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Changes in plasma ghrelin levels, gastric ghrelin production, and body weight after *Helicobacter pylori* cure

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Background. Ghrelin is a body weight-regulating peptide produced and secreted primarily by the gastric mucosa. *Helicobacter pylori* infection impairs gastric ghrelin production, leading to a lower plasma ghrelin concentration. However, the effect of *H. pylori* eradication on plasma ghrelin levels and its relation to body weight change after *H. pylori* cure are still uncertain. We examined the association of plasma ghrelin levels with gastric ghrelin production and body weight change before and after *H. pylori* eradication. ***Methods.*** Plasma ghrelin concentrations, gastric ghrelin expression, and body weight were determined in a total of 134 consecutive individuals before and 12 weeks after successful *H. pylori* eradication. Gastric ghrelin expression was evaluated by determining mRNA expression levels and the number of ghrelin-producing cells in gastric mucosa biopsy specimens by real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry, respectively. ***Results.*** Plasma ghrelin concentration increased in 50 patients and decreased in 84 patients after *H. pylori* eradication. After *H. pylori* cure, however, gastric preproghrelin mRNA expression was increased nearly fourfold ($P < 0.0001$), and the number of ghrelin-positive cells was increased or unchanged. In contrast, plasma ghrelin changes after *H. pylori* cure were inversely correlated with both body weight change ($P < 0.0001$) and initial plasma ghrelin levels ($P < 0.0001$). ***Conclusions.*** Changes in plasma ghrelin concentrations before and after *H. pylori* cure were inversely correlated with body weight change and initial plasma ghrelin levels but not with gastric ghrelin production in Japanese patients.

Key words: plasma ghrelin, gastric ghrelin, body weight, *Helicobacter pylori* cure

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Introduction

Ghrelin is a strong growth hormone-releasing peptide that controls food intake, facilitates fat storage, and regulates short- and long-term body weight.[1–5] The majority of circulating ghrelin is produced in the gastric mucosa.[6] Plasma ghrelin level is regulated by multiple factors, including food intake, body weight, and gastric ghrelin production.[7] In the short term, the plasma ghrelin concentration is increased after fasting and decreased after meals.[8,9] In the long term, the plasma ghrelin concentration is lower in obese and higher in lean subjects than in normal weight subjects.[8,10] Moreover, diet-induced or exercise-induced body weight loss increases the plasma ghrelin concentration,[5,11,12] and weight gain decreases elevated plasma ghrelin concentrations in anorexia nervosa.[13] In addition, attenuation of gastric ghrelin production through gastrectomy leads to a decrease in the plasma ghrelin concentration.[5]

Helicobacter pylori is a major cause of gastritis, peptic ulcer disease, and gastric carcinoma.[14–16] Eradication of *H. pylori* improves gastritis[16,17] and decreases the recurrence rate of peptic ulcer disease.[14,18] Much attention has recently been directed to the relationship between obesity and *H. pylori* infection. Previous studies showed that *H. pylori* infection is inversely related to obesity. For example, Wu et al.[19] reported that the seropositivity of *H. pylori* infection was significantly lower in morbidly obese patients.[19] Furuta et al.[20] have shown body weight gain after *H. pylori* cure. As ghrelin is mainly synthesized and secreted by the gastric mucosa, the inverse effect of *H. pylori* infection on body weight has been attributed to the difference in plasma ghrelin concentrations in patients with and without *H. pylori* infection.[21] This hypothesis states that the increase of gastric ghrelin production after *H. pylori* cure may elevate the plasma ghrelin concentration, resulting in a body weight gain. However, the hypothesis is still controversial.[22–24] We thus attempted to examine the effect of *H. pylori* eradi-

Table 1. Clinical characteristics before eradication therapy in patients with decreased or increased plasma ghrelin levels after treatment

Variable	Total (n = 134)	Change in plasma ghrelin levels after treatment		
		Decrease (n = 84)	Increase (n = 50)	P value*
Age	48.8 ± 0.6	48.3 ± 0.7	49.7 ± 0.8	0.25
Body mass index	22.5 ± 0.3	22.2 ± 0.3	23.0 ± 0.4	0.18
Initial plasma ghrelin (fmol/ml)	127 ± 7	149 ± 8	89 ± 8	<0.0001
Pepsinogen I (ng/ml)	73 ± 3	76 ± 4	68 ± 4	0.23
Pepsinogen I/II ratio	2.69 ± 0.10	2.69 ± 0.12	2.69 ± 0.16	0.86
Gastric preproghrelin mRNA	95 ± 1.5	12.8 ± 2.8	7.4 ± 1.9	0.17
Total cholesterol (mg/dl)	196 ± 3	197 ± 4	193 ± 4	0.46
HDL-cholesterol (mg/dl)	59.0 ± 1.5	60.0 ± 1.6	58.3 ± 1.9	0.51
Triglycerides (mg/dl)	99 ± 5	98 ± 6	101 ± 6	0.79
Fasting blood sugar (mg/dl)	95 ± 1	95 ± 1	95 ± 2	0.97

Data are means ± standard error
HDL, high-density lipoprotein
*Difference in clinical data before treatment between subjects with decreased and those with increased plasma ghrelin after treatment

cation on plasma ghrelin concentrations in a large number of patients to elucidate their relationship with body weight changes after *H. pylori* cure. To this end, we compared plasma ghrelin levels before and after *H. pylori* eradication. Furthermore, we examined the correlation between changes in plasma ghrelin with those in gastric ghrelin production and body weight after *H. pylori* cure. We report here that plasma ghrelin concentrations by *H. pylori* eradication were inversely correlated with body weight change and initial ghrelin levels but not with gastric ghrelin production in Japanese patients.

Methods

Participants

Subjects were selected from 144 consecutive *H. pylori*-infected men with normal body mass index (BMI)(calculated as weight in kilograms divided by the square of height in meters) identified in the gastric cancer surveillance program from June 2001 to March 2003 at Tochigi, Japan. Subjects underwent endoscopic biopsy at enrollment and 12 weeks after *H. pylori* treatment. Five adjacent biopsy specimens from the greater curvatures at the midcorpus of the stomach as well as five from the antrum were obtained endoscopically from all subjects. One biopsy specimen from the corpus of the stomach and one from the antrum were cultured individually to evaluate for the presence of *H. pylori* infection. Three biopsy specimens from the corpus and three from the antrum were immediately snap frozen and stored in liquid nitrogen for later use. The remaining corpus and antral specimens were fixed and stained with hematoxylin and eosin, Giemsa, and anti-ghrelin antibody. Histological assessments were performed by a single observer (H. Osawa.). *Helicobacter pylori* infection was evaluated by bacterial culture and histological examination.

All subjects received eradication therapy, and the eradication was successful in 134 subjects (mean age ± SE; 49.2 ± 0.5 years). Indications for *H. pylori* eradication included chronic gastritis accompanied by either adenoma, a family history of gastric cancer, hyperplastic polyps, severe atrophic gastritis (62 patients), gastric ulcer (47 patients), duodenal ulcer (23 patients), or enlarged fold gastritis (2 patients). Patient characteristics are shown in Table 1. All subjects were clinically stable at the time of evaluation and had no history of eradication therapy before the study. No subjects had evidence of a cachectic state such as advanced cancer, thyroid disease, liver disease, or infection. Subjects with diabetes mellitus or renal dysfunction (serum creatinine ≥1.5 mg/dl) were excluded. Written informed consent was obtained from the participants in accordance with the Declaration of Helsinki and its later revision. The Ethics Committee of Jichi Medical University, Japan, approved this study.

Eradication therapy and data collection

A triple regimen, composed of lansoprazole 30 mg twice daily, clarithromycin 200 mg twice daily, and amoxicillin 750 mg twice daily, was given for 7 days after the endoscopic examination. Body weight was measured at 4 p.m., and blood was collected at 8 a.m., after an overnight fast, before and 12 weeks after the treatment. There were no educational schedules provided for reducing body weight during the course of study.

Hormone assay and immunohistochemistry

Plasma ghrelin was measured using a radioimmunoassay for total ghrelin developed in our laboratory. Inter-

and intra-assay variation was less than 8% and 6%, respectively. The limit of detection of this assay is 12 fmol/tube of human ghrelin. We have described previously the properties of the antiserum for ghrelin used in this study.[6–8] Plasma ghrelin levels were 194 ± 15 fmol/ml (mean ± standard error) in healthy *H. pylori*-negative subjects.[7]

Immunohistochemical analysis was performed using anti-ghrelin antiserum as described previously.[6–8] Briefly, paraffin-embedded sections of the biopsy samples taken from the greater curvature at the midcorpus of the stomach were deparaffinized in xylene, immersed in citrate buffer, heated at 120°C for 20 min in an autoclave, and left at room temperature for 60 min. After incubation with a blocking reagent (Dako Japan, Kyoto, Japan) for 10 min, individual sections were incubated with ghrelin antiserum (1 : 500) in a moist chamber at 4°C overnight. The slides were then washed five times with phosphate-buffered saline and incubated with dextran polymer system/peroxidase (EnVision+; Dako Japan) at 37°C for 60 min. Slides were viewed at 100× magnification and digitized with a digital HD microscope (VH 7000; Keyence, Tokyo, Japan). Immunoreactive cells in the gastric mucosa were counted and calculated as the number of positive cells per branch of the oxyntic gland.

Real-time quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the biopsy specimen with ISOGEN (Nippon Gene, Tokyo, Japan). Two micrograms of total RNA from each sample was reverse-transcribed by using random nanomers and reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

The level of gastric preproghrelin mRNA was measured by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) on an ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, USA) as reported previously.[7] Briefly, the reaction contained preproghrelin sense (5′-GGCA GGCTCCAGCTTCCT-3′), and antisense (5′-TGGC TTCTTCGACTCCTTTCTC-3′) primers and preproghrelin probe labeled with a 6-carboxyfluorescein (5′-AGCCCTGAACACCAGAGA-3′). The thermal cycling conditions comprised 50°C for 2 min and 95°C for 10 min, followed by 15 s of denaturing at 95°C and 1 min of annealing/extension at 60°C for 40 cycles. The levels of preproghrelin mRNA were calculated as the ratio of preproghrelin mRNA/GAPDH mRNA and are shown as the mean ratio (×1000) of three corpus samples. As gastric ghrelin is produced predominantly in the corpus mucosa, preproghrelin mRNA levels in the gastric corpus mucosa were compared in subjects before and 12 weeks after *H. pylori* cure.

Statistical analysis

Statistical analyses were performed using Stat View, version 5.0 (SAS Institute, Cary, NC, USA). The level of preproghrelin mRNA was expressed as the median (first quartile to third quartile). The number of immunoreactive cells and clinical data are presented as means ± standard error. The Wilcoxon rank sum test was used to compare gastric preproghrelin mRNA levels before and after *H. pylori* cure. A two-tailed paired *t* test was used to compare the plasma ghrelin levels before and after *H. pylori* cure. A two-tailed unpaired *t* test was used to compare clinical data before eradication therapy between two groups classified according to the direction of change in the plasma ghrelin level after *H. pylori* cure. A *P* value of less than 0.05 was accepted as statistically significant.

Results

Changes in plasma ghrelin after H. pylori *cure*

To examine the effect of *H. pylori* eradication on plasma ghrelin concentration, we first compared plasma ghrelin concentrations before and 12 weeks after treatment. Interestingly, mean plasma ghrelin concentrations decreased from 120 ± 6.3 fmol/ml before *H. pylori* eradication to 103 ± 5.3 fmol/ml after *H. pylori* eradication ($P < 0.0001$). However, the direction of change in levels after treatment differed among enrolled patients: levels increased in 50 patients and decreased in 84 patients (Fig. 1A). To elucidate the potential mechanisms leading to these disparate changes in plasma ghrelin levels after *H. pylori* eradication, we analyzed the relationship between the initial plasma ghrelin levels and their changes at 12 weeks after *H. pylori* cure (Fig. 1B). Interestingly, elevated initial plasma ghrelin concentrations decreased after the cure, but lower initial plasma ghrelin concentrations did not change significantly. Moreover, the change in the plasma ghrelin concentration after 12 weeks was inversely correlated with the initial plasma ghrelin level ($r = -0.52$, $P < 0.0001$).

Gastric ghrelin increases after H. pylori *cure*

We next examined the effect of *H. pylori* eradication on ghrelin production by the gastric mucosa. Since circulating ghrelin is produced and secreted mainly by the gastric mucosa, we analyzed the relation between the changes in the plasma ghrelin concentration and gastric

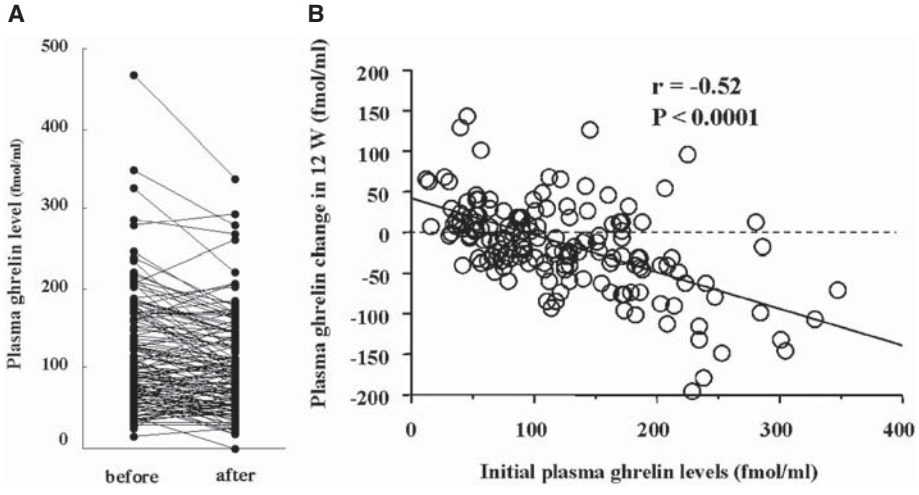

Fig. 1. A Comparison of plasma ghrelin concentrations before and 12 weeks after treatment. Plasma ghrelin levels increased in 50 patients and decreased in 84 patients. The averages before and after *Helicobacter pylori* eradication were 120 ± 6.3 and 103 ± 5.3 fmol/ml, respectively ($P < 0.0001$). **B** The relationship between the initial plasma ghrelin level and the change in plasma ghrelin at 12 weeks after *H. pylori* cure, calculated by subtracting the levels before the treatment from the levels at 12 weeks after treatment. The change at 12 weeks correlated inversely with the initial plasma ghrelin level ($r = -0.52$, $P < 0.0001$)

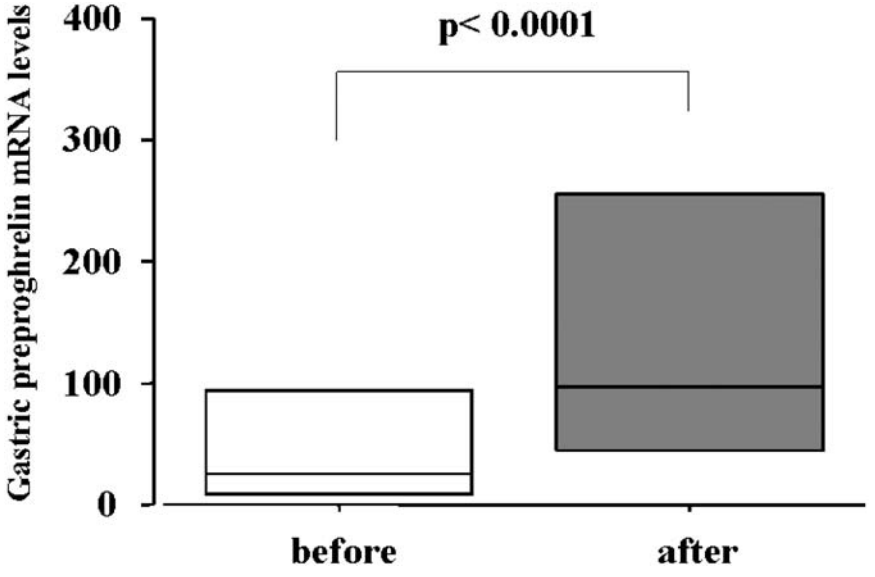

Fig. 2. Comparison of gastric preproghrelin mRNA expression levels before and 12 weeks after *H. pylori* cure. Gastric preproghrelin mRNA levels significantly increased after the eradication therapy [median (first quartile to third quartile); from 27 (8–94) to 98 (46–256); $P < 0.0001$ by Wilcoxon rank sum test]

ghrelin production after *H. pylori* eradication. We compared gastric preproghrelin mRNA expression levels in the corpus mucosa before and 12 weeks after treatment. As shown in Fig. 2, median preproghrelin mRNA expression was increased nearly fourfold ($P < 0.0001$) after *H. pylori* eradication. In addition, the number of ghrelin-positive cells was increased in 77 patients and was unchanged in 57 patients. No correlation was observed between the changes in plasma ghrelin and those in gastric preproghrelin mRNA or the number of ghrelin-positive cells after *H. pylori* cure. These data indicate that gastric ghrelin production is enhanced after *H. pylori* eradication even in patients with decreased plasma ghrelin concentrations.

Body weight changes correlate inversely with changes in the plasma ghrelin concentration

Body weight gain is a well-known effect of *H. pylori* eradication, and the plasma ghrelin concentration is influenced by body weight change.[5,12] Therefore, we examined the relationship between the changes in plasma ghrelin concentrations and body weight after *H. pylori* eradication. The change in plasma ghrelin was clearly inversely correlated with body weight change after *H. pylori* cure ($r = -0.50$, $P < 0.0001$) (Fig. 3). Plasma ghrelin decreased in 23 of 28 patients (82%) with more than 2 kg of weight gain, and in all 7 patients with more than 3 kg of weight gain. These data suggest that the plasma ghrelin concentration after *H. pylori* cure is more strongly influenced by body weight change than by the increase in gastric preproghrelin mRNA or the number of ghrelin-producing cells.

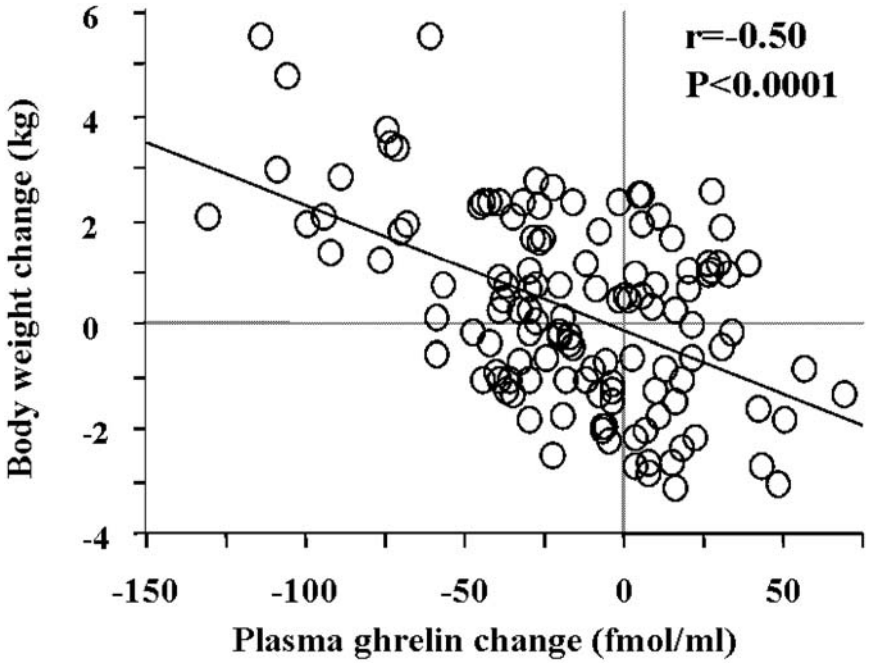

Fig. 3. The relationship between plasma ghrelin and body weight changes at 12 weeks after *H. pylori* cure. The change in plasma ghrelin levels correlated inversely with body weight changes after *H. pylori* cure ($r = -0.50$, $P < 0.0001$). Plasma ghrelin levels decreased in 82% of patients with more than 2 kg of weight gain, and in all patients with more than 3 kg of weight gain

Body weight changes correlate positively with initial plasma ghrelin concentrations

To clarify the differences in clinical characteristics among subjects in relation to changes in the plasma ghrelin level after *H. pylori* eradication, we classified the patients into two groups: patients with increased plasma ghrelin and those with decreased plasma ghrelin after *H. pylori* cure. The clinical characteristics before treatment in subjects of both groups are shown in Table 1. Initial plasma ghrelin levels were significantly higher in those whose plasma ghrelin decreased after treatment, although other clinical data showed no significant differences between the two groups. In addition, these subjects had a significantly greater increase in body weight than those with increased plasma ghrelin after treatment (0.7 ± 0.2 vs -0.3 ± 0.2 kg, $P = 0.003$), despite the short period after treatment. The initial plasma ghrelin levels and body weight changes after treatment were positively correlated ($r = 0.42$, $P < 0.0001$) (Fig. 4). In particular, 12 of 14 patients (86%) with initial ghrelin levels of more than 200 fmol/ml increased in body weight, suggesting that high levels of initial plasma ghrelin may be a predictive factor of body weight gain after *H. pylori* eradication.

Discussion

In the current study, we showed that despite an increase in gastric preproghrelin mRNA and ghrelin-producing cells after *H. pylori* eradication, the mean plasma ghrelin concentration decreased after treatment, but with wide variation. Moreover, we demonstrated that changes in plasma ghrelin concentrations after *H. pylori* eradication were inversely correlated with weight changes as well as with initial plasma ghrelin levels.

The majority of circulating ghrelin is synthesized in the gastric mucosa.[6] Gastric ghrelin production is decreased by *H. pylori* infection[7] and increased by eradication therapy.[25] As ghrelin is a body weight-regulating peptide, much attention has been paid to nutritional status and the dynamics of gastric and plasma ghrelin in response to *H. pylori* infection.[19,21] Nwokolo et al.[26] reported that plasma ghrelin levels increased after *H. pylori* cure in ten patients, a result that is inconsistent with our data. Since their report, it has been believed that plasma ghrelin concentrations increase after *H. pylori* cure owing to an increase in gastric ghrelin production, leading to body weight gain.[21,25] For example, Tatsuguchi et al.[25] reported that the number of gastric ghrelin-positive cells increased after *H. pylori* eradication, consistent with our present data. Although they did not measure plasma ghrelin concentrations or body weight after *H. pylori* eradication, they speculated, in accordance with the report of Nwokolo et al.,[26] that the increase in gastric ghrelin-positive cells would lead to increased plasma ghrelin levels, resulting in obesity. Another study, however, found that plasma ghrelin levels were unaffected.[27] In fact, the plasma ghrelin concentration is not regulated simply by the amount of gastric ghrelin production. Even in healthy humans, the plasma ghrelin concentration is tightly correlated with body weight.[8,10] Therefore, Peeters proposed in his re-

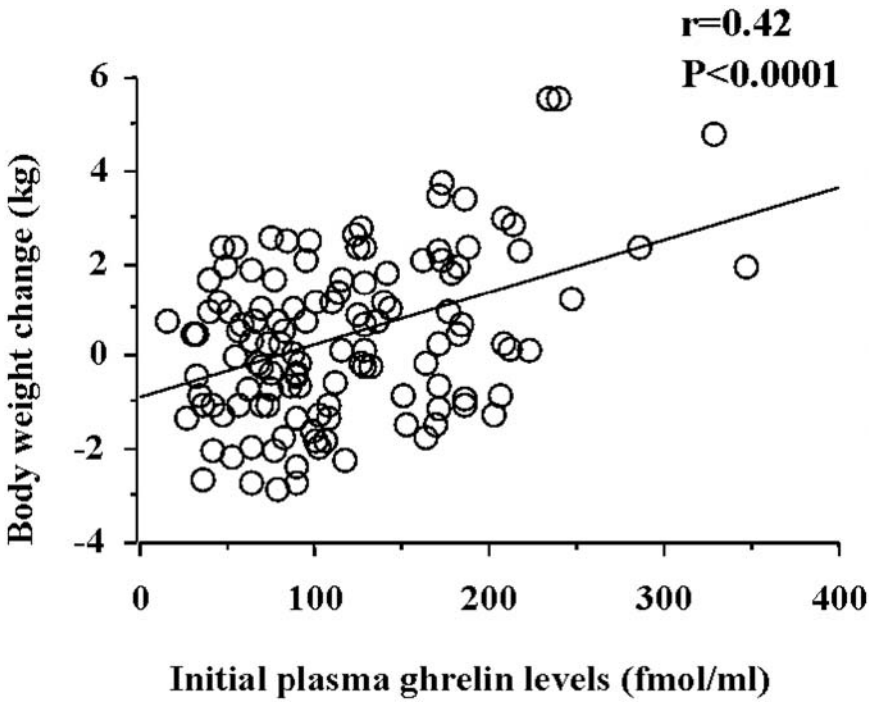

Fig. 4. The relationship between the initial plasma ghrelin level and body weight change. Initial plasma ghrelin levels correlated positively with body weight changes at 12 weeks after *H. pylori* cure ($r = 0.42$, $P < 0.0001$). Body weight increased in 86% of patients with initial plasma ghrelin levels of more than 200 fmol/ml

cent review[24] that the questions as to whether there is a rise in ghrelin following *H. pylori* eradication and whether such a rise can be an important determinant of body weight increase be reexamined. He also suggested that only a subpopulation of infected patients might show a rise in ghrelin following eradication. Thus, to clarify how plasma ghrelin concentrations changed after *H. pylori* cure and to elucidate how those changes affected body weight changes, we conducted the present study, in which we examined changes of plasma ghrelin concentration, gastric ghrelin production, and body weight before and after *H. pylori* eradication.

We clearly showed a significant inverse correlation between changes in plasma ghrelin and body weight, and found that these changes were not related to changes in gastric ghrelin production. Plasma ghrelin decreased in many patients with weight gain, in particular in all those who gained more than 3 kg of weight after treatment. Although the apparent discrepancy between the observations of Nwokolo's group and our group is difficult to explain, one might speculate that the discrepancy is related to differences in the study designs. The number of enrolled patients in our study was more than ten times the number in their study. In addition, racial differences in the enrolled subjects may account for the discrepancy. In this respect, Asians including Japanese are more prone to central adiposity than are Caucasians.[28–32] As body fat storage is closely associated with plasma ghrelin levels,[4] the racial difference in body fat distribution may account for the discrepancy.

It is important to note that, in our study, initial plasma ghrelin levels were negatively correlated with the change in plasma ghrelin levels and positively with the weight change after *H. pylori* eradication. Weight gain is a major effect of *H. pylori* eradication.[20,33,34] Those patients with high initial levels of plasma ghrelin gained in weight after *H. pylori* eradication. Thus, initial ghrelin levels can be a predictive factor of weight gain induced by *H. pylori* eradication. Previous studies have addressed the question as to whether ghrelin is involved in weight gain after *H. pylori* cure.[25,26] Although the correlation between initial plasma ghrelin levels and weight change suggests that ghrelin participates in the weight gain after *H. pylori* eradication, our present data do not definitely resolve this question. However, we suggest that the weight gain after *H. pylori* eradication does not result simply from an increase in plasma ghrelin by the recovery of gastric ghrelin production.

It is intriguing that plasma ghrelin concentrations decreased in many patients with weight gain after *H. pylori* cure in spite of the enhancement of gastric ghrelin production. We measured total plasma ghrelin, including octanoyl ghrelin and des-octanoyl ghrelin, using a radioimmunoassay.[6,7] Recently, the physiological roles of the two isoforms of ghrelin have been discussed. The discrepancy between gastric ghrelin transcription and plasma ghrelin concentration may be explained by ghrelin isoforms. Thompson et al.[35] reported that ghrelin and des-octanoyl ghrelin are present in plasma and affect growth hormone secretagogue receptors differently. Furthermore, des-octanoyl ghrelin, a major

circulating form of ghrelin, has adipogenic activity but does not stimulate growth hormone production, whereas octanoyl ghrelin does stimulate growth hormone production. Thus, it is reasonable to suggest that increased expression of preproghrelin mRNA in the stomach does not directly reflect the total plasma ghrelin level. However, the precise mechanism and regulation of gastric ghrelin secretion are yet to be elucidated. Further study on the ghrelin secretory machinery of gastric mucosal cells is warranted.

In conclusion, we have shown that changes in plasma ghrelin concentrations after *H. pylori* cure are inversely correlated with weight changes and initial ghrelin levels but not with gastric ghrelin production in Japanese patients. These observations provide novel insights for understanding ghrelin and its functions as it relates to various diseases.

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