The Preproghrelin 3056 TT Genotype is Associated with the Feeling of Hunger and Low Acylated Ghrelin Levels in Japanese Patients with *Helicobacter pylori*-negative Functional Dyspepsia

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Abstract

Objective An impairment of gastric motility is strongly associated with the pathophysiology of functional dyspepsia (FD). Plasma ghrelin is one of the key molecules linked to gastric motility. Therefore, this study aimed to evaluate whether ghrelin (GHL) gene polymorphisms are associated with clinical symptoms, the plasma ghrelin levels and gastric emptying in patients with FD as defined by the Rome III classification.

Methods We enrolled 74 *Helicobacter pylori*-negative patients presenting with typical symptoms of FD (epigastric pain syndrome (EPS), n=23; postprandial distress syndrome (PDS), n=51) and 102 healthy volunteers. Gastric motility was evaluated according to the Tmax value and T_{1/2} using the ¹³C-acetate breath test. We used the Rome III criteria to evaluate upper abdominal symptoms and SRQ-D scores to determine the depression status. The Arg51Gln(346G->A), preproghrelin3056T->C, Leu72Met(408C->A) and Gln90Leu(3412T->A) polymorphisms were analyzed in DNA in blood samples obtained from the enrolled subjects. Genotyping was performed using polymerase chain reaction.

Results There was a significant relationship (p=0.048) between the preproghrelin 3056TT genotype and the serum levels of acylated ghrelin in the *H. pylori*-negative FD patients. The preproghrelin 3056TT genotype was significantly (p=0.047) associated with the feeling of hunger in the *H. pylori*-negative FD patients.

Conclusion The preproghrelin 3056TT genotype is significantly associated with the acylated ghrelin levels and the feeling of hunger in *H. pylori*-negative FD patients. Further studies are needed to clarify the association between the preproghrelin 3056TT genotype and lower plasma acylated ghrelin levels and the impact of this relationship on the feeling of hunger in *H. pylori*-negative FD patients.

Key words: functional dyspepsia, gastric motility, polymorphism, preproghrelin, the feeling of hunger, acylated ghrelin

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Introduction

Functional dyspepsia (FD) is a common gastrointestinal disorder that has been subclassified into two new disease categories under the Rome III classification: epigastric pain

syndrome (EPS) and postprandial distress syndrome (PDS) (1). Impairment of gastric motility, including gastric emptying, is strongly associated with the pathophysiology of FD (2). Impaired physiological gastric emptying in patients with FD is accompanied by a variety of symptoms ranging from premature saturation, fullness, nausea, vomiting, epi-

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gastric pain and acid reflux accompanied by delayed emptying. We have previously reported that the T_{max} value, a marker of gastric emptying, is significantly greater in PDS patients compared to that observed in healthy volunteers (3). In addition, we have also reported that low levels of serum acylated ghrelin are significantly associated with disturbed T_{max} values (3).

Ghrelin (GHRL) is predominantly produced in the stomach, and substantially lower amounts are derived from the bowel, pancreas, pituitary, kidneys and placenta. GHRL is recognized to be an endogenous ligand for the growth hormone secretagogue receptor in the oxyntic gland of the stomach (4). The plasma ghrelin concentration rises during fasting and falls quickly after meals (5). Therefore, GHRL plays a role in the long-term as well as short-term regulation of feeding (6). In human studies, the administration of GHRL increases food intake and the sensation of hunger compared with saline infusion alone (7). In rodents, central or peripheral administration of GHRL stimulates gastric contraction and emptying (2) and exhibits prokinetic effects in a postoperative ileus model in rats (8). In pharmacologic studies, acylated ghrelin has been shown to accelerate gastric emptying, increase gastric tone and induce premature interdigestive migrating motor complex activity.

The preproghrelin gene codes for two short hormones, ghrelin and obestatin. The gene encoding preproghrelin, the ghrelin precursor, is located on chromosome 3(3p25-26) and comprises four exons and three introns (9). Three nonsynonymous single nucleotide polymorphisms (SNPs) in the GHRL gene have been reported. The Arg 51Gln SNP is associated with lower GHRL and insulin-like growth factor I concentrations (10). The Leu72Met SNP has been linked to obesity-related phenotypes; however, these findings are controversial (9-11).

In this study, we aimed to investigate whether GHRL gene SNPs contribute to FD symptoms by impairing gastric emptying and reducing the acylated ghrelin levels in *Helicobacter pylori* (*H. pylori*)-negative Rome III-based FD patients.

Materials and Methods

Patients

Seventy-four *H. pylori*-negative patients presenting with typical symptoms of FD (EPS, n=23; PDS, n=51) and 102 healthy volunteers were enrolled after undergoing upper gastrointestinal endoscopy and abdominal ultrasonography. The patients were diagnosed according to the Rome III criteria (12). The healthy volunteers were recruited from among the Japanese medical staff of Nippon Medical School who had no clinical history of gastroduodenal disease, including clinical symptoms of FD. Three of the healthy volunteers were *H. pylori*-positive. The exclusion criteria included severe heart disease, renal or pulmonary failure, liver cirrhosis, severe systemic illness and a history of malignant disease.

Patients with a history of previous gastroduodenal surgery, duodenal ulcer scars, diabetes mellitus and recent use of nonsteroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitor (PPIs) or anticoagulants at endoscopy were also excluded. The presence of *H. pylori* infection was determined using both the ¹³C-urea breath test and histological identification. Written informed consent was obtained from all subjects prior to undergoing upper gastrointestinal endoscopy and abdominal ultrasonography for an evaluation of their dyspeptic symptoms. The study protocol was approved by the Ethics Review Committee of Nippon Medical School Hospital.

Clinical symptoms

The clinical symptoms of FD were evaluated according to the Rome III criteria (12). The clinical symptoms must have involved at least one of the following: early satiation, bothersome postprandial fullness, epigastric pain or epigastric burning. The diagnostic criteria for PDS included bothersome postprandial fullness occurring after ordinary-sized meals and/or early satiation that prevented completion of a normal meal, with either symptom occurring at least several times a week. The diagnosis of EPS was made based on all of the following: pain or burning that is intermittent, localized to the epigastrium and of at least moderate severity occurring at least once per week. A diagnosis of PDS and EPS was fulfilled based on symptoms occurring for the last three months and the onset of symptoms occurring at least six months prior to diagnosis. In this study, we enrolled PDS patients without abdominal pain or epigastric burning. One patient diagnosed with EPS experienced symptoms that overlapped those of PDS. Abdominal symptoms, including the feeling of hunger, were assessed using a modified questionnaire that has been applied in previous studies and the feeling of hunger was evaluated according to three factors, including frequency, intensity and duration as follows: none, 5; one-two days/3 months, 4; one day/month, 3; one day/week, 2; half a day, 1; all day, 0 (1, 13, 14). We assessed abdominal symptoms using the modified Glasgow dyspepsia severity score (15), which evaluates the frequency of dyspeptic symptoms over the past six months (never, score 0; on only 1 or 2 days, score 1; on approximately 1 day per week, score 3; on approximately 50% of days, score 4; and on most days, score 5), the duration of symptoms (maximal score 5) and the intensity of symptoms (maximal score 3). The status of depression was evaluated using the SRQ-D (Self-Rating Questionnaire for Depression) score (16). The health-related quality of life (HR-QOL) was assessed using the Japanese version of the 8-item Short-Form Health Survey (SF-8) (17). Scores for the eight domains ("general health," "physical functioning," "role-physical," "bodily pain," "vitality," "social functioning," "mental health" and "role-emotional") and two summaries (physical component summary (PCS) and mental component summary (MCS)) were derived. A score <50 indicates an impaired QOL.

Measurement of gastric motility

Sodium acetate (water soluble) was used as a tracer to evaluate emptying of liquids (Cambridge Isotope Laboratories; MA, USA). The probes were analyzed using nondispersive infrared spectroscopy (IRIS, Wagner Analyzentechnik; Bremen, Germany). The subject's own production of 300 mmol CO₂ per m² body surface and per hour were set as the default. We used an Integrated Software Solutions program to calculate the half gastric emptying time (T_{1/2}) and the lag phase (T_{max}; min) as the point of maximum gastric emptying according to Hellmig et al. (18). The half gastric emptying time (T_{1/2}) represents the time when 50% of the initial gastric content is emptied. A T_{max} value greater than 60 minutes, representing the mean T_{max} plus SD in healthy volunteers, was defined as indicating relative disturbances in gastric emptying according to the diagnostic criteria of the Japan Society of Smooth Muscle Research and our study (3, 19).

Study protocol for gastric emptying of liquids

We used a liquid test meal consisting of 100 mg of ¹³C-acetate dissolved in 200 mL of liquid meal (Racol, 1 mL/L kcal; Otsuka Pharmacia Company, Tokyo, Japan). Breath samples were collected at 0 sec, 10 sec, 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, 60 min, 75 min and 90 min after ingestion of a test meal at 10:00 a.m. Patients were instructed not to drink, eat or smoke during the test.

Genotyping

We developed or optimized the following assays for genetic variation. Genotypes were confirmed or selectively assessed for GHRL (Arg51Gln 346G->A; rs:34911341), (preproghrelin 3056T->C; rs:2075356), (Leu72Met 408C->A; rs:696217) and (Gln90Leu 3412T->A; rs:4684677) genotypes using direct sequencing with the ABI 7500 Fast. Gene polymorphisms were determined according to methods reported in other studies. Real-time polymerase chain reaction using TaqMan chemistries (Applied Biosystems, Foster City, CA) was used to identify the alleles present in each sample. Real-time polymerase chain reactions were performed in an Applied Biosystems 7500 Fast machine (Applied Biosystems). Taq Man primer-probe assays for Arg51Gln SNPs 346G->A (rs:34911341; C-25607739-20), preproghrelin SNPs 3056T->C (rs:2075356; C-3229939-1), Leu72Met SNPs 408C->A (rs:696217; C-3151003-20) and Gln90Leu SNPs 3412T->A (rs:4684677; C-25607748-10) were purchased from Applied Biosystems. Briefly, each reaction volume was 10 µL and consisted of 5 µL of TaqMan Genotyping Master Mix (Applied Biosystems), 0.25 µL of a 40× primer probe assay mix (Applied Biosystems), 3.75 µL of H₂O and 1 µL (10 ng) of genomic DNA. The amplification conditions consisted of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds followed by 50°C for two minutes. The data were analyzed using an automated software program (SDS 2.1; Applied Biosystems)

to determine the genotype of each sample.

Measurement of the plasma ghrelin levels in the *H. pylori*-negative FD patients

We measured the plasma ghrelin levels to evaluate their association with polymorphisms of GHRL in the *H. pylori*-negative FD patients and healthy volunteers. Blood samples were obtained after an overnight fast of >12 hours, immediately transferred to chilled polypropylene tubes containing Na₂EDTA and aprotinin, then centrifuged at 4°C. One-tenth of the volume of 1NHCl was immediately added to the separated plasma. The acylated and des-acylated forms of ghrelin were measured using commercially available ELISA kits according to the manufacturer's instructions (Active Ghrelin ELISA Kit and Desaclyl-Ghrelin ELISA Kit, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). The intra- and inter-assay coefficients of variation (CV) were 6.5% and 9.8% for acylated ghrelin and 3.7% and 8.1% for des-acylated ghrelin, respectively.

Statistical analysis

For the statistical evaluation of group data, Students' *t*-test for paired data and an analysis of variance (ANOVA) for multiple comparisons were followed by Scheffe's *F* test. The Mann-Whitney *U* test was used for the analysis of categorical data. The distribution of alleles at each locus was assessed using the χ^2 statistic of the Hardy-Weinberg equilibrium. To determine factors associated with disturbance of gastric emptying, a multiple logistic regression analysis was used at 95% confidence intervals and associated *p* values. In addition, we evaluated the differences between two group factors (genotype of four SNPs of the GHRL gene, *H. pylori*-negative FD patients vs. healthy volunteers) for mean values of the acylated ghrelin levels using a two-way ANOVA analysis. The data analyses were performed using a standard software package (SPSS version 13.0, Chicago, IL). A *p* value of less than 0.05 was considered to be statistically significant.

Power of the study

In this study, we aimed to assess the potential association between the acylated ghrelin levels and symptoms of FD in patients with a preproghrelin 3056T->C allele status. In our study, we determined the sample size using the PS (Power and Sample size calculations program) software program, a gift from Vanderbilt University. The deviation of the acylated ghrelin levels in the healthy volunteers was approximately 3.2 ($\sigma=3.2$). Using the above data, setting $\alpha=0.05$, $\beta=0.80$, effect size=0.625 and the estimated mean ghrelin level in patients with FD=13.7 fmol/mL, 102 healthy volunteers and 74 FD patients would be sufficient to identify clinically relevant differences.

Table 1. Characteristics of the Patients

	FD	PDS	EPS	Healthy volunteers
Subjects (n)	74	51	23	102
Age	59.4±14.2	60.1±14.4	54.6±3.16	51.1±19.9
Sex(M/F)	36/38	24/27	12/11	54/48
BMI (kg·m ⁻²)	22.4±2.51	22.1±2.35	22.5±0.72	22.6±2.54
SRQ-D	9.87±0.84*	9.14±0.77**	12.6±1.61***	6.11±0.47
T _{1/2} (min)	95.4±3.28*	96.2±4.58**	93.3±5.63***	74.6±1.92
T _{max} (min)	59.3±1.68*	59.9±1.95**	59.0±3.78***	47.4±1.21

Summary values are mean ± SD.

T_{1/2} represents the time when 50% of the initial gastric content was emptied.T_{max} value > 60min was defined to represent relative disurbance in gastric emptying.

PDS : postprandial distress syndrome

EPS : epigastric pain syndrome

BMI : body mass index

SRQ-D : self-rating questionnaire for depression

T_{1/2} (min) : half gastric emptying timeT_{max} (min) : lag phase as the point of maximum gastric emptying

* p<0.001 vs.healthy volunteers

** p<0.001 vs.healthy volunteers

*** p<0.001 vs.healthy volunteers

Results

Characteristics of the *H. pylori*-negative FD, PDS and EPS patients and healthy volunteers

The age, sex and BMIs of the *H. pylori*-negative FD, PDS and EPS patients did not differ statistically from those of the healthy volunteers (Table 1). However, the SRQ-D scores and the T_{1/2} and T_{max} values in the *H. pylori*-negative FD, PDS and EPS patients were significantly (p<0.001) higher than those observed in the healthy volunteers. The rates of impaired gastric emptying in the FD patients (43.2%) and PDS patients (41.2%) were significantly (p<0.01) higher than that observed in the healthy volunteers (4.3%) (Table 1). In addition, the mean values of PCS and MCS determined according to the SF-8 method were 44.4±2.8 and 44.1±3.4 in the FD patients, 42.8±2.7 and 44.3±2.7 in the PDS patients and 43.9±3.9, and 43.5±3.6 in the EPS patients, respectively, indicating greater levels of impairment in the PDS and EPS patients compared to the scores of 48.9±3.3 and 47.1±3.7 observed in the healthy volunteers, respectively.

Genotypes of the GHRL gene in the FD patients

The Arg51Gln (346G->A), Leu72Met (408C->A), preproghrelin (3056 T->C) and Gln90Leu (3412T->A) genotype distribution in the *H. pylori*-negative FD patients was 73CC (98.6%), 1CT(1.4%); 21GT(28.4%), 52GG(70.3%), 1TT (1.3%); 3CC(4.1%), 29TC(39.2%), 42TT(56.7%); and 72TT (97.3%), 2TA(2.7%), respectively. The Arg51Gln (346G->A), Leu72Met (408C->A), preproghrelin (3056 T->C) and Gln90Leu (3412T->A) genotype distribution in the *H. pylori*-negative PDS patients was 51CC(100%); 16GT (31.4%), 34GG(66.7%), 1TT(1.9%); 3CC(5.9%), 20TC (39.2%), 28TT(54.9%); and 50TT(98.1%), 1TA(1.9%), respectively. Meanwhile, in the healthy controls, the Arg51Gln (346G->A), Leu72Met (408C->A), preproghrelin (3056 T->C) and Gln90Leu (3412T->A) genotype distribution was 101 CC(99%), 1CT(1%); 35GT(34.3%), 61GG(59.8%), 6TT (5.9%); 9CC(8.8%), 48TC(47.1%), 45TT(44.1%); and 96TT

Table 2. Genotypes of GHRL Gene in *H. pylori*-negative FD Patients, PDS Patients and Healthy Volunteers

Arg51Gln(346G->A) polymorphism and FD					
Variables (n)	genotype			OR (95%CI) CC vs others	p value
	CC	CT	TT		
Healthy volunteer(n=102)	101	1	0	Reference	
FD (n=74)	73	1	0	1.38 (0.085-22.43)	0.819
PDS (n=51)	51	0	0	0	0.478

Preproghrelin3056T->C polymorphism and FD					
Variables (n)	genotype			OR (95%CI) TT vs others	p value
	CC	TC	TT		
Healthy volunteer(n=102)	9	48	45	Reference	
FD (n=74)	3	29	42	0.53 (0.328-1.097)	0.146
PDS (n=51)	3	20	28	0.65 (0.330-1.278)	0.282

Leu72Met(408C->A) polymorphism and FD					
Variables (n)	genotype			OR (95%CI) GG vs others	p value
	GG	GT	TT		
Healthy volunteer(n=102)	61	35	6	Reference	
FD (n=74)	52	21	1	0.63 (0.333-1.191)	0.129
PDS (n=51)	34	16	1	0.74 (0.366-1.496)	0.274

Gln90Leu(3412T->A) polymorphism and FD					
Variables (n)	genotype			OR (95%CI) TT vs others	p value
	TT	TA	AA		
Healthy volunteer(n=102)	96	6	0	Reference	
FD (n=74)	72	2	0	0.44 (0.086-2.244)	0.318
PDS (n=51)	50	1	0	0.32 (0.038-2.732)	0.274

PDS : postprandial distress syndrome

EPS : epigastric pain syndrome

OR : odds ratio

p value for deviation from Hardy-Weinberg equilibrium

(94.1%), 6TA(5.9%), respectively. There were no significant differences between the genotype distributions observed in the *H. pylori*-negative FD patients and those observed in the healthy volunteers (Table 2).

Relationship between the genotypes of GHRL gene SNPs and the acylated ghrelin levels in the *H. pylori*-negative FD patients and healthy volunteers

To investigate whether the genotypes of the GHRL gene are associated with the serum levels of acylated ghrelin and des-acylated ghrelin, we compared the relationships between the four GHRL gene SNPs and the serum levels of acylated ghrelin and des-acylated ghrelin levels in the *H. pylori*-negative FD patients and healthy volunteers. We measured the mean values of plasma acylated ghrelin (FD patients, 13.77±3.56 fmol/mL; healthy volunteers, 16.14±3.12 fmol/mL) and des-acylated ghrelin (FD patients, 74.34±6.97 fmol/mL; healthy volunteers, 93.75±19.2 fmol/mL). The acylated ghrelin levels in the Arg51Gln 346CC genotype (dominant model) group were similar to those observed in the Arg51Gln346CT/TT genotype group as well as those observed in the Gln90Leu3412 TT genotype (dominant model) group (Table 3). The acylated ghrelin levels (12.25±1.81 fmol/mL) in the Leu72 Met408GG (dominant model) group were also similar to those observed in the Leu72 Met 408 GT/TT genotype group (11.37±2.67 fmol/mL). The acylated ghrelin levels (9.02±1.75 fmol/mL) observed in the preproghrelin 3056 TT genotype (dominant model) group were

Table 3. Association between Acylated Ghrelin Levels and Genotypes of GHRL Gene in *H. pylori*-negative FD Patients and Healthy Volunteers

Genotypes	<i>H. pylori</i> -negative FD patients				Healthy volunteers					
	Acylated ghrelin (fmol/min)	Des-acylated ghrelin (fmol/min)	<u>p¹ value</u>		Acylated ghrelin (fmol/min)	Des-acylated ghrelin (fmol/min)	<u>p¹ value</u>		<u>p² value</u>	
									<i>H. pylori</i> -negative FD vs HV	Genotype
Arg51Gln346CC	11.97±1.48	71.00±6.44	0.253 (R=0.198)	0.118 (R=0.278)	14.94±1.59	82.41±18.44	0.397 (R=0.121)	0.321 (R=1.08)	0.132	0.259
CT/TT	12.78±0.79	78.14±7.92			15.88±1.29	94.76±14.51				
Preproghrelin3056TT	9.02±1.75	62.29±6.64	0.048* (R=0.39)	0.599 (R=0.069)	14.93.±2.02	89.22±18.09	0.236 (R=0.197)	0.138 (R=0.245)	0.025**	0.521
TC/CC	13.87±1.51	76.76±12.0			15.75±2.93	97.37±21.40				
Leu72Met408GG	12.25±1.81	75.51±7.05	0.889 (R=0.056)	0.594 (R=0.067)	14.08±1.98	88.35±16.02	0.534 (R=0.104)	0.109 (R=0.264)	0.347	0.600
GT/TT	11.37±2.67	66.46±13.49			16.12±2.68	98.56±25.55				
Gln90Leu3412TT	11.78±1.46	70.69±6.31	0.441 (R=0.097)	0.332 (R=0.115)	15.49±2.63	101.00±25.73	0.324 (R=0.164)	0.723 (R=0.059)	0.099	0.465
TA/AA	12.23±1.01	67.81±4.09			12.30±4.95	112.15±38.05				

HV: healthy volunteers

* There was a significant relationship between preproghrelin 3056TT and acylated ghrelin levels in *H. pylori*-negative FD patients.** In two-way ANOVA analysis, in preproghrelin 3056 genotype, there was a significant difference in acylated ghrelin levels between *H. pylori*-negative FD patients and healthy volunteers.¹P value refer to the difference in each genotype for acylated ghrelin and des-acylated ghrelin levels by logistic regression analysis.²P value refer to the difference in two group factors (genotype; *H. pylori*-negative FD patients vs healthy volunteers) for mean values of acylated ghrelin levels by two-way ANOVA.**Table 4.** Comparison of Tmax Value with Genotypes of GHRL Gene in *H. pylori*-negative FD Patients

Genotypes	Tmax>60min	Tmax<60min	p value
Arg51Gln346G->A CC	31	42	0.249
CT/TT	1	0	
Preproghrelin3056T->C TT	16	26	0.624
TC/CC	14	18	
Leu72Met408C->A GG	20	32	0.202
GT/TT	12	10	
Gln90Leu3412T->A TT	32	41	0.891
TA/AA	0	1	

Comparison of Tmax value with genotypes of GHRL gene in healthy volunteers

Genotypes	Tmax>60min	Tmax<60min	p value
Arg51Gln346G->A CC	11	90	0.727
CT/TT	0	1	
Preproghrelin3056T->C TT	2	43	0.269
TC/CC	7	50	
Leu72Met408C->A GG	3	59	0.160
GT/TT	5	35	
Gln90Leu3412T->A TT	1	7	0.576
TA/AA	9	85	

significantly lower than those (13.87±1.51 fmol/mL) observed in the preproghrelin 3056 TC/CC genotype group. There was a significant relationship ($p=0.048$) between the preproghrelin 3056TT and acylated ghrelin levels in the *H. pylori*-negative FD patients (Table 3). In contrast, we could not find any significant correlations ($p=0.253$; $p=0.889$; $p=0.441$) between the acylated ghrelin levels and the Arg51Gln (346G->A), Leu72Met (408C->A) or Gln90Leu (3412T->A) genotypes in the *H. pylori*-negative FD patients (Table 3). There were also no significant correlations between any of the four GHRL gene SNPs and the des-acylated ghrelin levels in the *H. pylori*-negative FD patients (Table 3). There were also no significant associations between any of the four GHRL gene SNPs and the acylated or des-acylated ghrelin levels in the healthy volunteers (Table 3). Moreover,

we also determined whether the acylated ghrelin levels were different between the *H. pylori*-negative FD patients and the healthy volunteers for each genotype of the GHRL gene. According to a two-way ANOVA analysis of the preproghrelin 3056 T->C genotype, there was a significant ($p=0.025$) difference in the acylated ghrelin levels between the *H. pylori*-negative FD patients and healthy volunteers.

Comparison of the four GHRL gene SNPs with the Tmax value in the FD patients and healthy volunteers

Since low levels of serum acylated ghrelin were found to be linked to impaired gastric emptying in PDS patients in our previous study, we attempted to clarify whether any of the four GHRL gene SNPs were associated with the Tmax values in the *H. pylori*-negative FD patients and healthy volunteers. In the current study, we could not find any significant relationships ($p=0.249$; $p=0.624$; $p=0.202$; $p=0.891$) between any of the four GHRL gene SNPs, including Arg51 Gln (346G->A), preproghrelin 3056T->C, Leu72Met (408C->A) and Gln90Leu (3412T->A), and the Tmax values in the *H. pylori*-negative FD patients (Table 4). In addition, there were no significant relationships ($p=0.727$; $p=0.269$; $p=0.160$; $p=0.576$) between any of the four GHRL gene SNPs and the Tmax values in the healthy volunteers (Table 4).

Comparison of the four GHRL gene SNPs with clinical parameters and symptoms in the *H. pylori*-negative FD patients

Since the serum acylated ghrelin levels have been previously reported to be linked to BMI and eating disorders, we also investigated whether any of the four GHRL gene SNPs were associated with any of the clinical parameters or clinical

Table 5. Comparison of Genotypes of GHRL Gene with Clinical Parameters in FD Patients

Variable	Arg51Gln346G->A		p value	Preproghrelin3056T->C			p value
	CC	CT/TT		TT	TC/CC		
Age	59.2±14.4	68	0.547	59.8±13.8	54.4±14.9	0.761	
Sex(M/F)	36/37	1/0	0.294	19/18	17/19	0.599	
BMI (kg·m ⁻²)	22.2±2.53	20	0.388	22.6±2.45	21.6±2.56	0.096	
SRQ-D	9.87±0.72	15	0.402	10.73±0.79	9.16±1.05	0.222	

Variable	Leu72Met408C->A		p value	Gln90Leu3412T->A			p value
	GG	GT/TT		TT	TA/AA		
Age	60.0±13.9	57.7±15.5	0.534	59.7±14.4	62	0.084	
Sex(M/F)	22/20	14/18	0.294	36/37	0/1	0.599	
BMI (kg·m ⁻²)	22.9±1.19	20.1±1.01	0.018 [*]	22.2±2.53	22.4	0.922	
SRQ-D	9.87±0.72	15	0.402	9.76±0.69	2	0.058	

Summary values are mean ± SD.

BMI: body mass index

SRQ-D: self-rating questionnaire for depression

cal symptoms, including the feeling of hunger, in the Japanese FD patients diagnosed according to the Rome III classification. There were no significant relationships between the preproghrelin 3056TT genotype and clinical parameters, such as age ($R=0.036$, $p=0.761$), sex ($R=0.062$, $p=0.599$), BMI ($R=0.195$, $p=0.096$) and the SRQ-D score ($R=0.146$, $p=0.222$) (Table 5). In addition, there were no significant relationships between either the GHRL Gln90Leu (3412T->A) genotype or the Arg51Gln346G->A genotype and the clinical parameters. In contrast, there was a significant relationship ($R=0.274$, $p=0.018$) between the GHRL Leu72Met 408C->A genotype and BMI (Table 5). In the *H. pylori*-negative FD patients, the preproghrelin 3056TT genotype was significantly ($p=0.047$) associated with the feeling of hunger compared to the preproghrelin 3056 TC/CC genotype (Figure A). However, in the *H. pylori*-negative FD patients, there were no significant relationships between clinical symptoms and the Arg51Gln (346G->A), Leu72Met (408C->A) or Gln90Leu (3412T->A) genotypes. In the *H. pylori*-negative PDS patients, the preproghrelin 3056TT genotype was significantly ($p=0.045$; $p=0.016$) associated with the feeling of hunger and abdominal fullness after meals compared to the preproghrelin 3056 TC/CC genotypes (Figure B). There were no significant relationships between clinical symptoms and the genotypes of Arg51Gln (346G->A), Leu72Met (408C->A) or Gln90Leu (3412T->A) in the *H. pylori*-negative PDS patients (Figure B).

Discussion

The major findings of this study are as follows: 1) there was a significant relationship between the preproghrelin 3056TT genotype and the serum levels of acylated ghrelin in the *H. pylori*-negative FD patients, 2) in the preproghrelin 3056T->C genotype group, there was a significant ($p=0.025$) difference in the acylated ghrelin levels between the *H. pylori*-negative FD patients and the healthy volunteers and 3) the preproghrelin 3056TT genotype was found to be significantly associated with the feeling of hunger in the *H.*

pylori-negative FD patients.

Altered gut-brain interactions may underlie symptom generation in patients with FD. We herein reported that the acylated ghrelin levels are significantly lower in PDS patients than in healthy volunteers. In addition, there is a significant inverse relationship between the plasma acylated ghrelin levels and the Tmax values in PDS patients (3). Lee et al. reported that the total plasma ghrelin levels are significantly lower in dysmotility-like FD patients than in controls (20). Shinomiya et al. reported that the plasma acylated ghrelin and des-acylated ghrelin levels are not significantly different between FD patients and controls and that the plasma acylated ghrelin levels are correlated with subjective symptom scores in FD patients (21). Takamori et al. suggested that there are no significant associations among gastric emptying, the plasma ghrelin levels and psychological factors in FD patients (22). These results suggest that the relationship between the plasma ghrelin levels and FD symptoms remains uncertain. However, ghrelin may play an important role in the etiology of FD because it has well-established roles in increasing appetite and food intake and stimulating gastric emptying and acid secretion (6, 23). In our data, there were no significant differences in the distributions of the genotypes of the four GHRL gene SNPs between the *H. pylori*-negative FD patients and the healthy volunteers (Table 2).

Brain-gut interactions modulate appetite, feeding and digestion. The gut hormone blood levels are altered in patients with dyspeptic disorders, including those with FD, and may be involved in the regulation of appetite. Patients with dysmotility-like dyspepsia have higher serum concentrations of leptin, which are associated with gastritis and *H. pylori* infection (24). Tahara et al. reported that a CCK genotype is associated with PDS in Japanese men (25). In addition, we have also previously reported that there is a significant relationship between low levels of acylated ghrelin linked to appetite and the Tmax values (3). Stanghellini et al. reported that disturbed gastric emptying is associated with satiation and impaired food intake (26). Therefore, it is critical to clarify whether the genotypes of ghrelin affect the Tmax values in FD patients and healthy volunteers. In our study, there were no significant relationships between the Tmax values as a marker of gastric motility and any of the four GHRL gene SNPs, including Arg51Gln (346G->A), preproghrelin 3056T->C, Leu72Met (408C->A) and Gln90Leu (3412T->A) in the FD patients and healthy volunteers. Gastric motility is affected by various gut hormones, including motilin, ghrelin, cholecystokinin, GLP-1 and peptide YY (27). It is of clinical importance to consider the relationship between gastric acidity and gastric motility because Lee et al. and Schwartz et al. both reported that intraduodenally administered acid affects gastroduodenal motility as well as visceral hypersensitivity (28, 29). Furthermore, Saito et al. reported that acceleration of gastric emptying was observed in an *H. pylori*-infected animal model (30). In contrast, in our previous study, *H. pylori* infection reduced the number

Figure. Associations between the four preproghrelin genotypes and clinical symptoms in the *H. pylori*-negative FD and PDS patients. **A:** There were no significant relationships between Arg51Gln (346G->A), Leu72Met (408C->A), Gln90Leu (3412T->A) and various clinical symptoms. Among the Japanese subjects, the preproghrelin3056TT genotype was significantly ($p=0.0485$) associated with the feeling of hunger in the *H. pylori*-negative FD patients compared to the preproghrelin 3056TC and CC genotypes. Arg51Gln 346CC ($n=73$), Arg 51Gln 346CT/TT ($n=1$); Leu72Met 408GG ($n=52$), Leu72Met 408GT/TT ($n=22$); Gln90Leu 3412TT ($n=72$), Gln90Leu 3412TA/AA ($n=2$); preproghrelin 3056TT ($n=42$), preproghrelin 3056TC/CC ($n=32$) *vs. preproghrelin 3056 TC/CC genotypes. **B:** There were no significant relationships between Arg 51Gln (346G->A), Leu72Met (408C->A), Gln90Leu (3412T->A) and various clinical symptoms. In the Japanese *H. pylori*-negative PDS patients, the preproghrelin 3056 TT genotype was significantly ($p=0.045$; $p=0.016$) associated with the feeling of hunger and abdominal fullness compared to the preproghrelin 3056 TC and CC genotypes. Arg51Gln 346CC ($n=51$); Leu72Met 408GG ($n=34$), Leu72Met 408GT/TT ($n=17$); Gln90Leu 3412TT ($n=50$), Gln90Leu 3412TA/AA ($n=1$); preproghrelin 3056TT ($n=28$), preproghrelin 3056TC/CC ($n=23$) *vs. preproghrelin 3056 TC/CC genotypes. **vs. preproghrelin 3056 TC/CC genotypes.

of ghrelin-producing cells that are linked to gastric emptying (31). Suzuki et al. reported that the plasma ghrelin levels correlate well with the serum levels of the pepsinogen I/II (PGI/II) ratio (32) and are decreased in association with advancing gastric mucosal atrophy. Taking together the find-

ings of previous reports and our data, the serum ghrelin levels may be affected by *H. pylori* infection (30-32). In our data, there was a significant relationship between preproghrelin 3056T->C genotype and the acylated ghrelin levels in the *H. pylori*-negative FD patients. Therefore, in future studies, we should investigate why the preproghrelin 3056T->C genotype could not be linked to the Tmax value as a marker of gastric emptying in the *H. pylori*-negative subjects. In addition, due to the small number of subjects in the Rome III subgroup, type II errors cannot be excluded. Therefore, our results should be treated with caution until they are replicated in different populations and other races.

There is increasing evidence that susceptibility to functional gastrointestinal disorders (FGIDs) is also influenced by hereditary factors (33-35). Holtmann et al. reported that homozygous GN β 3 825CC is associated with upper abdominal symptoms unrelated to meals in patients in Germany (36). We also first reported that the GN β 3 CC variant is significantly associated with disturbance of gastric emptying in PDS patients without gastroesophageal reflux symptoms (37). In this study, since the serum ghrelin level has been reported to be associated with food intake and gastric motility, we focused on the relationships between the genotypes of the GHRL gene SNPs and the feeling of hunger in FD patients. The preproghrelin 3056T->C genotype was found to be significantly associated ($p=0.045$) with the feeling of hunger and abdominal fullness in the *H. pylori*-negative PDS patients. Miwa et al. reported that FD patients eat meals irregularly and do not have an appetite (38). The authors speculated that eating habits associated with the feeling of hunger are related to the pathophysiology of FD, especially PDS (38). Therefore, we also speculate that the feeling of hunger linked to appetite is a very important factor in considering the etiology of FD in certain patients with GHRL gene SNPs, such as preproghrelin 3056T->C. In contrast, in this study, there were no significant relationships between the three other GHRL gene SNPs (Arg51Gln (346G->A), Leu72Met (408C->A) and Gln90Leu (3412T->A)) and the feeling of hunger in the *H. pylori*-negative PDS patients. Considering that ghrelin infusion also increases food intake and the sensation of hunger compared with saline infusion alone (7), measuring the genotypes of the GHRL gene may significantly reflect the activity of serum ghrelin compared to that of ghrelin. Ukkola et al. reported that Arg51GlnSNP is associated with lower ghrelin levels (10). However, in our data, the Arg51Gln(346G->A) genotype was almost entirely of the CC type in both the Japanese FD patients and healthy volunteers. Ando et al. reported that the preproghrelin gene 3056T->C SNP is associated with changes in the basal ghrelin concentrations (39). It is controversial whether any of the ghrelin gene SNPs alter the levels of ghrelin and/or obestatin (40, 41). In this study, we also confirmed that the preproghrelin 3056TT genotype is significantly linked to low levels of acylated ghrelin in *H. pylori*-negative FD patients compared to healthy volunteers. The active form of ghrelin is acylated ghrelin, the serin3

residue of which is posttranslationally octanoylated. Recently, the enzyme that octanoylates the serine3 residue of ghrelin has been discovered to be ghrelin *O*-acyltransferase (GOAT) (42). Considering that there were no significant differences in the distributions of the genotypes of the four GHRL gene SNPs between the *H. pylori*-negative FD patients and healthy volunteers, we speculate that the acylated ghrelin levels in healthy volunteers expressing the preproghrelin 3056 TT genotype may be modified by other factors, such as upregulation of the activity of GOAT. In addition, the Met 72 allele has been shown to be associated with an earlier age of self-reported onset of obesity in several studies (9, 11) and with higher BMI values. However, a recent Danish study concluded that the Leu 72 Met polymorphism of the ghrelin gene is not associated with metabolic syndrome or related quantitative traits (43). The Gln90Leu SNP has also been linked to obesity (44). In our data, there were no significant relationships ($p=0.21$; $p=0.94$) between BMI and the genotypes of Arg51Gln (346G→A) or Gln90Leu (3412T→A) in the *H. pylori*-negative FD patients. In contrast, the genotype of Leu72Met (408C→A) was found to be significantly ($p=0.018$) associated with BMI in the *H. pylori*-negative FD patients. Further studies are needed to investigate the precise mechanisms through which the genotypes of preproghrelin are linked to dysfunction of acylated ghrelin and whether the preproghrelin 3056T→C genotype contributes to the long-term regulation of appetite and BMI (6).

Akamizu et al. reported the ability of repeated ghrelin administration to increase appetite and food intake in patients with FD (45). Recently, the acyltransferase responsible for ghrelin acylation, GOAT, was discovered (42). The post-translational modification of octanoylation appears to be essential for GH-releasing ability (46) as well as acylated ghrelin's regulation of food intake and insulin secretion (47). Considering the present results and those of a previous study, ghrelin administration may be more useful for therapy in certain FD patients expressing the preproghrelin 3056TT genotype. The relationship between the ghrelin signaling system and eating behavior makes identifies ghrelin, growth hormone secretagogue receptor (GHSR) and GOAT as good candidate genes for hunger sensation and the risk of eating disorders (45, 48).

Taken together, in this study, the preproghrelin 3056TT genotype is significantly associated with reductions in the levels of acylated ghrelin and the feeling of hunger in *H. pylori*-negative FD patients. Further studies are needed to clarify the mechanisms through which the preproghrelin 3056TT genotype is associated with reduction of the acylated ghrelin levels and the feeling of hunger in *H. pylori*-negative FD patients.

The authors state that they have no Conflict of Interest (COI).

References

1. Tack J, Talley NJ, Camilleri M, et al. Functional gastroduodenal disorders. *Gastroenterology* **130**: 1466-1479, 2006.
2. Quigley EM. Gastric emptying in functional gastrointestinal disorders. *Aliment Pharmacol Ther* **20** (Suppl 7): 56-60, 2004.
3. Shindo T, Futagami S, Hiratsuka T, et al. Comparison of gastric emptying and plasma ghrelin levels in patients with functional dyspepsia and non-erosive reflux disease. *Digestion* **79**: 65-72, 2009.
4. Fischer H, Heidemann T, Hengst K, Domschke W, Konturek JW. Disturbed gastric motility and pancreatic hormone release in diabetes mellitus. *J Physiol Pharmacol* **49**: 529-541, 1998.
5. Shiya T, Nakazato M, Mizuta M, et al. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J Clin Endocrinol Metab* **87**: 240-244, 2002.
6. Cummings DE. Ghrelin and the short-and long-term regulation of appetite and body weight. *Physiol Behav* **89**: 71-84, 2006.
7. Quigley EM. Gastric motor and sensory function, and motor disorders of the stomach. In: *Gastrointestinal and Liver Disease, Pathophysiology/Diagnosis/Management*. Feldman M, Friedman LS, Sleisenger MH, Eds. WB Saunders, Philadelphia, 2002: 691-714.
8. Talley NJ, Stanghellini V, Heading RC, Koch KL, Malagelada JR, Tytgat GN. Functional gastroduodenal disorders. *Gut* **45** (Suppl 2): II37-II42, 1999.
9. Ukkola O, Ravussin E, Jacobson P, et al. Mutations in the preproghrelin/ghrelin gene associated with obesity in humans. *J Clin Endocrinol Metab* **86**: 3996-3999, 2001.
10. Ukkola O, Ravussin E, Jacobson P, et al. Role of ghrelin polymorphisms in obesity based on the three different studies. *Obes Res* **10**: 782-791, 2002.
11. Korbonsits M, Gueorguiev M, O'Grady E, et al. A variation in the ghrelin gene increases weight and decreases insulin secretion in tall, obese children. *J Clin Endocrinol Metab* **87**: 4005-4008, 2002.
12. Drossman DA. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* **130**: 1377-1390, 2006.
13. McColl K, Murray L, El-Omar E, et al. Symptomatic benefit from eradicating *Helicobacter pylori* infection in patients with nonulcer dyspepsia. *N Engl J Med* **339**: 1869-1874, 1998.
14. Portincasa P, Altomare DF, Moschetta A, et al. The effect of acute oral erythromycin on gallbladder motility and on upper gastrointestinal symptoms in gastrectomized patients with and without gallstones: a randomized, placebo-controlled ultrasonographic study. *Am J Gastroenterol* **95**: 3444-3451, 2000.
15. El-Omar EM, Banerjee S, Wirz A, McColl KE. The Glasgow dyspepsia severity score—a tool for the global measurement of dyspepsia. *Eur J Gastroenterol Hepatol* **8**: 967-971, 1996.
16. Rockliff BW. A brief self-rating questionnaire for depression (SRQ-D). *Psychosomatics* **10**: 236-243, 1969.
17. Fukuhara S, Suzukamo Y. Instruments for measuring Health-related Quality of Life-SF8 and SF36. *J Clin Exp Med [Igaku no Ayumi]* **213**: 133-136, 2005 (in Japanese).
18. Hellmig S, Von Schoning F, Gadow C, et al. Gastric emptying time of fluids and solids in healthy subjects determined by ¹³C breath tests: influence of age, sex and body mass index. *J Gastroenterol Hepatol* **21**: 1832-1838, 2006.
19. Futagami S, Shindo T, Kawagoe T, et al. Migration of eosinophils and CCR2-/CD68-double positive cells into the duodenal mucosa of patients with postinfectious functional dyspepsia. *Am J Gastroenterol* **105**: 1835-1842, 2010.
20. Lee KJ, Cha DY, Cheon SJ, Yeo M, Cho SW. Plasma ghrelin levels and their relationship with gastric emptying in patients with dysmotility-like functional dyspepsia. *Digestion* **80**: 58-63, 2009.
21. Shinomiya T, Fukunaga M, Akamizu T, et al. Plasma acylated

- ghrelin levels correlate with subjective symptoms of functional dyspepsia in female patients. *Scand J Gastroenterol* **40**: 648-653, 2005.
22. Takamori K, Mizuta Y, Takeshima F, et al. Relation among plasma ghrelin level, gastric emptying, and psychologic condition in patients with functional dyspepsia. *J Clin Gastroenterol* **41**: 477-483, 2007.
 23. Wren AM, Bloom SR. Gut hormones and appetite control. *Gastroenterology* **132**: 2116-2130, 2007.
 24. Lankarani KB, Moghadami M, Masoumpoor M, Geramizadeh B, Omrani GR. Serum leptin level in patients with functional dyspepsia. *Dig Liver Dis* **36**: 717-721, 2004.
 25. Tahara T, Arisawa T, Shibata T, et al. 779TC of CCK-1 intron 1 is associated with postprandial syndrome (PDS) in Japanese male subjects. *Hepatogastroenterology* **56**: 1245-1248, 2009.
 26. Stanghellini V, Tosetti C, Paternico A, et al. Risk indicators of delayed gastric emptying of solids in patients with functional dyspepsia. *Gastroenterology* **110**: 1036-1042, 1996.
 27. Khoo J, Rayner CK, Feinle-Bisset C, Jones KL, Horowitz M. Gastrointestinal hormonal dysfunction in gastroparesis and functional dyspepsia. *Neurogastroenterol Motil* **22**: 1270-1278, 2010.
 28. Lee KJ, Vos R, Janssens J, Tack J. Influence of duodenal acidification on the sensorimotor function of the proximal stomach in humans. *Am J Physiol Gastrointest Liver Physiol* **286**: G278-G284, 2004.
 29. Schwartz MP, Samsom M, Smout AJ. Chemospecific alterations in duodenal perception and motor response in functional dyspepsia. *Am J Gastroenterol* **96**: 2596-2602, 2001.
 30. Saito Y, Suzuki H, Tsugawa H, et al. Dysfunctional gastric emptying with down-regulation of muscle-specific microRNAs in *Helicobacter pylori*-infected mice. *Gastroenterology* **140**: 189-198, 2011.
 31. Tatsuguchi A, Miyake K, Gudis K, et al. Effect of *Helicobacter pylori* infection on ghrelin expression in human gastric mucosa. *Am J Gastroenterol* **99**: 2121-2127, 2004.
 32. Suzuki H, Masaoka T, Hosoda H, et al. Plasma ghrelin concentration correlates with the levels of serum pepsinogen I and pepsinogen I/II ratio-a possible novel and non-invasive marker for gastric atrophy. *Hepatogastroenterology* **51**: 1249-1254, 2004.
 33. Morris-Yates A, Talley NJ, Boyce PM, Nandurkar S, Andrews G. Evidence of a genetic contribution to functional bowel disorder. *Am J Gastroenterol* **93**: 1311-1317, 1998.
 34. Levy RL, Jones KR, Whitehead WE, Feld SI, Talley NJ, Corey LA. Irritable bowel syndrome in twins; heredity and social learning both contribute to etiology. *Gastroenterology* **121**: 799-804, 2001.
 35. Kalantar JS, Locke GR 3rd, Zinsmeister AR, Beighley CM, Talley NJ. Familial aggregation of irritable bowel syndrome: a prospective study. *Gut* **52**: 1703-1707, 2003.
 36. Holtmann G, Siffert W, Haag S, et al. G-protein $\beta 3$ subunit 825 CC genotype is associated with unexplained (functional) dyspepsia. *Gastroenterology* **126**: 971-979, 2004.
 37. Shimpuku M, Futagami S, Kawagoe T, et al. G-protein $\beta 3$ subunit 825CC genotype is associated with postprandial distress syndrome with impaired gastric emptying and with the feeling of hunger in Japanese. *Neurogastroenterol Motil* **23**: 1073-1080, 2011.
 38. Miwa H. Life style in persons with functional gastrointestinal disorders: large-scale internet survey of lifestyle in Japan. *Neurogastroenterol Motil* **24**: 464-471, e217, 2012.
 39. Ando T, Ichimaru Y, Konjiki F, Shoji M, Komaki G. Variations in the preproghrelin gene correlate with higher body mass index, fat mass, and body dissatisfaction in young Japanese women. *Am J Clin Nutr* **86**: 25-32, 2007.
 40. Ogiso K, Asakawa A, Amitani H, Inui A. Ghrelin: a gut hormonal basis of motility regulation and functional dyspepsia. *J Gastroenterol Hepatol* **26** (Suppl 3): 67-72, 2011.
 41. Zou CC, Huang K, Liang L, Zhao ZY. Polymorphisms of the ghrelin/obestatin gene and ghrelin levels in Chinese children with short stature. *Clin Endocrinol* **69**: 99-104, 2008.
 42. Yang J, Brown M, Liang G, et al. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* **132**: 387-396, 2008.
 43. Bing C, Ambye L, Fenger M, et al. Large-scale studies of the Leu 72Met polymorphism of the ghrelin gene in relation to the metabolic syndrome and associated quantitative traits. *Diabet Med* **22**: 1157-1160, 2005.
 44. Hinney A, Hoch A, Geller F, et al. Ghrelin gene: identification of missense variants and a frameshift mutation in extremely obese children and adolescents and healthy normal weight students. *J Clin Endocrinol Metab* **87**: 2716-2719, 2002.
 45. Akamizu T, Iwakura H, Ariyasu H, et al. Repeated administration of ghrelin to patients with functional dyspepsia: its effects on food intake and appetite. *Eur J Endocrinol* **158**: 491-498, 2008.
 46. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**: 656-660, 1999.
 47. Gutierrez JA, Solenberg PJ, Perkins DR, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci USA* **105**: 6320-6325, 2008.
 48. Liu B, Garcia EA, Korbonits M. Genetic studies on the ghrelin, growth hormone secretagogue receptor (GHSR) and ghrelin O-acyl transferase (GOAT) genes. *Peptides* **32**: 2191-2207, 2011.