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Author contributions

Both authors contributed to all aspects of this article.

Original Article

Improvement of reflux symptom related quality of life after *Helicobacter pylori* eradication therapy

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The relationship between *Helicobacter pylori* (*H. pylori*) eradication therapy and the risk of developing gastroesophageal reflux disease (GERD) is controversial. We investigated the influence of *H. pylori* eradication on the risk of GERD by focusing on the quality of life (QOL) and evaluating reflux symptoms. Patients with *H. pylori* infection were administered triple therapy for *H. pylori* eradication. At 3 months and 1 year after the eradication therapy, surveys were conducted to determine the health-related QOL by quality of life in reflux and dyspepsia-Japanese version, (QOLRAD-J) and the severity of GERD symptoms by Carlsson-Dent questionnaire (CDQ). Forty patients were included in the analysis. Although no significant changes of these scores were apparent 3 months after *H. pylori* eradication, the QOLRAD-J and CDQ scores were significantly improved after 1 year. The degree of improvement was even more marked in cases with initially low scores. In conclusion, improved GERD-related QOL and reflux symptoms were noted 1 year after *H. pylori* eradication therapy. In addition, the degree of improvement was more marked in cases with severe reflux symptoms.

***Key Words*:** ***Helicobacter pylori*, eradication therapy, reflux symptoms, quality of life, questionnaire**

Helicobacter pylori (*H. pylori*) eradication therapy has been reported as an effective strategy in the treatment of peptic ulcers and gastric mucosa-associated lymphoid tissue lymphoma, in addition to the prevention of recurrence of gastric cancer after endoscopic resection.[1,2] On the other hand, the influence of *H. pylori* eradication in the management of gastroesophageal reflux disease (GERD) is controversial. Some researchers have suggested that *H. pylori* eradication leads to a more resilient GERD.[3–5] Decreased acid secretion in patients with *H. pylori* infection occurs as a result of progressive gastric mucosal atrophy.[6] Thus, reflux symptoms were thought to be exacerbated after *H. pylori* eradication therapy because of the recovery of acid secretion. Meanwhile, other researchers have reported that *H. pylori* eradication does not exacerbate GERD symptoms.[7–9] Sasaki *et al.*[10] reported that it was rare for reflux esophagitis that develops after *H. pylori* eradication therapy to become severe or cause long-term GERD symptoms.

Quality-of-life (QOL) is an important determinant of symptom generation in GERD patients. The endoscopic severity of GERD is not always correlated with heartburn severity.[11,12] Regardless of the endoscopic findings, QOL may be greatly reduced by the presence of strong symptoms. It has been reported that GERD-related QOL may be worse than that of mild heart failure or angina.[13] Laine *et al.*[14] reported no significant change in QOL 6 months after *H. pylori* eradication therapy, and concluded that *H. pylori* eradication did not worsen the GERD-related QOL. However, how the GERD-related QOL might change on long-term follow-up has not yet been explored.

Talking medical history is one of the most useful means of diagnosing GERD. The presence of GERD can be diagnosed only by history taking in many cases, although endoscopy, 24 h pH monitoring, etc., have been developed to assist in diagnosis. The heartburn version of QOLRAD[15] (quality of life in reflux and dyspepsia) is a self-administered questionnaire. QOLRAD was created with an emphasis on the GERD-related QOL. QOLRAD is a disease-specific instrument, including 25 items classified into 5 domains: emotional distress, sleep disturbance, food/drink problems, physical/social functioning, and vitality. The scores for each QOLRAD domain are expressed on a scale of +1 to +7: the lower the QOLRAD score, the more severe the effect on daily QOL. The QOLRAD has been extensively documented in international studies in patients with heartburn for its reliability, validity, and responsiveness, and in the assessment of GERD-related QOL.[15,16] The Japanese version of QOLRAD (QOLRAD-J) has demonstrated utility in the evaluation of GERD-related QOL in Japanese patients.[17]

The Carlsson-Dent questionnaire (CDQ) is a self-administered questionnaire designed for screening GERD.[18] CDQ contains 7 kinds of questions about regurgitation, stomach discomfort and chest discomfort. The response to each question is chosen from among 3 or 4 alternatives. A score ranging from −7 to +18 is calculated by adding the individual positive and negative scores for the items in the questionnaire: the higher the CDQ score, the stronger the reflux symptoms. Dent *et al.*[19] reported that the CDQ is useful for the diagnosis of GERD and a cut-off level of 4 points is frequently used for a clinical diagnosis of GERD. In addition, the Japanese version of the CDQ is useful as a diagnostic tool for GERD in Japan.[20]

The serum pepsinogen (PG) test is sensitive for atrophic gastritis.[21–23] Serum PG consists of 2 biochemically and immunologically distinct types, pepsinogen I (PGI) and pepsinogen II (PGII).[24] The levels of PGI and the PG I/II ratio are useful sero-

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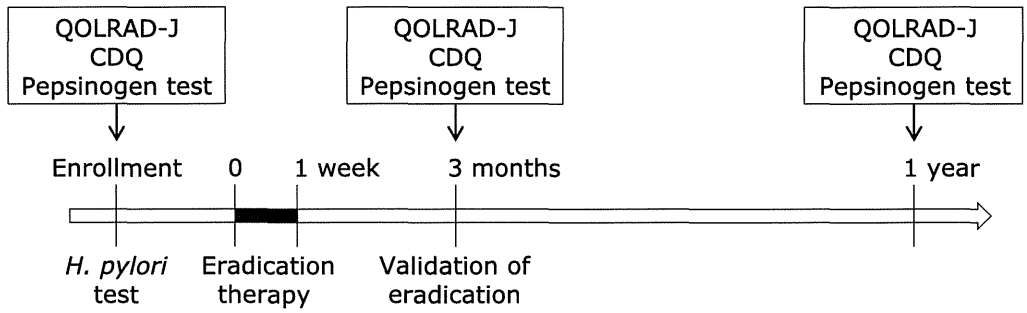

Fig. 1. Study design. All patients were given *H. pylori* eradication therapy (omeprazole 20 mg b.i.d., clarithromycin 400 mg b.i.d., and amoxicillin 750 mg b.i.d.) for 1 week, and eradication status was validated 3 months later. The QOLRAD-J questionnaire survey, the CDQ questionnaire surveys, and serum pepsinogen test were performed before, and at 3 months and 1 year after eradication therapy.

logical markers for chronic atrophic gastritis.[25–27] Kitahara *et al.*[28] reported that it is possible to detect gastric cancer by serum PG screening, using a PGI concentration of less than 70 ng/mL and a PG I/II ratio of less than 3.0 as the cut-off point for diagnosing severe atrophic gastritis.

This study was designed to identify the time course of changes in the GERD-related QOL and GERD symptoms using the QOLRAD-J and CDQ self-administered questionnaires after *H. pylori* eradication therapy.

Materials and Methods

Study design. This was a 2-center prospective cohort study. Outpatients with *H. pylori* infection at Keio University Hospital (Tokyo, Japan) and Eiju General Hospital (Tokyo, Japan) were enrolled from September 2008 to March 2009. *H. pylori* infection status was determined by the ^{13}C urea breath test, microaerobic bacterial cultivation, or histopathological examination of endoscopic biopsy specimens. All patients were given eradication therapy for 1 week with omeprazole 20 mg b.i.d., clarithromycin 400 mg b.i.d., and amoxicillin 750 mg b.i.d. Eradication status was validated 3 months after eradication therapy. The QOLRAD-J and CDQ surveys were conducted and PG levels were measured before eradication (BE) therapy, and at 3 months (3M) and 1 y (1Y) after (Fig. 1). Using QOLRAD-J, the scores for the following 5 domains were determined: emotional distress, sleep disturbance, food/drink problems, physical/social functioning, and vitality. The questionnaires were mailed to the patients who did not come to the outpatient clinic. Patients who did not receive eradication therapy, dropped out during treatment because of side effects from the eradication therapy, did not undergo evaluation of the effect of eradication therapy, or showed eradication failure were excluded from the study. The study protocol was approved by the ethics committees of Keio University School of Medicine and Eiju General Hospital, and written informed consent was obtained prior to subject enrollment. The UMIN Clinical Trials Registry number for this study is UMIN000001399 [http://www.umin.ac.jp/ctr/]. The study was performed in accordance with the principles of the Declaration of Helsinki.

Patient background. Height, weight, body mass index (BMI), alcohol consumption status, smoking status, presence/absence of dyspepsia, and previous history of peptic ulcer before eradication therapy were obtained from a review of medical records and medical interview sheets. Alcohol consumption status was defined as a positive/negative history of daily alcohol consumption. Smoking status was defined as a positive/negative history of smoking cigarettes. The presence/absence of dyspepsia was defined as a positive/negative history of epigastralgia, discomfort, or feeling of fullness in the epigastrium. Patients' prescription histories of antisecretory agents (histamine type-2 receptor antagonist or proton pump inhibitor) at 1 week (1W), 3M, 6 months (6M), and 1Y after eradication therapy were also reviewed.

Statistical analysis. The average QOLRAD-J and CDQ scores and PG expression were compared using one-way repeated-measures analysis of variance and the Bonferroni post-hoc test. Associations between the clinical background factors—age, height, weight, and BMI—and changes in the QOLRAD-J and CDQ scores were compared using the Student's *t* test, and the associations between clinical background factors—sex, the presence/absence of dyspepsia, previous history of peptic ulcer, alcohol consumption status, smoking status, and PG—and changes in the QOLRAD-J and CDQ scores were compared using the chi-square test. The correlation between changes in QOLRAD-J and CDQ scores were evaluated by a linear regression model. Statistical significance was defined as a p value of less than 0.05. All statistical analyses were performed using PASW Statistics (SPSS Inc., Chicago, IL).

Results

Patient characteristics. Fifty-seven patients were enrolled with informed consent. From these 57, 17 patients were excluded: 3 did not receive eradication therapy, 2 dropped out halfway owing to the appearance of side effects (nausea and hemorrhagic colitis), 6 did not undergo evaluation of the effect of eradication therapy, and 6 showed eradication failure (Fig. 2). Finally, data from 40 patients (55.7 ± 11.3 years old, range 22–76 y; 21 men and 19 women) were included. Patient characteristics are shown in Table 1. The number and percentage of subjects for whom data were collected was 40 (100.0%) at BE, 39 (97.5%) at 3M, and 35 (87.5%) at 1Y for estimation of the QOLRAD-J scores, and 37 (92.5%) at BE, 38 (95.0%) at 3M, and 34 (85.0%) at 1Y for the CDQ scores.

Changes in the QOLRAD-J and CDQ scores after *H. pylori* eradication therapy. Improvement in both the QOLRAD-J and CDQ scores was observed at 1Y, although no significant differences were noted between the levels at BE and at 3M. With regard to the sub-domains of QOLRAD-J, significant improvements were observed in emotional distress, sleep disturbance, and food/drink problems at 1Y. On the other hand, improvement in PG score was identified not only in 1Y but in 3M (Table 2.).

A positive history of gastric antisecretory agent prescription during the follow-up period was identified in 32 of 40 patients. The number of patients prescribed antisecretory agents at 1W was 9 (28.1%), at 3M was 4 (12.5%), at 6M was 3 (9.4%), and at 1Y was 3 (9.4%). Improvement in both the QOLRAD-J and CDQ scores was observed at 1Y, even when the 3 patients who were taking antisecretory agents from 6M to 1Y were excluded from the analysis.

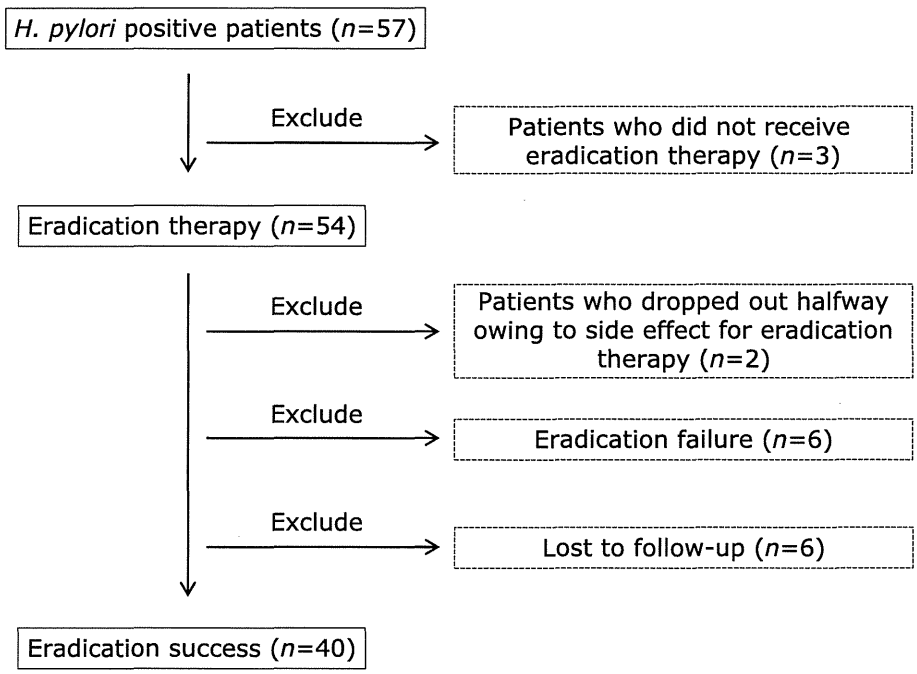

Fig. 2. Exclusion criteria. Fifty-seven outpatients with *H. pylori* infection were enrolled after obtaining their written informed consent. Patients who did not receive the eradication therapy, dropped out halfway due to side effects, did not undergo evaluation of the effect of the eradication therapy, or showed eradication failure were excluded from the study.

Table 1. Characteristics of subjects (n = 40)

Sex, No.	
Male (%)	21 (52.5)
Female (%)	19 (47.5)
Age, years	
Mean ± SD (range)	55.7 ± 11.3 (22–76)
Body Height, cm	
Mean ± SD (range)	163.9 ± 10.3 (150.0–184.0)
Body Weight, kg	
Mean ± SD (range)	58.7 ± 12.6 (41.0–110.0)
Body Mass Index, %	
Mean ± SD (range)	21.9 ± 3.3 (17.1–32.5)
Dyspepsia, No.	
Presence (%)	19 (47.5)
Absence (%)	13 (32.5)
Unknown (%)	8 (20.0)
Peptic ulcer, No.	
Presence (%)	15 (37.5)
Absence (%)	18 (45.0)
Unknown (%)	7 (17.5)
Alcohol Habit, No.	
Everyday (%)	13 (32.5)
Social drinker or nothing (%)	22 (55.0)
Unknown (%)	5 (12.5)
Smoking Habit, No.	
Presence (%)	6 (15.0)
Absence (%)	29 (72.5)
Unknown (%)	5 (12.5)

The proportions of patients in which no worsening of the QOLRAD-J scores was observed were 82.1% (32 of 39 patients) at 3M and 85.7% (30 of 35 patients) at 1Y, whereas those in which no worsening of the CDQ scores was observed were 73.0% (27 of 37 patients) at 3M and 88.2% (30 of 34 patients) at 1Y. In addition, worsening of the QOLRAD-J and CDQ scores was observed in 18.0% (7 of 39 patients) and 27.0% (10 of 37 patients) of the patients, respectively, at 3M, although score improvement was observed in 57.1% (4 of 7 patients) and 80.0% (8 of 10 patients) of the patients, respectively, at 1Y (Table 3).

Changes from the initial scores. Fig. 3A shows the changes in the QOLRAD-J score and Fig. 3B shows the changes in the CDQ score relative to the initial score. The CDQ scores were divided into groups with initial scores of <4 and ≥4, which represents the clinical cutoff. QOLRAD-J does not have a cut-off level because of digitizing of the QOL, whereas CDQ was developed for screening GERD. In this study, the average QOLRAD-J score at BE was 6.51 ± 0.15. Therefore, for the QOLRAD-J score, we set a cut-off level of 6 points for descriptive purposes to analyze the group with lower QOL scores. In this study, 7 of the 40 patients had QOLRAD-J scores <6 points at BE and 17 of 37 patients had CDQ scores ≥4 points at BE. In the group of patients (n = 7) with QOLRAD-J scores of <6, 5 patients showed improvement, while 2 became worse at 3M. However, at 1Y, all 7 patients showed significant improvement. In addition, the group with QOLRAD-J scores ≥6 hovered around the same high scores throughout the study period. Meanwhile, the group of patients with CDQ scores <4 points at BE showed significant improvement even at 3M. In particular, 29.4% (5 of 17 patients) of the patients with scores <4 became completely asymptomatic within 3M. On the other hand, among the patients in whom the CDQ scores were <4 at BE, the scores became worse at 3M, even though the average score was better than 4 at that time. However, no significant changes were noted between BE and 1Y.

Correlations between the QOLRAD-J and CDQ scores. Fig. 4A shows the correlations between the changes in QOLRAD-

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Table 2. Alteration of QOLRAD-J and CDQ score after *H. pylori* eradication therapy

	BE	3M†	1Y†
QOLRAD-J score			
Overall average	6.51 ± 0.15	6.71 ± 0.12	6.85 ± 0.06*
Emotional distress	6.55 ± 0.13	6.75 ± 0.10	6.86 ± 0.06*
Sleep disturbance	6.57 ± 0.16	6.78 ± 0.12	6.91 ± 0.05*
Food/Drink problems	6.29 ± 0.20	6.54 ± 0.17	6.77 ± 0.08*
Physical/Social functioning	6.72 ± 0.13	6.87 ± 0.66	6.97 ± 0.03
Vitality	6.58 ± 0.15	6.59 ± 0.16	6.72 ± 0.11
CDQ score	4.00 ± 0.69	4.43 ± 0.68	3.03 ± 0.72*
Serum pepsinogen level			
Pepsinogen I (ng/ml)	100.97 ± 22.91	51.94 ± 12.99*	48.54 ± 3.62*
Pepsinogen II (ng/ml)	32.97 ± 5.85	8.80 ± 1.70**	8.72 ± 0.44**
Pepsinogen I/II ratio	2.94 ± 0.36	5.55 ± 0.45**	5.60 ± 0.38**

Each value represents the mean ± SE. BE: before the eradication therapy, 3M: 3 months after the eradication therapy, 1Y: 1 year after the eradication therapy. †one-way repeated-measures analysis of variance and the Bonferroni post-hoc test compared to BE. *$p<0.05$, **$p<0.01$.

Table 3. The proportions of change in patients' scores

	BE-3M	BE-1Y
QOLRAD-J score		
Improvement	13/39 (33.3%)	13/35 (37.1%)
No change	19/39 (48.7%)	17/35 (48.6%)
Aggravation	7/39 (18.0%)	5/35 (14.3%)
CDQ score		
Improvement	13/37 (35.1%)	16/34 (47.1%)
No change	14/37 (37.8%)	14/34 (41.2%)
Aggravation	10/37 (27.0%)	4/34 (11.8%)

Values are *n* (%). BE: before the eradication therapy, 3M: 3 months after the eradication therapy, 1Y: 1 year after the eradication therapy.

J and CDQ scores at BE and 3M, and Fig. 4B shows the correlations between 3M and 1Y. Significant correlations were identified between the scores at 3M and 1Y, although no such correlation was identified between the scores at BE and 3M.

Association between clinical background factors and the QOLRAD-J and CDQ scores. Table 4 shows the association between clinical background factors and changes in the QOLRAD-J and CDQ scores. In order to identify the difference between the groups in which the scores worsened or did not worsen, the associations with clinical background factors were analyzed; however, no significant correlation was identified. When the association between clinical background factors and the baseline QOLRAD-J and CDQ scores were analyzed, no significant correlations were identified.

Changes of CDQ scores in initial positive/negative PG test group. In addition, we evaluated the degree of atrophic gastritis by performing the PG test at BE. The number and percentage of subjects for whom data were collected at BE was 31 of 40 (77.5%). Eight patients were positive for the PG test (PGI >70 and PG I/II ratio <3), and 23 were negative. Changes of CDQ scores in positive/negative PG test groups were shown in Fig. 5. The CDQ score varied from 2.88 ± 1.55 to 3.25 ± 1.76 (from BE to 1Y) in the positive-PG test group. On the other hand, the CDQ score varied from 4.65 ± 0.85 to 3.40 ± 0.95 (from BE to 1Y) in the negative-PG test group. Therefore, CDQ score tended to improve in the negative-PG test group than in the positive-PG test group ($p = 0.065$). No significant differences were observed in the QOLRAD-J score.

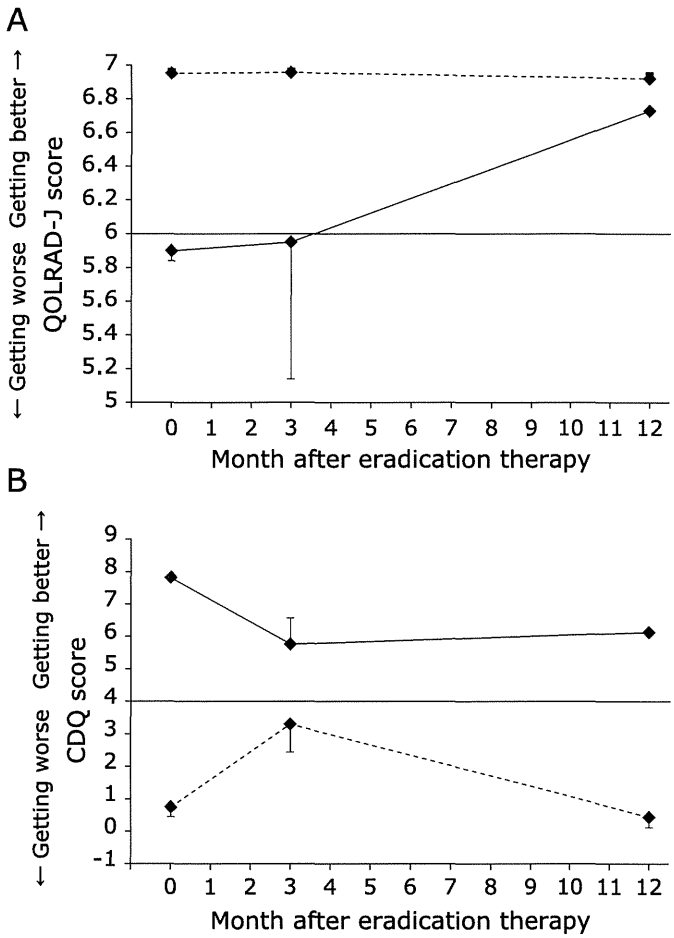

Fig. 3. Changes in QOLRAD-J and CDQ scores with low or high initial scores. The solid lines represent the low-score group and the dashed lines the high-score group. Changes in the QOLRAD-J score according to the initial score dichotomized at a cut-off of 6 [≥6 ($n = 33$); <6 ($n = 7$)] (A). Changes in the CDQ score according to initial score dichotomized at a cut-off of 4 [≥4 ($n = 17$); <4 ($n = 20$)] (B). *$p<0.05$ compared to BE using one-way repeated-measures analysis of variance and the Bonferroni post-hoc test.

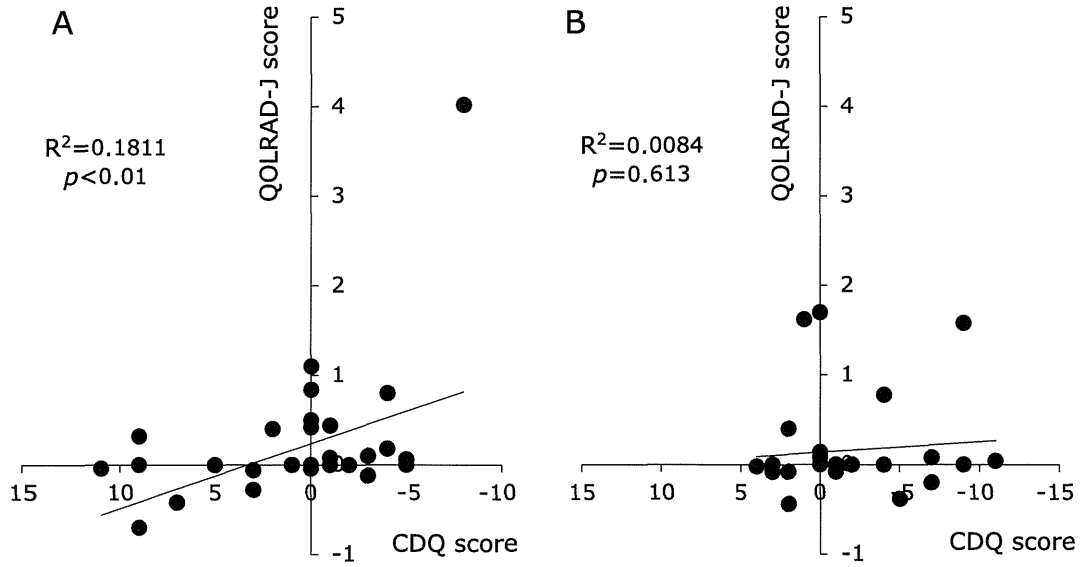

Fig. 4. Correlations between the changes in the QOLRAD-J and CDQ scores. The changes in the QOLRAD-J score are plotted on the y axis, and those in the CDQ score are plotted on the x axis. Correlation between the changes in the 2 scores from BE to 3M. (A) Correlation between the changes in the 2 scores from 3M to 1Y. (B) Significant correlation was identified between the changes in QOLRAD-J and CDQ scores from BE to 3M; however, no such significant correlation was identified from 3M to 1Y.

Table 4. Association between clinical background factors and change of QOLRAD-J and CDQ score

Clinical background factor	*p* value			
	QOLRAD-J score		CDQ score	
	BE-3M	BE-1Y	BE-3M	BE-1Y
Sex[1)]	0.671	0.682	0.863	0.052
Age[2)]	0.342	0.424	0.435	0.859
Body height[2)]	0.873	0.914	0.729	0.419
Body weight[2)]	0.511	0.501	0.872	0.814
Body Mass Index[2)]	0.748	0.551	0.693	0.223
Dyspepsia[1)]	0.132	0.971	0.976	0.606
Peptic ulcer[1)]	0.732	0.286	0.400	0.128
Alcohol habit[1)]	0.192	0.686	0.491	0.901
Smoking habit[1)]	0.218	0.424	0.882	0.464

BE: before the eradication therapy, 3M: 3 months after the eradication therapy, 1Y: 1 year after the eradication therapy. [1)]chi-square test, [2)]Student's *t* test.

Discussion

This study showed significant improvement in both the QOLRAD-J and CDQ scores at 1Y after *H. pylori* eradication therapy, even though no significant changes were observed at 3M after therapy. In a previous study, no significant change in QOL was identified at 6M after *H. pylori* eradication therapy.[14] However, our data showed improvement in the GERD-related QOL at 1Y after *H. pylori* eradication therapy, the period of follow-up being longer in this than in the previous report. On the other hand, no change in the GERD-related QOL was observed at 3M after *H. pylori* eradication therapy.

The degree of improvement was even more marked in cases with low initial scores. On the other hand, in patients with high initial scores, the scores remained high even after eradication therapy. On the basis of these results, we conclude that *H. pylori* eradication therapy may be more effective in patients with severe reflux symptoms or low QOL scores. Furthermore, strong reflux symptoms or reduction in the QOL may not occur after *H. pylori* eradication therapy.

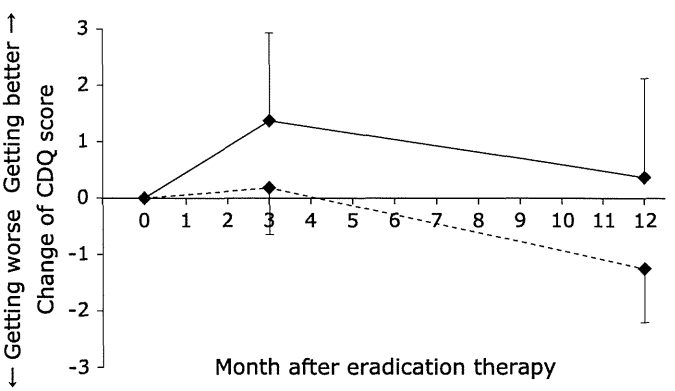

Fig. 5. Changes in CDQ scores with initial PG positive/negative test group. The solid lines represent the PG positive test group (n = 8) and the dashed lines the PG negative test group (n = 23). CDQ score tended to improve in the PG-negative test group than in the PG-positive test group (p = 0.065).

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Antisecretory agents, such as histamine type-2 receptor antagonist or proton pump inhibitor, are thought to primarily protect gastric mucosa by inhibiting gastric acid secretion.[29] Therefore, the effect of antisecretory agents should be considered as a confounding factor, because antisecretory agents might improve the QOL and/or the symptoms of reflux. Yoshida *et al.*[30] reported that omeprazole improved symptoms and QOL in patients with reflux esophagitis. In this study, significant improvement in the QOLRAD-J and CDQ scores were noted at 1Y, even among patients who did not take antisecretory agents from 6M to 1Y. Furthermore, the number of patients who were prescribed antisecretory agents at their own request decreased in a time-dependent fashion after the eradication therapy. This result also suggests that the symptoms of reflux and GERD-related QOL improved gradually after *H. pylori* eradication therapy.

This study also showed that the changes in the QOLRAD-J and CDQ scores were not correlated during the period from 3M to 1Y, whereas a significant correlation was identified during the period from BE to 3M. This result suggests that exacerbation of symptoms might not always imply decreased QOL after 3M. GERD-related QOL might improve following recovery from *H. pylori* infection, even if the reflux symptoms worsen after 3M. The announcement of eradication success at 3M might possibly influence the improvement in the QOL.

In this study, no significant correlation was identified between clinical background factors and the QOLRAD-J and CDQ scores. Hunt *et al.*[31] reported that there was no association between *H. pylori* eradication and the risk of GERD in a population of dyspeptic patients, while a 2-fold higher risk of erosive GERD was observed in patients with peptic ulcer disease. No significant association was identified between dyspepsia and the development of reflux symptoms or GERD-related QOL in our study as well; however, no association between the presence of peptic ulcer disease and the risk of developing reflux symptoms or GERD-related QOL was observed, although our study population included 15 patients with a previous history of peptic ulcer and 18 patients without a history of peptic ulcer. Patients with peptic ulcer might develop erosive GERD following *H. pylori* eradication therapy; however, the GERD-related QOL and severity of reflux symptoms may remain unchanged.

Our data show that the CDQ score tended to improve in the negative-PG group than in the positive-PG group. This means that milder atrophic gastritis may indicate improval of reflux symptoms by *H. pylori* eradication therapy. Atrophic gastritis spreads from an antral-predominant phenotype to a pangastritis phenotype as it progresses. Because of *H. pylori* eradication therapy, recovery of acid secretion may strongly affect reflux symptoms in patients with severe atrophic gastritis.

The limitation of this study is that we could not analyze the groups with *H. pylori* eradication failure. There were only 6 patients with eradication failure in our study. We performed a second eradication as soon as eradication failure was identified. Therefore, we did not follow the eradication failure group.

In summary, improvement in GERD-related QOL and reflux symptoms was observed at 1Y after *H. pylori* eradication therapy. While some patients showed worsening of GERD-related QOL and reflux symptoms 3M after eradication therapy, all patients showed improvement at 1Y. In addition, the degree of improvement was even more pronounced in cases with severe symptoms. Thus, *H. pylori* eradication therapy may be a valid therapeutic option for improving the GERD-related QOL and reflux symptoms.

Acknowledgments

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Conflict of Interest

No potential conflicts of interest were disclosed.

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PANCREAS, BILIARY TRACT, AND LIVER

Biliary Findings Assist in Predicting Enlargement of Intraductal Papillary Mucinous Neoplasms of the Pancreas

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BACKGROUND & AIMS: **There is controversy over the optimal management strategy for patients with branch-duct type intraductal papillary mucinous neoplasms of the pancreas (BD-IPMNs), precursors to pancreatic cancer. We aimed to identify factors associated with the presence of BD-IPMNs and changes in their diameter.**

METHODS: **Two separate analyses were conducted in a cohort of patients who underwent magnetic resonance cholangiopancreatography (MRCP) in a single year (2006). MRCP findings and clinical outcomes of these patients were followed for a maximum of 6 years. We evaluated initial MRCP findings and demographics associated with the presence of BD-IPMNs at baseline and increase in BD-IPMN diameter over time.**

RESULTS: **During the follow-up period, 154 patients developed BD-IPMN and 322 patients did not. Older age, diabetes mellitus, gallbladder adenomyomatosis, and absence of gallstones were associated with the presence of BD-IPMNs at baseline. Increases in diameter of BD-IPMNs were associated with 3 baseline factors: BD-IPMN diameter greater than 17 mm, gallbladder adenomyomatosis, and a common bile duct diameter less than 5.5 mm. Patients with BD-IPMNs could be stratified into 4 groups with varying risk for the enlargement of BD-IPMNs over time: those with 3 risk factors (hazard ratio [HR], 11.4; 95% confidence interval [CI], 3.4–37.8), 2 risk factors (HR, 4.7; 95% CI, 1.7–12.8), or 1 risk factor (HR, 3.1; 95% CI, 1.2–8.2) compared with those without risk factors.**

CONCLUSIONS: **For patients with BD-IPMNs, careful follow-up evaluation is particularly important for those with BD-IPMN >17 mm in size, common bile duct diameter <5.5 mm, or gallbladder adenomyomatosis.**

Keywords: Prognostic Factors; Tumor Development; Imaging Results; Progression.

See editorial on page 555.

Intraductal papillary mucinous neoplasms (IPMNs) are characterized by tall columnar mucin-producing epithelium with or without papillary projections. IPMNs are precursor lesions from which neoplastic progression along an adenoma-carcinoma sequence occurs. The incidence of IPMN has increased rapidly, representing 1% of all pancreatic adenocarcinomas, mainly because of an increase in diagnostic scrutiny.[1] IPMNs involve the main duct (MD), branch ducts (BD), or both (mixed IPMNs). Because MD-IPMNs and mixed IPMNs have a significant risk of malignancy, resection is recommended.[2] In comparison, the natural history of BD-IPMN suggests slower progression to cancer.[3] For patients with BD-IPMNs, the decision to recommend surgery or surveillance is based on factors that predict malignant behavior. Previous studies showed that invasive malignancy was more common in patients with symptoms, larger diameter of BD-IPMN, mural nodules, dilated main pancreatic duct (MPD), and positive cytology, although the specificity was low (23%–31%).[2,4] Increased specificity of

Abbreviations used in this paper: BD, branch-duct; CBD, common bile duct; CI, confidence interval; HR, hazard ratio; IPMC, intraductal papillary mucinous carcinoma; IPMN, intraductal papillary mucinous neoplasms; MD, main-duct; MPD, main pancreatic duct; MRCP, magnetic resonance cholangiopancreatography; MRI, magnetic resonance imaging; PBM, pancreaticobiliary maljunction; ROC, receiver operator characteristic; SSFSE, single shot fast spin-echo.

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clinical prognostic markers could lead to a more tailored approach to patients with BD-IPMNs.

Enlargement of diameter of BD-IPMN is one of the most reliable predictors of malignant transformation of BD-IPMN.[5,6] A recent study showed that growth rate of BD-IPMN could be used to predict malignancy in patients with BD-IPMNs.[7] Therefore, factors associated with increased IPMN diameter might also be risk factors for the development of pancreatic malignancy. The aim of this study was to identify clinical factors that predict the presence of or increase in diameter of BD-IPMNs.

Methods

Study Population

The protocol for this study was approved by the ethics committee of the Keio University School of Medicine (no. 2012-035). Patients who received care from the Division of Gastroenterology and Hepatology at Keio University Hospital and who had undergone abdominal magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP) in 2006 were retrospectively enrolled in this study, and an imaging diagnosis of BD-IPMN was made. MRCP was considered diagnostic when a cystic pancreatic mass communicating with MPD through a small channel was identified. On the basis of initial MRI and MRCP findings, patients diagnosed with MD-IPMN, mixed IPMN, pancreatic cancer, which included pancreatic ductal adenocarcinoma and pancreatic neuroendocrine tumor, and chronic pancreatitis were excluded from the analyses. Patients with BD-IPMN were followed according to the international consensus guidelines for the management of IPMN/mucinous cystic neoplasm published in 2006.

Magnetic Resonance Imaging and Image Analysis

MR examinations were performed with a 1.5 Tesla clinical scanner (Signa Excite HD or Signa Advantage LX; GE Healthcare, Waukesha, WI) by using an 8-channel body coil as a receiver. To reduce the signals in the stomach and bowel, oral negative contrast material was administered approximately 10 minutes before the start of MR examination (ferric ammonium [FerriSeltz; Otsuka Pharmaceutical, Tokyo, Japan] in 2006 or manganese chloride tetrahydrate [Bothdel oral solution 10; Kyowa Hakko Kirin, Tokyo, Japan] after 2006). Transverse and coronal heavy T2-weighted images were obtained by using single shot fast spin-echo (SSFSE) with following parameters to cover the whole pancreas: repetition time, infinite; effective echo time, 90–180 milliseconds; field of view, 32–38 cm; section thickness, 5–7 mm with slice gap of 1 mm; and matrix size, 288 × 192. MRCP images were obtained by using a thick-slab, single-slice SSFSE sequence in rotating coronal oblique orientations with the following parameters: repetition time, infinite; effective echo time, 1200 milliseconds; field of view, 32–36 cm; slice thickness, 50 mm; and matrix size, 512 × 320. Additional sequences were obtained including breath-hold fat-suppressed T2-weighted images, 2-dimensional or 3-dimensional T1-weighted images with fat suppression, diffusion-weighted images, and steady state coherent imaging; however, only transverse and coronal SSFSE and MRCP images were served for the evaluation in the current study. Images were reviewed and evaluated retrospectively on a PACS system (Centricity; GE Healthcare) by 2 independent investigator teams each consisting of 1 radiologist and 1 gastroenterologist. The images were interpreted by 2 radiologists who had 24 and 30 years of experiences in hepatobiliary imaging. The κ coefficient was calculated to assess interobserver agreement.

Study Design and Measurement of Variables

We conducted 2 analyses by using this population, a cross-sectional study and a retrospective cohort study. In the cross-sectional study, analyzed patients were divided into patients with and without BD-IPMN in 2006. By using initial MRI and MRCP findings, the presence/absence of gallbladder adenomyomatosis, gallstones, chronic pancreatitis, and malignant pancreaticobiliary diseases were assessed. Demographic information including age, gender, smoking habits, height, weight, and presence/absence of diabetes mellitus was collected in 2006 by using medical records. Body mass index (weight/height^2) was calculated. MRI findings and demographics of patients were compared between these 2 groups.

Patients were followed by using MRCP for a maximum of 6 years. In a retrospective cohort study, alteration of BD-IPMN diameter was serially examined among patients with BD-IPMN. To identify possible risk factors for enlargement of BD-IPMN, characteristics were compared between patients in whom BD-IPMN diameter had increased and had not increased. For this analysis, the diameters of largest BD-IPMN, common bile duct (CBD), and MPD were used as predictors. In addition, the presence/absence of mural nodule was assessed by using initial MRCP images among patients with BD-IPMN. Follow-up MRCP films of patients without BD-IPMN were reviewed to identify development of BD-IPMN or pancreatic cancer and increasing diameter of BD-IPMN.

Statistical Analysis

To identify risk factors for the presence of BD-IPMN at the baseline, univariate and multivariate regression models were constructed. All variables that were significant ($P < .05$) in the univariate analysis were included in a multivariate model. In the retrospective cohort study, baseline characteristics were compared between patients who did or did not experience increasing diameter of BD-IPMN by using the Fisher exact test for categorical variables or Student t test for continuous variables. Receiver operator characteristic (ROC) analyses were constructed to determine the optimal cutoff values of extracted risk factors. Hazard ratios (HRs) for increasing diameter of BD-IPMN were calculated for each risk factor by using Cox proportional hazards model. Possible risk factors that were worthwhile to include in the risk prediction model for the enlargement of BD-IPMN were determined by using ROC analysis. Finally, patients with BD-IPMN were stratified into risk groups by counting the number of risks. The cumulative proportion of BD-IPMN that enlarged during follow-up in each risk group was computed by Kaplan–Meier method. HRs in each risk group were calculated by using Cox proportional hazards model.

All statistical analyses were performed by using SPSS version 18.0 for Windows (SPSS Inc, Chicago, IL). The data were expressed as mean ± standard deviation. Two-sided P values were considered to be statistically significant at a level of less than .05.

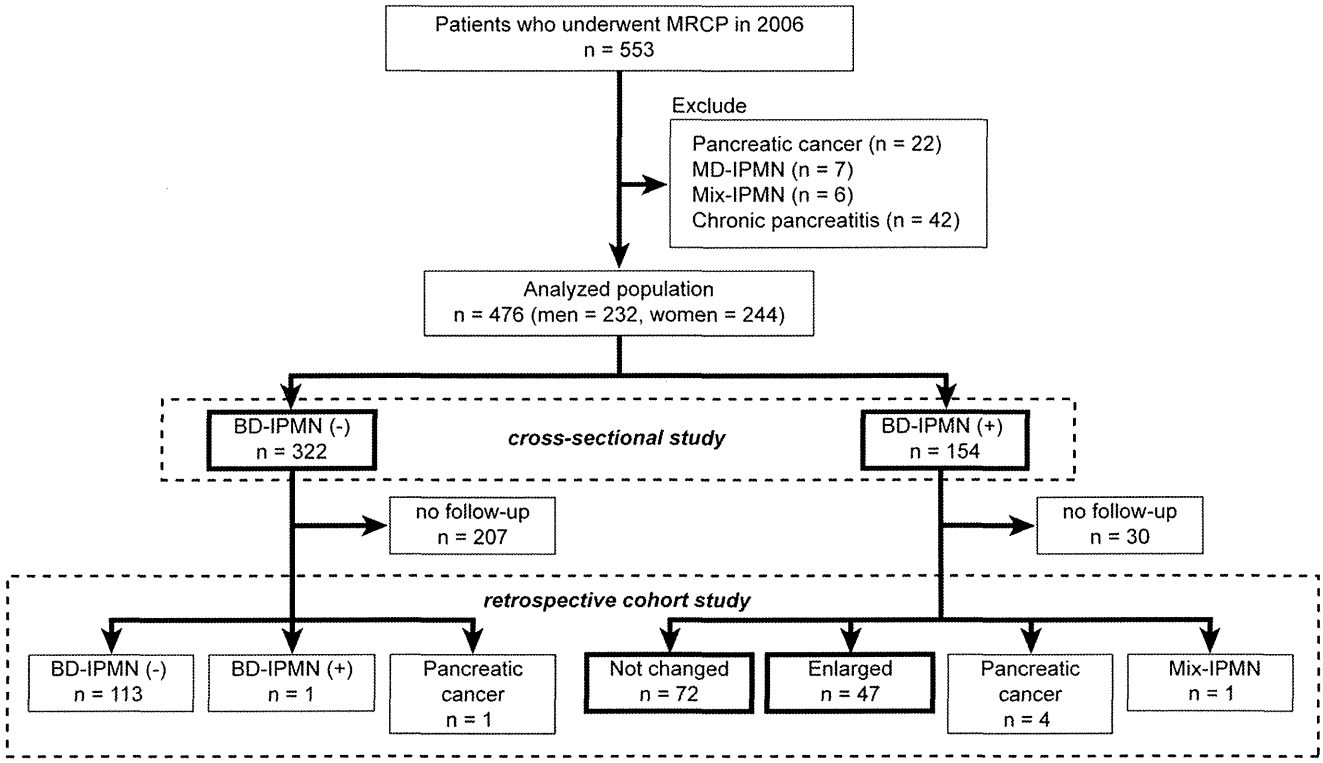

Figure 1. The study population. Two separate analyses were conducted in a cohort of patients who underwent MRCP in a single year (2006). In the cross-sectional study, MRCP findings and demographics were compared between patients with and without BD-IPMN in 2006. In the retrospective cohort study, baseline characteristics were compared between patients who did or did not experience the enlargement of BD-IPMN. Mix-IPMN, mixed type intraductal papillary mucinous neoplasm.

Results

Participant Characteristics

Of 553 patients who underwent MRCP in 2006, 22 with pancreatic cancer, 7 with MD-IPMN, 6 with mixed IPMN, and 43 with chronic pancreatitis were excluded. A total of 476 patients were included in this analysis, which was divided into 154 patients with BD-IPMN and 322 patients without BD-IPMN (Figure 1). Among patients with BD-IPMN at baseline, 3 developed pancreatic ductal adenocarcinoma, 1 developed intraductal papillary mucinous carcinoma (IPMC), 1 developed mixed IPMN, 47 showed an increased diameter of BD-IPMN, 72 did not show any change, and 30 were not followed up. Among patients without BD-IPMN at baseline, 1 developed pancreatic ductal adenocarcinoma, 1 developed BD-IPMN, 113 did not show any change in terms of BD-IPMN, and 207 were not followed up. The incidence of pancreatic cancer, which included pancreatic ductal adenocarcinoma and IPMC, was numerically higher among patients with BD-IPMN compared with patients without BD-IPMN, although the difference did not reach statistical significance (P = .37, Fisher exact test). Interobserver agreement between the 2 MRCP reviewer teams was κ = 0.63, which indicates substantial agreement.

Among 154 patients with BD-IPMN, BD-IPMN had already been identified in 135 patients (87.7%), all of whom underwent MRCP in 2006 as part of their regular checkups. BD-IPMNs were incidentally identified in the other 19 patients; 9 patients were required to undergo MRCP as a further examination based on the results of general health checkup, 8 patients underwent MRCP for the evaluation of biliary tract diseases such as CBD stones and cholangitis; and 2 patients underwent MRCP for the evaluation of liver diseases such as chronic hepatitis and metastatic liver tumor. None of the patients with BD-IPMN had any symptoms that were thought to be of pancreatic origin.

Among patients with BD-IPMN, 93 patients (60.4%) also underwent abdominal ultrasound or computed tomography scanning in addition to MRCP, and pancreatic cysts having an appearance compatible with BD-IPMN were identified, although abdominal ultrasound and computed tomography often could not detect a ductal connection, estimate main duct involvement, or identify small branch duct cysts.[8] Twenty-three of 154 patients with BD-IPMN underwent endoscopic retrograde cholangiopancreatography or endoscopic ultrasound, and all diagnoses of BD-IPMN were confirmed, which showed that MRCP has excellent accuracy in diagnosing BD-IPMN.

Results of Cross-sectional Analysis

The presence of BD-IPMN was associated with older age, diabetes mellitus, gallbladder adenomyomatosis, and absence of gallstones (Table 1). All patients with biliary cancer or gallbladder cancer did not have BD-IPMN. Results of multivariate logistic regression model with adjustment for age, diabetes mellitus, adenomyomatosis, and gallstones showed that these 4 factors were independently associated with the presence of BD-IPMN. The association between adenomyomatosis or gallstones and BD-IPMN suggests that some altered condition in the biliary tract would contribute to the development of BD-IPMN.

Table 1. Initial Clinical Characteristics of Patients and Association for Presence of BD-IPMN

			Univariate analysis	Multivariate analysis[a]
	BD-IPMN (–) (n = 322)	BD-IPMN (+) (n = 154)	Odds ratio (95% CI)	Odds ratio (95% CI)
Age (*y*), mean ± SD	59.0 ± 16.8	68.9 ± 10.0	1.05 (1.03–1.07)[b]	1.05 (1.04–1.07)[b]
Sex (male)	173 (53.7%)	71 (46.1%)	0.74 (0.50–1.08)	
Smoking				
Current smokers	47 (14.6%)	16 (10.4%)	0.61 (0.33–1.13)	
Ex-smokers	59 (18.3%)	20 (13.0%)	0.61 (0.35–1.07)	
BMI (kg/m^2), mean ± SD	21.7 ± 3.7	21.8 ± 2.9	1.01 (0.95–1.07)	
Diabetes mellitus	30 (9.3%)	27 (17.5%)	2.07 (1.18–3.62)[b]	1.92 (1.05–3.51)[b]
Adenomyomatosis	27 (8.4%)	22 (14.3%)	1.82 (1.00–3.32)[b]	02.21 (1.14–4.31)[b]
Gallstones	96 (29.8%)	30 (19.5%)	0.57 (0.36–0.91)[b]	0.38 (0.23–0.63)[b]
Biliary cancer	15 (4.7%)	0 (0%)	NA	
Gallbladder cancer	3 (0.9%)	0 (0%)	NA	
Chronic hepatitis	28 (8.7%)	16 (10.4%)	1.22 (0.64–2.32)	
Primary sclerosing cholangitis	10 (3.0%)	0 (0%)	NA	

BMI, body mass index; NA, could not be analyzed.
[a]Analyzed by multivariate logistic regression model with adjustment for age, diabetes mellitus, adenomyomatosis, and gallstones.
[b]Significant difference.

Results of Retrospective Cohort Analysis

Median follow-up duration was 4.76 years among patients in whom BD-IPMN diameter increased and 5.13 years among patients in whom BD-IPMN diameter did not increase. The mean follow-up interval was 0.79 ± 0.44 years among patients in whom BD-IPMN diameter increased and 0.94 ± 0.59 years among patients in whom BD-IPMN diameter did not increase. There was no significant difference between the intervals. Compared with patients in whom BD-IPMN diameter did not increase during the follow-up period, there was a higher prevalence of diabetes mellitus, gallbladder adenomyomatosis, baseline larger diameter of BD-IPMN, and smaller diameter of CBD among patients in whom BD-IPMN diameter increased during the follow-up period (Table 2). A priori, the optimal cutoff values of diameter of IPMN and CBD were hypothesized to be 17 and 5.5 mm, respectively, for the prediction of increasing diameter of BD-IPMN (Figure 2). Univariate Cox regression analysis revealed that the enlargement of BD-IPMN could be predicted by the following 3 factors: diameter of IPMN greater than 17 mm, adenomyomatosis, and diameter of CBD less than 5.5 mm (Table 3). A multivariate Cox regression model showed that a baseline diameter of BD-IPMN greater than 17 mm was the strongest predictive factor for the enlargement of BD-IPMN. In addition, presence of adenomyomatosis and diameter of CBD less than 5.5 mm trended with the enlargement of BD-IPMN, although these 2 factors lost significance in the multivariate model ($P < .1$). Therefore, ROC analysis was performed to identify the optimal prediction model for the enlargement of BD-IPMN. Area under the curves were 0.641 (95% confidence interval [CI], 0.538–0.744) for the model that used only a baseline diameter of BD-IPMN greater than 17 mm, 0.685 (95% CI, 0.585–0.785) for the model that used both a baseline diameter of BD-IPMN greater than 17 mm and presence of adenomyomatosis, 0.690 (95% CI, 0.594–0.787) for the model that used both a baseline diameter of BD-IPMN greater than 17 mm and diameter of CBD less than 5.5 mm, and 0.723 (95% CI, 0.630–0.816) for the model that used all 3 factors. This showed that a model that incorporated all 3 factors yielded the best prediction of increasing diameter of BD-IPMN.

Patients with BD-IPMN could be stratified into 4 risk groups: those who had none of these factors, those who had 1 factor, those who had 2 factors, and those who had all 3 factors. Patients were also categorized according to the latest international guidelines of IPMN[2] into 4 groups by using only the size of BD-IPMN (<1, 1–2, 2–3, and >3 cm). The cumulative proportion of patients in whom BD-IPMN diameter increased is shown in Figure 3. Although both risk stratification models predicted enlargement of BD-IPMN (log rank test, $P < .05$), risk stratification including adenomyomatosis and the diameter of

Table 2. Characteristics of Patients in 2006 Stratified by Enlargement of BD-IPMN

	Not changed (n = 72)	Enlarged (n = 47)	*P* value
Median follow-up period (*y*)	4.76	5.13	
Age (*y*)	67.7 ± 10.3	69.9 ± 9.4	.26[a]
Sex (male)	33 (45.8%)	19 (40.4%)	.58[b]
Smoking			
Current smokers	6 (8.3%)	3 (6.4%)	1.00[b]
Ex-smokers	11 (16.2%)	4 (9.3%)	.40[b]
BMI (kg/m^2)	21.7 ± 3.0	22.4 ± 2.9	.28[a]
Diabetes mellitus	8 (11.1%)	12 (25.5%)	.048[b,c]
Diameter of IPMN (*mm*)	14.8 ± 11.3	22.1 ± 11.8	.001[a,c]
Diameter of CBD (*mm*)	6.6 ± 2.9	5.6 ± 2.0	.027[a,c]
Diameter of MPD (*mm*)	2.4 ± 0.9	2.3 ± 1.0	.90[a]
Mural nodule	2 (2.8%)	4 (8.5%)	.21[b]
Adenomyomatosis	5 (6.9%)	13 (27.7%)	.003[b,c]
Gallstones	14 (19.4%)	8 (17.0%)	.81[b]
IPMN location			
Head	43 (59.7%)	32 (68.1%)	.44[b]
Body	41 (56.9%)	33 (70.2%)	.18[b]
Tail	21 (29.2%)	14 (29.8%)	1.00[b]

BMI, body mass index.
[a]Student *t* test.
[b]Fisher exact test.
[c]Significant difference.

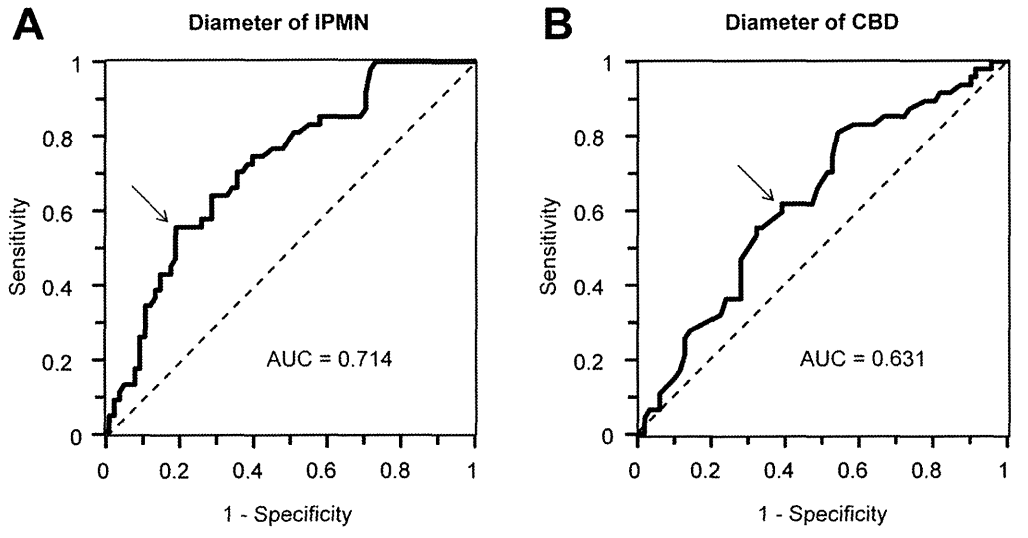

Figure 2. ROC curves illustrating optimal cutoff points of BD-IPMN diameter (*A*) and of CBD diameter (*B*) to predict the enlargement of BD-IPMN. *Arrows* indicate optimal cutoff points.

CBD in addition to baseline diameter of BD-IPMN illustrated superior separation of the Kaplan-Meier curves (Figure 3).

Characteristics of Patients Who Developed Pancreatic Cancer During Follow-up

Four patients developed pancreatic cancer among patients with BD-IPMN during follow-up (Figure 1). None were included in the lowest risk group; however, 2 of 3 patients who developed pancreatic ductal adenocarcinoma had a baseline BD-IPMN diameter greater than 17 mm, and 1 had gallbladder adenomyomatosis. One patient who developed IPMC had a baseline BD-IPMN diameter greater than 17 mm. One patient who developed mixed IPMN had 3 risk factors such as baseline BD-IPMN diameter greater than 17 mm, gallbladder adenomyomatosis, and CBD diameter less than 5.5 mm.

Discussion

In a cross-sectional analysis, we identified an association of older age, diabetes mellitus, gallbladder adenomyomatosis, and absence of gallstones with the presence of BD-IPMN. Moreover, this study provides evidence that adenomyomatosis and baseline narrow CBD (<5.5 mm) in addition to large diameter BD-IPMN (>17 mm) are risk factors for increased diameter of BD-IPMN. Categorizing patients with BD-IPMN into risk groups on the basis of the number of risk factors improved prediction for BD-IPMN diameter increase over current guidelines. Because the presence/absence of these factors can be evaluated with not only MRCP but also abdominal ultrasound, the results of the present study can improve the detection and management of BD-IPMN.

Large diameter of BD-IPMN is a known risk factor for malignant IPMN and concomitant ductal carcinoma.[9] Although presence of mural nodules and dilated MPD are reliable findings for the prediction of malignancy,[2] these 2 factors were not identified as risk factors for the enlargement of BD-IPMN in the present study. This may be because MPD dilatation and mural nodules are not the "cause" of malignancy but are the "result" of malignancy. On the other hand, the association of adenomyomatosis and narrow CBD with enlargement of BD-IPMN is novel. Although the mechanism of this association could not be elucidated in the present study, reflux of biliary or duodenal contents into the pancreatic duct might be a possible cause to explain these findings. The CBD and the MPD frequently form a common channel. In patients with a pancreaticobiliary maljunction (PBM), the sphincter of Oddi does not function properly, and two-way regurgitation (pancreaticobiliary and biliopancreatic refluxes) occurs. Some clinical reports have demonstrated the occurrence of IPMC in patients with PBM.[10,11] Animal experiments also confirmed that bile reflux into the pancreatic ducts is a significant factor predisposing to the development of IPMC.[10] Furthermore, it has become obvious that pancreaticobiliary reflux can occur in individuals without PBM,[12] suggesting that biliopancreatic reflux might also occur and lead to the development of IPMN in those without PBM.[13] On

Table 3. Regression Coefficients for Enlargement of BD-IPMN From a Cox Hazard Regression Model

	Univariate analysis				Multivariate analysis[a]			
	B	SE	HR (95% CI)	*P* value	B	SE	HR (95% CI)	*P* value
IPMN >17 mm	0.954	0.30	2.60 (1.45–4.64)	.001[b]	0.859	0.31	2.39 (1.29–4.42)	.006[b]
Adenomyomatosis (+)	0.824	0.33	2.28 (1.20–4.33)	.012[b]	0.608	0.33	1.84 (0.96–3.53)	.069
CBD <5.5 mm	0.599	0.30	1.82 (1.01–3.29)	.047[b]	0.556	0.31	1.74 (0.96–3.18)	.070
Diabetes mellitus (+)	0.582	0.34	1.79 (0.93–3.45)	.082	0.159	0.36	1.17 (0.58–2.37)	.580

B, unstandardized regression coefficient; SE, standard error.
[a]Analyzed by multivariate logistic regression model with adjustment for diameter of IPMN >17 mm, adenomyomatosis, diameter of CBD <5.5 mm, and diabetes mellitus.
[b]Significant difference.

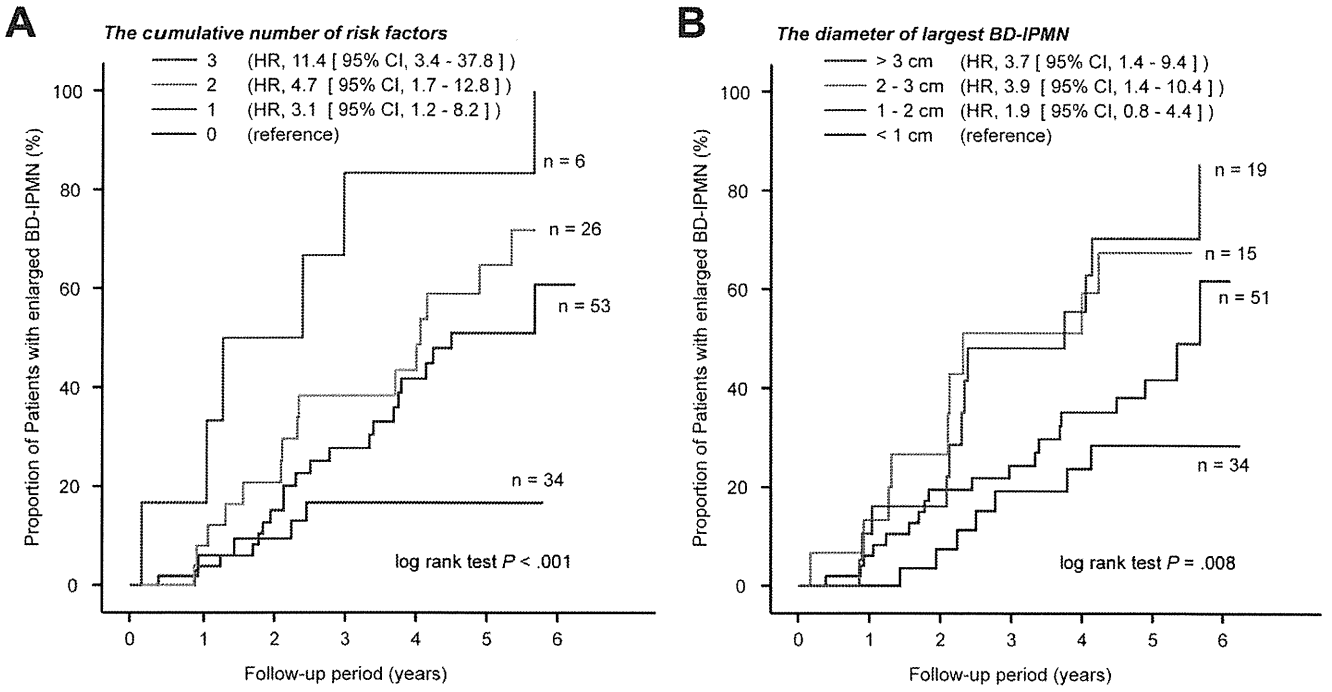

Figure 3. Kaplan–Meier curves of patients with enlarging BD-IPMN and HRs of the enlargement. (*A*) Patients with BD-IPMN were divided into 4 groups according to the number of risk factors consisting of diameter of IPMN more than 17 mm, gallbladder adenomyomatosis, and diameter of CBD less than 5.5 mm. (*B*) Patients with BD-IPMN were divided into 4 groups according to the size of BD-IPMN.

the other hand, gallbladder adenomyomatosis is frequently observed in patients with PBM. Tanno et al[14] reported that adenomyomatosis is more common among patients with undilated type PBM. Even among patients without PBM, the association between occult pancreaticobiliary reflux and adenomyomatosis has been demonstrated.[12] Thus, two-way regurgitation between CBD and MPD could explain the association between BD-IPMN and adenomyomatosis. In addition, among individuals in whom two-way regurgitation occurs, a narrow CBD might contribute to divert the direction of flow (pancreaticobiliary reflux to biliopancreatic reflux), because the flow volume in narrow tubes is decreased as the diameter of the tubes decreases. If occult pancreaticobiliary and biliopancreatic refluxes would contribute to the enlargement of BD-IPMN, endoscopic biliary sphincterotomy to abolish the common channel might prevent enlargement of BD-IPMN. In fact, 3 patients with BD-IPMN underwent endoscopic sphincterotomy before 2006, and none of their BD-IPMNs were enlarged.

Limitations of our study include generalizability; our patients without BD-IPMN may not be representative of the general population because this is a hospital-based study. Another criticism is that the duration of time required for enlargement of BD-IPMN might be longer than the duration that subjects were observed in this study. Time-to-event data were calculated by using the date of MRCP examination in which the enlargement of BD-IPMN was observed; however, follow-up intervals were different among participants. To establish a proper follow-up strategy for BD-IPMN, further prospective studies should be performed, and the results of the present study can contribute to the design of such studies.

In conclusion, this study demonstrates that BD-IPMN >17 mm in diameter, presence of gallbladder adenomyomatosis, and CBD <5.5 mm in diameter predict the enlargement of BD-IPMN. These factors might be able to improve specificity in prediction of pancreatic cancer. Careful follow-up is particularly necessary among patients with these risk factors.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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PLOS ONE

Musashi-1 Post-Transcriptionally Enhances Phosphotyrosine-Binding Domain-Containing m-Numb Protein Expression in Regenerating Gastric Mucosa

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Abstract

Objective: Upregulation of the RNA-binding protein Musashi-1 (Msi1) has been shown to occur in rat gastric corpus mucosa after ethanol-induced mucosal injury. However, there is no direct evidence linking Msi1 with gastric regeneration. We examined the process of tissue repair after acute gastric mucosal injury with Msi1-knock-out (KO) mice to clarify the role of Msi1 and Msi1-dependent regulation of m-Numb expression in regenerating gastric mucosa.

Methods: Acute gastric injury was induced in Msi1-KO and wild-type ICR mice by administering absolute ethanol. Expression of the splicing variants of *m-Numb* mRNA and protein in the gastric mucosa were analyzed by quantitative RT-PCR and western blotting, respectively.

Results: We demonstrated that phosphotyrosine-binding domain-containing m-Numb expression was significantly upregulated at both the mRNA and protein levels in wild-type mice at 3 h after ethanol-induced acute gastric injury. In contrast, in Msi1-KO mice, the m-Numb protein was expressed weakly, and was associated with delayed regeneration of the injured gastric mucosal epithelium. In the Msi1-KO mouse, the ratio of *m-Numb* mRNA to total *m-Numb* mRNA in the heavy polysome fractions was lower than that in the wild-type mouse. Further, we showed that m-Numb-enhancement in gastric mucous cells induced the expression of prostate stem cell antigen and metallothionein-2. Under the m-Numb enhancing condition, the gastric cells exhibited enhanced cell proliferation and were significantly more resistant to H_2O_2-induced cell death than control cells.

Conclusions: Msi1-dependent post-transcriptional enhancement of m-Numb is crucial in gastric epithelial regeneration.

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Introduction

Epithelia in the digestive tract exhibit a remarkable capacity for regeneration. The gastric mucosa, in particular, shows continuous regenerative activity, mediated through the differentiation of stem cells, which may be localized in a proliferating area called the isthmus, and migration of the differentiating cells towards the gastric surface and into the base of the fundic glands. Gastric mucosal architecture is restored within a very short period following injury [1,2,3]. However, the mechanisms underlying this rapid restoration of the gastric mucosal architecture have not yet been fully clarified.

An RNA-binding protein, Musashi-1 (Msi1) [4,5,6] was isolated as a mammalian homologue of the *Drosophila* Musashi; this protein is required for the asymmetric cell division of the sensory neural precursor cells [7,8]. In the mammalian central nervous system, Msi1 is known to regulate progenitor cell function through the post-transcriptional regulation of its target RNA [4,5,6]. In the rat gastric corpus, Msi1 expression has been shown to be upregulated after ethanol-induced mucosal injury [9]. However, currently, there is no direct evidence linking Msi1 with gastric regeneration.

Msi1 is known to bind to the 3′-untranslated region (UTR) of several target mRNAs and to regulate these genes post-transcriptionally [10,11,12,13], in particular, those encoding Numb and p21. Msi1-dependent suppression of Numb translation has been reported to contribute to the self-renewal of neural stem cells [11]. Recent studies indicated that deregulation of the Musashi/Numb pathway is likely to be involved in tumor development [14,15,16].

Numb expression has been demonstrated in the gastric epithelium during the early stages of chicken gastric gland formation [17], although its expression and translational regulation by Msi1 in the postnatal gastric mucosa remains unclear.

Numb was first identified in *Drosophila* as a protein playing a role during asymmetric cell division of the neural precursors [18,19]. The mammalian homolog of *Drosophila* Numb (m-Numb) and Numb-like (a second mammalian Numb protein) [20] have been shown to be key factors involved in the regeneration of the brain after ventricular damage, since severe damage was observed in mice with conditional deletion of these genes in the postnatal brain [21]. The corresponding *m-Numb* gene has splicing sites affecting the phosphotyrosine-binding (PTB) domain and proline-rich region (PRR), and gives rise to at least 4 alternatively spliced transcripts [22], which produce 4 protein isoforms: Numb1 (72 kDa), Numb2 (66 kDa), Numb3 (71 kDa), and Numb4 (65 kDa) (Figure S1). These m-Numb splicing variants are not considered components of a single protein but rather as a family of stage-specific proteins with distinct regulatory roles in neural development [23,24]. Furthermore, the expression of the individual splicing variants of *m-Numb mRNA* have been reported to differ in the adult testis, liver, lung, spleen, thymus, and brain [24,25]. This indicates that splicing variant-specific m-Numb expression may be regulated in both temporal and a tissue-specific manner. The postnatal roles of each m-Numb variant in the gastric mucosa remain unclear.

In the present study, we demonstrate that splicing variant-specific m-Numb expression is induced during gastric mucosal regeneration after acute damage, and that the stomachs of Msi1-knock-out (Msi1-KO) mice, which lack the m-Numb expression response, show delayed gastric regeneration. The m-Numb protein induces expression of regeneration-related genes such as prostate stem cell antigen (PSCA) and metallothionein-2 (Mt2). We report that this enhancement of m-Numb protein expression by Msi1 in the stomach mucosal regeneration is occurred by post-transcriptional regulation.

Results

Delayed Gastric Mucosal Repair after Ethanol Administration in Msi1-KO Mice

Msi1 is up-regulated in rat gastric corpus mucosa after ethanol-induced mucosal injury [9]. We firstly examined the histochemical analysis of gastric mucosa of wild-type and Msi1-KO mice in the water-treated control group. There was no significant difference in the erosive lesions of gastric mucosa (Figure 1A) and the number of H^+, K^+-ATPase-positive parietal cells, Muc6-positive mucous neck cells, and pepsinogen-positive mucosal zymogenic cells between wild-type (n = 5) and Msi1-KO (n = 3) mice in the control group (Figure S2). Thus, we investigated mucosal regeneration after ethanol-induced acute gastric injury in Msi1-KO mice. Five hours after ethanol administration, the erosive and ulcerative lesions in the gastric mucosa were more enhanced in the Msi1-KO mice than in the wild-type mice (Figure 1A). The area of erosive and ulcerative lesions was significantly larger in the stomach of ethanol-treated Msi1-KO mice (n = 3) than in the stomach of ethanol-treated wild-type mice (n = 6) (Figure 1B). Furthermore, at this time-point, an abundant decrease in the number of H^+, K^+-ATPase-positive parietal cells, Muc6-positive mucous neck cells, and pepsinogen-positive mucosal zymogenic cells was observed in the superficial area of the gastric fundus of the Msi1-KO mice (above on dotted line in Figures 2B, D, and F), even in areas of undetached epithelium.

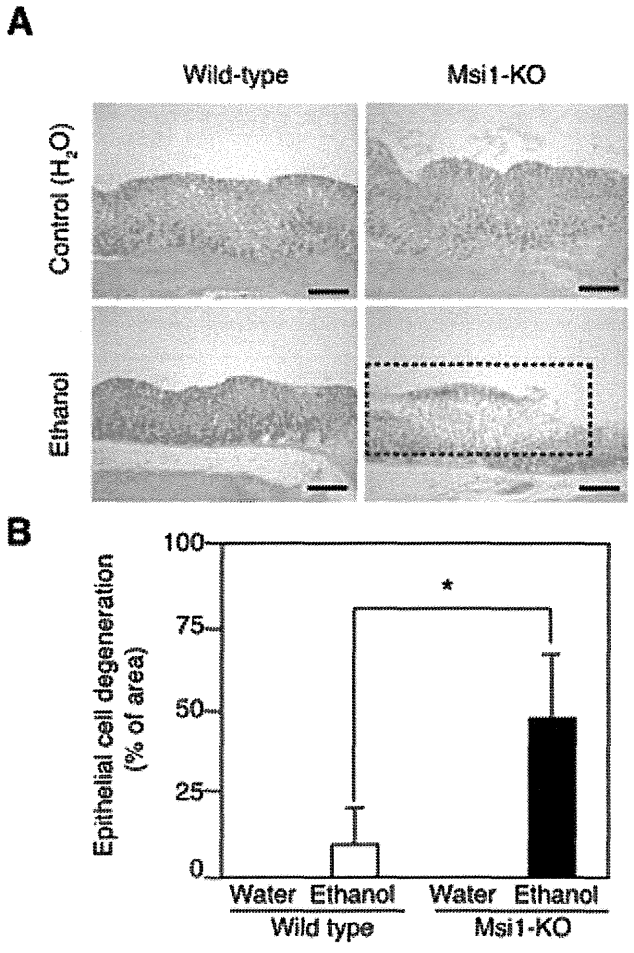

Figure 1. Gastric epithelial cell degeneration after gastric damage. (A) Hematoxylin and eosin (H&E)-stained sections of the stomachs of wild-type and Msi1-KO mice. Control group (water administration) and ethanol administration group. Bars = 100 μm (B) Gastric epithelial cell degeneration in the fundic area was noted in the H&E-stained sections. *$P<0.05$.
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Post-transcriptional Enhancement of m-Numb Expression by Msi1 in the Stomach

To investigate whether the *Msi1* target gene was deregulated in the gastric mucosa of Msi1-KO mice, the expressions of p21 and m-Numb proteins, known to be the major targets of Msi1 in the stomach and cerebrum, were compared between wild-type and Msi1-KO mice. There was no difference in the expression of p21 protein in both the stomach and the cerebrum of Msi1-KO mice compared to that in the wild-type mice (Figure 3A). On the other hand, although the expression of m-Numb protein in the cerebrum was higher in Msi1-KO mice than in the wild-type mice, the expression of m-Numb protein in the stomach was markedly downregulated in the Msi1-KO mice (Figure 3A). The m-Numb protein was normally expressed in the gastric epithelium and slightly expressed in the zymogenic region (Methods S1 and Figure S3). And the decreased m-Numb expression in the Msi1-KO mice was not observed in other tissue like cerebrum, cerebellum, colon, testis, liver and lung (Figure S4).

The antibody used here for western blotting analysis can distinguish between the PRRL (Numb1 and/or Numb3) and PRRS (Numb2 and/or Numb4) forms of the m-Numb protein

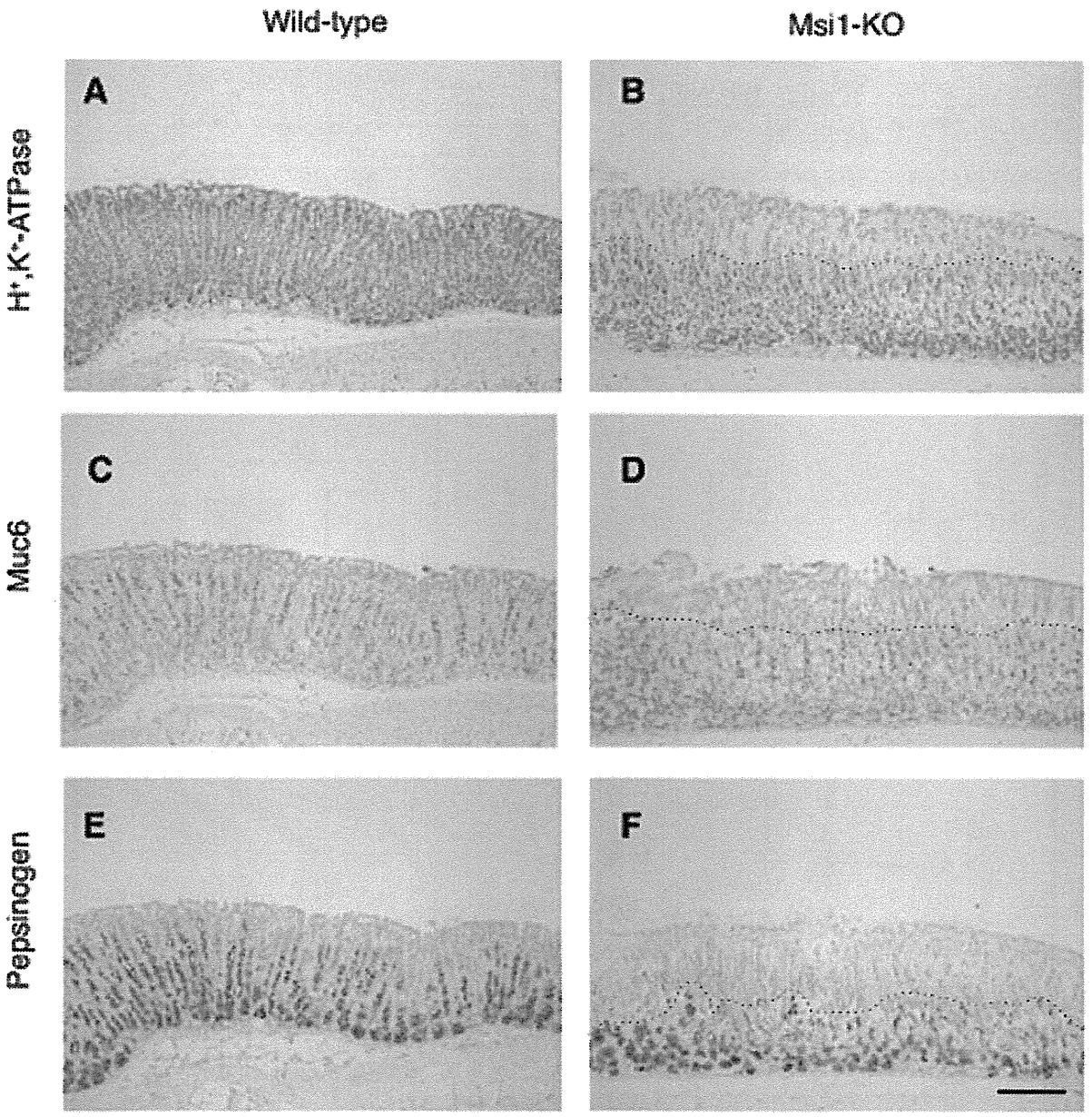

Figure 2. Immunohistochemical analysis revealed a defect in cell differentiation in Msi1-KO stomach. Wild-type (A, C, and E) and Msi1-KO (B, D, and F) mice were administered ethanol. Sections of the gastric mucosa from each mouse at 5 h after ethanol administration were then stained using anti-H^+, K^+-ATPase- (A and B), anti-Muc6- (C and D), and anti-pepsinogen- (E and F) antibodies. Bar = 100 μm.
doi:10.1371/journal.pone.0053540.g002

(Figure 3B), but not the PTB form, because of its small molecular size (11-amino acids) compared to PRR (49-amino acids); thus, western blotting with this antibody would yield 2 bands according to the presence or absence of PRR. In the stomach, the expression of PRRL and PRRS m-Numb proteins were significantly decreased in Msi1-KO mice as compared to that in wild-type mice (Figure 3C, n = 3, respectively).

The total *m-Numb* mRNA detected with the common primers for all the *m-Numb* variants was not decreased in the stomach of Msi1-KO mice as compared with that of wild-type mice, indicating post-transcriptional regulation of m-Numb protein expression by Msi1. Since the translated RNA forms a functional translational initiation complex, which consists of matured RNA and numerous 80S ribosomes, the polysome profiles in 15–40% sucrose gradients were established for lysates of wild-type and Msi1-KO mouse stomachs, and the pattern of *m-Numb* mRNA was determined by quantitative RT-PCR. The ratio of *m-Numb* mRNA in the heavy polysome fraction (fractions 1–9) was decreased in Msi1-KO mice (Figure 3D), indicating that translation of *m-Numb* mRNA was reduced in the stomach of Msi1-KO mouse.

The mammalian Musashi family consists of 2 genes, *Msi1* and *Msi2*. To investigate a possible compensatory effect of *Msi2* on the regulation of m-Numb in the stomach of Msi1-KO mice, *in vitro* knockdown analysis was performed in human gastric cell line N87 by using shRNA-containing lentivirus. Silencing *Msi1* reduced the expression of m-Numb in accordance with the results observed in the stomachs of Msi1-KO mice (Figure 3E).

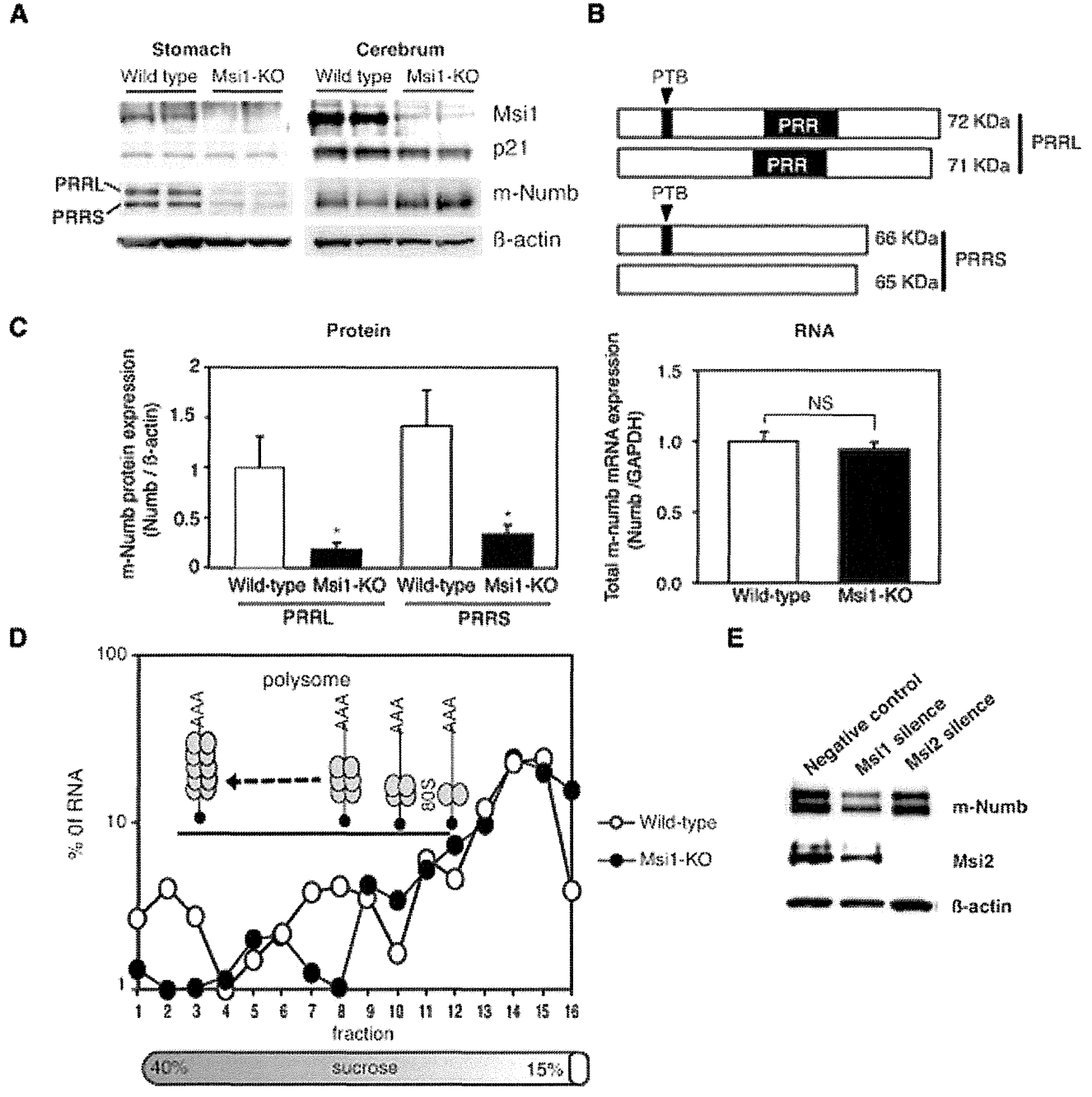

Figure 3. Expression of p21 and m-Numb in the stomach of wild-type and Msi1-KO mice. (A) Western blots indicating expression of Msi1, p21, and m-Numb protein in the stomach and cerebrum of sham-treated wild-type and Msi1-KO mice. (B) Classification of m-Numb protein by proline-rich region (PRR). (C) m-Numb protein and total RNA expression. Western blotting and quantitative RT-PCR for the expression analysis of m-Numb protein and mRNA in sham-treated wild-type and Msi1-KO mice was performed in triplicate. The density of each m-Numb protein band in western blotting is normalized to actin and represented as the fold-change relative to expression of the protein in wild-type mice. White bars: wild-type mice; black bars: Msi1-KO mice. *$P<0.01$ compared to wild-type mice. (D) Polysome analysis of *m-Numb* mRNA from mouse stomach. Fractions of a polysome gradient prepared from the stomachs of wild-type (empty circles) and Msi1-KO (filled circles) mice. RNA was extracted from each fraction and used for quantitative RT-PCR. The results are shown as the percentage of the total amount of RNA in each fraction. (E) Knockdown of human *Msi1* and *Msi2* in N87 cells by shRNA-containing lentiviral particles. Western blotting was performed using primary antibodies specific for Msi1, Msi2, m-Numb, and β-actin.
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The Msi2 expression level was slightly decreased by *Msi1* silencing; however, silencing *Msi2* did not alter the expression of m-Numb, indicating that the enhanced expression of m-Numb protein is independent of *Msi2*.

Enhanced m-Numb Protein Expression in the Regenerating Mouse Gastric Tissue

To investigate changes in the expression of m-Numb protein after gastric injury, total protein was extracted from the gastric

tissues of wild-type and Msi1-KO mice at 1, 3, and 5 h after ethanol administration, and these extracts were used in western blotting. The expression of both the PRRL and PRRS forms of m-Numb increased in a time-dependent manner after the induction of gastric damage in the wild-type mice (Figures 4A and B). In contrast, in the Msi1-KO mice, only a weak and insignificant induction of m-Numb expression was observed, and the m-Numb expression levels were significantly lower than those in wild-type mice at 1, 3, and 5 h after ethanol administration (Figures 4A and B). The expression of p21 did not change after the gastric damage (Figure 4A).

Association of PTB-containing Splicing Variants of m-Numb mRNA with Mucosal Injury

Although m-Numb protein expression was confirmed after gastric injury, western blotting analysis could not distinguish the presence or absence of the PTB domain of the Numb protein. To distinguish the *m-Numb* splicing variants responsible for mucosal regeneration, total RNA was extracted from the gastric tissues of the wild-type (n = 5 each) and Msi1-KO mice (n = 3 each) at 1, 3, and 5 h after ethanol administration, and quantitative RT-PCR was performed using primers specific for each splicing variant (Figure 5A). The mRNA expression of the PTBS form of *m-Numb* did not change after ethanol-induced gastric damage (Figure 5B). The possibility of genomic contamination in the PCR results for PTBS was ruled out by reverse transcription in the absence of the sample (Figure S5). In contrast, the expression of the PTBL form of *m-Numb* mRNA was specifically increased after ethanol-induced gastric mucosal damage in both wild-type and Msi1-KO mice (Figure 5B).

Two forms of PTB-site-conserved complete *m-Numb* mRNA have been described, namely, Numb1 (PTBL-PRRL) and Numb2 (PTBL-PRRS), but the expression of each of these mRNAs could not be analyzed by quantitative PCR, because the PTB domain is distant from the PRR domain. Therefore, semi-quantitative PCR with a long-range DNA polymerase was performed for wild-type control (n = 5) and ethanol-administered (n = 5) mice. This indicated that the expression levels of both PTB-domain-containing *m-Numb* mRNAs were significantly increased at 5 h after ethanol administration (Figure 5C). Interestingly, as we demonstrated in Figure 4, the protein translation of the increased transcripts in ethanol administration was required for Msi1 activity.

m-Numb Induced the Regeneration-related Gene Expression

To investigate the role of the induced m-Numb protein in regenerating gastric mucosa, changes in gastric regeneration-related genes were examined in the gastric mucosa of wild-type (n = 5) and Msi1-KO mice (n = 3). The mRNA expression of *leucine-rich repeat-containing G protein-coupled receptor 5* (*LGR5*) and *doublecortin and* Ca^{2+}*/calmodulin-dependent kinase-like-1* (*DCLK1*), which are putative stem cell markers, did not change in either group of mice (Figure 6A). On the other hand, the mRNA expression of PSCA, which is expressed in the isthmus of gastric mucosa [26],

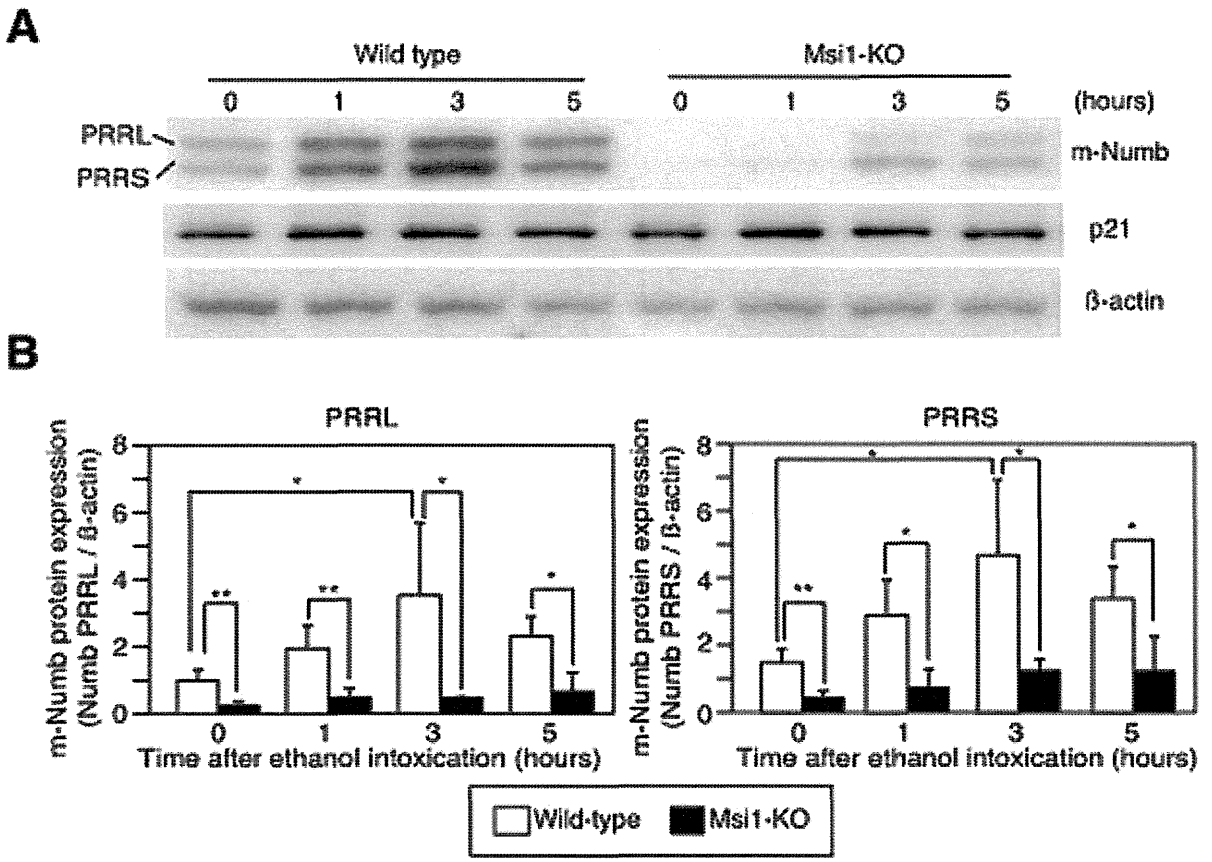

Figure 4. Expression of m-Numb protein in the stomach of wild-type and Msi1-KO mice after ethanol-induced gastric damage. (A) Expression of m-Numb and p21 protein in the stomach of wild-type and Msi1-KO mice. (B) The intensity of each band in the western blot of m-Numb was analyzed and the results were statistically compared. White bars: wild-type; black bars: Msi1-KO. *$P<0.05$, **$P<0.01$.
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