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Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion

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Mogami S, Suzuki H, Fukuhara S, Matsuzaki J, Kangawa K, Hibi T. Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion. *Am J Physiol Gastrointest Liver Physiol* 302: G359–G364, 2012. First published December 8, 2011; doi:10.1152/ajpgi.00297.2011.—The gastrointestinal (GI) tract is one of the most susceptible organs to ischemia. We previously reported altered gastric motility after gastric ischemia and reperfusion (I/R). However, there have also been few reports of alterations in the eating behavior after gastric I/R. Ghrelin is a GI peptide that stimulates food intake and GI motility. Although ghrelin itself has been demonstrated to attenuate the mucosal injuries induced by gastric I/R, the endogenous ghrelin dynamics after I/R has not yet been elucidated. The present study was designed to investigate the relationship between food intake and the ghrelin dynamics after gastric I/R. Wistar rats were exposed to 80-min gastric ischemia, followed by 12-h or 48-h reperfusion. The food intake, plasma ghrelin levels, gastric preproghrelin mRNA expression levels, and the histological localization of ghrelin-immunoreactive cells were evaluated. The effect of exogenous ghrelin on the food intake after I/R was also examined. Food intake, the plasma ghrelin levels, the count of ghrelin-immunoreactive cells corrected by the percentage areas of the remaining mucosa, and the expression levels of preproghrelin mRNA in the stomach were significantly reduced at 12 h and 48 h after I/R compared with the levels in the sham-operated rats. Intraperitoneal administration of ghrelin significantly reversed the decrease of food intake after I/R. These data show that gastric I/R evoked anorexia with decreased plasma ghrelin levels and ghrelin production, which appears to be attributable to the I/R-induced gastric mucosal injuries. The decrease in the plasma ghrelin levels may have been responsible for the decreased food intake after gastric I/R.

food intake; ghrelin; mucosal injury

GASTROINTESTINAL (GI) TRACT is one of the most susceptible organ systems to ischemia. Various investigations have demonstrated that ischemia and reperfusion (I/R) contribute significantly to the gastric mucosal injuries caused by stress, such as burn stress (17) or hemorrhagic shock (35), nonsteroidal anti-inflammatory drugs (30), and *Helicobacter pylori* (*H. pylori.*) infection (26, 27). We previously demonstrated, not only postischemic mucosal injury, but also transient delay in gastric emptying in a rat model of gastric I/R (28). These changes were found to be associated with disruption of the network of the interstitial cells of Cajal and decrease in neuronal nitric oxide synthase-positive neurons in the smooth muscle layer. On the other hand, there have been no reports on alterations in eating behavior after gastric I/R, at least to our knowledge.

Ghrelin, a 28-residue octanoylated peptide, is an endogenous ligand of the growth hormone secretagogue receptor (18) and is produced and secreted from the A-like cells found mainly in the oxyntic glands of the gastric fundus (8). Gastric ghrelin accounts for the major part of circulating ghrelin, as an ~80% reduction in the circulating levels of ghrelin has been demonstrated after gastrectomy or fundectomy (10). Ghrelin is now known to play a role, not only in growth-hormone release, but also in stimulating gastric motility and food intake (1, 21, 32). Recent studies have also reported the gastroprotective effect of ghrelin; ghrelin has been demonstrated to reduce ethanol-induced gastric ulceration (23), acetic acid-induced chronic gastric and duodenal ulceration (6), and I/R-induced gastric ulceration (11) in rats. Although changes in the plasma ghrelin levels and association with various GI diseases have been reported such as in functional dyspepsia (22), chronic gastritis and gastric ulcer (14), the ghrelin dynamics after gastric I/R has yet to be elucidated.

The present study was designed to investigate the influences of gastric I/R injuries on the food intake and ghrelin dynamics in a rat model of gastric I/R injury.

MATERIALS AND METHODS

I/R. Six-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). All rats were handled according to the guidelines of the Keio University Animal Research Committee (approved protocol No. 078086) and the Experimental Animal Ethics Committee of Tsumura & Co. (approved protocol No. 09–155, 09–157, 10–096, 10–110, 10–156). All rats were used after acclimation for 1 wk and denied access to food for 22–24 h (but allowed free access to water) before the operation. The rats were anesthetized with pentobarbital sodium (50 mg/ kg ip) during the surgery. The abdomen was opened by a midline incision, and the celiac artery was occluded with a small clamp for 80 min. Reperfusion was established for 12 h or 48 h by removal of the clamp. For comparison, some rats were subjected to a sham operation (surgery, but no clamping). Rats were supplied with food after the surgery (returned to normal feeding). Food intake was measured at 12 h after I/R (when gastric emptying was delayed compared with sham-operated rats) and at 48 h after I/R (when gastric emptying was restored) (Fig. 1*A*). In the fasting condition, food deprivation was continued after the surgery when reperfusion was established for 12 h. When reperfusion was established for 48 h, the rats were fed after the operation (normal feeding), but were again deprived of food for 24 h before euthanasia to establish the fasted condition (Fig. 1*B*). To measure plasma ghrelin levels in the fed condition at 48 h after I/R, I/R rats were fed ad libitum after the surgery. Sham-operated rats were given the same amount of food as

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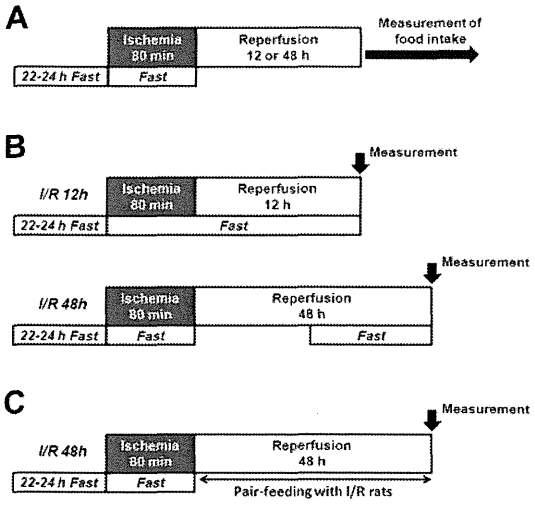

Fig. 1. Experimental protocol. *A*: measurement of cumulative food intake at 12 h or 48 h after gastric ischemia and reperfusion (I/R) in fed condition. *B*: measurement of gastric emptying rates or plasma ghrelin levels at 12 h or 48 h after I/R in fasted condition. *C*: measurement of plasma ghrelin levels at 48 h after I/R in the fed condition.

the I/R rats to eliminate the effect of the difference in the food intake (Fig. 1*C*).

Measurement of food intake. All rats were housed in individual hanging-wire cages. After reperfusion for 12 h or 48 h, the rats were supplied with preweighed food, and the cumulative food intake of each rat was calculated as the difference between the food weights before and after the feeding period. In the experiment to determine the effect of exogenous ghrelin, rat ghrelin (30 nmol/0.5 ml saline per rat; Peptide Institute, Osaka, Japan) or 0.5 ml saline was administered intraperitoneally, immediately before the supply of the preweighed food (Fig. 1*A*).

Evaluation of gastric emptying of solid food. Solid gastric emptying was evaluated using powdered food (13) and glass beads (24, 34). After 24-h food deprivation (Fig. 1*B*), 1 ml of the test meal containing powdered food and glass beads (0.2-mm diameter, BZ-02; AS One, Osaka, Japan) was orally administered to the rats through a Teflon tube (AWG-14) attached to a 1-ml syringe, using a 10Fr Nelaton's catheter. The test meal contained 32 g of ground meal, 40 g of glass beads, and 80 ml of distilled water. Rats were then killed by decapitation 2.5 h after the test meal administration, except for the animals that were killed immediately after the injection to recover the entire dose of the test meal. The gastric contents were then recovered from the stomach, dried, and weighed. The gastric emptying of solid food was calculated as follows: Gastric emptying (%) = [1 − (dried weight of food recovered from stomach / dried weight of food recovered from the stomach immediately after the test meal administration)] × 100.

Measurement of the plasma ghrelin levels. After 24-h food deprivation (Fig. 1*B*) or after 48-h pair feeding (Fig. 1*C*), whole blood samples were obtained from the right ventricle under ether anesthesia in tubes containing EDTA-2Na (1 mg/ ml) and aprotinin (500 kIU/ ml). Samples were promptly centrifuged at 4°C, and the supernatant was acidified with 1 mol/l HCl (1/10 volume) and stored at −80°C until use. The ghrelin level was determined using the Active Ghrelin ELISA Kit, and the desacylghrelin (ghrelin without octanoyl acid modification) level was determined using the Desacyl Ghrelin ELISA Kit (Mitsubishi Chemical Medience, Tokyo, Japan).

Immunohistochemistry. Stomach tissue specimens were fixed in 10% neutralized formalin and embedded in paraffin. After deparaffinization and hydration, the antigens were retrieved by heating for 20 min at 97°C in Dako REAL Target Retrieval Solution (DAKO Japan, Tokyo, Japan). Nonspecific binding was blocked by Protein Block (DAKO Japan). All sections were incubated overnight at 4°C with anti-ghrelin (13–28) antiserum (7) (1:10,000). After being washed with TBS-T, the slides were incubated with peroxidase-labeled dextran polymer conjugated anti-rabbit IgG in Tris-HCl (EnVision/HRP; Dako Japan) for 30 min at room temperature and then visualized after color development using 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 3 min. Counterstaining was performed with hematoxylin. The stained sections were observed under a light microscope equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan), and the photomicrographs were obtained in areas without gastric I/R-induced mucosal injuries. DAB-stained ghrelin immunoreactive cells were counted by visual inspection, and hematoxylin-stained nuclei were counted using the ImageJ program (National Institutes of Health, Bethesda, MD). The numbers of ghrelin-IR cells were normalized by dividing by the total number of cells counterstained with hematoxylin. The numbers of ghrelin-IR cells were further corrected by the percentages of the remaining mucosal areas without erosive lesions, which were quantified using the image analysis software. The erosive lesions are indicated by dashed lines in Fig. 4*A*. Corrected IR cells = % of number of ghrelin-IR cells × [(mucosal area without the erosive lesion)/(total area)]. Hematoxylin-eosin (HE) staining was also conducted to evaluate the severity of the injuries induced by I/R.

Preparation of total RNA and quantitative RT-PCR analysis. Total RNA was extracted from the stomach tissue using RNeasy Mini kit (Qiagen, Valencia, CA), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). Quantitative RT-PCR analysis was performed using Dice (Takara) with SYBR Premix Ex TaqII (Takara). The primer sequences used were as follows; preproghrelin mRNA: 5′-GGA ATC CAA GAA GCC ACC AGC′ and 5′-GCT CCT GAC AGC TTG ATG CCA-3′; GAPDH mRNA: 5′- GGC ACA GTC AAG GCT GAG AAT G -3′, 5′- ATG GTG GTG AAG ACG CCA GTA -3′. The mRNA expression levels were normalized using the GAPDH mRNA expression levels.

Statistical analysis. All values were expressed as means ± SD. The statistical significance of any differences between two groups was evaluated using unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$, unless otherwise indicated.

RESULTS

Food intake after gastric I/R. Cumulative food intakes were significantly reduced at 12 h after gastric I/R compared with that in the sham-operated rats in the fed condition (Fig. 2*A*). No significant difference was observed in the cumulative food intakes of shorter period, probably because 12 h was not sufficient for recovery from the surgical stress, and the food intake was very small even in the sham-operated rats. Cumulative food intakes (2, 4, 6, and 24 h) were also significantly reduced at 48 h after gastric I/R compared with those in the sham-operated rats in the fed condition (Fig. 2*B*). Decreased food intakes were also observed in the fasting condition after I/R (data not shown).

Gastric emptying of solids after gastric I/R. Gastric emptying rates were investigated using powdered food and glass beads at 48 h after I/R because decreased gastric emptying of liquids at 12 h after I/R was restored at 48 h although food intake was reduced in the I/R rats compared with that in the sham-operated rats. Figure 2*C* shows that the gastric emptying rates of solids in the I/R rats (50.1 ± 15.5%) were comparable with those in the sham-operated rats (57.0 ± 16.9%).

Plasma ghrelin levels. Plasma ghrelin and desacylghrelin levels were measured at 12 and 48 h after gastric I/R in the fasting (Fig. 3, *A* and *B*) and pair-fed (Fig. 3*C*) conditions to eliminate the effect of food intake. As shown in Fig. 3*A*, fasting

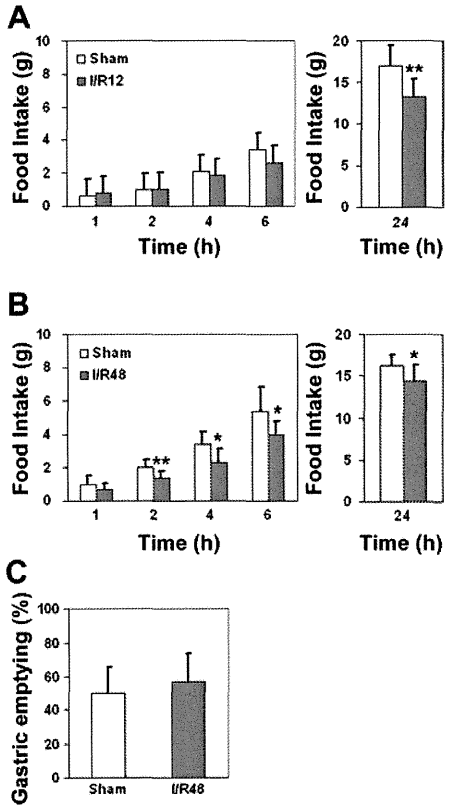

Fig. 2. *A*: effect of gastric I/R on the cumulative food intakes at 12 h after I/R in the fed condition. Sham-operated rats, open bar (n = 8); I/R rats, solid bar (n = 10). *B*: effect of gastric I/R on the cumulative food intakes at 48 h after I/R in the fed condition. Sham-operated rats, open bar (n = 9); I/R rats, solid bar (n = 10). *C*: gastric emptying rates of solids in the sham-operated rats (open bar) and I/R rats (solid bar) at 48 h (Sham, n = 6; I/R, n = 7) after I/R. Data are means ± SD. $*P < 0.05$, $**P < 0.01$ compared with the sham-operated rats by Student's t-test.

plasma ghrelin levels at 12 h and 48 h after I/R were significantly lower than those in the sham-operated rats at the corresponding time-points (sham 12 h, 64.5 ± 13.5 fmol/ml; I/R 12 h, 46.3 ± 9.25 fmol/ml; sham 48 h, 97.2 ± 30.3 fmol/ml; I/R 48 h, 70.9 ± 18.4 fmol/ml). Fasting plasma desacylghrelin levels at 12 h and 48 h after I/R were also significantly lower than those in the sham-operated rats at the corresponding time-points (sham 12 h, 834 ± 137 fmol/ml; I/R 12 h, 663 ± 113 fmol/ml; sham 48 h, 1,092 ± 150 fmol/ml; I/R 48 h, 835 ± 187 fmol/ml), as shown in Fig. 3*B*. Plasma ghrelin (sham, 115 ± 30.0 fmol/ml; I/R, 45.4 ± 23.7 fmol/ml) and desacylghrelin (sham, 1,311 ± 118 fmol/ml; I/R, 577 ± 201 fmol/ml) levels in the fed condition were also significantly decreased compared with those in the pair-fed sham-operated rats at 48 h after I/R (Fig. 3*C*).

Immunohistochemical staining for ghrelin-producing cells. Ghrelin-immunoreactive (IR) cells were counted in the mucosal layer of the fundic gland region (Fig. 4*A*). In case of counting in the mucosal layers of I/R group, the places without the mucosal injuries induced by gastric I/R were selected. The count of ghrelin-IR cells was decreased at 12 h after I/R (Sham, 0.92 ± 0.18%; I/R, 0.58 ± 0.11%, P = 0.0017) but recovered by 48 h (Sham, 0.86 ± 0.17%; I/R, 0.92 ± 0.20%) (Fig. 4*B*). However, because erosive lesion areas were observed at 12 h and 48 h after I/R (Fig. 4*A*, *right*), we corrected the numbers of ghrelin-IR cells by the percentages of the remaining mucosal areas not showing erosive lesions (Fig. 4*C*). The corrected numbers of ghrelin-IR cells were significantly decreased throughout the observation period (44.7 ± 11.1% at 12 h and 78.4 ± 18.6% at 48 h after I/R relative to the value in the sham-operated rats).

Ghrelin production after gastric I/R. The expression levels of preproghrelin mRNA were significantly reduced at 12 h and 48 h (53.4 ± 22.7% and 42.3 ± 16.8% relative to the value in the sham-operated rats) after I/R compared with the levels in the sham-operated rats at the corresponding time points (Fig. 5*A*). Mucosal injuries in the fundic gland regions, where ghrelin-IR cells are mainly distributed, persisted throughout the observation period, as visualized in the HE-stained sections (Fig. 5*B*).

Restoration of decreased food intake by exogenous ghrelin administration. In Fig. 6, ghrelin was administered intraperitoneally (30 nmol/rat) to sham-operated and I/R rats to investigate the effect of exogenous ghrelin on the decreased food intake at 48 h after I/R. In sham-operated rats, food intake was enhanced for 1 h, but not at 2- and 3-h cumulative food intake (Fig. 6*A*). However, administration of ghrelin significantly restored the decreased cumulative food intake (2 and 3 h) in I/R rats (Fig. 6*B*). The effect of decreased food intake restoration by exogenous ghrelin waned 4 h after administration. Administration of 10 nmol ghrelin per rat failed to increase food intake in both sham-operated and I/R rats (data not shown).

DISCUSSION

In the present study, we demonstrated that anorexia was induced after gastric I/R associated with decreased plasma ghrelin levels in rats. Not only the plasma ghrelin level but also ghrelin production was reduced by continuous mucosal injuries. Exogenous ghrelin administration significantly restored the food intake, indicating that it was the decrease in the levels of the orexigenic hormone that induced the anorexia after gastric I/R.

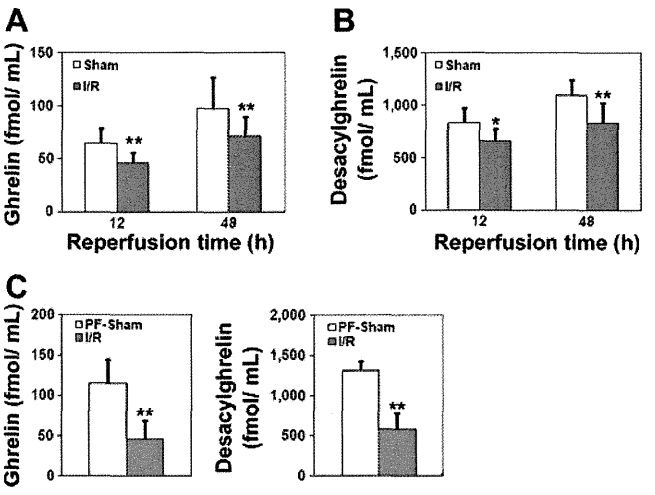

Fig. 3. Fasting plasma ghrelin (*A*) and desacylghrelin (*B*) levels in the sham-operated rats (open bar) and I/R rats (solid bar) at 12 h (Sham, n = 18; I/R, n = 16) and 48 h (Sham, n = 12; I/R, n = 13) after I/R. *C*: plasma ghrelin levels of I/R rats in the fed condition (solid bar, n = 9) and of sham-operated rats in the pair-fed condition (open bar, n = 6) at 48 h after I/R. Data are means ± SD. $*P < 0.05$, $**P < 0.01$ compared with the sham-operated rats by Student's t-test.

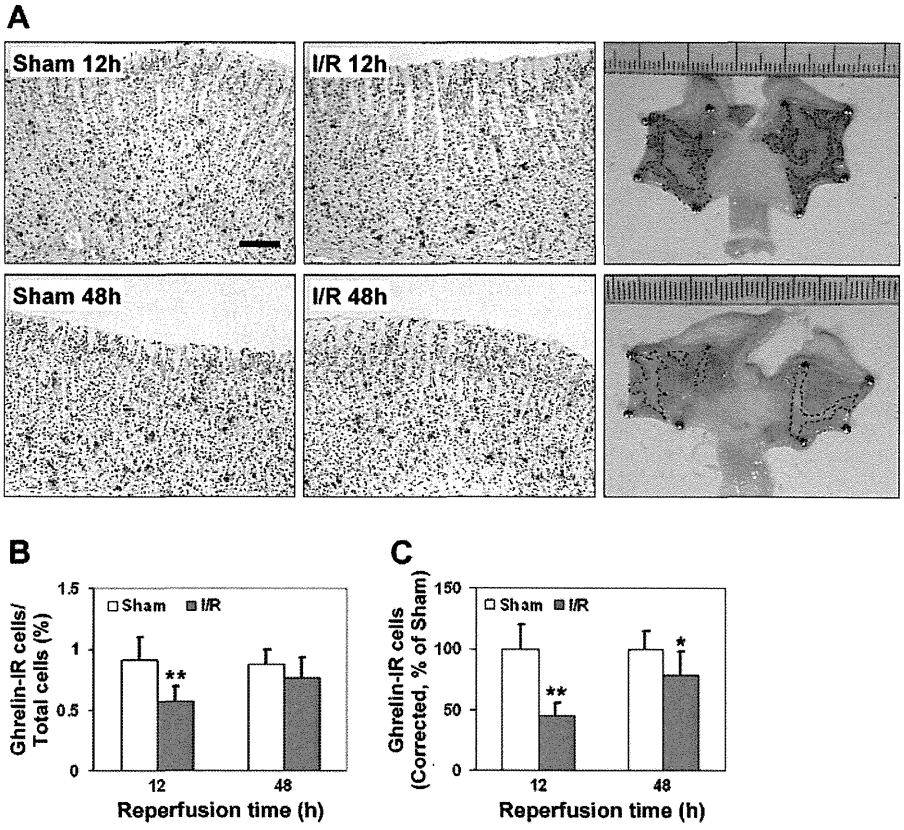

Fig. 4. *A*: representative photomicrograph of ghrelin-immunoreactive (IR) cells in the gastric fundic mucosa of the sham-operated rats and I/R rats at 12 h and 48 h after I/R. The brown-colored cells represent the ghrelin-IR cells. Bar = 100 μm. *Right* photographs of the gastric mucosa obtained from the I/R rats at 12 h and 48 h after I/R. The erosive lesion areas are shown by the dashed lines. *B*: numbers of ghrelin-IR cells in the images from the sham-operated rats (open bar) and I/R rats (solid bar) were counted and normalized by the total number of cells counterstained with hematoxylin, which was quantified using the image analysis software. *C*: numbers of ghrelin-IR cells in the images were corrected by the percentages of the remaining mucosal areas without the erosive lesions, which was quantified using the image analysis software. Sham, $n = 6$; I/R; $n = 7$. Data are means ± SD. $^*P < 0.05$, $^{**}P < 0.01$ compared with the sham-operated rats by Student's *t*-test.

Thermal injuries (3) have been reported to induce decreased food intake, and aspirin treatment led to a further and significant decrease of food intake compared with that in the controls (16). A previous study reported that the restoration of gastric ghrelin production was associated with ulcer healing and improvement of the appetite in patients with *H. pylori*-associated active duodenal or gastric ulcer (15). Although these events are reported to induce gastric I/R (17, 26, 27, 30, 35), whether anorexia can be induced by gastric I/R alone remains unclear. The present study is the first report documenting decreased food intake associated with reduced production of ghrelin after gastric I/R.

We previously reported transient delay in gastric emptying of liquids at 12 h after I/R (28), which may be considered as inducing early satiety and contribute to the anorexia. However, the delayed gastric emptying of liquids was normalized at 48 h after I/R. The gastric emptying rates of solids at 48 h were also not significantly different between the sham-operated rats and I/R rats in the present study. The normalized gastric emptying rates do not explain the decrease of food intake at 48 h after I/R; therefore, other factors may also be associated with the anorexia.

Significant decrease in the plasma levels of ghrelin, an orexigenic hormone, in the fasting condition were observed at 12 h and 48 h after I/R in this study. Decreased plasma ghrelin levels are reported to induce anorexia, such as in the lipopolysaccharide-induced food intake and gastric emptying-altered model (31) and cisplatin-induced anorexia model (29). Therefore, we assumed that the decrease in the plasma ghrelin levels may have contributed to the persistent decrease of food intake after I/R in this study. This is also supported by our observation that intraperitoneal administration of exogenous ghrelin restored the food intake at 48 h after I/R.

Ghrelin has been reported to attenuate mucosal injuries induced by gastric I/R (11) and intestinal I/R (33). In the present study, single ghrelin administration, after the formation of mucosal injuries (mucosal injuries were already present after 1-h reperfusion, and exogenous ghrelin was administered at 48

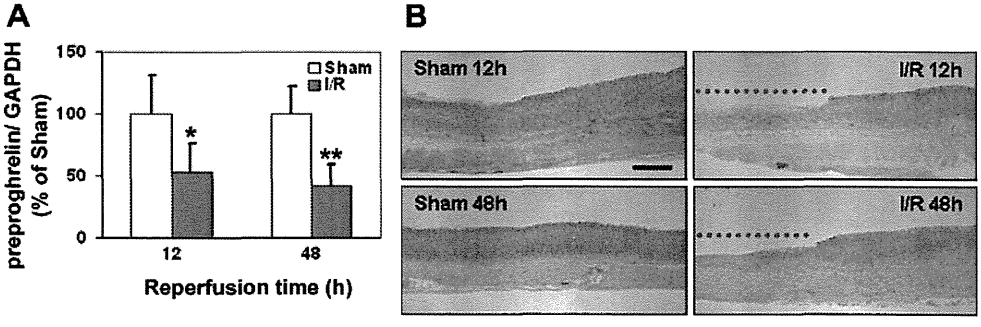

Fig. 5. *A*: expression levels of preproghrelin mRNA in the stomach of the sham-operated rats (open bar) and I/R rats (solid bar) at 12 h (Sham, $n = 6$; I/R, $n = 7$) and 48 h (Sham, $n = 6$; I/R, $n = 7$) after I/R. Data are means ± SD. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the sham-operated rats by Student's *t*-test. *B*: hematoxylin-eosin staining of gastric tissue (Bar = 500 μm).

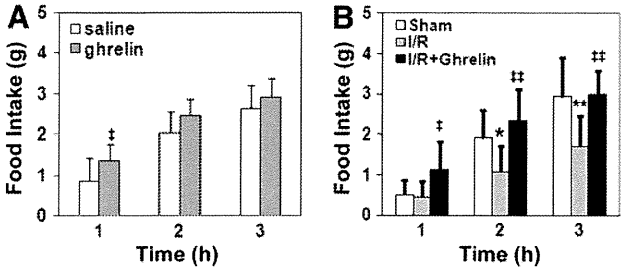

Fig. 6. *A*: effect of exogenous ghrelin (30 nmol/rat ip) in sham-operated rats in the fed condition. Saline-injected sham-operated rats, open bar (n = 10); ghrelin-injected sham-operated rats, shaded bar (n = 9). Data are means ± SD. ‡$P < 0.05$ compared with the saline group by Student's t-test. *B*: effect of exogenous ghrelin (30 nmol/rat ip) in I/R rats in the fed condition. Sham-operated rats, open bar (n = 9); I/R rats, shaded bar (n = 9); ghrelin-administered I/R rats, solid bar (n = 7). Data are means ± SD. *$P < 0.05$; **$P < 0.01$ compared with the sham-operated rats by Student's t-test. ‡‡$P < 0.01$ compared with the I/R rats by Student's t-test.

h after I/R in this study) restored the gastric I/R-induced decreased food intake; however, the restoration effect lasted only 3 h. Although ghrelin has the potential to attenuate mucosal injuries, it is unlikely that ghrelin can regenerate the gastric mucosa in 3 h. Also, if the restoration effect of exogenous ghrelin in this study is attributable to the attenuation of gastric mucosal injuries, the restoration effect should continue and should not wane after 3 h. Therefore, in this study, we considered that ghrelin administration restored the decreased food intake without attenuating mucosal injuries, indicating that decreased plasma ghrelin level, rather than mucosal injury itself, induces anorexia. However, because ghrelin is expressed in the gastric mucosa, gastric mucosal injury may induce decreased ghrelin production and subsequently induce anorexia. In a previous study, it was reported that lower concentrations of ethanol (but not absolute ethanol) induced increased plasma ghrelin levels despite the increase in the area of hemorrhagic erosions. This may represent the phenomenon of adaptive cytoprotection mediated by mild irritants, and 1 h after ethanol administration was not enough to decrease the ghrelin production in this model (5). Another report showed that the plasma total and active ghrelin levels were significantly higher in cysteamine-treated duodenal ulcer model rats probably attributable to the inhibition of somatostatin secretion, not to the formation of ulcers (12).

We cannot deny the possibility that gastric I/R-induced damages in central ghrelin production and peripherally administered ghrelin might have penetrated the blood-brain barrier and restored the decreased central ghrelin production. However, it is unlikely that a single administration of ghrelin abrogated central injury in 3 h. Ghrelin has an orexigenic effect by activating neuropeptide Y/AgRP (agouti-related protein) neurons through vagal afferent nerves. This signaling pathway is believed to be retained after gastric I/R because ghrelin administration increased food intake in I/R rats in this study although some damages are undeniable.

The ghrelin-IR cells were significantly decreased in number compared with that in the sham-operated rats at 12 h after gastric I/R in this study. According to previous studies, the number of gastric A-like cells is decreased by gastric mucosal injury induced by *H. pylori* infection (25, 29), probably attributable to the large amounts of reactive oxygen species produced during the process of colonization of the host by the bacteria (2, 9). Oxidative stress produced by the xanthine-xanthine oxidase system after gastric I/R may damage the A-like cells. The number of ghrelin-IR cells was restored in the remaining mucosa at 48 h, and we did not detect any cell death by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling in the mucosal layers, except in the areas adjacent to the erosive lesion area (data not shown). Therefore, it is unlikely that the A-like cells were destroyed after I/R and regenerated within 48 h. The expression of ghrelin in the A-like cells might be transiently decreased and the stores of ghrelin in each cell reduced, thereby making the number of ghrelin-immunoreactive cells appear to be decreased, although the precise mechanisms remain to be elucidated. The percentages of ghrelin-immunoreactive cells relative to the total number of cells were restored at 48 h after I/R. However, the numbers of ghrelin-IR cells corrected by the percentage areas not showing the erosive lesions were significantly decreased compared with those in the sham-operated rats at 48 h after I/R. This decrease might have induced the decreased ghrelin production and consequently, decreased plasma ghrelin levels.

The expressions of preproghrelin mRNA in the total stomach were significantly downregulated after I/R, which may explain the decreased plasma ghrelin levels throughout the observation period. We investigated the expression of preproghrelin mRNA in the total stomach, including the mucosal layer and muscle layer, which would reflect the total gastric production. Ghrelin is expressed only in the mucosal layer, thus mucosal injuries alone may decrease the mRNA expression of ghrelin. Erosive legion areas were observed predominantly in the fundic gland region, which is the region in which ghrelin-producing cells are predominantly identified. Thus mucosal injuries might induce decreased ghrelin production and, consequently, decreased plasma ghrelin levels, after gastric I/R.

Expressions of preproghrelin mRNA in the gastric mucosa were previously reported to be increased in response to mucosal injuries, such as those induced by gastric I/R (reperfusion 3 h) (20) and 1 h after ethanol exposure (19), and 3.5-h water-restraint stress (4). Not only preproghrelin mRNA, but also ghrelin protein expression was demonstrated to be increased at 1 h after the ethanol exposure. The expressions were investigated within a short time after the occurrence of the mucosal injuries. In this study, we examined the preproghrelin mRNA expression at 12 h and 48 h after I/R, which could have yielded different results. If the ghrelin protein expression continued to increase after I/R, the number of ghrelin-IR cells in the remaining mucosa would be unlikely to decrease, which was observed in this study. The density of the ghrelin-IR cells did not appear to differ between the sham-operated rats and I/R rats. Therefore, the expression of preproghrelin mRNA might have transiently increased at 3 h but decreased at 12 h after I/R. Also, the expression was determined only in the remaining mucosal layer in previous studies, different from the case in our present study, because we used total stomach to evaluate the total gastric ghrelin production.

In conclusion, gastric I/R caused anorexia associated with a significant decrease of the plasma ghrelin levels, which is attributed to the gastric mucosal injuries induced by I/R. The decrease in the plasma ghrelin levels may have been responsible for the decrease in the food intake after gastric I/R, as it

was restored by exogenous ghrelin administration. The results of this study show that ghrelin can stimulate food intake in rats with mucosal injuries induced by gastric I/R, suggesting that ghrelin or its analogs may also prove useful for attenuating I/R-induced dysfunctions.

GRANTS

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DISCLOSURES

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Antimicrobial Agents and Chemotherapy

Efficacy of Sitafloxacin-Based Rescue Therapy for Helicobacter pylori after Failures of First- and Second-Line Therapies

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Efficacy of Sitafloxacin-Based Rescue Therapy for *Helicobacter pylori* after Failures of First- and Second-Line Therapies

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Sitafloxacin-based triple therapy achieved 83.6% (per-protocol) and 78.2% (intention-to-treat) success in eradicating *Helicobacter pylori* among 78 Japanese patients after clarithromycin-based first-line and metronidazole-based second-line triple therapies failed. Eradication succeeded in 32 out of 43 patients, even with *gyrA* mutation-positive *Helicobacter pylori* (per protocol). The position of the *gyrA* mutation (N87 or D91) was determined to be a better marker than MIC levels for predicting outcomes of sitafloxacin-based treatment.

The emergence of *Helicobacter pylori* strains resistant to both clarithromycin and metronidazole has generated an urgent need for other treatment options for third-line rescue therapy. Possible candidates for such rescue eradication regimens include fluoroquinolones. Quinolone resistance in *H. pylori* is caused by point mutations (N87 and D91) in the quinolone resistance-determining region of the *gyrA* gene of *H. pylori*. The presence of a *gyrA* mutation is predictive of treatment failure with triple therapy, including commonly used quinolones, such as levofloxacin (3, 6). Eradication rates of levofloxacin-based therapies against levofloxacin-resistant strains (MIC, ≥1 μg/ml) or *gyrA* mutation-positive strains hover around 33.3% to 41.7% (3, 7). However, a high incidence of *gyrA* mutation was found especially in patients with previous eradication failures (5, 6, 9). Recently, we have shown that a newly developed quinolone, sitafloxacin (STFX), can overcome the resistance of *H. pylori* strains carrying *gyrA* mutations *in vitro* (8). The present study was designed to investigate the efficacy and safety of STFX-based third-line *H. pylori* eradication therapy, especially in *gyrA* mutation-positive strains.

The present study was a prospective trial conducted in Keio University Hospital from April 2009 to October 2011. Eighty-seven patients in whom eradication treatment with clarithromycin-based first-line therapy (triple therapy with clarithromycin [800 mg/day], amoxicillin [1,500 mg/day], and proton pump inhibitors [PPIs] for 7 days) and metronidazole-based second line therapy (triple therapy with metronidazole [500 mg/day], amoxicillin [1,500 mg/day], and PPIs for 7 days) failed were enrolled after obtaining informed consent (UMIN000001558). Before treatment, *H. pylori* isolates were obtained from gastric biopsy specimens. The MICs of STFX against *H. pylori* isolates and the *gyrA* mutation status were determined by the method described previously (5, 6). Seventy-eight patients (37 men and 41 women; mean age, 50.7 ± 13.4 years) were administered STFX-based therapy combined with rabeprazole (10 mg, four times a day [q.i.d.]), amoxicillin (500 mg, q.i.d.), and STFX (100 mg, two times a day [b.i.d.]) for 7 days (intention-to-treat [ITT] population). Three patients with penicillin allergy, 1 patient with loss of follow-up, and 5 patients in whom *H. pylori* could not be detected by culture were excluded from the study. For 73 patients, eradication results were confirmed (per-protocol [PP] population), whereas 5 patients were lost to follow-up. Among 73 patients, 38 had dyspepsia, 22 had peptic ulcer, 2 had early gastric cancer, 1 had mucosa-associated lymphoid tissue (MALT) lymphoma, 1 had idiopathic thrombocytopenic purpura, and 11 received PPIs (rabeprazole, $n = 5$; lansoprazole, $n = 4$; omeprazole, $n = 2$). Successful eradication was confirmed using a [^{13}C]urea breath test (^{13}C-UBT) 12 weeks after the end of therapy. The cutoff value for negative ^{13}C-UBT was less than 2.5%. At least 1 month before performing the ^{13}C-UBT, PPIs and antibiotics were not given. For two patients who showed a borderline value (2.5% to 5.0%) of ^{13}C-UBT, an *H. pylori* stool antigen test was also performed. No severe side effects to this treatment were reported. Mild and transient adverse effects, such as diarrhea (33.3%), soft stool (25.3%), abdominal pain (6.9%), epigastric fullness (6.9%), and dysgeusia (6.9%), were reported. Characteristics of the 73 patients are shown in Table 1.

The eradication rates determined by PP and ITT analyses were 83.6% (61/73 patients) and 78.2% (61/78 patients), respectively. Among 31 patients with *gyrA* mutation-negative *H. pylori*, a PP eradication rate of 96.7% (29/30) and an ITT eradication rate of 93.5% (29/31) were achieved. Moreover, even among 47 patients with *gyrA* mutation-positive *H. pylori*, the PP and ITT eradication rates were 74.4% (32/43) and 68.1% (32/47), respectively. The average MIC of STFX was higher in patients with eradication failure than in patients with eradication success (Table 1). Interestingly, the MICs of STFX in *gyrA* mutation-positive strains differed, depending on the position of the *gyrA* mutation (Fig. 1A). The MICs of STFX were higher in N87-mutated strains (0.21 ± 0.16 μg/ml) than in D91-mutated strains (0.12 ± 0.11 μg/ml) ($P = 0.03$). In fact, eradication rates were lower in patients with N87-mutated strains (61.9% for PP) than in patients with D91-mutated strains (86.4% for PP) ($P = 0.09$). Receiver-operating characteristic (ROC) curves based on the positions of *gyrA* muta-

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TABLE 1 Participant characteristics and predictive accuracy of treatment outcome

Parameter[a]	No. (%) with infection: Eradicated (n = 61)	Not eradicated (n = 12)	OR (95% CI)[b]
Demographic information			
Age (yr [mean ± SD])	51.4 ± 13.4	51.2 ± 10.8	1.00 (0.95–1.05)
Gender			
Men	31 (50.8)	4 (33.3)	0.48 (0.13–1.78)
Women	30 (49.2)	8 (66.7)	
Smokers	17 (27.9)	4 (33.3)	1.65 (0.48–5.74)
Alcohol drinkers	23 (37.7)	6 (50.0)	1.29 (0.34–4.87)
BMI (kg/m^2 [mean ± SD])	22.1 ± 2.9	21.0 ± 2.4	0.85 (0.66–1.10)
H. pylori status			
MIC of STFX (μg/ml [mean ± SD]	0.09 ± 0.13	0.17 ± 0.14	40.55 (0.76–2172.43)
Presence of mutation in *gyrA*	32 (52.5)	11 (91.7)	**9.97 (1.21–82.05)**
Presence of *gyrA* mutation at:			
D91	19 (31.1)	3 (25.0)	0.74 (0.18–3.03)
N87	13 (21.3)	8 (66.7)	**7.39 (1.92–28.42)**
Specific change at D91 or N87:			
D91N	6 (9.8)	0 (0)	
D91G	6 (9.8)	0 (9.8)	
D91Y	7 (11.5)	3 (25.0)	2.57 (0.56–11.82)
N87T	0 (0)	1 (8.3)	
N87K	12 (19.7)	5 (41.7)	2.92 (0.79–10.81)
N87I	1 (1.6)	2 (16.7)	12.00 (0.99–145.03)
CLR resistance (MIC, ≥1 μg/ml)	54 (88.5)	11 (91.7)	1.01 (0.98–1.03)
MNZ resistance (MIC, ≥8 μg/ml)	48 (78.7)	9 (75.0)	1.00 (0.97–1.03)

[a] BMI, body mass index; STFX, sitafloxacin; CLR, clarithromycin; MNZ, metronidazole.
[b] Boldface values indicate significant association with the treatment outcome, analyzed using a univariable logistic regression model.

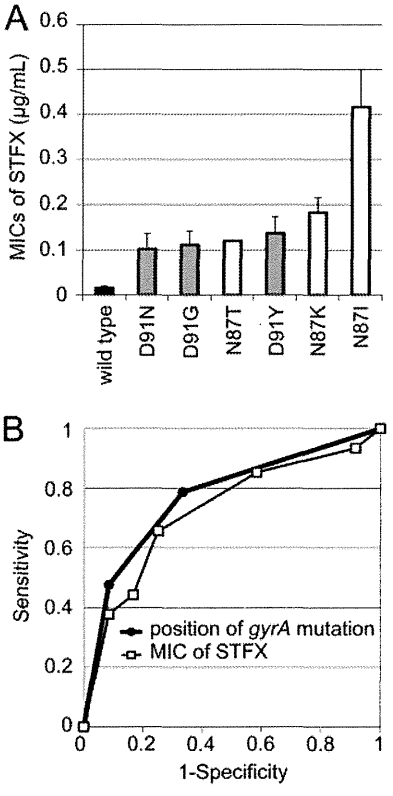

FIG 1 (A) The average MICs of sitafloxacin were higher in *H. pylori* strains with a *gyrA* mutation at N87 than in those with a *gyrA* mutation at D91. (B) Receiver-operating characteristic (ROC) curves show that the position of the *gyrA* mutation is a better marker than MIC levels for predicting outcomes of sitafloxacin-based treatment.

tions and MICs of STFX demonstrated that the diagnostic accuracy of the position of *gyrA* mutations (area under the curve [AUC], = 0.773 ± 0.070) for predicting eradication success is higher than that of MICs of STFX (AUC = 0.725 ± 0.076) (Fig. 1B). When the cutoff value for the MICs of STFX was defined as more than 0.12 μg/ml, an odds ratio (OR) of 5.7 (95% confidence interval [CI], 1.4 to 23.4), a positive predictive value (PPV) of 93.0%, a negative predictive value (NPV) of 30.0%, and an accuracy of 67.1% were yielded for predicting eradication success. On the other hand, the presence of N87 mutations achieved an OR of 7.4 (95% CI, 1.9 to 28.4), a PPV of 92.3%, an NPV of 38.1%, and an accuracy of 76.7%. These results show that prediction of treatment outcomes was better using the positions of *gyrA* mutations than using the MICs of STFX.

According to a systematic review and meta-analysis, the mean eradication rate with 7-day levofloxacin-based rescue therapies was 73% (1). The present study showed that the STFX-based therapy is a marked improvement on quinolone-based rescue therapy, especially for *H. pylori* strains with a *gyrA* mutation. We also discovered that the position of the *gyrA* mutation affects the outcome of the STFX-based therapy. The MICs of the other quinolones, such as levofloxacin and moxifloxacin, have also been demonstrated to be different between N87 and D91 mutations (2, 4). This suggests that detection of N87 mutations will be useful for predicting eradication failure in broad-spectrum quinolone-based therapies and not just in STFX-based therapy. Since stool specimens can be used noninvasively to obtain *H. pylori* DNA, the position of the *gyrA* mutation can be checked more easily and rapidly than drug susceptibility testing in clinical practice. In conclusion, the STFX-based rescue therapy was highly effective even in patients infected with *gyrA* mutation-positive *H. pylori* and is a promising

candidate for third-line therapy. Furthermore, the *gyrA* mutation at N87 is a better marker than MIC levels for predicting outcomes of quinolone-based treatment. To confirm the efficacy of the STFX-based rescue therapy, randomized controlled trials are warranted.

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Classification of functional dyspepsia based on concomitant bowel symptoms

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Abstract

Background *Functional dyspepsia (FD) is a heterogeneous disease, and categorized into postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS). However, many FD patients have overlap of both PDS and EPS. The present study was designed to examine whether FD could be categorized based on the presence of concomitant gastrointestinal symptoms.* ***Methods*** *A web survey comprised of the Gastrointestinal Symptom Rating Scale (GSRS), Rome III criteria of FD, and demographic information was sent to public participants who have no history of severe illness. Factor and cluster analyses were conducted to identify sub-categories of FD based on GSRS.* ***Key Results*** *A total of 8038 participants completed the survey. A total of 563 participants met the criteria for FD, whereas 6635 participants did not have dyspepsia symptoms. The remainder had either organic disease (377) or uninvestigated dyspepsia (463). The cluster analysis categorized participants as constipation predominant (cluster C), diarrhea predominant (cluster D), or having neither diarrhea nor constipation (cluster nCnD). Cluster C and D were significantly associated with the presence of FD [odds ratio (OR) 2.57, 95% confidence interval (CI) 2.06–3.21; OR 2.80; 95% CI 2.27–3.45, respectively]. In FD, especially in PDS cases, the scores of upper gastrointestinal symptoms were higher in cluster C or D than in cluster nCnD.*

Conclusions & Inferences *The severity of dyspepsia symptoms is associated with the presence of bowel symptoms especially in PDS. This novel categorization of FD based on concomitant constipation or diarrhea may improve classification of patients.*

Keywords *cluster analysis, constipation, diarrhea, dyspepsia, factor analysis.*

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INTRODUCTION

Functional dyspepsia (FD) is a common clinical syndrome characterized by chronic and recurrent gastroduodenal symptoms in the absence of any organic or metabolic disease that is likely to explain the symptoms.[1,2] FD is a heterogeneous condition consisting of different subgroups. According to Rome III criteria of FD, FD is divided into two subgroups: postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS), to distinguish between meal-induced symptoms and meal-unrelated symptoms believed to be pathophysiologically and clinically relevant.[1,3] Although it has been postulated that symptom subgroups could be used to identify more homogenous subgroups that would respond to targeted medical therapy, up to half of FD patients have overlap of both PDS and EPS.[4] In addition, to the best of our knowledge, there is no evidence that PDS or EPS should be treated differently.

Overlap among functional gastrointestinal disorders (FGIDs) is extremely common. Specifically, dyspepsia and bowel symptoms, such as diarrhea and constipation, often coexist.[5] A recent meta-analysis reported that the prevalence of irritable bowel syndrome (IBS) among participants of dyspepsia was 37% compared with 7% in those without dyspepsia.[6] In addition, the prevalence of esophageal symptoms, such as heartburn, is also high in FD patients, although esophageal reflux

symptoms may co-exist, but are not consider typical FD symptoms in the Rome III criteria.[1] Savarino *et al.*[7] showed that patients with functional heartburn had more frequent postprandial fullness, bloating, early satiety, and nausea than patients with non-erosive reflux disease (NERD). These recent studies reinforce the concept that FGIDs extend beyond the boundaries suggested by the anatomical location of symptoms.

Although subclasses of FD based on symptom clusters have been proposed,[1,8] subclustering based on bowel symptoms or esophageal symptoms has not been investigated. The aim of this study was to use factor and cluster analyses to determine whether FD could be characterized based on the presence of concomitant other gastrointestinal (GI) symptoms, including bowel symptoms and esophageal symptoms.

MATERIALS AND METHODS

Study participants

The protocol for this study was approved by the ethics committee of Tokyo Ekimae Building Clinic (TEC-0801, September 24, 2008). We conducted a web-based cross-sectional study. Participants were solicited from a list of public participants who are invited previously to participate in the clinical studies conducted by the Tokyo Ekimae Building Clinic with informed consent. No participants in the list have a severe chronic or life-threating illness such as malignancy or systemic autoimmune diseases, and a serious mental illness, such as major depression or schizophrenia. Participants who used prescribed medicines or over-the-counter (OTC) drugs were not excluded from the present study. The questionnaires were comprised of items including the Japanese version of the Gastrointestinal Symptom Rating Scale (GSRS)[9] and the Rome III criteria of FD;[1] in addition, prior receipt of upper GI screening examination was elicited. If either of the latter two were identified, the presence/absence of structural disease was also abstracted. The Japanese version of GSRS questionnaire is a validated, self-administered questionnaire that includes 15 questions, which assess severity of GI symptoms, including esophageal reflux symptoms, dyspepsia symptoms, and bowel symptoms, using a 7-point Likert scale.[10] Demographic information, such as age, gender, smoking habit, alcohol habit, height, and weight, were also obtained, and body mass index (BMI) (weight height^{-2}) was calculated. Smoking was categorized into 'none', 'light' (1–15 cigarettes day^{-1}), and 'heavy' (>16 cigarettes day^{-1}) according to a number of cigarettes consumed per day. Alcohol intake was also categorized into 'none', 'light' (1–3 days week^{-1}), and 'heavy' (4–7 days week^{-1}) according to a number of days of alcohol consumption per week.

Definition of FD cases and non-dyspepsia controls

Based on Rome III criteria, participants were defined as having dyspepsia when they have one or more of symptoms, such as postprandial fullness, early satiation, or epigastric pain or burning for at least 6 months prior to the survey. Participants without dyspepsia symptoms were defined as a 'non-dyspepsia' control group. Participants with dyspepsia who had undergone the upper GI examination and had no evidence of structural disease in the stomach and duodenum were defined as 'FD' cases. Participants with dyspepsia who had not undergone upper GI examination were classified as 'uninvestigated dyspepsia'. Participants with dyspepsia who had undergone upper GI examination and had structural disease were classified as 'organic disease' patients.

The FD subjects with postprandial fullness or early satiation were defined as those with PDS, whereas FD subjects with epigastric pain or burning were defined as those with epigastric pain syndrome (EPS). Using these definitions, FD subjects were subcategorized into three groups as follows: subjects with PDS alone, subjects with EPS alone, and subjects with both PDS and EPS.

Statistical analysis

Exploratory factor analysis was conducted in all web responders to identify the latent pathologic conditions, named 'symptom factors', and to reduce the dimensionality of subsequent analyses. Principal factor method with Varimax rotation was used. Subsequently, a non-hierarchical k-means cluster analysis for a three-cluster solution was performed using the symptom factor scores derived from the preceding factor analysis. Three 'symptom clusters' were extracted. The differences in the prevalence of FD between three symptom clusters were evaluated using univariable and multivariable logistic regression. In the multivariable model, age, gender, smoking, alcohol use, and BMI were included. The differences of the symptom factor scores between three symptom clusters in FD cases were examined using one-way ANOVA and Tukey's *post hoc* analysis. The differences of life-style characteristics between three symptom clusters were examined for each gender separately, as participant characteristics were significantly different between men and women. The differences between the three symptom clusters in age and BMI were examined using one-way ANOVA and Tukey's *post hoc* analysis. The differences between the three symptom clusters in smoking and alcohol habits were examined using Fisher's exact test.

All statistical analyses were conducted using the SPSS Statistics version 18.0 for Windows software (SPSS Japan, Tokyo, Japan; SPSS Inc., Chicago, IL, USA). The data in the tables were expressed as mean ± standard deviation. Two-sided P-values were considered as statistically significant at a level of 0.05.

RESULTS

Participant characteristics

A total of 8038 participants (3462 men and 4576 women; mean age 40.8 ± 9.7 years) completed the questionnaire. A total of 563 participants were defined as FD cases, whereas 6635 participants without dyspepsia symptoms were identified as non-dyspepsia controls. A total of 463 participants were classified as uninvestigated dyspepsia, and 377 had organic disease (Fig. 1). Participant characteristics are shown in Table 1. Mean age was higher in FD cases than in non-dyspepsia controls. There was greater proportion of women among FD cases than among non-dyspepsia controls. Alcohol consumption and smoking was greater in FD than in non-dyspepsia. BMI was lower in FD than in non-dyspepsia. BMI was especially lower

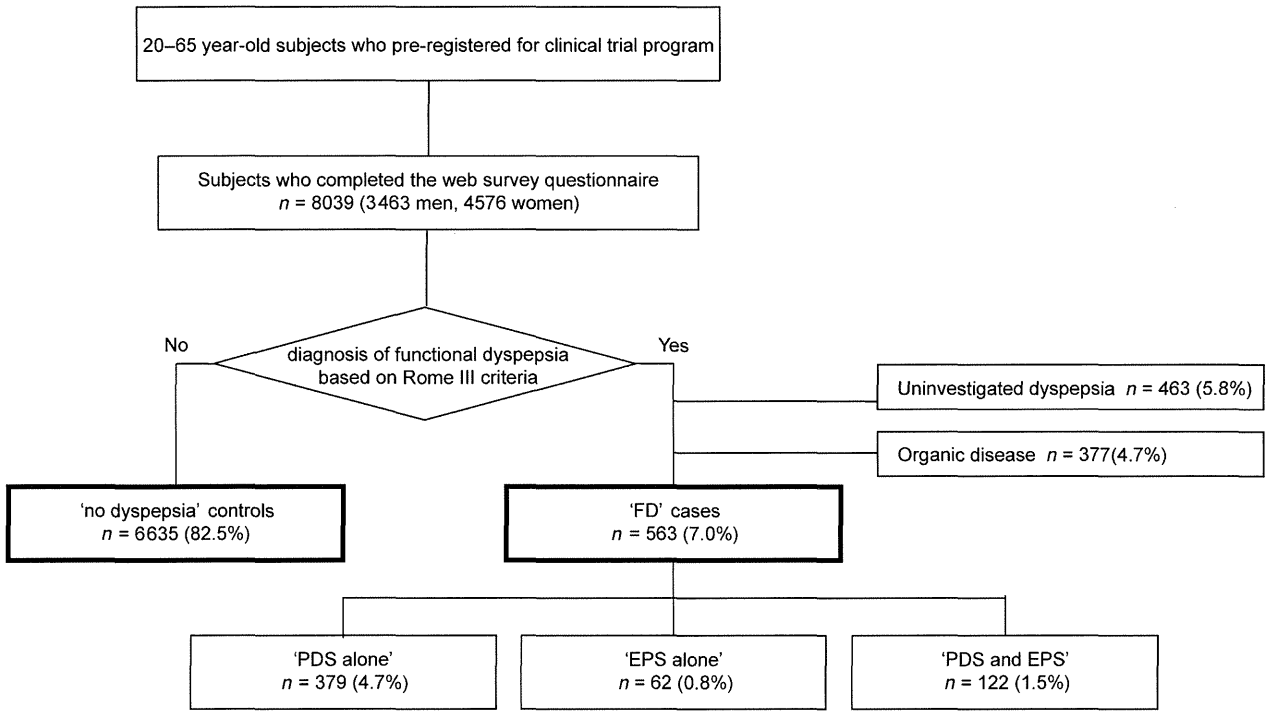

Figure 1 The study population.

Table 1 Participant characteristics

	All (*n* = 8038)	Non-dyspepsia (*n* = 6635)	FD (*n* = 563)	Uninvestigated dyspepsia (*n* = 463)	Organic disease (*n* = 377)	*P* value (non-dyspepsia *vs* FD)
Age						
Mean ± SD (years) (Mean ± SD)	40.8 ± 9.7	40.9 ± 9.8	42.8 ± 7.8	34.4 ± 9.0	44.0 ± 8.7	<0.001*
20–29 [No. (%)]	1028	842 (12.7)	21 (3.7)	148 (32.0)	17 (4.5)	
30–39 [No. (%)]	2639	2183 (32.9)	157 (27.9)	199 (43.0)	100 (26.5)	
40–49 [No. (%)]	2894	2372 (35.7)	282 (50.1)	80 (17.3)	160 (42.4)	
50–59 [No. (%)]	1201	999 (15.1)	90 (16.0)	33 (7.1)	79 (21.0)	
60–65 [No. (%)]	276	239 (3.6)	13 (2.3)	3 (0.6)	21 (5.6)	
Gender [No. (%)]						
Men	3462	2920 (44.0)	228 (40.5)	142 (30.7)	172 (45.6)	0.111†
Women	4576	3715 (56.0)	335 (59.5)	321 (69.3)	205 (54.4)	
Smoking habit [No. (%)] (number of consumptions per day)						
None (0)	5924	4947 (74.6)	392 (69.6)	332 (71.7)	253 (67.1)	0.037†
Light (1–15)	1062	848 (12.8)	85 (15.1)	74 (16.0)	55 (14.6)	
Heavy (>16)	1052	840 (12.7)	86 (15.3)	57 (12.3)	69 (18.3)	
Alcohol habit [No. (%)] (number of days of consumption per week)						
None (0)	2743	2281 (34.4)	181 (32.1)	159 (34.3)	122 (32.4)	<0.001
Light (1–3)	2956	2482 (37.4)	178 (31.6)	186 (40.2)	110 (29.2)	
Heavy (4–7)	2339	1872 (28.2)	204 (36.2)	118 (25.5)	145 (38.5)	
BMI (kg m^{-2}) (Mean ± SD)	22.6 ± 3.9	22.7 ± 3.9	22.2 ± 3.8	21.7 ± 3.9	22.6 ± 4.5	0.008*

BMI, body mass index; FD, functional dyspepsia.
*Analyzed by unpaired Student's *t*-test.
†Analyzed by Pearson's Chi-squared test.

in 'PDS alone' group (22.1 ± 3.7 kg m^{-2}) than in non-dyspepsia (22.7 ± 3.9 kg m^{-2}, $P = 0.003$). This suggests that participants with PDS alone may avoid food because it precipitates their symptoms.

The differences between FD cases and non-dyspepsia controls in the average scores of the 15 GI symptom assessed by GSRS were compared using unpaired Student's *t*-test. All of the 15 GI symptoms were

significantly more severe in FD cases than in non-dyspepsia controls (See Table S1 online). Scores in participants with uninvestigated dyspepsia or organic disease were also higher than in non-dyspepsia. These results showed that not only upper GI symptoms, but also bowel symptoms and esophageal symptoms were more severe in participants with dyspepsia.

Factor analysis

Factor analysis revealed that the 15 items could be reduced to three GI symptom factors, namely factor EGD (esophagogastroduodenal symptoms), factor C (constipation), and factor D (diarrhea) (Table 2). Factor EGD mainly reflects the severity of upper GI symptoms, such as heartburn, abdominal pains, and abdominal distension. Factor C reflects constipation-related symptoms. Factor D reflects diarrhea-related symptoms.

To examine potential associations between demographic factors (exposure variables) and the three symptom factors (outcome variables), linear regression analyses were performed (See Table S2 online). Younger age was associated with increased scores of all three symptom factors. Factor EGD and factor C scores were greater in women, whereas factor D scores were greater in men. Smoking was associated with factor EGD score in a dose-dependent manner. Heavy smoking was also associated with factor D score. Heavy alcohol consumption positively associated with factor EGD and factor D scores; conversely it was inversely correlated with factor C score. BMI was inversely associated with factor C score.

Table 2 Factor loading of the severity of 15 gastrointestinal symptoms (n = 8038)

	Factor EGD	Factor C	Factor D
Heartburn	**0.718**	0.139	0.132
Acid regurgitation	**0.701**	0.092	0.165
Abdominal pains	**0.681**	0.157	0.150
Sucking sensations in the epigastrium	**0.651**	0.200	0.166
Nausea and vomiting	**0.591**	0.169	0.228
Abdominal distension	**0.555**	0.335	0.181
Eructation	**0.498**	0.233	0.218
Borborygmus	0.396	0.314	0.276
Increased flatus	0.306	0.399	0.280
Feeling of incomplete evacuation	0.251	**0.624**	0.307
Urgent need for defecation	0.237	0.192	**0.668**
Increased passage of stools	0.227	0.064	**0.835**
Loose stools	0.209	0.088	**0.818**
Hard stools	0.188	**0.772**	0.047
Decreased passage of stools	0.161	**0.820**	0.010

Bold values indicate the loading values of higher than 0.5 for each symptom factor.
Factor EGD: the severity of upper gastrointestinal symptoms.
Factor C: the severity of constipation-related symptoms.
Factor D: the severity of diarrhea-related symptoms.

Cluster analysis

Cluster analysis based on the three symptom factor scores showed that FGIDs could be categorized into three clusters, namely cluster nCnD (non-constipation and non-diarrhea), cluster C (constipation), and cluster D (diarrhea). Cluster C was characterized by high scores of factor C (factor EGD 0.31; factor C 1.31; factor D −0.38). Cluster D was characterized by high scores of factor D (factor EGD 0.28; factor C −0.08; factor D 1.34). Cluster nCnD was not associated with any of the three symptom factors (factor EGD −0.22; factor C −0.43; factor D −0.39). The scores of the three symptom factors are plotted on the 3D coordinate systems to illustrate the distribution of three clusters in Fig. 2.

Based on the result of cluster analysis, FD cases and non-dyspepsia controls could be categorized into three clusters. Among 6635 non-dyspepsia controls, 4101 (61.8%) were categorized to cluster nCnD, 1218 (18.4%) were to cluster C, and 1316 (19.8%) were to cluster D. On the other hand, among 563 FD cases, 217 (38.5%) were categorized to cluster nCnD, 160 (28.4%) were to cluster C, and 186 (33.0%) were to cluster D. Multivariable logistic regression analysis revealed that both cluster C and D were significantly associated with the presence of FD (Table 3). Association between cluster C and FD were almost same level as association between cluster D and FD, suggesting that constipation and diarrhea were equally contributed to the onset of FD.

The prevalence of PDS or EPS was similar among the three symptom clusters: 217 FD participants in cluster nCnD were 146 (67.3%) with PDS alone, 26 (12.0%) with EPS alone, and 45 (20.7%) with both PDS and EPS; 160 in cluster C were 113 (70.6%) with PDS alone, 16 (10.0%) with EPS alone, and 31 (19.3%) with both PDS and EPS; 186 in cluster D were 120 (64.5%) with PDS alone, 20 (10.8%) with EPS alone, and 46 (24.7%) with both PDS and EPS. This illustrates that overlap of constipation or diarrhea was not associated with the presence/absence of PDS or EPS. In 'PDS alone' and 'PDS and EPS' groups, factor EGD score was higher in cluster C or D than in cluster nCnD. These results showed that upper GI symptoms, such as reflux or dyspepsia, were more severe in participants with bowel symptoms than without bowel symptoms especially in participants with PDS. On the other hand, in 'EPS alone' group, factor EGD score was not significantly different among the three symptom clusters (Fig. 3).

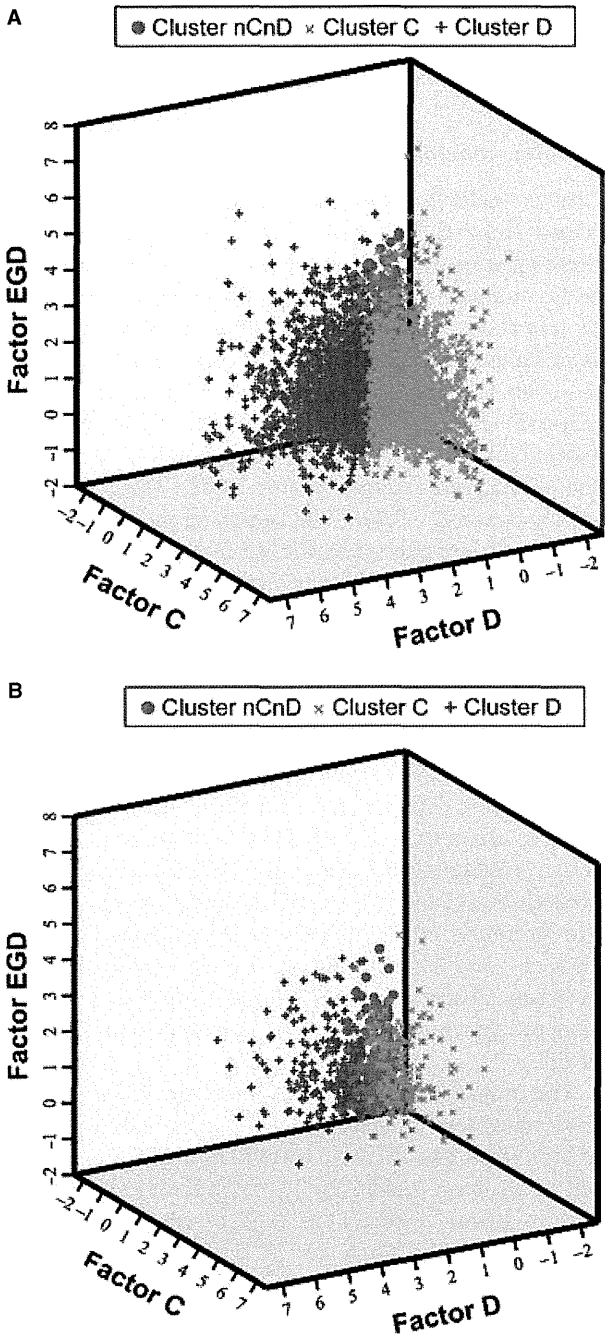

Figure 2 Distribution of cluster nCnD, cluster C, and cluster D. The 3D spatial distribution of overall 8038 participants (A) and 563 functional dyspepsia participants (B) with three symptom factor scores derived from factor analysis showed that the three symptom clusters were well separated.

Demographic factors in FD cases were significantly different between the three clusters (See Table S3 online). As there was a greater proportion of women in cluster C, subsequent analyses were examined for each gender separately. In both genders, alcohol consumption was associated with cluster nCnD and cluster D, but not with cluster C. In women, lower BMI was associated with cluster C.

DISCUSSION

This population based, large-scale cross-sectional study was conducted to identify GI symptom clusters in FGIDs. Cluster analysis in the present study revealed that all FGIDs, including FD, could be subcategorized based on concomitant bowel symptoms. As IBS is classified as constipation predominant IBS (IBS-C), diarrhea predominant IBS (IBS-D), and mixed IBS (IBS-M) in Rome III criteria,[11] FD could be categorized into three clusters: absence of bowel symptoms (cluster nCnD), constipation predominant (cluster C), and diarrhea predominant (cluster D). Esophageal reflux symptoms, postprandial distress, and epigastric pain symptoms could not be separated using factor analysis, suggesting that overlaps between functional esophageal disorders, PDS, and EPS occur frequently. Classification of FD based on concomitant lower GI symptoms is a novel concept and may improve our ability to discriminate between subgroups of FD. Recent study showed that psychosocial factors, such as anxiety, depression, and somatization are also important variables for subgrouping FD.[12] Classification of FD based on a combination of bowel symptoms and psychosocial factors would be a promising alternative for gastroduodenal symptom-based classification as proposed by the Rome III criteria.

In the present study, FD was more prevalent in participants with bowel symptoms (cluster C or cluster D) than those without bowel symptoms (cluster nCnD). This result is consistent with the observed high frequency of overlap between FD and IBS. Moreover, concomitant bowel symptoms were associated with demographic factors, such as gender, alcohol consumption, and BMI, among FD participants. These results suggest that the etiology of dyspepsia symptoms may differ among participants classified as cluster nCnD, cluster C, and cluster D. Corsetti *et al.*[13] showed that FD-IBS overlap is more prevalent among women and is associated with a greater weight loss, overall symptom severity, and with hypersensitivity to distention than FD alone. The present study confirmed that FD with constipation is more prevalent among women, and is associated with lower BMI among women. On the other hand, these associations were not observed in FD with diarrhea (See Table S3 online).

When FD subjects were subcategorized into 'PDS alone', 'EPS alone', and 'PDS and EPS' groups, a significant association between these three groups

Table 3 Relationship between the three symptom clusters and diagnosis of FD

	Non-dyspepsia (n = 6635)	FD (n = 563)	Univariable analysis*	Multivariable analysis†
	No. (%)	No. (%)	Odds ratio (95% CI)	Odds ratio (95% CI)
Cluster nCnD (n = 4318)	4101 (95.0)	217 (5.0)	Ref.	Ref.
Cluster C (n = 1378)	1218 (88.4)	160 (11.6)	2.48 (2.00–3.08)	2.57 (2.06–3.21)
Cluster D (n = 1502)	1316 (87.6)	186 (12.4)	2.67 (2.18–3.28)	2.80 (2.27–3.45)

CI, confidence interval; FD, functional dyspepsia.
*Analyzed by univariable logistic regression model.
†Analyzed by multivariable logistic regression model with adjustment for cluster C, cluster D, age, gender, smoking habit, alcohol habit, and body mass index.

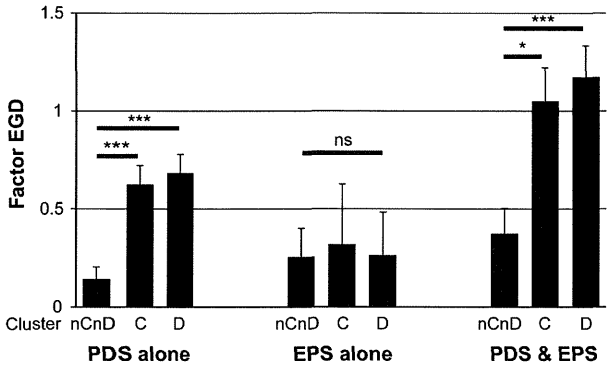

Figure 3 Associations between upper gastrointestinal symptoms and the three symptom clusters in each subgroup of functional dyspepsia. ***P < 0.001, *P < 0.05 significant difference using one-way ANOVA and Tukey's *post hoc* analysis. EPS, epigastric pain syndrome; ns, not significant; PDS, postprandial distress syndrome.

and the three symptom clusters was not observed. However, the association between the severity of upper GI symptoms (factor EGD score) and concomitant bowel symptoms among PDS participants differed from the association among participants with EPS alone. Some previous studies also demonstrated that FD-IBS overlap patients have worse quality of life than FD-alone and IBS-alone patients.[14,15] Results of the present study revealed that FD participants with bowel symptoms have greater symptoms severity than those without bowel symptoms especially in PDS, but not in EPS alone. This suggests that while PDS might be associated with the bowel symptoms, EPS without PDS might be independent of the presence/absence of bowel symptoms. Patients with constipation or diarrhea tend to have a general motor disturbance throughout the GI tract, including abnormal colonic transit and delayed gastric emptying, especially in patients with concomitant FD and IBS.[16–18] GI motility disorders are likely to induce symptoms of PDS rather than those of EPS.[19] The other study showed that patients with both FD and IBS are associated with hypersensitivity to distention of the stomach using gastric barostat.[13] Gastric hypersensitivity was more prevalent when patients suffered from both EPS and PDS.[20] These previous reports also support that concomitant constipation or diarrhea is associated with PDS, but not EPS alone.

Criticisms of the present study include possible differences between web-survey responder population and general population (generalizability). Web-based assessment may select participants from comparatively young and socially advantaged groups characterized by high literacy, and high internet access.[21] In the present study, mean age in FD cases were older than that in non-dyspepsia controls. This might be because our population contains a higher proportion of young people (<40 years old) than general population. This participant bias might affect the prevalence of FD, as FD was more prevalent in those with lower household income, lower educational levels, larger household membership, and those who were unemployed.[22–25] However, a previous study showed that participation bias is thought to have little effect on associations with putative risk factors.[21] In addition, web-based survey has advantages related to the speed and cost of data collection.[21] Therefore, it would be a powerful tool for studying characteristics of diseases and overlaps of the other disorders in FGIDs.

The disadvantage of the k-means cluster analysis is that the number of clusters must be supplied as a parameter. In the present study, we selected a three-cluster solution, as the results in three-cluster solution were the most understandable not only for gastroenterologists but also general practitioners. This categorization of FD can be determined just by the presence/absence of constipation or diarrhea which can be obtained from medical history taking. Whether treatments for bowel symptoms would improve dyspepsia symptoms in FD patients with constipation or diarrhea has not been examined[26], warranting future research.

In conclusion, GI symptoms, including FD, can be categorized into three clusters based on the presence

and type of bowel symptoms, suggesting differences in etiology between FD patients with constipation, with diarrhea, or neither. Constipation and diarrhea contribute almost equally to the presence of FD. PDS patients with bowel symptoms have greater symptoms severity than those without bowel symptoms. This categorization of FD is easy to use for general practice, and may improve classification of patients and identify subgroups that have differing pathophysiology or who may respond differently to treatment.

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AUTHOR CONTRIBUTIONS

HS & YF designed the research study. HS & YF conducted the web survey and collected the data. JM, HS & KA analyzed and interpreted the data. JM & HS drafted the article. KA & JMI revised the manuscript. TT & TH supervised and approved to be published.

CONFLICT OF INTEREST

The authors have no competing interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Average scores of 15 gastrointestinal symptoms.

Table S2. Associations between demographic factors and symptom factor scores (n = 8038).

Table S3. Difference of life-style characteristics between three symptom clusters in FD cases.

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Original Contribution

FecA1, a bacterial iron transporter, determines the survival of *Helicobacter pylori* in the stomach

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ABSTRACT

Helicobacter pylori encodes a single iron-cofactored superoxide dismutase (SodB), which is regulated by the ferric uptake regulator (Fur). Ferrous ion (Fe^{2+}) is necessary for the activation of SodB. The activity of SodB is an important determinant of the capability of *H. pylori* for long-term colonization of the stomach and of the development of metronidazole (Mtz) resistance of the bacterium. This study is conducted to characterize the Fe^{2+}-supply mechanisms for the activation of SodB in *H. pylori*, which, as mentioned above, is associated with the host-colonization ability and Mtz resistance of *H. pylori*. In this study, we demonstrate that *fecA1*, a Fe^{3+}-dicitrate transporter homolog, is an essential gene for SodB activation, but not for the biogenic activity of *H. pylori*. *H. pylori* with SodB inactivation by *fecA1* deletion showed reduced resistance to H_2O_2, reduced gastric mucosal-colonization ability in Mongolian gerbils, and also reduced resistance to Mtz. Our experiment demonstrated that FecA1 is an important determinant of the host-colonization ability and Mtz resistance of *H. pylori* through Fe^{2+} supply to SodB, suggesting that FecA1 may be a possible target for the development of a novel bactericidal drug.

Helicobacter pylori is a spiral-shaped, flagellated, microaerophilic gram-negative bacterium that colonizes the gastric epithelium of humans and is a major cause of peptic ulcers and also a key risk factor for gastric cancer and gastric MALT lymphoma [1]. *H. pylori* encodes only one single iron-cofactored superoxide dismutase (Fe-SOD; SodB) (HP0389), which catalyzes the conversion of superoxide anions to hydrogen peroxide, with the latter degraded into oxygen and water by catalase [2-4]. The *sodB* mRNA expression in *H. pylori* is directly regulated by the ferric uptake regulator (Fur) protein [5]. Recently, we reported that clinically isolated metronidazole (Mtz)-resistant strains (KS0048 and KS0145) showed derepression of *sodB* mRNA expression by amino acid mutations of Fur (C78Y and P114S; mutant Fur), which led to the development of Mtz resistance [6]. In addition, it has been reported that *sodB* deletion in *H. pylori* causes the bacterium to lose its ability to colonize the gastric mucosa in mice [7]. These results demonstrate that SodB is an important determinant of Mtz resistance and of the host-colonization ability of *H. pylori*.

Ferrous ion (Fe^{2+}) is necessary for the basal functioning of all cells, as a cofactor for enzymes and metalloproteins, and is also required for SodB activation [3,8]. On the other hand, iron (Fe^{2+} and Fe^{3+}) overload produces toxic oxygen radicals in the presence of oxygen [9]. Therefore, there exists an ingenious regulatory system for intracellular iron (Fe^{2+} and Fe^{3+}) uptake. *H. pylori* has three *fecA*-like genes (HP0686, *fecA1;* HP0807, *fecA2;* and HP1400, *fecA3*), each encoding a high-affinity transporter of Fe^{3+}-dicitrate. The expression of *fecA1* and *fecA2* is regulated by Fur, while the transcription of *fecA3* is regulated by nickel-responsive regulator (NikR) [10-12]. Additionally, Danielli et al. [13] reported that the expression patterns of *fecA1* and *fecA2* throughout the period of growth were different: whereas *fecA1* showed sustained expression over time, *fecA2* expression was derepressed only in the late phase of growth. This report suggested that the role of FecA1 was different from that of FecA2, although they were both regulated by Fur [13].

Fur dimers, as global transcriptional regulators, are formed by binding of Fur to Fe^{2+} (iron-bound Fur), and these dimers bind to the Fur-binding consensus sequences (Fur-box) of the target genes [14,15]. The expression of the *fecA1* and *fecA2* genes is repressed by binding of iron-bound Fur to each Fur-box under iron-replete conditions (normal cultivation conditions; +Fe) (Fig. 1). On the other hand, under iron-restricted conditions (−Fe), iron-bound Fur is absent; thus, the expression is derepressed by a decrease in the binding of iron-free Fur (apo-Fur) to each Fur-box (Fig. 1) [10,15].

The mechanisms of detoxification of ROS by antioxidant enzymes are of particular interest in understanding the development of Mtz resistance and also the capability of *H. pylori* for long-term gastric mucosal colonization [2,6,16,17]. Therefore, examination of the SodB activation process is required for understanding the mechanisms of detoxification of ROS in *H. pylori*. This study was designed to examine

Abbreviations used: FecA1, ferric citrate transporter homolog protein; Fur, ferric uptake regulator; Mtz, metronidazole; ROS, reactive oxygen species; SodB, iron-cofactored superoxide dismutase.

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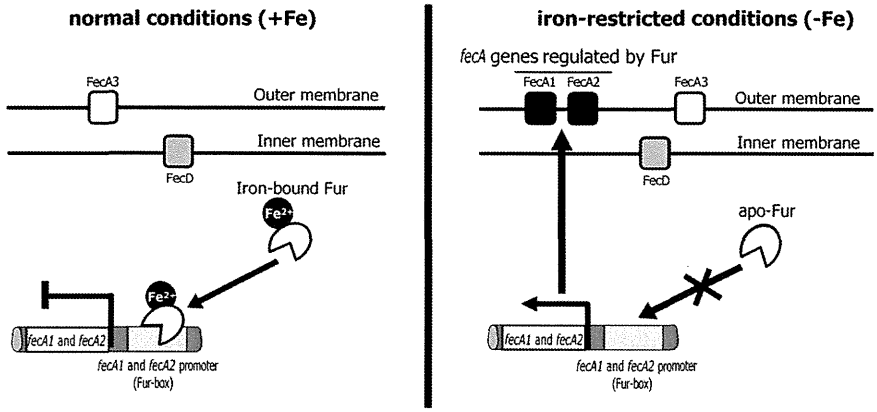

Fig. 1. Regulation of the *fecA* genes for the Fe^{3+}-dicitrate transporter by Fur in *H. pylori*. The transcription of *fecA3* is not regulated by Fur. Iron-bound Fur binds to the promoter of *fecA1* and *fecA2*, leading to transcriptional repression under normal cultivation conditions (iron-replete conditions). Under iron-restricted conditions, iron-bound Fur is absent; thus the *fecA1* and *fecA2* genes are derepressed by iron-free Fur (apo-Fur).

the Fe^{2+} supply system associated with SodB activation, by analyzing the *H. pylori* strains KS0048 and KS0145.

Materials and methods

Bacterial strains and culture conditions

H. pylori strains ATCC700392 and KS0189 were used as the Mtz-susceptible strains with wild-type Fur; ATCC430504 was used as the Mtz-resistant strain with deletion of the oxygen-insensitive NADPH nitroreductase (RdxA) [18]; the KS0048 and KS0145 strains were used as the Mtz-resistant strains with mutant Fur. KS0048 showed an amino acid mutation of Fur (Pro 114 replaced by Ser and Asn 118 replaced by His; mutant Fur); KS0145 also showed mutant Fur (Cys 78 replaced by Tyr and Asn 118 replaced by His) [6]. The KS strains were clinically isolated strains and were kept at −80 °C in *Brucella* broth (Becton–Dickinson, Franklin Lakes, NJ, USA) containing 25% (vol/vol) glycerol. The bacteria were cultured on *Brucella* agar containing 7% sheep blood and 7% fetal bovine serum (FBS) for 2 days at 37 °C under microaerobic conditions maintained with AnaeroPack MicroAero (Mitsubishigas, Tokyo, Japan).

Construction of SodB-overexpressing and fecA1-deletion mutants

The construction of a SodB-overexpressing strain of *H. pylori* using the shuttle vector pHel3 [19] has been described previously [6]. Briefly, the pHel3::sodB construct was electroporated into *H. pylori*, which was grown on 30 μg/ml kanamycin to obtain a SodB-overexpressing strain (ATCC700392 pHel3::sodB). Only the pHel3 vector was electroporated into *H. pylori*, which was grown on 30 μg/ml kanamycin to obtain a control strain (ATCC700392 pHel3 control).

The target-region gene cassette (5′*fecA1*–chloramphenicol acetyltransferase (*cat*)–3′*fecA1*) for construction of a *fecA1*-deletion mutant was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), and the sequences were determined (target vector). The target-region gene cassette was constructed for insertion into the open reading frame of FecA1. The construction of the target-region gene cassette was carried out using the PCR-based overlap extension method [20]. The primer sequences used for construction of the target-region gene cassette were as follows: 5′*fecA1* region, forward 5′-ATGAAAAGAATTTTAGTCTCTTTGGCTG and reverse 5′-GGTGGTATATC-CAGTGATTTTTTTCTCCATATTCATCATGCCCCCTGTTCTTAAG; *cat* region, forward 5′-ACTTAAGAACAGGGGGCATGATGAATATGGAGAAAAAAAT-CACTGGATATACCACC and reverse 5′-CCATACCGCTGTATAGTTGTTAAA-CAGTTACGCCCCGCCCTGCCACTCATCGC; 3′*fecA1* region, forward 5′-GCGATGAGTGGCAGGGCGGGGCGTAACTGTTTAACAACTATACAGCGGTAT GG and reverse 5′-AATACCAAAAGTCGTGTGCTTGTAAG. For the overlap extension, the forward primer was the 5′*fecA1* region forward primer and the reverse primer was the 3′*fecA1* region reverse primer. The target vector was electroporated into *H. pylori* ATCC700392, ATCC43504, KS0048, and KS0145, respectively, which were grown on 20 μg/ml chloramphenicol to obtain the *fecA1*-deletion mutants (ATCC700392 *fecA1*-deletion mutant, ATCC43504 *fecA1*-deletion mutant, KS0048 *fecA1*-deletion mutant, and KS0145 *fecA1*-deletion mutant).

Expression and purification of Fur

The expression and purification methods for recombinant Fur protein from *Escherichia coli* BL21(DE3) using a pET-30b(+) vector (Novagen, Madison, WI, USA) (*E. coli* pET::Fur) have been described previously [6]. Briefly, the expression of Fur protein was induced by 0.5 mM IPTG for 6–8 h at 30 °C, and then the Fur protein expressed in the *E. coli* pET::Fur was purified using the MagneHis protein purification system (Promega, Madison, WI, USA).

RNA isolation and quantitative reverse transcription–polymerase chain reaction (RT-PCR)

The bacteria, normalized to an OD_{600} of 1.0, were incubated under normal cultivation conditions (*Brucella* broth containing 7% FBS) and iron-restricted conditions (normal cultivation conditions with 20 μM deferoxamine mesylate, which is a ferric-iron chelator) for 5 h. Because *fecA* genes (*fecA1* and *fecA2*) were derepressed by apo-Fur under iron-restricted conditions, the bacteria were incubated under normal cultivation conditions. The total RNA of the bacteria was isolated using the SV total RNA isolation system (Promega). The reverse transcription was performed using the PrimeScript RT reagent kit (TaKaRa, Ohtsu, Japan). The quantitative RT-PCR was performed using the SYBR Premix Ex Taq Perfect Real-Time Kit (TaKaRa) in a Dice thermal cycler real-time system (TaKaRa). The primer sequences used were as follows: *fecA1* mRNA, forward 5′-GGTGGAAGCTTCAGGGGTG and reverse 5′-GCTTCTTCAATGCTCT-GATTGG; *fecA2* mRNA, forward 5′-AGCTCTCGCACGGTGATTTCCAAC and reverse 5′-CTATGCCCGTTACCGCCCC; *pfr* mRNA, forward 5′-TTGATCATGCGGCTGAAGAATACG and reverse 5′-TGATGTGTTGCT-CATGTTCATAGGC. The 16S rRNA gene mRNA primers used as the internal control for the quantitative RT-PCR have been described in detail previously [21].

Binding assays by surface plasmon resonance assay (BIAcore2000)

A BIAcore2000 instrument (Biacore AB, Uppsala, Sweden) was used to perform the surface plasmon resonance assay in accordance with the manufacturer's guidelines. First of all, each promoter region

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