).
*Genotype frequencies for controls are from: Serretti and colleagues,[49] Small and colleagues,[46] and Tsai and colleagues.[50]

5'-TCACACCGGA GGTTACTTCCCTCG 3' and antisense position 1165: 5' -TCCGACGACA GCGCGAGTT 3'. The Msp1 restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) was used at 100 units per reaction.

(B) A single nucleotide polymorphism at position 753 consists of a C to G transversion that changes amino acid 251 of the third intracellular loop of the α_{2A} adrenoceptor from asparagine to lysine (α_{2A}Lys251). This polymorphism results in enhanced Gi protein coupling and increased agonist promoted receptor function. The polymorphism is identified by the restriction enzyme Sty1, and it shows a higher prevalence in African-Americans (0.04) than in European Americans (0.004).[41] GenBank accession number was #AF281308. A 310 bp region containing the polymorphic site was amplified by PCR using the following primers: sense position 581: 5' -AGTGGTACGTCATCTCGTCG 3' and antisense position 871: 5' -GAGCTCTCCTCCAGGTCCAG 3'. The Sty1 restriction enzyme (Invitrogen, Carlsbad, California, USA) was used at 10 units per reaction.

(C) There is a 12 bp deletion beginning with nucleotide 964 in the region coding for the third intracellular loop of the α_{2C} adrenoceptor.[42] It results in loss of four amino acids (Gly-Ala-Gly-Pro) at positions 322–325 (denoted α_{2C} Del 322–325), and loss of an Nci1 restriction site. The ethnic distribution of this polymorphism in controls in a US population showed an allele frequency of 41.1% in African-Americans and 3.8% in European Americans. The genotype distribution for the two groups were, respectively: wild-type 34.5% and 94.3%; heterozygous 48.8% and 3.8%, and homozygous deleted 16.6% and 1.9%. GenBank accession number was #J03853. A 723 bp region containing the polymorphic site was amplified by PCR using the following primers: sense position 547: 5'-CCACCATCGT CGCCGTGTGG CTCATCT 3' and antisense position 1246: 5'-AGGCCTCGCG GCAGATGCCG TACA 3'. The Nci1 restriction enzyme (New England Biolabs) was used at 20 units per reaction.

Detection of norepinephrine transporter (NET) gene mutation

A guanine (G) to cytosine(C) missense mutation in nucleotide 237 of the coding region of NET is associated with Ala457Pro mutation.[17] For examination of the NET gene, upstream and downstream primers were synthesised as follows: sense 5' CCGGAAACTCTTCACATTTG 3' and antisense 5' CGCTGAATTGAGGATGCTGG 3'.[16] GenBank accession number was #X76753. PCR products were visualised on a 1% agarose gel and confirmed by direct sequencing.

Detection of polymorphism in the serotonin transporter protein promoter

A polymorphism in the 5' promoter region (5'-HTTLPR) of SLC6A4 (42) consists of a repetitive sequence of 22 bp located 1 kb upstream of the SLC6A4 transcription start site.[29] This polymorphism is biallelic in most populations. The allele with the smallest number of repeats, commonly called the short (S) allele, has lower transcriptional activity, leading to marked reductions in messenger RNA levels, 5-HT binding, and 5-HT uptake in both platelets and lymphoblasts compared with the long (L) allele.[29] In European-Americans, the S and L alleles have generally been observed to occur at frequencies of approximately 0.43 and 0.57 with wild-type (LL) 32%, heterozygous 49%, and homozygous short 19%.[29]

Identification of the polymorphisms in the promoter for SLC6A4, the serotonin transporter protein, was by PCR based fragment length polymorphisms.[22] GenBank accession number was #X76753. We synthesised oligonucleotide primers flanking the long polymorphic region corresponding to the nucleotide positions 1671 sense 5' -GCCGCTCTGAATGCC-AGCAC 3' and position 2219 antisense 5' -GGAGGAACTG-ACC-CCTGAAAACTG 3' to generate 572 bp PCR amplified fragments.

Data and statistical analysis

Logistic regression models were used to estimate the associations (odds ratios (OR)) for specific phenotypes of IBS and high somatic symptom scores with the different adrenergic and serotonergic polymorphisms.

A high somatic symptom score was defined based on the subject's mean score greater than the 90th percentile of healthy participants. The odds ratios (95% confidence interval CI)) for a specific phenotype were computed from the estimated logistic regression model coefficients (and their standard errors) examining individual polymorphisms or their combinations relative to the homozygous type. Race and sex were included as covariates in each of the logistic regression models. The statistical software used for all analyses was SAS.

After the study was completed, we estimated the effect size detectable given the number of subjects in different subgroups included in this study.

RESULTS

Lower FGID symptoms and high somatic symptom scores

The symptom phenotypes of patients with lower FGID were: 90 IBS-C, 128 IBS-D, 38 IBS alternating bowel function, and

Table 2 Distributions (%) of irritable bowel syndrome and chronic abdominal pain phenotypes, and controls, in each genotype category

	n*	Controls (%)	IBS-C (%)	IBS-D (%)	IBS-Alt (%)	CAP (%)
SLC6A4 wild-type	137	27	26	30	12	5
Heterozygous	185	32	21	33	9	5
Homozygous polymorphism	74	33	20	35	7	5
Any polymorphism	259	32	21	34	8	5
α_{2C} Del 322–325 wild-type	348	32	21	33	9	5
Heterozygous	31	23	32	29	13	3
Homozygous polymorphism	8	25	38	25	12	0
Any polymorphism	39	23	33	28	13	3
α_{2A} −1291 (C→G) wild-type	210	33	19	34	9	5
Heterozygous	160	27	26	31	10	6
Homozygous polymorphism	22	36	32	32	0	0
Any polymorphism	182	28	26	31	10	5

*A total of 396 (276 patients and 120 controls) had SLC6A4, 387 had α_{2C} Del 322−325, and 392 had α_{2A} −1291(C→G) genotypes assayed.
IBS-C, irritable bowel syndrome with predominant constipation; IBS-D, irritable bowel syndrome with predominant diarrhoea; IBS-Alt, irritable bowel syndrome with alternating bowel function; CAP, chronic abdominal pain.

Table 3 Distributions (%) of different phenotypes by combinations of different serotonergic and adrenergic genotype. n refers to number of people (patients and controls) with a specified genotype or genotype combination

n	SLC 6A4	α_{2C} Del 322–325	α_{2A} −1291 (C→G)	Controls (%)	IBS-C (%)	IBS-D (%)	IBS-Alt (%)	CAP (%)
58	Wt	Wt	Wt	31	24	28	12	5
59	Wt	Wt	Het/hom	24	27	32	10	7
9	Wt	Het/hom	Wt	22	44	33	0	0
7	Wt	Het/hom	Het/hom	29	0	42	29	0
126	Het/hom	Wt	Wt	36	13	37	9	5
103	Het/hom	Wt	Het/hom	31	25	32	7	5
12	Het/hom	Het/hom	Wt	25	34	25	8	8
11	Het/hom	Het/hom	Het/hom	18	46	18	18	0

Wt, wild-type; Het, heterozygous; hom, homozygous polymorphism.
IBS-C, irritable bowel syndrome with predominant constipation; IBS-D, irritable bowel syndrome with predominant diarrhoea; IBS-Alt, irritable bowel syndrome with alternating bowel function; CAP, chronic abdominal pain.

20 CAP. The demographics of patients in the different subgroups are shown in table 1. Female participants predominated in the patient (82%) and control (79%) groups. Caucasian patients were 89% of controls and 97% of patients; there were 6% Asian and 3% Hispanic healthy participants. Other racial groups were <1% in the two groups.

Lower FGID was associated with significantly higher somatic symptom scores relative to healthy controls (p<0.05).

Prevalence of polymorphisms in lower FGID

α_{2A}Lys251 and *NET* polymorphisms were not identified in the first 100 patients and 20 controls, and were not measured in the remaining participants.

The distributions of polymorphisms for SLC6A4, α_{2C} Del 322–325, and α_{2A} −1291 (C→G) were not significantly different between patients with lower FGID compared with controls (table 1). The distribution of these polymorphisms in the controls of the present study was similar to the distribution in control populations from previous studies.[43–45] In tables 2 and 3, we provide the distribution of lower FGID phenotypes and controls in each genotype category or combinations of genotypes.

Odds ratios for the associations between the different polymorphisms and FGID phenotypes are shown in table 4 and fig 2. The presence of either α_{2C} Del 322–325 or α_{2A} −1291 (C→G) polymorphism appeared to be associated with an increased OR (95% CI) for the phenotype of IBS-C versus controls: α_{2C} Del 322–325, OR = 2.48 (0.98, 6.28), p = 0.05 and α_{2A} −1291 (C→G) OR = 1.66 (0.94, 2.92), p = 0.08. There were no significant associations between the genetic polymorphisms tested and other phenotypes of IBS or CAP.

Association of polymorphisms with somatic symptoms in lower FGID

The mean somatic symptom scores, percentage of high somatic scores, and the associations with different polymorphisms are shown in table 5. Overall, the α_{2C} Del 322–325 polymorphism (alone or combined with other polymorphism) was significantly associated with a high somatic symptom score (OR = 2.2 (1.06, 4.64); p = 0.03). Combinations of polymorphisms were also associated with high somatic symptom scores: α_{2C} Del 322–325 with SLC6A4 (OR = 5.0 (1.11, 22.22); p = 0.04) and α_{2C} Del 322–325 with α_{2A} −1291 (C→G) (OR = 11.1 (1.15, 108.1); p = 0.04) relative to respective wild-type genotype(s). The significant OR for combined α_{2C} Del 322–325 with α_{2A} −1291 (C→G) polymorphisms had a wide confidence interval due to the small sample size.

Statistical power to detect associations

Table 6 summarises the "degree of association" between the presence/absence of specific polymorphisms in α_{2C} Del 322–325, α_{2A} −1291 (C→G), and *SLC6A4* genes versus specific symptom subgroups (including overall FGID). This assessment was based on a comparison of the observed proportion of patients in the group without a specific polymorphism (number of patients divided by the sum of the number of patients plus number of controls) and the proportion that could have been detected among those with the corresponding polymorphism.

The data indicate that clinically meaningful associations of these three candidate genotypes could have been detected with at least 80% power—for example, a difference in prevalence of wild-type versus polymorphic genotypes of 12% for all lower FGID, and 19–20% for IBS-C and IBS-D.

Table 4 Odds ratio (95% CI) for functional gastrointestinal disorder phenotype versus controls in polymorphic (heterozygous or homozygous polymorphism) relative to respective wild-type genotypes

	IBS-C	IBS-D	IBS-Alt	CAP
SLC6A4	0.7 (0.4, 1.2)	1.0 (0.6, 1.7)	0.7 (0.3, 1.4)	0.9 (0.3, 2.5)
α_{2C} Del 322–325	2.5 (1.0, 6.3)	1.3 (0.5, 3.4)	2.2 (0.7, 7.1)	0.8 (0.1, 6.8)
α_{2A} −1291 (C→G)	1.7 (0.9, 2.9)	1.2 (0.7, 2.0)	1.3 (0.6, 2.8)	1.2 (0.5, 3.2)
α_{2A} −1291 (C→G) and SLC6A4	1.1 (0.5, 2.7)	1.4 (0.6, 3.2)	0.7 (0.2, 2.2)	1.2 (0.3, 5.6)
α_{2C} Del 322–325 and SLC6A4	2.2 (0.4, 12.1)	1.3 (0.2, 7.5)	1.0 (0.1, 11.7)	2.4 (0.2, 32.4)
α_{2A} −1291 (C→G) and α_{2C} Del 322–325	NE	2.3 (0.3, 16.0)	3.3 (0.4, 30.4)	NE
α_{2A} −1291 (C→G) and SLC6A4 and α_{2C} Del 322–325	4.1 (0.6, 25.8)	1.5 (0.2, 12.7)	3.5 (0.4, 31.8)	NE

SLC6A4 (heterozygous or homozygous polymorphism).
α_{2C} *Del 322–325* (heterozygous or homozygous polymorphism).
α_{2A} *−1291 (C→G)* (heterozygous or homozygous polymorphism).
IBS-C, irritable bowel syndrome with predominant constipation; IBS-D, irritable bowel syndrome with predominant diarrhoea; IBS-Alt, irritable bowel syndrome with alternating bowel function; CAP, chronic abdominal pain; NE, non-estimable.
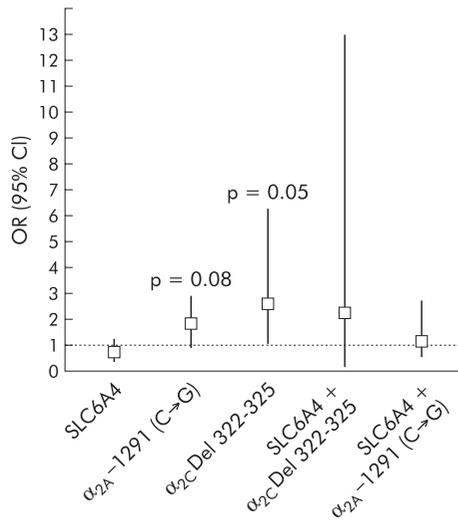
Figure 2 Odds ratio (95% confidence interval (CI)) for irritable bowel syndrome (IBS) constipation phenotype versus controls in polymorphic relative to respective wild-type genotypes. Note the significant association between the constipation predominant phenotype of IBS and the α_{2C} Del 322–325 deletion, which alters the coding region of the gene.

DISCUSSION

We have observed associations between functionally distinct genetic polymorphisms (α_{2A} and α_{2C} adrenoceptor and *SLC6A4*) and specific phenotypes in patients with IBS. Our data suggest that the α_{2C} Del 322–325 adrenoceptor polymorphism is associated with increased odds for constipation (p = 0.05) and, separately, increased odds for higher frequency and severity of somatic symptoms in participants with lower functional gastrointestinal disorders. These studies are quite challenging in a functional disorder such as the irritable bowel syndrome, given the fluctuation in symptoms that characterise the phenotype; in future studies, we also perceive that attention needs to be given to the stability of symptoms over time as the phenotype is characterised.

Combinations of α_{2A} and α_{2C} polymorphisms or α_{2C} Del 322–325 adrenoceptor and *SLC6A4* polymorphisms also showed significant associations with high somatic scores. Combinations of polymorphisms may be functionally important. For example, the combined β1 adrenoceptor and α_{2C} adrenoceptor polymorphisms were reported to predispose to heart failure.[46] Norepinephrine causes 5-HT release from enterochromaffin cells in mouse ileal tissues via α_2

adrenoceptors.[23] Functional alteration of both serotonin and noradrenergic control by the concurrence of α_{2C} Del 322–325 adrenoceptor and *SLC6A4* polymorphisms may also influence gastrointestinal functions.

The α_2 adrenoceptor polymorphisms tested alter the third intracellular loop (which binds to G proteins) or synthesis of the receptor; association of distinct α_2 adrenoceptor genotypes with the IBS constipation phenotype supports a role for genetic predisposition in IBS in twin studies and epidemiological reports.[10–12] These genetic variations may alter manifestations of the disorder. α_{2C} Del 322–325 and α_{2A} −1291 (C→G) polymorphisms result in reduced prejunctional α_2 adrenoceptor function, and norepinephrine released from the prejunctional site is not effectively inactivated by reuptake and subsequent monoamine oxidation. Increased synaptic norepinephrine may inhibit cholinergic enteric motor neurones,[47] reducing gastrointestinal motility (for example, constipation). Moreover, α_{2A} mechanisms directly alter gut motor function.[26]

Genetic variation in α_2 adrenoceptor functions may influence visceral sensation and behaviour in IBS. α_{2A} Adrenoceptors on primary visceral afferents facilitate the transmission of pain to the dorsal horn neurone in the spinal cord, and those in high density in locus coeruleus neurones influence the sedative effects of α_2 agonists,[27] which may relieve psychological or somatisation disorders in IBS. α_{2C} Adrenoreceptors are involved in control of behaviour and those on spinal interneurones modify descending inhibitory pathways from the brainstem[27] that downregulate the dorsal horn neurones and peripheral sensation.[48] Thus loss of normal α_{2C} adrenoceptor function may reduce descending modulation, thereby increasing the "sensitivity" of the dorsal horn neurone.[27] Higher somatic scores with the α_{2C} adrenoceptor polymorphisms may reflect a somatisation disorder, or altered peripheral pathways or central sensation (for example, spinal pathway sensitivity or induction of affective disturbance). Loss of function α_{2C} adrenoreceptor function may also result in reduced feedback through presynaptic receptors, thereby allowing continued release of norepinephrine which results in inhibition of excitatory (cholinergic) neurones in the gut and hence colonic transit delay.

The allele frequencies and genotype distributions observed in the healthy controls in this study illustrate the importance of having ethnic controls in these studies. Thus distributions for the α_{2A} −1291 (C→G) polymorphism is very different in Japanese, Swedish men, and the predominantly female European American populations in our study. On the other hand, distributions for α_{2C} Del 322–325, α_{2A}Lys251, and *SLC6A4* were very similar to those for Caucasians in the published literature.

Table 5 Mean somatic scores (SoSc) and percentage of abnormal SoSc for each genotype combination in patients with lower functional gastrointestinal disorders and healthy controls

N	SLC6A4	α_{2C} Del 322–325	α_{2A} −1291 (C→G)	SoSC score (mean) (SD)	% High score	OR (95% CI)*
51	Wt	Wt	Wt	0.67 (0.57)	33%	1.0 (ref)
51	Wt	Wt	Het/hom	0.74 (0.49)	41%	1.4 (0.6,3.1)
9	Wt	Het/hom	Wt	0.77 (0.65)	44%	1.7 (0.4,7.3)
6	Wt	Het/hom	Het/hom	0.94 (0.24)	83%	11.1 (1.1, 100.1)
108	Het/hom	Wt	Wt	0.59 (0.57)	30%	0.8 (0.4, 1.7)
90	Het/hom	Wt	Het/hom	0.76 (0.58)	40%	1.3 (0.6, 7.8)
10	Het/hom	Het/hom	Wt	1.23 (0.79)	70%	5.0 (1.1, 22.2)
9	Het/hom	Het/hom	Het/hom	0.53 (0.43)	22%	0.6 (0.1, 3.4)

The high somatic symptom score refers to >0.75, the 90th percentile for healthy controls.
*Estimated odds ratio (95% confidence interval) from logistic regression model adjusting for race and sex.
Wt, wild-type; Het, Heterozygous; hom, homozygous polymorphism.

Table 6 Statistical power to detect genotype differences with this study sample

No with genotype SLC6A4 (Wt)	% with phenotype	No with genotype SLC6A4 (het/hom)	≥80% power† to detect this proportion (%) with phenotype
137	Any FGID: 73%	259	Any FGID: ≥85% (or ≤61%)
73	IBS-C: 49%	137	IBS-C: ≥69% (or ≤29%)
78	IBS-D: 53%	170	IBS-D: ≥72% (or ≤32%)
No with genotype α_{2C} Del 322–325 (Wt)	**% with phenotype**	**No with genotype α_{2C} Del 322–325 (het/hom)**	**≥80% power† to detect this proportion (%) with phenotype**
348	Any FGID: 68%	39	Any FGID: ≥88% (or ≤48%)
183	IBS-C: 40%	22	IBS-C: ≥71% (or ≤9%)
224	IBS-D: 51%	20	IBS-D: ≥82% (or ≤20%)
No with genotype α_{2A} −1291 (C→G) (Wt)	**% with phenotype**	**No with genotype α_{2A} −1291 (C→G) (het/hom)**	**≥80% power† to detect this proportion (%) with phenotype**
210	Any FGID: 67%	182	Any FGID: ≥80% (or ≤54%)
109	IBS-C: 37%	99	IBS-C: ≥57% (or ≤17%)
140	IBS-D: 51%	108	IBS-D: ≥69% (or ≤33%)
No with genotype α_{2A} −1291 (C→G) (Wt) and SLC6A4 (Wt)	**% with phenotype**	**No with genotype α_{2A} −1291 (C→G) (het/hom) and SLC6A4 (het/hom)**	**≥80% power† to detect this proportion (%) with phenotype**
68	Any FGID: 71%	115	Any FGID: >88% (or <52%)
39	IBS-C: 49%	66	IBS-C: >76% (or <22%)
39	IBS-D: 49%	69	IBS-D: >76% (or <22%)

†Based on two sample test for proportions using a two sided alpha level of 0.05.
IBS-C, irritable bowel syndrome with predominant constipation; IBS-D, irritable bowel syndrome with predominant diarrhoea; FGID, functional gastrointestinal disorders.

The association (p = 0.04) of the combination of α_{2C} Del 322–325 and *SLC6A4* polymorphisms with IBS is consistent with evidence that modulation of α_2 adrenoceptor function may alter the biological effects of serotonin.[43] An increased availability of 5-HT at the synapse resulting from less effective reuptake due to the *SLC6A4* polymorphism may result in greater stimulation of afferent pathways. Decreased α_{2C} function by the α_{2C} Del 322–325 polymorphism might reduce 5-HT release from enterochromaffin cells.[23 43] The lack of a significant association observed between IBS phenotype and *SLC6A4* polymorphism does not negate a potential role of this genetic variation in responses to stress (as recently demonstrated in depression, see below) or to responses to serotonergic medications.[22] *SLC6A4* polymorphisms have been associated with a variety of psychological disorders and their response to therapy.[29 43]

Further studies are needed to assess the biological effects of the combined α_{2C} and *SLC6A4* polymorphisms. Specific hypotheses that are plausible and should be addressed in future studies are: firstly, that α_{2C} Del 322–325 is associated with a greater prevalence and colonic transit delay in IBS than in IBS patients without the deletion; and secondly, that there is greater severity and frequency of pain and lower thresholds for sensation of rectal or colonic distension in patients with functional lower gastrointestinal disorders with the combined α_{2C} and *SLC6A4* polymorphisms compared with patients without the genetic polymorphisms.

Our study followed the standards recommended for appraising genotype prevalence and gene-disease association[45]: analytical validity by DNA sequencing to confirm results by PCR and restriction; blinding of investigators with laboratory personnel assessing genotype and clinicians categorising the phenotype; confirmation that genotype frequencies conform to those reported in controls[46 49 50] from the same source population; and presentation of genotype frequencies. There were no patients with the mutation tested for *NET* or for the α_{2A}Lys251 polymorphism in the first 100 patients and 20 controls. However, the investigated *NET*

mutation had been identified in a specific family with orthostatic intolerance syndrome.[17] This mutation was not confirmed in a recent study of 14 patients with orthostatic intolerance syndrome.[51] Therefore, we cannot exclude the possibility that alternative genetic alterations in the *NET* gene may be relevant to disorders of gastrointestinal function.

We assessed the power to detect associations between phenotypes and candidate genes. We detected no NET mutation or α_{2A}Lys251 polymorphisms in 120 predominantly Caucasian patients and controls. Absence of any genetic variation in these two candidate genes suggests that they are unlikely to play significant roles in determining IBS or somatic symptom phenotype. However, given the low prevalence of the α_{2A}Lys251 polymorphism in European Americans, further studies in African-Americans are required. Table 6 summarises the "degree of association" between the presence/absence of specific polymorphisms in α_{2C} Del 322–325, α_{2A} −1291 (C→G), and *SLC6A4* genes or combinations versus specific symptom subgroups (including overall FGID). It indicates that clinically meaningful associations of these three candidate genotypes could have been detected with at least 80% power.

In summary, we have shown that two functionally distinct α_2 adrenoceptor polymorphisms, alone or in combination with a *SLC6A4* polymorphism, are associated with constipation and high somatic symptom scores in patients with lower functional gastrointestinal disorders. These data suggest that genetic factors may interact with environmental factors and contribute to the manifestations of IBS; however, the strength of the genetic contribution to the phenotype is unclear. A gene-by-environment interaction was recently demonstrated in depression as *SLC6A4* polymorphism influences the effect of life event stress on depression.[52] Similar gene-environment interaction studies are needed in IBS in view of the evidence that life event stress is associated with IBS,[2–4] models of visceral hypersensitivity,[53] and depression.[54] Moreover, in view of the reduced global IBS symptoms and bowel dysfunction in IBS patients treated with an α_2 agonist[55]

and modulation of µ opioid responses to pain by genetic variation in activation of norepinephrine by catechol-o-methyl transferase,[56] pharmacogenomic studies would be of significant interest and potential clinical relevance.

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Authors' affiliations

H J Kim, M Camilleri, P J Carlson, F Cremonini, I Ferber, D Stephens, S McKinzie, R Urrutia, Clinical Enteric Neuroscience Translational and Epidemiological Research (CENTER) Program, Gastroenterology Research Unit, Mayo Clinic College of Medicine, Rochester, Minnesota, USA

A R Zinsmeister, Department of Health Sciences Research, Division of Biostatistics, Mayo Clinic College of Medicine, Rochester, Minnesota, USA

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