Superiority of pulmonary administration of mepenzolate bromide over other routes as treatment for chronic obstructive pulmonary disease.	Tanaka, K., Kurotsu, S., Asano, T., Yamakawa, N., Kobayashi, D., Yamashita, Y., Yamazaki, H., Ishihara, T., Watanabe, H., Maruyama, T., Suzuki, H., Mizushima, T.	Sci. Rep. 4:4510, 2014	2014年3月	国外
Mechanisms of Helicobacter pylori antibiotic resistance and molecular testing.	Nishizawa, T., Suzuki, H.	Front. Mol. Biosci. 1:19. doi: 10.3389/fmolb.2014.00019.	2014年10月	国外
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様式第１９

学　会　等　発　表　実　績

委託業務題目「胃薬テプレノンのアルツハイマー病治療薬としての開発」
機関名　公益社団法人鹿児島共済会南風病院

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Imaging discrepancies between magnetic resonance imaging and brain perfusion single-photon emission computed tomography in the diagnosis of Alzheimer's disease, and verification with amyloid positron emission tomography.	Yokoyama S, KajiyaY, Yoshinaga T, Tani A, Hirano H	Psychogeriatrics	2014	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
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様式第１９

学　会　等　発　表　実　績

委託業務題目「テプレノン服用が認知症発症に与える影響：久山町研究」
機関名：九州大学大学院医学研究院環境医学分野

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発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
日本人の糖尿病合併症～久山町研究から考える～．口頭	清原　裕	大阪市（第57回日本糖尿病学会年次学術集会）	2014.5	国内
血管性認知症の疫学：久山町研究．口頭	小原知之，清原裕	東京都（第29回日本老年精神医学会）	2014.6	国内
心血管病と認知症の疫学：久山町研究．口頭	秦　淳，清原　裕	福岡市（第56回日本老年医学会）	2014.6	国内
地域住民における中年期および老年期の喫煙と認知症発症との関連：久山町研究．口頭	小原知之，秦淳，吉田大悟，福原正代，永田雅治，岸本裕歩，北園孝成，神庭重信，清原　裕	福岡市（第56回日本老年医学会）	2014.6	国内
生活習慣病と認知症：久山町研究．口頭	清原　裕	福岡市（第55回日本人間ドック学会学術大会）	2014.9	国内
Dietary patterns and risk of dementia in an elderly Japanese population: the Hisayama Study. 14th Inernational College of Geriatric Psychoneuropharmacology.口頭	Tomoyuki Ohara, Toshiharu Ninomiya, Yutaka Kiyohara	つくば市（14th Inernational College of Geriatric Psychoneuropharmacology）	2014.10	国内
Secular trends in dementia and its risk factors in a Japanese Community: the Hisayama Study.口頭	Toshiharu Ninomiya, Yutaka Kiyohara	東京都（Global Dementia Legacy Event Japan）	2014.11	国内
変貌する日本人の生活習慣病の現状と課題：久山町研究．口頭	清原　裕	福岡市（第61回日本臨床検査医学会学術集会）	2014.11	国内

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Milk and dairy consumption and risk of dementia in an elderly Japanese population: the Hisayama Study.	Ozawa M, Ohara T, Ninomiya T, Hata J, Yoshida D, Mukai N, Nagata M, Uchida K, Shirota T, Kitazono T, Kiyohara Y	J Am Geriatr Soc	2014	国外
Altered expression of diabetes-related genes in Alzheimer's disease brains: the Hisayama Study.	Hokama M, Oka S, Leon J, Ninomiya T, Honda H, Sasaki K, Iwaki T, Ohara T, Sasaki T, Laferla FM, Kiyohara Y, Nakabeppu Y	Cereb Cortex	2014	国外

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ORIGINAL ARTICLE

Mucosal expression of aquaporin-4 in the stomach of histamine type 2 receptor knockout mice and *Helicobacter pylori*-infected mice

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Key words
acid suppression, aquaporin-4, SPEM, *Helicobacter pylori*.

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Abstract

Background and Aim: Basolateral water channel, aquaporin-4 (AQP4), is known to be expressed in gastric parietal cells, especially in the basal side of gastric mucosa. However, the role of AQP4 in the stomach is still unknown. Histamine type 2 receptor (H_2R) knockout mice, which are characterized by suppressed gastric acid secretion, are known as formation of mucosal hyperplasia with cystic dilatation and spasmolytic polypeptide-expressing metaplasia (SPEM) in the stomach. The aim of the present study is to investigate whether the expression of AQP4 is changed by the condition of acid suppression and *Helicobacter pylori* infection.

Methods: Male H_2R knockout mice and their controls (C57BL/6) were used. *H. pylori* was orally infected at the age of 5 weeks. The distributions of AQP4 and H^+/K^+-ATPase in the gastric mucosa were investigated by fluorescent immunohistochemistry. The mRNA expressions of *AQP4*, *H^+/K^+- ATPase*, *sonic hedgehog* (*Shh*), and *trefoil factor-2* (*TFF2*) were investigated by quantitative reverse transcription polymerase chain reaction (RT-PCR).

Results: In the H_2R knockout mice, the distribution of AQP4-positive parietal cells was extended toward the surface of the fundic glands. Although the mRNA expression levels of *AQP4* and *H^+/K^+ ATPase* were elevated in H_2R knockout mice at the age of 20 weeks, the elevations were not maintained by aging or *H. pylori* infection. In H_2R knockout mice with *H. pylori* infection, the expression level of *TFF2* mRNA was elevated while the ratio between *AQP4* and *H^+/K^+ ATPase* mRNA expression was decreased compared with the H_2R knockout mice without *H. pylori* infection.

Conclusions: In the H_2R knockout mice, massive SPEM was induced by *H. pylori* colonization and the ratio between AQP4 and *H^+/K^+ ATPase* mRNA expression was decreased.

Introduction

The use of histamine type 2 receptor (H_2R) antagonists and proton-pump inhibitors (PPIs) has become widespread for the treatment of peptic ulcer disease and gastroesophageal reflux disease.[1] Although the influence of long-term acid suppression is controversial in the stomach, some reports indicated that usage of PPIs has known to be associated with the formation of gastric sporadic fundic gland polyps[2,3] and hyperplastic polyps.[4] In addition, there are reports indicating that long-term usage of H_2R antagonists or PPI may facilitate the formation of gastric malignant lesions such as gastric carcinoid tumors and cancers.[5,6]

The administration of PPIs strongly suppresses acid secretion by inhibition of H^+/K^+-ATPase in the gastric parietal cells. Gastric acid secretion is known to be potently stimulated by histamine, acetylcholine, and gastrin. Histamine, which is secreted from enterochromaffin-like cells, acts via the H_2R on the parietal cells to stimulate gastric secretion. Furthermore, histamine enhances the differentiation of gastric mucosal lineages through the secretion of paracrine and autocrine regulators including sonic hedgehog (Shh), transforming growth factor-α, and heparin binding-epidermal growth factor-like growth factor.[7–10] Especially, Shh is an important morphogen to guide gastrointestinal epithelium into specific lineages for differentiation from progenitor cells. Previous reports showed that the gastric mucosa of Shh null mice exhibits intestinal-type differentiation.[11] Furthermore, decreased expression of Shh has been reported to be associated with carcinogenesis in the stomach.[12–14] *H. pylori* infection, one of the major causes of gastric cancer, is known to decrease the expression of Shh[15] and then spasmolytic polypeptide-expressing metaplasia (SPEM) is induced. SPEM is characterized by loss of parietal cells, oxyntic atrophy, and then replacement of trefoil factor family 2 (TFF2)

immunoreactive cells which is morphologically similar to deep antral gland cells or Brunner's gland cells. Previously, SPEM has been reported to associate with the development of gastric cancer following chronic inflammation caused by *H. pylori*.[16,17] Moreover, to explore the physiological functions of the H_2R, a line of H_2R knockout mice was previously generated, and it was characterized by a higher gastric pH, hypergastrinemia, and formed hyperplasia of gastric mucosa which TFF2 is highly expressed like SPEM.[18]

Aquaporins are a family of small integral plasma membrane proteins that primarily transport water across the plasma membrane.[19–21] Aquaporin-4 (AQP4) is expressed predominantly on the basolateral membrane of the parietal cells. In AQP4 knockout mice, a tendency toward decrease in fluid secretion in the fundic glands has been observed.[22] However, the function of AQP4 in the stomach still remains unknown. Recently, the expression of AQP4 was reported to be remarkably decreased in the gastric cancer tissue.[23] These findings suggest that the functions of parietal cells may differ depending on their vertical localization in the gastric fundic glands, and AQP4 could be useful markers to investigate impaired parietal cell differentiation and the formation of precarcinogenic lesion. We previously reported that the administration of PPI affected the expression of AQP4.[24] However, the dynamics of AQP4 in H_2R knockout mice has not been fully examined.

In the present study, we showed the aberrant parietal cell differentiation by focusing the expression of AQP4 in the H_2R knockout mice, associated with *H. pylori* infection.

Methods

Animals. Male H_2R knockout mice, and C57BL/6J mice which are their controls, were purchased from Sankyo Labo (Tokyo, Japan). All mice were used for the study after habituation for 1 week. The mice in the *H. pylori*-infected group were administered with *H. pylori* at a concentration of 10^6 colony-forming units per milliliter by per-oral injection for three times on alternate days at the age of 6 weeks. The mice in the control group were administered vehicle (0.5% carboxymethyl cellulose (CMC) solution) by per-oral injection for three times on alternate days at the age of 6 weeks. The experimental mice were sacrificed at the age of 20 weeks. Furthermore, to evaluate the influence of aging, H_2R knockout mice and their controls were also sacrificed at the age of 40 and 60 weeks. After 24-h food deprivation, although access to water was not restricted, the stomachs were resected. All experiments and procedures in the present study were approved by the Keio University Animal Research Committee (08083-[10]).

Histology and fluorescent immunohistochemistry. The lesser curvature of the stomach tissue specimens from the experimental mice were fixed in 10% neutralized buffered formalin, embedded in paraffin, placed on poly-L-lysine-pretreated slides, and then stained with HE for histological examination. Rabbit polyclonal anti-AQP4 antibody (sc-20812, 1:100, Santa Cruz, CA, USA) and mouse monoclonal anti-H^+/K^+-ATPase α subunit antibody (clone 1H9, 1:50, Fitzgerald, MA, USA) were used for the fluorescent immunohistochemistry. For immunofluorescent staining, after deparaffinization and rehydration, the antigens were retrieved by heating in citrate buffer (10 mM, pH 6.0) for anti-AQP4 antibody (121°C, 3 min). Nonspecific binding was blocked by a blocking reagent (Protein Block, Dako Japan, Tokyo, Japan). Sections were incubated overnight with the primary antibodies at 4°C. After rinsing in Tris-buffered saline with Tween 20 (TBS-T), the slides were incubated with Alexa Flour 488 donkey anti-rabbit IgG (1:1000, Life Technologies, Carlsbad, CA, USA). For double staining, after rinsing in TBS-T, anti-H^+/K^+-ATPase was digested with proteinase K solution (Dako Japan) for 4 min at room temperature. Subsequently, blocking reagent was applied for blockade of nonspecific binding, and then sections were incubated for overnight with anti-H^+/K^+-ATPase α subunit antibody. After rinsing in TBS-T, the slides were incubated with Alexa Fluor 568 goat anti-mouse IgG (1:1000, Molecular Probes).

Table 1 The primer sequence

Primer	Primer sequence 5′-3′
Mouse *AQP4* forward	TTTGGACCCGCAGTTATCA
Mouse *AQP4* reverse	CCACATCAGGACAGAAGACATAC
Mouse *H^+/K^+-ATPase* forward	AGATGTCCTCATCCGCAAGACAC
Mouse *H^+/K^+-ATPase* reverse	CAGCCAATGCAGACCTGGAA
Mouse *Shh* forward	AGCAGGTTTCGACTGGGTCT
Mouse *Shh* reverse	GCCACGGAGTTCTCTGCTT
Mouse *TFF2* forward	CTTGGTGTTTCCACCCACTT
Mouse *TFF2* reverse	GGAAAAGCAGCAGTTTCGAC
Mouse *β-actin* forward	GCCTTCCTTCTTGGGTATGG
Mouse *β-actin* reverse	AGGTCTTTACGGATGTCAACG

RNA purification and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNAs of tissue specimens from each stomach tissue specimens were extracted by using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA). RNA was converted into cDNA using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). The cDNA was used for quantitative PCR analysis with Dice (Takara) using SYBR Premix Ex TaqII (Takara). The mRNA expressions of mouse *AQP4*, *H^+/K^+-ATPase α subunit*, *Shh*, *TFF2*, and *β-actin* were measured. The primer sequences are shown in Table 1. Data for each gene were normalized to the expression level of *β-actin*.

Statistical analysis. All data were expressed as mean ± standard error. Statistical significance of the differences between two groups was evaluated using unpaired Student's *t*-test. All statistical analyses were performed using the SPSS Statistics version 20.0J software for Windows (SPSS Japan, Tokyo, Japan). A two-sided P value of < 0.05 was considered as denoting statistical significance.

Results

Alteration of AQP4 expression through aging in the H_2R knockout mice. To investigate the progression of gastric lesion, HE staining was performed (Fig. 1a,b). The gastric mucosa in the H_2R knockout mouse showed glandular hyperplasia with multiple cystic dilatations with increased mucous cells and parietal cells as well as SPEM. Also, the hyperplasia was more

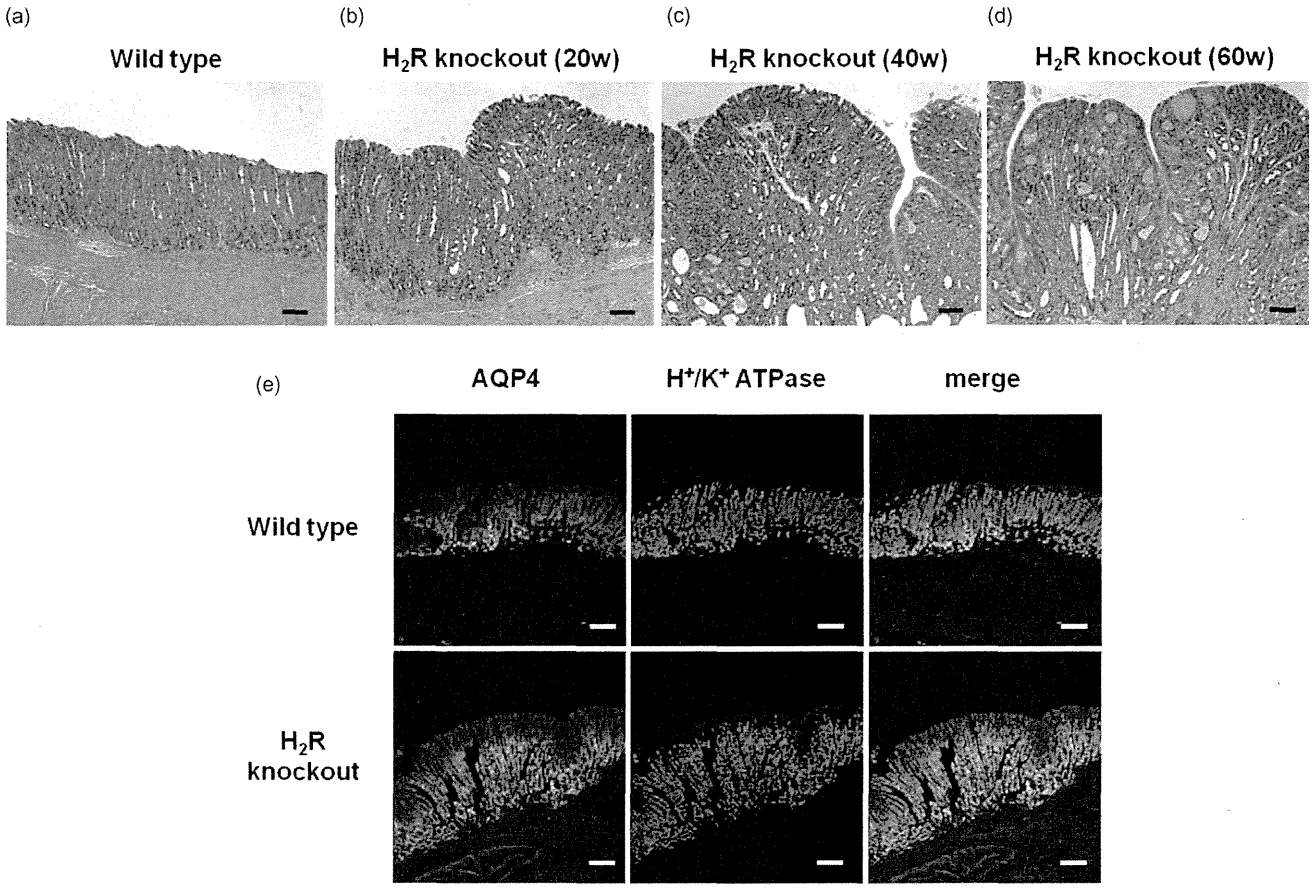

Figure 1 Histological findings of gastric mucosa in histamine type 2 receptor (H_2R) knockout mice.
(a)–(d) The representative HE staining of gastric mucosa. As compared with wild type (a), the gastric mucosal hyperplasia with multiple cystic dilatations was gradually getting more severe in the H_2R knockout mouse (b; 20 weeks, c; 40 weeks, d; 60 weeks). Bar indicates 100 micrometers. (e) At the age of 20 weeks, in wild type, the aquaporin-4 (AQP4)-positive parietal cells (green) was located in basal side of gastric mucosa compared H^+/K^+-ATPase-positive parietal cells (red). On the other hand, the AQP4-positive parietal cells in H_2R knockout mouse were increased and extended to the apical side as compared with those in wild type. Therefore, the area of AQP4- and H^+/K^+-ATPase-positive cells (yellow) was extended in H_2R knockout mouse compared with wild type.

thickened with increase of cystic dilatation at the age of 40 and 60 weeks than at the age of 20 weeks (Fig. 1c,d). However, no gastric carcinoma lesion was observed even at the age of 95 weeks (data not shown). For the purpose of characterization of parietal cells along with vertical localization, fluorescent immunohistochemistry of H^+/K^+-ATPase and AQP4 was performed (Fig. 1e). In the wild type at the age of 20 weeks, H^+/K^+-ATPase-positive parietal cells were diffusely observed in the gastric mucosa, whereas AQP4-expressing parietal cells were mainly localized in basal area of the mucosa. In the H_2R knockout mouse, however, AQP4-positive parietal cells were more broadly observed from the basal area to the apical side of the mucosa.

Along with the histological findings, the mRNA expression level of *AQP4* in the stomach of the H_2R knockout mouse was significantly higher than that of wild type regardless of the aging period (Fig. 2a). However, the mRNA expression level of *AQP4* in 60 weeks old was significantly lower than those in 20 weeks old. The mRNA expression level of *H^+/K^+-ATPase* in the stomach of the H_2R knockout mouse was higher than that of wild type at the age of 20 weeks and 40 weeks (Fig. 2b). However, it was gradually decreased through the aging in the H_2R knockout mouse. In addition, the ratio of the mRNA expression between *AQP4* and *H^+/K^+-ATPase* were higher in the H_2R knockout mouse regardless of the aging period compared with wild type (Fig. 2c).

To summarize these data, the mucosal hyperplasia was induced in the H_2R knockout mouse and was aggravated by aging along with the decrease of the mRNA expression of *H^+/K^+-ATPase*. The mRNA expression of AQP4 was significantly higher in the H_2R knockout mouse but was decreased by aging. The higher ratio of mRNA expression between *AQP4* and *H^+/K^+-ATPase* was kept in the H_2R knockout mouse, suggesting that the decrease of *AQP4* mRNA levels by aging was caused by reduced viability of gastric parietal cells.

Alteration of AQP4 expression in the H_2R knockout mice with* H. pylori *infection. Subsequently, the influence on *H. pylori* infection for the gastric mucosal status of

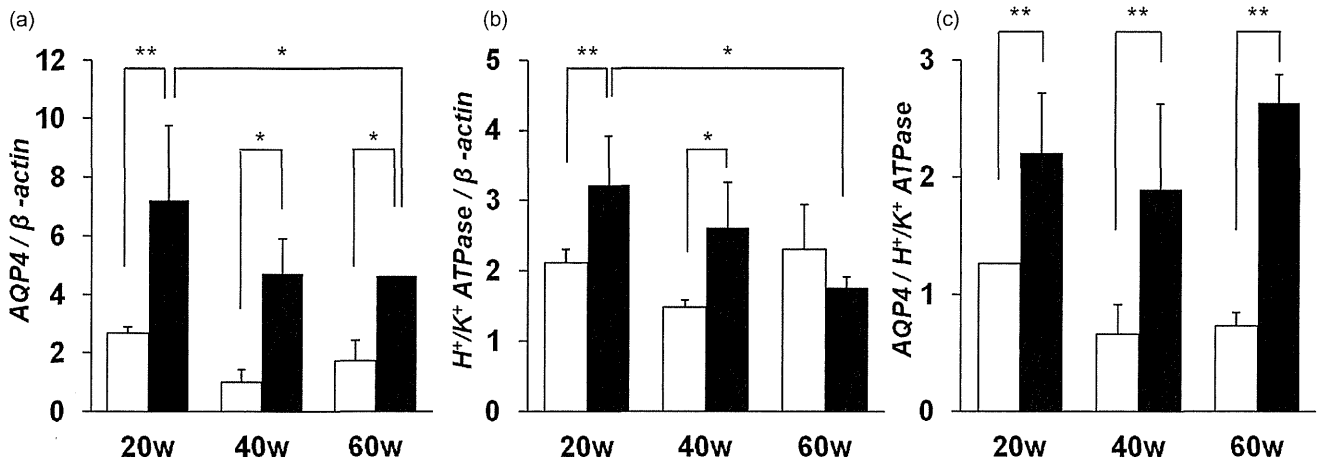

Figure 2 The aquaporin-4 (*AQP4*) and *H⁺/K⁺- ATPase* mRNA expression in histamine type 2 receptor (H_2R) knockout mouse.
(a) The *AQP4* mRNA expression was significantly increased in H_2R knockout mice (black bar) compared with that in wild type (white bar). The increased *AQP4* mRNA expression in H_2R knockout mice was gradually decreased depend on aging. (b) The *H⁺/K⁺* ATPase mRNA expression was also significantly increased in H_2R knockout mice compared with that in wild type. It was significantly decreased along aging as same as *AQP4* mRNA expression. (c) In 20, 40, and 60 weeks, the ratio between *AQP4* and *H⁺/K⁺ ATPase* mRNA expression in H_2R knockout mice was significantly increased compared with wild type (*$P < 0.05$, **$P < 0.01$). □, Wild type; ■, H_2R knockout.

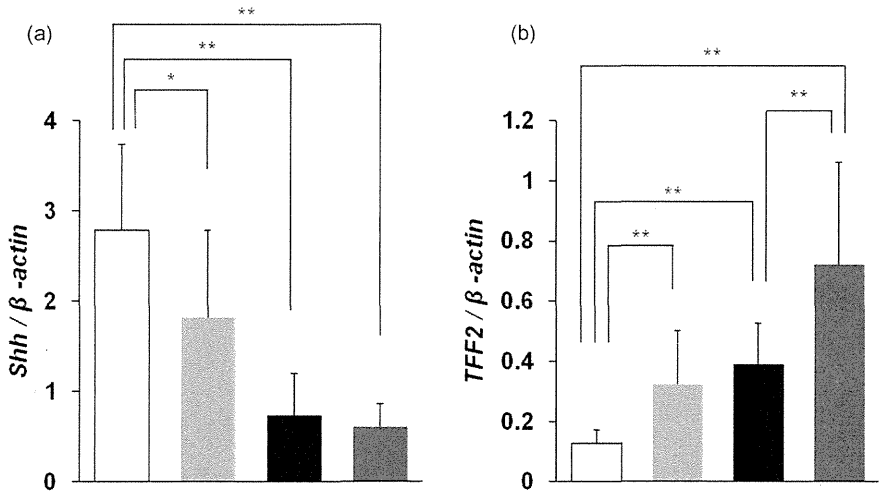

Figure 3 The *Shh* and *TFF2* mRNA expression in histamine type 2 receptor (H_2R) knockout mouse.
(a) Compared with wild type (white bar), the *Shh* mRNA expression was significantly decreased in H_2R knockout mice (black bar) and *H. pylori* infection (light gray bar). (b) The *TFF2* mRNA expression in the H_2R knockout mouse was significantly increased compared with that of wild type. Furthermore, the infection of *H. pylori* in the H_2R knockout mouse (dark gray bar) significantly decreased the mRNA expression level of *TFF2* compared with that of H_2R knockout mouse without *H. pylori* infection (*$P < 0.05$, **$P < 0.01$). □, Wild type; ▒, Wild type + *H. pylori*; ■, H_2R knockout; ▒, H_2R knockout + *H. pylori*.

SPEM was assessed in wild type or the H_2R knockout mice. In the wild type mice, the mRNA expression level of *Shh*, which is a morphogen for differentiation of gastric mucosal cells, in the stomach was significantly decreased by *H. pylori* infection (Fig. 3a). In the H_2R knockout mouse, the mRNA expression level of *Shh* was lower than that of wild type. The mRNA expression level of *Shh* was the lowest in the H_2R knockout mouse with *H. pylori* infection. The mRNA level of *TFF2*, which is an indicator of SPEM, was significantly higher in the H_2R knockout mouse compared with that of wild type (Fig. 3b). Furthermore, it was increased by *H. pylori* infection in the wild type and in the H_2R knockout mouse. These data suggest that SPEM in the H_2R knockout mouse with *H. pylori* infection would be the most severe among these mice.

The fluorescent immunochemistry of AQP4 and H^+/K^+-ATPase was performed using these mice. In the wild type mice, the infection of *H. pylori* decreased the expression of AQP4 in the stomach (Fig. 4). Similarly, in the H_2R knockout mouse, the infection of *H. pylori* suppressed the expression of AQP4 as compared with that without the infection of *H. pylori*, while mucosal hyperplasia with multiple cystic dilatations was observed regardless of the infection of *H. pylori*. The mRNA expression of *AQP4* was significantly decreased by infection of *H. pylori* in the wild type as well as in the H_2R knockout mouse (Fig. 5a). The mRNA expression level of *H⁺/K⁺-ATPase* was also decreased by infection of *H. pylori* in wild type and in the H_2R knockout mouse (Fig. 5b). Interestingly, the ratio between AQP4 and H^+/K^+-ATPase was significantly decreased by *H. pylori* infection in the H_2R knockout mouse, but not in the wild type (Fig. 5c). Since the mRNA expression levels of TFF2 was significantly higher in the *H. pylori*-infected H_2R knockout mouse compared with H_2R knockout

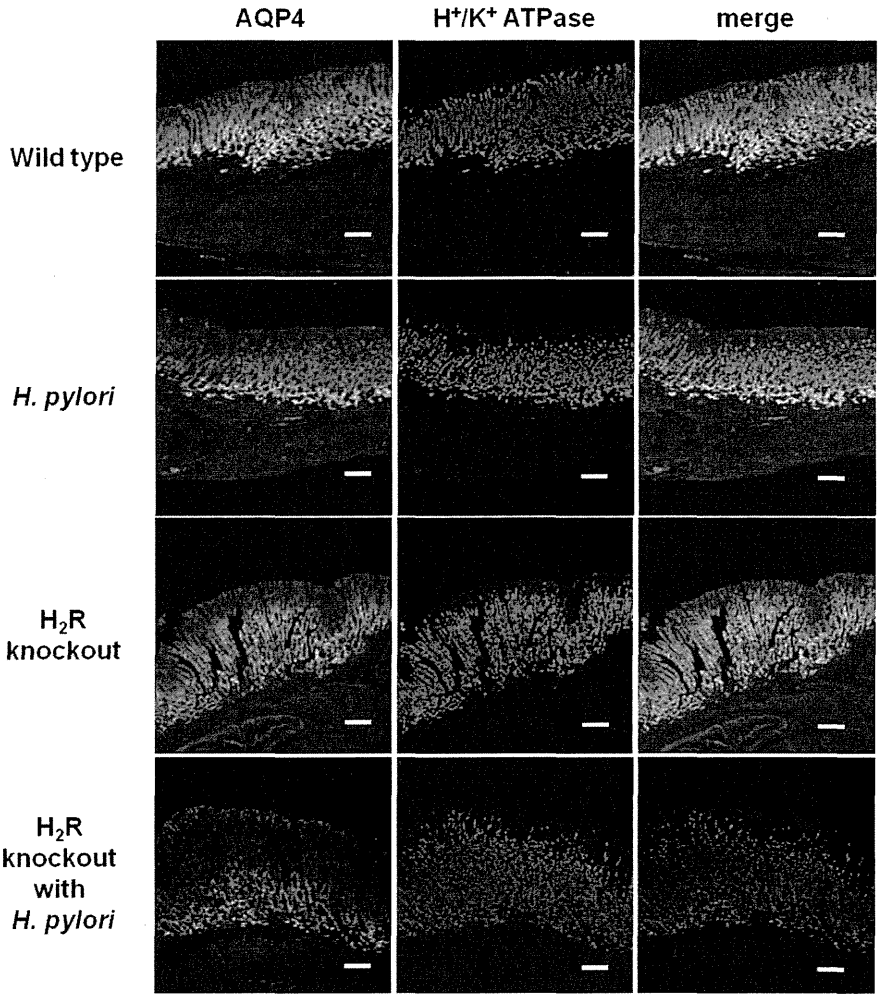

Figure 4 Immunohistochemistry of parietal cell-specific membrane protein.
The aquaporin-4 (AQP4)-positive parietal cells in histamine type 2 receptor (H_2R) knockout mouse were increased and extended to the apical side as compared with those in wild type. The marked extension of the distribution of AQP4-positive parietal cells in H_2R knockout mouse was not observed by *H. pylori* infection.

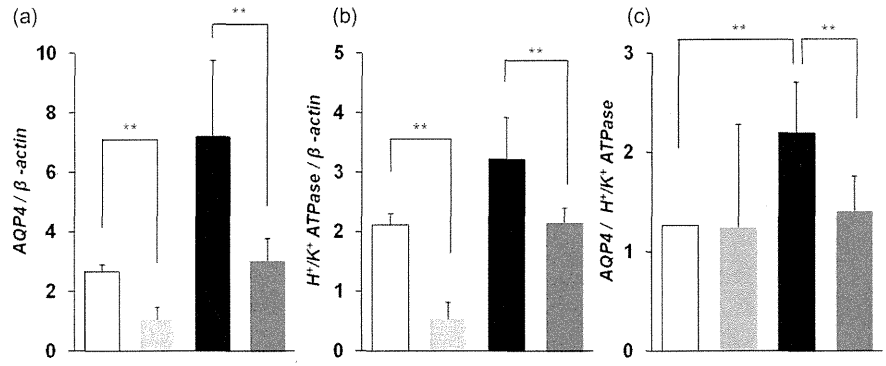

Figure 5 The dynamics of aquaporin-4 (*AQP4*) and *H⁺/K⁺ ATPase* mRNA expression by *H. pylori* infection.
(a) Compared with wild type (white bar), the *AQP4* mRNA expression was significantly decreased by *H. pylori* infection (light grey bar). In addition, the increased expression of the *AQP4* mRNA expression in histamine type 2 receptor (H_2R) knockout mice (black bar) was significantly decreased by infection of *H. pylori* (dark grey bar). (b) The *H⁺/K⁺ ATPase* mRNA expression in H_2R knockout mice was also decreased by infection of *H. pylori* significantly, as same as *AQP4*. (c) The ratio between *AQP4* and *H⁺/K⁺ ATPase* mRNA expression in H_2R knockout mice was significantly increased compared with wild type. However, *H. pylori* infection significantly decreased the ratio compared with H_2R knockout mice without *H. pylori* infection. (*$P < 0.05$, **$P < 0.01$) □, Wild type; ▒, Wild type + *H. pylori*; ■, H_2R knockout; ▒, H_2R knockout + *H. pylori*.

mouse without the infection of *H. pylori*, the decreased ratio between AQP4 and H^+/K^+-ATPase was supposed to be one of the indicators on the process of cancer development from SPEM.

Discussion

In the present study, the distribution of the AQP4-positive parietal cells which is localized in the basal part of the fundic gland in wild type was extended toward the apical side of the mucosa in the H_2R knockout mouse. Furthermore, the mRNA expression level of *AQP4* was significantly higher in the H_2R knockout mouse compared with that of wild type. We previously reported that PPI treatment, which induces acid suppression, encounters mucosal hyperplasia and enhances the expression of AQP4 while the expression of Shh was decreased.[24] Similarly, the expression of Shh and hedgehog signaling reported to depend on gastrin and gastric acidity.[25] Furthermore, the expression of AQP4 was reported to be significantly decreased in gastrin knockout mouse compared with wild type and was restored by the supplementation of gastrin.[7] In both PPI-treated mouse and H_2R knockout mouse, the plasma level of gastrin was known to be elevated through the acid suppression.[26] Thus, it was suggested that acid suppression might disturb the differentiation process of gastric mucosal epithelial cells including parietal cells and the expression of AQP4 followed by the formation of mucosal hyperplasia through the increase of gastrin. However, long-term acid suppression also leads to the development of SPEM through the decrease of parietal cells and the increase of TFF2-positive cells.[27] The decrease of *AQP4* mRNA expression by aging might reflect the loss of viability of whole parietal cells.

Meanwhile, the expression of *AQP4* mRNA was significantly decreased by the infection of *H. pylori* in both of wild type and the H_2R knockout mouse. Although the expression of *H^+/K^+-ATPase* was also decreased by the infection of *H. pylori*, the increase in the ratio between *AQP4* and *H^+/K^+-ATPase* mRNA expression was only observed in the H_2R knockout mouse without *H. pylori* infection. Immunohistochemistry showed almost all of the AQP4-positive parietal cells are co-stained with H^+/K^+-ATPase, suggesting the ratio between AQP4 and H^+/K^+-ATPase mRNA expression indicate the proportion of AQP4-positive parietal cells. Interestingly, previous report revealed that the infection rate of *H. pylori* was significantly higher in patients with anti-AQP4 antibody-positive neuromyelitis optica that is one of the demyelinating diseases of central nerve system.[28] The infection of *H. pylori* is known to produce *H. pylori* neutrophil-activating protein which is one of the major proinflammatory proteins and supposed to be associated with systemic inflammation through the activation of neutrophils. Thus, the infection of *H. pylori* would strongly affect the mRNA expression of *AQP4* rather than *H^+/K^+-ATPase* nevertheless the aberrant differentiation of parietal cells.

The mRNA expression of *Shh* was significantly decreased by the *H. pylori* infection in the wild type. In addition, in the H_2R knockout mouse, the *Shh* expression was further decreased nevertheless the infection of *H. pylori*. Moreover, the mRNA expression of *TFF2* was significantly increased in the H_2R knockout mouse with *H. pylori* infection compared with wild type, wild type with *H. pylori* infection, and H_2R knockout mouse without *H. pylori* infection. We previously reported that the decreased expression level of Shh was observed in the H_2R knockout mouse showing the formation of SPEM.[18] Since abnormal TFF2 expression has been reported in gastric cancer,[29] an increase in TFF2 expression may be a subtle indicator of potential malignancy. We also reported that suppressed Shh expression caused abnormal mucous neck-to-zymogenic cell lineage differentiation in the *H. pylori*-colonized stomach of Mongolian gerbils.[15,30] SPEM is thought to be an early change of gastric metaplasia and then it gradually develops to intestinal metaplasia.[31] The present study demonstrated that SPEM was formed in the H_2R knockout mouse at the age of 20 weeks. However, no malignant lesions such as gastric adenocarcinoma were observed even at the age of 60 weeks, while high ratio between *AQP4* and *H^+/K^+-ATPase* mRNA expression was preserved. On the other hand, the H_2R knockout mouse with *H. pylori* infection showed the highest mRNA level of TFF2 and suppressed expression of AQP4. Only in the H_2R knockout mouse, the ratio between *AQP4* and *H^+/K^+-ATPase* mRNA expression was suppressed by *H. pylori* infection. Previous report showed that decrease of AQP4 was observed in gastric adenocarcinoma tissue.[23] In this study, while the expressions of both AQP4 and *H^+/K^+-ATPase* mRNA are decreased in old age of the H_2R knockout mouse and H_2R knockout mouse with *H. pylori* infection, the ratio between AQP4 and *H^+/K^+-ATPase* was decreased only in the H_2R knockout mouse with *H. pylori* infection which is the most prominent for SPEM. Taken together, the ratio between *AQP4* and *H^+/K^+-ATPase* mRNA expression might be a possible biomarker for the severe SPEM, which would be more likely to link to the gastric cancer development (Fig. 6).

In conclusion, although AQP4-positive parietal cell is localized in the basal side of gastric mucosa in wild type, acid suppression

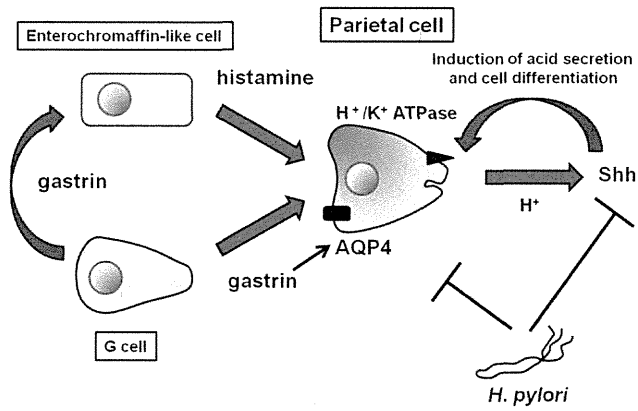

Figure 6 The hypothesis of interlink between histamine, gastrin, parietal cell, Shh, and aquaporin-4 (AQP4).
In normal, gastric acid is secreted from H^+/K^+ ATPase in the parietal cell. The secretion is induced by gastrin from G cells and histamine from enterochromaffin cell. Gastric acid facilitates the expression of Shh and then secreted Shh also induces gastric secretion from parietal cell and mucosal cell differentiation properly. The expression of AQP4 in parietal cell, which is located in basal side of gastric mucosa, is controlled by gastrin. However, the infection of *H. pylori* is supposed to decrease the expression of Shh and AQP4. Although acid suppression enhances gastric hyperplasia and temporarily enhances the expression of AQP4 through the increase of gastrin, long term acid suppression with the infection of *H. pylori* facilitate the formation of SPEM through the decrease of parietal cells, especially the AQP4-positive cells.

like H_2R knockout mouse causes the disturbance of parietal cell. Extended distribution of AQP4-positive cells in H_2R knockout mouse is not preserved by *H. pylori* infection. As the expression of TFF2, a marker of SPEM, is elevated in the H_2R knockout mouse with *H. pylori* infection, the decrease of the ratio between *AQP4* and H^+/K^+-*ATPase* mRNA expression might be related to preneoplastic lesion through the aggravation of SPEM.

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Randomized clinical trial: rikkunshito in the treatment of functional dyspepsia—a multicenter, double-blind, randomized, placebo-controlled study

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Key Messages

- This study is the first multicenter, randomized, double-blind, placebo-controlled, parallel-group trial for the efficacy and safety of rikkunshito for the treatment of functional dyspepsia (FD) diagnosed upon Rome III criteria.
- This study demonstrated a potential efficacy of rikkunshito for the relief of FD, particularly for the symptom of epigastric pain.
- In addition, postprandial fullness tended to improve by rikkunshito.
- These results serve as a valuable basis for further studies of rikkunshito and other Japanese herbal medicines in the treatment of FD.

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#See Appendix.
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Abstract

Background *Rikkunshito, a standardized Japanese herbal medicine, is thought to accelerate gastric emptying and relieve dyspepsia, although no large-scale, randomized, placebo-controlled trials of rikkunshito have been conducted. This study aimed to determine the efficacy and safety of rikkunshito for treating functional dyspepsia (FD).* ***Methods*** *FD*

patients received 2.5 g rikkunshito or placebo three times a day for 8 weeks in this multicenter, randomized, placebo-controlled, parallel-group trial. The primary end point was the proportion of responders at 8 weeks after starting test drug, determined by global patient assessment (GPA). The improvement in four major dyspepsia symptoms severity scale was also evaluated. In addition, plasma ghrelin levels were investigated before and after treatment. ***Key Results*** *Two hundred forty-seven patients were randomly assigned. In the eighth week, the rikkunshito group had more GPA responders (33.6%) than the placebo (23.8%), although this did not reach statistical significance ($p = 0.09$). Epigastric pain was significantly improved ($p = 0.04$) and postprandial fullness tended to improve ($p = 0.06$) in the rikkunshito group at week 8. Rikkunshito was relatively more effective among Helicobacter pylori-infected participants (rikkunshito: 40.0% vs placebo: 20.5%, $p = 0.07$), and seemed less effective among H. pylori-uninfected participants (rikkunshito: 29.3% vs placebo: 25.6%, $p = 0.72$). Among H. pylori-positive individuals, acyl ghrelin levels were improved just in rikkunshito group. There were no severe adverse events in both groups.* ***Conclusions & Inferences*** *Administration of rikkunshito for 8 weeks reduced dyspepsia, particularly symptoms of epigastric pain and postprandial fullness. (UMIN Clinical Trials Registry, Number UMIN000003954).*

Keywords *epigastric pain, functional dyspepsia, Helicobacter pylori, postprandial fullness, rikkunshito.*

INTRODUCTION

Functional dyspepsia (FD) is a pathological condition involving symptoms such as epigastric pain, epigastric burning, uncomfortable postprandial fullness, and early satiation, without evidence of organic diseases documented by the esophagogastroduodenoscopy (EGD).[1] Drug therapies such as antisecretory agents, prokinetics, and antidepressant agents have been used to treat FD in clinical practice; however, strong placebo effects have been identified and a reliable treatment strategy has not yet been established.

Rikkunshito is an herbal medicine consisting of a standardized, evenly compounded formulation that has been prescribed to patients in Japan with various upper gastrointestinal symptoms, including dyspepsia, for more than 20 years.[2,3] Although the precise mechanisms underlying its effects have not been clarified, rikkunshito is thought to improve relaxation of the gastric fundus, which maintains the gastric storage capacity, and to improve gastric antral peristalsis, which facilitates emptying of the stomach. These effects improve gastric accommodation and emptying.[4–11] However, our previous systematic review of randomized clinical trials of the efficacy of Japanese herbal medicines for treating FD revealed that efficacy assessments produced inconsistent data.[4] There was therefore a need for a large-scale, randomized, controlled clinical trial of rikkunshito in FD patients.

Several previous studies have demonstrated that rikkunshito enhanced plasma ghrelin levels in animals and humans.[12–14] Ghrelin is an endogenous 28-amino acid peptide ligand of the growth hormone (GH) secretagogue receptor, and is mainly produced and secreted by the A-like cells in the stomach. Ghrelin has two major molecular forms: acyl ghrelin and desacyl ghrelin. Acyl ghrelin is thought to be an active form that specifically stimulates the release of GH from the pituitary, and also stimulates gastric motor activity and food intake.[15] Desacyl ghrelin has opposing effects, decreasing food intake and delaying gastric emptying.[16]

The present multicenter, randomized, double-blind, placebo-controlled, parallel-group study investigated the efficacy of rikkunshito for treatment of dyspepsia in a Japanese population with investigated FD. FD was categorized into epigastric pain syndrome (EPS) or postprandial distress syndrome (PDS) based on the Rome III criteria to examine whether rikkunshito had any sub-group-specific effects.[1] Furthermore, plasma ghrelin levels were examined during the intervention.

MATERIALS AND METHODS

Trial design

The present trial was a multicenter, randomized, double-blind, placebo-controlled, parallel-group trial (UMIN Clinical Trials Registry number: UMIN000003954 [http://www.umin.ac.jp/ctr/]). The trial was designed by the authors and conducted at 31 institutions in Japan. Twenty of these were hospitals and 11 were primary care clinics. The study protocol was approved by the ethics committees of Keio University School of Medicine (No. 20100051; 1 October 2010) and all the other institutions, and written informed consent was obtained before subject enrollment. Participants were randomly assigned to receive rikkunshito or placebo in a 1 : 1 ratio. The study was performed in accordance with the principles of the Declaration of Helsinki.

Participants

Participants aged >20 years who had FD, as defined by the Rome III classification, were included. The participants had one or more symptoms, including bothersome postprandial fullness, early

satiation, epigastric pain, or epigastric burning. Epigastric pain and burning were defined as a pain and burning sensation localized to the epigastrium, not extending up to the poststernal regions. None of the participants had any evidence of organic diseases such as peptic ulcer disease, reflux esophagitis greater than grade A on the LA-classification, gastric cancer, or gastritis with multiple varioliform erosions, based on the results of EGD or upper gastrointestinal series conducted within 6 months of the start of the study. The participants' symptoms had been documented at least 6 months prior to the study, and they were present in the 3 months before the start of treatment. Only participants with any dyspepsia symptom with scores of 3 or higher on the 5-point Likert scale for more than 4 of the 14 days prior to registration were included in the study. Participants scoring 1 or 2 on the 5-point Likert scale for all four dyspepsia symptoms at baseline were therefore not eligible for this study.

Participants were excluded if they had a history of upper gastrointestinal tract surgery; severe liver, heart, or kidney diseases; a history of malignant disease; *Helicobacter pylori* eradication therapy within the last 6 months; a diagnosis of irritable bowel syndrome; or current or past evidence of uncontrolled diabetes mellitus, psychosomatic disorders, such as depressive and anxiety disorders, and drug or alcohol abuse. Pregnant or breastfeeding women were also excluded. Participants with documented symptom induction or aggravation due to self-recognized deregulated food habits or acute psychological stress arising from a clear personal or social circumstance were excluded. Participants were not allowed to be treated with proton pump inhibitors (PPIs), H_2-receptor antagonists, antacids, prokinetics, non-steroidal anti-inflammatory drugs, antidepressant drugs, anticholinergic agents (except as pretreatment for endoscopic examination), cholinergic agents, or tranquilizers within 1 week of study commencement or during the study. Participants who had used these drugs discontinued the treatment at least 1 week before the intervention as a wash-out period. Participants who had experienced heartburn within 12 weeks before the baseline period were also excluded. Participants already receiving mucosal protectants were eligible for inclusion, provided the investigator considered that a constant dose of such therapy was needed throughout the study.

Interventions

Rikkunshito or placebo (2.5 g powder) was taken three times a day before each meal for 8 weeks (Fig. 1A). The appearance, packaging, and labeling of rikkunshito and placebo were identical to maintain blinding to investigators and participants. Treatment compliance was assessed by counting the returned unused drugs at the outpatient clinic visit; and acceptable compliance was defined as an intake of ≥75% of the study medication. Each week, all participants were required to complete a patient diary comprising the global patient assessment (GPA) score, the 5-point Likert dyspepsia severity scale of each symptom, and the Japanese version of the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire.[17]

The GPA score was used to assess whether dyspepsia symptoms remained the same, improved, or deteriorated, compared to the pretreatment phase according to a previous study.[18] The question asked was 'How were your overall symptoms during the past week, compared with your symptoms before the trial?' This was scored on a 7-point Likert scale, ranging from '1: extremely improved', '2: improved', '3: slightly improved', '4: not changed', '5: slightly aggravated', '6: aggravated' and '7: extremely aggravated'. The 5-point Likert dyspepsia severity scale was used to measure the severity of the four dyspepsia symptoms separately. The scoring was performed as follows: 1: no complaints, 2: few complaints, 3: moderate complaints, 4: many complaints, and 5: extremely serious complaints. Overall gastrointestinal symptoms were assessed using GSRS, which included 15 scoring criteria: abdominal pain, heartburn, acid regurgitation, sucking sensations in the epigastrium, nausea and vomiting, borborygmus, abdominal distension, eructation, increased flatus, decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation, and feeling of incomplete evacuation. These 15 symptoms were grouped into the following five domains: reflux, abdominal pain, constipation, indigestion, and diarrhea.[17]

The attending physician discontinued drug administration upon observation of any serious adverse events, patient withdrawal from the study, realization that the patient did not meet inclusion criteria or met exclusion criteria, discovery of concomitant medication not permitted for the study, or any other clinical judgement indicating that administration should be discontinued. Overall safety was assessed by the participants and investigators at the clinic visits and by telephone contact 30 days after termination of the study drug. Vital signs were evaluated at all study visits or at early termination.

Before the intervention, participants filled out a questionnaire including demographic information, comorbidities, and previous history of eradication for *H. pylori*. Charlson comorbidity index was calculated to compare comorbid conditions.[19] Charlson comorbidity index contains 19 categories of comorbidities, such as myocardial infarction, cerebrovascular disease, diabetes mellitus, solid tumor, and age class.

Blood analysis was performed before administration of rikkunshito or placebo, and in the eighth week of treatment (Fig. 1A). Blood was analyzed for anti-*H. pylori* IgG antibodies before administration (visit 1). Biochemical analyses including measurement of potassium, total bilirubin, aspartate aminotransferase, alanine transaminase, and γ-glutamyl transpeptidase (γ-GTP) levels were carried out in the eighth week of treatment (visit 3). Acyl and desacyl ghrelin were measured in plasma both before administration, and in the eighth week of treatment (visits 1 and 3). Measurement of desacyl ghrelin was conducted at 28 study sites, whilst acyl ghrelin measurements were conducted at only 5 hospitals that had the instruments necessary for sample centrifugation and hydrochloric acid treatment. The presence of serum anti-*H. pylori* IgG (>10 U/mL) was considered as present infection or eradicated before the study. When previous history of eradication therapy for *H. pylori* was taken into account, participants' *H. pylori* status (negative, eradicated, or positive) could be obtained.

Outcomes

Most analyses were conducted in the intention-to-treat (ITT) population, which included all eligible participants. Participants who were lost to follow-up or who discontinued the intervention were treated as censored cases in the ITT analysis, and excluded from the per-protocol (PP) population. Participants with insufficient data during the eighth week of intervention were also excluded from the PP population.

Primary efficacy end point The primary efficacy end point was the proportion of responders in the ITT population in the eighth week of treatment, determined using GPA scores. Participants who answered '1: extremely improved' or '2: improved' were defined as responders.

Secondary efficacy end points

1. The elimination rate for each dyspepsia symptom was assessed in the ITT population in the eighth week of treatment by using

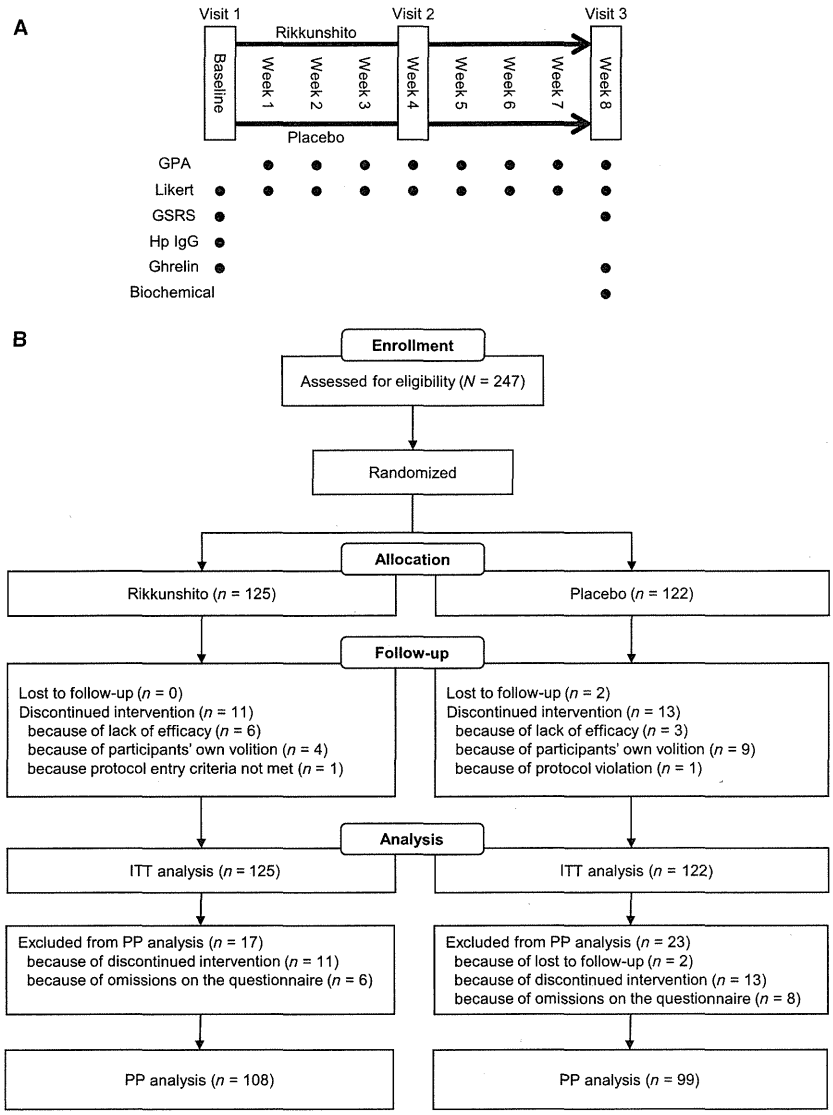

Figure 1 (A) Protocol of the trial. GPA, global patient assessment; GSRS, gastrointestinal symptom rating scales; Hp IgG, anti-*Helicobacter pylori* IgG antibody. (B) Trial flow and patient disposition. ITT, intention-to-treat; PP, per-protocol.

the 5-point Likert scale. Elimination of a dyspepsia symptom was defined as a response of '1: no complaints' or '2: few complaints' on the 5-point Likert scale following treatment, associated with a response of '3: moderate complaints', '4: many complaints', or '5: extremely serious complaints' at baseline.

2. The mean changes (%) in the overall GSRS values and in the five GSRS domains were calculated using the baseline GSRS and those in the eighth week of treatment. The mean changes following drug administration were compared between the rikkunshito and placebo groups.
3. The weekly change in the proportion of responders (GPA scores) and relief of each dyspepsia symptom (5-points Likert scale) was analyzed using the Kaplan–Meier method, and compared between the rikkunshito group and the placebo group. The length of time required to relieve the dyspepsia symptoms was determined using the study participants' diaries.

Sub-group analyses Sub-group analyses were conducted based on FD subcategory (both EPS and PDS, EPS alone, or PDS alone); the *H. pylori* infection status (negative, positive, or eradicated); the type of recruiting institution (hospital or primary care clinic); and gender. For each sub-group, the responder proportion (GPA scores) and each symptom relief rate (5-point Likert scale) were assessed in the ITT population.

Adverse events Adverse events were defined as any undesirable medical symptoms or conditions that emerged in participants during test drug administration (including changes in laboratory values), regardless of an apparent causal relationship. An increase in laboratory values to higher than twice the upper limit of the reference range was considered an adverse reaction. The frequency of adverse events was compared between the rikkunshito and placebo groups.

Sample size and study conduct

According to a previous trial,[20] dyspepsia symptom relief rates were 23.3% in placebo group, 36.0% in H_2 receptor antagonist group, and 51.1% in PPI group in dyspepsia population. We

anticipated that a similar percentage of patients should be treated rikkunshito in this study as that treated with the H_2 receptor antagonist in the previous study. Assuming an effective rikkunshito ratio of 36.0% and a placebo effective ratio of 23.3%, an α-error of 0.05, a β-error of 0.20, and power of 0.80, 416 participants would be required, with 208 of these in the placebo group and 208 in the rikkunshito group. Taking into consideration for the censored cases, 430 participants (215 in the placebo group and 215 in the rikkunshito group) therefore needed to be recruited to the study.

However, as patient recruitment was slow and the study period exceeded 2 years, we decided to decrease the recruitment goal to 220 participants. Based on a 0.05 significance level and 0.50 power, an enrolled population of 110 participants per arm was required. Finally, 247 participants were enrolled on 28 February 2013. The sample size was calculated using Stata version 11.0 (Stata Corp, College Station, TX, USA).

Randomization

Eligible participants were sequentially randomized in a 1 : 1 ratio according to a computer-generated random assignment program. The computer-generated randomization code was managed in the data management department of the Center for Clinical Research at Keio University School of Medicine (CCR). Concealed allocation was assured by an encrypted code kept in the Site Management Organizations (SMO) for clinical trials (EPS Co. Ltd., Tokyo, Japan). After all the study data had been collected by the CCR data management department, the data code-key was requested from EPS Co. Ltd, enabling data analysis.

Statistical methods

Baseline characteristics of participants in the rikkunshito and placebo groups were compared using Pearson's chi-square test or Fisher's exact test for categorical variables, or an unpaired Student's *t*-test for continuous variables. The proportion of responders (GPA scores) and the proportion of participants with individual symptom relief (5-point Likert scales) were compared between the rikkunshito and placebo groups by using the Fisher's exact test. Weekly changes in these parameters in the two study groups were compared using the log-rank test. The mean GSRS reduction rates in the rikkunshito and placebo groups were compared using Student's *t*-test. Individual plasma ghrelin levels before and after the intervention were analyzed using paired *t*-tests in the rikkunshito and placebo groups separately. The frequency of adverse events in the rikkunshito and placebo groups was compared using Student's *t*-test. Statistical analyses were performed using SPSS statistics 21 for Windows (SPSS Inc., Chicago, IL, USA). The data were expressed as means ± SD. Two-sided *p*-values of <0.05 were considered to be statistically significant.

RESULTS

Participant disposition

Two hundred forty-seven participants were randomized to receive either rikkunshito (n = 125) or placebo (n = 122) three times a day for 8 weeks (Fig. 1B). The first patient was enrolled on 1 March 2011, and the last patient completed the trial on 30 April 2013. In the rikkunshito group, 11 participants discontinued the intervention. Six participants discontinued because of a lack of efficacy and four of their own volition; one participant discontinued because the individual did not meet the primary inclusion criteria. These 11 participants were included in the ITT population (n = 125), but were excluded from the PP population. In the placebo group, two participants were lost to follow-up and 13 participants discontinued the intervention. Three of these discontinued because of a lack of efficacy, nine of their own volition, and one because of a protocol violation. These 15 participants were included in the ITT population (n = 122), but excluded from the PP population. Six participants in the rikkunshito group and eight in the placebo group were also excluded from the PP population because of omissions on questionnaires. The PP analysis therefore included 207 participants, 108 in the rikkunshito group, and 99 in the placebo group.

The two treatment groups were well balanced with respect to demographic and clinical characteristics at baseline for both the ITT populations (Table 1) and the PP populations (Table S1). In both the rikkunshito and placebo groups, about 63% of the participants were women, about 80% were non-smokers, and 50% did not consume alcohol. Approximately 45% of each group had both EPS and PDS symptoms, 35% had PDS only, and 20% had EPS only. The presence of *H. pylori* infection, determined using serum anti-*H. pylori* antibody levels and medical interviews, did not differ between the groups (18.4% in the rikkunshito and 17.4% in the placebo groups). The baseline 5-point Likert scale results were also well balanced.

Primary efficacy end point

In the ITT population, the proportion of responders in the eighth week of the intervention (last survey point), as assessed by GPA scores, tended to be higher in the rikkunshito group (33.6%) than in the placebo group (23.8%), although this did not reach statistical significance (p = 0.09; Table 2). Weekly improvement rates on GPA scores in the PP population were shown in Fig. 2. The efficacy of rikkunshito for dyspepsia symptoms seemed to be apparent from 4 weeks after the administration.

Secondary efficacy end points

Elimination rate for each dyspepsia symptom at week 8 The proportion of patients with epigastric pain symptom relief at week 8 was significantly higher in the rikkunshito group (44.0%) than in the placebo

Table 1 Characteristics of analyzed patients (intention-to-treat population)

	Rikkunshito (n = 125)	Placebo (n = 122)	p value
Age (years) (mean ± SD)	54.5 ± 16.2 (22–85)	53.6 ± 16.0 (21–85)	0.67*
Gender			
Men	46 (36.8%)	45 (36.9%)	1.00†
Women	79 (63.2%)	77 (63.1%)	
Smoking habits			
Non-smokers	108 (86.4%)	94 (77.0%)	0.07‡
Ex-smokers	12 (9.6%)	14 (11.5%)	
Current smokers	5 (4.0%)	14 (11.5%)	
Alcohol habits			
Abstainers	66 (52.8%)	58 (47.5%)	0.57‡
Social drinkers	57 (45.6%)	63 (51.6%)	
Heavy drinkers	2 (1.6%)	1 (0.8%)	
BMI (kg/m^2) (mean ± SD)	21.5 ± 3.3	21.3 ± 2.9	0.49*
Charlson comorbidity index	2.0 ± 1.6	1.9 ± 1.5	0.65*
Helicobacter pylori infection			
Negative	75 (60.0%)	82 (67.8%)	0.34‡
Eradicated	27 (21.6%)	18 (14.9%)	
Positive	23 (18.4%)	21 (17.4%)	
Unknown	0 (0%)	1 (0.8%)	
Sub-group of functional dyspepsia			
Overlap of EPS and PDS	50 (40.0%)	56 (45.9%)	0.34‡
EPS alone	26 (20.8%)	29 (23.8%)	
PDS alone	49 (39.2%)	37 (30.3%)	
Previous medication use			
Proton pump inhibitors	17 (13.6%)	23 (18.9%)	0.30‡
Histamine 2 receptor antagonists	18 (14.4%)	10 (8.2%)	0.16‡
Prokinetics	8 (6.4%)	13 (10.7%)	0.26‡
Mucosal protectants	14 (11.2%)	21 (17.2%)	0.20‡
Never use	86 (67.2%)	82 (67.2%)	0.89‡
Baseline 5-point Likert scale (mean ± SD)			
Epigastric pain	2.84 ± 1.19	3.09 ± 1.37	0.13*
Epigastric burning	2.30 ± 1.24	2.26 ± 1.16	0.84*
Postprandial fullness	3.90 ± 0.94	3.73 ± 1.03	0.18*
Early satiation	3.22 ± 1.34	3.17 ± 1.39	0.81*

BMI, body mass index: EPS, epigastric pain syndrome; PDS, postprandial distress syndrome; *Student's t-test; †Fisher's exact test; ‡Pearson's chi-square test.

Table 2 The efficacy end points

	Rikkunshito	Placebo	p value
Intention-To-Treat population	(n = 125)	(n = 122)	
Primary end point			
Elimination rate for global patient assessment score	42 (33.6%)	29 (23.8%)	0.09†,§
Secondary end points			
Elimination rate for Likert scale of each symptom			
Epigastric pain	55 (44.0%)	37 (30.3%)	0.04†,*
Epigastric burning	30 (24.0%)	25 (20.5%)	0.54†
Postprandial fullness	63 (50.4%)	46 (37.7%)	0.06†,§
Early satiation	45 (36.0%)	36 (29.5%)	0.28†
Per-Protocol population	(n = 108)	(n = 99)	
Secondary end points			
Reduction rate of GSRS (%) (mean ± SD)			
Overall	73.4 ± 20.6	76.6 ± 25.7	0.32‡
Reflux domain	76.8 ± 38.3	81.7 ± 43.0	0.39‡
Abdominal pain domain	65.7 ± 27.0	72.3 ± 32.4	0.12‡
Indigestion domain	77.1 ± 26.7	77.2 ± 31.1	0.99‡
Diarrhea domain	90.8 ± 41.7	90.8 ± 44.8	1.00‡
Constipation domain	82.5 ± 39.2	82.8 ± 36.6	0.97‡

GSRS, gastrointestinal symptom rating scale; †Fisher's exact test; ‡Student's t-test; *$p < 0.05$; §$p < 0.1$.

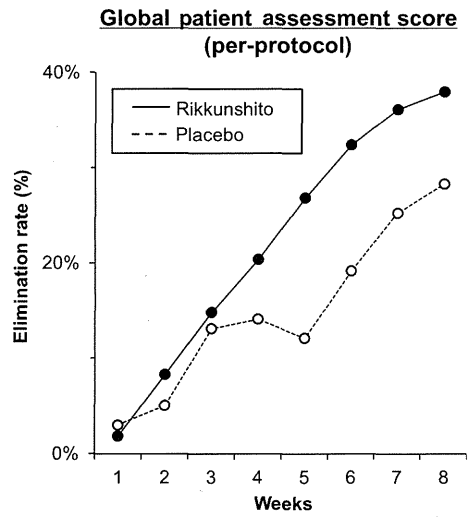

Figure 2 Weekly improvement rates assessed by global patient assessment scores in per-protocol population.

group (30.3%; $p = 0.04$). In addition, the proportion of patients with symptomatic relief of postprandial fullness at week 8 tended to be higher in the rikkunshito group (50.4%) than in the placebo group (37.7%; $p = 0.06$). On the other hand, there were no significant differences in the proportion of patients with relief of epigastric burning or early satiation at week 8 between the rikkunshito and placebo groups.

Reduction rates for GSRS scores at week 8 There was no significant difference in the mean reduction rate of the overall GSRS score, or in any of the five GSRS domains, in the PP population in the rikkunshito and placebo groups (Table 2).

Weekly changes in the proportion of responders The proportion of responders, assessed using weekly GPA scores, tended to be higher in the rikkunshito group than in the placebo group ($p = 0.08$; Fig. 3A). Analysis of the weekly changes in the proportion of

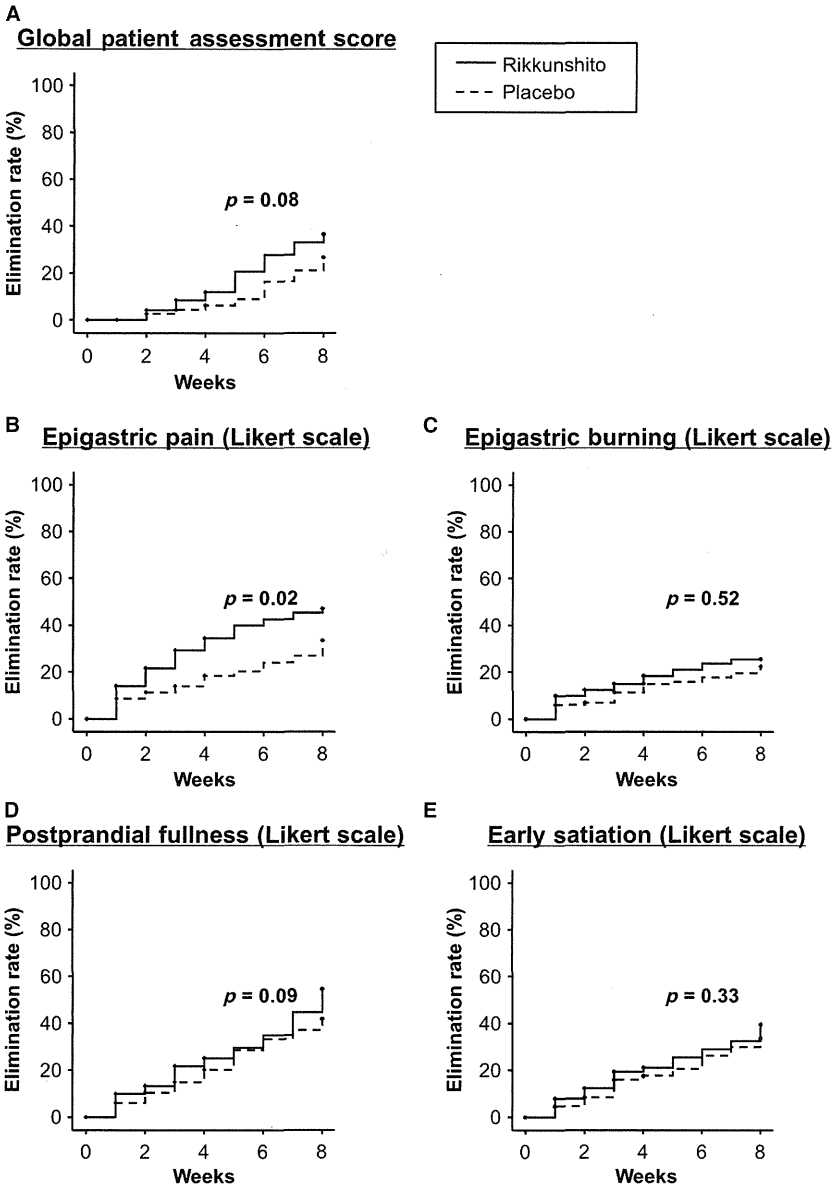

Figure 3 Symptom relief rates during the study period shown by the Kaplan–Meier method. (A) Global patient assessment scores. (B) Epigastric pain based on 5-point Likert dyspepsia severity scales. (C) Epigastric burning based on 5-point Likert dyspepsia severity scales. (D) Postprandial fullness based on 5-point Likert dyspepsia severity scales. (E) Early satiation based on 5-point Likert dyspepsia severity scales; Weekly changes in these parameters were compared using the log-rank test.

participants showing relief of individual symptoms was performed using the 5-point Likert scale. The weekly change in the proportion reporting relief of epigastric pain was significantly higher in the rikkunshito group than in the placebo group ($p = 0.02$; Fig. 3B). The weekly change in the proportion of individuals reporting symptomatic relief of postprandial fullness also tended to be higher in the rikkunshito group than in the placebo group ($p = 0.09$; Fig. 3D). On the other hand, no significant differences in the weekly proportion of responders reporting relief of epigastric burning ($p = 0.52$) or early satiation ($p = 0.33$) were found between the rikkunshito and placebo groups (Fig. 3C and E). Of the four dyspepsia symptoms analyzed, the placebo effect was the strongest for the relief of postprandial fullness.

Sub-group analyses

Stratification of the rikkunshito *vs* placebo analysis by FD subcategory (EPS and PDS, EPS alone, or PDS alone) revealed that there were no significant differences between the proportions of responders in the eighth week of the intervention (assessed by GPA scores) in these sub-groups (Table 3). However, in the group of participants with PDS alone, the proportion showing symptomatic relief of postprandial fullness at week 8

Table 3 Sub-group analysis (intention-to-treat population)

	Rikkunshito	Placebo	*p* value†
Overlap of EPS and PDS	(*n* = 50)	(*n* = 56)	
Elimination rate for global patient assessment score	15 (30.0%)	14 (25.0%)	0.66
Elimination rate for Likert scale of each symptom			
Epigastric pain	25 (50.0%)	20 (35.7%)	0.17
Epigastric burning	14 (28.0%)	15 (26.8%)	1.00
Postprandial fullness	21 (42.0%)	24 (42.9%)	1.00
Early satiation	19 (38.0%)	21 (37.5%)	1.00
EPS alone	(*n* = 26)	(*n* = 29)	
Elimination rate for global patient assessment score	13 (50.0%)	9 (31.0%)	0.18
Elimination rate for Likert scale of each symptom			
Epigastric pain	16 (61.5%)	12 (41.4%)	0.18
Epigastric burning	7 (26.9%)	4 (13.8%)	0.32
Postprandial fullness	13 (50.0%)	12 (41.4%)	0.59
Early satiation	7 (26.9%)	6 (20.7%)	0.75
PDS alone	(*n* = 49)	(*n* = 37)	
Elimination rate for global patient assessment score	14 (28.6%)	6 (16.2%)	0.21
Elimination rate for Likert scale of each symptom			
Epigastric pain	14 (28.6%)	5 (26.3%)	0.12
Epigastric burning	9 (18.4%)	6 (16.2%)	1.00
Postprandial fullness	29 (59.2%)	10 (27.0%)	0.004**
Early satiation	19 (38.8%)	9 (24.3%)	0.17
Helicobacter pylori: Negative‡	(*n* = 75)	(*n* = 82)	
Elimination rate for global patient assessment score	22 (29.3%)	21 (25.6%)	0.72
Elimination rate for Likert scale of each symptom			
Epigastric pain	33 (44.0%)	27 (32.9%)	0.19
Epigastric burning	15 (20.0%)	16 (19.5%)	1.00
Postprandial fullness	38 (50.7%)	30 (36.6%)	0.08§
Early satiation	28 (37.3%)	26 (31.7%)	0.50
H. pylori: Positive or Eradicated‡	(*n* = 50)	(*n* = 39)	
Elimination rate for global patient assessment score	20 (40.0%)	8 (20.5%)	0.07§
Elimination rate for Likert scale of each symptom			
Epigastric pain	22 (44.0%)	10 (25.6%)	0.08§
Epigastric burning	15 (30.0%)	9 (23.1%)	0.63
Postprandial fullness	25 (50.0%)	16 (41.0%)	0.52
Early satiation	17 (34.0%)	10 (25.6%)	0.49
Hospitals	(*n* = 86)	(*n* = 86)	
Elimination rate for global patient assessment score	26 (30.2%)	20 (23.3%)	0.39
Elimination rate for Likert scale of each symptom			
Epigastric pain	35 (40.7%)	28 (32.6%)	0.34
Epigastric burning	18 (20.9%)	16 (18.6%)	0.85
Postprandial fullness	39 (45.3%)	32 (37.2%)	0.35
Early satiation	27 (31.4%)	26 (30.2%)	1.00
Primary care clinics	(*n* = 39)	(*n* = 36)	
Elimination rate for global patient assessment score	16 (41.0%)	9 (25.0%)	0.22
Elimination rate for Likert scale of each symptom			
Epigastric pain	20 (51.3%)	9 (25.0%)	0.03*
Epigastric burning	12 (30.8%)	9 (25.0%)	0.62
Postprandial fullness	24 (61.5%)	14 (38.9%)	0.07§
Early satiation	18 (46.2%)	10 (27.8%)	0.15

EPS, epigastric pain syndrome; PDS, postprandial distress syndrome; †Fisher's exact test; $^{**}p < 0.01$; $^{*}p < 0.05$; §$p < 0.1$; ‡A patient with unknown *H. pylori* infection status was excluded from this sub-group analysis.

was significantly higher in the rikkunshito group (59.2%) than in the placebo group (27.0%; $p = 0.004$). In the group of participants with both EPS and PDS, or in the group of those with EPS alone, high relief rates of epigastric pain at week 8 were shown (50.0% and 61.5% respectively), although there were no statistical differences between the rikkunshito and the placebo groups.

In the group of *H. pylori*-positive participants or those with previous eradication of an *H. pylori* infection, the proportion of responders (assessed using GPA scores at week 8) tended to be higher in the rikkunshito group than in the placebo group ($p = 0.07$; Table 3). In this group, the proportion showing relief of epigastric pain tended to be higher in the rikkunshito group than in the placebo group ($p = 0.08$). In the groups of *H. pylori*-negative participants, the proportion of responders with symptomatic relief of postprandial fullness at week 8 tended to be higher in the rikkunshito group than in the placebo group ($p = 0.08$).

Interestingly, the efficacy of rikkunshito differed between participants in hospitals and those in primary care clinics. Among the participants recruited in primary care clinics, the proportion of responders with symptomatic relief of epigastric pain ($p = 0.03$) was significantly higher and that of postprandial fullness ($p = 0.07$) tended to be higher in the rikkunshito group than in the placebo group at week 8. On the other hand, there were no significant differences between the rikkunshito and placebo groups among participants recruited in hospitals (Table 3). This difference in rikkunshito efficacy suggested some differences in the characteristics of the participants recruited in hospitals and those recruited in primary care clinics. As shown in Table S2, the participants recruited in primary care clinics were younger, and showed a higher prevalence of PDS. Of patients, 61.0% in hospitals and 84.0% in primary care clinics had never taken some drugs for dyspepsia ($p < 0.001$), suggesting that more patients with treatment-resistant dyspepsia were included in hospitals than in primary care clinics. On

the other hand, baseline scores of postprandial fullness and early satiation were worse in patients in primary care clinics than those in hospitals. Comorbidity indexes were not different between participants in primary care clinics and those in hospitals when the age class was excluded from the calculation.

According to a sub-group analysis of gender, the responder proportions (GPA scores) were no significant differences between the rikkunshito and placebo groups both among men and among women (Table S3). In the female, the proportion of participants showing symptom relief for epigastric pain at week 8 in the rikkunshito group tended to be higher than in the placebo group ($p = 0.07$).

Plasma ghrelin levels

There were no significant differences in plasma acyl ghrelin levels at week 8, as compared to baseline levels, in the rikkunshito or placebo groups (Table S4). As plasma ghrelin levels were shown to be suppressed by *H. pylori* infection,[21,22] a sub-group analysis based on *H. pylori* infection status was also conducted. Notably, plasma acyl ghrelin levels tended to increase in the *H. pylori*-positive participants who received rikkunshito, but not in those who received placebo. There were no significant changes in plasma desacyl ghrelin levels following treatment in the rikkunshito or placebo groups.

Safety assessment

There were 19 cases (15.2%) of adverse events recorded in the rikkunshito group and 14 cases (11.5%) in the placebo group during the trial period. There was no significant difference in the incidence of adverse events between the rikkunshito and placebo groups ($p = 0.46$). In the rikkunshito group, only mild symptoms or mild elevation of laboratory values were reported during the test period. The symptoms comprised diarrhea (seven cases), nausea (three cases), headache (three cases), γ-GTP elevation (two cases), upper abdominal pain (two cases), alanine transaminase elevation (one case), abdominal bloating (one case), abdominal discomfort (one case), nasopharyngitis (one case), tinnitus (one case), skin dysesthesia (one case), oral dysesthesia (one case), dizziness (one case), and urticaria (one case). In the placebo group, the following mild symptoms were reported: γ-GTP elevation (four cases), cold symptoms (three cases), dermatitis (two cases), diarrhea (one case), upper abdominal pain (one case), abdominal bloating (one case), lower back pain (one case), and sudden deafness (one case).

DISCUSSION

This study was a multicenter, double-blind, randomized, placebo-controlled, parallel comparative study on the efficacy of rikkunshito for the treatment of FD, diagnosed using Rome III criteria, in a Japanese population. The data obtained were exploratory because of the lower-than-projected sample size resulting from slow recruitment of participants. This led to termination of study recruitment before the target sample size was reached. However, the proportion of responders identified using GPA tended to be higher in the rikkunshito group (33.6%) than in the placebo group (23.8%) at week 8 ($p = 0.09$, Table 2). In addition, the study also showed that rikkunshito significantly improved epigastric pain ($p = 0.04$) and tended to improve postprandial fullness ($p = 0.06$). These results suggested that rikkunshito could be a new and valuable treatment option for FD.

According to previous reports, rikkunshito has a lot of effects which might improve dyspepsia. Rikkunshito accelerates gastric emptying through nitric oxide-mediated mechanisms and antagonism of serotonin 5-HT_3 receptors.[7,23] Rikkunshito also improves gastric accommodation reflex and reduces epigastric fullness following gastric distension induced by a gastric barostat.[8,9,24] Furthermore, some researchers showed that rikkunshito improves the dyspepsia symptoms at least in part by facilitating the secretion of ghrelin through serotonin 2-$HT_{2B/2C}$ receptor antagonism.[10,11]

Thus, as rikkunshito is considered to improve gastric motility, we hypothesized that it would be more effective against PDS-related dyspepsia symptoms. In fact, sub-group analysis by FD subcategory showed that rikkunshito significantly improved postprandial fullness in the participants with PDS only (Table 3). However, this study also showed that rikkunshito was effective for epigastric pain. In addition, we could not show that rikkunshito has significant effect on plasma ghrelin levels in large cohorts. The efficacy of rikkunshito for epigastric pain did not seem to depend on any alteration of plasma ghrelin levels. This finding indicated that rikkunshito probably had additional effects, besides enhancement of ghrelin secretion, on gastro-duodenal function. For instance, because epigastric pain is known to be associated with visceral hypersensitivity, rikkunshito might also affect visceral hypersensitivity in the stomach.[25] Araki *et al.* reported that rikkunshito strongly absorbed hydrophobic bile salts.[26] As hydrophobic bile salts are known to induce oxidative stress and inflammation in the upper gastrointestinal tract, decreased levels of these might improve visceral hypersensitivity in the stomach.

Traditionally, rikkunshito also has effects on loose stools, diarrhea, and heartburn. More recently, Gunji *et al.* reported that 4-week administration of rikkunshito improved symptoms of gastroesophageal reflux, abdominal pain, diarrhea, and constipation among 10 patients after proximal gastrectomy.[27] They also showed that these effects of rikkunshito were independent of the plasma acyl and desacyl ghrelin levels. These various effects of rikkunshito might be explained by its ability to alter bile acid bioavailability in the gastrointestinal tract.

Unexpectedly, the efficacy of rikkunshito differed between *H. pylori*-uninfected and *H. pylori*-infected participants. The overall response, assessed by GPA, showed that rikkunshito was relatively more effective among *H. pylori*-infected participants (rikkunshito: 40.0% *vs* placebo: 20.5%, $p = 0.07$), and seemed less effective among *H. pylori*-uninfected participants (rikkunshito: 29.3% *vs* placebo: 25.6%, $p = 0.72$; Table 3). Interestingly, plasma levels of acyl ghrelin (active form) were only elevated following treatment with rikkunshito among *H. pylori*-infected participants (Table S4). The severity of gastric mucosal atrophy induced by *H. pylori* infection is known to be correlated with plasma ghrelin levels.[21,22] In line with this knowledge, the present data also showed that baseline plasma ghrelin levels were lower in *H. pylori*-infected participants. Rikkunshito might act to restore acyl ghrelin to normal levels just when ghrelin secretion is inhibited by some stresses. This effect of rikkunshito on plasma acyl ghrelin levels might explain its different efficacy in *H. pylori*-uninfected participants, compared to that in *H. pylori*-infected participants. Another possibility is that rikkunshito might have bacteriostatic action against *H. pylori*, although there were no previous data to support this hypothesis. We have thought *H. pylori*-positive and -negative patients with FD should be managed separately and that *H. pylori*-positive dyspepsia should be categorized as a separate disease entity from FD if the symptoms are relieved long after the *H. pylori* eradication therapy.[28–30] The present results support that underlying mechanism for the development of dyspepsia symptoms would be different between *H. pylori*-positive and -negative patients. Therefore, management strategy should be discussed separately.

A second sub-group analysis suggested that rikkunshito was more effective in patients recruited from primary care clinics than in those recruited from hospitals (Table 3). Rikkunshito only produced significant improvement in epigastric pain among participants from primary care clinics. Postprandial fullness also tended to be more improved in primary care clinic patients. As there were several differences in the baseline characteristics of participants recruited in hospitals and those from primary care clinics, it was difficult to determine the reason(s) for this difference in efficacy, although it may relate to the higher prevalence of patients with treatment-resistant FD in hospitals than in primary care clinics.

The primary limitation of this study was the reduced statistical power as a result of the smaller enrollment numbers. Suggested effects of rikkunshito shown as marginal statistical significances were not proven. In addition, the measurement of acyl ghrelin was not available at all sites. It makes more difficult to detect the association of acyl ghrelin levels with the effect of rikkunshito, as the reliability of sub-group analysis is comparatively poor because of greater α and β errors. Further investigation with an increased number of study subjects will be necessary to confirm and extend these results with sufficient statistical power. The other criticisms include that there were no data for psychological status in participants before and during the intervention.

In conclusion, the results of this study showed potential efficacy of rikkunshito for the treatment of FD, particularly for the symptom of epigastric pain. These results serve as a valuable basis for further studies of rikkunshito or other Japanese herbal medicines in the treatment of Rome III-based FD.

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CONFLICTS OF INTEREST

During the last 2 years, Hidekazu Suzuki received scholarship funds for the research from Astellas Pharm Inc., Astra-Zeneca K.K., Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd. Yuji Naito received scholarship funds for the basic research from Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Eisai Co., Ltd., and Tanabe-Mitsubishi Co., Ltd. Naomi Uemura has received service honoraria for the lecture from Takeda, Eisai and Astra-Zeneca Pharmaceutical Co. Ltd. During the last 2 years, Takeshi Kamiya has received service honoraria from AstraZeneca K.K., Daiichi-Sankyo Co., Takeda Pharmaceutical Co. Ltd., and Astel-