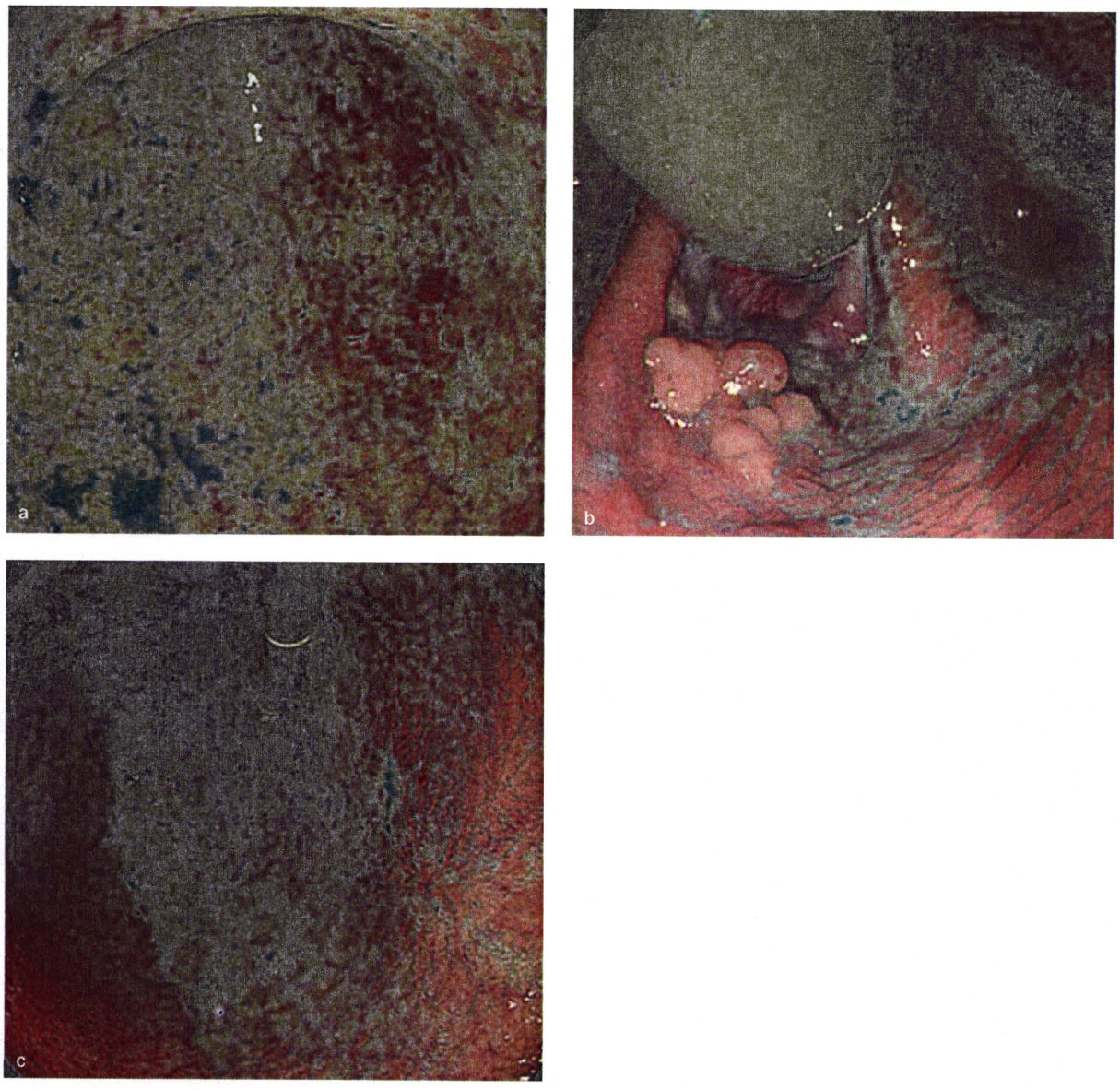

Figure 1 Representative cases for a comparison between predictive and actual procedural time. (a) 50 mm in maximal diameter located in the lower-third of the stomach without ulcerative findings. Predictive time is 125 min and the actual time is 130 min. (b) 15 mm in maximal diameter located in the upper-third of the stomach without ulcerative findings. Predictive time is 77.5 min and the actual time is 80 min. (c) 15 mm in maximal diameter located in the middle-third of the stomach with ulcerative findings. Predictive time is 77.5 min and the actual time is 90 min.

procedural time with a significant difference or correlation. The multivariate analysis using these variables revealed that the location in the upper-third of the stomach, presence of ulcerative findings, and > 20 mm in size were independent factors with a significant difference (Table 2), with a procedural time exceeding 120 min considered a long time.[10]

To compose a predictive formula of procedural time, categorical data were changed as follows: location in the upper-third of the stomach, 1; the middle or the lower third of the stomach, 0; presence of ulcerative findings, 1; and the absence of ulcerative findings, 0. After these variables were included in a linear regression model, the following formula was obtained: predictive procedural time (min) = 2.384 × (tumor size, mm) + 38.568 × (location) + 40.333 × (ulcerative findings) ($R^2 = 0.767$, $P = < 0.0001$). The simplified predictive formula is shown in Table 3 with some examples of clinical cases (Fig. 1).

Table 3 Predictive formula of procedural time and some examples for a comparison between predictive and actual procedural time

	Predictive procedural time (min) = 2.5 × (tumor size, mm)				
Case	Tumor size (mm)	Location	+ 40 × (Location)† Ulcerative findings	+ 40 × (Ulcerative findings)‡ Predictive time (min)	Actual time(min)
a	50	Lower	Absence	125	130
b	15	Upper	Absence	77.5	80
c	15	Middle	Presence	77.5	90

†Upper-third = 1, middle- or the lower-third = 0; ‡Presence = 1, absence = 0.

Discussion

In the present study, we elucidated that the prediction of procedural time was possible by determining tumor size, location, and ulcerative findings. The findings that these parameters affected the procedural time of gastric ESD were consistent with previous reports[7–10] and our own experiences. Oda *et al.* reported that these factors were equal to characteristics technically difficult to resect, by investigating the en bloc resection rate, and that ESD was prolonged depending on the presence of these factors, with no statistical analysis.[7] Although Onozato *et al.*[8] and Imagawa *et al.*[9] also demonstrated that procedural time was significantly prolonged, influenced by tumor size, location, and ulcerative findings, these data were only from univariate analyses. The present study is considered to be valuable with regard to the disclosure of the determinant factors, as determined by multivariate analysis.

In the predictive formula of procedural time, each coefficient of predictors indicates a degree of influence on procedural time. The formula implies that procedural time (min) is nearly equal to maximal tumor size (mm) multiplied by 2.5, and an additional 40 min is required when the tumor is located in the upper-third of the stomach or has ulcerative findings (in both situations, an additional 80 min is needed). By quantifying the influence of these parameters, we can easily calculate the predictive procedural time.

The prediction of procedural time is useful for the patient. We can arrange some safer conditions for the patient when ESD is expected to take a long time. First, treatment by an ESD expert can contribute to a faster operation. Second, the use of intermittent compression of the calf with an external pressure cuff may prevent deep vein thrombosis. Third, the attendance of an anesthesiologist can make the operator concentrate on the treatment. Fourth, airway management by the insertion of an endotracheal tube assists secure ventilation and may prevent intraoperative aspiration or postoperative pneumonia.

Because the primary objective of this study is to elucidate predictors of procedural time before ESD, we used factors that could be known before ESD. In practice, intraoperative conditions, such as unexpected massive bleeding, perforation, piecemeal resection, patient's compliance to venous anesthesia, and the application of an electrocautery snare in the final step of dissection, are expected to be influential on the actual procedural time to some extent. However, this study revealed a strong correlation with actual time ($R^2 = 0.767$) by using only three preoperative parameters.

Some shortcomings may be raised in the generalization our findings. First, these outcomes were obtained from ESD experts; second, a Flex knife was used as a main electrosurgical knife (resection with an IT knife is supposed to be quicker); and third, there are some technical differences, including equipment, among hospitals. The most important thing in this study, however, lies in the predictability of the procedural time before ESD. Accumulation of the predictive formula with various conditions in various hospitals is expected to confirm the possibility to predict procedural time.

In summary, we found a formula to predict the procedural time of gastric ESD, based on tumor size, location, and ulcerative findings. This may be useful in determining an appropriate operation schedule for the patient when the operation is expected to take a long time.

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Digestive Endoscopy

Technical feasibility of endoscopic submucosal dissection for early gastric cancer in patients taking anti-coagulants or anti-platelet agents

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ABSTRACT

Background: Endoscopic submucosal dissection is a novel technique that is expected to be a curative treatment for early gastric cancers. Anti-coagulants and anti-platelet agents are widely used, especially in elderly patients, to prevent thromboembolic disease. However, the feasibility of endoscopic submucosal dissection for such patients has not been investigated.
Aims: To determine the feasibility of endoscopic submucosal dissection for patients using anti-coagulant and anti-platelet agents via retrospective investigation of clinical outcomes.
Methods: Of 408 patients with 444 early gastric cancers consecutively treated by endoscopic submucosal dissection from January 2000 to December 2007 in our hospital, 47 patients with 56 early gastric cancers were receiving anti-coagulants or anti-platelet agents. All patients were classified into groups for high and low risk of thromboembolism. In 44 low-risk patients, these agents were stopped for 1 week before and after treatment. Only three high-risk patients underwent intravenous heparin replacement during the cessation period.
Results: Comparison with other patients showed no significant differences in complete en-bloc resection (96.4%) or perforation (1.8%). Postoperative bleeding requiring endoscopic treatment occurred for six early gastric cancers (10.7%) in the anti-coagulant and anti-platelet group; this frequency was slightly higher than that observed for other patients (5.2%). The healing of endoscopic submucosal dissection ulcers was not delayed by anti-coagulant and anti-platelet treatment (91% in the scarring stage) when checked at the 8th week after endoscopic submucosal dissection.
Conclusion: The clinical outcomes of endoscopic submucosal dissection for early gastric cancers in patients receiving anti-coagulants or anti-platelet agents indicated that endoscopic submucosal dissection for low-risk patients could be a reliable technique with equivalent efficacy and risk in comparison with that for other early gastric cancer patients.

1. Introduction

Successful outcomes for endoscopic mucosal resection (EMR) have resulted in its implementation as the standard treatment for small and non-ulcerative early gastric cancers (EGCs), as it is less invasive than surgical gastrectomy [1,2]. Recently, owing to establishment of criteria for node-negative tumours [3] and development of endoscopic submucosal dissection (ESD) [4–10], endoluminal treatments have also been recommended for large-sized or ulcerative EGCs.

ESD has become a more acceptable option than gastrectomy, especially for elderly patients who often have several comorbidities [11], such as medication with anti-coagulants or anti-platelet agents for primary and secondary prevention of cerebro- and cardiovascular disease. These agents are considered to enhance the tendency to bleed, which may result in increased intra- and post-ESD bleeding. Additionally, ESD-induced ulcers may delay the healing process due to their inhibition of the regeneration of epithelial cells. Thus, the cessation during the perioperative period has been principally recommended without solid evidence; this practice may cause life threatening cerebro- and cardiovascular events.

Because the feasibility of ESD for patients receiving anti-coagulant and anti-platelet therapy has not been investigated in detail, we here investigated the clinical outcomes of ESD in EGC patients receiving these drugs.

2. Methods

Amongst 408 patients with 444 EGCs consecutively treated by ESD from January 2000 to December 2007 in the University of Tokyo hospital, 47 patients (males: 37; females: 10) with 56 EGCs were receiving anti-coagulants or anti-platelet agents. All patients were

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Table 1
Summary of anti-platelet agents and anti-coagulants in 47 patients[a].

	N
Anti-coagulants	
Warfarin	5
Anti-platelet agents	
Aspirin	35
Ticlopidine	11
Icosapentate ethyl	9
Cilostazol	6
Dipyridamole	2
Prostaglandin	2
Others	4

[a] Some patients were receiving more than one agent.

classified into groups for high and low risk of thromboembolism as classified by the American Society for Gastrointestinal Endoscopy. High-risk criteria included conditions of atrial fibrillation associated with valvular heart disease, mechanical valve in the mitral position, and mechanical valve with prior thromboembolic event. In this study, patients with coronary stents, including drug-eluting stents that require double anti-platelet therapy, were also included in the high-risk group. Other patients were assigned to the low-risk group [12].

We defined the following as anti-platelet agents: cyclooxygenase inhibitors (e.g., aspirin), phosphodiesterase inhibitors (e.g., cilostazol), purinergic receptor antagonists (e.g., ticlopidine), serotonin receptor antagonists (e.g., sarpogrelate), and eicosapentaenoic acid preparations (e.g., icosapentate) [13]. These agents were principally stopped for 1 week before and after treatment if the patients were considered to be at low risk for thromboembolism. For patients at high risk for thromboembolism, intravenous heparin was administered until 6 h before treatment and restarted once haemostasis was confirmed by stable vital signs and laboratory data, and after consultation with the prescribing doctors.

All indicated lesions were preoperatively confirmed to be adenocarcinomas or lesions suspected to be adenocarcinomas by endoscopic biopsy. In our study, no lymphovascular invasion in addition to the following were criteria for node-negative EGCs [3]: intramucosal intestinal-type cancer without ulcerative findings, regardless of size (M-UI[−]); intramucosal intestinal-type cancer with ulcerative findings, 3 cm or less in size (M-UI[+]); SM1 (slight invasion into submucosa less than 500 μm from muscularis mucosa) intestinal-type cancer, 3 cm or less in size (SM1).

The above preoperative diagnosis was principally predicted by white-light conventional and choromoendoscopy with indigo carmine. Endoscopic ultrasonography was additionally performed for lesions suspected to present submucosal invasion and/or ulcers. Some lesions that did not meet the above criteria preoperatively were also resected by ESD if they were technically resectable upon consideration of patient comorbidities and/or requests [14]. The ESD procedure was performed as previously reported elsewhere [5,6,8]. All patients provided written informed consent before receiving treatment.

Table 2
Summary of comorbidities of patients receiving anti-coagulants or anti-platelet agents.

Comorbidity	N
Ischaemic heart disease	21
Cerebral vascular disturbance	12
Arteriosclerosis obliterans	5
Atrial fibrillation	3
Abdominal aortic aneurysm	1
Unknown	12

Table 3
Clinicopathological findings of early gastric cancers resected by endoscopic submucosal dissection in patients receiving anti-coagulants or anti-platelet agents.

Findings	N = 56
Mean size of resected mucosa (mm, mean ± S.D.)	21.3 ± 16.2
Location	
Upper	11 (19.6%)
Middle	20 (35.7%)
Lower	22 (39.3%)
Remnant	3 (5.4%)
Macroscopic type	
Protruded	19 (33.9%)
Flat or depressed	31 (55.4%)
Mixed	6 (10.7%)
Depth of invasion	
M	42 (75%)
SM1	6 (10.7%)
SM2	8 (14.3%)
Histological type	
Diffuse	5 (8.9%)
Intestinal	51 (91.1%)
Angiolymphatic invasion	
Yes	7 (12.5%)
No	49 (87.5%)

Endoscopic characteristics of the lesions were classified according to the Paris endoscopic classification [15]. Histological assessment was performed according to the revised Vienna classification of gastrointestinal epithelial neoplasia [16].

The parameters assessed in this study were en-bloc resection, en-bloc resection with tumour-free lateral and basal margins (R0 resection), and frequency of complications; these included postoperative bleeding and perforation as well as ulcer healing after ESD. These parameters were compared amongst patients who were using or not using anti-coagulants or anti-platelet agents and who were treated during the same period in our hospital. Statistical analyses were conducted using the χ^2-test with Yates' modification and Student's t-tests. $P \leq 0.05$ was considered significant.

3. Results

The mean age of the 47 patients was 72.4 ± 7.7 years (range, 57–90 years), which was significantly higher than that of the other 361 patients in the same period (mean age, 66.3 ± 9.9 years). All of these 47 patients were receiving anti-platelet agents, and warfarin was additionally prescribed to five patients. In three patients, intravenous heparin replacement was performed during the cessation period due to high risk for thromboembolism. Intravenous heparin was restarted in these three high-risk patients at 18, 14 and 11 h after ESD, respectively. The most common anti-platelet agent was aspirin (74%), followed by ticlopidine (23%) (**Table 1**). Thirty-five patients had apparent comorbidities requiring these agents. The most common comorbidity was ischaemic heart disease (45%), followed by cerebral vascular disturbance (26%). Twelve patients

Table 4
Clinical outcomes according to use of anti-coagulants or anti-platelets.

	With anti-coagulants or anti-platelet agents	Without anti-coagulants or anti-platelet agents	P-value
En-bloc resection (%)	96.4 (54/56)	94.3 (366/388)	NS
Plus R0 resection	82.1 (46/56)	93.0 (361/388)	NS
Postoperative bleeding (%)	10.7 (6/56)	5.2 (20/388)	NS
Perforation (%)	1.8 (1/56)	4.4 (17/388)	NS

NS, not significant.

Table 5
Characteristics of tumours and patients experiencing postoperative bleeding.

Case no.	Age (years)	Sex	Location	Diameter of lesions (mm)	Diameter of specimen (mm)	Depth of invasion	Scar	Pathological feature	Timing of bleeding	Anti-coagulants	Anti-platelet drugs	Restart of drugs	Comorbidity
1	64	F	Middle	47	113	M	+	Intestinal	Within 24 h	–	Aspirin, ticlopidine, Icosapentate	19th day	Cerebral vascular disturbance
2	76	M	Lower	17	36	M	–	Intestinal	Within 24 h	–	Icosapentate	Not restarted	Hypertension
3	65	M	Lower	5	20	M	–	Intestinal	7th day	–	Aspirin	28th day	Ischaemic heart disease
4	81	M	Lower	16	24	M	–	Intestinal	2nd day	–	Cilostazol	21st day	Hypertension
5	70	M	Middle	20	26	SM1	–	Intestinal	3rd day	–	Aspirin	Not restarted	Ischaemic heart disease
6	67	M	Upper	35	50	SM1	–	Intestinal	14th day	–	Aspirin, ticlopidine[a]	3rd day	Ischaemic heart disease

[a] A patient with a coronary stent in the high-risk group. Intravenous heparin was alternatively administered.

receiving anti-platelet agents had no certain comorbidities such agents (**Table 2**). One patient was receiving aspirin in spite of liver cirrhosis with thrombocytopenia.

Histopathological findings of the 56 resected lesions are summarised in **Table 3**. There were no significant differences in mean size, location, macroscopic type, depth, histological type, and lymphovascular invasion of the lesions in the two groups (P = not significant).

The frequency of en-bloc and en-bloc plus R0 resections as well as complications are summarised in **Table 4**. The frequency of en-bloc resection was 96.4%, and that of en-bloc resection plus R0 resection was 82.1%. A mean change in haemoglobin levels -0.44 ± 0.77 g/dL (range, -1.9 to $+1.2$ g/dL) was observed between pre- and post-ESD, and the haemoglobin level dropped by more than 1 g/dL in 9 (19%) of 47 patients. Postoperative bleeding requiring endoscopic treatment occurred in six EGCs (10.7%) in five low-risk patients and one high-risk patient. Postoperative bleeding was observed within 24 h in two patients, within 24–72 h in two patients, and at more than 72 h in two patients. Only one high-risk patient had postoperative bleeding after restarting anti-platelet agent treatment, and in five low-risk patients, bleeding occurred during the cessation period of these agents (**Table 5**). Perforation during ESD occurred in one low-risk patient (1.8%). There were no significant differences in the frequency of complications in the two types of patients evaluated in the same period.

Twenty-two low-risk patients underwent follow-up endoscopy at the 8th week after ESD in our hospital following our usual follow-up schedule. Examination of the 22 low-risk patients revealed 20 reddish scars without ulcers and 2 reduced ulcers surrounded by regenerative mucosa. There seemed to be no delay of healing for gastric ulcers created by ESD in low-risk patients when compared to the other patients without anti-coagulants or anti-platelet agents, as previously described elsewhere [17].

4. Discussion

As the populations of developed countries age, older high-risk patients with EGCs are being treated with ESD. Our previous study demonstrated the technical feasibility of ESD treatment for these patients, with outcomes comparable to those in younger patients [11]. However, elderly patients have higher incidences of comorbidities including cerebro- and cardiovascular diseases that require anti-coagulants or anti-platelet agents, as shown in the present study. Increased treatment of EGCs in elderly patients with ESD techniques will therefore result in increased likelihood of gastroenterologists encountering patients receiving those agents.

From this study, a reliable outcome can be expected for ESD in patients receiving such agents, assuming that a sufficient cessation period is secured. However, a longer cessation period could increase the risk of thromboembolism, although we did not observe any cases of cerebro- or cardiovascular events during cessation. Some cases of adverse events during cessation have been reported in Japan [18]; therefore, shorter cessation periods are preferable even for patients at low risk for thromboembolism. However, shorter cessation periods may also cause significant increases in postoperative bleeding and delayed healing of artificial ulcers. To balance the risk of thromboembolism in the perioperative period with complications related to the agents, it is necessary to definitely identify the optimal cessation time.

Although this study revealed similar clinical outcomes of ESD for EGCs in patients taking and not taking anti-coagulants or anti-platelet agents, several limitations must be considered when interpreting the data. These include the retrospective design and the insufficient number of patients to analyse risk factors for complications. In particular, there was a very limited number of patients

at high risk for thromboembolism who required intravenous heparin; this implies that our study may actually only represent the feasibility of ESD for EGCs in patients at low risk for thromboembolism.

A non-concurrent, long-term follow-up study of EGC revealed that median duration of the early stage was estimated as 44 months and the cumulative 5-year corrected survival was estimated as 62.8% in unresected patients [19]. Because the treatment strategy should be determined by weighing risks against obtainable benefits, it might be one of options for high-risk patients to be followed without any treatment for EGC. The limited number of such patients in the present study may indicate that primary physicians managing the high-risk patients made the treatment decision before consulting endoscopic surgeons, as we did not intentionally exclude high-risk patients from ESD treatment in this study.

In summary, ESD for EGC can be performed with satisfactory outcomes with permissible risk even in patients receiving anti-coagulants or anti-platelet agents when a sufficient cessation period of these agents is obtained. The indication criteria for ESD may therefore be expanded to such patients, at least to those with a low risk of thromboembolism. However, further studies with a larger number of high-risk cases are needed to conclusively assess the feasibility of ESD for EGCs in this subgroup.

Conflict of interest statement

None declared.

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Outcomes of endoscopic submucosal dissection for early gastric cancer with special reference to validation for curability criteria

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Background and study aims: Endoscopic submucosal dissection (ESD) is a novel, promising endoscopic technique for gastrointestinal neoplasms. We aimed to elucidate the feasibility of ESD as curative treatment for intestinal-type early gastric cancer (EGC) potentially without lymph-node metastases.

Patients and methods: For the short-term analysis, 276 consecutive, intestinal-type EGCs, which fulfilled the criteria for node-negative EGC in 231 patients who had undergone ESD from January 2000 to March 2007, were retrospectively investigated. For the long-term analysis, 212 lesions checked by endoscopy later than 1 year or recurrence within 1 year after ESD were assessed for local recurrence, and 208 patients followed for over 1 year or to death within 1 year after ESD were assessed for metastases and survival. All lesions/patients were divided into three groups: intramucosal cancer without ulcerative findings (M-UI[–]); intramucosal cancer with ulcerative findings, ≤ 3 cm (M-UI[+]); and slight invasive cancer into submucosa (< 500 µm), ≤ 3 cm (SM1).

Results: En bloc and complete resection rates were 96.7 % and 91.7 %, respectively. During a median follow-up of 36 months (range 2–93 months), two local recurrences occurred (0.9 %), which were detected at 2 and 6 months after ESD, respectively. During a median follow-up of 38 months (range 6–97 months), the 5-year overall and disease-specific survival rates were 96.2 % and 100 %, respectively, with neither lymph node nor other-organ metastasis; one patient died due to other disease 6 months after ESD. No disease-related death occurred. No significant differences were found between the groups in short- and long-term analyses.

Conclusions: The prognostic analyses demonstrated the validity of the criteria of node-negative intestinal-type EGC as curability criteria for ESD. ESD can be proposed as an alternative method to gastrectomy for the treatment of these EGCs.

Introduction

Endoscopic submucosal dissection (ESD), which is characterized by circumferential mucosal incision and submucosal dissection beneath the lesion, is a recently developed endoscopic method used mainly for intramucosal neoplasms of the gastrointestinal tract [1–3]. This technique enables en bloc resection even for large or ulcerative lesions.

However, "resectability of the primary site" is quite different from "curability of the entire lesion," because an endoluminal approach can only completely resect a primary site. Although the "tentative" curability criteria for ESD in the treatment of early gastric cancer (EGC) has been established by the condition of absence of nodal metastasis in the surgically resected stomach and regional lymph nodes [4], there are, at least, three considerable drawbacks to curability criteria. First, investigation of metastatic lymph nodes in the operative cases in the study [4] may be insufficient, because the nodal metastases were only investigated at one central section of each lymph node stained by hematoxylin and eosin. Second, cancer cells may remain in the gastric wall between the primary site and the lymph nodes, which may result in a intraluminal recurrent tumor, such as a submucosal tumor, after ESD. Finally, the tumor may have metastasized to organs other than lymph nodes, because nodal metastasis is not the only way to metastasize.

Accordingly, the prognostic analyses in patients with EGC who have undergone ESD are warranted, to evaluate whether the condition of node-negative EGC can be regarded as a curability criterion for ESD. In prognosis studies of patients with gastrectomy, the 5-year disease-specific

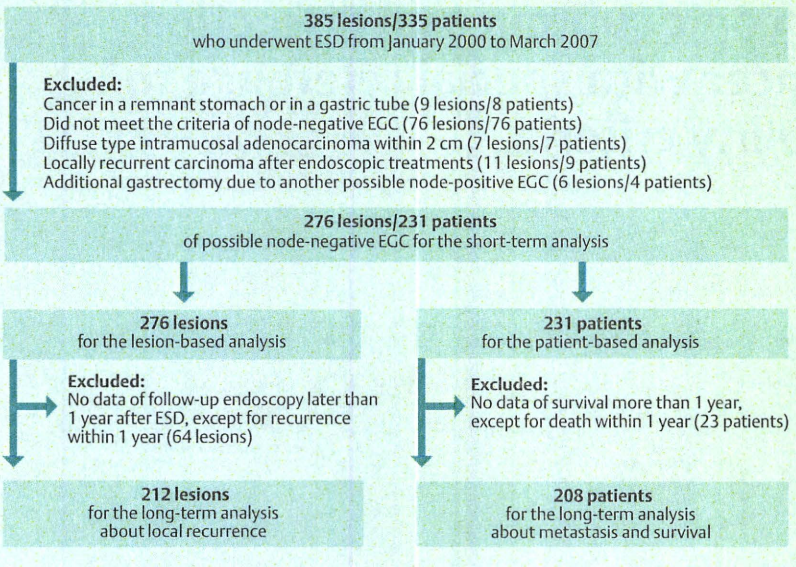

Fig. 1 Flowchart showing the patient inclusion in this study: 276 lesions from 231 patients were eligible for the short-term analysis, and 212 lesions from 208 patients were eligible for the long-term analysis.

survival rate was 99% in mucosal cancer groups and 96% in submucosal cancer groups [5–7]. However, little is known about the prognosis of endoscopic resection, particularly ESD [8–10]. Therefore, we performed this retrospective, long-term analysis to investigate the feasibility of ESD as curative treatment for possible node-negative EGC.

Patients and methods

From January 2000 to March 2007, 335 patients with 385 EGCs underwent ESD in our hospital after giving informed consent. All the indicated lesions were preoperatively either proven or suspected as adenocarcinoma by endoscopic biopsy. The following criteria, which are without angiolymphatic invasion, were the curability criteria for ESD used in our hospital, and reported by Gotoda et al. as node-negative EGCs [4]:

- intramucosal intestinal-type cancer without ulcerative findings, regardless of size (M-UI[–])
- intramucosal intestinal-type cancer with ulcerative findings, 3 cm or less in size (M-UI[+])
- SM1 (slight invasion into submucosa less than 500 µm from muscularis mucosa) intestinal-type cancer, 3 cm or less in size (SM1).

The original criteria by Gotoda et al. also included small intramucosal diffuse-type cancers without ulcer findings. In our hospital, however, these cancers are principally resected by gastrectomy for reasons such as difficulty in demarcation of the tumor margin, possibility of rapid growth of residual cancer cells, or difficulty in deciding on ulcerative status in some cases.

The above histological diagnosis was principally predicted by white-light conventional endoscopy and chromoendoscopy with indigo carmine. Endoscopic ultrasonography was also performed for lesions with suspicion of submucosal invasion and/or ulcerative lesions. After ESD, some of the lesions were excluded from further assessment; a total of 276 lesions in 231 patients, which met the above curability criteria, were eligible to be assessed for this study (**Fig. 1**). Excluded were: nine lesions in eight patients in a remnant stomach after gastrectomy or in a gastric tube after esophagectomy because a previous operation might have changed the angiolymphatic flow and the previous carcinoma might have influenced the patient's survival; 76 lesions in 76 patients that did not meet the criteria of node-negative EGC; seven diffuse-type adenocarcinomas within 2 cm in seven patients; 11 locally recurrent carcinomas in nine patients; and six lesions in four patients, in whom post-ESD lesions were resected by additional gastrectomy due to another possible node-positive EGC because the outcome of current ESD could not reflect the survival of these patients accurately. Clinicopathological features of the eligible lesions are shown in **Table 1**.

For the short-term analysis, all the EGCs (246 M-UI[–]s, 17 M-UI[+]s, 13 SM1s) were analyzed in terms of en bloc resection (resection in a one-piece fashion with endoscopically no residual tumor), complete resection (resection in a one-piece fashion with histologically no tumor on the lateral and vertical margins), and two major complications (delayed bleeding and perforation). Additionally, the subanalysis targeting the M-UI(-) group (179 small tumors [≤2 cm in size] and 67 large tumors [>2 cm in size]) was performed to verify the technical feasibility of ESD for large tumors.

For the long-term analysis, 212 lesions (76.8% of the 276 lesions), which were checked by esophagogastroduodenoscopy at least once later than 1 year post-ESD or had recurred within 1 year, were analyzed for local recurrence. In the subanalysis, the frequency of local recurrence was compared between the lesions with and without en bloc/complete resection. In terms of metastases and survival, data from 208 patients (90.0% of the 231 patients) whose survival information was obtained later than 1 year or whose death occurred within 1 year, were analyzed. For the 117 patients who had no medical records for the most recent year up to the end of March 2008, the following questionnaire was sent in order to obtain the necessary information.

- When did you have your last endoscopy?
- Was the local recurrence identified by endoscopy?
- When did you have your last abdominal computed tomography (CT) scan?
- Was any metastasis identified by CT?

At total of 78 patients (66.7%) had responded to our questionnaire by the end of April 2008, and the obtained data were included in the analyses.

	Cancer type			
	Total	M-UI(-)	M-UI(+)	SM1†
Number of patients	231	203	15	13
Age, mean ± SD, years	66.8 ± 9.6	66.7 ± 9.4	67.0 ± 13.7	68.8 ± 8.3
Sex, male/female	192/39	168/35	13/2	11/2
Number of lesions	276	246	17	13
Location				
Upper	43	36	2	5
Middle	89	79	4	6
Lower	144	131	11	2
Circumference				
Anterior wall	51	48	1	2
Posterior wall	71	61	3	7
Lesser curve	112	98	11	3
Greater curve	42	39	2	1
Macroscopic type				
Protruded or elevated	81	75	2	4
Flat or depressed	171	151	15	5
Combined	24	20	0	4

† Slight invasion into submucosa < 500 μm from muscularis mucosa.

Table 1 Clinicopathological features of possible node-negative cancers resected by endoscopic submucosal dissection.

	Cancer type				
	Total	M-UI(-)	M-UI(+)	SM1‡	*P*-value
Number of lesions	276	246	17	13	
En bloc resection rate, %	96.7	97.6	88.2	92.3	0.0731
Complete resection rate, %*	91.7	92.3	82.4	92.3	0.3575
Delayed bleeding rate, %†	5.1	4.5	11.8	7.7	0.3770
Perforation rate, %	4.0	3.7	5.9	7.7	0.7063

* En bloc with histologically tumor-free on the edge of lateral/vertical surgical margins.
† The rate of cases needing emergency endoscopy due to hematemesis or melena.
‡ Slight invasion into submucosa < 500 μm from muscularis mucosa.

Table 2 Short-term outcomes of endoscopic submucosal dissection for possible node-negative early gastric cancers.

ESD techniques have been described precisely elsewhere [2,3,11–13]. In brief, a flex-knife (KD-630L; Olympus, Tokyo, Japan) was used as the main electrosurgical knife [3,11]. An insulation-tipped diathermic knife (IT knife) [12] or a hook-knife [13] was used as the lesion required. These knives were used for cutting the surrounding non-neoplastic mucosa and for submucosal dissection beneath the lesion. A mixture of 10% glycerin plus 5% fructose and 0.9% saline preparation (Glyceol, Chugai Pharmaceutical Co., Tokyo, Japan) or 20% glucose, which contained 0.005% indigo carmine and 0.0005% epinephrine, was injected into the submucosa under the lesion to make a submucosal fluid cushion [14]. Hyaluronic acid was added to the injection solution for lesions with ulcerative findings or those located in a difficult area [15]. Hemostatic forceps (HDB2422W; Pentax, Tokyo, Japan) were used for bleeding during the procedure or for ablation of visible vessels on the mucosal defect after resection [16]. An electrocautery snare 15 mm (SD-210L-15, Olympus) or 25 mm (SD-210L-25, Olympus) in diameter was used at the final step of ESD when appropriate, following consideration of several factors (e.g. procedure time, technical difficulty, complications, patient's comorbidity).

If the patient's symptoms, laboratory findings, and chest and abdominal radiographs were unremarkable the day after ESD, a light meal was permitted, and the patient was then discharged within 1 week. If complications occurred, the schedules were changed according to the individual patient's condition. Follow-up endoscopy for surveillance of recurrence was carried out at 2 months after ESD in the first year, and annually thereafter. If tumor-free margins of the resected specimens had not been obtained at ESD, an additional endoscopy at 6 months post-ESD was scheduled. Abdominal CT was also performed annually, or more frequently, according to the judgment of doctors in charge. For the statistical analyses, the chi-squared test was used for the analysis of short-term outcomes, and Fisher's exact probability test was used for the subanalyses. The Kaplan-Meier method was used for the analysis of long-term outcomes. If we could not calculate the difference between the groups due to no event in either group, the chi-squared test or Fisher's exact probability test was used to assess those differences in proportions. Statistical significance was set at a *P*-value of less than 0.05.

Results

The short-term outcomes of ESD are summarized in ● **Table 2**. In total, favorable outcomes could be shown in the en bloc resection rate (96.7%) and the complete resection rate (91.7%). Delayed bleeding rate (5.1%) and perforation rate (4.0%) were also acceptable and all complications were managed without surgical intervention. In the analyses according to EGC group, there were no significant differences between the groups. Even in the subanalysis of tumor size in the M-UI(-) group, there were no significant differences in the resectability and complication rates (● **Table 3**).

	≤2 cm	>2 cm	*P*-value
Number of lesions	179	67	
En bloc resection rate, %	97.8	97.0	0.6650
Complete resection rate, %*	93.3	89.6	0.4200
Delayed bleeding rate, %†	5.0	3.0	0.7320
Perforation rate, %	3.4	4.5	0.7075

* En bloc with histologically tumor-free on the edge of lateral/vertical surgical margins.
† The rate of cases needing emergency endoscopy due to hematemesis or melena.

Table 3 Short-term outcomes of endoscopic submucosal dissection for intramucosal cancers without ulcerative findings.

For the lesion-based long-term analysis during a median endoscopic follow-up of 36 months (range 2–93 months), locally recurrent carcinoma occurred in two of 212 lesions (0.9%). Both cases had been resected in an en bloc fashion without tumor-free lateral margins. In one case, local recurrence was diagnosed at 2 months after ESD for M-UI(-). Additional ESD was performed in an en bloc fashion without tumor-free lateral margins; local recurrence was indicated again after a further 7 months, but no further treatment was performed because of coexisting uncontrollable hepatocellular carcinoma, which caused death at 25 months after the initial ESD. In the other case, local recurrence was diagnosed at 6 months after ESD for M-UI(+). Additional ESD was performed with complete resection and no further recurrence was seen after a further 10 months. No recurrence was seen in lesions that underwent complete resection. Between the lesions with and without en bloc resection, there was no significant difference in local recurrence rate (1.0% [2/203] vs. 0% [0/9]; $P > 0.9999$), whereas in the lesions with incomplete resection local recurrence was significantly more frequent than in the lesions with complete resection (8.7% [2/23] vs. 0% [0/189]; $P = 0.011$).

For the patient-based long-term analysis during a median follow-up of 38 months (range 6–97 months), no metastasis to lymph nodes or other organs was seen and nine patients died due to diseases other than gastric cancer. Of those, one patient died 6 months after ESD. The 3- and 5-year overall/disease-specific survival was 96.2%/100% and 96.2%/100%, respectively, with no significant differences between the three groups (**Fig. 2**).

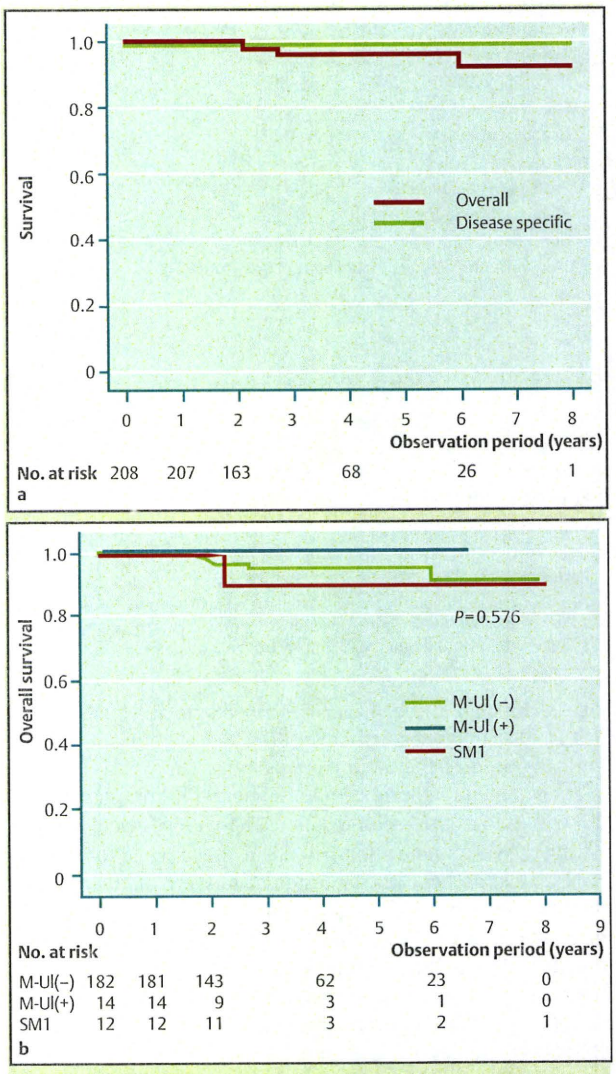

Fig. 2 Kaplan-Meier estimation of survival for early gastric cancer patients: **a** in total; **b** in the three groups. There was no significant difference between the groups.

Discussion

In the present study, the overall rates of en bloc resection and complete resection, which were major parameters for the evaluation of the short-term outcomes of ESD, were high (96.7% and 91.7%). In addition, technical feasibility to expand the indication criteria for ESD to include large intramucosal intestinal-type EGCs without ulcerative findings and small intramucosal intestinal-type EGCs with ulcerative findings may be acceptable in terms of short-term outcomes. Furthermore, the favorable short-term outcomes have led to excellent long-term prognoses in this study. In particular, the 3- and 5-year disease-specific survival rates (100%, 100%) are as high as those in reported data for gastrectomy [5–7]. When considering the preservation of stomach physiology in cases of ESD, it is quite obvious that ESD will win an advantage over gastrectomy.

In small EGCs, some reports have demonstrated the long-term efficacy of endoscopic resection, showing satisfactory data of 3- or 5-year disease-specific survival rates (> 99%) [8,9]. The comparative studies between endoscopic mucosal resection (EMR) and ESD have revealed some advantages and disadvantages of ESD and EMR [9,10]. Further studies will be needed to elucidate the best endoscopic treatments for small EGCs in each case.

Additionally, another advantage of ESD, to minimize local recurrence even when piecemeal resection is performed, was noticed in this study. From this study, only two cases with en bloc resection resulted in local recurrence, whereas none of nine lesions that underwent piecemeal resection recurred. On the contrary, previous studies of EMR have revealed that piecemeal resection could be a major causal factor for local recurrence [1,17,18]. Procedural differences between EMR and ESD may influence the re-

sults, which means that circumferential mucosal incision around the tumor with an appropriate margin during ESD may prevent the occurrence of residual cancer cells even when ESD is completed in a piecemeal fashion. Further studies are warranted to determine whether en bloc resection by ESD is essential for a favorable prognosis. However, from the aspect of precise histological investigation, piecemeal resection should be avoided even when ESD is applied [19], and the number of pieces should be kept to a minimum.

In contrast to intestinal-type EGC, ESD for diffuse-type EGC should be discussed cautiously [20,21]. Even though Gotoda et al. [4] reported that there was no lymph node metastasis in diffuse-type EGCs of less than 2 cm in size without ulcerative findings, the results were based on relatively small numbers (141 cases), and there remain a number of identified difficulties in using ESD for this indication (e.g. difficulty in demarcation of tumor margin, possibility of rapid growth of residual cancer cells, and uncertainty over ulcerative findings). More evidence is needed to clarify the use of ESD in this indication.

The limitations of this study are its retrospective design and single-center analysis, with recall bias to some extent, although the results were obtained from consecutive data. To increase the follow-up rate, questionnaires were sent to those patients lost during follow-up. However, there are approximately 20% of local recurrence and 10% of survival data still missing. A prospective, multicenter study will be necessary to confirm our results.

In conclusion, the present study with favorable long-term prognoses corroborated the feasibility of ESD for possible node-negative intestinal-type EGC and the validity of the criteria of node-negative intestinal-type EGC as curability criteria for ESD. We would propose that ESD can become an alternative method to gastrectomy in cases of possible node-negative intestinal-type EGC, with an invaluable advantage over gastrectomy of preservation of the stomach.

Competing interests: None

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ORIGINAL ARTICLE

LONG-TERM RESULTS OF GASTRIC CANCER SCREENING USING THE SERUM PEPSINOGEN TEST METHOD AMONG AN ASYMPTOMATIC MIDDLE-AGED JAPANESE POPULATION

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Background and Aim: In order to reduce gastric cancer death, mass screening for gastric cancer has been established in Japan for several decades. Only photofluorography is considered to be an acceptable screening method so far, but recent evidence may show the usefulness of serum pepsinogen (PG) measurement for gastric cancer screening. The aim of the present study was to elucidate the feasibility of measuring serum PG levels for detection of gastric cancers.

Methods: Serum PG levels (PGI/PGII) were measured in asymptomatic middle-aged Japanese between 1991 and 2005. Those with a PGI ≤ 70 ng/mL and PGI/PGII ≤ 3 were defined as having a positive PG test. According to the obtained results of serum PG levels and previous individual records, those with a positive PG test and those with a negative PG test took gastroendoscopy every 2 and 5 years, respectively.

Results: The total number of participating individuals was 101 892 (mean age of 48.7 years). In a total of 21 178 planned gastroendoscopies (20.8%), 13 789 (65.1%) underwent gastroendoscopy and 125 gastric cancers were detected, which corresponded to 0.12% of all participants and to 0.91% of those with gastroendoscopy. Early-stage cancers and intestinal-type intramucosal cancers accounted for 80% and 39% of all the detected cancers, respectively.

Conclusions: Serum PG measurement for mass screening of gastric cancer enabled us to achieve high recruitment for gastroendoscopy in intended individuals, a favorable detection rate of gastric cancer and, in particular, an extremely high proportion of early-stage gastric cancer in all the detected cancers.

Key words: endoscopy, gastric cancer, mass screening, serum pepsinogen.

INTRODUCTION

Gastric cancer is a major public health burden. Globally, it is the fourth most common cancer and the second leading cause of cancer-related death, with 700 000 deaths annually worldwide. The risk of gastric cancer varies among the countries and populations and it is well known that Japan is one of the highest-risk areas, where the age-standardized incidence rate (ASR) is greater than 20 per 100 000.[1] Early gastric cancer is typically small and asymptomatic, and the high mortality from gastric cancer is mainly due to late presentation. Therefore, early detection is an important way to reduce deaths from gastric cancer.

Screening for gastric cancer using photofluorography began in the 1960s and is now an established nationwide program in Japan. Asymptomatic individuals older than 40 years are eligible for this government-sponsored (nowadays municipality sponsored) mass screening. However, actual participation among eligible individuals is only approximately 20%.[2] Thus, a new wave to encourage the individuals to participate in screening has been warranted.

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Measurement of serum pepsinogen (PG) has been gradually accepted as a non-invasive serological screening test for gastric cancer, particularly in Japan. Serum PG has recently gained attention as a candidate for a new screening test for gastric cancer in several Asian-Pacific countries,[3–5] as well as in Japan.[6–14] Serum PG consists of two types: PGI and PGII. PGI levels decrease with loss of fundic gland mucosa, whereas PGII remains constant. Therefore, a low PGI level or a low PGI/II ratio, or both, are good indicators of atrophic gastritis – a preneoplastic gastric lesion and a PGI level ≤ 70 ng/mL and a PGI/PGII ratio ≤ 3 are common cut-offs used for the identification of patients with atrophic gastritis.[6–14] In Japanese patients, a PGI/II ratio > 3 has a sensitivity of 93% and a specificity of 88% for the diagnosis of a normal fundic gland.[11] In a pooled analysis of Japanese studies that assessed approximately 300 000 people, the sensitivity of serum PG testing for gastric cancer screening was 77% and the specificity was 73%.[12]

Early detection using the serum test in an asymptomatic stage may result in minimal invasive surgery of the detected gastric cancer, including endoscopic resection, which can save complete stomach physiology.[13] In the present study, we investigated the feasibility of measuring serum PG levels in asymptomatic middle-aged individuals for the detection of gastric cancers and the possibility of the application of endoscopic resection for the detected cancers by PG measurement.

METHODS

Serum PG levels for mass screening of gastric cancer were measured as part of the annual medical check-up for employees of several companies which commissioned the Mitsubishi Clinic, Tokyo, Japan between 1991 and 2005. Blood samples were taken after fasting, and serum PG levels were assayed by a modified radioimmunoassay method using PGI and PGII RIA BEAD Kits (Dainabot Co., Tokyo, Japan) or the latex enhanced immunoturbidimetric assay using PGI and the PGII LZ-test EIKEN (Eiken Chemical Co., Tokyo, Japan). Those with a PGI $\leq$ 70 ng/mL and PGI/PGII $\leq$ 3 were defined as having a positive PG test.[6–14] Individuals with renal failure were precluded from this screening due to interference of the results. Individuals who had been prescribed medication that might affect gastrointestinal function, such as proton pump inhibitors or non-steroidal anti-inflammatory drugs before examination and who had eradication therapy for *Helicobacter pylori* were also precluded.

According to the results of serum PG levels and previous individual records, those with a positive PG test and without gastroendoscopy during the previous year, and those with a negative PG test and without gastroendoscopy during the previous 4 years were recommended to undergo gastroendoscopy. In brief, those with a positive PG test and those with a negative PG test underwent gastroendoscopy every 2 and 5 years, respectively. The decision to undertake gastroendoscopy or not was finally judged by the individuals who were informed of the necessity.

Gastroendoscopy was carried out at an endoscopy unit of Mitsubishi Clinic with topical pharyngeal lidocaine anesthesia by skilled endoscopists (over 5 years experience), using standard gastroendoscopes (GIF-P30, GIF-XQ260; Olympus, Tokyo, Japan). If mucosal appearances were suspected of cancers during gastroendoscopy, endoscopic biopsies were taken from the areas. This mass-screening method for gastric cancer was approved by board members of the clinic and all the commissioning companies also agreed to carry out this mass-screening method for gastric cancer screening. Statistical analyses between groups were carried out using the chi-squared test and a P value <0.05 was considered a significant difference.

RESULTS

During the study period of 15 years, the total number of individuals who participated in this screening program was 101 892 (85 578 men and 16 314 women; mean age, 48.7 years). Among those, 22 987 (22.6%) (19 988 men and 2999 women) individuals were allocated into positive PG tests and, furthermore, 12 874 and 8304 individuals planned to undergo gastroendoscopy due to a positive PG test without gastroendoscopy for 2 years and a negative PG test without gastroendoscopy for 5 years, respectively. For the total of the planned gastroendoscopies, 13 789 individuals (65.1%) (9136 with a positive PG test and 4653 with a negative PG test) underwent gastroendoscopy. There was a significant difference ($P < 0.05$) between those with a positive PG test and those with a negative PG test (71.0% *vs* 56.0%).

Gastric cancer was detected in 125 patients (121 men and four women), which corresponded to 0.12% of all participants and to 0.91% of those who had a gastroendoscopy. When the cancer patients were divided into 110 patients with a positive PG test and 15 patients with a negative PG test, the numbers corresponded to 0.48% and 0.019% of the total participants of each group, respectively, and to 1.2% and 0.32% of the participants of each group who had gastroendoscopy, respectively. The detection rates between participants with positive and negative PG tests were significantly different ($P < 0.05$). The inducements for gastroendoscopy were: a positive PG test as the first enrollment of this mass screening (47 patients: 38%); continuous positive PG tests during follow up (53 patients: 42%); change to a positive PG test during follow up (10 patients: 8%); and continuous negative PG tests during follow up (15 patients: 12%). The characteristics of the detected gastric cancers are shown in Table 1 and Fig. 1. Early-stage (intramucosal or submucosally invasive) cancers and differentiated-type cancers accounted for 80% and 62% of all detected cancers, respectively. Among 72 early-stage differentiated-type cancers, 49 (68%) were intramucosal cancers, which were potentially curable by endoscopic resection such as endoscopic mucosal resection and endoscopic submucosal dissection.[15] Subanalyses of the detected cancers according to inducements for gastroendoscopy revealed that there was no significant difference among the groups in terms of histology and depth of cancer invasion.

Table 1. Characteristics of detected gastric cancers using the serum pepsinogen test method among an asymptomatic middle-aged Japanese population

	Differentiated -type	Undifferentiated -type	Total
Early-stage (intramucosal)	72 (49)	28 (23)	100 (72)
Advanced-stage	6	19	25
Total	78	47	125

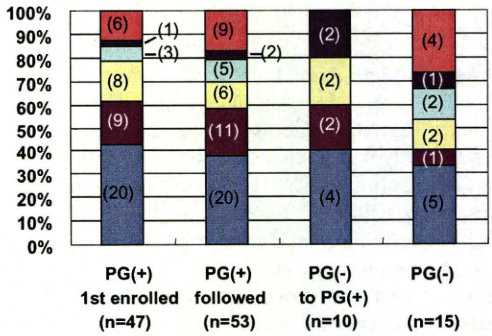

Fig. 1. Distribution of the detected cancers according to inducements for gastroendoscopy. Numbers in parentheses are those of the corresponding cases.

DISCUSSION

This long-term, large-scale study revealed the usefulness of serum PG measurement for gastroendoscopy in terms of

early detection of gastric cancer among asymptomatic middle-aged Japanese. Nearly half a century has passed since a mass photofluorography screening program for gastric cancer were initiated, which has no doubt contributed to lessen gastric cancer deaths in Japan. A population-based, case–control study showed that screening for gastric cancer at 5-year intervals might reduce mortality by 60%.[16] However, recent demands are in the direction of cure without gastrectomy; that is, endoscopic resection, or less radical treatment, if possible, for obtaining a better quality of life. Miki *et al.* reported the incidence of gastric cancer to be 0.05% by photofluorography and 0.18% by PG measurement in populations who underwent photofluorography and PG measurement simultaneously, and that 90% of gastric cancers detected by the PG measurement were in the early stage.[10] Another advantage of PG measurement may be no radiation exposure, no adverse events, much less man-power or reduced costs[17] etc. When considering these factors, serum PG measurement is considered to be quite reasonable for mass screening of gastric cancer.

In spite of these advantages over photofluorography, there has been a major criticism to prevent widespread use of PG measurement for mass screening of gastric cancer, pointing to a lack of data for reduced gastric mortality using this method. Finally, a recent study revealed the reduction in gastric cancer mortality by screening based on PG measurement, which showed that odds ratios for death from gastric cancer among control subjects screened within 1 and 2 years before the individuals were diagnosed versus those who not screened were 0.238 and 0.375, respectively.[18] Thus, we are convinced that PG measurement for mass screening of gastric cancer seems superior to photofluorography, although further studies are needed to obtain final conclusions.

We recognize that a limitation of this study may be that it is impossible to elucidate the accuracy of this screening method from the cross-sectional study setting. The major reason why we used detection rate as an outcome measurement in this study is the difficulty in following most participating individuals due to their being transferred to another workplace branch within a few years. In spite of the drawback, we believe that this study was meaningful for the following reasons. First, all individuals of the mass-screening were enrolled for PG measurement by serum samples obtained at an annual medical check-up and 65% of individuals intended for gastroendoscopy did undergo gastroendoscopy, which is much higher than the proportions who underwent photofluorography; second, gastric cancer was detected in 0.1% of all participants and in 0.9% of those who underwent gastroendoscopy, which is not less than the proportions obtained with photofluorography; third, early-stage cancers accounted for 80% of all detected cancers, which is also much higher than the proportions obtained with photofluorography; fourth, 39% of all the detected cancers were intestinal-type intramucosal cancers, which are considered to be potentially curable by endoscopic resection.

In summary, 15 years experience of serum PG measurement for mass screening of gastric cancer revealed high recruitment for gastroendoscopy in intended individuals, a favorable detection rate of gastric cancer in asymptomatic individuals and, in particular, an extremely high proportion of early-stage gastric cancer in all the detected cancers. Further studies are needed to evaluate our new mass-screening method, namely, the serum pepsinogen test method, as one of the world standards for gastric cancer screening within Japan and worldwide.

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LETTER TO THE EDITOR

Cautious comparison between East and West is necessary in terms of the serum pepsinogen test

Dear Editor,

It is well known that *Helicobacter pylori* (*H. pylori*) infection plays a major role in the carcinogenesis of gastric cancers. The hypothesis established by Correa is widely accepted and various types of evidence to support it have been accumulated.[1] According to the hypothesis, chronic atrophic gastritis (CAG) induced by *H. pylori* infection is considered to be the first step of sequential mucosal changes leading to the gastric cancer.

Our previous study showed that progression of CAG was well correlated with a stepwise reduction in serum pepsinogen (PG) levels, which consisted of two types: PGI and PGII. The PGI level decreases with loss of fundic gland mucosa, whereas PGII remains constant.[2] Therefore, a low PGI level and a low PGI/II ratio are good indicators of CAG and a PGI level ≤70 ng/mL and a PGI/PGII ratio ≤3 are common cut-offs used for the identification of patients with CAG.[3] In a pooled analysis of Japanese studies that assessed approximately 300 000 people using the criteria, the sensitivity of serum PG testing for gastric cancer screening was 77% and the specificity was 73%.[4] This type of meta-analysis is possible in Japan, because both a correlation coefficient and an inclination among all the available kits for the measurement of serum PG levels from Japanese companies show values nearly equal to 1, which gives almost the same results in absolute values of PG levels.[5]

Recently, a convenient set of kits for four biomarkers, PGI, PGII, gastrin-17, and *H. pylori* antibody, has been released as the GastroPanel examination (Biohit Plc., Helsinki, Finland) to evaluate dyspepsia, *H. pylori* infection, CAG and related risks such as gastric cancer.[6] Although the concept is reasonable and should be forwarded, one of our major concerns is the use of different calibrations to obtain the absolute levels of PG compared with Japanese kits. Figure 1 shows correlations of the PG levels between a representative Japanese kit (LZ-test EIKEN; Eiken Chemical Co., Tokyo, Japan) and the GastroPanel examination. For 304 blood samples obtained from asymptomatic individuals living in Tokyo, the correlation coefficients of PGI, PGII and the PGI/II ratio were 0.981, 0.976 and 0.920, respectively. These values showed very strong correlations between the two kits, but inclinations of PGI, PGII and the PGI/II ratio were 1.3804, 0.8321 and 2.1997, respectively. These findings imply that PGI, PGII and the PGI/II ratio obtained by the GastroPanel examination is approximately 40% higher, 20% lower, and twofold higher than those obtained by the Japanese kits, respectively. Thus, cautious comparison between East and West is necessary in terms of interpreting the results of measurements of PG levels obtained using kits with different calibration. This may cause some confusion in daily practice when evidence-based medicine is promoted.

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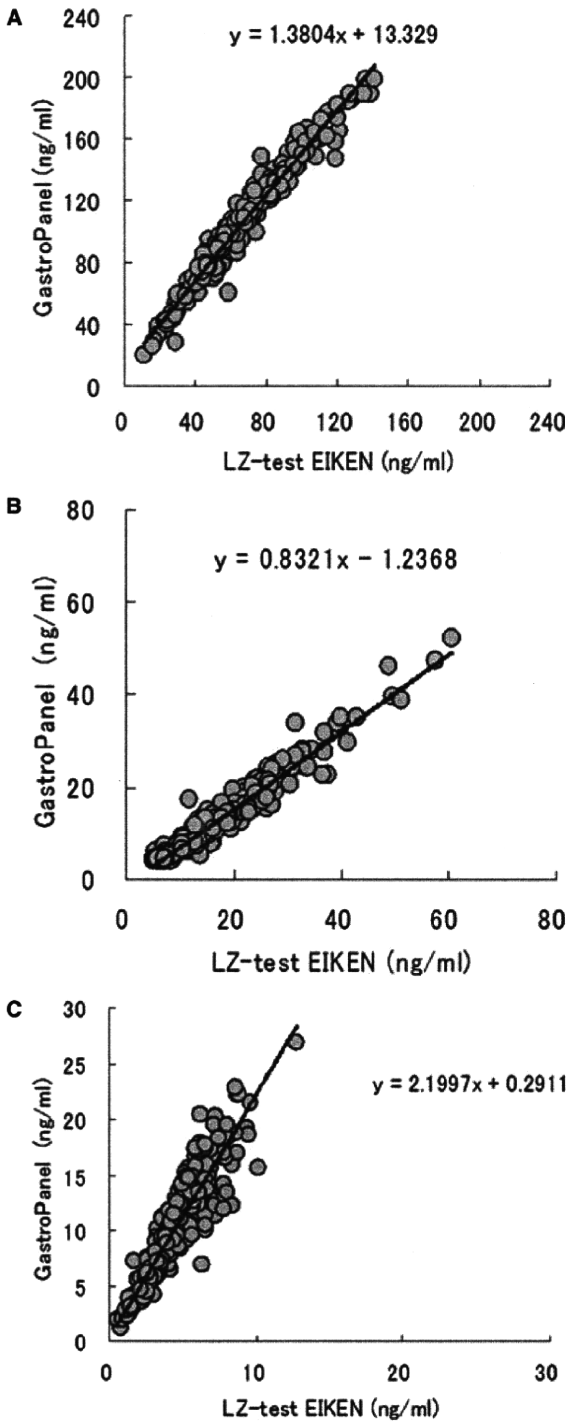

Fig. 1. Correlations of the pepsinogen test between LZ-test EIKEN and GastroPanel. (a) Correlation of the pepsinogen I level, (b) correlation of the pepsinogen II level, (c) correlation of the pepsinogen I/II ratio.

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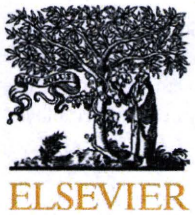

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Research Article

Cdx2 and the Brm-type SWI/SNF complex cooperatively regulate *villin* expression in gastrointestinal cells☆

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ABSTRACT

In our recent study showing a correlation between Brm-deficiency and undifferentiated status of gastric cancer, we found that the Brm-type SWI/SNF complex is required for *villin* expression. To elucidate intestinal *villin* regulation more precisely, we here analyzed structure and function of the promoter of human *villin*. About 1.1 kb upstream of the determined major transcription start site, we identified a highly conserved region (HCR-Cdx) among mammals, which contains two binding sites for Cdx. Expression analyses of 30 human gastrointestinal cell lines suggested that *villin* is regulated by Cdx2. Introduction of *Cdx* family genes into colorectal SW480 cells revealed that *villin* is strongly induced strongly by Cdx2, moderately by Cdx1, and marginally by Cdx4. Knockdown of Cdx2 in SW480 cells caused a clear downregulation of *villin*, and reporter assays showed that HCR-Cdx is crucial for Cdx2-dependent and Brm-dependent *villin* expression. Immunohistochemical analyses of gastric intestinal metaplasia and cancer revealed that villin and Cdx2 expression are tightly coupled. GST pull-down assays demonstrated a direct interaction between Cdx2 and several SWI/SNF subunits. Chromatin immunoprecipitation analyses showed the recruitment of Cdx2 and Brm around HCR-Cdx. From these results, we concluded that Cdx2 regulates intestinal *villin* expression through recruiting Brm-type SWI/SNF complex to the *villin* promoter.

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Introduction

Villin is a calcium-regulated actin-binding protein that modulates the structure and assembly of actin filaments [1–3]. In normal adult tissues, the expression of villin is observed in epithelial cells of the small and large intestinal mucosa, kidney proximal tubules, intrahepatic bile ducts, pancreatic ducts, gall bladder epithelium, and so on [1,4]. It has also been reported that villin is expressed in the proliferative stem cells of the intestinal crypts [5]. In terms of its pathophysiology, villin expression is induced in the intestinal metaplasia observed in Barrett's esophagus and in chronic atrophic gastritis [1,6]. In addition, villin is also frequently found to be expressed in human adenocarcinomas, especially those of intestinal origin [1,7]. Based upon the evidence produce to date, villin is considered as one of the most important intestinal differentiation markers.

In our previous study, we examined the expression of Brm and BRG1 in human gastric cancer cell lines and primary stomach cancers, and found that only Brm is frequently deficient in gastric malignancies [8]. Brm and BRG1 are alternative catalytic subunits of the SWI/SNF chromatin remodeling complex, which involves expression of a vast majority of genes such as *CD44* [9,10], *MMP-1* [10], *E-cadherin* [9], etc. We also demonstrated that the Brm-type SWI/SNF complex plays crucial roles in regulating *villin* expression in these gastric cancer cells; *villin* transcripts are undetectable in most of the Brm-deficient cell lines that were tested, and we observed a clear induction of *villin* in these cells upon the exogenous introduction of Brm but not BRG1 [8]. We have also observed a clear correlation between the Brm-deficiency status and the histologic appearance of gastric malignancy. Of note, in the major gastric cancer types (well or moderately differentiated tubular adenocarcinoma and poorly differentiated adenocarcinoma), frequent loss of Brm expression was found to positively correlate with the undifferentiated status.

In our present study, we analyzed the regulation of intestinal *villin* expression in a more precise fashion using 32 human cancer cell lines and clinical tissues from 39 gastric cancer patients. We anticipate that such accurate analyses of *villin* expression will facilitate the future elucidation of not only the regulatory pathways in intestinal differentiation, but also the roles of Brm in determining the histologic features of gastric cancer.

Materials and methods

Cell culture

Six gastric cancer cell lines (KE-39, KE-97, HuG1-N, HuG1-PI, ECC-10, and ECC-12) were maintained in RPMI1640 with 10% fetal calf serum (Gibco/Invitrogen Corp., Carlsbad, CA) at 37 °C. Other 14 gastric cancer cell lines, 10 colorectal cancer cell lines, and two non-gastrointestinal cancer cell lines (HeLa-S3 and MDA-MB435) were grown in DMEM with 10% fetal calf serum (Gibco/Invitrogen) at 37 °C.

RT-PCR

Total cellular RNAs were prepared using the Isogen RNA isolation reagent (Wako Pure Chemical Industries, Osaka, Japan). Semi-quantitative RT-PCR was performed via a Superscript One-Step reaction using the Platinum Taq (Invitrogen). The primers used to map the TSS of *villin* are shown in the Supplementary materials and methods. The primer pairs and RT-PCR procedures employed to detect the expression levels of the *villin*, *GAPDH*, *Cdx2*, *Cdx1*, *Cdx4*, and *LI-cadherin* are also shown.

Western blotting

Immunoblotting was performed as described previously [11], using the antibodies anti-Cdx2 mouse monoclonal (CDX2-88, Biogenex, San Ramon, CA, 1:200), anti-Brm rabbit polyclonal (Transgenic Inc., Kumamoto, Japan, 1:300), and anti-β-actin mouse monoclonal (Ab-5, BD Transduction, Lexington, KY, 1:2000). Specific bands were detected using the ECL kit (Amersham, Piscataway, NJ).

5′-Rapid amplification of cDNA ends (5′-RACE)

Total RNA was extracted from NUGC-4 cells and subjected to 5′-RACE as described previously [8].

Primer extension and sequencing

To determine the TSSs of the *villin* gene, primer extension analyses were undertaken in parallel with sequencing of the same region by dideoxy chain-termination reaction. Aliquots equaling 9 μg of total RNA from NUGC-4 and AZ521 cells were reverse transcribed with SuperScript III (Invitrogen) and with a 5′-^{32}P-labeled 5′-tggtgat-gttgagagagcctttt-3′ primer (designed using the sequence just downstream of the ATG translation start site of *villin*). To obtain DNA templates for use in the dideoxy chain-termination reaction, NUGC-4 genomic DNA was amplified with the primers 5′-gcagaacagagttcaaaggcact-3′ and 5′-ctgtcttggggaggcagctgc-3′. The resulting PCR products were then fused to the 5′-RACE products inserted in the pCR2.1 TA cloning vector (Invitrogen). The PCR2.1 construct containing an upstream sequence (1183 bp), exon 1, exon 2, exon 3 and a portion of exon 4 of the *villin* gene was thus made. Single-stranded DNAs were synthesized from the same 5′-^{32}P-labeled primer described above using ΔTth DNA polymerase (TOYOBO Co., Tokyo, Japan) in the presence of dideoxynucleotides. Primer extension products and DNA ladders were separated on 10% polyacrylamide gels containing 7 M urea. This gel was then dried and visualized with an image analyzer (FLA5100, Fujifilm, Tokyo, Japan).

Immunohistochemistry

Deparaffinization, endogenous peroxidase inactivation, and antigen retrieval of FFPE clinical tissues were performed as described previously [8]. Immunostaining and signal amplification with anti-villin (Clone-12, BD Transduction) or anti-Cdx2 (CDX2-88, Biogenex) antibodies were also undertaken as described previously [12]. The immunostained sections were evaluated independently by two pathologists in conjunction with the hematoxylin and eosin stained sections from the same lesions.

Retrovirus vectors

Vesicular stomatitis virus G protein (VSV-G)-pseudotyped, MuLV-based retrovirus vectors were prepared using the PLAT prepackaging cell line [13]. To generate vectors expressing Cdx, cDNA inserts for