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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Neutrophil-activating Protein Polymorphism of *Helicobacter pylori* Determines the Host Risk of Dyspepsia

Helicobacter pylori infection is an accepted cause of dyspepsia, according to the Rome IV criteria,[1] because its eradication significantly resolves dyspepsia; however, no *H pylori* virulence factor that can cause dyspepsia has been identified. Here, we identified a polymorphism in *H pylori* neutrophil-activating protein A (NapA) closely associated with dyspepsia occurrence.

There is evidence that chronic gastric inflammation caused by reactive oxygen species (ROS) is associated with dyspepsia severity in individuals with *H pylori* infection.[2] ROS production is characteristic during innate immune responses to *H pylori*, and *H pylori* uses various enzymes to counteract ROS, which facilitate the establishment of persistent infection.[3,4] We investigated the sequences of genes encoding 2 antioxidant proteins, NapA and alkyl hydroperoxide reductase (AhpC),[5] in patient-derived *H pylori* strains.

Patients with and without dyspepsia (n = 33 and 88, respectively) were enrolled (Supplementary Methods), and *H pylori* strains were isolated from their stomachs (Supplementary Figure 1*A*). The baseline characteristics of participants are presented in Supplementary Table 1. *H pylori* sequencing analysis revealed 2 polymorphisms in each of *napA* (encoding amino acids 70 and 73) and *ahpC* (encoding amino acids 126 and 140). *H pylori* with NapA including serine at amino acid 70 (Ser 70-NapA) was isolated from dyspeptic patients more frequently than *H pylori* carrying threonine at the same position (Thr 70-NapA) (age-adjusted odds ratio, 2.88; 95% confidence interval, 1.19–6.94; $P = 0.019$) (Figure 1). The prevalence of Ser 70-NapA *H pylori* infection did not differ between patients with meal-related and meal-unrelated dyspepsia (Supplementary Figure 1*B* and *C*).

According to the homo-12-mer 3D structure of NapA (RCSB Protein Data Bank ID: 1JI4, http://www.rcsb.org),[6] amino acid 70 lies close to the monomer junction and is exposed on the dodecameric complex surface (Supplementary Figure 2*A*); therefore, it likely contributes to the stability of 12-mer formation and NapA neutrophil activation function, which may influence dyspepsia. NapA must form a dodecameric complex to sequester and store iron, which are functions associated with the antioxidant abilities of *H pylori*.[7] NapA has dual roles, enhancing bacterial antioxidant ability and stimulating ROS production by neutrophil recruitment.[8] To examine phenotypic differences in *H pylori* with polymorphisms of NapA amino acid 70, we cultured five Ser 70-NapA *H pylori* strains isolated from dyspeptic patients and five Thr 70-NapA *H pylori* strains from non-dyspeptic patients (Supplementary Figure 2*B*). Investigation of susceptibility to H_2O_2 and *t*-BuOOH demonstrated that Ser 70-NapA *H pylori* strains had significantly smaller inhibition zones ($P = .021$ and .047, respectively), indicating that Ser 70-NapA *H pylori* strains are better adapted to ROS exposure than those with Thr 70-NapA (Supplementary Figure 2*C*). When we exposed mouse neutrophils to *H pylori* culture supernatants, intracellular ROS production was marginally higher in those exposed to Ser 70-NapA *H pylori* than Thr 70-NapA *H pylori* supernatants ($P_{\text{for trend}} = .06$) (Supplementary Figure 2*D*), although migration activity did not differ ($P_{\text{for trend}} = .20$) (Supplementary Figure 2*E*).

We also compared changes in Mongolian gerbils infected with Ser 70-NapA and Thr 70-NapA *H pylori*. Four months after infection, gastric emptying was significantly delayed in gerbils infected with Ser 70-NapA *H pylori* relative to those with Thr 70-NapA *H pylori* or uninfected controls ($P_{\text{for trend}} = .004$) (Supplementary Figure 3*A*). Seven months after infection, hematoxylin-eosin staining of the gastric antrum revealed more severe infiltration of inflammatory cells in gastric mucosa infected with Ser 70-NapA *H pylori*, and more severe inflammatory cell infiltrations around the myenteric plexus (MP) were detected after Ser 70-NapA *H pylori* infection, compared with Thr 70-NapA *H pylori* or uninfected controls (Figure 2). The activity of myeloperoxidase (MPO), a hemoprotein secreted during inflammatory cell activation, was higher in gastric mucosa infected with Ser 70-NapA *H pylori* strains ($P_{\text{for trend}} = .0003$), resulting in a tendency toward increased levels of thiobarbituric acid reactive substances (TBARS), an indicator of free radical–mediated lipid peroxidation injury ($P_{\text{for trend}} = .08$) (Supplementary Figure 3*B*). These results suggest that Ser 70-NapA *H pylori* infection delays gastric emptying through induction of ROS by enhancing inflammatory cell recruitment.

Finally, we used a conventional mail survey to compare symptom relief rates of patients after eradication of Ser 70-NapA or Thr 70-NapA *H pylori* (Supplementary Figure 3*C*). More than 1 year after successful eradication therapy, we sent 33 dyspeptic patients a questionnaire regarding the presence/absence of dyspepsia, and 18 responses were received. Symptom improvement rates were 26.6% greater after eradication of Ser 70-NapA *H pylori* than Thr 70-NapA *H pylori* (61.5% [8/13] vs 40.0% [2/5]), although the difference was not statistically significant because of the small sample size (Supplementary Figure 3*D*). Treatment response in dyspeptic patients with Thr 70-NapA *H pylori* (40.0%) was comparable to a previously reported placebo effect.[9] To prove this effect, a double-blind study comparing eradication and non-eradication groups would provide more conclusive results; however, because of the cancer-preventive effect of eradication therapy, it would be

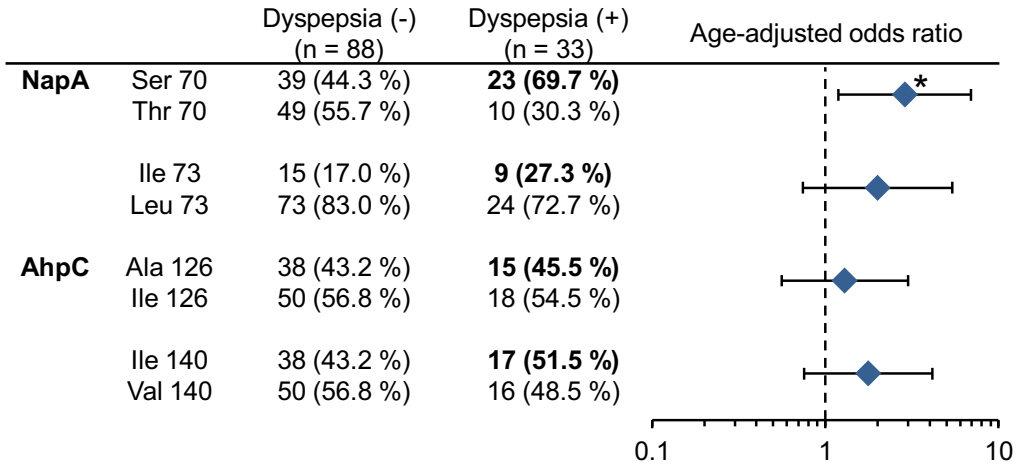

Figure 1. Associations between dyspepsia and polymorphisms in *H pylori* NapA or AhpC. *Error bars* indicate 95% confidence intervals. *$P < .05$ by logistic regression analysis.

ethically inappropriate to include a non-eradication group.

Notably, 2 previous reports support a potential key role for polymorphism of amino acid 70 in *H pylori* NapA.[7,10] We did not perform whole genome sequencing of isolated *H pylori* strains; therefore, experiments using genetically modified *H pylori* strains expressing Thr 70-NapA or Ser 70-NapA on the same genetic background could confirm our hypothesis. Thus, although there is a possibility that differences at other *H pylori* loci contribute to the development of dyspepsia, our data represent the first molecular-level evidence of the mechanism underlying *H pylori*–associated dyspepsia. These findings could assist in efficient diagnosis and prediction of treatment responses in patients with *H pylori*–associated dyspepsia.

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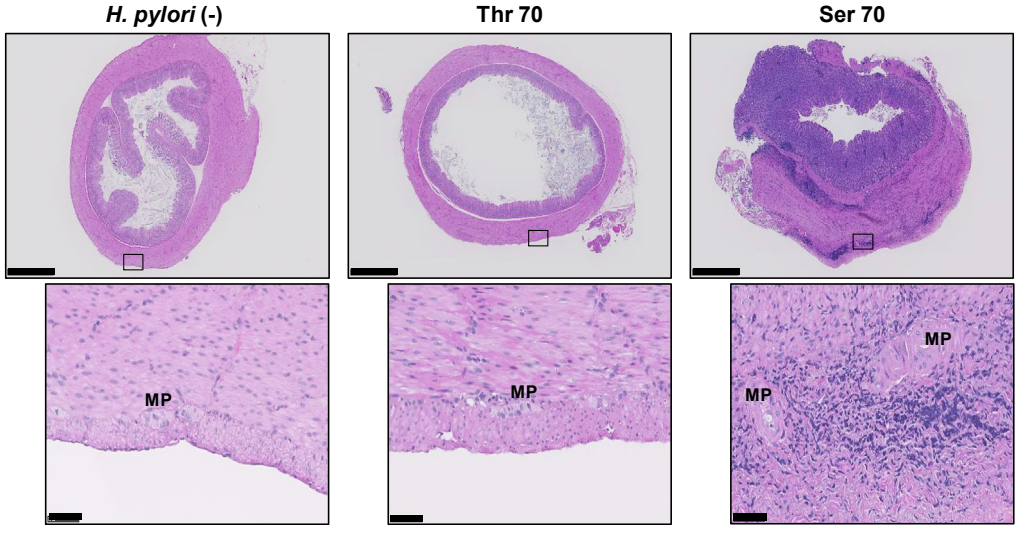

Figure 2. Light microscopy images of hematoxylin-eosin–stained normal gastric mucosa and Thr 70- or Ser 70-NapA *H pylori*–infected gastric mucosa at 7 months after infection. MP, myenteric plexus. Scale bars in *upper and lower panels* indicate 1 mm and 50 μm, respectively.

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Abbreviations used in this letter: AhpC, alkyl hydroperoxide reductase; MPO, myeloperoxidase; MP, myenteric plexus; NapA, neutrophil-activating protein A; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances

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Author contributions

J.M., H.T., and H.S. designed the study. J.M., H.S., and H.M. were involved in the collection of cases. J.M. and Y.I. were involved in the collection of control data. J.M. performed sequencing analyses. H.T., Y.K., H.M., and T.M. performed animal experiments. Y.Y., N.K., and T.M. conducted the postal mail survey. J.M. performed statistical analyses. J.M. and H.T. wrote the manuscript. H.S., M.S., and T.K. critically revised the manuscript. All authors reviewed the manuscript and approved the final version.

Conflicts of interest

These authors disclose the following: H.S. received scholarship funds for this research from Daiichi-Sankyo, EA Pharma, Otsuka Pharmaceutical, and Tsumura and service honoraria from Astellas, AstraZeneca, Daiichi-Sankyo, Otsuka Pharmaceutical, Mylan EPD, Takeda Pharmaceutical, and Tsumura. T.K. received scholarship funds from Astellas Pharma Inc, AstraZeneca K.K., Otsuka Pharmaceutical Co Ltd, Takeda Pharmaceutical Co Ltd, Eisai Pharmaceutical Co Ltd, Zeria Pharmaceutical Co Ltd, Tanabe Mitsubishi Pharmaceutical Co Ltd, JIMRO Co Ltd, and Kyorin Pharmaceutical Co Ltd to conduct this research. T.K. received service honoraria from Astellas Pharma Inc, Eisai Pharmaceutical Co Ltd, JIMRO Co Ltd, Tanabe Mitsubishi Pharmaceutical Co Ltd, Otsuka Pharmaceutical Co Ltd, Takeda Pharmaceutical Co Ltd, Miyarisan Pharmaceutical Co Ltd, and Zeria Pharmaceutical Co Ltd. The remaining authors disclose no conflicts.

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Supplementary Methods

Study Population

Patients for whom both first-line (proton pump inhibitors, amoxicillin, and clarithromycin) and second-line (proton pump inhibitors, amoxicillin, and metronidazole) eradication therapies had failed, and who had participated in a trial for a third-line treatment (UMIN000006483),[1] were included. Those who had structural diseases that could explain dyspeptic symptoms were excluded. *H pylori* was isolated from gastric specimens. Participants in whom *H pylori* could not be detected by culture were excluded.

The definition of dyspepsia used was that for functional dyspepsia in the Rome III classification.[2] Participants were defined as having dyspepsia when they had dyspeptic symptoms for at least 6 months before the survey. Meal-related dyspepsia was defined as the presence of postprandial fullness or early satiation, whereas meal-unrelated dyspepsia was defined as the presence of epigastric pain or burning and the absence of postprandial fullness or early satiation.

DNA sequencing and Three-dimensional Structure of Helicobacter pylori Neutrophil-activating Protein A

The *napA* and *ahpC* genes were polymerase chain reaction–amplified by using specific primers (*napA* forward 5′-GTTTGCGGACATGTTTGATG and reverse 5′-TCGCCTTCTTTTTCAGCAGT; *ahpC* forward 5′-GATCCAACAAAATTTTAAAACACTTA and reverse 5′-GCGAAGAATCAAAAAGAAAGGA). Polymerase chain reaction and sequencing reactions were performed as described previously.[3] NapA protein three-dimensional structures were visualized by using the RCSB Protein Data Bank (ID: 1JI4) and DeepView-Swiss-PdbViewer.

Bacterial Strains and Culture Conditions

Five Ser70-NapA strains isolated from dyspeptic patients and five Thr70-NapA strains isolated from non-dyspeptic patients were used for the following experiments. Bacterial isolates were maintained as described previously.[4] To collect culture supernatants, *H pylori* were cultured in RPMI1640 containing 10% fetal bovine serum for 24 hours at 37°C under microaerobic conditions. Culture supernatants were filtered through 0.22-μm filters and stored at −30°C until use.

Inhibition Zone Assay

H pylori, normalized to an OD_{600} of 1.0, were plated on Brucella agar and grown until confluent. Sterile 5-mm disks saturated with 10 μL of 10 mol/L H_2O_2 (Wako, Osaka, Japan) or 7 mol/L *t*-BuOOH (Sigma-Aldrich, St Louis, MO) were placed onto the plates. After 3 days, the zones of inhibition around the disks were measured.[5]

Mouse Neutrophil Isolation

C57BL/6J mice were purchased from Japan Clea (Tokyo, Japan). Neutrophils were isolated by using a Neutrophil Isolation Kit (Cayman, Ann Arbor, MI). Intraperitoneal sterile inflammation was induced by injection of 7.5% casein. Neutrophils were enriched by using Percoll density gradient centrifugation.

Transwell Migration Assay

Twelve-well culture plate chemotaxis chambers containing polycarbonate membrane filters (pore size, 3 μm) (Corning, NY) were prepared. Membranes were pre-coated with fibrinogen. The upper chambers contained mouse neutrophils in 400 μL RPMI1640 (1×10^6 cells/well), and lower chambers each contained 1000 μL of *H pylori* culture supernatant. The cells were allowed to migrate for 2 hours at 37°C in 5% CO_2. EDTA (100 μL, 0.5 mol/L) was added to the lower chambers, and plates were incubated for 15 minutes at 4°C. Samples in lower chambers were enumerated.

Assessment of Intracellular Reactive Oxygen Species Production in Neutrophils

Mouse neutrophils were incubated with *H pylori* culture supernatants at 37°C in 5% CO_2 for 24 hours. Subsequently, cells were incubated with 1 μmol/L CM-H2DCFDA (C6827; Invitrogen, Carlsbad, CA) for 1 hour at 37°C. After washing with phosphate-buffered saline, the fluorescence signal of CM-H2DCFDA in the cells was measured by flow cytometry (BD FACSAria II; BD Biosciences, Bedford, MA).

Helicobacter pylori Infection of Mongolian Gerbils

Mongolian gerbils (MON/Jms/Gbs Slc) were purchased from Japan SLC (Hamamatsu, Japan). Animals were inoculated with *H pylori* by administration of 0.6 mL of bacterial suspension (8×10^8 colony-forming units/mL) by using an orogastric catheter. Gastric emptying was measured by using a breath test with ^{13}C-labeled acetic acid (CLM-317; Wako) 4 months after inoculation.[6,7] The time of peak $\Delta^{13}CO_2$ excretion (T_{max}) was determined by using an exponential power model for curve fitting.

Four and 7 months after inoculation, animals were killed, and their stomachs were excised. Proteins were extracted from tissues by incubation in RIPA buffer. MPO activity and TBARS were measured by using MPO Activity Assay Kit (Abcam, Cambridge, UK) and TBARS Assay Kit (Cayman), respectively.

Statistical Analysis

Age-adjusted odds ratios were calculated by logistic regression analysis. The other statistical methods are indicated in the legends. Box-and-whisker plots show the full range of variation, interquartile range, and median values. Outliers are points that fell more than 1.5 times the interquartile range above the third quartile. All tests for statistical significance were two-sided at $P = .05$ using IBM SPSS Statistics version 23 (IBM Japan, Tokyo, Japan).

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki and with the approval of the ethics committee of Keio University School of

Medicine (20130040). Animal experiments were conducted in accordance with the Guidelines for Laboratory Animal Experiments in Research and with the approval of the Keio University Animal Research Committee (08080-12) and the Committee for Ethics of Animal Experimentation of the National Cancer Center (T18-011).

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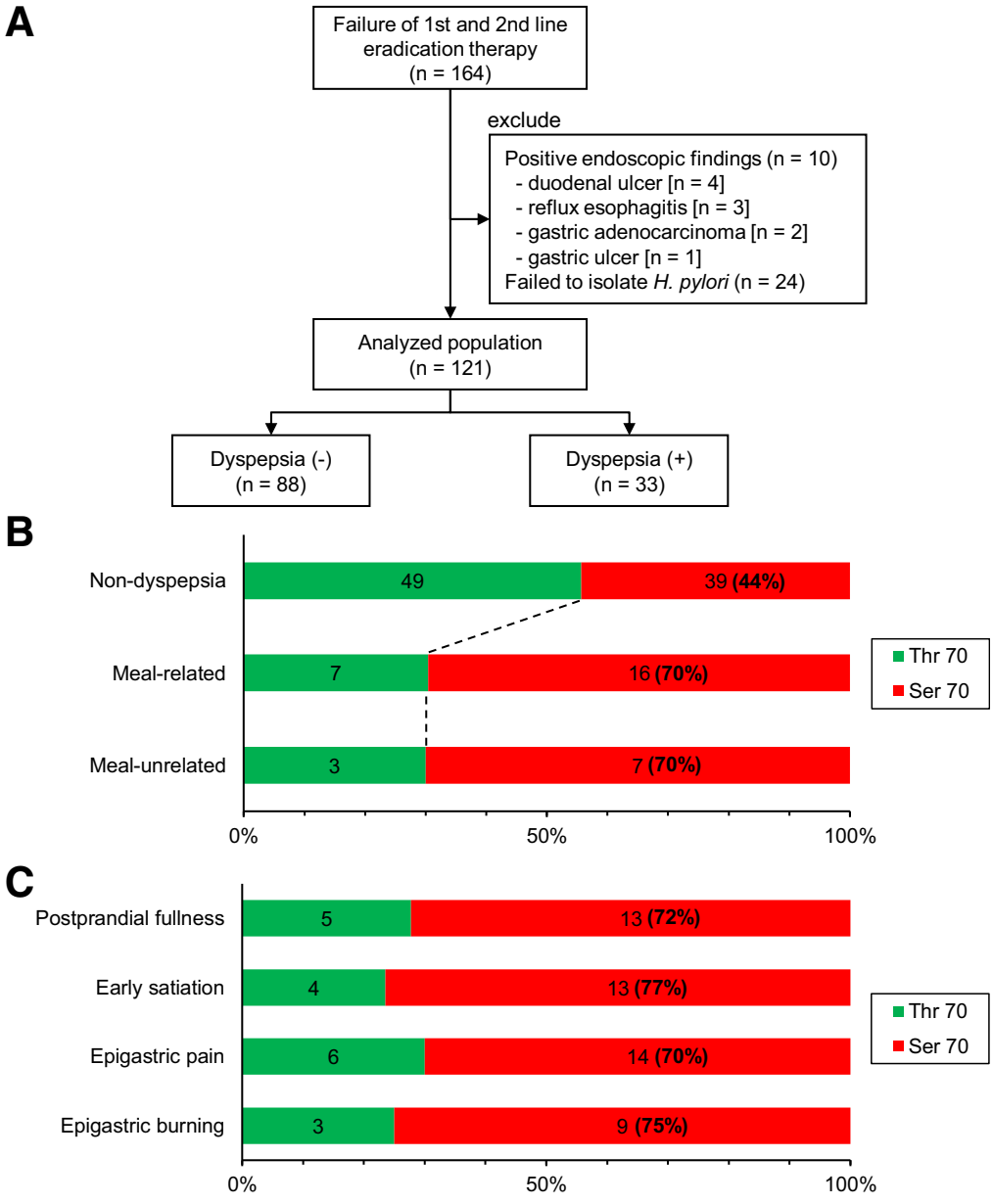
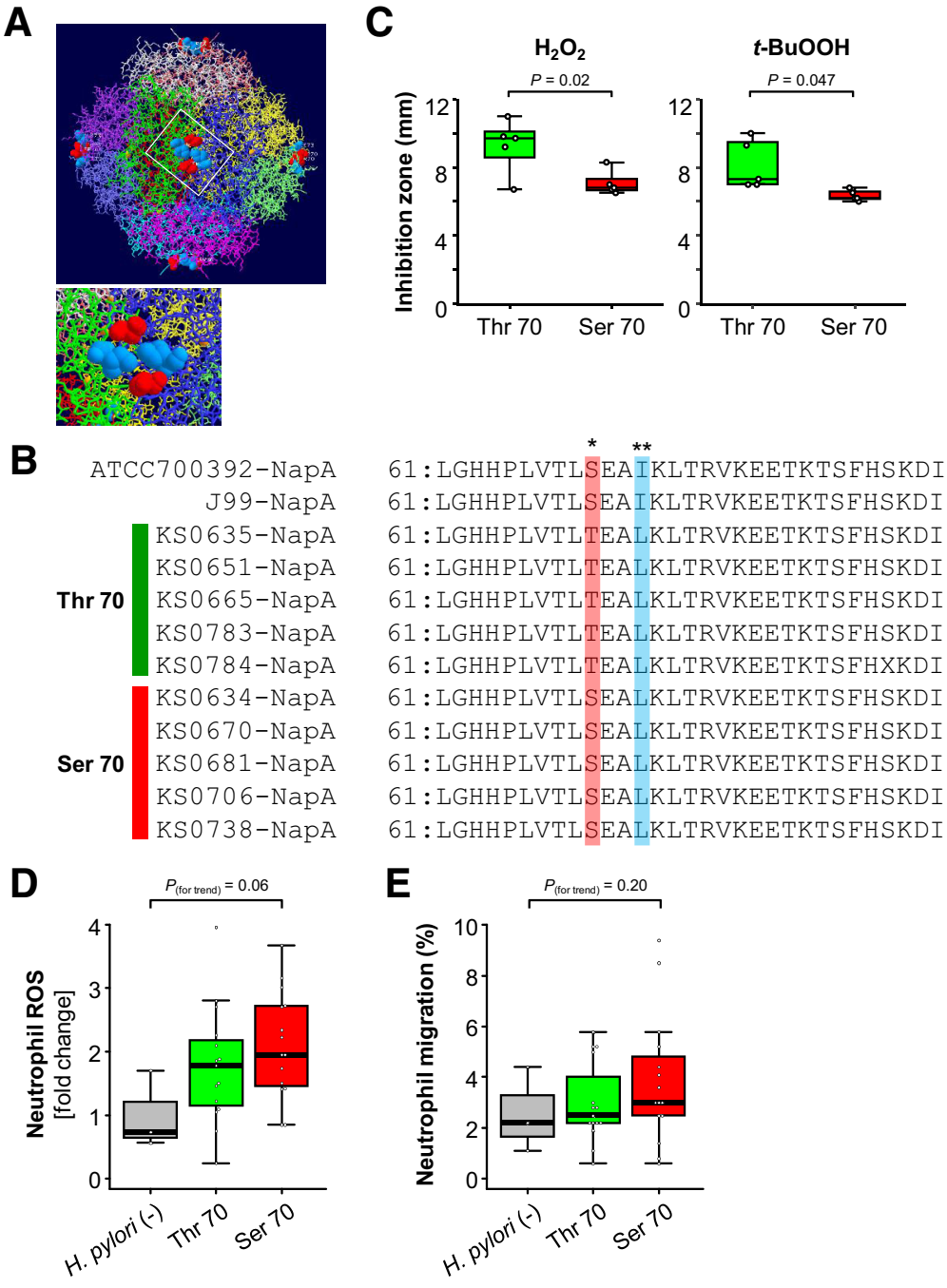
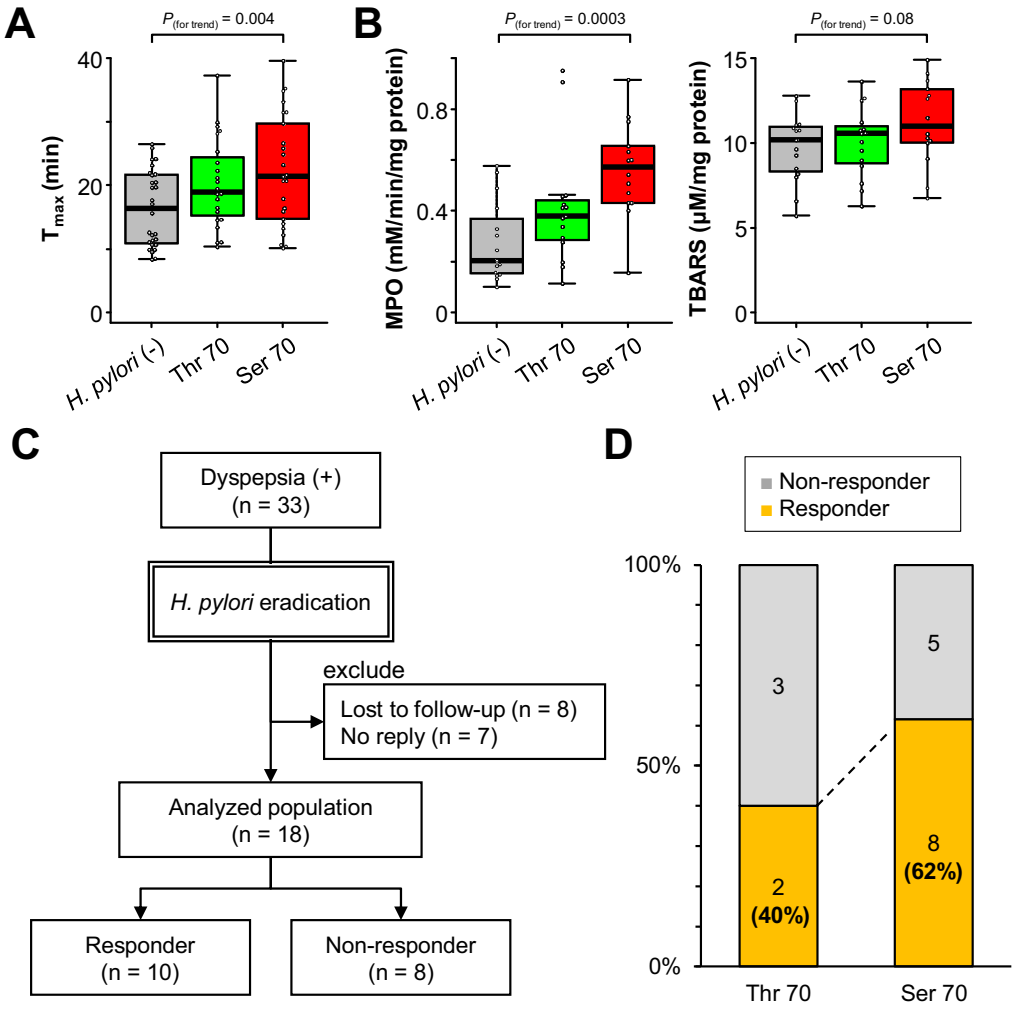


Supplementary Figure 1. (*A*) Patient recruitment flowchart. (*B* and *C*) Prevalence of *H pylori* NapA polymorphisms in patients without dyspepsia and with each subgroup of dyspepsia.



Supplementary Figure 2. (*A*) **Homo-12-mer structure of NapA.** *Red and blue spheres* indicate locations of amino acids 70 and 73, respectively. (*B*) NapA amino acid sequences. ATCC700392 and J99 are genome-sequenced references. (*C*) Inhibition zone assay data were collected by 5 strains from each group in 1 independent experiment. *P* values, Student *t* test. Data of neutrophil ROS induced by *H pylori* culture supernatants (*D*) and neutrophil migration (*E*) were collected by 3 independent experiments for five Thr70-NapA and five Ser70-NapA strains. *P* values for trend, Pearson correlation analysis.



Supplementary Figure 3. (*A*) Gastric emptying in Mongolian gerbils infected with Thr70- or Ser70-NapA *H pylori* or uninfected controls (n = 23, 25, and 28, respectively). (*B*) MPO activity (*left panel*) and TBARS levels (*right panel*) in gastric mucosa from Mongolian gerbils (n = 15 for each). P values for trend, Pearson correlation analysis. (*C*) More than 1 year after successful eradication therapy for 33 dyspeptic patients, a questionnaire was posted to them about the presence/absence of dyspepsia. Among the 18 analyzed patients, 10 reported the resolution of dyspepsia after eradication therapy. (*D*) Symptom relief rates were 40.0% (2/5) and 61.5% (8/13) after eradication of Thr70-NapA and Ser70-NapA *H pylori*, respectively.

Supplementary Table 1. Participant Characteristics

	Dyspepsia (–) (n = 88)	Dyspepsia (+) (n = 33)	*P* value
Age (*y*), mean ± SD	54.8 ± 13.2	**47.1 ± 13.3**	.005[a]
Sex			
Male	37 (42.0%)	12 (24.5%)	.68[b]
Female	51 (58.0%)	21 (63.6%)	
BMI (kg/m^2), mean ± SD	22.0 ± 3.2	22.0 ± 4.1	.97[c]
Frequency of alcohol consumption			
Social drinker (<5 days/wk)	43 (48.9%)	19 (57.6%)	.11[c]
Frequent drinker (5–7 days/wk)	15 (17.0%)	1 (3.0%)	
Smoking habit			
Ex-smoker	21 (23.9%)	7 (21.2%)	.20[c]
Current smoker	6 (6.8%)	6 (18.2%)	
Medication			
Proton pump inhibitor	10 (11.4%)	9 (27.3%)	.052[b]
Histamine 2 receptor antagonist	12 (13.6%)	5 (15.2%)	1.00[b]
Dyspeptic symptom categories			NA
Postprandial fullness	0 (0%)	18 (54.5%)	
Early satiation	0 (0%)	17 (51.5%)	
Epigastric pain	0 (0%)	20 (60.6%)	
Epigastric burning	0 (0%)	12 (36.4%)	

NOTE. Bold values indicate significant differences.
BMI, body mass index; NA, not applicable; SD, standard deviation.
[a]Student *t* test.
[b]Fisher exact test.
[c]Pearson χ^2 test.