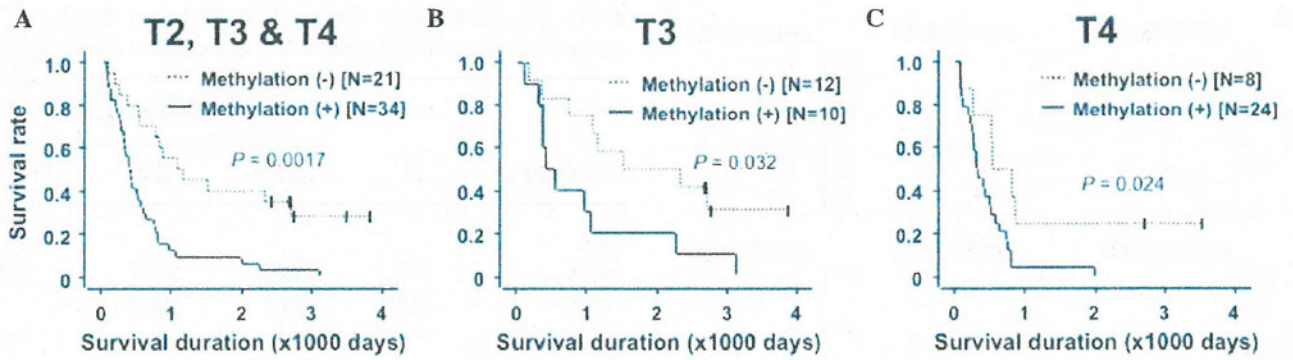

Figure 3. Kaplan-Meier analysis of 55 GC patients with LNM. (A) Overall survival in all the patients with LNM. (B and C) The survival curves of patients categorized into T3 and T4. Methylation (+), GCs with PMR of the region around cg06436185>28.8; Methylation (-), GCs with the PMR<28.8. GC, gastric cancer; LNM, lymph node metastases; PMR, percentage of the value of methylated DNA reference.

and clinicopathological characteristics (age, gender and T category) were analyzed in 157 GC patients with LNM and 30 without LNM. No difference in methylation levels according to age, gender or T category was found (Table III). Using 55 of the 157 GC patients with LNM, whose prognostic information was available (T2, one patient; T3, 22 patients; T4, 32 patients), a correlation between the methylation level and survival rate was analyzed. Patients with high methylation levels (>28.8%; the value used to detect the presence or absence of LNM) had a significantly poorer overall survival rate compared to those with low methylation levels (P=0.0017; Fig. 3A). Since the T category is known to be the major prognostic factor in GC patients (26), patients in the T3 and T4 categories were analyzed separately. In the T3 and T4 subgroups, the patients with high methylation levels demonstrated a significantly poorer overall survival rate than those with low methylation (P=0.032 and 0.024, respectively; Fig. 3B and C). These results revealed that the high methylation level of the genomic region around cg06436185 was associated with an unfavorable prognosis, regardless of the depth of tumor invasion.

Discussion

Using a genome-wide methylation analysis using metastatic lymph nodes and primary GCs without LNM, a genomic region (around cg06436185) whose methylation level in primary GCs was associated with the presence of LNM was successfully identified. Notably, the association was also significant in an independent validation set (P=0.033). Generally, markers isolated by genome-wide analyses need to be validated in a different set of samples due to the overfitting issues caused by multiple testing (27). Even in the present study, 9 of the 10 candidate genomic regions that revealed significant hypermethylation in GCs with LNM in the screening set (P=0.0005-0.048) were not reproduced in the validation set. This observation emphasizes the value of the methylation level of the genomic region around cg06436185. Since it had a sensitivity of 43% and specificity of 85%, the combined use of this novel methylation marker with imaging tools is predicted to improve the diagnostic accuracy of LNM of GCs.

The mean methylation levels of GCs with and without LNM were 18.7 and 27.5%. This small difference is extremely difficult to detect by a genome-wide screening method. Our strategy in the present study was to benefit from the monoclonal growth of cells in metastatic lymph nodes and compare metastatic lymph nodes and GCs without LNM. The methylation levels of the genomic regions around cg06436185 were 13.2 and 54.3%, respectively, in these samples. This relatively significant difference was identified using genome-wide screening, which has a relatively low accuracy in the analysis of methylation levels. Using a more accurate and sensitive method, qMSP, the small difference between GCs with and without LNM (18.7 and 27.5%, respectively) was clearly demonstrated.

A method to measure methylation levels in CpG-poor genomic regions, qPTMR, was developed using a combination of digestion with a methylation-dependent restriction enzyme and qPCR. qPTMR had an error range of 5% in this study. It is difficult to measure methylation levels in CpG-poor genomic regions by qMSP, a well-established method with a high accuracy, due to the difficulty in designing primers. Alternatively, *Msp*JI, a recently developed methylation-sensitive restriction enzyme, recognizes ^{m}CNNR (N=A, T, G or C; R=G or C) sequences and cleaves DNA when the C is methylated (24,25). Since the recognition sequence is applicable to the majority of CpG sites and cytosines in non-CpG sites are not methylated in somatic cells, the positive cleavage by *Msp*JI is used to determine methylation status of most CpG sites. Using qPTMR, the methylation levels of all the 19 candidate regions with few CpG sites were quantified. This new method is predicted to have various applications.

The methylation status of the genomic region around cg06436185 was unlikely to affect transcription of a known nearby gene (*PRKAG2*). However, its high methylation level in GCs, namely large fractions of cancer cells with methylation in cancer tissue, was associated with the presence of LNM and also with a poorer prognosis of the GC patients. One possible reason is that the region is located in a promoter region of unknown genes, including microRNA genes, or in enhancer regions whose methylation is critical for the regulation of gene expression levels. Another possible reason is that the methylation of the region is caused by an abnormality of unknown methylation regulation and that this abnormality is critical for tumor metsastasis or malignancy. In this case, other genomic regions are likely to be methylated in GCs with LNM or a poorer prognosis.

In conclusion, we identified one genomic region with a methylation status in primary GCs that was associated with the presence of LNM and a poorer prognosis of GC patients.

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ORIGINAL ARTICLE

PROSPECTIVE SINGLE-ARM TRIAL OF TWO-WEEK RABEPRAZOLE TREATMENT FOR ULCER HEALING AFTER GASTRIC ENDOSCOPIC SUBMUCOSAL DISSECTION

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Aim: Endoscopic submucosal dissection (ESD) causes artificial ulcers, and there is no consensus regarding the degree of healing in ESD-induced ulcers or the optimal duration of proton pump inhibitor (PPI) treatment. The aim of the present study was to investigate the healing rates of post-ESD ulcers in response to the protective effect of 2-week PPI treatment.
Methods: Between February 2007 and March 2010, 75 patients/75 lesions and 55 patients/55 lesions were enrolled as interim and per-protocol groups, respectively. All patients were prescribed rabeprazole (10 mg/day) orally for 16 days beginning on the day before ESD. Follow-up endoscopy was carried out 8 weeks after ESD to evaluate ulcer healing. The primary end-point was the healing rate of post-ESD ulcers at 8 weeks after ESD. Secondary end-points were the rate of post-ESD bleeding with emergency endoscopy and the rate of other severe adverse effects during the study period.
Results: The transitional rate to scarring-stage ulcers was 80.0% (44/55). Location in the lesser curve and large resected size (>40 mm) were statistically significant predictors for delayed ulcer healing by univariate analysis and the latter was still significant by the multivariate analysis. Post-ESD bleeding occurred within 2 weeks in two cases (2.7%), but both cases were successfully managed with endoscopic hemostasis only. Severe adverse effects did not occur.
Conclusions: Two-week administration of PPI for post-ESD gastric ulcers may be sufficient to aid healing without increasing any adverse effects in cases where there are no possible deteriorating factors on ulcer healing, although large resection and/or resection in the lesser curve may result in delayed healing even after 8 weeks of ESD.

Key words: endoscopic submucosal dissection, gastric intraepithelial neoplasm, postoperative bleeding, proton pump inhibitor, ulcer healing.

INTRODUCTION

After the recognition of *Helicobacter pylori* (*H. pylori*) and several non-steroidal anti-inflammatory drugs (NSAIDs) as causative factors of ulcers, the pathogenesis of gastric ulcer diseases has been studied mainly from these two major etiological categories.[1–4] The recommended treatment strategies for gastric ulcer diseases are *H. pylori* eradication, cessation of NSAIDs, cyclooxygenase (COX)-2 inhibitors, antisecretory drugs and prostaglandin analogs with NSAIDs, depending on the case.[5,6]

Early exposure to gastroscopy to detect curable gastric neoplasia, as a nationwide gastric cancer screening program, has rapidly increased the number of endoscopic resections for these lesions, especially in Asian countries.[7] The chronological trend in the number of operations for early gastric cancers (EGC) at National Cancer Center Hospital, Tokyo, Japan, reveals that the number of endoscopic resections exceeded that of surgical gastrectomies from 2001.[8] As a new category of gastric ulcer diseases, an artificial ulcer after endoscopic resection (e.g. endoscopic mucosal resection [EMR] and endoscopic submucosal dissection [ESD]) has attracted its own field of study. However, little is known about artificial ulcers, especially ESD, and so far they have been treated as similar to *H. pylori*-negative and NSAID-negative peptic ulcers.[6] Therefore, they are treated with proton pump inhibitors (PPI) for approximately 8 weeks after ESD in most hospitals.[9,10]

In a previous preliminary case series, we found that 8 weeks of PPI and sucralfate seemed to be sufficient to cure even a large ESD ulcer when the treated tumors had no submucosal fibrosis.[11,12] In cases of EMR, Lee *et al.* reported that treatment with PPI for 1 week was equivalent to treatment for 4 weeks in terms of ulcer reduction ratio or ulcer stage at 4 weeks after EMR, although the created ulcer size was approximately 2 cm in diameter.[13] This finding suggests that artificial ulcers caused by endoscopic resection might heal faster than *H. pylori*- or NSAID-related peptic ulcers,

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even if the larger artificial ulcer is created by ESD, and spontaneous healing might be expected after postoperative bleeding complications are successfully managed. Several studies have revealed that postoperative bleeding occurs frequently within 24 h and, at the latest, 7 to 10 days after ESD.[10,14–16] Therefore, we prospectively evaluated the healing rate of post-ESD ulcers and the factors influencing delayed healing by 2-week PPI therapy at index endoscopy carried out 8 weeks after ESD.

METHODS

Study design

The present study was a single-arm prospective trial. The study protocol was approved by the institutional review board (IRB) of the University of Tokyo Hospital, Tokyo, Japan, and was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) as number UMIN000000688. There was no pharmaceutical industry support for this study. The authors vouch for the completeness and veracity of the data and data analyses.

Patients

Patients were enrolled in this study if they were scheduled to undergo ESD for gastric epithelial neoplasms at the University of Tokyo Hospital, Tokyo, Japan, between February 2007 and March 2010 after receiving IRB approval for this study. ESD became one of the treatment options for lesions with preoperative diagnosis of gastric adenoma or possible node-negative EGC: intramucosal intestinal-type cancer without ulcerative findings, regardless of size (M-UL[−]); intramucosal intestinal-type cancer with ulcerative findings, 3 cm or less in size (M-UL[+]); or intestinal-type cancer with slight submucosal invasion of less than 500 μm from the muscularis mucosae, 3 cm or less in size (SM1).[17] Patients were informed of the risks and benefits of several treatments, including ESD, conventional EMR, ablation therapy, and surgical gastrectomy.

Among all the patients who provided written informed consent for ESD, patients with any of the following were excluded from the study: concomitant administration of antithrombotic agents, corticosteroids or NSAIDs; drug allergy to PPI; severe comorbidity (i.e. liver cirrhosis, renal failure, or respiratory failure); multiple primary lesions that caused more than one post-ESD ulcer; or lesions with ulcerative findings.[18–20] Lesions with ulcerative findings were exclusion criteria because our previous study revealed that ulcerative lesions delayed artificial ulcer healing even if PPI and sucralfate were given for 8 weeks.[12]

Interim registration was permitted for the patients who provided written informed consent for participation in this study according to Good Clinical Practice guidelines, and final registration was 2 weeks after ESD, when histopathological diagnosis of the resected specimens was obtained and rabeprazole administration for 2 weeks after ESD was completed, without severe perioperative complications, such as perforation or massive bleeding. Patients with histopathological diagnoses potentially requiring additional treatment were excluded from the final registration as follows: SM2 (massive submucosal invasion of more than 500 μm from the muscularis mucosae) or deeper; vessel infiltration; diffuse-type neoplasia; or tumor exposure on the vertical or horizontal margins of the resected specimens. Lesions with ulcerative findings by histological evaluation were also excluded because of possible delays in ulcer healing, as previously described.[12]

Study protocol

The bleeding rates of post-ESD were 3.4% (13/383) in our previous case series and 6.1% (59/945) in another leading center,[14,21] and all bleeding events occurred within 2 weeks after ESD. Therefore, we considered 2 weeks of PPI necessary and sufficient to prevent postoperative bleeding. Patients were prescribed 10 mg oral rabeprazole daily from the day before ESD to the 14th day after ESD. Other antisecretory drugs that may promote ulcer healing and NSAIDs, including aspirin, which may diminish ulcer healing, were prohibited until 8 weeks after ESD, when the second follow-up (index) endoscopy was carried out.

ESD was carried out as previously reported.[22–24] In brief, the procedure consisted of marking around the lesion, injection into the submucosal layer, mucosal incision around the marking, and submucosal dissection beneath the lesion. Submucosal injection solution, endosurgical knives and other equipment were not predetermined for this study but were selected by the operators based on their experience and the characteristics of the lesions. Perioperative patient care, except for administration of antisecretory drugs, was standard.[16] Patients without complications were usually allowed to eat a light meal the day after ESD.

The first follow-up endoscopy was carried out once within a week to check whether there was a recent hemorrhage or a possible bleeding spot that should be treated on the post-ESD ulcer. The day of the first follow-up endoscopy was decided by the operator according to the patient's condition. When bleeding or non-bleeding visible vessels were seen (sometimes in removing adherent clots by forceps or water jet) during the endoscopy, prophylactic hemostasis was carried out. Clipping with hemostatic clips (HX-610–135 or HX-610–090L; Olympus, Tokyo, Japan) was carried out for large non-bleeding vessels, and thermocoagulation with hemostatic forceps was carried out for bleeding vessels or small non-bleeding vessels or in locations where it was difficult to place a clip because of consolidation of the ulcer bed. If the hospitalization was uneventful, patients were discharged within 1 week after ESD.

Post-ESD bleeding was defined when emergency endoscopy because of apparent hematemesis or melena, changes of vital signs, and/or progressive anemia (e.g. a rapid fall in hemoglobin level of 2 g/dL or more) revealed a post-ESD ulcer with evidence of recent or ongoing bleeding. The patients with post-ESD bleeding were treated with the best available endoscopic hemostasis and supportive tools.

Index endoscopy was carried out at 8 weeks after ESD to evaluate the healing process of the ulcer and to check whether there was a recent hemorrhage or a possible bleeding spot. Patients were advised to contact the doctor in charge in case of hematemesis or melena, even after discharge. The ulcer-healing stage was evaluated using the six-stage classification known as "Sakita-Miwa classification",[25] and ulcer healing

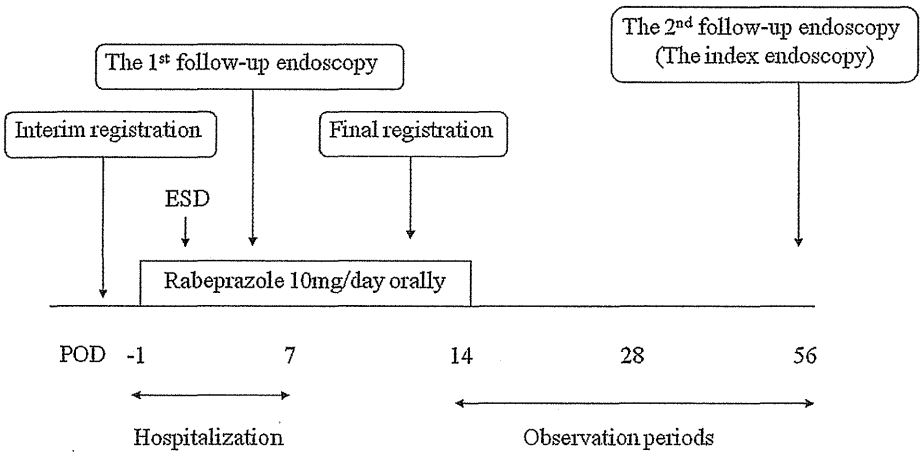

Fig. 1. Treatment protocol. POD, post-operative day.

was defined as reaching the S1 stage. Patients completed the study period at index endoscopy, and additional management was left to the doctors in charge (Fig. 1).

End-points

Our primary end-point was the healing rate of post-ESD ulcers at 8 weeks after ESD among the finally registered patients. Secondary end-points included the rate of post-ESD bleeding with emergency endoscopy and the rate of other severe adverse effects during the study period in all preregistered patients.

Sample size

We did not make a control arm for this study because we had already reported that the healing rate of post-ESD ulcers by giving PPI and sucralfate for 8 weeks was 100% (95/95) as a historical control from our preliminary study. Additionally, the healing rates of peptic ulcers by giving PPI for 6 to 8 weeks have been reported to be more than 80%, which is clinically acceptable for gastric ulcer treatment.[26] Therefore, we considered it clinically acceptable if 2-week administration of rabeprazole resulted in a >80% healing rate at 8 weeks after ESD. Assuming that 90% of our patients healed at 8 weeks after ESD by 2-week administration of rabeprazole, which was 10% higher than the acceptable level, the required number of patients was 34. Additionally, when post-ESD bleeding was considered to determine the sample size with a statistical power of 95%, the required number of patients was 50, assuming 6% of our patients had post-ESD bleeding. We therefore aimed to recruit 55 patients for a valid analysis.

Statistical analysis

Statistical analysis was carried out using JMP version 8.0 software (SAS Institute, Cary, NC, USA). Patient data were statistically compared by using the chi-squared test for categorical data and Student's *t*-test for numerical data as univariate analysis. *P*-values less than 0.05 were considered significant. If there was more than one predictor with a significant difference by univariate analysis, multivariate analysis was carried out. Predictors with *P*-values less than 0.2 by univariate analysis were included in multivariate analysis using a stepwise logistic regression model.

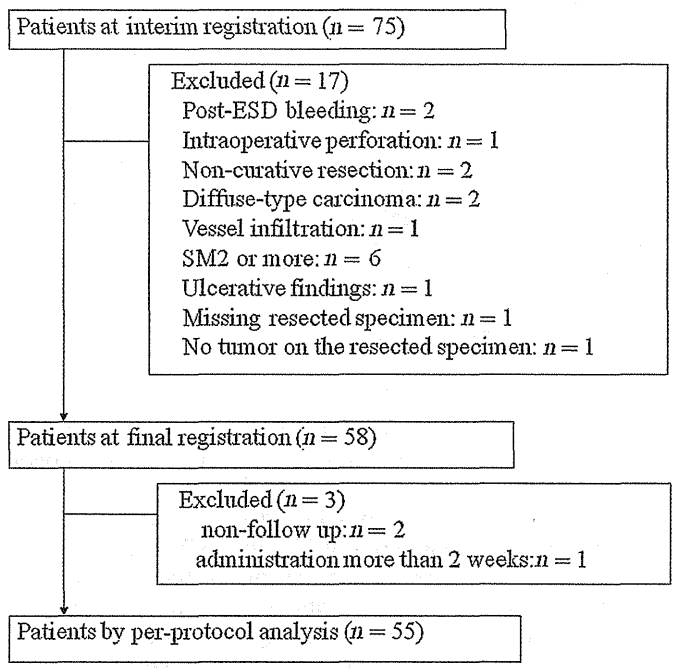

Fig. 2. Flowchart of the participants.

RESULTS

Between February 2007 and March 2010, 75 patients/75 lesions and 58 patients/58 lesions were enrolled as the interim group and the final group, respectively (Fig. 2). Seventeen patients/17 lesions were excluded from the final registration for the following reasons: two for post-ESD bleeding within 2 weeks, one for intraoperative perforation, two for non-curative resection, two for diffuse-type carcinoma, one for vessel infiltration, six for SM2 invasion, one for ulcerative findings, one for lack of the resected specimen before retrieving from the stomach, and one for no tumor on the resected specimen. Among 58 patients/58 lesions in the final group, two patients did not visit our hospital for the index endoscopy, one patient took rabeprazole

Table 1. Demographic and clinicopathological characteristics of patients in the current study†

	75 patients	55 patients
Age, mean (range), years	67.1 (41–85)	67.9 (50–85)
Sex (Female/Male)	24/51	17/38
Smoking (Yes/No)	18/57	14/41
Alcohol (Yes/No)	37/38	25/30
Helicobacter pylori (positive/negative)	48/27	35/20
Diabetes mellitus (Yes/No)	8/67	4/51
Hypoalbuminemia (Yes/No)	4/71	3/52
Lesion size, mean (range), mm	18.6 (1–120)	17.2 (1–60)
<10/–20/–30/–40/–50/–60/61< , mm	26/27/11/4/4/1/2	20/18/10/3/2/2/0
Location (U/M/L)	15/35/25	15/24/16
Circumference (AW/GC/LC/PW)	13/9/27/26	7/7/22/19
Macroscopic type		
Protruded type (0–I, 0–IIa)	26	19
Depressed type (0–IIc)	46	33
Flat type (0–IIb)	3	3
Tumor depth		
Adenoma	2	1
M	56	48
SM1	8	6
SM massive	6	0
Unknown	3	0
En bloc resection (Yes/No)	72/3	52/3
Resected size, mean (range), mm	33.9 (14–134)	32.7 (15–73)
Procedure time, mean (range), min	94.8 (20–230)	91.3 (20–220)

†75 patients/75 lesions in the interim group and 55 patients/55 lesions in the per-protocol analysis.
AW, anterior wall; GC, greater curve; L, lower third; LC, lesser curve; M, middle third; PW, posterior wall; SM1, minimally invasive carcinoma with infiltration depth ≦500 μm; U, upper third.

more than 2 weeks after ESD by mistake, and 55 patients/55 lesions could be investigated as the per-protocol analysis.

Table 1 summarizes the baseline demographic and clinicopathological characteristics of 75 patients/75 lesions in the interim group and 55 patients/55 lesions in the per-protocol analysis. *H. pylori* status was evaluated by either serological testing or urea breath test. Procedure time was measured from marking to the end of tumor removal. Histology was unknown in three lesions because of lack of resected specimen, no tumor on the resected specimen, and severe burn effect of the resected specimen. In order to investigate the predictors for delayed healing as a post-hoc analysis, we divided patients into two groups for each possible parameter.

Primary end-point

Among 55 valid ulcers for the per-protocol analysis of ulcer healing, 11 and 44 improved to H2 stage and S1 stage, respectively, at 8 weeks after ESD. The transitional rate to ulcer scar was 80.0% (44/55). Circumferential location (lesser curve *vs* others) and resected size (≦40 mm *vs* 40< mm) were significantly associated with differences in ulcer healing at 8 weeks by univariate analysis (Tables 2,3). When we carried out logistic multivariate analysis using seven possible variables derived by univariate analysis, only the resected size was statistically significant (odds ratio, 6.26; 95% confidence interval, 1.36–32.10; $P = 0.0207$). The third follow-up endoscopy was carried out for 33 of the 55 patients. During the follow-up period (mean, 10.3 months; range, 4–16 months), all post-ESD ulcers improved to S1 stage without additional administration of PPI, which included six H2-stage ulcers at the index endoscopy.

Table 2. Post-ESD ulcer healing stage at 8 weeks, according to patient factors

	H2 stage	S1 stage	*P*-value
Age			0.3153
≦70 years	4	25	
>70 years	7	19	
Sex			0.2858
Male	6	32	
Female	5	12	
Smoking			0.8820
Yes	3	11	
No	8	33	
Alcohol			0.3262
Yes	3	22	
No	8	22	
Diabetes mellitus			0.1748
Yes	2	2	
No	9	42	
Hypoalbuminemia			0.4952
Yes	1	2	
No	10	42	
Helicobacter pylori			0.1810
Positive	5	30	
Negative	6	14	

ESD, endoscopic submucosal dissection.

Table 3. Post-ESD ulcer healing stage at 8 weeks, according to lesion characteristics

	H2 stage	S1 stage	*P*-value
Lesion diameter			0.1607
≤20 mm	5	31	
>20 mm	6	13	
Resected diameter			0.0017
≤40 mm	4	38	
>40 mm	7	6	
Location			0.6097
U	4	11	
M	5	19	
L	2	14	
Circumference			0.0186
LC	8	14	
Others: GC	0	7	
AW	0	7	
PW	3	16	
Histological depth			0.3669
Adenoma	0	1	
M	11	37	
SM1	0	6	
Macroscopic type			0.2338
Protruded type (0–I, 0–IIa)	2	17	
Depressed type (0–IIc)	9	24	
Flat type (0–IIb)	0	3	
En bloc resection			0.0985
Yes	9	43	
No	2	1	
Procedure time			0.1607
≤90 min	5	31	
>90 min	6	13	

AW, anterior wall; ESD, endoscopic submucosal dissection; GC, greater curve; L, lower third; LC, lesser curve; M, middle third; PW, posterior wall; SM1, minimally invasive carcinoma with infiltration depth ≦500 μm; U, upper third.

Secondary end-point

The first follow-up endoscopy of 75 lesions in the interim group was carried out for 74 lesions (median, 1 day after ESD; range, 1–8 days), excluding one case of postoperative bleeding before the first follow-up endoscopy. Prophylactic hemostasis for bleeding or non-bleeding visible vessels during the endoscopy was carried out for 18/74 lesions (24.3%), according to the judgment of the endoscopist in charge. Overall rates of perforation and postoperative bleeding requiring emergency endoscopy were 1.3% (1/75) and 2.7% (2/75), respectively, and these bleeding complications occurred on 2 days and 7 days after ESD, respectively. The former occurred before the first follow-up endoscopy, and the latter occurred after the first follow-up endoscopy without prophylactic hemostasis. No patients needed emergency surgery for complications or blood transfusions for massive bleeding. In the 55 patients in the per-protocol analysis, post-ESD bleeding with emergency endoscopy and severe adverse effects did not occur.

DISCUSSION

The present study is the first prospective study showing that PPI treatment for 2 weeks after gastric ESD may be sufficient for post-ESD ulcers to heal, when the patients have no severe comorbidities and no concomitant use of possible ulcer-promoting drugs and the target lesions have no submucosal involvement, such as ulcerative findings or deep tumor invasion.[12] These results are meaningful not only for the possibility of reducing the cost of post-ESD management but also for understanding the differences between the healing processes of peptic ulcers and artificial ulcers.

In our preliminary observational study using endoscopy, ESD ulcers under 8-week PPI and sucralfate administration were covered by a white mucoid cap at 1 week, followed by reduction to almost half of the initial size by 4 weeks. There was a slight appearance of regenerative mucosa at the border at 4 weeks, and the remaining mucosal defect was fully covered by 8 weeks, regardless of resected size or location.[11] Another study using the surgically resected specimens after gastric ESD revealed that the mechanism of size reduction of these ulcers was contraction of the ulcer for the first few weeks, followed by slow coverage by regenerative mucosa. The remarkable contraction was assumed to be due to the contraction of the intact proper muscle layer.[27] Enhancement of gastric mucosal blood flow at the ulcer margin is an important factor for mucosal regeneration and Hashimoto *et al.* found that blood flow at the margin of EMR-induced ulcer was higher compared with that in a corresponding area of peptic ulcers, the latter being more prone to relapse.[28,29] These findings suggest that artificial ulcer after EMR or ESD may have a more self-healing process.

In addition, the present study showed that the healing process could spontaneously continue without the help of PPI at least as early as 2 weeks after ESD. There is no clear reason for the cut-off time of 2 weeks in terms of the healing effect of PPI, but we speculated that the most serious complications after ESD were postoperative bleeding and perforation which may occur within 2 weeks after ESD. So, it is still controversial to set the length of PPI for a shorter duration (e.g. 1 week), similar to that of EMR.[13] In terms of ulcer-related symptoms, we did not investigate them in detail, because Lee *et al.* reported that no significant differences in ulcer-related symptoms at 4 weeks after EMR were observed between the 1-week PPI group and the 4-week PPI group.[13] In fact, we did not get any complaints from the participating patients requesting painkillers during the study period.

Before the role of *H. pylori* was known, the multifactorial pathogenesis of gastric ulcer had been studied mainly from the perspective of an imbalance of aggressive gastric luminal factors (acid and pepsin) and defensive mucosal barrier function (mucus and local mucosal blood flow).[30] According to the imbalance theory, aggressive factors are heavily suppressed in the gastric environment where gastric cancer emerges because atrophic gastritis with low acidity and low pepsin production pre-exists in most patients with gastric cancer, as a precancerous condition. Thus, the healing efficacy of acid suppression by PPI may be limited in cases of post-ESD ulcers.

However, the efficacy of PPI for post-ESD bleeding has to be considered as a different clinical entity from that for ulcer healing. A few studies have investigated the risk factors of post-ESD bleeding: large tumor size (e.g. >2 cm), scar in the tumor, and middle or lower tumor location.[9,10,31] When the target lesion is small, without a scar, and located in the upper stomach, shorter PPI therapy may be acceptable from both aspects of post-ESD bleeding and ulcer healing.

In the present study, not all of the post-ESD ulcers healed at 8 weeks after ESD, although an additional endoscopy 8 weeks or more after ESD revealed ulcer healing without any specific medication in the non-healing cases. When we consider that the clinical significance of achieving ulcer healing may be to relieve any adverse effects and prevent bleeding from the created ulcer, the obtained healing effect, without any significant adverse effects or any post-ESD bleeding later than 14 days after ESD in this study, must be acceptable in daily practice. In our post-hoc analysis to investigate the predictors related to delayed healing under preconditioning of this study with no deteriorating factors on ESD ulcer healing, location of the lesser curve and large resected size (>40 mm) were significant by univariate analysis and large resected size was still only a predictor by multivariate analysis.

In terms of location, the theory of a dual-control mechanism of ulcer formation, as effected by the mucosa and musculature of the stomach, which was established in 1969 by Oi *et al.*,[32] might explain the possible reason. In agreement with the dual-control mechanism, nearly all gastric ulcers were located adjacent to a mucosal boundary on the side opposite the fundic gland area in relation to the gastric mucosa and/or were located within identifiable special muscular areas where oblique muscle bundles were lacking in relation to the gastric musculature. The latter existed at the narrow lesser curvature of the corpus of the stomach, and gastric motility creates severe kinetic strain in this area. Thus, in case of the lesser curve, the shorter duration of PPI could not counterbalance the negative force for ulcer healing in relation to the gastric musculature. In terms of the resected size, it is quite reasonable for the larger ulcer to need more time for healing. Even in both conditions, however, it is still controversial to use PPI for a longer duration, because there was no adverse effect on patients with delayed healing by 2 weeks of PPI treatment.

After *H. pylori* had been known to be an important factor in healing of peptic ulcer, eradication of *H. pylori* has been recommended to prevent peptic ulcer recurrence. Moreover, Fukase *et al.* reported that eradication of *H. pylori* after endoscopic resection of EGC resulted in an odds ratio of 0.353 for metachronous gastric cancer in comparison with non-eradication in the following 3 years.[33] Although eradication of *H. pylori* should be considered to lessen a metachronous gastric cancer, it may be unnecessary to eradicate it in order to lessen artificial ulcer recurrence, because the pathogenesis is completely mechanical, not due to degradation of gastric mucosa by gastric juice or apoptosis by *H. pylori* and this study also revealed no significant effect on delayed healing.

The major limitation of the present study is that patients with concomitant use of possible ulcer-promoting drugs were excluded. Antithrombotic agent usage has been increasing recently and our consecutive cases of gastric ESD between January 2000 and December 2007 revealed that 11.5% of them were receiving antithrombotic agents.[34]

Patients with ulcerative findings in the tumor were also excluded due to possible delayed healing. When delayed healing does not cause any significant events for the patients, however, these patients may be managed similarly to patients without ulcerative findings (i.e. 2 weeks of PPI administration). Further studies are necessary to determine the appropriate administration of PPI or whether another ulcer-healing drug is better for these patients, from the aspects of both post-ESD bleeding and ulcer healing.

In conclusion, this study revealed that 2-week administration of PPI for post-ESD gastric ulcers may be effective and sufficient to help them heal without increasing any adverse effects in cases where there are no deteriorating factors on ESD ulcer healing. Taken together, our results indicate that artificial ulcers caused by endoscopic resection may be categorized as a new entity of gastric ulcer disease and that their healing process and management should be investigated differently from peptic ulcer disease in some respects.

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Rebamipide induces dendritic cell recruitment to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-exposed rat gastric mucosa based on *IL-1β* upregulation

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ABSTRACT

Rebamipide is usually used for mucosal protection, healing of gastric ulcers, treatment of gastritis, etc., but its effects on gastric malignancy have not been elucidated. Using Lewis and Buffalo rat strains treated with peroral administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), we evaluated the effect of rebamipide on the induction of tumor-suppressive dendritic cells, which are known to be heterogeneous antigen-presenting cells of bone marrow origin and are critical for the initiation of primary T-cell responses. Using CD68 as a marker for dendritic cells, the stomach pyloric mucosae of Lewis and Buffalo rats were immunohistochemically analyzed in the presence or absence of rebamipide and MNNG. After a 14-day treatment of rebamipide alone, no significant change in number of CD68-expressing cells was detected in either rat strain. However, after concurrent exposure to MNNG for 14 days, treatment with rebamipide slightly increased CD68-positive cells in the Lewis strain, and significantly increased them in the Buffalo strain. Analysis of two chemotactic factors of dendritic cells, IL-1β and TNF-α, in the gastric cancer cells showed that expression of *IL-1β*, but not *TNF-α*, was induced by rebamipide in a dose-dependent manner. A luciferase promoter assay using gastric SH-10-TC cells demonstrated that an element mediating rebamipide action exists in the *IL-1β* gene promoter region. In conclusion, rebamipide has potential tumor-suppressive effects on gastric tumorigenesis via the recruitment of dendritic cells, based on the upregulation of the *IL-1β* gene in gastric epithelial cells.

1. Introduction

Rebamipide, an amino acid analog of 2(1H)-quinolinone, is clinically used for mucosal protection, healing of gastric ulcers, and treatment of gastritis [1,2]. The healing effects depend on the enhancement of mucosal defense, scavenging free radicals, and activation of genes such as cyclooxygenase-2 and some growth factors [2]. In the view of carcinogenesis, however, reports concerning the effects of rebamipide on gastric malignancy have been scarce. In this study, we focused on the recruitment of dendritic cells to examine whether rebamipide has a tumor-suppressive effect on gastric mucosa.

Dendritic cells are heterogeneous antigen-presenting cells of bone marrow origin that are critical for the initiation of primary T-cell responses [3,4]. They are thought to play key roles in tumor-specific immune responses via (1) cross-priming of tumor cells and dendritic cells, (2) presentation of tumor antigens through MHC class I, and (3) the generation of CD8+ cytolytic T-cells [4]. We have previously demonstrated that dendritic cells infiltrate the mesenchymal layer of rat stomach during chemical carcinogenesis induced by administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [5]. In our previous study using ACI (sensitive to chemically induced carcinogenesis) and Buffalo (resistant to chemically induced carcinogenesis) rat strains, we observed

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

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a negative correlation between the susceptibility to MNNG-induced carcinogenesis and induction of dendritic cells [5,6].

In the present study, we examined the effect of rebamipide on the recruitment of dendritic cells to the gastric mucosa, using CD68 as a dendritic cell marker [7,8]. In addition, focusing on the candidate chemotactic factors of dendritic cells, we examined the effect of rebamipide on gastric cells by analyzing the gene expression of two cytokines, IL-1β and TNF-α, involved in dendritic cell mobilization. Our study should shed light on a novel effect of rebamipide on the precancerous gastric mucosa.

2. Materials and methods

2.1. Animals

Forty male 6-week-old Lewis rats (LEW/Crj; Charles River Japan, Inc., Yokohama, Japan) and 40 male 6-week-old Buffalo rats (BUF/NacJCl; Nihon Clea, Tokyo, Japan) were used. Based upon the combination of drinking water and food (CRF1, Oriental. Yeast Co., Ltd., Tokyo, Japan) provided, the rats were divided into four groups, with 10 rats in each group, as follows: (1) water with 120 mg/l of MNNG (Sigma–Aldrich, St. Louis, MO) and food with 0.25% w/w rebamipide (Sigma–Aldrich), (2) water with 120 mg/l of MNNG and normal food, (3) normal water and food with 0.25% w/w rebamipide, and (4) normal water and normal food. Treatment of animals used in this study adhered to the Declaration of Helsinki.

2.2. Immunohistochemistry

Deparaffinization, endogenous peroxidase inactivation, and antigen retrieval of rat stomach tissues were performed as described previously [9,10]. Immunostaining with anti-CD68 mouse monoclonal antibody (Clone: KP1; DAKO, Tokyo, Japan) at a 1:200 dilution was performed, followed by visualization with 20 mg/dl 3,3′-diaminobenzidine tetrahydrochloride (DAKO) solution containing 0.006% hydrogen peroxide. The immunostained sections were evaluated independently by two pathologists along with hematoxylin and eosin-stained sections from the same lesions.

2.3. Cell cultures

Twenty gastric cancer cell lines (NUGC-4, AZ521, KATO-III, SH-10-TC, MKN-7, H-III-TC, MKN-1, MKN-45, MKN-74, TGBC11TKB, KE-39, KE-97, GCIY, HGC-27, HuG1-PI, HuG1-N, AGS, NCI-N87, ECC-10, ECC-12), 10 colorectal cancer cell lines (WiDr, DLD-1, SW480, COLO320DM, HCT116, HCT-15, SW620, LS174T, LOVO, HT-29), and two non-gastrointestinal cancer cell lines (HeLa-S3 and MDA-MB435) were maintained in DMEM with 10% fetal calf serum (Gibco/Invitrogen, Carlsbad, CA) at 37 °C, as previously reported [11]. Rebamipide at concentrations of 6, 2, 0.7, and 0 mM in culture medium (DMEM with 10% fetal calf serum) was used for treatment.

2.4. RT-PCR

Total cellular RNAs were prepared using the Isogen RNA isolation reagent (Wako Pure Chemical Industries, Osaka, Japan) as previously reported [12]. Semi-quantitative RT-PCR was performed via a Superscript One-Step reaction using the Platinum Taq (Invitrogen). The primer pairs used to detect the transcripts of *TNF-α*, *IL-1β*, and *GAPDH* were as follows: 5′-gtgcttgttcctcagcctct-3′ and 5′-ttgatggcagagaggaggtt-3′ for *TNF-α*, 5′-tccagggacaggatatggag-3′ and 5′-ccctagggattgagtccaca-3′ for *IL-1β*, and 5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′ for *GAPDH*. RNA was reverse-transcribed for 30 min at 50 °C, and after an initial denaturation at 94 °C for 3 min, cDNA amplification procedures were performed as follows: for *TNF-α* and *IL-1β*, 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min; for *GAPDH*, 24 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min.

2.5. Luciferase promoter assay

SH-10-TC cells were seeded in 96-well (0.32 cm^2) plates in a semi-confluent manner in the presence or absence of 6 mM rebamipide in medium for 24 h. They were then transfected with 6.5 ng of Renilla luciferase control vector pGL4.74 (Promega, Madison, WI), and either 200 ng of firefly luciferase experimental vector pGL4.12 (Promega, negative control), pGL4.12_IL-1β-P1 (upstream 1062 bp from the exon 1 of *IL-1β*), pGL4.12_IL-1β-P2 (upstream 513 bp), pGL4.12_IL-1β-P3 (upstream 131 bp), or pGL4.12-TK (positive control). All transfections were performed with Lipofectamine Reagent and Lipofectamine PLUS (Invitrogen). Luciferase activities were measured at 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega).

2.6. Constructions

To construct the vectors for reporter assays, a 1500-bp upstream sequence from the 5′-end of exon 1 of the *IL-1β* gene was first amplified from genomic DNA derived from SH-10-TC cells using the primers 5′-atacttgcacagaggctcac-3′ and 5′-gattggctgaagagaatccc-3′. The amplified products were cloned into pT7blue T-vector cloning vectors (TAKARA Bio Inc., Shiga, Japan) to generate pT7Blue-IL-1β-P0. The 1.6-kb DNA BamHI/SpeI fragment was blunted with Klenow fragment (TAKARA) and inserted into the EcoRV site of pGL4.12 (Promega) to generate pGL4.12-IL-1β-P0. After the 5.5-kb XhoI/SmaI fragment derived from pGL4.12-IL-1β-P0 was blunted with Klenow fragment, self-ligation was performed to generate pGL4.12-IL-1β-P1 (upstream 1062 bp). The 5.0-kb SacI fragment derived from pGL4.12-IL-1β-P0 was also self-ligated to generate pGL4.12-IL-1β-P2 (upstream 513 bp). The 0.15-kbp HindIII/BamHI fragment derived from pT7Blue-IL-1β-P0 was inserted into EcoRV site of pGL4.12 to generate pGL4.12-IL-1β-P3 (upstream 131 bp). For positive control, the 0.8 kb HindIII/KpnI fragment (human simplex virus thymidine kinase gene promoter) derived from pGL4.74 (Promega) was inserted into HindIII/KpnI site of pGL4.12 to generate pGL4.12-TK.

3. Results

3.1. Treatment with rebamipide increases the number of dendritic cells in MNNG-exposed rat gastric mucosa

To evaluate the effect of rebamipide on the gastric mucosa, we analyzed the expression of CD68-positive cells in the stomach pyloric mucosa of Lewis and Buffalo rat strains. CD68 is an established marker of dendritic cells, and CD68-positive cells in the gastric mucosa can be considered as dendritic cells [7,8,13]. After the 14-day treatment of rebamipide alone, an obvious microscopic change of the gastric mucosa could not be detected in both Lewis and Buffalo strains. There were also no significant differences in the number of CD68-positive cells with or without the 14-day treatment of rebamipide (Fig. 1A and 1B).

We next analyzed the effect of rebamipide on the recruitment of CD68-positive cells in the stomach pyloric mucosa of the MNNG-exposed Lewis and Buffalo rats. Consistent with previous reports [14,15], neither intestinal metaplasia nor adenocarcinoma could be observed in the gastric mucosa after the treatment of MNNG for only 14 days. We have already reported that the number of dendritic cells in the rat stomach pyloric mucosa increase after

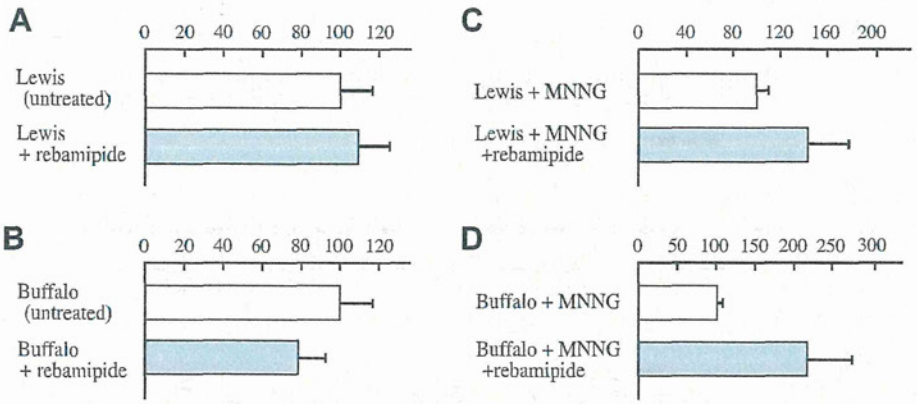

Fig. 1. Numbers of CD68-positive cells in the gastric pyloric mucosa of Lewis and Buffalo rat strains in a single microscopic view (200×). Data were obtained from the eight groups of 10 rats as follows: (A) Lewis untreated (control) and Lewis treated with rebamipide for 14 days; (B) Buffalo untreated (control) and Buffalo treated with rebamipide for 14 days; (C) Lewis treated with MNNG alone for 14 days and Lewis treated with a combination of MNNG and rebamipide for 14 days; and (D) Buffalo treated with MNNG alone for 14 days and Buffalo treated with a combination of MNNG and rebamipide for 14 days. In each panel, the number of CD68-positive cells was standardized to that of the rats without rebamipide treatment. About 120 mg/l of MNNG in drinking water and 0.25% w/w of rebamipide in food were used as treatments. The error bars indicate the standard errors based on the data obtained from corresponding 10 rats.

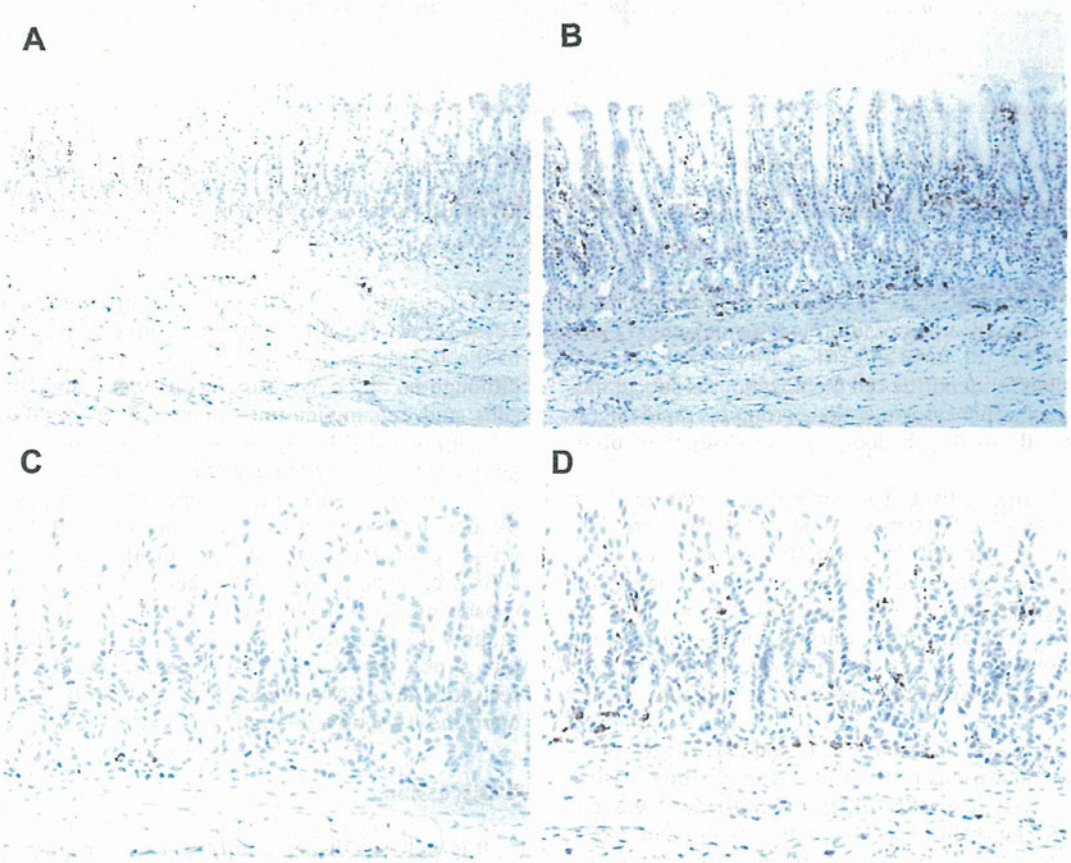

Fig. 2. Immunohistochemical staining of CD68 in gastric pyloric tissue sections obtained from Lewis and Buffalo rats. Magnification 200×. (A) Lewis strain rats treated with MNNG alone for 14 days. (B) Lewis strain rats treated with a combination of MNNG and rebamipide for 14 days. (C) Buffalo strain rats treated with MNNG alone for 14 days. (D) Buffalo strain rats treated with a combination of MNNG and rebamipide for 14 days. About 120 mg/l of MNNG in drinking water and 0.25% w/w of rebamipide in food were used as treatments.

exposure to MNNG [5]. In the Lewis strain, induction of CD68-positive cells seemed to be stronger with rebamipide treatment (Fig. 2A and 2B), although statistical significance could not be obtained (Fig. 1C). As for the Buffalo strain, a significant increase in CD68-positive dendritic cells was detected with the rebamipide treatment (Figs. 2C, 2D and 1D). From these results, we concluded that oral rebamipide intake has the potential to induce the migration of dendritic cells to the gastric mucosa. Rebamipide and MNNG possibly have an addictive or multiplier effect on the dendritic cell migration.

3.2. Treatment with rebamipide activates the transcription of IL-1β, a potent chemotactic factor for dendritic cells, in various gastric cell lines

It is well established that dendritic cells are mobilized in response to a large variety of chemical, physical, or biological stimuli.

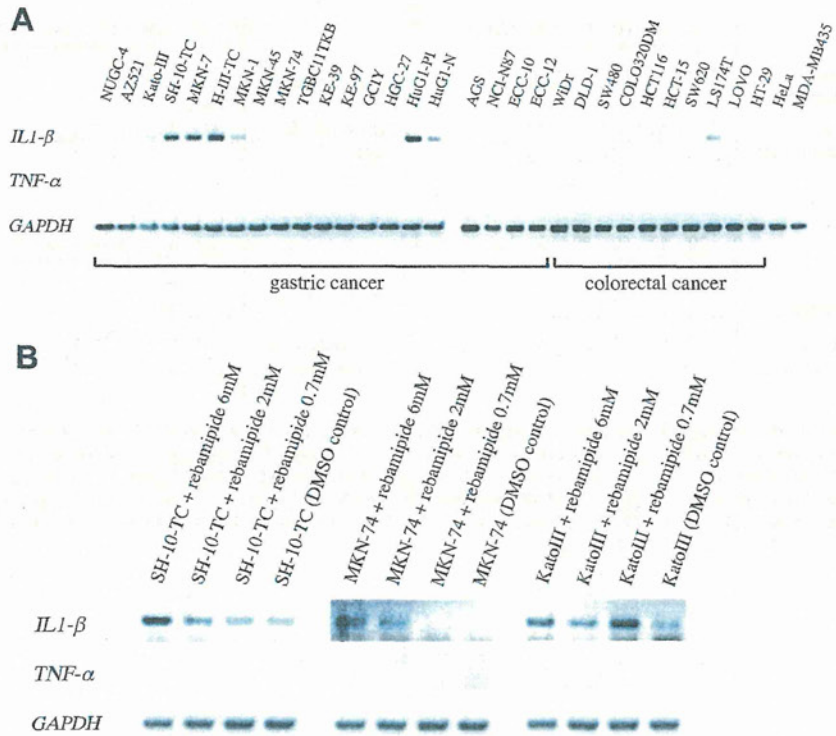

Fig. 3. (A) Expression patterns of *IL-1β*, *TNF-α*, and *GAPDH* mRNAs in a panel of 32 human cancer cell lines. Twenty gastric cancer cell lines, 10 colorectal cell lines, and two non-gastrointestinal cell lines (HeLa-S3 and MDA-MB435) were analyzed by RT-PCR. (B) Expression of *IL-1β*, *TNF-α*, and *GAPDH* after a 2-day treatment of rebamipide at various concentration (6, 2, 0.7, or 0 mM). Three gastric cancer cell lines (SH-10-TC, MKN-74, and KATO-III) were analyzed by RT-PCR.

Despite their diversity, most of the mobilization signals appear to exert their activity through a pair of intermediate messenger cytokines, IL-1β and TNF-α [16]. IL-1β and TNF-α are not only required but are also sufficient for dendritic cell mobilization, as subcutaneous administration of either cytokines alone promotes rapid migration of dendritic cells to lymph nodes in the absence of other stimuli [17,18].

To elucidate the mechanism of rebamipide-induced dendritic cell recruitment, expression patterns of both cytokines were analyzed using twenty gastric cell lines and 10 colorectal cell lines. *TNF-α* mRNA was barely detected in these gastrointestinal tumor cells (Fig. 3A), suggesting that the expression of TNF-α is strongly suppressed in cells of the alimentary tract. In contrast, *IL-1β* gene transcript was detected in several gastric cancer cells (Fig. 3A); we speculate that IL-1β may be a major inducer of dendritic cells during gastric canceration.

Next, we analyzed the expression of *IL-1β* and *TNF-α* in the presence of rebamipide at various concentrations using three gastric cancer cell lines: SH-10-TC, MKN-74, and KATO-III. As was expected, marked upregulation of *IL-1β* expression, but not *TNF-α* expression, was detected in an approximately dose-dependent manner (Fig. 3B). From these results, we concluded that rebamipide treatment activates transcription of *IL-1β* in gastrointestinal cells.

3.3. The element mediating rebamipide action in gastric cells exists in the promoter region of IL-1β gene

To elucidate the mechanism of rebamipide-induced *IL-1β* upregulation, we designed a series of reporter constructs using the upstream sequence of *IL-1β* gene and performed a luciferase assay in SH-10-TC cells (Fig. 4). The upstream 131 bp (P3) alone did not express the reporter luciferase gene, whereas the upstream 513 bp region (P2) had an obvious promoter activity (Fig. 4B). These results suggest that the sequence between −513 and −131 of the *IL-1β* promoter has promoter activity in the gastric cells, although no difference was seen between the rebamipide-treated cells and rebamipide-untreated cells (Fig. 4B). The upstream 1062 bp region (P1) also had an evident promoter activity, which got stronger in the presence of rebamipide.

As was expected, the three reporter constructs (P1, P2, and P3) did not show any transcriptional activity in IL-1β-deficient MDA-MB435 cells originated from breast cancer (Fig. 4B). From these results, we concluded that an element mediating rebamipide action, capable of increasing transcriptional activity in gastric cells, exists in the *IL-1β* promoter region from −1062 to −513. This result reinforces our speculation that rebamipide-induced dendritic cell recruitment to MNNG-exposed rat gastric mucosa should be based upon the *IL-1β* upregulation.

4. Discussion

It is well established that *Helicobacter pylori*-associated chronic gastritis is a precancerous condition of gastric malignancy [19,20]. Although the effects of rebamipide on gastric malignancy have been barely elucidated, there have been some reports concerning its tumor-suppressive effects via an influence on gastritis or gastric cancer cells. For instance, it was reported that rebamipide inhibits the production of neutrophil chemokines (CINC/KC) and TNF-α, and consequently prevents the development of chronic gastritis [21]. In the gastric cancer mouse model induced by *N*-methyl-*N*-nitrosourea (NMU) treatment and *Helicobacter pylori* infection, it was also reported that long-term rebamipide administration inhibits upregulation of oncogenic proteins and down-regulation of anti-oncogenic proteins in gastric cells [22]. In addition, it was recently reported that rebamipide treatment inhibits gastric cancer

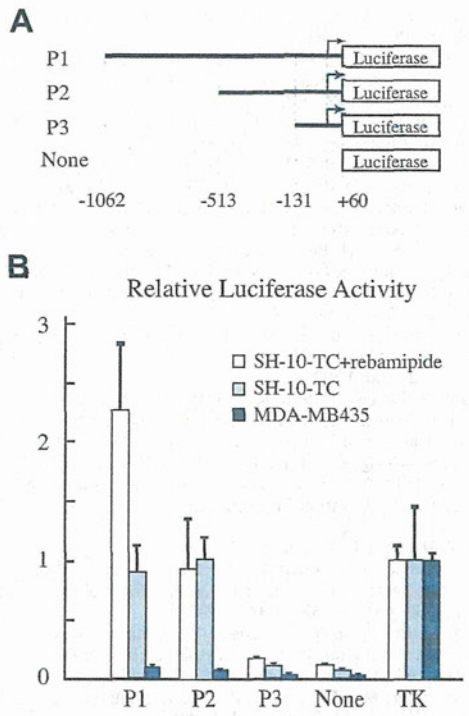

Fig. 4. (A) Schematic representation of the human *IL-1β* gene reporter constructs used in this study. The numbers represent base pairs measured from the 5′-end of the first exon of the *IL-1β* gene (+1), which is denoted as a rectangular arrow in each construct. (B) Transcriptional activities of a series of *IL-1β* promoter constructs in gastric SH-10-TC cells in the presence or absence of rebamipide. As a positive control, pGL4.12-TK (TK) harboring the human simplex virus thymidine kinase gene promoter was used. The promoter activities of MDA-MB435 cells deficient in *IL-1β* expression are also shown. Luciferase activities were measured 24 h after transfection, and the data shown are the mean values of triplicate experiments with the error bars corresponding to the standard errors.

cell growth [23,24]. These results suggest that rebamipide has some tumor-suppressive effects on gastric canceration.

In our present study, we discovered another possible anti-oncogenic effect of rebamipide upon gastric tumorigenesis, through the recruitment of dendritic cells capable of inducing tumor-specific immune responses [4]. There is a strong correlation between infiltration of dendritic cells to the tumor lesion and better prognosis in various cancers, including lung cancer [25], breast cancer [26], and hepatocellular carcinoma [27]. As for gastric cancer, there have also been several reports showing a strong correlation between the recruitment of dendritic cells and better prognosis [28–30]. In the MNNG-induced gastric cancer model rats treated with rebamipide, we detected a significant increase in dendritic cells in the precancerous gastric mucosa, which suggests a tumor-suppressive effect of the rebamipide administration.

We speculate that *IL-1β* transcriptional activation in gastric cells is one of the key mechanisms of the rebamipide-induced dendritic cell migration, but the precise mechanism of rebamipide on *IL-1β* gene promoter in gastric cells has not been elucidated. Contrary to our results, it was previously reported that rebamipide suppresses *IL-1β* expression in blood mononuclear cells [31]. The opposing results suggest that the transcriptional regulation of *IL-1β* gene in gastric epithelial cells is different from that of bone marrow-derived mononuclear cells. Considering the high-dose and long latent period required for the transcriptional activation of *IL-1β* gene by rebamipide, we presume that rebamipide-induced transactivation of *IL-1β* gene in gastric cells is due to an indirect effect of rebamipide via inducing some other transcription factors to the promoter region from −1062 to −513. Including validation of the possibility for direct binding of rebamipide to the *IL-1β* promoter region, the detailed mechanism of rebamipide on *IL-1β* gene transactivation needs to be resolved in the future. The variation of *IL-1β* gene regulation in different cell types is also a major problem that should be concurrently elucidated.

MNNG-treated rat stomachs and chronically *Helicobacter pylori*-infected human stomachs are quite different, but gastric mucosae in both cases are in a precancerous condition with chronic inflammation and upregulation of various cytokines [32]. We are planning to conduct a prospective cohort study of patients with chronic *Helicobacter pylori* infection, who will be treated with rebamipide or a placebo. Evaluation of dendritic cell migration in the presence or absence of rebamipide should be informative and suggestive for predicting its preventive effect on gastric tumorigenesis. Long-term follow-up focusing on gastric cancer incidence will finally provide clear evidence for our hypothesis of the tumor-suppressive effect of rebamipide.

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ORIGINAL ARTICLE

Relationship between vomiting reflex during esophagogastroduodenoscopy and dyspepsia symptoms

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Aim: Although frequent vomiting reflexes during esophagogastroduodenoscopy (EGD) causes suffering in patients, very few studies have investigated the characteristics of subjects who frequently develop vomiting reflexes. This study examined the incidence of the vomiting reflex and related factors, especially upper gastrointestinal symptoms, among individuals undergoing transoral EGD.

Methods: Subjects included 488 consecutive adults (mean age, 56.1 ± 8.9 years) who underwent transoral EGD for gastric cancer screening between February 2010 and March 2011. All procedures were performed by an endoscopist with 15 years of experience. Based on a questionnaire survey using the frequency scale for the symptoms of gastroesophageal reflux disease (FSSG), symptoms (dyspepsia and acid reflux symptoms) and the number of vomiting reflexes during EGD were recorded.

Results: Of the 488 subjects, 271 (56%) developed vomiting reflexes (mean, 4.2 times). This reflex-positive group was younger (54.3 ± 9.5 years) than the reflex-negative group (58.3 ± 7.7 years, $P < 0.001$). The number of subjects in the reflex-positive group with a high FSSG dyspepsia score (2.27 ± 2.57 *vs* 1.23 ± 1.84; $P < 0.001$), acid reflux symptom score (1.96 ± 2.22 *vs* 1.34 ± 2.14; $P < 0.01$) or an esophageal hiatal hernia (14.8% *vs* 4.6%; $P < 0.001$) was significantly higher than in the reflex-negative group. Multivariate analysis also showed a significant correlation between these four factors and the occurrence of vomiting reflexes. Using an FSSG dyspepsia score of 1 as the cut-off offered 68% sensitivity and 57% specificity for predicting the occurrence of vomiting reflexes.

Conclusion: Based on FSSG questionnaire responses on upper gastrointestinal symptoms, dyspepsia symptoms, in particular, are related to presence of vomiting reflexes during EGD.

Key words: conscious sedation, dyspepsia, gag reflex, transnasal endoscopy, vomiting reflex.

INTRODUCTION

Japan has a higher *Helicobacter pylori* infection rate than Western countries, and *Helicobacter pylori*-related chronic gastritis is a main background factor in many cases of gastric cancer. To reduce the related high mortality and morbidity rates, mass screening for gastric cancer has been conducted as a public health service since the mid-1960s.[1] Gastric cancer screening with esophagogastroduodenoscopy (EGD) has gradually gained popularity in Japan in place of conventional screening with barium X-ray.[2,3] However, EGD is an invasive procedure that entails considerable discomfort, so minimizing the distress of subjects who undergo gastric cancer screening with EGD is important.

The vomiting reflex that occurs frequently during EGD is one response encountered in patients who feel discomfort. Transnasal EGD is reportedly associated with fewer vomiting reflexes and less discomfort compared to conventional transoral EGD.[4–7] Therefore, transnasal EGD has increased in popularity recently in Japan, with the aim of alleviating discomfort. However, as the number of EGD for gastric cancer screening increases, problems are increasingly encountered regarding the manpower needed for the complex pretreatment for transnasal EGD and patient safety (i.e. adverse effects from sedatives). These issues make transnasal EGD and conscious sedation difficult to apply in all patients. In addition, many endoscopists have encountered a fair number of patients who show absolutely no vomiting reflex during transoral EGD. However, very few reports have examined which subjects are more likely to show a vomiting reflex, and the details of this reflex remain unclear.

Functional dyspepsia (FD) is a typical functional gastrointestinal disorder,[8] and it has been suggested that it occurs as a result of many factors,[9] including impaired gastric accommodation. Gastric accommodation is thought to reflect gastric retention function and to be related to dyspepsia symptoms such as the amount of food that can be ingested and sense of satiation.[10–13] In EGD, factors associated with the vomiting reflex are thought to be air insufflation and gastric

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irritation caused by the endoscope, which, in turn, cause the gastric walls to distend and stimulate the vomiting center via sympathetic and vagal afferents.[14] Impaired gastric accommodation in response to distention of the gastric wall by insufflation and the endoscope during the procedure may be one cause of the vomiting reflex.

The purpose of this study was to investigate the relationship between the presence or absence of the vomiting reflex and various background and clinical factors in subjects who underwent transoral EGD for gastric cancer screening. In particular, the relationship between upper gastrointestinal symptoms and the vomiting reflex were closely examined.

METHODS

Study population

In Japan, health checkups are performed to identify selected diseases (e.g. gastric cancer) in the early stage of development. Subjects included 488 consecutive adults (409 men, 79 women; mean age, 56.1 ± 8.9 years) who underwent transoral EGD for gastric cancer screening. A single endoscopist (SE; 15 years of experience, during which he performed transoral EGD in more than 5000 patients) performed all procedures at the Wakayama Wellness Foundation (Wakayama, Japan) between February 2010 and March 2011. The study protocol was approved by the ethics committee of Wakayama Medical University (Wakayama, Japan) and informed consent was obtained from all subjects.

Endoscopic procedure

EGD was performed in a standardized fashion for the main purpose of gastric cancer screening, but also for differential diagnosis of other esophageal, gastric and duodenal diseases. The endoscope was pushed to the esophagogastric junction via the mouth, and then through the stomach and pylorus into the descending duodenum. The endoscope was then pulled back into the stomach for inspection of the gastric antrum and corpus. Gastric angulation and gastric cardia were inspected in inversion. The endoscope was removed slowly, after careful inspection of the stomach and esophagus. Three endoscopes were used, including two GIF-XP240 (tip outer diameter, 7.7 mm) and one GIF-XQ230 (tip outer diameter, 9.2 mm) from Olympus Medical Systems (Tokyo, Japan).

For EGD preparations, 22.5 mL water was added to 2.5 mL 4% lidocaine hydrochloride solution (Xylocaine; AstraZeneca, Osaka, Japan) and then gargled to a total of 25 mL (10-fold dilution). Next, 100 mL water was added to 5 mL 2% dimethicone syrup (Gascon; Kissei Pharmaceutical, Nagano, Japan), and 1 g sodium bicarbonate (Sioe Pharmaceutical, Hyogo, Japan) and 0.5 g pronase (Pronase MS; Kaken Pharmaceutical, Tokyo, Japan) were dissolved and administered. Third, 8% lidocaine hydrochloride spray (AstraZeneca) was sprayed into the pharynx. Subjects who desired conscious sedation during the test were given intravenous diazepam (Cercine; Takeda Pharmaceutical, Osaka, Japan) at 5–7.5 mg/body (5 mg/body for patients ≥70 years old or with body weight ≤50 kg) immediately before EGD.

Assessment of upper gastrointestinal symptoms

A medical questionnaire was used for objective evaluation of upper gastrointestinal symptoms. Various medical questionnaires have been devised for gastroesophageal reflux disease (GERD).[15] In Japan, the frequency scale for the symptoms of GERD (FSSG) developed by Kusano *et al.* is often used.[16] The FSSG questionnaire uses 12 questions,scored to indicate the frequency of symptoms as follows: never = 0, occasionally = 1, sometimes = 2, often = 3, and always = 4. Of the 12 questions on the FSSG, seven relate to acid reflux symptoms and five to dyspepsia symptoms. Evaluations can be performed separately for these two symptom systems.[17–19] This makes the FSSG useful as a diagnostic tool not only for GERD, but also for FD. FSSG was used in this study, and subjects completed the questionnaire with regards to upper gastrointestinal symptoms encountered in the preceding 2–3 weeks. Evaluations were performed for acid reflux symptom score, dyspepsia symptom score, presence or absence of acid reflux symptoms, presence or absence of dyspepsia symptoms, and the 12 questions on the FSSG.

Parameters assessed

The number of vomiting reflexes by each patient during the procedure (from endoscope insertion into the pharynx until withdrawal) was recorded by the endoscopist or nursing staff. Anything thought to be vomiting, belching or retching was recorded in the number of positive reflexes. Obvious coughs were not recorded in the number of reflexes. Factors related to the presence or absence of the vomiting reflex were investigated. In addition to upper gastrointestinal symptoms assessed by the FSSG, parameters investigated were age, sex, body mass index, current drinking habits, current smoking habits, history of gastrectomy, administration of acid reducers (proton pump inhibitor or histamine-2 receptor antagonist), past experience of EGD, differences in endoscope used, whether the subjects desired conscious sedation during the procedure, time for EGD, presence or absence of reflux esophagitis (Los Angeles classification system:[20] Grades A–D indicate erosive esophagitis), and presence or absence of hiatal hernia (hiatal hernia was diagnosed endoscopically when the distance between the crural impression and gastroesophageal junction was ≥2 cm).

Statistical analysis

Univariate analysis was performed using the unpaired *t*-test for numerical data and Fisher's exact test for categorical data. Multivariate analysis was performed using a logistic regression model. All tests were two-sided, and values of $P < 0.05$ were considered statistically significant. All analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

RESULTS

Of the 488 subjects, 271 (56%) experienced ≥1 vomiting reflex during EGD. The mean number of vomiting reflexes in these subjects was 4.2. Subjects with ≥1 vomiting reflex were placed in a reflex-positive group, and those without vomiting reflex in a reflex-negative group. Various factors were

compared between reflex-negative and reflex-positive groups (Tables 1,2). Age was significantly lower in the reflex-positive group ($P < 0.001$). The reflex-positive group also had significantly higher FSSG scores for acid reflux symptoms ($P < 0.01$) and dyspepsia symptoms ($P < 0.001$), and significantly higher rates of acid reflux symptoms ($P < 0.001$) and dyspepsia symptoms ($P < 0.001$). Findings of hiatal hernia were present at a higher rate in the reflex-positive group ($P < 0.001$). The percentage of cases in which conscious sedation was performed according to the wishes of the patient was significantly higher in the reflex-positive group ($P < 0.001$). Since effects of conscious sedation on vomiting reflex could not be ignored in this study, a separate investigation of subjects without conscious sedation (reflex-negative, $n = 125$; reflex-positive, $n = 106$) and with conscious sedation (reflex-negative, $n = 92$; reflex-positive, $n = 165$) was performed (Table 1,2). The results of analysis of these subgroups did not differ markedly from the results of the overall analysis. Age was significantly lower, FSSG acid reflux symptom and dyspepsia symptom scores were significantly higher, and the rate of findings of hiatal hernia was significantly higher in the reflex-positive group than in the reflex-negative group for subjects both with and without conscious sedation. However, findings of erosive esophagitis were significantly higher in the reflex-positive group than in the reflex-negative group among subjects with conscious sedation ($P < 0.05$), whereas no significant difference was apparent among subjects without conscious sedation.

A comparison of each of the 12 FSSG questions between groups showed that scores for all five questions related to dyspepsia symptoms (Questions 2, 3, 5, 8, and 11) were higher in the reflex-positive group than in the reflex-negative group (Table 3).

Multivariate analysis was performed for vomiting reflex using the four factors of age, presence or absence of hiatal hernia, acid reflux symptoms and dyspepsia symptoms. Age was a significant negative independent factor for vomiting reflex ($P < 0.001$), and hiatal hernia ($P < 0.001$), acid reflux symptoms ($P < 0.05$) and dyspepsia symptoms ($P < 0.01$) were significant positive independent factors (Table 4).

Using a cutoff value of 1 for the FSSG dyspepsia symptom score achieved 68% sensitivity and 57% specificity with respect to predicting presence of the vomiting reflex.

DISCUSSION

In this study, the vomiting reflex was seen in the majority of subjects who underwent gastric cancer screening using transoral EGD. Although conscious sedation with diazepam or midazolam is used to overcome the vomiting reflex, these medications are sometimes not helpful and most sedated subjects are still able to vomit.[21] Similarly, the vomiting reflex-positive group in the present study included a larger number of subjects with conscious sedation using diazepam. Many of the present subjects had undergone EGD in the past and may have chosen conscious sedation because of the discomfort they had experienced from the vomiting reflex at that time. During endoscopy and fine endoscopic examinations, procedures take a substantial amount of time, and sedation is necessary. However, in the present study, the average time for transoral EGD examination for the purpose of

Table 1. Comparison of subjects' background factors according to presence of vomiting reflex

	Total subjects			Subjects without conscious sedation			Subjects with conscious sedation		
	Reflex-negative ($n = 217$)	Reflex-positive ($n = 271$)	*P*-value	Reflex-negative ($n = 125$)	Reflex-positive ($n = 106$)	*P*-value	Reflex-negative ($n = 92$)	Reflex-positive ($n = 165$)	*P*-value
Age (years, mean ± SD)	58.30 ± 7.66	54.32 ± 9.45	<0.001	59.12 ± 7.56	56.00 ± 9.94	0.009	57.17 ± 7.70	53.24 ± 8.98	<0.001
Sex (men/women)	185/32	224/47	0.460	106/19	88/18	0.722	79/13	136/29	0.598
Body mass index (mean ± SD)	23.38 ± 3.12	23.66 ± 3.19	0.326	23.60 ± 3.06	23.59 ± 3.03	0.991	23.09 ± 3.20	23.71 ± 3.29	0.144
Current drinking (−/+)	73/144	100/171	0.505	44/81	41/65	0.588	29/63	59/106	0.584
Current smoking (−/+)	155/62	177/94	0.172	94/31	82/24	0.758	61/31	95/70	0.185
Past history of gastrectomy (−/+)	214/3	267/4	1.000	124/1	104/2	0.594	90/2	163/2	0.619
Medication of PPI or H2RA (−/+)	214/3	265/6	0.737	123/2	104/2	1.000	91/1	161/4	0.657

H2RA, histamine-2 receptor antagonist; PPI, proton pump inhibitor.

Table 2. Comparison of subjects' clinical factors according to presence of vomiting reflex

	Total subjects			Subjects without conscious sedation			Subjects with conscious sedation		
	Reflex-negative ($n = 217$)	Reflex-positive ($n = 271$)	*P*-value	Reflex-negative ($n = 125$)	Reflex-positive ($n = 106$)	*P*-value	Reflex-negative ($n = 92$)	Reflex-positive ($n = 165$)	*P*-value
FSSG									
Acid reflux symptom score (mean ± SD)	1.34 ± 2.14	1.96 ± 2.22	0.002	1.56 ± 2.18	2.19 ± 2.29	0.034	1.03 ± 2.06	1.82 ± 2.17	0.005
Acid reflux symptoms (−/+)	118/99	89/182	<0.001	61/64	30/76	0.002	57/35	59/106	<0.001
Dyspepsia symptom score (mean ± SD)	1.23 ± 1.84	2.27 ± 2.57	<0.001	1.30 ± 1.76	2.14 ± 2.34	0.003	1.13 ± 1.94	2.35 ± 2.71	<0.001
Dyspepsia symptoms (−/+)	123/94	87/184	<0.001	66/59	37/69	0.007	57/35	50/115	<0.001
EGD									
Past experience of EGD (−/+)	14/203	14/257	0.562	8/117	7/99	1.000	6/86	7/158	0.553
Endoscope (GIF-XP240 [7.7 mm]/GIF-XQ230 [9.2 mm])	182/35	222/49	0.630	102/23	87/19	1.000	80/12	135/30	0.379
Conscious sedation (−/+)	125/92	106/165	<0.001	–	–	–	–	–	–
Endoscopic biopsy (−/+)	201/16	259/12	0.175	115/10	99/7	0.803	86/6	160/5	0.208
Time for EGD (s: mean ± SD)	424.98 ± 21.80	422.88 ± 14.78	0.226	425.28 ± 24.12	424.53 ± 19.77	0.798	424.57 ± 18.30	421.81 ± 10.32	0.187
Erosive esophagitis (−/+)	182/35	211/60	0.107	101/24	84/22	0.869	81/11	127/38	0.032
Hiatal hernia (−/+)	207/10	231/40	<0.001	119/6	92/14	0.033	88/4	139/26	0.007

EGD, esophagogastroduodenoscopy; FSSG, frequency scale for the symptoms of gastroesophageal reflux disease.

gastric cancer screening was approximately 8 min, which is relatively not long. In addition, close to half of the subjects did not have a vomiting reflex, and given the poor effect of diazepam in reducing the vomiting reflex and problems such as the occurrence of accidental symptoms with sedation, the need for conscious sedation in all patients remains highly debatable.

In this study, many subjects with dyspepsia symptoms were in the reflex-positive group, suggesting that impaired gastric accommodation may be a factor in the vomiting reflex. However, dyspepsia symptoms are not only seen in FD patients. The results of this study showed no relationship between the vomiting reflex and reflux esophagitis. Moreover, while a stronger relationship was seen with dyspepsia symptoms than with acid reflux symptoms, the finding that younger people and hiatal hernias were more common in the reflex-positive group also suggests a relationship with GERD. However, the fact that no previous reports have described a high frequency of impaired gastric accommodation or FD among younger people is inconsistent with the finding of many young people being reflex-positive in the present study. This suggests that the vomiting reflex is influenced by multiple factors. Accordingly, there is a high likelihood that FD and GERD are complicating conditions, and in fact, physiological assessment using tests,[22] such as fundic barostat, single photon emission computed tomography, or ultrasonography, is probably necessary to demonstrate a relationship between impaired gastric accommodation and the vomiting reflex.

The vomiting reflex during EGD is not thought to have a single trigger. The vomiting reflex is also called the gag reflex or pharyngeal reflex, and may even be produced by touching the pharynx (especially the base of the tongue) with an endoscope. As the transnasal endoscope is inserted without touching the tongue and does not induce the reflex, several studies have reported this approach as being significantly more patient-friendly than conventional transoral endoscopy.[4–7] Contrary to the results of those investigations, other studies have reported no significant differences in the number of occurrences of vomiting reflex between transnasal and transoral groups.[23–25] Given that no vomiting reflex was seen in about half of subjects who underwent transoral EGD in our study, contact stimulus at the base of the tongue alone is not likely induce vomiting in all cases.

The mean age of subjects in this study was relatively low. Age was a negative independent factor for the vomiting reflex, but the percentage of subjects showing a vomiting reflex may differ greatly depending on the age makeup of the subject cohort. Subjects in this study were people who underwent gastric cancer screening, typically because of suspicious findings from the FSSG questions. Generally, people who undergo gastric cancer screening do not have strong subjective symptoms that prompt a hospital visit for an examination. In studies like this one that look at people who undergo EGD for both general screening and for close investigation of existing symptoms, the percentage of people with a vomiting reflex may be increased.

The present study had several limitations that must be considered when interpreting the results. First, the investigation was based on results from EGD performed by a single endoscopist. However, if the procedure had been performed by multiple endoscopists, varying EGD skill level resulting

Table 3. Comparison of FSSG score according to presence of vomiting reflex

FSSG question			Vomiting reflex-negative group ($n=217$)	Vomiting reflex-positive group ($n=271$)	*P*-value
Q1	A	Do you get heartburn?	0.295 ± 0.598	0.428 ± 0.679	0.022
Q2	D	Does your stomach get bloated?	0.309 ± 0.602	0.542 ± 0.773	<0.001
Q3	D	Does your stomach ever feel heavy after meals?	0.286 ± 0.586	0.564 ± 1.030	<0.001
Q4	A	Do you sometimes subconsciously rub your chest with your hand?	0.111 ± 0.405	0.170 ± 0.480	0.140
Q5	D	Do you ever feel sick after meals?	0.065 ± 0.281	0.182 ± 0.488	0.001
Q6	A	Do you get heartburn after meals?	0.217 ± 0.475	0.373 ± 0.588	0.001
Q7	A	Do you have a burning sensation in your throat?	0.194 ± 0.561	0.277 ± 0.684	0.140
Q8	D	Do you feel full while eating meals?	0.180 ± 0.518	0.284 ± 0.612	0.042
Q9	A	Do some things get stuck when you swallow?	0.148 ± 0.437	0.214 ± 0.529	0.129
Q10	A	Do you get bitter liquid coming up into your throat?	0.346 ± 0.613	0.483 ± 0.660	0.017
Q11	D	Do you burp a lot?	0.364 ± 0.740	0.657 ± 0.863	<0.001
Q12	A	Do you get heartburn if you bend over?	0.051 ± 0.259	0.207 ± 1.856	0.174

A, acid reflux symptom; D, dyspepsia symptom; FSSG, frequency scale for the symptoms of gastroesophageal reflux disease.

Table 4. Factors associated with vomiting reflex: results of multivariate logistic analysis

Factor	*P*-value	Odds ratio	95%CI
Age (years)	0.001	0.96	0.93–0.98
Hiatal hernia (–/+)	0.001	3.5	1.67–7.35
Acid reflux symptoms (–/+)	0.046	1.56	1.01–2.39
Dyspepsia symptoms (–/+)	0.002	1.99	1.29–3.06

CI, confidence interval.

from differences in the number of years of experience or other factors would be unavoidable. Investigations performed by a single endoscopist have the advantage of eliminating this point.

Second, no special instruments were used for measurements in recording the number of vomiting reflexes, and objectivity may thus have suffered. The vomiting reflex has a variety of causes, including insufflation during the procedure, gastric distention by the endoscope and stimulation of the base of the tongue; it cannot be clearly distinguished which one produces the reflex in each case. Similarly, vomiting, belching, retching or just excretions of air are totally different phenomena, and fundamentally distinguishing between them would be useful in pathological analysis of the vomiting reflex. However, in practical terms, making these distinctions is quite difficult, and attempting to do so may have the effect of decreasing accuracy. Therefore, anything thought to represent one of these symptoms as a positive reflex was recorded. However, the vomiting reflex was compared between a reflex-negative group, in which no vomiting reflexes were encountered, and a reflex-positive group showing ≥1 vomiting reflexes. An assessment of no vomiting reflexes at all is thought to offer a relatively high degree of objectivity, and in an investigation dividing patients into two groups such, this is not considered a particularly large problem.

A third limitation was that the relationship between endoscopic manipulation and the vomiting reflex could not be analyzed. Thus, the records do not clearly distinguish between vomiting reflex due to air insufflation of the stomach and vomiting reflex due to pharyngeal stimulation on insertion of the endoscope. Endoscopic manipulations such as insufflation or insertion of the endoscope represent coordinated manipulations, and since they are not always performed independently, discriminating between these two operations is likely to prove difficult. However, if the relationship between endoscopic manipulation and the vomiting reflex could be recorded and analyzed, more accurate evaluation of the cause of the reflexes would be possible. Studies from this perspective will be necessary in the future.

For the vomiting reflex, mainly background factors were investigated, but no objective evaluation of distress in subjects was performed. For example, no evaluation was undertaken of whether there was truly little distress in the vomiting reflex-negative group or whether little distress was seen as a result of conscious sedation even in the reflex-positive group. In the future, evaluation of distress using a visual analog scale or other tools will be necessary.[26]

In conclusion, this study revealed a relationship between the vomiting reflex, dyspepsia and other upper gastrointestinal symptoms. Symptoms can be objectively assessed with the use of medical questionnaires such as the FSSG, and prediction of the presence or absence of the vomiting reflex during EGD may be feasible. Relationships were suggested between the vomiting reflex and FD or GERD. With conventional transoral EGD, a majority of subjects showed a vomiting reflex, but some showed no reflex at all. Further investigation is warranted regarding the kinds of subjects in whom conscious sedation or transnasal EGD are appropriate.

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