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Serotonin, *ghrelin*, and *motilin* gene/receptor/transporter polymorphisms in childhood functional constipation

Bengu Arslan[1*], Guzide Dogan[2], Seda Orenay-Boyacioglu[3], Metin Caliskan[4], Murat Elevli[5]

SUMMARY

OBJECTIVE: Functional constipation is the most common form of constipation, and its exact aetiology is still unclear. However, it is known that deficiencies in hormonal factors cause constipation by changing physiological mechanisms. Motilin, ghrelin, serotonin acetylcholine, nitric oxide, and vasoactive intestinal polypeptide are factors that play a role in colon motility. There are a limited number of studies in the literature where hormone levels and gene polymorphisms of *serotonin* and *motilin* are examined. Our study aimed to investigate the role of motilin, ghrelin, and serotonin gene/receptor/transporter polymorphisms in constipation pathogenesis in patients diagnosed with functional constipation according to the Rome 4 criteria.

METHODS: Sociodemographic data, symptom duration, accompanying findings, the presence of constipation in the family, Rome 4 criteria, and clinical findings according to Bristol scale of 200 cases (100 constipated patients and 100 healthy control) who applied to Istanbul Haseki Training and Research Hospital, Pediatric Gastroenterology Outpatient Clinic, between March and September 2019 (6-month period) were recorded. Polymorphisms of *motilin-MLN* (rs2281820), *serotonin receptor-HTR3A* (rs1062613), *serotonin transporter-5-HTT* (rs1042173), *ghrelin-GHRL* (rs27647), and *ghrelin receptor-GHSR* (rs572169) were detected by real-time PCR.

RESULTS: There was no difference between the two groups in terms of sociodemographic characteristics. Notably, 40% of the constipated group had a family history of constipation. The number of patients who started to have constipation under 24 months was 78, and the number of patients who started to have constipation after 24 months was 22. There was no significant difference between constipation and control groups in terms of genotype and allele frequencies in *MLN*, *HTR3A*, *5-HTT*, *GHRL*, and *GHSR* polymorphisms ($p>0.05$). Considering only the constipated group, the rates of gene polymorphism were similar among those with/without a positive family history of constipation, constipation onset age, those with/without fissures, those with/without skin tag, and those with type 1/type 2 stool types according to the Bristol stool scale.

CONCLUSION: Our study results showed that gene polymorphisms of these three hormones may not be related to constipation in children.

KEYWORDS: Child. Constipation. *Ghrelin*. *Motilin*. *Serotonin*. Polymorphism, single nucleotide.

INTRODUCTION

Constipation is defined as difficulty in passing stools that may be infrequent (≤2 per week), painful, and associated with stool retention. Chronic constipation is a common health problem, especially in children, and has a great impact on physical/mental health. Chronic constipation is divided into two groups, namely, organically caused and functional constipation. Notably, 95% of constipation is of the functional type in childhood, and the prevalence rates are reported to be 32.2% worldwide. In recent years, research on aetiology and pathogenesis of childhood constipation has focused on environmental factors, behavioural problems, and genetic factors. Various environmental factors are associated with a higher prevalence of childhood constipation which include diet and mobility of the children, low maternal educational level, and social circumstances. The relationship of constipation with behavioural problems is also complex, partly because constipation can be both a cause and product of behavioural problems. Furthermore, constipation has been reported more frequently in children with specific behavioural phenotypes, such as autism spectrum disorder[1,2]. The role of genetics in the aetiology of constipation is still largely unknown. So far, linkage studies, association studies, and direct gene sequencing have yet to find mutations in genes specifically associated with constipation. However, more than 40% of constipated children have a family history of constipation, and a genetic

[1]University of Health Sciences, Basaksehir Cam and Sakura City Hospital, Department of Pediatric Inherited Metabolic Diseases – Istanbul, Turkey.
[2]Bezmialem Vakıf Unıversity, Department of Pediatric Gastroenterology – Istanbul, Turkey.
[3]Aydin Adnan Menderes University, Faculty of Medicine, Department of Medical Genetics – Aydın, Turkey.
[4]Usak University, Faculty of Medicine, Department of Medical Biology – Usak, Turkey.
[5]Istanbul Haseki Training and Research Hospital, Department of Pediatric Gastroenterology – Istanbul, Turkey.
*Corresponding author: benguarslan.ba@gmail.com

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predisposition is suggested in which monozygotic twins have six times more constipation than dizygotic twins[2].

Deficiencies in hormonal factors cause changes in physiological mechanisms and lead to constipation. Motilin, ghrelin, serotonin, acetylcholine, nitric oxide, and vasoactive intestinal polypeptide play a role in colon motility. The most important task of the motilin hormone is to speed up bowel movements. Motilin binds to motilin receptors (MTLR) located on the cell surface to show its effect[3]. Ghrelin has been shown to have a prokinetic effect on gastrointestinal motility through the vagus and pelvic nerves. Centrally acting GRLN-R agonists stimulate defecation in animals and humans and accelerate gastrointestinal passage[4]. Serotonin plays a role in motility and secretion, slows gastric emptying, and increases colonic motor activity[5].

In the literature, there are limited studies involving only serotonin/motilin hormone levels and gene polymorphisms, and no study have evaluated the polymorphisms of three factors (*motilin*, *ghrelin*, and *serotonin*) together. Our study aimed to clarify the effectiveness of these factors in the pathogenesis of functional constipation and to reveal the underlying genetic cause in many cases monitored by functional constipation.

METHODS

Ethics

Ethical approval was obtained from the Corporate Ethics Committee of Istanbul Haseki Training and Research Hospital in 2019 (document number: 2011-KAEK-50). Informed consent was obtained from the families of all study participants before the study.

Samples and sample size

Our study included 100 children with constipation (constipated group) and 100 children without constipation and gastrointestinal system problems (control group) in Istanbul Haseki Training and Research Hospital, Pediatric Gastroenterology Outpatient Clinic, between March and September 2019 (6-month period). Inclusion criteria of the constipated group were as follows: (1) those with chronic constipation, who meet the criteria for functional constipation according to the Rome 4 criteria; (2) children aged 6 months to 18 years; and (3) those who read the patient informed consent form and agreed to participate in the study. exclusion criteria were as follows: (1) congenital anomalies of the anorectal region and Hirschprung's disease; (2) those with neurological disorders such as cerebral palsy and spina bifida; (3) those diagnosed with hypothyroidism, diabetes mellitus, and diabetes insipidus; (4) those who have had previous abdominal surgery; and (5) those who read the patient informed consent form and did not agree to participate in the study.

Sociodemographic data, duration of symptoms, accompanying findings, presence of constipation in the family, Bristol stool scale findings, complete physical examination findings, and body weight, body length, and weight and height percentiles were recorded in the patient's forms.

The question of encopresis and urinary incontinence evaluation was asked to the families of children older than 3.5 years, who had urinary and stool control and had the ability to urinate and defecate, and the answers were recorded in our case form. When the family answered "yes" to the question of repeated (usually involuntary) passing stool on the child's clothes, encopresis or faecal incontinence was detected and the answers were recorded. The families were asked about the enuresis of the children and the answers were recorded. Anal fissure and skin tag were detected during anal examination in children with constipation and the data were recorded in our case form.

The sample size in the study was determined using G-power. Considering similar studies on the subject in the literature, the minimum sample size was calculated as 200 people, with a statistical power of 89.8%, a confidence level of 95%, and a type 1 error rate of 0.05[6].

Selection of *MLN*, *HTR3A*, *5-HTT*, *GHRL*, and *GHSR* polymorphisms

In the study, polymorphisms [*motilin-MLN* (rs2281820), *serotonin receptor-HTR3A* (rs1062613), *serotonin transporter-5-HTT* (rs1042173), *ghrelin-GHRL* (rs27647), and *ghrelin receptor-GHSR* (rs572169)] that are thought to be related to patients diagnosed with functional constipation and are found to be associated with some gastrointestinal diseases were selected.

DNA isolation

DNA isolation from blood samples was performed using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the kit usage procedures of the commercial company. A volume of 200 µl of blood sample was placed in a 1.5-ml tube, and 200 µl of binding buffer and 40 µl of proteinase K were added, mixed, and incubated at 70°C for 10 min. Then, 100 µl of isopropanol and 200 µl of elution buffer were added, mixed, and incubated at 70°C. All of this prepared mixture was transferred to a tube and centrifuged at 8,000 *g* for 1 min. Then, 500 µl of inhibitor removal buffer was added to the filter tube and centrifuged at 8,000 *g* for 1 min. A volume of 500 µl wash buffer was added and centrifuged at 8,000 *g* for 1 min. This process was repeated 2 times. The tubes were emptied and centrifuged

at 13,000 *g* for 1 min. Elution buffer kept at 70°C was added to the filter tube by placing an Eppendorf tube under the filter tube. Pure DNA was obtained by centrifugation at 8,000 *g* for 1 min.

DNA concentration and purity measurement

DNA purity and concentration were measured using NanoDrop 1000 Spectrophotometer version 3.7 (Thermo Scientific, USA). The concentration and purity of the obtained DNA samples were observed at wavelengths of 260 and 280 nm determined by measuring their absorbance. Generally, 5–50 ng of DNA per reaction was considered sufficient to detect a single-nucleotide polymorphism (SNP).

Determination of genotypes

SNPs were detected in LightCycler 480 (Roche) using a LightSNiP assay (TIBMOLBIOL GmbH, Berlin, Germany) panel based on SimpleProbe® (Roche). Probes provided by the manufacturer detect single-base mismatches and polymorphisms, making the analysis possible. SNPs were observed by a melting curve analysis at the end of amplification.

For each SNP, there is a tube of primer and a tube containing the simple probes in lyophilized form. A volume of 105 µl of water was added to each tube separately and vortexed.

It was prepared according to real-time mix separately for each SNP. After 7.5 µl per mix plate, the final volume of 10 µl was reached by distributing the sample and adding 2.5 µl of DNA to it. The device protocol was entered and worked on the LightCycler 480 device.

Data analysis

Data analysis was done by melting curve genotyping using the LightCycler 480 software.

Statistical analysis

Differences between the patients and controls in terms of categorical variables, such as demographic and clinical data, were analyzed using chi-square (χ^2) tests, while continuous variables were analyzed with Student's t-test. Differences in allelic distribution of the SNPs were also examined using χ^2 test. The statistical significance level was accepted as $p<0.05$.

RESULTS

The demographic characteristics of the groups are shown in Table 1. The average duration of constipation was 19.5±19.3 months (3–120 months), and the median duration of constipation was 12 months in the constipated group. The mean duration of defecation time was 4.4±1.9 days (2 days–11 days) in the constipated group. According to the Bristol stool scale evaluations of the patient group, 56 patients had type 1 stool and 44 patients had type 2 stool. Those with and without encopresis, urinary incontinence, anal fissure, and skin tag were compared with the duration of constipation. There was no statistically significant difference between the groups ($p>0.05$).

Genotype and allele frequency comparisons of constipation and control groups are given in Table 2. According to these comparisons, there was no significant difference in genotype and allele frequencies in terms of gene polymorphisms investigated between constipation and control groups ($p>0.05$). No statistically significant difference was found in the families of children with constipation according to the history of constipation and the onset age of symptoms compared to the allele and genotype frequencies of gene polymorphisms (Table 3) ($p>0.005$).

Table 1. Demographic features of groups.

Demographic features	Constipated group n=100	Control group n=100	p-value
Average age (months)	70.9±54.9	68.9±54.0	0.799
Gender (male/female)	45/55	53/47	0.258
Weight percentile	49.4±32.5	49.6±27.0	0.955
Height percentile	48.1±30.0	48.9±27.8	0.835
Faecal incontinence (yes/no %)	17/83		
Urinary incontinence (yes/no %)	9/91		
Recurrent urinary tractinfection (yes/no %)	14/86		
Family history of constipation (yes/no %)	40/60		
Anal fissure (yes/no %)	33/67		
Skin tag (yes/no %)	28/72		

Table 2. Genotype and allele frequency of constipation and control groups.

Gene and SNP	Constipated group (n=100)	Control group (n=100)	p-value
5HTT-rs1042173			
Genotype GG	31	28	
Genotype GT	45	46	0.885
Genotype TT	24	26	
Allele G	107	102	0.689
Allele T	93	98	
HTR3A-rs1062613			
Genotype CC	70	68	
Genotype CT	27	27	0.768
Genotype TT	3	5	
Allele C	167	163	0.693
Allele T	33	37	
MLN-rs2281820			
Genotype CC	46	43	
Genotype CT	14	9	0.384
Genotype TT	17	8	
Allele C	126	139	0.204
Allele T	74	61	
GHRL-rs27647			
Genotype CC	17	8	
Genotype CT	35	36	0.133
Genotype TT	48	56	
Allele C	71	52	0.070
Allele T	129	148	
GHSR-rs572169			
Genotype GG	59	65	
Genotype GA	38	27	0.109
Genotype AA	3	8	
Allele G	156	157	0.070
Allele A	44	43	

DISCUSSION

Although studies have concluded that constipation develops mainly between the ages of 2–4 years, in 17–40% of children, symptoms begin within the first year[7]. The data obtained in our study show that the average age of the patient group is 70.9±54.9 months, similar to the literature. In our study, the mean constipation time was 19.5±19.3 months (3–120 months), and the median constipation time was 12 months. Misra et al. in their study on 101 constipated cases reported the average duration of complaints of the patients to be 32.2–40.7 months[8]. In our study, the duration of constipation was found to be shorter than in the literature. This may be due to the fact that the patient's family awareness is high and that the family receives polyclinic service in a shorter time.

Functional urinary incontinence is the most frequently investigated clinical finding accompanying constipation[9,10]. There are studies in the literature showing that recurrent urinary infections and the urge to urinate in children with constipation are significantly increased, but bladder functions are positively affected after constipation treatment[11,12]. Benninga et al. reported the rate of urinary incontinence as 41% in children with constipation[9]. In our study, urinary incontinence, urinary tract infections, and urinary system symptoms were found at lower rates compared to the literature. This may be due to ethnic origin or the low number of cases. In our study, it was observed that the complaints of enuresis decreased in children who participated in the study and treated for constipation, similar to the literature.

Table 3. Comparison of genotype distributions of constipation patients with/without a positive family history of constipation and symptom onset age.

Gene and SNP Genotype		With constipation positive family history n=60	Without constipation positive family history n=40	P-value	Symptom onset age <24 months n=78	Symptom onset age >24 months n=22	p-value
5HT rs1042173	GG	21	10	0.187	24	6	0.622
	GT	25	20		36	9	
	TT	14	10		18	7	
HTR3A rs1062613	CC	43	27	0.587	57	13	0.445
	CT	14	13		19	8	
	TT	3	0		2	1	
MLN rs2281820	CC	22	18	0.505	34	6	0.250
	CT	30	16		35	11	
	TT	8	6		9	5	
GHRL rs27647	CC	8	9	0.392	11	6	0.289
	CT	22	13		29	6	
	TT	30	18		38	9	
GHSR rs572169	GG	36	23	0.344	49	10	0.153
	GA	23	15		26	12	
	AA	1	2		3	0	

Genetic and environmental factors are also thought to play a role in constipation. In many studies, constipation has been questioned in the family. Edan and Yahya found the rate of history of constipation in the family was 21% in the constipated group and 0.7% in the control group[13]. Similar studies in the literature found the rate of family history of constipation to be between 41 and 70.8%[14]. In our study, a history of familial constipation was found in 40 (40%) patients, the rate similar to the literature.

Camilleri, who evaluated the effects of serotonin on the gastrointestinal tract, found that serotonin increased intestinal motility. In addition, he reported that serotonin polymorphism was high in patients with diarrhoea and low in patients with constipation[5]. Some studies showed association of serotonin levels and functions in the gastrointestinal tract and even in cases of increased intestinal peristalsis, others failed to show relationship of serotonin or its receptors with gastrointestinal diseases. For example, no difference was found in terms of polymorphism in the integral membrane protein SLC6A4 (5-HTT) involved in the presynaptic neuronal transfer of serotonin from the synaptic area in irritable bowel disease[15]. In our study, allele and genotype frequency states of *5-HTT* and *HTR3A* polymorphisms were examined in patients and control groups, and no significant difference was found between the groups. Similarly, when the patient group was evaluated within itself, no significant difference was found between the patient and control groups in terms of allele and genotype frequency states of *5-HTT* and *HTR3A* genes according to the constipation onset time, and positive family history and defecation frequency.

Recent studies in animals and humans related to ghrelin show that it also has a prokinetic activity in the lower gastrointestinal tract[16]. In another study, it has been shown that serum ghrelin levels are essential in response to lactulose treatment in patients with constipation[17]. In this study, patients' serum ghrelin levels were measured at the beginning of constipation treatment, and those with low serum ghrelin levels initially responded better to the lactulose treatment. In contrast, those who had high serum ghrelin levels did not respond adequately to the other group than lactulose treatment. The results of treatment suggest that the effect of ghrelin on intestinal motility may be more effective on functional constipation aetiology. There is no study in the literature investigating and evaluating ghrelin polymorphisms in patients with constipation. In our study, allele and genotype frequencies of GHRL and GHSR polymorphisms were examined in the patient and control groups, and no significant difference was found between the groups.

The most important task of the motilin hormone is to speed up bowel movements. Peak levels of motilin in plasma are correlated with peristaltic solid contractions[18]. Motilin binds to MTLRs on the cell surface to show its effect. In humans, MTLR density is highest in the gastroduodenal region and decreases gradually towards the colon[19]. In the literature, there are studies in which motilin levels are measured in patients with constipation, in which motilin and agonists are given to patients, and the efficacy is evaluated. In the study by Hirabayashi et al., dogs were given mitemcinal (GM-611), an agonist of motilin, and found that colon motility and the number of defecations increased[20]. Although motilin is a factor that may affect constipation, the motilin levels measured in constipated individuals are similar to those of healthy controls. For example, Penning et al. found that in adult patients with slow-pass constipation, fasting and toughness did not detect a change in plasma motilin level compared to the healthy control group, while Aydın et al. also found a significant reduction in the level of motilin compared to the control group in adult constipated patients[21,22]. A study on the relationship between polymorphism and serum motilin level found that there was no significant difference in *MLN* gene polymorphism between paediatric patients with constipation and those without[6]. In our study, allele and genotype frequencies of *MLN* gene polymorphisms were examined in the patient and control groups, and no significant difference was found between the groups.

LIMITATIONS

Studies involving larger patient groups are needed to support this result.

CONCLUSION

Our study results showed that gene polymorphisms of these three hormones may not be related to constipation in children. As in our study, the fact that the results of genetic studies in the literature do not provide a relationship with constipation in children reveals the need for epigenetic-based studies on this issue.

AUTHORS' CONTRIBUTIONS

BA: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **GD:** Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft. **SOB:** Resources, Formal Analysis, Data curation. **MC:** Resources, Formal Analysis, Data curation. **ME:** Project administration, Supervision, Visualization Investigation.

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