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Address requests for reprints to: Hidekazu Suzuki, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. e-mail: hsuzuki@sc.itc.keio.ac.jp; fax: (81) 3-5363-3967.

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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*

Hitoshi Tsugawa [a], Hidekazu Suzuki [a,*], Hiroe Muraoka [b], Fumiaki Ikeda [b], Kenro Hirata [a], Juntaro Matsuzaki [a], Yoshimasa Saito [a], Toshifumi Hibi [a]

[a] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan
[b] Chemotherapy Division, Mitsubishi Chemical Medience, Tokyo, Japan

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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.

* Corresponding author. Address: Division of Gastroenterology and Hepatology, Department of Internal Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81 3 5363 3967.
E-mail address: hsuzuki@sc.itc.keio.ac.jp (H. Suzuki).

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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

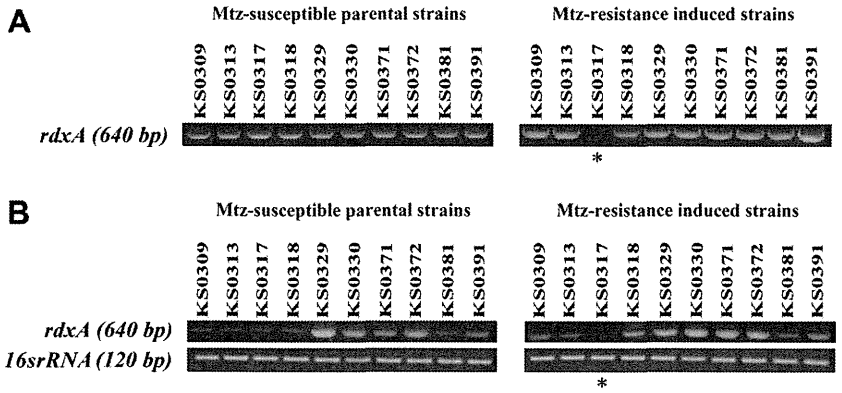

Fig. 1. *rdxA* expression after passaging for induction of Mtz-resistance *in vitro*. (A) The *rdxA* coding region in the Mtz-susceptible parental strains and Mtz-resistance-induced strains was detected by the PCR using a genome DNA as the template. (B) The mRNA expression of *rdxA* was detected by RT-PCR.

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

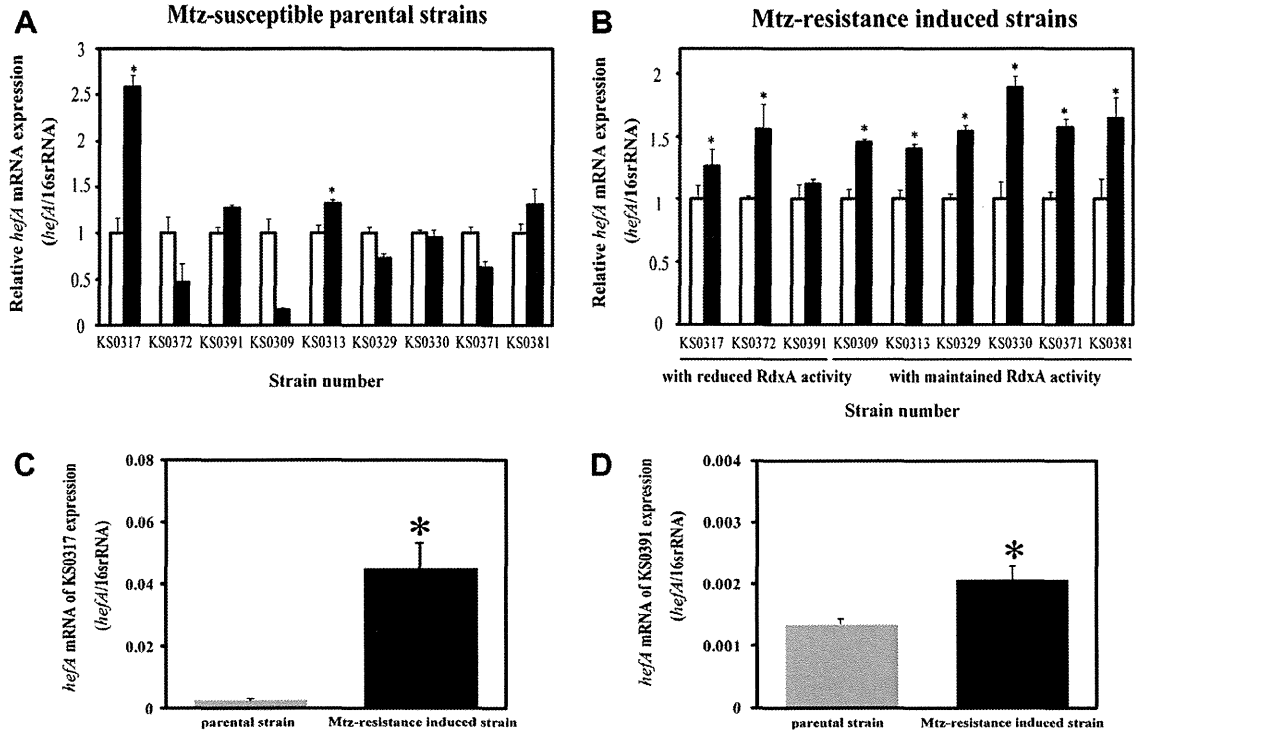

Fig. 2. Alterations in the expression of the *hefA in H. pylori* after passaging for induction of Mtz-resistance *in vitro*. Expression of *hefA* mRNA in the Mtz-susceptible parental strains (A) and Mtz-resistance-induced strains (B) exposed to 0 (white) and 0.5 μg/mL (black) Mtz was measured by quantitative 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

Juntaro Matsuzaki · Hidekazu Suzuki · Keiko Asakura · Yoshimasa Saito · Kenro Hirata · Toru Takebayashi · Toshifumi Hibi

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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

J. Matsuzaki · H. Suzuki (✉) · Y. Saito · K. Hirata · T. Hibi
Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: hsuzuki@sc.itc.keio.ac.jp

K. Asakura · T. Takebayashi
Department of Preventive Medicine and Public Health, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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

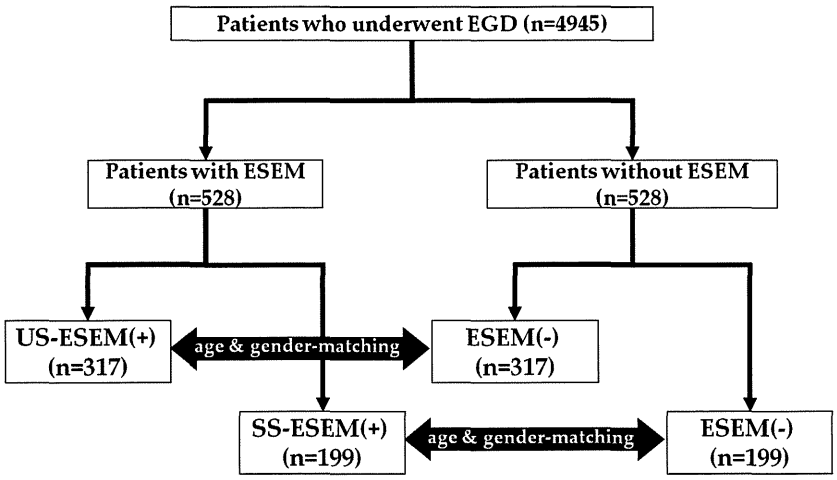

Fig. 1 Flow diagram of the case–control study. Among 4945 patients who underwent esophagogastroduodenoscopy (EGD) at Keio University Hospital between November 2007 and April 2008, 528 with ESEM and 528 age- and gender-matched subjects without ESEM were selected [10]. Of the 528 patients with ESEM, 317 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

Table 4 Association of gastric corpus atrophy and gallstones with the presence of US-ESEM or SS-ESEM

	US-ESEM			SS-ESEM		
	ESEM(+) No./total no. (%)	ESEM(−) No./total no. (%)	Odds ratio (95% CI)[c]	ESEM(+) No./total no. (%)	ESEM(−) No./total no. (%)	Odds ratio (95% CI)[c]
Gastric corpus atrophy (−)[a], gallstones (−)	107/218 (49.1)	139/233 (59.7)	0.61 (0.41–0.89)*	69/143 (48.3)	88/151 (58.3)	0.63 (0.39–1.02)▲
Gastric corpus atrophy (+)[b], gallstones (−)	64/218 (29.4)	51/233 (21.9)	1.56 (1.01–2.40)*	37/143 (25.9)	42/151 (27.8)	0.92 (0.54–1.56)
Gastric corpus atrophy (−)[a], gallstones (+)	27/218 (12.4)	31/233 (13.3)	0.92 (0.53–1.61)	17/143 (11.9)	13/151 (8.6)	1.45 (0.68–3.11)
Gastric corpus atrophy (+)[b], gallstones (+)	20/218 (9.2)	12/233 (5.2)	1.99 (0.94–4.21)▲	20/143 (14.0)	8/151 (5.3)	2.99 (1.26–7.06)*

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

▲ $p < 0.1$, *$p < 0.05$

[a] Severity of gastric mucosal atrophy, none or mild

[b] Severity of gastric mucosal atrophy, moderate or severe

[c] Adjustment for age and gender

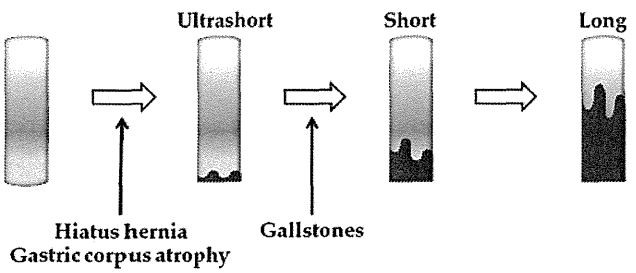

Fig. 2 Risk factors for the elongation of ESEM. The risk factors for US-ESEM and SS-ESEM were different. Hiatus hernia was associated with the presence of both US- and SS-ESEM. Gastric corpus atrophy was associated with the presence of US-ESEM, but its association with the presence of SS-ESEM was not statistically significant. Gallstones were associated with the presence of SS-ESEM, but not with that of US-ESEM

gastric mucosal atrophy was marginally associated with the presence of US-ESEM, but not with that of SS-ESEM. Presence of gastric corpus atrophy without gallstones was significantly associated with the presence of US-ESEM, but not with that of SS-ESEM. On the other hand, the presence of gallstones was significantly associated with the presence of SS-ESEM, but not with that of US-ESEM. The presence of severe reflux esophagitis was also associated with the presence of SS-ESEM, but not with that of US-ESEM (Fig. 2). The presence of *H. pylori* infection was not associated with the presence of either US- or SS-ESEM.

These results suggest that the etiology of US-ESEM may differ from that of SS-ESEM. The presence of gastric corpus atrophy appeared to be a necessary condition for the development of US-ESEM. El-Serag et al. [24] also reported that while USBE was not associated with the presence of erosive esophagitis, it showed a positive association with the presence of gastric mucosal atrophy and metaplasia. Known risk factors for intestinal metaplasia at the gastric cardia include advanced age, male sex, and severe bile reflux [25]. Ye et al. [26] reported that adenocarcinoma of the gastric cardia was not associated with the presence of *H. pylori* infection, but it showed a positive association with the presence of gastric mucosal atrophy, which can be caused by exposure of the stomach to bile. The etiological association of USBE may be similar to that of adenocarcinoma of the gastric cardia, and the condition appears to be caused by duodenogastric reflux of bile into the gastric corpus.

On the other hand, the epidemiology of SS-ESEM appeared to be similar to that of esophageal adenocarcinoma. The presence of gallstones and reflux esophagitis has been reported to be associated with abnormal acid and bile reflux into the esophagus [27, 28]. Akiyama et al. [29] reported that the prevalence of erosive esophagitis was higher in subjects with a circumferential length of Barrett's epithelia of at least 2 cm than in those of less than 2 cm. Okita et al. [30] reported that the severity of reflux esophagitis, reflux symptoms, and hiatus hernia was positively correlated with the length of SSBE. These studies also suggest that the presence of severe gastroesophageal reflux is a necessary condition for the elongation of BE.

The limitation of the present study was potential selection bias due to it being a hospital-based study. Subjects in the present study were older than the general population, and most of them had some diseases. The prevalence of gallstones would also be higher. To verify the results of the

present study, a prospective study based on a health check-up cohort would be needed.

In conclusion, the epidemiology of US-ESEM and SS-ESEM was divergent. In Japan, USBE might account for the majority of patients with BE. US-ESEM was associated with the presence of gastric corpus atrophy, but not with the presence of *H. pylori* infection, suggesting that US-ESEM may be associated with duodenogastric bile reflux, which is also known to be associated with the development of gastric corpus atrophy. On the other hand, SS-ESEM was not associated with the presence of gastric mucosal atrophy, suggesting that a large amount of gastroesophageal acid reflux was necessary for the development of SS-ESEM. Further studies are needed for histological confirmation of our findings.

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Review

Ghrelin and oxidative stress in gastrointestinal tract

Hidekazu Suzuki,* Juntaro Matsuzaki and Toshifumi Hibi

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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Oxidative stress is a major cause of the gastrointestinal damage under physical or psychological stress. Ghrelin exhibits gastroprotective effects and they are supposed to be derived from antioxidant effects. In gastroduodenal mucosal injury, the plasma ghrelin levels increase in response to the demand for gastroduodenal cytoprotection. However, in the condition of *Helicobacter pylori*-induced gastric mucosal severe atrophy, the plasma ghrelin concentration shifted to lower levels. In diabetic gastroparesis, the regulation of ghrelin secretion is impaired with vagal nerve dysfunction. Selective ghrelin agonist is expected to represent a new class of prokinetic agent. In addition, the plasma ghrelin levels are also enhanced by systemic oxidative stress, and ghrelin exhibits antioxidant effects in many organs, such as heart, pancreas, and lung. This suggests that ghrelin would be an important player as a sensor of systemic oxidative stress.

Key Words: **oxidative stress, ghrelin, peptic ulcer, gastroparesis**

The physiological response to stressor includes an increased activity of the hypothalamic-pituitary-adrenal axis as well as changes in gastrointestinal damage. According to Selye's formulation of the general adaptation syndrome, an increase in adrenocortical activity should be related to an increase in the incidence of gastric ulceration.[1] The strong candidate for the cause of stress ulcer would be oxidative stress. There are some evidences that not only physical stress, such as surgery and infection, but also psychological stress leads to oxidative stress.[2,3]

Oxidative stress, which refers to a state of elevated levels of reactive oxygen species (ROS), forms a variety of conditions that stimulate either ROS production or a decline in antioxidant defenses. Oxidative stress is involved in the pathogenesis of lifestyle-related diseases, including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and malignancies.[4] Several gastrointestinal diseases, such as peptic ulcer disease and gastroparesis, are known to be related with the dysfunction of the antioxidative properties.[5]

Ghrelin, produced and secreted by the A-like cells of the oxyntic glands of the stomach, stimulates growth hormone (GH) secretion, gastric motility, and food intake.[6] Many researchers reported the relationship between oxidative stress and the expression or function of ghrelin.[7,8] Moreover, ghrelin administration is expected to reduce oxidative stress and control diseases.[9] Previous studies have reported that ghrelin has an anti-inflammatory action on the oxidative injury of the diverse organs, such as the heart, liver and pancreas.[10–14] In the present article, we discuss the association of oxidative gastrointestinal damages with the potential role of ghrelin.

Effects of Ghrelin against Gastric Mucosal Injury

Recent studies have shown that ghrelin exhibits gastroprotective effects.[15–19] Ghrelin administration reduced ethanol-induced gastric ulceration,[15,17,18] acetic acid-induced chronic gastric and duodenal ulceration,[16] and ischemia-reperfusion (I/R)-induced gastric ulceration[18,19] in rats. In addition, ghrelin administration increased mucosal cell proliferation[16] and mucosal microvascular flow[16,18,20] in rats. These effects could be observed by intracerebroventricular,[15,17,18] subcutaneous,[15] intraperitoneal,[18] and peripheral intravascular [19] administration of synthetic ghrelin.

The mechanism of the gastroprotective effects of ghrelin remains unclear. Sibilia *et al.* reported that such effects of ghrelin are mediated by endogenous nitric oxide (NO) release and requires the integrity of sensory nerve fibers.[15] Sibilia *et al.*[17] also reported that cyclooxygenase-1-derived prostaglandins (PGs) are mainly involved in ghrelin-associated gastroprotection and that NO derived from constitutive source, together with PGE_2, are involved in its activity. Ceranowicz *et al.*[16] reported that the gastroprotective effects of ghrelin are mediated by the release of endogenous GH and insulin-like growth factor-1. Brzozowski *et al.*[18] reported that these effects involved vagal nerve integrity, partially depending upon afferent nerves and hyperemia mediated by sensory neuropeptides such as calcitonin gene-related peptide released from these nerves.

The most remarkable gastroprotective effects of ghrelin are supposed to be derived from its antioxidant effects. Eter *et al.*[19] reported that peripheral administration of ghrelin attenuated I/R-induced gastric mucosal injury by reducing ulceration, tissue congestion, cellular infiltration and vascular permeability in rats. Serum level of LDH and tissue content of tumor necrosis factor α (TNFα) were markedly reduced by the ghrelin administration. In their study, a decrement of thiobarbituric acid reactive substance (TBARS) and an increment in glutathione were observed, which suggested that ghrelin has an antioxidant activity. *In vitro* studies, using human polymorphonuclear cells incubated with ghrelin, showed that ghrelin inhibited ROS generation as measured by chemiluminecence.[19] Iseri *et al.*[21] reported that although alendronate induces oxidative gastric damage by a local irritant effect, ghrelin ameliorates this damage by its possible antioxidant and anti-inflammatory powers.

Ghrelin Secretion and Gastric Mucosal Injury

The most common causes of gastric mucosal injury and peptic ulceration are *Helicobacter pylori* (*H. pylori*) infection and the consumption of non-steroidal anti-inflammatory drugs (NSAIDs). *H. pylori* induces a strong inflammatory response, generating large amounts of ROS during the process of colonizing the host.[20,22–29] In the pathogenesis of NSAID-induced gastric mucosal injury, oxygen radicals also play an important role.[30]

*To whom correspondence should be addressed.
E-mail: hsuzuki@sc.itc.keio.ac.jp

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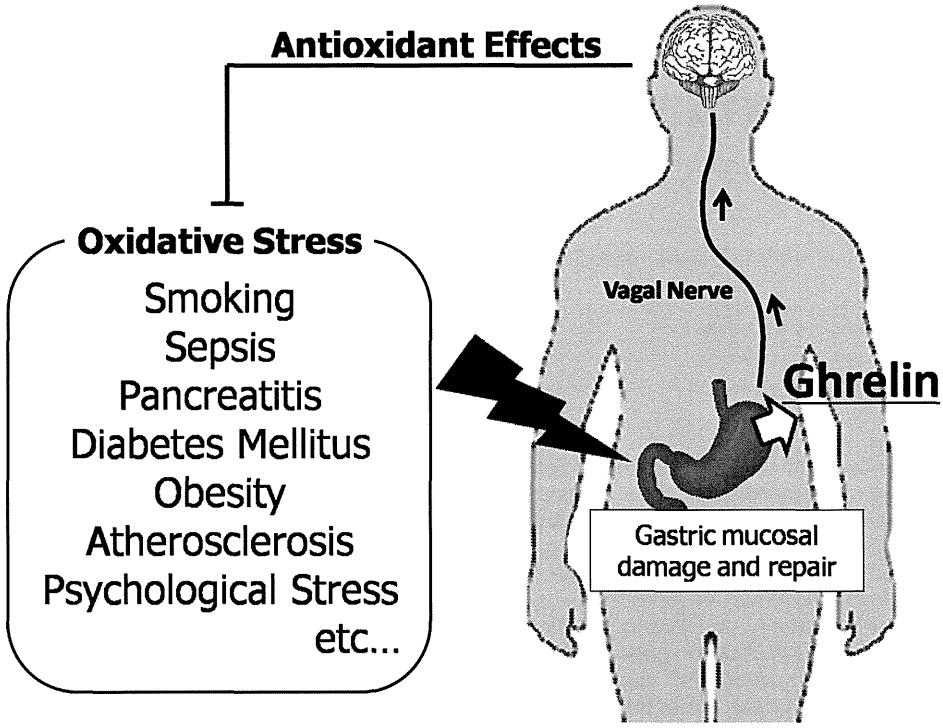

Fig. 1. Ghrelin has antioxidant effects on systemic oxidative stress. Many kinds of systemic oxidative stress could lead to gastric mucosal injury. Ghrelin would be released when the A-like cells were damaged or repaired. Secreted ghrelin send a signal to the brain through the vagal nerve, and enhance the antioxidant reaction in the body.

Although ghrelin secretion would be required to protect gastric mucosa against ROS-induced injury, the number of the gastric A-like cells is decreased simultaneously by gastric mucosal injury.[31,32] Therefore, the status of ghrelin secretion in gastric mucosal injury is complicated. The plasma total and active ghrelin levels are known to be increased by the formation of duodenal ulcers, which induced by administration of cysteamine, a somatostatin inhibitor, in rats.[33] In a human study, enhanced plasma ghrelin levels were observed in patients, not only with duodenal ulcers, but also with gastric ulcers.[34] According to the report by Isomoto *et al.*,[35] amomg plasma ghrelin levels of the patients with chronic gastritis, gastric ulcer, duodenal ulcer, acute gastritis, and normal mucosa, the levels of acute gastritis group were the highest, and then that of chronic gastritis group were the lowest. Within the *H. pylori*-positive population, the plasma ghrelin levels of duodenal ulcer group were higher than gastric ulcer group or chronic gastritis group.[35] The plasma total and active ghrelin levels correlated with the serum pepsinogen I levels, as well as the serum pepsinogen I/II ratio, and decreased with increasing extent of gastric mucosal atrophy.[36,37] These results suggest that the plasma ghrelin levels increase in response to the severe gastric mucosal oxidative stress. However, in the condition of *H. pylori*-induced gastric mucosal severe atrophy, the number of A like cells as well as the plasma ghrelin concentration shifted to lower levels with the reduction of other component cells in gastric fundic gland due to inflammatory cell infiltrations.

Gastric Motility Dysfunction and Ghrelin

Oxidative stress induces not only gastric mucosal injury, but also gastric motility dysfunction, such as diabetic gastroparesis. Gastroparesis are thought to be caused by ROS-induced damage of the networks of the interstitial cells of Cajal.[38] The authors reported that the plasma ghrelin levels and gastric preproghrelin mRNA expression levels are increased in rats with streptozotocin-induced diabetes, that is known for hyperphagia.[39] In a human study, however, the fasting plasma ghrelin level was significantly lower in diabetes mellitus with diabetic gastroparesis than in healthy controls.[40] The change in the plasma ghrelin levels with sham feeding in diabetic gastroparesis patients and postsurgical gastroparesis patients were significantly lower than in normal subjects, although the plasma ghrelin levels increased in idiopathic gastroparesis.[41] Impaired regulation of the plasma ghrelin levels in diabetic gastroparesis would be caused by vagal nerve dysfunction.[41]

Ghrelin administration accelerated gastric emptying of a meal in humans even in the presence of a neural dysregulation by diabetes or surgical vagotomy.[42] Also in idiopathic gastroparesis, administration of ghrelin enhances gastric emptying and improves meal-related symptoms.[43] Therefore, analogues of ghrelin are expected to represent a new class of prokinetic agents.[44] TZP-101, the synthetic, selective ghrelin agonist, is now tested in clinical trials. This new agent was well-tolerated in diabetes patients with moderate-to-severe chronic gastroparesis and showed statistically significant improvements in gastric emptying.[45,46]

Systemic Oxidative Stress and Ghrelin

Systemic oxidative stress is induced by various reasons. In diabetes mellitus, NADPH oxidases, endothelial NO synthase uncoupling, and protein kinase C signaling plays an important roles for mediating increased vascular superoxide production and endothelial dysfunction.[47] Smoking stimulates pulmonary alveolar macrophages and increased superoxide production.[48] During sepsis, multiple intracellular sites, such as mitochondrial, NADPH oxidase, and Rac1 pathways, are responsible to the superoxide production.[49]

Several studies suggested that systemic oxidative stress enhance the plasma ghrelin levels. The plasma ghrelin levels were correlated with vascular super oxide. production and NADPH oxidase

activity in patients with atherosclerosis.[50] Plasma ghrelin was elevated in cachectic patients with chronic heart failure, associated with increases in GH and TNFα.[51] Smoking acutely increased the plasma ghrelin levels.[52] On the other hand, in the early stage of sepsis, ghrelin levels decreased, although the activity of its receptor was markedly elevated in rats.[53] In patients with acute pancreatitis, the plasma ghrelin levels increased after patients' recovery, as compared with the levels before therapy.[54] Decreased ghrelin levels in the early phase of sepsis or pancreatitis would be caused by the damage of gastric A like cells. With repairment of the A like cells, the plasma ghrelin levels would recover after sepsis or pancreatitis.

Taken together, it is considered that gastric mucosa would play an important role for sensing a systemic oxidative stress (Fig. 1). Exposure to oxidative stress could lead to gastric mucosal injury, and ghrelin would be released when the A like cells were damaged or repaired. Secreted ghrelin would have an anti-inflammatory action on the oxidative injury of the several organs, such as increasing cardiac output,[55] vasorelaxation,[56] attenuation of acute pancreatic damage,[57] and attenuation of acute lung injury,[58] as well as rapid repairment of gastric epithelial cells. Ghrelin would be also secreted from the stomach as an anti-inflammatory player for the systemic oxidative injury.

Conclusions

Ghrelin has the possible antioxidant and anti-inflammatory effects. Selective ghrelin agonist would be expected as a new agent to treat not only gastrointestinal motility dysfunction, but also gastric mucosal injury, cardiovascular disease, and various systemic diseases induced by oxidative stress. The stomach would be an important organ as a sensor of systemic oxidative stress.

Abbreviations

ROS	reactive oxygen species
I/R	ischemia-reperfusion
GH	growth hormone
TBARS	thiobarbituric acid reactive substance
NSAIDs	non-steroidal anti-inflammatory drugs

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GASTROENTEROLOGY

Can *Helicobacter pylori*-associated dyspepsia be categorized as functional dyspepsia?

Hidekazu Suzuki,* Toshihiro Nishizawa† and Toshifumi Hibi*

*Division of Gastroenterology and Hepatology,Department of Internal Medicine, Keio University School of Medicine, Shinjuku-k, and †Division of Gastroenterology, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

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Correspondence
Hidekazu Suzuki, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Email: hsuzuki@sc.itc.keio.ac.jp

Conflict of interest
No potential conflict of interest to disclose.

Abstract

Evidence for an association between *H. pylori* and functional dyspepsia (FD) is uncertain. In the present review, we focused the special relevance of *H. pylori* infection to the development dyspepsia from the aspects of pathogensis, clinical efficacy of eradication of *H. pylori* in the West and in the East. Although clinical trials conducted to evaluate the efficacy of *H. pylori* eradication treatment for FD, including non-ulcer dyspepsia (NUD), have yielded conflicting results, it is quite clear that *H. pylori* eradication treatment is effective at least in a subset of FD patients. In contrast to the previous results obtained in studies of Western populations, the result of a double-blind, randomized, placebo-controlled trial conducted in a Singapore suggests that patients with FD could benefit from *H. pylori* eradication therapy, with as much as a 13-fold increase in the chance of symptom resolution. Especially in Asia, *H. pylori* should not be overlooked when considering the pathophysiology of FD. *H. pylori*-associated dyspepsia might be dealt as a different disease entity from FD.

Introduction

Functional dyspepsia (FD) refers to a broad range of chronic upper abdominal symptoms associated with food intake (pain or discomfort centered in the upper abdomen, early satiety, fullness, bloating sensation in the upper abdomen, nausea, etc),[1] and is a condition that is frequently encountered in clinical practice. It has been reported to occur at a prevalence rate of 15% in adults, and about 25% of those with the condition are estimated to be receiving some form of treatment.[2]

Since the etiology of dyspepsia is uncertain, it is little surprise that *H. pylori* (*Helicobacter pylori*) has been implicated in the pathogenesis of dyspepsia. Large population-based studies have shown that the bacterium is found more frequently in the gastric mucosa of dyspeptic patients than in that of controls.

Evidence for an association between *H. pylori* and FD is uncertain. Trials conducted to evaluate the efficacy of *H. pylori* eradication treatment for FD, including non-ulcer dyspepsia (NUD), have yielded conflicting results, however, it is quite clear that *H. pylori* eradication treatment is effective at least in a subset of patients with FD or NUD.[3,4]

Pathogenesis

There is some evidence to suggest that *H. pylori*-associated dyspepsia is caused by the effect of *H. pylori* infection on acid secretion. *H. pylori* infection is associated with increased fasting and post-prandial serum gastrin levels and decreased gastric mucosal concentrations of somatostatin, abnormalities which are corrected following *H. pylori* eradication.[5] Gastrin-releasing peptide (GRP) is a neuropeptide that induces responses mimicking the physiological responses of the stomach to a meal. After intravenous infusion of GRP, patients with peptic ulcers and *H. pylori* infection show a six-fold increase of acid secretion, patients with NUD and *H. pylori* infection show a four-fold increase of acid secretion, whereas asymptomatic *H. pylori*-positive individuals show only a $2^1/_2$ fold increase in stimulated acid secretion, as compared to asymptomatic controls without *H. pylori* infection.[6] This would suggest that NUD may represent a component of the spectrum of *H. pylori*-induced gastric disease. The spectrum ranges from an asymptomatic carrier state, through an ulcer-free dyspeptic period, finally to the development of peptic ulcer.

Ghrelin, a novel growth hormone-releasing peptide, has been reported to accelerate food intake and gastrointestinal motility. We investigated the plasma ghrelin levels in 47 patients with FD and 17 healthy controls. The plasma ghrelin levels were significantly higher in the FD patients, especially in those with dysmotility-like FD, based on the Rome II classification, as compared with those in the controls, suggesting that this parameter could become a useful novel supportive marker for the diagnosis of FD.[7] As the patient cohort with dysmotility-like FD is almost compatible to that with postprandial distress syndrome (PDS) described by the more recent Rome III classification, plasma ghrelin levels (total and active) are thought to be increased in subjects with PDS.

We recently investigated the role of microRNAs (miRNAs) in gastric motility disorders associated with *H. pylori* infection. The gastric motility was compared between mice with long-term *H. pylori* infection and uninfected mice. Gastric emptying was significantly accelerated in the mice with chronic *H. pylori* infection as well as those with infection by one of the other *Helicobacter* species, *H. felis*. Histologic examination showed that the muscular layer of the stomach was significantly thickened, with hyperplasia of the myocytes, in *H. pylori*-infected mice. The miRNA expression profile revealed that the muscle-specific miRNAs, miR-1, miR-133a and miR-133b were significantly downregulated in the stomachs of mice with long-term *H. pylori* infection. However, the expressions of histone deacetylase 4 (HDAC4) and serum response factor (SRF), which are reported as target genes of miR-1 and miR-133, and are known to enhance muscular hyperproliferation, were increased. Downregulation of miR-1 and miR-133 and increased cell proliferation were also observed in C2C12 mouse myoblast cells co-cultured with *H. pylori*. Chronic *H. pylori* infection is associated with downregulated expressions of muscle-specific miRNAs and upregulated expression of HDAC4 and SRF. These might cause hyperplasia of the muscular layer of the stomach and dysfunction of gastric emptying, especially accelerated gastric emptying, possibly through disturbed gastric accomodation. These findings provide a novel insight into the molecular pathogenesis of gastric motility disorders associated with *H. pylori* infection, and might show an organic aspect of *H. pylori*–associated dyspepsia.[8]

Further work has suggested that mast cells are found in patients with *H. pylori*- negative dyspepsia but not in *H. pylori*- positive patients, suggesting a different mechanism underlying the development of symptoms in patients with *H. pylori*-negative dyspepsia.[9]

H. pylori eradication

Many studies and several meta-analyses have attempted to establish a relationship between *H. pylori* infection and the development of FD (Table 1).

In 1998, McColl *et al.* performed a randomized, placebo-controlled trial comparing the efficacy of 2 weeks' treatment with omeprazole + antibiotics (160 patients) and omeprazole alone (158 patients) against the symptoms of dyspepsia in patients with *H. pylori* infection but no endoscopic evidence of ulcer disease. They reported that one year later, dyspepsia had resolved in 33 of the 154 patients (21%) given omeprazole plus antibiotics, as compared with 11 of the 154 (7%) patients given omeprazole alone (95% CI for the difference, 7 to 22%; $P < 0.001$), and that among the patients given omeprazole plus antibiotics, the symptoms resolved in 26 of the 98 patients (27%) who had had symptoms for five years or less, as compared with 7 of the 56 patients (12%) who had had symptoms for more than five years ($P = 0.03$). Their results clearly suggest that in patients with *H. pylori* infection and FD, *H. pylori* eradication treatment is more likely to resolve the symptoms than treatment with omeprazole alone.[10] On the other hand, Blum *et al.* conducted a double-blind, multicenter trial of patients with *H. pylori* infection and dyspeptic symptoms and showed that treatment was successful in 27.4% of the patients in the eradication treatment group and 20.7% in the group treated with omeprazole alone ($P = 0.17$) and concluded that in patients with FD, eradication of *H. pylori* is not likely to relieve symptoms.[11]

The different conclusions reached by two high-quality meta-analyses may be likely explained by which trials were included and which were excluded in each review.[12,13] Then, Talley *et al.*[14] randomly assigned 170 *H. pylori*-infected patients with non-ulcer dyspepsia (NUD) to receive twice-daily treatment with 20 mg of omeprazole, 1000 mg of amoxicillin, and 500 mg of clarithromycin for 14 days, and 167 such patients to receive identical-appearing placebos; all patients were then followed through regular visits for 12 months. They showed that at 12 months, there was no significant difference in the rate of successful treatment between the two groups (46% in the active-treatment group and 50% in the placebo group; relative likelihood of success with active treatment, 0.93; 95 percent confidence interval, 0.73 to 1.18; $P = 0.56$), and also found no evidence to suggest that eradication of *H. pylori* infection in patients with NUD would lead to relief of symptoms. Moayyedi *et al.*[15] also investigated the possibility of lowering the prevalence of dyspepsia in the community and improving the quality of life of the subjects by *H. pylori* eradication in a double-blind randomized controlled trial, and reported that community screening and treatment for *H. pylori* produced

Table 1 RCT for the effect of *H. pylori* eradication therapy on the treatment of functional dyspepsia (non-ulcer dyspepsia)

Authors	McColl *et al.*	Blum *et al.*	Talley *et al.*	Moayyedi *et al.*	Gwee *et al.*	Dhali *et al.*
Patient number (eradicated/non-eradicated)	154/154	164/164	150/143	880/871	41/41	32/30
Place	UK	Europe, Canada, South Africa	USA	UK	Singapore	India
Single/multi center	single center	multi-center	multi-center	field study	single center	single center
Eradication protocol	OAM	OAC	OAC	OCT	OCT	BTM
Control	omeprazole 40 mg/day	omeprazole 40 mg/day	placebos	placebos	placebos	sucralphate 4 g/day
Eradication period	14 days	7 days	14 days	7 days	7 days	14 days
Observation period	1 year	1 year	1 year	2 years	1 year	3 months
Effective rates (%) (eradicated/non-eradicated)	21/7	27.4/20.7	46/50	72/67	24/7	81/33
Efficacy	effective			effective	effective	effective

BTM, bismuth, tetracycline, metronidazole; OAC, omeprazole, amoxicillin, clarithromycin; OAM, omprazole, amoxicillin, metronidazole; OCT, omeprazole, clarithromycin, tinidazole.

only a 5% reduction in the prevalence of dyspepsia, with no impact on the QOL.

A recently published meta-analysis suggests that *H. pylori* eradication may have a small but statistically significant benefit in the treatment of FD at 12 months (relative risk of remaining dyspeptic after *H. pylori* eradication therapy = 0.91; 95% *CI* = 0.87–0.99). While statistically significant, the clinical significance of this finding is not so clear, because the effect is small, that is,15 *H. pylori-positive* dyspeptic patients will need to be treated to achieve just one case of relief from dyspepsia.[16]

H. pylori eradication in Asia

Several randomized controlled trials involving populations in the West have no statistically significant advantage of *H. pylori* eradiation therapy over placebo in dyspepsia patients. However, none of these studies involved Asian populations which show high infection rates.

In Singapore, Gwee *et al.* conducted a double-blind, randomized, placebo-controlled trial of *H. pylori* eradication therapy for FD in a Singapore-based Asian population. Forty-one patients received active treatment consisting of a 1-week course of omeprazole 20 mg once daily, clarithromycin 250 mg twice daily and tinidazole 500 mg twice daily, while another 41 patients received matching placebo tablets. On ITT analyses, symptom resolution was observed in 24% of the patients on active treatment and 7% of those on placebo; the difference in the proportion of patients showing symptom resolution between the two groups was statistically significant ($P = 0.02$, 95% confidence interval: 1.1–17.7). Among patients with *H. pylori* successful eradication, the symptom resolution rate was 39% (10/ 26 patients), whereas among patients in the placebo group who had persistent *H. pylori* infection, it was only 3% (1/35 patients).[17] In contrast to the results obtained in studies of Western populations, the results suggest that patients with FD in Asia could benefit from *H. pylori* eradication therapy, with as much as a 13-fold increase in the chance of symptom resolution following *H. pylori* eradication.

In India, Dhali *et al.*[18] performed a randomized study for evaluating the efficacy of anti-*H. pylori* treatment versus sucralphate in 62 patients with *H. pylori*-positive NUD. According to their report,[18] in patients of NUD and *H. pylori* infection, triple therapy eradicated *H. pylori* in 88% of the patients and was superior to sucralphate in producing symptom relief (81 *vs* 33%, $P = 0.0003$).

We have previously reported that successful *H. pylori* eradication improved the QOL of patients with FD, in particular *H. pylori*-positive patients with ulcer-like FD or dysmotility-like FD, in Japan.[19] The Gastrointestinal Symptom Rating Scale (GSRS) questionnaire was administered to the patient just before the start of the eradication therapy and at 3 months after the therapy, just before the UBT was performed. In successfully eradicated patients, significant decrease of the total GSRS and abdominal pain score was observed. In particular, significant decreases of the abdominal pain score and indigestion score were observed after successful *H. pylori* eradication in patients with ulcer-like FD or dysmotility-like FD.

Most studies on *H. pylori* in relation to dyspepsia have been performed on Western populations, and the results cannot be directly extrapolated to Asian populations, who differ from the western populations in many respects, including points about the most prevalent strains, average level of acid production, as well as the average severity and pattern of gastritis. *H. pylori* should not be overlooked when considering the pathophysiology of FD, especially in Asia.

Since FD is a highly heterogeneous disorder, traditional pathophysiological paradigms are still inadequate to explain the variations in the symptoms observed. Actually, current symptom classifications also largely failed to identify responders to specific therapies.

On the other hand, *H. pylori* infection evokes a significant level of inflammatory changes, not only in the gastric mucosa, but also in the gastric muscular layer as well as in the duodenum. The diagnosis of *H. pylori* infection seems rather homogeneous and it is not simple to categorize *H. pylori*-associated dyspepsia as an inorganic disease. Then, there might be a reason to consider *H. pylori*-associated dyspepsia as an organic disease and to deal with it as a different disease entity from FD. A new classification based on the mechanisms and specific symptoms needs to be considered to further the diagnostic and therapeutic advances in this field.

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