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BASIC—ALIMENTARY TRACT

Dysfunctional Gastric Emptying With Down-regulation of Muscle-Specific MicroRNAs in *Helicobacter pylori*-Infected Mice

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BACKGROUND & AIMS: Little is known about the pathogenic mechanisms of functional dyspepsia. We investigated the role of microRNAs (miRNAs) in gastric motility disorders associated with *Helicobacter pylori* infection. **METHODS:** Male C57BL/6 mice were infected with *H pylori*. After long-term infection, gastric emptying was examined and compared with that of uninfected mice (controls). The miRNA expression profile was analyzed by miRNA microarray and quantitative reverse-transcriptase polymerase chain reaction. The results obtained from the animal study were confirmed by in vitro experiments. **RESULTS:** Gastric emptying was significantly accelerated in mice after chronic infection with *H pylori*. Histologic examination showed that the muscular layers of the stomachs of *H pylori*-infected mice were significantly thickened. The miRNA expression profile revealed that the muscle-specific miRNAs *miR-1* and *miR-133* were significantly down-regulated in the stomachs after long-term infection with *H pylori*. However, expression of histone deacetylase 4 and serum response factor, which are reported target genes of *miR-1* and *miR-133*, increased. Down-regulation of *miR-1* and *miR-133* and increased cell proliferation were observed in C2C12 mouse myoblast cells after coculture with *H pylori*. **CONCLUSIONS: Chronic infection with *H pylori* down-regulates expression of muscle-specific miRNAs and up-regulates expression of histone deacetylase 4 and serum response factor. These might cause hyperplasia in the muscular layer of the stomach and dysfunction in gastric emptying. These findings provide insight into the molecular pathogenesis of gastric motility disorders, including functional dyspepsia.**

Keywords: Functional Gastrointestinal Disorder; Noncoding RNA; Muscle Cell; Liquid Gastric Emptying.

Helicobacter pylori has been shown to be involved not only in the pathogenesis of chronic gastritis, peptic ulcer, and gastric cancer[1–5] but also in gastric motility disorders such as functional dyspepsia (FD).[6–8] Although delayed gastric emptying has been reported in patients with FD,[9–11] several studies have failed to confirm such a relationship.[12,13] Recent studies have shown that accelerated gastric emptying in patients with FD is associated with postprandial fullness, bloating, nausea, and stomach pain[14] and that gastric emptying is significantly accelerated in *H pylori*-positive children with nonulcer dyspepsia in comparison with noninfected children.[15] Lee et al[16] have reported that spontaneous duodenal acid exposure is increased in a subset of FD patients who are characterized with more severe dyspeptic symptoms. They suggested that increased duodenal acid may enhance the pathophysiology of FD, producing symptoms such as delayed gastric emptying. Despite many clinical studies of affected patients, the pathogenesis of FD is still poorly understood.

MicroRNAs (miRNAs) are small noncoding RNAs that function as endogenous silencers of target genes, thus playing critical roles in cell proliferation, apoptosis, and differentiation during mammalian development.[17] Links between miRNAs and human cancers are becoming increasingly apparent, and aberrant expression of miRNAs is known to be involved in the initiation and progression of gastrointestinal (GI) cancers.[18–24] Moreover, recent studies have revealed that *miR-29a* and *miR-510* are involved in the pathophysiology of irritable bowel syndrome (IBS), indicating that miRNAs play important roles not only in GI cancers but also in functional GI disorders such as IBS.[25,26] To investigate the molecular mechanism underlying the pathogenesis of functional gastric disorders associated with *H pylori* infection, we analyzed the miRNA expression profile in the stomachs of mice after long-term infection with *H pylori*.

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; BrdU, bromodeoxyuridine; CFU, colony-forming unit; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; FD, functional dyspepsia; GI, gastrointestinal; HDAC4, histone deacetylase 4; *H pylori*, *Helicobacter pylori*; IBS, irritable bowel syndrome; IL, interleukin; miRNA, microRNA; RT-PCR, reverse-transcription polymerase chain reaction; SRF, serum response factor; TNF, tumor necrosis factor.

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In the present study, we found that gastric emptying was accelerated in *H pylori*-infected mice. Interestingly, the miRNA expression profile revealed that *miR-1* and *miR-133* were markedly down-regulated in the stomach after chronic infection with *H pylori*. It has been reported that *miR-1* and *miR-133* are highly expressed in differentiated muscle tissues and control muscle differentiation and proliferation.[27,28] In addition, it has been shown that the expression of *miR-1* and *miR-133* is decreased in mouse and human cardiac hypertrophy, thus playing an essential role in the development of heart disease.[29-32] Here, we present novel data that offer insights into the molecular pathogenesis of gastric motility disorder associated with chronic *H pylori* infection via aberrant expression of muscle-specific miRNAs.

Materials and Methods

Animals and H pylori *Infection*

All experiments and procedures in this study were approved by the Keio University Animal Research Committee. A total of 14 male C57BL/6 mice were infected with *H pylori* (the Sydney strain; SS1) at a concentration of 10^6 colony-forming units (CFUs)/mL by oral gavage. Fourteen uninfected male wild-type C57BL/6 mice were used as controls. After 40 weeks of infection, the mice with *H pylori* infection (n = 7) and age-matched control mice (n = 7) were killed, and then their stomachs were dissected out and subjected to histologic examination and extraction of RNA and protein. Gastric emptying of mice chronically infected with *H pylori* and *Helicobacter felis* (53 weeks after infection; n = 7 and n = 13, respectively) and control mice (n = 7) was evaluated as described below.

Helicobacter infection was confirmed in all the stomachs of *Helicobacter*-infected mice by microaerobic bacterial culture of stomach homogenates on Nissui Plate *Helicobacter* Agar (Nissui, Tokyo, Japan) and histologic examination.

Evaluation of Gastric Emptying

Gastric emptying in mice was evaluated by measuring the amount of phenol red remaining in the stomach after oral administration. The experiment was conducted according to the method reported by Suzuki et al.[33] Each mouse received 200 μL of phenol red (100 μg/mL) orally and was killed 15 minutes later, except for some mice that were killed immediately after administration to recover the entire dose of phenol red. The stomach was removed immediately and washed in 10 mL of Na_2HPO_4 solution (0.1 mol/L) to collect the gastric contents and phenol red, and then 1 mL of the rinse solution was added to 0.5 mL of Na_2HPO_4 solution (0.1 mol/L) (S1: the rinse solution and Na_2HPO_4 solution). The residual rinse solution was added to 1 mL of phenol red (100 μg/mL) solution and then diluted 5-fold with Na_2HPO_4 solution (0.1 mol/L) (S2: diluted solution of the residual rinse solution and phenol red). The absorbances of solutions S1 and S2 were measured at a wavelength of 570 nm with a microplate reader (Bio-Rad, Hercules, CA). Gastric emptying was calculated as follows:

$$\text{Gastric emptying}(\%) = 100 - (A/B) \times 100$$

A: Amount of phenol red remaining in the stomach (micrograms)

$$= (100 - [1.5 \times <S1>])/(5 \times <S2>/[1.5 \times <S1>] - 1)$$

Note: <S1> and <S2> are the phenol red concentrations in solutions S1 and S2.

B: The amount of phenol red recovered from the stomach immediately after phenol red administration (micrograms).

Histologic Examination

Tissues were stained with H&E and subjected to histologic examination. Thickness of smooth muscle was expressed as the averaged value of the thickness of the muscularis propria layer at 3 points in the corpus (oral side, middle, anal side) determined by microscopy (×40) from comparison with a size scale.

4′,6-Diamidino-2-phenylindole (DAPI) staining was performed, and the nuclei of myocytes were counted using the fluorescence microscope (Eclipse E600; Nikon Corporation, Tokyo, Japan). The average number of myocytes in the muscular layer in 2 randomly selected microscopic fields (×200) was examined.

RNA Extraction and Microarray Analysis

Total RNAs of tissue specimens from the stomachs of *H pylori*-infected mice and control mice were extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX). Three micrograms of total RNA from the stomachs of 3 *H pylori*-infected mice were pooled, and the same was done for 3 control mice. miRNA microarray analysis with the pooled RNA samples from *H pylori*-infected mice and control mice was conducted by LC Sciences (www. lcsciences.com: Houston, TX). All data are Minimum Information About a Microarray Experiment (MIAME) compliant and have been deposited in the ArrayExpress database (accession number: E-MEXP-2239).

Quantitative Reverse-Transcription Polymerase Chain Reaction of miRNAs

miRNA expression levels were analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using the TaqMan microRNA assay for *miR-1*, *miR-133a*, and *miR-133b* (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions.

Expression levels were normalized against U6 RNA expression.

Western Blotting

Protein extracts were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were hybridized with rabbit anti-human histone deacetylase 4 (HDAC4) polyclonal antibody (H-92: sc-11418; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human serum response factor (SRF) polyclonal antibody (G-20: sc-335; Santa Cruz Biotechnology). β-Actin was used as the internal control.

Culture of C2C12 Mouse Myoblast Cells

C2C12 mouse myoblast cells were obtained from the American Type Culture Collection (Manassas, VA), and cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (growth medium). Myogenic differentiation was induced by changing the growth medium to Dulbecco's modified Eagle medium containing 2% heat-inactivated horse serum (differentiation medium), as described previously.[29]

Coculture With H pylori, *Transfection of anti-miRNA Inhibitors, and Treatment With Cytokines*

H pylori bacteria (SS1) were added to C2C12 cells cultured with differentiation medium at a multiplicity of infection of 50 and cocultured for 12 hours, followed by isolation of total RNAs and proteins. Anti-*miR-1* and anti-*miR-133* inhibitors and a negative control were purchased from Ambion. Each anti-miRNA inhibitor is a chemically modified, single-stranded nucleic acid designed to bind to, and inhibit, a specific endogenous miRNA molecule. The inhibitors were transfected into C2C12 cells at a final concentration of 100 nmol/L using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Forty-eight hours after transfection, the cells were collected and analyzed.

C2C12 cells were treated with the cytokines interleukin (IL)-1β, IL-13, and tumor necrosis factor (TNF)-α (R&D Systems, Inc, Minneapolis, MN) at concentrations of 10 pg/mL, 5 ng/mL, and 50 pg/mL, respectively, for 12 hours in accordance with the manufacturer's instructions.

Immunostaining of C2C12 Cells With Ki-67

C2C12 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. They were then incubated with rat anti-mouse Ki-67 monoclonal antibody (Dako; diluted 1:50) overnight at 4°C, followed by Alexa Fluor 488 goat anti-mouse immunoglobulin G (Invitrogen), and examined using the fluorescence microscope. The cells were counterstained with DAPI.

Cell Proliferation Enzyme-Linked Immunosorbent Assay Using Bromodeoxyuridine

C2C12 cells were cultured in differentiation medium in a 96-well plate. They were cocultured with *H pylori* (SS1) at a multiplicity of infection of 50, and labeled with bromodeoxyuridine (BrdU) for 12 hours. BrdU incorporation was quantified by colorimetric enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions (Cell Proliferation ELISA, BrdU; Roche, Basel, Switzerland).

Gastric Tissue Samples From Patients With H pylori *Infection*

Gastric tissue samples of the antral region of the stomach were obtained from patients with or without *H pylori* infection by endoscopic biopsy at Keio University Hospital (Tokyo, Japan). *H pylori* infection status was identified by the ^{13}C-urea breath test and/or serologic examination. Patients who had *H pylori* eradication therapy were excluded. The average age of *H pylori*-negative patients was 69.1 years (male/female, 6/2) and that of *H pylori*-positive patients was 70.6 years (male/female, 11/

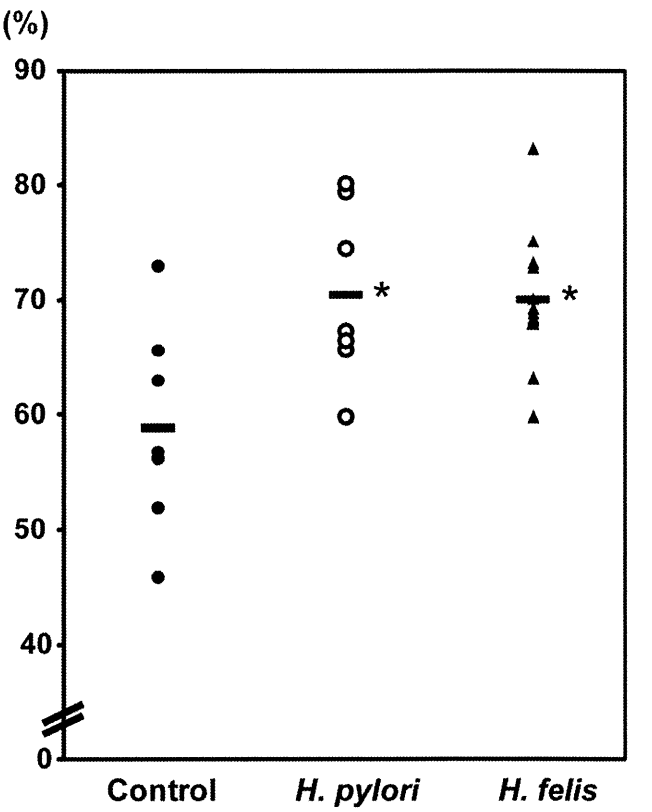

Figure 1. Gastric emptying in control mice and *Helicobacter*-infected mice. Gastric emptying rates (%) in control mice (n = 7) and mice chronically infected with *H pylori* (n = 7) and *Helicobacter felis* (n = 13) were evaluated by measuring the amount of phenol red remaining in the stomach after oral administration. Gastric emptying was significantly increased in the stomachs of mice chronically infected with *H pylori* and *H felis* relative to control mice (*$P < .05$).

3). This study was approved by Keio University School of Medicine Ethics Committee (No. 19-68-5) and entered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN 000001057). Informed consent was obtained from all patients before the examination.

Statistical Analysis

Data were analyzed using the SPSS statistical software package version 17.0 (SPSS Inc, Chicago, IL). Differences at P values of less than .05 were considered significant.

Results

Significant Acceleration of Gastric Emptying in Helicobacter-*Infected Mice*

To evaluate gastric motility in *Helicobacter*-infected mice, we analyzed the gastric emptying rate of mice chronically infected with *H pylori* and *H felis* by measuring the amount of phenol red remaining in the stomach after oral administration. As shown in Figure 1, the gastric emptying rate was significantly increased in mice chronically infected with *H pylori* and *H felis* in comparison with control mice.

Muscular Layer Hyperplasia in the Stomach of H pylori-*Infected Mice*

Histologic examination with H&E staining revealed active gastritis with infiltration of neutrophils and prominent thickening of the muscular layer in the gastric corpus of *H pylori*-infected mice (Figure 2*A*). The muscularis propria layer was significantly thicker in the stomachs of *H pylori*-infected mice than in those of control mice (Figure 2*B*).

To analyze proliferation of myocytes in the stomach, DAPI staining was performed, and the nuclei of myocytes were counted (Figure 3*A*). As shown in Figure 3*B*, the average number of myocytes in the muscular layer of the stomach was significantly increased in *H pylori*-infected mice in comparison with control mice, indicating the presence of myocyte hyperplasia in the former.

Down-regulation of Muscle-Specific miRNAs in the Stomachs of H pylori-*Infected Mice and* H pylori-*Positive Patients*

To identify miRNAs that play important roles in *H pylori*-associated gastric disorders, we performed miRNA microarray analysis using gastric tissue samples

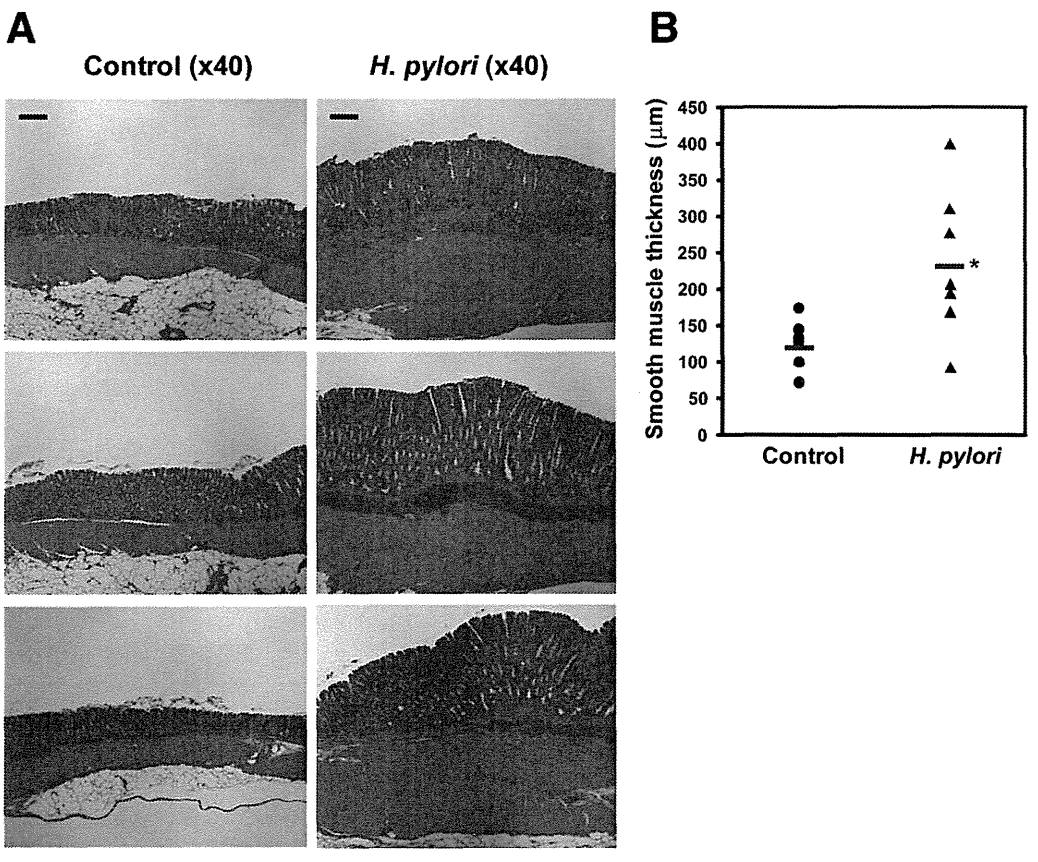

Figure 2. Histologic examination of the stomachs of control mice and mice chronically infected with *H pylori*. (*A*) Stomach tissues from uninfected control mice and mice infected with *H pylori* were stained with H&E and subjected to histologic examination (original magnification: 40×). The representative stomach tissues of 3 control mice and 3 infected mice are shown (*scale bars*, 100 μm). (*B*) Comparison of gastric smooth muscle thickness between control mice (n = 7) and *H pylori*-infected mice (n = 7). The thickness of the muscularis propria layer was significantly increased in *H pylori*-infected mice relative to control mice (*P < .05).

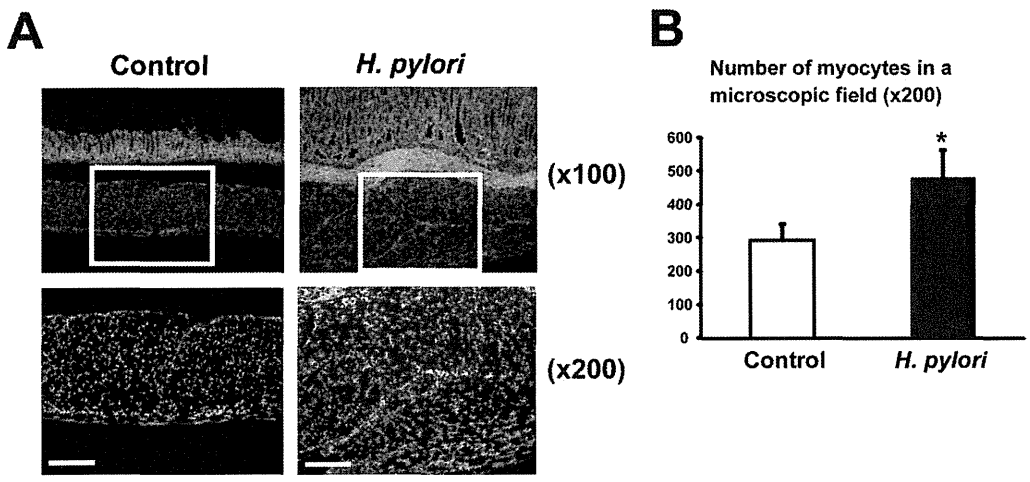

Figure 3. 4′,6-Diamidino-2-phenylindole (DAPI) staining of the stomach of uninfected control mice and mice chronically infected with *H pylori*. (*A*) Representative DAPI staining of control mice and infected mice. The muscular layer was randomly selected, as shown by the *box* (original magnification: 100×), in which the number of myocyte nuclei was counted at a magnification of 200× (*scale bars*, 100 μm). (*B*) The average number of myocytes in 2 randomly selected microscopic fields in the muscular layer of the stomach. The average number of myocytes in the muscular layer was significantly increased in *H pylori*-infected mice (n = 7) relative to control mice (n = 7) (*$P < .05$).

from *H pylori*-infected mice and uninfected control mice. miRNA expression profiling revealed that 47 out of 470 miRNAs were differentially expressed in the gastric tissues of *H pylori*-infected mice relative to control mice. Table 1 shows the 10 miRNAs with the greatest difference in expression. Interestingly, 3 of these 10 miRNAs—*miR-1*, *miR-133a*, and *miR-133b*—are known to be highly expressed in differentiated muscle tissues.[27,28] To confirm the microarray data, we performed quantitative RT-PCR for muscle-specific miRNAs. As shown in Figure 4*A*, the expression levels of *miR-1*, *miR-133a*, and *miR-133b* were markedly reduced in the stomachs of *H pylori*-infected mice.

With respect to other miRNAs that were differentially expressed in the stomach of *H pylori*-infected mice, *miR-206* is reported to be expressed in muscle tissues and down-regulated in estrogen receptor α-positive human breast cancer.[34,35] Recent studies have shown that *miR-217* and *miR-290* modulate cell senescence[36,37] and that *miR-122a* and *miR-146* function as novel tumor suppressors in cancer.[38,39]

We also examined the expression levels of muscle-specific miRNAs in clinical samples of the gastric antrum obtained by endoscopic biopsy from patients with or without *H pylori* infection. These samples included muscularis mucosae and/or myoblast cells in the gastric epithelia. As shown in Figure 4*B*, the expression levels of *miR-1*, *miR-133a*, and *miR-133b* were significantly decreased in patients positive for *H pylori* infection in comparison with those who were negative. These findings suggest that, in the muscularis mucosa and myoblast cells in gastric epithelia, as well as in the muscularis propria, muscle-specific miRNAs are down-regulated after *H pylori* infection.

Table 1. Summary of the Differentially Expressed miRNAs in the Stomach of *Helicobacter pylori*-Infected Mice

No.	miRNAs	Control	*H pylori*	Fold change
1	*miR-206*	4013.2	17.5	0.004
2	*miR-217*	357.0	8.7	0.024
3	*miR-216b*	289.7	8.8	0.030
4	*miR-216a*	94.7	6.5	0.069
5	*miR-1*	18,214.4	4905.5	0.269
6	*miR-290*	100.4	365.1	3.635
7	*miR-122a*	83.5	269.8	3.230
8	*miR-133b*	3197.9	1228.2	0.384
9	*miR-133a*	2806.8	1080.3	0.385
10	*miR-146b*	397.3	955.7	2.406

NOTE. Data in control and *H pylori* are average values of signal intensities of microarray analysis. Fold change represents the ratio of signal intensities of *H pylori*/control.
miRNA, microRNA.

Increased Expression of HDAC4 and SRF in the Stomachs of H pylori*-Infected Mice*

A recent study has shown that *miR-1* and *miR-133* specifically repress HDAC4 and SRF as their targets, respectively, thus contributing to the regulation of myoblast proliferation and differentiation.[29,40] Moreover, decreased expression of *miR-1* and *miR-133* has been observed in mouse and human cardiac hypertrophy.[32] These findings prompted us to investigate the expression levels of HDAC4 and SRF in the stomachs of *H pylori*-infected mice. The results of Western blotting shown in Figure 5*A* demonstrate that the expression of HDAC4 and SRF was increased in the stomachs of *H pylori*-infected mice relative to that in uninfected control mice, which is consistent with our finding that the expression levels of *miR-1* and *miR-133* were significantly reduced in the former.

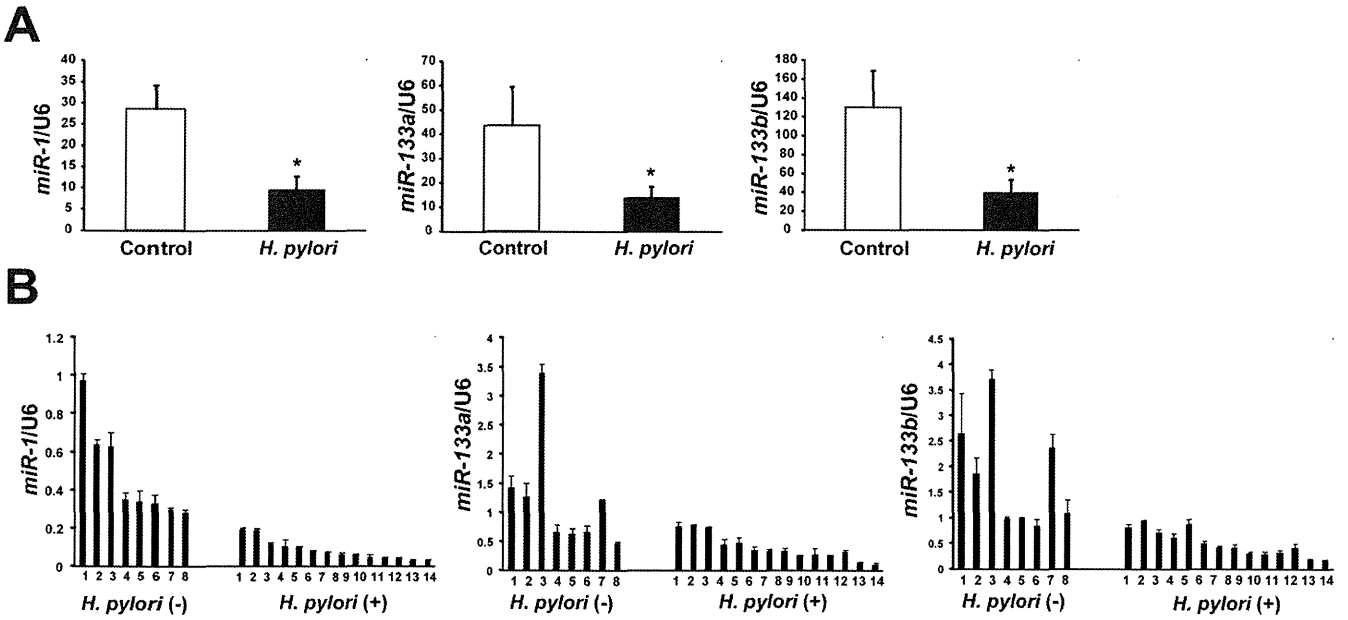

Figure 4. Expression levels of *miR-1* and *miR-133* in the stomachs of mice with *H pylori* infection. (*A*) Expression levels of *miR-1*, *miR-133a*, and *miR-133b* were analyzed by quantitative RT-PCR using the TaqMan microRNA assay in the stomachs of control (*open bar*; n = 7) and *H pylori*-infected mice (*solid bar*; n = 7). Expression levels were normalized against U6 RNA expression. All reactions were done in duplicate and expressed as mean ± standard deviation. *$P < .005$ compared with control. (*B*) Expression levels of *miR-1*, *miR-133a*, and *miR-133b* in clinical samples from the gastric antrum of patients with or without *H pylori* infection. Expression levels of *miR-1*, *miR-133a*, and *miR-133b* were significantly decreased in patients positive for *H pylori* infection (n = 14), relative to patients who were negative (n = 8) (*miR-1*, $P < .005$; *miR-133a*, $P < .05$; *miR-133b*, $P < .01$).

To confirm these findings, we conducted in vitro validation with C2C12 mouse myoblast cells, which had been established by D. Yaffe and O. Saxel.[41] C2C12 is a mesenchymal cell line widely used as an in vitro model of muscle cell differentiation. The muscle-specific miRNAs, *miR-1* and *miR-133*, are induced during the differentiation of C2C12, thus allowing analysis of the expression patterns of muscle-specific miRNAs.[28,29] We transfected C2C12 cells with anti-*miR-1* and anti-*miR-133a* inhibitors and assessed the expression levels of the miRNAs and their targets by quantitative RT-PCR and Western blotting, respectively. As shown in Figure 5*B*, the expression levels of *miR-1* and *miR-133a* were significantly reduced in C2C12 cells after transfection with anti-*miR-1* and anti-*miR-133a*. After knockdown of *miR-1* and *miR-133a*, up-regulation of their targets, HDAC4 and SRF, was observed, indicating that inhibition of *miR-1* and *miR-133a* induced activation of HDAC4 and SRF (Figure 5*B*).

Significant Increase of C2C12 Myoblast Cell Proliferation After Coculture With H pylori

The expression levels of muscle-specific miRNAs were analyzed in C2C12 myoblast cells after coculture with *H pylori*. As shown in Figure 6*A*, the expression levels of *miR-1*, *miR-133a*, and *miR-133b* were significantly reduced in C2C12 cells cocultured with *H pylori* relative to untreated C2C12 cells.

H pylori infection induces an inflammatory response with increased levels of proinflammatory cytokines such as IL-1β and TNF-α.[42] In addition, a recent study has shown that *miR-1* is down-regulated in IL-13-transgenic mice.[43] We investigated the expression levels of muscle-specific miRNAs after exogenous application of the cytokines IL-1β, IL-13, and TNF-α. Although *miR-1* expression was increased after application of TNF-α, and that of *miR-133a* was decreased after application of IL-13, the differences in miRNA expression induced by inflammatory cytokines were smaller than those induced by coculture with *H pylori*. There was no significant difference in the expression level of *miR-133b* after application of cytokines. These results suggest that down-regulation of the muscle-specific miRNAs *miR-1*, *miR-133a*, and *miR-133b* is caused mainly by *H pylori* itself, rather than inflammatory cytokines.

To further confirm the association between *H pylori* infection and proliferation of myoblast cells, C2C12 myoblasts in active phases of the cell cycle were assessed by immunostaining with Ki-67. As shown in Figure 6*B*, Ki-67-positive C2C12 cells were sufficiently observed in cells cocultured with *H pylori*, whereas there were a few positive cells in untreated cells. The number of Ki-67-positive cells was significantly increased upon coculture with *H pylori* in comparison with cells cultured alone (Figure 6*B*).

We also performed ELISA with the BrdU assay to confirm the proliferation of C2C12 cells. As shown in Figure 6*B*, the rate of BrdU incorporation was signifi-

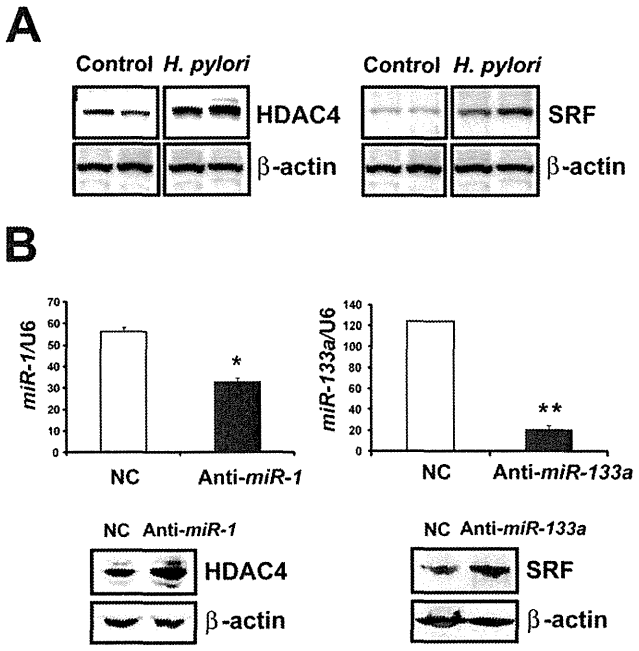

Figure 5. Expression levels of HDAC4 and SRF in the stomachs of control and *H pylori*-infected mice and C2C12 mouse myoblast cells. (*A*) Expression levels of HDAC4 and SRF in the stomachs of control and *H pylori*-infected mice were analyzed by Western blotting. β-Actin was used as the internal control. (*B*) C2C12 mouse myoblast cells were transfected with a negative control (*NC*; *open bar*) and inhibitors of *miR-1* and *miR-133a* (*solid bar*), and the expression levels of *miR-1* and *miR-133a* were confirmed by quantitative RT-PCR. Expression levels of HDAC4 and SRF were assessed by Western blotting. U6 RNA and β-actin were used as the internal controls. The quantitative RT-PCR reactions were done in triplicate and expressed as mean ± standard deviation. $^{*}P < .05$, $^{**}P < .01$ compared with NC.

cantly increased in C2C12 cells cocultured with *H pylori* relative to cells that were cultured alone, indicating that coculture with *H pylori* induces proliferation of C2C12 myoblasts.

Discussion

Little is known about the molecular mechanism underlying the pathogenesis of functional GI disorders, including FD. Our present results demonstrate that gastric emptying was accelerated in mice infected with *Helicobacter*. Moreover, the miRNA expression profile revealed that the muscle-specific miRNAs, *miR-1* and *miR-133*, were significantly down-regulated in the stomachs of *H pylori*-infected mice. We were able to show, for the first time, that chronic infection with *H pylori* induces down-regulation of muscle-specific miRNAs and hyperplasia of muscle cells in the stomach, which may lead to dysfunction of gastric emptying.

Although recently it has been reported that patients with FD frequently have delayed gastric emptying,[9-11] our study using a mouse model showed that gastric emptying was accelerated after chronic infection with *H pylori*. It should be borne in mind that the gastric emptying rate examined in the present study was liquid emptying, which is physiologically different from emptying of a solid meal. Therefore, the clinical implications of our results may not necessarily reflect the clinically more relevant gastric emptying of nutrient solids. Rapid gastric emptying of a liquid meal has been reported in patients with type 2 diabetes, even though that of nutrient solids may be normal or delayed.[44-46] Recent clinical studies indicate that a subset of FD patients may have impaired gastric accommodation, which may be associated with liquid gastric emptying.[47,48] These findings suggest that enhanced liquid emptying may be due to impaired gastric accommodation rather than increased muscular propulsive contraction. Impaired gastric accommodation may contribute to symptom generation such as early satiety observed in postprandial distress syndrome. Studies of the gastric emptying of solids will be necessary to further investigate the gastric motility disorders associated with *H pylori* infection.

We showed that reduced expression of muscle-specific miRNAs led to activation of their targets, HDAC4 and SRF, in the stomachs of *H pylori*-infected mice and also in C2C12 mouse myoblast cells. HDAC4, a transcriptional repressor of muscle gene expression, has been shown to inhibit muscle differentiation.[49,50] SRF is a transcription factor that binds to the serum response element, a sequence that mediates the transient response of many cellular genes to growth stimulation. Increased expression of HDAC4 and SRF is thought to impair the differentiation and proliferation of muscle cells, possibly causing hyperplasia of smooth muscle cells and disorders of gastric motility.

The in vitro study using C2C12 myoblast cells also demonstrated that coculture with *H pylori* induced down-regulation of *miR-1 and miR-133* and increased cell proliferation. Our data indicate that down-regulation of muscle-specific miRNAs is probably caused by *H pylori* itself rather than inflammatory cytokines. These findings raise the possibility that *H pylori* alone may elaborate mediators capable of enhancing the proliferation of muscle cells. Further studies will be necessary to clarify the systems responsible for regulation of miRNA expression and the proliferation and differentiation of muscle cells in the stomach through the direct and indirect effects of chronic *H pylori* infection.

We obtained samples of gastric tissue from mice in which *miR-1* had been knocked out by targeted deletion and examined them histologically by H&E staining.[30] Although this model is characterized by a striking myocyte cell-cycle abnormality that leads to hyperplasia of the heart, we observed no significant difference in thickness of muscular layer in the stomach between control mice and mice lacking *miR-1* (data not shown). This suggests that hyperplasia of myocytes in the stomach might require down-regulation of other muscle-specific

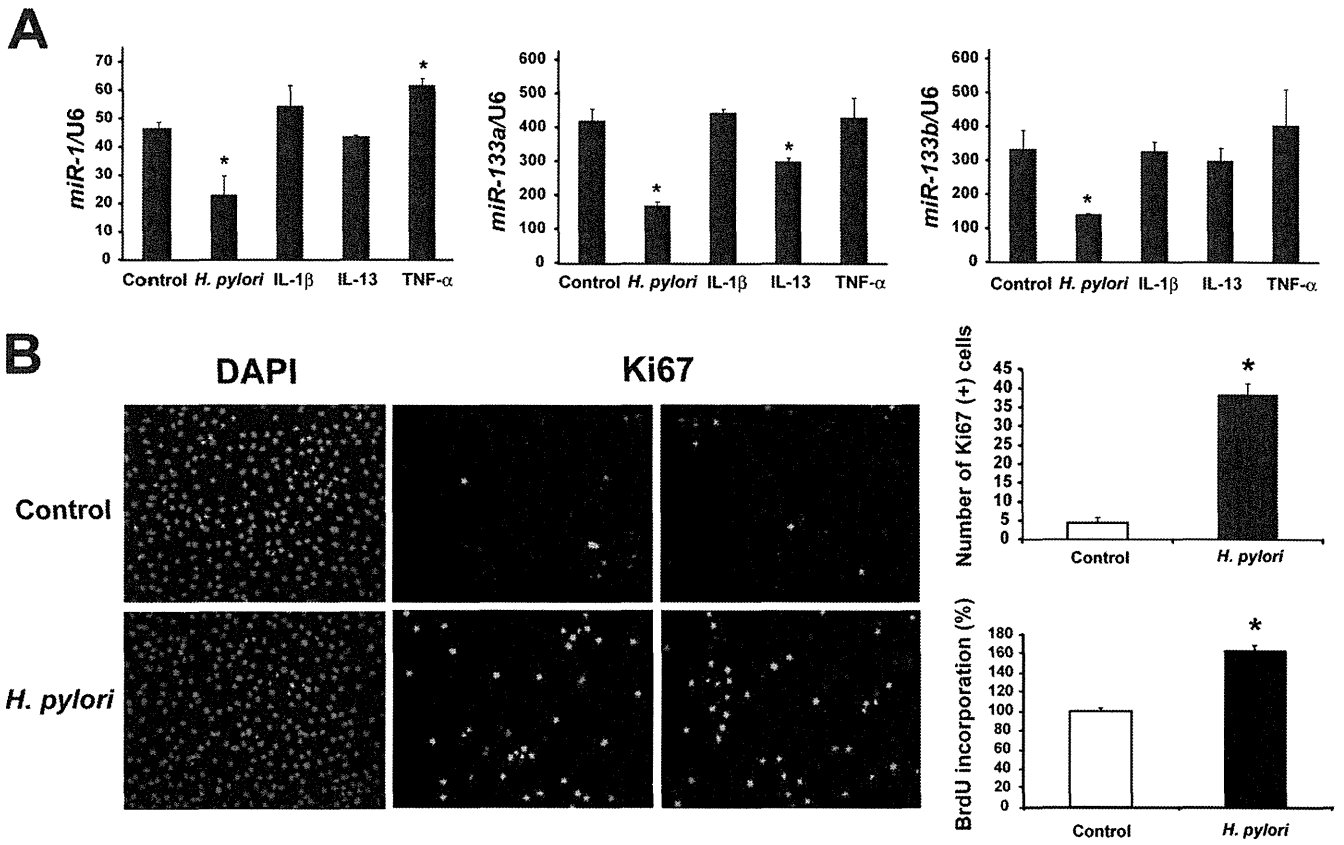

Figure 6. Expression levels of muscle-specific miRNAs and proliferation of C2C12 cells after coculture with *H pylori*. (*A*) Expression levels of *miR-1*, *miR-133a*, and *miR-133b* were analyzed by quantitative RT-PCR in C2C12 cells after coculture with *H pylori* and treatment with IL-1β, IL-13, and TNF-α. Expression levels were normalized against that of U6 RNA. All reactions were done in duplicate and expressed as mean ± standard deviation. *$P < .05$ compared with control. (*B*) Cell proliferation assay was performed by immunostaining of C2C12 cells for Ki-67 (original magnification: 200×). The cells were counterstained with DAPI. The numbers of Ki-67-positive cells among control cells and cells cocultured with *H pylori* were counted. BrdU assay was performed in C2C12 cells cocultured with *H pylori* at a multiplicity of infection of 50. BrdU incorporation was quantified by colorimetric ELISA assay. *$P < .001$ compared with control.

miRNAs such as *miR-133a* and *miR-133b*, in addition to disruption of *miR-1*.

A recent study has shown that expression of *miR-29a* was increased in the small bowel and colon tissues of patients with IBS. Increased expression of *miR-29a* suppresses the *glutamine synthetase* (*GLUL*) gene as its target and regulates intestinal membrane permeability in patients with IBS.[26] Moreover, Kapeller et al[25] have indicated an association of functional variants in the *miR-510* binding site of the *serotonin receptor type 3E* (*HTR3E*) gene with diarrhea-predominant IBS. These findings, taken together with our present results, suggest that miRNAs may play important roles in the pathogenesis of functional GI disorders and could be novel therapeutic targets in patients with FD and IBS.

In conclusion, chronic infection with *H pylori* has been shown to induce down-regulation of muscle-specific miRNAs with activation of their targets HDAC4 and SRF, which may lead to muscular layer hyperplasia and dysfunction of gastric emptying. Further human studies will be necessary to validate the association between aberrant expression of muscle-specific miRNAs and the gastric motility disorder associated with *H pylori* infection.

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Conflicts of interest

The authors disclose no conflicts.

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Enhanced bacterial efflux system is the first step to the development of metronidazole resistance in *Helicobacter pylori*

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ABSTRACT

Although metronidazole (Mtz) is an important component of *Helicobacter pylori* eradication regimens, it has been pointed out that the increasing use of Mtz may result in increase in the incidence of Mtz-resistant strains. The present study was designed to examine the initial mechanism of resistance acquisition of *H. pylori* to Mtz. After 10 Mtz-susceptible strains were cultured on plates containing sub-inhibitory concentrations of Mtz, the MIC of Mtz for 9 of the 10 strains increased to levels of the Mtz-resistant strains. In the Mtz-resistance-induced strains, the expression of the TolC efflux pump (*hefA*) was significantly increased under Mtz exposure, without the reduction of the Mtz-reductive activity. Our finding suggests that overexpression of *hefA* may be the initial step in the acquisition of Mtz resistance in *H. pylori*.

1. Introduction

Helicobacter pylori (*H. pylori*), a gram-negative bacterium, has been recognized to cause life-long infection of the gastric mucosa in billions of people worldwide, and is a major cause of peptic ulcers and also a key risk factor for gastric cancer and gastric MALT lymphoma [1].

Metronidazole (Mtz) has been demonstrated to exert activity against a wide variety of prokaryotic and eukaryotic pathogens, including *H. pylori*. Recently, Mtz resistance has been demonstrated among clinical isolates of *H. pylori*, especially those isolated from geographic regions with high Mtz usage [2]. It has been reported that Mtz resistance of *H. pylori* mainly arises from a decrease in NADPH nitroreductase (RdxA) activity which is a member of the Mtz-reductive activator enzyme in *H. pylori* [3]. By contrast, some clinical isolates of Mtz-resistant strains have been found to express functional RdxA and encode full-length *rdxA*, suggesting that other factors than RdxA may be involved in the resistance acquisition to Mtz [4,5]. We recently reported that overexpression of *H. pylori*-SOD (SodB) by amino acids mutation of Ferric uptake regulator (Fur) was associated with the development of Mtz resistance [6]. In addition, Amsterdam et al. reported that discharge of Mtz by active TolC homolog efflux pumps was associated with resistance [7]. Taken together these reports, it is supposed that there are three Mtz-resistance mechanisms in *H. pylori*.

Kim et al. suggested that since Mtz is widely prescribed for many bacterial infections, excessive use of this inexpensive drug may have contributed to the increase in the emergence of Mtz resistance [8]. In addition, it has been reported from *in vitro* studies that Mtz-susceptible *H. pylori* became Mtz-resistant after several passages on an agar plate containing sub-inhibitory concentrations of Mtz [9]. From these reports, it can be easily assumed that patients infected with Mtz-susceptible *H. pylori* may become Mtz-resistant under repeated exposure of sub-inhibitory concentrations of Mtz. Thus, to prevent the increase in the incidence of Mtz-resistant *H. pylori*, it is important to understand the first anti-Mtz response for resistance acquisition of Mtz-susceptible *H. pylori*. The present study was focused on the initial response to the development of Mtz resistance.

Generally, in bacteria, five families of multidrug efflux transporters have been described [10]. The RND family of the one of the five efflux systems has three components, namely, the inner membrane efflux proteins, a periplasmic efflux protein and an outer membrane efflux protein (the TolC or TolC homolog protein). In *H. pylori*, four RND families have been identified (HP0605 to HP0607; HefABC, HP0971 to HP0969; HefDEF, HP1327 to HP1329; HefGHI and HP1489 to HP1487) and the participation of these proteins in the development of multidrug resistance has been reported in *H. pylori* [7,11,12].

Abbreviations: Mtz, metronidazole; RdxA, NADPH nitroreducatse; MIC, minimum inhibitory concentration; RND, resistance nodulation-cell division; EPI, efflux pump inhibitor.

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The present study was designed to explore the transcriptional variation of the RND efflux pump systems in the initial phase during the *in vitro* development of Mtz resistance.

2. Materials and methods

2.1. Bacterial strains

H. pylori strains KS0309, KS0313, KS0317, KS0318, KS0329, KS0330, KS0371, KS0372, KS0381 and KS0391 were isolated from patients as Mtz-susceptible strains. The bacteria were cultured on Brucella Broth agar supplemented with 7% FBS and 5% Sheep Blood for 2 days at 37 °C, under micro-aerobic conditions maintained with AnaeroPack MicroAero (MITSUBISHIGAS, Tokyo, Japan).

2.2. In vitro induction of Mtz resistance

H. pylori strains susceptible to Mtz were used for *in vitro* induction of Mtz resistance, using a previously described method [13]. The Mtz-susceptible strains were cultivated under micro-aerobic conditions at 37 °C and transferred to agar plates containing one-half-, one- or two-times the MIC of Mtz, with 10 passages every 3 days.

2.3. DNA sequencing of rdxA

The complete *rdxA* gene was PCR-amplified with specific primers (forward 5′-GGAAAATCAATGAAATTTTTGGATCAAG, and reverse 5′-GATTTTGTTTAATCACAACCAAGTAATC) using Ex Taq DNA polymerase (TaKaRa, Ohtsu, Japan). The specific PCR products were direct-sequenced using the BigDye terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the deduced amino acid sequences were aligned using GENETYX Version 5.1.

2.4. Construction of the rdxA deletion mutant strain

The target-region gene cassette (5′*rdxA-chloramphenicol acetyltransferase* (*cat*)-3′*rdxA*) for construction of *rdxA* deletion mutant strain were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA), and then the sequences were determined (target-vector). The target-vector was electroporated into *H. pylori* ATCC700392, which was grown on 20 μg chloramphenicol (SIGMA) to obtain an *rdxA* deletion mutant strain of *H. pylori* ATCC700392.

2.5. Measurement of Mtz-reductive activity in H. pylori

After sonication (1.5 min at 25% power) of the bacteria, the resultant bacterial lysates were centrifuged. Mtz-reductive activity was measured spectrophotometrically with reduction of Mtz observed at 320 nm [6]. The reaction mixture contained Tris/acetate (100 mM Tris–HCl, 50 mM acetate), pH 7.0, 0.05 mM Mtz and 0.3 mM NADH, as described by Goodwin et al. [3].

2.6. Total RNA isolation and quantitative RT-PCR

After the bacteria (at an OD_{600} of 0.5) were incubated with 0.5 μg/mL Mtz for 5 h in the Brucella Broth supplemented with 7% FBS, the total RNA was isolated using a SV Total RNA Isolation system (Promega, Madison, WI). The RT reaction was performed using the PrimeScript RT reagent Kit (TaKaRa), in accordance with the manufacture's guideline. The real-time PCR amplification was performed using a SYBR Premix Ex taq Perfect Real Time Kit (TaKaRa) in a Thermal Cycler Dice Real Time System (TaKaRa). The primer sequences of the TolC homolog efflux pump genes constructed by Hirata et al. were used for the real-time RT-PCR [14]. The 16S rRNA gene was used as the internal control for the quantitative RT-PCR [15].

2.7. Measurement of the MIC of Mtz

Measurement of the MIC of Mtz for *H. pylori* strains was performed by the twofold agar dilution method, as described previously [16]. The bacteria (at an OD_{600} of 0.1) were inoculated on an agar plate containing twofold dilutions of Mtz (0.5–128 μg/mL). All the plates were incubated at 37 °C under micro-aerobic conditions, and the MIC values were determined. Phe-Arg-β-naphthylamide (PAβN), which has been shown to be active against the RND efflux pump system (efflux pump inhibitor; EPI), was used for the subsequent MIC determinations [17].

3. Results

3.1. In vitro resistance development by sequential subculturing in the presence of Mtz

Induction of Mtz-resistance was performed using 10 Mtz-susceptible strains for which the MIC of Mtz was 0.5–2 μg/mL (Table 1). After 10 passages of the Mtz-susceptible strains on the agar plates containing one-half-, one- or two-times the MICs of Mtz, the MICs increased to levels seen for the Mtz-resistant strains (MIC ⩾ 8 μg/mL) for nine of the 10 strains (8–64 μg/mL) (Table 1). Mtz resistance could not be induced only in the KS0318 strain (Table 1). Thus, nine of the 10 susceptible *H. pylori* strains became Mtz-resistant after 10 passages on the agar plate containing sub-inhibitory concentrations of Mtz.

3.2. Contribution of Mtz-reductive activity to the development in vitro of resistance to Mtz

To assess the contribution of Mtz-reductive activity in the Mtz-resistance-induced strains to the development of Mtz-resistance, the *rdxA* mRNA expression, RdxA amino acids sequences and Mtz-reductive activity after the development of Mtz resistance were examined. Fig. 1 shows that only the *rdxA* gene of KS0317 was deleted after the development of Mtz resistance, and the full-length *rdxA* gene was encoded in the remaining nine strains. The Mtz-reductive activity of KS0317, as well as that of the *rdxA*-deleted strain (ATCC700392*ΔrdxA*) used as negative control, was significantly decreased as compared with that of the parental strains (Table 2). Then, we investigated the amino acid mutations of RdxA in the Mtz-resistance-induced strains. Amino acid mutations of RdxA were observed in five of the strains (KS0372, KS0391, KS0309, KS0329 and KS0381) in which Mtz resistance was induced (Table 1). Furthermore, only two (KS0372 and

Table 1
MICs (μg/mL) of Mtz-susceptible strains before and after 10-passages for development of resistance.

Strain numbers	Parental strains (before 10 passages)	Mtz-resistance-induced strains (after 10 passages)
KS0309	1	64
KS0313	2	64
KS0317	1	64
KS0318	0.5	2
KS0329	1	16
KS0330	0.5	32
KS0371	1	64
KS0372	1	64
KS0381	1	64
KS0391	0.5	8

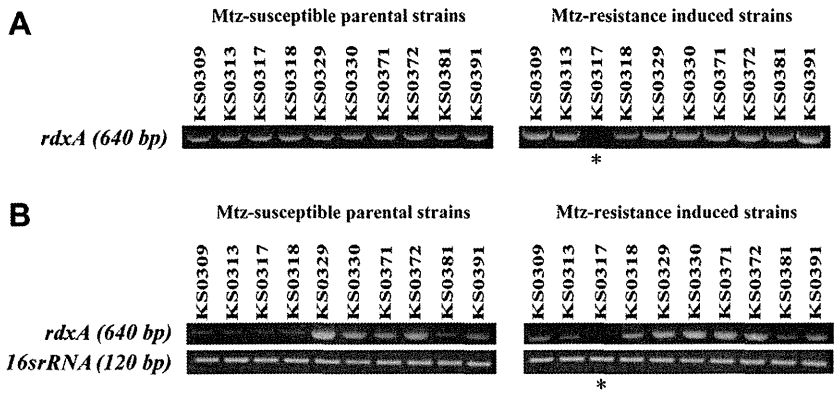

Fig. 1. *rdxA* expression after passaging for induction of Mtz-resistance *in vitro*. (A) The *rdxA* coding region in the Mtz-susceptible parental strains and Mtz-resistance-induced strains was detected by the PCR using a genome DNA as the template. (B) The mRNA expression of *rdxA* was detected by RT-PCR.

Table 2
Alterations of the Mtz-reductive activity and the RdxA amino acids after the development of Mtz resistance.

Strain numbers	Mtz sensitivity	Mtz-reductive activity (nmol/min/mg protein)	*p* values	Amino acids mutations of RdxA
ATCC700392	S	4.66 ± 1.47		
ATCC700392 ΔrdxA	R	2.55 ± 0.95	0.0023**	Delete
KS0317	Parental strain (S)	5.43 ± 2.20		
	Resistance induction (R)	2.59 ± 1.04	0.0034**	Delete
KS0372	Parental strain (S)	6.98 ± 2.64		
	Resistance induction (R)	4.21 ± 1.51	0.0149*	G189D
KS0391	Parental strain (S)	6.90 ± 2.41		
	Resistance induction (R)	3.04 ± 2.07	0.0022**	P44S E194K
KS0309	Parental strain (S)	5.63 ± 2.76		
	Resistance induction (R)	4.40 ± 1.09	0.232	R9I
KS0329	Parental strain (S)	4.88 ± 2.73		
	Resistance induction (R)	3.71 ± 1.63	0.286	H127P C14SW
KS0381	Parental strain (S)	5.77 ± 1.92		
	Resistance induction (R)	4.43 ± 2.01	0.166	M561 V111A A118T V192A
Nitroreductase from *Escherichia coli*		11.03 ± 0.38		

Asterisks indicate statistical significance for the comparison with each parental strain as determined by Student's *t*-test (**$p < 0.01$, *$p < 0.05$).

KS0391) of five strains showed a decrease of the Mtz-reductive activity (Table 2). These results indicate that reduced Mtz-reductive activity participated in the development of the Mtz resistance in three (KS0317, KS0372 and KS0391) of nine strains, and Mtz resistance in the remaining six strains (KS0309, KS0313, KS0329, KS0330, KS0371 and KS0381) was conferred by a factor(s) other than the reduced Mtz-reductive activity.

3.3. Contribution of the bacterial efflux pumps to the development of Mtz resistance

To assess the factors associated with the induction of Mtz resistance, we focused on the expression of the TolC homolog efflux pump genes (*hefA*, *hefD*, *hefG* and HP1489). The *hefA* expression was scarcely induced in any of the Mtz-susceptible parental strains under Mtz exposure, except KS0317 and KS0313 (Fig. 2A). After the development of Mtz resistance, *hefA* expression was significantly induced under Mtz exposure in all strains except KS0391 (Fig. 2B). In particular, the *hefA* expression was significantly induced in six Mtz-resistance-induced strains in which the Mtz-reductive activity remained under Mtz exposure (Fig. 2B). In addition, the *hefA* expression was significantly increased after the acquisition of Mtz resistance in KS0317 and KS0391, even in the absence of Mtz exposure (Fig. 2C, D).

The *hefG* expression was not induced even after the acquisition of the Mtz resistance in any of the strains (data not shown). Thus, *hefD* and HP1489 expressions were not detected (data not shown).

3.4. Effect of inhibition of the efflux pump on the Mtz-resistance-induced strains

To assess the effect of EPI on the induction of Mtz resistance, we measured the MICs in the presence of 40 mg/L PAβN without growth inhibition. The MICs of Mtz for the KS0309, KS330, KS0371 and KS0381 strains, which retained the ability to reduce Mtz were decreased in the presence of 40 mg/L of PAβN (Table 3). In contrast, the MICs of KS0317 and KS0391 which exhibited decreased Mtz-reductive activity did not further decrease in the presence of PAβN (Table 3).

4. Discussion

Recently, in gram-negative bacteria, the RND efflux systems contributing to antibiotic resistance have been described from a number of clinically important bacteria, including *H. pylori* [7,18,19]. In the present study, the Mtz-resistance-induced strains without the reduced Mtz-reductive activity showed overexpression of *hefA* (Table 2 and Fig. 2). Moreover, the Mtz-resistance-induced strains both with the decreased Mtz-reductive activity and the enhanced expression of *hefA* were also identified (Table 2 and Fig. 2). From these results, it is assumed that *H. pylori* develop resistance to Mtz by following two steps; the first step is overexpression of *hefA* to prevent Mtz accumulation in the bacterial cell, and then, further excess Mtz exposure could cause amino acids

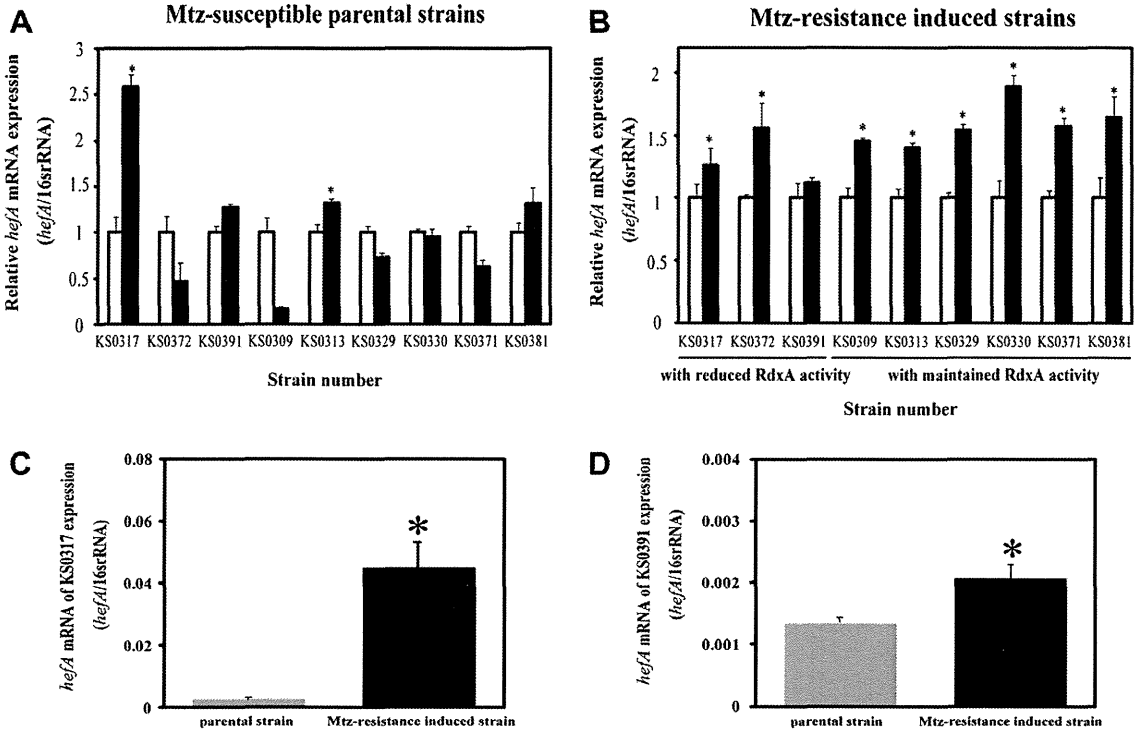

Fig. 2. Alterations in the expression of the *hefA in H. pylori* after passaging for induction of Mtz-resistance *in vitro*. Expression of *hefA* mRNA in the Mtz-susceptible parental strains (A) and Mtz-resistance-induced strains (B) exposed to 0 (white) and 0.5 μg/mL (black) Mtz was measured by quantitative RT-PCR. The basal level expression of *hefA* mRNA in the KS0317 (C) and KS0391 (D) strains were measured by quantitative RT-PCR. Results are means ± SE of three independent assays. Asterisks indicate statistical significance as determined by Student's *t*-test (*$p < 0.05$).

Table 3
Effect of efflux pump inhibitor on MICs (μg/mL) of Mtz-resistance induced strains.

Strain numbers	Without PAβN	With 40 mg/L PAβN
KS0317	64	64
KS0372	64	16*
KS0391	8	16
KS0309	64	16*
KS0313	64	64
KS0329	16	16
KS0330	32	16*
KS0371	64	32*
KS0381	64	32*

Asterisks of each strain indicate decrease of MICs of Mtz.

mutation of enzymes involved in Mtz-reductive activation such as RdxA.

In *H. pylori*, it has been reported that a ferredoxin-like protein (HP1508; FdxB), NADH flavin oxidoreductase (HP0642; FrxA) and NADPH nitroreductase (HP0954; RdxA) are involved in the Mtz-reductive activity, and Mtz resistance is known to be conferred by the inactivation of these genes [20]. Since the majority of clinically isolated Mtz-resistant strains show amino acid mutations of RdxA, the inactivation of the Mtz-reductive activity by the RdxA mutation is considered as the most important mechanism underlying the development of Mtz resistance [3,21]. However, after the 10 passages under sub-inhibitory concentrations of Mtz, only three strains (KS0317, KS0372 and KS0391) showed inactivation of the Mtz-reductive activity (Table 2), indicating that the Mtz resistance in the remaining strains (KS0309, KS0313, KS0329, KS0330, KS0371 and KS0381) was dependent on *hefA* overexpression.

Interestingly, although the *hefA* transcription was not induced by Mtz in the Mtz-susceptible parental strains, overexpression of *hefA* began to be observed only in the Mtz-resistance-induced strains (Fig. 2). Therefore, it was considered that the regulatory systems of *hefA* in the Mtz-resistance-induced strains had changed. The mechanisms underlying overexpression of *hefA* have been shown to fall into four groups; (i) mutations in the local repressor gene, (ii) mutations in a global regulatory gene, (iii) mutations in the promoter region, (iv) insertion elements upstream of the efflux gene [19].

The efflux pump inhibitor (EPI) is expected to increase the intracellular concentration of antibiotics that are expelled by efflux pumps, decrease the intrinsic bacterial resistance to antibiotics, reverse the acquired resistance associated with efflux pump overexpression [22]. The MICs of KS0309, KS330, KS0371, KS0372 and KS0381, all of which showed the *hefA* overexpression, were decreased by 50–75% (Table 3). On the other hand, although KS0313 and KS0329 showed the *hefA* overexpression and Mtz-reductive activity, EPI did not reduce the MICs for these strains (Table 3), suggesting the following two possibilities; (i) the inhibitory specificity of EPI is different among applied antimicrobial agents. As reported by Payot et al., the EPI effect was more efficient in decreasing erythromycin resistance than in decreasing quinolone resistance in *Campylobacter coli* [17]. It has been thought that different antibiotics may have different binding sites on the efflux pumps and that the inhibition by EPI could occur only by specific binding [23]. Therefore, the magnitude of the inhibitory effect of EPI is strongly dependent on its substrates, namely antimicrobials. (ii) Recently, overexpression of Fe-SOD was reported to be associated with Mtz resistance in the protozoan parasite *Entamoeba histolytica* [24]. In addition, we recently reported that overexpression of SodB by amino acids mutation of Fur (C78Y and P114S), which is also associated with the development of Mtz resistance [6]. However, in the present study, the amino acids mutation of Fur in the Mtz-resistance-induced strains was not detected (data not shown). Based on these results, it is considered that there is a novel and poorly known mechanism associated with Mtz resistance development.

The MICs of KS0317 and KS0391 which show a decrease in the Mtz-reductive activity and overexpression of *hefA* were not reduced by EPI (Table 3), suggesting the major contribution of inactivation of RdxA in the development of Mtz resistance in *H. pylori*.

Recently, it has been reported that aspirin increases the endocellular concentrations of antimicrobials and increases the susceptibility of *H. pylori* to Mtz [25]. The development of high-specific EPI might be effective for the prevention of development of Mtz-resistance.

In conclusion, overexpression of *hefA*, which is Mtz-responsive, is the initial step in the acquisition of Mtz resistance in *H. pylori*. During antimicrobial therapy with Mtz, it is important to prevent the evolution of Mtz resistance, by ensuring that Mtz use is limited to a reasonable amount for bactericidal action to be existed over the short term before the overexpression of *hefA*.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Etiological difference between ultrashort- and short-segment Barrett's esophagus

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Abstract
Background Barrett's esophagus has been divided into three categories based on the extent of the metaplasia: long-segment (LSBE), short-segment (SSBE), and ultrashort-segment Barrett's esophagus (USBE). While both LSBE and SSBE are thought to be induced by gastroesophageal reflux, the etiology of USBE is still unclear.
Methods We conducted a case–control study to identify the differences in the pathogenesis between SSBE and USBE in a hospital-based population. The endoscopic findings and clinical factors of 199 patients with short-segment endoscopically suspected esophageal metaplasia (SS-ESEM) and 317 patients with ultrashort-segment ESEM (US-ESEM) were compared with those of 199 and 317 age- and gender-matched patients without ESEM.
Results The severity of gastric mucosal atrophy was marginally associated with the presence of US-ESEM [odds ratio (OR) 1.20, 95% confidence interval (CI) 0.98–1.46, $p = 0.08$], but not with that of SS-ESEM. On the other hand, the presence of gallstones and that of severe reflux esophagitis were associated with the presence of SS-ESEM (OR 2.19, 95% CI 1.21–3.98; OR 1.72, 95% CI 1.08–2.75), but not with that of US-ESEM. Presence of gastric corpus atrophy without gallstones was associated with the presence of US-ESEM, but not with that of SS-ESEM.
Conclusions Presence of gastric corpus atrophy was associated with an increased likelihood of the presence of US-ESEM, whereas the presence of gallstones was associated with an increased likelihood of the presence of SS-ESEM, suggesting difference in etiology between US- and SS-ESEM.

Keywords Short-segment Barrett's esophagus · Gastric corpus atrophy · Gallstone

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Introduction

Barrett's esophagus (BE) is a premalignant lesion that is detected in the majority of patients with esophageal adenocarcinoma. The risk of esophageal adenocarcinoma is 30–40 times higher in patients with BE than in patients without BE. The incidence of esophageal adenocarcinoma is rising rapidly in Western countries [1–3]. In Japan, esophageal adenocarcinoma accounts for only 1% of all esophageal carcinomas, although the incidence has been gradually increasing [4]. The known risk factors for BE include advanced age, male sex, white race, symptoms of reflux, and obesity [5].

BE has been divided into three categories on the basis of the length: long-segment (LSBE), short-segment (SSBE), and ultrashort-segment Barrett's esophagus (USBE). The prevalence of SSBE is known to be significantly higher than that of LSBE [6]. The prevalence of SSBE has been estimated to be in the range of 6.0–20.6%, while that of LSBE has been estimated to be in the range of 0.2–0.4% in Japan [4, 7]. USBE is thought to be a premalignant lesion for adenocarcinoma arising within 1 cm of the gastroesophageal junction. The association of esophagogastric junctional cancers with gastric mucosal atrophy or *Helicobacter pylori*

(*H. pylori*) infection is still controversial [8]. According to a meta-analysis conducted by the Eurogast Study Group, while there is no global association between *H. pylori* infection and the development of junctional cancers, a strong tendency towards a negative association between the two conditions has been reported in studies from the Western world, whereas a positive association has been reported in studies from the East [9].

We recently conducted a case–control study and reported that the presence of gallstones was associated with an increase in the risk of BE [10]. In addition, a positive association was also observed between the presence of gastric corpus atrophy and that of BE [10]. However, several studies have shown an inverse association between the presence of gastric mucosal atrophy and of *H. pylori* infection and that of BE [11]. Since many of patients with USBE were included in cohorts of BE in the previous study, the authors re-evaluated the association between the presence of gastric mucosal atrophy and the length of BE by separating USBE from SSBE.

The present study was designed to investigate the difference in etiology between USBE and SSBE through reanalysis of a previously generated database for an age- and gender-matched case–control study conducted by us. Here, the authors demonstrated the existence of a positive association between the presence of gastric corpus atrophy and that of USBE, but not of SSBE.

Methods

Definition of ultrashort-, short-, and long-segment ESEM

The authors investigated the epidemiology of endoscopically suspected esophageal metaplasia (ESEM), which is considered as an endoscopic finding consistent with BE pending histological confirmation according to the Montreal definition [12]. The presence/absence of ESEM was examined in the lower portion of the esophagus, including the esophagogastric junction (EGJ), during inflation of the esophagus. The EGJ was defined as the oral end of the fold continuous with the gastric lumen [13], or the anal end of the palisade vessels, because the veins in the lower part of the esophagus are distributed uniformly, running longitudinally and in a parallel fashion in the lamina propria [14–18]. In our hospital, using standard endoscopes (GIF-H260, Olympus Medical Systems, Tokyo, Japan), since endoscopists were instructed to observe the patient's EGJ during deep inhalation, the distal end of the lower esophageal palisade vessels were recorded more clearly than the proximal margin of the gastric folds in patients with ESEM. Therefore, most patients were evaluated by using the distal end of the lower esophageal palisade vessels. The squamo-columnar junction (SCJ) was defined by a clear change in the color of the mucosa. ESEM was defined as the area between the SCJ and the EGJ [14].

Ultrashort-segment ESEM (US-ESEM) is defined as ESEM over a maximum length of less than 1 cm. Long-segment ESEM (LS-ESEM) is defined as ESEM over a circumferential length of more than 3 cm. Short-segment ESEM (SS-ESEM) is defined as ESEM over a length of the esophagus that is intermediate between ultrashort- and long-segment ESEM. The lengths of ESEM were determined retrospectively by three gastrointestinal endoscopists; cases that were difficult to categorize into one of the three aforementioned categories because their lengths were around 1 or 3 cm had been already excluded in the previous study.

Study population

The authors had previously conducted a hospital-based age- and gender-matched case–control study for determining the risk factors associated with ESEM. The design and the primary results of the case–control study have been reported [10]. Among the 4945 patients who underwent esophagogastroduodenoscopy (EGD) at Keio University Hospital between November 2007 and April 2008, 528 patients with ESEM were allocated to the case group, while 528 age- and gender-matched (frequency-matching) subjects without ESEM were allocated to the control group.

Among the 528 patients with ESEM, 317 patients (60%) with US-ESEM, 199 patients (38%) with SS-ESEM, and 12 patients (2%) with LS-ESEM were identified. The data of the 12 patients with LS-ESEM were excluded from the present analysis because of the small number of subjects. The 317 and 199 age- and gender-matched patients without ESEM were allocated to the respective control groups (Fig. 1). The characteristics of the subjects are shown in Tables 1 and 2. Findings detected on EGD (hiatus hernia, reflux esophagitis, and gastric mucosal atrophy) and clinical factors, including alcohol habit, smoking habit, *H. pylori* infection status, body mass index (BMI), and presence/absence of hypertension, diabetes mellitus, dyslipidemia, and gallstones were compared between each of the case and control groups.

The severity of gastric mucosal atrophy was assessed endoscopically by the Kimura–Takemoto classification of the atrophic pattern [14, 19, 20]. This classification divides the severity of gastric mucosal atrophy into seven types (C-0, C-1, C-2, C-3, O-1, O-2, and O-3) according to the location of the atrophic border as detected by endoscopy, as follows: C-0, absence of atrophy; C-1, pyloric mucosal atrophy; C-2, atrophy extending over the lesser curvature of the lower third of the stomach; C-3, the atrophy extending over the lesser curvature of the middle third of the stomach; O-1, border of the atrophy between the lesser

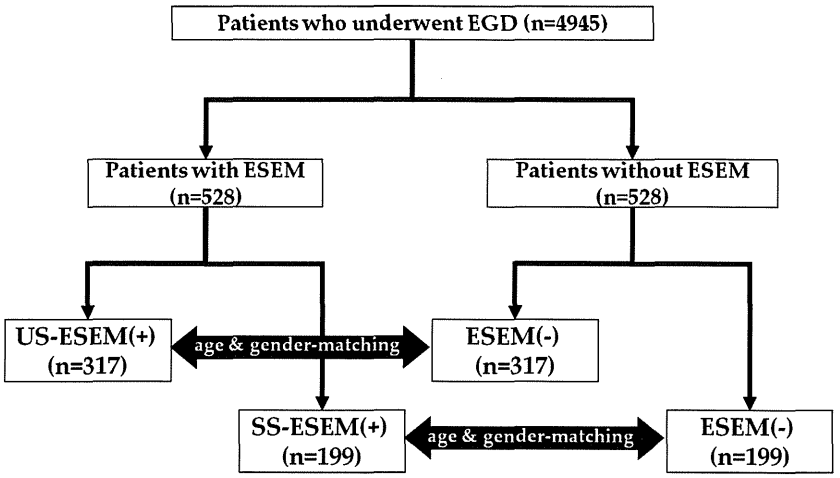

Fig. 1 Flow diagram of the case–control study. Among 4945 patients who underwent esophagogastroduodenoscopy (EGD) at Keio University Hospital between November 2007 and April 2008, 528 with ESEM and 528 age- and gender-matched subjects without ESEM were selected [10]. Of the 528 patients with ESEM, 317 were assigned to the US-ESEM group, and of the 528 subjects without ESEM, 317 age- and gender-matched subjects were assigned to the non-ESEM control group. In the same way, of the 528 subjects with ESEM, 199 were assigned to the SS-ESEM group, and of the 528 subjects without ESEM, 199 age- and gender-matched subjects were assigned to the non-ESEM control group. The data obtained from the medical records and EGD images of these 1032 cases and controls were reviewed in this study

curvature and anterior wall of the stomach; O-2, atrophy within the limits of the anterior wall of the stomach; O-3, atrophic area extending from the anterior wall to the major curvature of the stomach. Using this classification, we divided the severity of the gastric mucosal atrophy into four grades: none (C-0), mild (C-1 and C-2), moderate (C-3 and O-1), and severe (O-2 and O-3). The presence/absence of hiatus hernia was examined by valvular appearance of the cardia visualized from below using the retroflexed endoscope during gastric inflation [21]. Reflux esophagitis was defined as the presence of gross mucosal injury, ranging from red longitudinal streaks with associated friability, to erosion or ulceration in the distal esophagus or breakage in the lower portion of the esophagus. The severity of reflux esophagitis was graded according to the Los Angeles classification [22].

The alcohol consumption status was defined as a positive/negative history of daily alcohol consumption. The smoking status was defined as a positive/negative history of smoking cigarettes. The presence of *H. pylori* infection was defined as a history of *H. pylori* infection, including both pre- and post-eradication. The presence of *H. pylori* infection was detected by serological test, ^{13}C-urea breath test, culture or histology of the gastric mucosal biopsy specimen [23]. Obesity was defined as a BMI of more than 25 kg/m^2. Hypertension was defined as systolic blood pressure of over 140 mmHg and/or diastolic blood pressure of over 90 mmHg, or a history of use of antihypertensive drugs for the treatment of hypertension. Diabetes mellitus was defined as a serum hemoglobin A_1c (HbA_1c) value of over 6.5% or a history of use of antidiabetic agents. Dyslipidemia was defined as a serum level of low-density lipoprotein cholesterol (LDL-C) of over 140 mg/dl, high-density lipoprotein cholesterol (HDL-C) of under 40 mg/dl, a fasting triglyceride level of over 150 mg/dl, or a history of use of lipid-lowering agents. The presence/absence of gallstones was determined by abdominal CT or ultrasonography, including subjects who underwent cholecystectomy.

Since the clinical factors could not be determined for all of the subjects from the medical records, the associations between the clinical factors and the presence of ESEM were analyzed by using data from a proportion of the subjects for whom the data were available. The study was performed in accordance with the Declaration of Helsinki.

Statistical analyses

The associations of the endoscopic findings or clinical factors with the presence of ESEM were evaluated by a logistic regression model with adjustment for age and gender. All of the statistical analyses were performed using the SPSS, Statistics version 17.0 for Windows (SPSS Japan, Tokyo, Japan). Two-sided p values of less than 0.05 were considered to be indicative of statistical significance.

Results

Association of endoscopic findings and clinical factors with the presence of US-ESEM

Using a logistic regression model with adjustments for age and gender, we detected a strong association between the

Table 1 Characteristics of the subjects with ultrashort-segment ESEM and without ESEM

	US-ESEM(+) cases (n = 317)	ESEM(−) controls (n = 317)
Age		
Mean ± SD (year)	63.8 ± 12.7	63.8 ± 12.7
20–29	4 (1.3%)	4 (1.3%)
30–39	15 (4.7%)	15 (4.7%)
40–49	23 (7.3%)	23 (7.3%)
50–59	55 (17.4%)	55 (17.4%)
60–69	98 (30.9%)	98 (30.9%)
70–79	102 (32.2%)	102 (32.2%)
80–89	20 (6.3%)	20 (6.3%)
Gender		
Male	217 (68.5%)	217 (68.5%)
Female	100 (31.5%)	100 (31.5%)
Hiatus hernia		
Presence	265 (83.6%)	190 (59.9%)
Absence	52 (16.4%)	127 (40.1%)
Reflux esophagitis		
None	303 (95.6%)	304 (95.9%)
Grade A	10 (3.2%)	8 (2.5%)
Grade B	4 (1.3%)	4 (1.3%)
Grade C	0 (0%)	1 (0.3%)
Grade D	0 (0%)	0 (0%)
Gastric mucosal atrophy		
None	66 (20.8%)	66 (20.8%)
Mild	136 (42.9%)	167 (52.7%)
Moderate	96 (30.3%)	69 (21.8%)
Severe	19 (6.0%)	15 (4.7%)
Clinical factors[a] [no./total no. (%)]		
Alcohol habit	87/225 (38.7%)	73/209 (34.9%)
Smoking habit	110/225 (48.9%)	97/212 (45.8%)
H. pylori	57/86 (66.3%)	50/69 (72.5%)
Overweight	44/172 (25.6%)	36/189 (19.0%)
BMI ± SD (kg/m^2)	22.5 ± 4.0	22.3 ± 3.5
Hypertension	107/207 (51.7%)	87/177 (49.2%)
Diabetes	47/232 (20.3%)	47/224 (21.0%)
Dyslipidemia	85/142 (59.9%)	71/122 (58.2%)
Gallstones	47/218 (21.6%)	43/233 (18.5%)

US-ESEM ultrashort-segment endoscopically suspected esophageal metaplasia, *BMI* body mass index

[a] Some of the clinical information could not be collected from the subjects' medical records; therefore, the total numbers of each collected data are indicated in the "Clinical factors" section

Table 2 Characteristics of the subjects with short-segment ESEM and without ESEM

	SS-ESEM(+) cases (n = 199)	ESEM(−) controls (n = 199)
Age		
Mean ± SD (year)	67.6 ± 11.2	67.6 ± 11.2
20–29	2 (1.0%)	2 (1.0%)
30–39	1 (0.5%)	1 (0.5%)
40–49	10 (5.0%)	10 (5.0%)
50–59	35 (17.6%)	35 (17.6%)
60–69	47 (23.6%)	47 (23.6%)
70–79	83 (41.7%)	83 (41.7%)
80–89	21 (10.6%)	21 (10.6%)
Gender		
Male	131 (65.8%)	131 (65.8%)
Female	68 (34.2%)	68 (34.2%)
Hiatus hernia		
Presence	169 (84.9%)	131 (65.8%)
Absence	30 (15.1%)	68 (34.2%)
Reflux esophagitis		
None	179 (89.9%)	192 (96.5%)
Grade A	6 (3.0%)	3 (1.5%)
Grade B	12 (6.0%)	2 (1.0%)
Grade C	1 (0.5%)	2 (1.0%)
Grade D	1 (0.5%)	0 (0%)
Gastric mucosal atrophy		
None	34 (17.1%)	36 (18.1%)
Mild	88 (44.2%)	95 (47.7%)
Moderate	55 (27.6%)	56 (28.1%)
Severe	22 (11.1%)	12 (6.0%)
Clinical factors[a] [no./total no. (%)]		
Alcohol habit	51/138 (36.9%)	54/145 (37.2%)
Smoking habit	58/140 (41.4%)	58/148 (39.2%)
H. pylori	47/63 (74.6%)	34/44 (77.3%)
Overweight	31/114 (27.2%)	28/119 (23.5%)
BMI ± SD (kg/m^2)	22.5 ± 4.1	22.7 ± 3.3
Hypertension	70/135 (51.9%)	59/124 (47.6%)
Diabetes	26/152 (17.1%)	16/141 (11.3%)
Dyslipidemia	38/75 (50.7%)	46/83 (55.4%)
Gallstones	37/144 (25.7%)	21/151 (13.9%)

SS-ESEM short-segment endoscopically suspected esophageal metaplasia, *BMI* body mass index

[a] Some of the clinical information could not be collected from the subjects' medical records; therefore, the total numbers of each collected data are indicated in the "Clinical factors" section

presence of hiatus hernia and that of US-ESEM [odds ratio (OR) 3.62, 95% confidence interval (CI) 2.47–5.32, $p < 0.001$]. The severity of gastric mucosal atrophy was marginally associated with the presence of US-ESEM (OR 1.20, 95% CI 0.98–1.46, $p = 0.08$). Neither the presence of reflux esophagitis nor that of gallstones was associated with the presence of US-ESEM. Multivariate logistic regression analysis with adjustments for age, gender, presence/absence of hiatus hernia, and severity of gastric mucosal atrophy revealed that the presence of hiatus hernia was independently associated with the presence of US-ESEM (Table 3).

Table 3 Association of endoscopic findings and clinical factors with the presence of ESEM: odds ratio (95% CI) from logistic regression analysis

	US-ESEM (n = 317)		SS-ESEM (n = 199)	
	Age- and gender-adjusted analysis	Multivariate analysis[a]	Age- and gender-adjusted analysis	Multivariate analysis[b]
Hiatus hernia	3.62 (2.47–5.32)***	3.60 (2.45–5.28)***	2.98 (1.83–4.88)***	2.34 (1.31–4.19)***
Reflux esophagitis	0.97 (0.57–1.63)		1.72 (1.08–2.75)*	1.36 (0.81–2.27)
Gastric mucosal atrophy	1.20 (0.98–1.46)▲	1.17 (0.95–1.44)	1.17 (0.92–1.48)	
Alcohol habit	1.15 (0.74–1.78)		0.93 (0.53–1.62)	
Smoking habit	1.15 (0.75–1.76)		1.05 (0.63–1.77)	
H. pylori	0.73 (0.36–1.47)		0.80 (0.31–2.07)	
Overweight	1.47 (0.89–2.43)		1.31 (0.71–2.40)	
Hypertension	1.21 (0.79–1.85)		1.23 (0.74–2.04)	
Diabetes	0.97 (0.61–1.55)		1.62 (0.82–3.19)	
Dyslipidemia	1.21 0.73–2.02)		0.79 (0.42–1.50)	
Gallstones	1.25 (0.78–1.99)		2.19 (1.21–3.98)*	2.18 (1.19–4.00)*

CI confidence interval, *US-ESEM* ultrashort-segment endoscopically suspected esophageal metaplasia, *SS-ESEM* short-segment endoscopically suspected esophageal metaplasia

▲ $p < 0.1$, *$p < 0.05$, ***$p < 0.001$

[a] Adjustment for age, gender, presence/absence of hiatus hernia, and severity of gastric mucosal atrophy

[b] Adjustment for age, gender, presence/absence of hiatus hernia, presence/absence of reflux esophagitis, and presence/absence of gallstones

Association of endoscopic findings and clinical factors with the presence of SS-ESEM

Using a logistic regression model with adjustments for age and gender, we detected a strong association between the presence of hiatus hernia and that of SS-ESEM (OR 2.98, 95% CI 1.83–4.88). The presence of reflux esophagitis was also significantly associated with the presence of SS-ESEM (OR 1.72, 95% CI 1.08–2.75, $p = 0.02$). No association was observed between the severity of gastric mucosal atrophy and that of SS-ESEM (OR 1.17, 95% CI 0.92–1.48, $p = 0.21$). Among the clinical factors, only the presence of gallstones was significantly associated with the presence of SS-ESEM (OR 2.19, 95% CI 1.21–3.98, $p = 0.01$). Multivariate logistic regression analysis with adjustment for age, gender, presence/absence of hiatus hernia, presence/absence of reflux esophagitis, and presence/absence of gallstones revealed that the presence of hiatus hernia and that of gallstones were independently associated with the presence of SS-ESEM (Table 3).

Association of gastric corpus atrophy with/without gallstones with US-ESEM or SS-ESEM

To assess the relationship among the presence of gastric corpus atrophy, gallstones, and US/SS-ESEM, the subjects were re-allocated into four groups, as follows: subjects with neither gastric corpus atrophy nor gallstones, subjects with gastric corpus atrophy but without gallstones, subjects with gallstones but without gastric corpus atrophy, and subjects with both gastric corpus atrophy and gallstones. The association of each set of clinical factors with the presence of US/SS-ESEM was analyzed by using a logistic regression model with adjustment for age and gender (Table 4).

The results of this additional analysis could reveal the association of the presence of gastric corpus atrophy with US-ESEM more precisely. The presence of neither gastric corpus atrophy nor gallstones was negatively associated with the presence of US-ESEM (OR 0.61, 95% CI 0.41–0.89, $p = 0.01$) and marginally associated with the presence of SS-ESEM (OR 0.63, 95% CI 0.39–1.02, $p = 0.06$). Presence of gastric corpus atrophy without gallstones was associated with the presence of US-ESEM (OR 1.56, 95% CI 1.01–2.40, $p = 0.045$), but not with that of SS-ESEM (OR 0.92, 95% CI 0.54–1.56, $p = 0.76$). Presence of gallstones without gastric corpus atrophy was associated with neither the presence of US-ESEM nor that of SS-ESEM. Presence of both gastric corpus atrophy and gallstones was strongly associated with the presence of SS-ESEM (OR 2.99, 95% CI 1.26–7.06, $p = 0.01$) and marginally associated with the presence of US-ESEM (OR 1.99, 95% CI 0.94–4.21, $p = 0.07$).

Discussion

The present study showed that the risk factors for US-ESEM and SS-ESEM were different. The presence of