Predictable Marker for Regression of Barrett’s Esophagus by Proton Pump Inhibitor Treatment in Korea

Hyun Jin Jo,1 Hye Seung Lee,2 Nayoung Kim,1,3* Ryoung Hee Nam,1 Hyun Chang,1 Min Soo Kim,1 Sung Eun Kim,1 Jane C Oh,4 Dong Ho Lee1,3 and Hyun Chae Jung3

Departments of 1Internal Medicine and 2Pathology, Seoul National University Bundang Hospital, Seongnam, Gyeonggi-do, Korea; 3Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Korea; and 4Yonsei Plus Clinic, Seongnam, Gyeonggi-do, Korea

Background/Aims
There has been no report regarding the regression of Barrett’s esophagus (BE) by continuous treatment of proton pump inhibitor (PPI). The aim of this study was to determine the regression rate of BE by PPI and predictable markers related to regression.

Methods
Thirty-five patients diagnosed as BE were consecutively enrolled and most of them took continuous PPI. The 25 patients underwent endoscopic surveillance and received biopsy. If the specialized intestinal metaplasia (SIM) was lost at any point of surveillance and did not recur, the case was regarded as the regression group. The proportion of SIM was graded and the mucin phenotype was decided using immunohistochemistry for MUC2, MUC5AC and MUC6. To assess the cell proliferation indexes and the degree of intestinal maturation, immunohistochemistry for Ki67 and CDX2 were performed.

Results
The regression of BE occurred in the 11 (44%) patients. The clinical and demographic factors showed no difference between the regression (n = 11) and persistence group (n = 14). The lower grade of SIM (P < 0.001) and gastric predominant mucin phenotype (P = 0.018) were more frequent, and the number of Ki67 positive cell per gland (P = 0.008) and the mean extent of CDX2 (P = 0.022) were lower in the regression group than in the persistence group.

Conclusions
The regression of BE by PPI treatment was frequent in Korea. The immunohistochemical detection of mucin phenotype, grade of SIM, Ki67 and CDX2 expression in Barrett’s mucosa could be useful as a predictable marker for regression of SIM in BE.

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Key Words
Barrett esophagus; Biologic markers; Regression
Introduction

Although controversies over the definition of Barrett’s esophagus (BE) have continued, BE is commonly diagnosed when there is an endoscopically irregular Z-line and the replacement of the normal stratified squamous epithelium by columnar epithelium with specialized intestinal metaplasia (SIM) containing goblet cells in a biopsy of the distal esophagus. Recently, the prevalence and incidence of BE in Asia has been expected to increase with the availability of gastroscopy and with the increased prevalence of reflux esophagitis related to obesity. Moreover some studies showed that the mean age at the time of diagnosis of BE was getting younger below the age of 50 years. BE is well known as a precancerous lesion. That is, the annual risk of development of esophageal adenocarcinoma from BE was estimated to be approximately 0.5% and 5-year survival rate of esophageal adenocarcinoma was reported as low as below 15%. For this reason, endoscopic surveillance according to grade of dysplasia was considered as indispensable exam to detect complication of BE as early as possible.

The appropriate predictable marker is considered to play an important role in the surveillance of BE patients. However, there has been no clinically established biomarker to predict of the progression or regression of BE, including clinical, demographic and histopathologic factors.

Materials and Methods

Patients and Endoscopic Examination

A total of 35 patients (25 male and 10 female) diagnosed as BE by the presence of SIM at initial EGD were consecutively enrolled from April 2005 to June 2012. These patients had an average age of 35 ± 2.2 years (range 32-78). They completed a validated gastroesophageal reflux questionnaire concerning presence or absence of gastroesophageal reflux disease (GERD) symptoms, predominant GERD symptoms, grade and frequency of predominant symptom and use of PPIs. Diagnosis of Helicobacter pylori was performed by histology (by modified Giemsa staining), and Campylobacter-like organism (CLO) test (Delta West, Bentley, Australia), which were tested with the mucosa of antrum and corpus, respectively. The GERD symptoms were prospectively analyzed using GERD impact score. Patients with systemic diseases requiring chronic medication (except hypertension and diabetes mellitus) were excluded (1 patient with a history of gastrointestinal surgery and 1 patient with liver cirrhosis). Finally, 25 patients of remaining 33 patients underwent endoscopic surveillance and received biopsy. All endoscopies were performed and recorded by one experienced endoscopist (N.K.). The distal portion of the esophagus was evaluated carefully to determine the presence of mucosal injury. The presence of endoscopic BE was determined by the identification of the detectable upward displacement of the squamous-columnar junction. The squamous-columnar junction was defined as the location at which the light-pink colored mucosa of the squamous-lined esophagus joined the red columnar-lined esophagus. Esophagogastric junction was defined as the level of the proximal margin of the gastric mucosal folds. In patients with hiatal hernia, this junction was defined by the proximal margin of gastric folds. The length of the BE was defined as the distance between the esophagogastric junction and squamous-columnar junction. Reflux esophagitis was defined according to the Los Angeles classification. Principally the biopsy samples were taken according to a stepwise four-quadrant biopsy procedure with 1-cm interval from just below the squamous-columnar junction. However, 2 biopsy samples were taken from just below the squamous-columnar junction, mainly because all had the SSBE and most of them showed tongue like projection rather than the circumferential finding. If the BE showed no change at surveillance, biopsy specimens were taken at the previous biopsy site re-
ferring to Web PACS (Picture Archiving Communication System) images of previous endoscopic examination.

All medical records of enrolled patients were reviewed, retrospectively, including the duration and dose of PPI treatment. PPI dose was divided into the following 3 categories; half-dose, standard-dose and double-dose. Standard dose of PPI was composed rabeprazole 20 mg, omeprazole 20 mg, pantoprazole 40 mg, lansoprazole 30 mg or esomeprazole 40 mg, all of them once per day. To overcome the diversity of dose and duration of PPI treatment, “total dose of PPI” was calculated in the following manner: multiply the weighted value (half-dose = 0.5, standard-dose = 1 and double-dose = 2) by the treatment duration (months).

**Histology and Immunohistochemistry Analysis**

A total of 93 biopsies were taken from endoscopic examination. Biopsy samples were immediately placed in a 10% buffered formalin solution. Samples were embedded in paraffin, and sectioned and stained with hematoxylin and eosin (H&E) and/or alcian blue. The pathologic diagnosis of BE was performed according to the guidelines of “The Practice Parameters Committee of the American College of Gastroenterology.” Based on the guidelines, the histologic component requires that biopsies taken from the endoscopically identified columnar mucosa contain metaplastic or intestinalized columnar epithelium with goblet cells.

Additionally, immunohistochemistry (IHC) was performed on paraffin embedded sections of formalin-fixed biopsy samples. Sections, cut to 4 μm thickness, were deparaffinized and rehydrated by standard methods. After deactivation of endogenous peroxidase with 3% hydrogen peroxide and blocking of non-specific binding sites, sections were incubated with the following primary antibody: anti-Ki67 antibody (mouse monoclonal antibody; 1:500 dilution, Dako, Denmark) for checking the cellular proliferation potential; anti-MUC2 antibody (mouse monoclonal antibody; 1:100 dilution, Novocastra, United Kingdom), anti-MUC5AC antibody (mouse monoclonal antibody; 1:100 dilution, Novocastra) and anti-MUC6 antibody (mouse monoclonal antibody; 1:100 dilution, Novocastra) for phenotyping of Barrett’s mucosa and anti-CDX2 antibody (mouse monoclonal antibody; BioGenex, India) for determining the degree of intestinal maturation in Barrett’s mucosa. The immunostain was performed using an automatic immunostainer (BenchMark XT, Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer’s instructions. UltraView Universal DAB detection kit (Ventana Medical Systems, Inc.) was used as secondary antibody.

The proportion of SIM in total columnar cells was graded as grade 0 (0%), grade 1 (1-29%), grade 2 (30-69%) and grade 3 (≥ 70%). Ki67 stained cells were counted in at least 300 gland cells and the Ki67 index was expressed as the average number of stained cells per one gland of Barrett’s mucosa. The extent of CDX2 and MUC staining was assessed as percentage of positively stained areas within total columnar cells. According to the result of MUC staining, the mucin phenotype of BE was decided as follows. When over 50% of the glands were stained by anti-MUC2 antibody, the Barrett’s mucosa was classified as an intestinal predominant mucin phenotype. When less than 50% of the glands were stained by anti-MUC2 antibody, the Barrett’s mucosa was classified as a gastric predominant mucin phenotype. All the biopsy sections and IHC slides were examined by the experienced gastrointestinal pathologist (H.S.L.) blinded to the endoscopic findings and the results of questionnaires. This study was approved by the ethical committee of Seoul National University Bundang Hospital. Written informed consent was obtained from all participants.

**Statistical Methods**

All statistical calculations were performed using SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). Continuous variation was expressed as mean with standard error of mean. Analysis of continuous parametric data was performed with Mann-Whitney U test and Chi-square test for discrete variate were used. A P-value below 0.05 was considered statistically significant.

**Results**

**The Clinical and Demographic Characteristics**

The data of 25 patients who had at least one more than endoscopic surveillance were analyzed. Seventeen (68%) were male and the mean age at the diagnosis of BE was 56 ± 2.7 years. Total and mean number of endoscopic examination was 84 and 3.4 ± 0.3, respectively. The sum and mean of duration of follow-up from first endoscopic examination was 836 months and 33.4 ± 3.8 months, respectively. There was no LSBE longer than 3 cm.

The 25 patients were grouped to regression or persistence group by the result of surveillance endoscopic exam and pathology. If the SIM was lost at any point of surveillance and did not recur, the cases were grouped as the regression group. If the SIM
was found consistently, then the cases were categorized as the persistence group.

The clinical and demographic characteristics of 2 groups are described in Table 1. Fourteen patients (56%) showed the persistent BE and the remaining 11 (44%) patients had the regression of BE. The proportion of hiatal hernia was higher in the persistence group (43%) than in the regression group (9%), but the difference did not reach statistical significance ($P = 0.062$). The circumferential finding at initial EGD showed the different proportion between regression and persistent groups. Eight patients (73%) showed the circumferential finding in the regression group, while one patient (7%) had the circumferential finding in

| Table 1. Comparison of Regression and Persistence Group in the Patients with Barrett’s Esophagus |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Regression group (n = 11) | Persistence group (n = 14) | $P$-value |
| Gender (M:F) | 6:5 | 11:3 | 0.389 |
| Age at diagnosis (mean ± SE [range], yr) | 56.6 ± 4.1 (32-70) | 55.5 ± 3.6 (33-78) | 0.850 |
| No. of EGD surveillance (mean ± SE [range]) | 2.4 ± 0.3 (1-4) | 2.4 ± 0.5 (1-8) | 0.992 |
| Duration of follow-up (mean ± SE [range], mo) | 36.6 ± 4.7 (13-65) | 31.0 ± 5.9 (12-88) | 0.485 |
| Hiatal hernia at initial EGD (n [%]) | 1 (9.1) | 6 (42.9) | 0.062 |
| Reflux esophagitis at initial EGD (n [%]) | 2 (18.2) | 5 (35.7) | 0.332 |
| Initial EGD finding (n [%]) | | | 0.002 |
| Only circumferential finding | 3 (27) | 0 (0) | |
| Circumferential finding with projection | 5 (45) | 1 (7) | |
| Only projectile finding | 3 (27) | 13 (93) | |
| State of $H. pylori$ infection | | | 0.175 |
| Non-infection | 5 (45.5) | 11 (78.6) | |
| Eradicated after initial EGD | 3 (27.3) | 3 (21.4) | |
| Persistent infection (not eradicated) | 1 (9.1) | 0 (0.0) | |
| Unknown | 2 (18.2) | 0 (0.0) | |
| Major symptom of reflux (n [%]); symptom frequency | | 0.144 |
| Heartburn | 2 (18.2):0/1/1/0 | 0 (0.0) | |
| Acid regurgitation | 1 (9.1):1/0/0/0 | 3 (21.4):1/0/1/1 | |
| Chest pain | 3 (27.3):0/0/2/1 | 0 (0.0) | |
| Hoarseness | 1 (9.1):0/0/1/0 | 4 (28.6):0/1/2/1 | |
| Globus sensation | 0 (0.0) | 1 (7.1):1/0/0/0 | |
| Cough | 0 (0.0) | 0 (0.0) | |
| Epigastric soreness | 3 (27.3):1/0/1/1 | 3 (21.4):1/0/1/1 | |
| No symptom | 1 (9.1) | 3 (21.4) | |
| Smoking (n [%]) | | 0.230 |
| Current | 9 (81.8) | 8 (57.1) | |
| Ex or non-smoker | 0 (0.0) | 3 (21.4) | |
| No answer | 2 (18.2) | 3 (21.4) | |
| Alcohol use (n [%]) | | 0.970 |
| Yes | 6 (54.5) | 7 (50.0) | |
| No | 3 (27.3) | 4 (28.6) | |
| No answer | 2 (18.2) | 3 (21.4) | |
| PPIs after BE diagnosis (n [%]) | | 1.000 |
| $\geq 1$, $< 3$ (mo) | 2 (20.0) | 2 (15.4) | |
| $\geq 3$, $< 6$ (mo) | 1 (10.0) | 2 (15.4) | |
| $\geq 6$, $< 12$ (mo) | 3 (30.0) | 0 (0.0) | |
| $\geq 12$ (mo) | 4 (40.0) | 9 (69.2) | |
| Total dose of PPIs (mean ± SE [range], value = weighted value$^b$ × month(s)) | | 0.288 |
| $\geq 1$, $< 3$ (mo) | 7.3 ± 1.6 (2-17) | 9.4 ± 1.2 (1-14) | |
| $\geq 3$, $< 6$ (mo) | | |
| $\geq 6$, $< 12$ (mo) | | |
| $\geq 12$ (mo) | | |

$^a$Symptom frequency (1-2 per week/3-4 per week/daily/difficult to answer due to irregularity), $^b$Weighted value was demarcated according to daily proton pump inhibitor (PPI) dose (half-dose = 1, standard-dose = 1 and double-dose = 2).

EGD, esophagogastroduodenoscopy; $H. pylori$, Helicobacter pylori; PPIs, proton pump inhibitors; BE, Barrett’s esophagus.
Figure 1. Comparison of the grade of specialized intestinal metaplasia (SIM) and mucin phenotype between regression and persistence group. (A) The proportion of grade 1 SIM was higher in regression group than in persistence group ($P < 0.001$). The grade was classified by the proportion of SIM as follows; grade 1 (1-29%), grade 2 (30-69%) and grade 3 ($\geq 70$%). (B) There was no intestinal mucin phenotype in regression group, while the intestinal mucin phenotype was 45% in persistent group ($P = 0.018$)

Table 2. Comparison of Immunohistochemical Results Between Regression and Persistence Group

|                        | Regression group (n = 11) | Persistence group (n = 12) | $P$-value |
|------------------------|---------------------------|---------------------------|-----------|
| SIM (mean ± SE [range], %) | 9.2 ± 2.7 (2.0-30.0)      | 63.3 ± 9.0 (10.0-100.0)   | $< 0.001$ |
| Ki67 proliferative index (mean ± SE [range], cell No. of positive immunostain/gland) | 19.6 ± 4.9 (0.6-58.5) | 35.4 ± 3.4 (13.2-53.0) | 0.008 |
| Percentage of Ki67 (mean ± SE [range], %) | 44.1 ± 9.9 (0.0-80.8) | 49.8 ± 4.7 (22.0-74.6) | 0.602 |
| Percentage of MUC2 (mean ± SE [range], %) | 5.8 ± 2.8 (0.0-25.0) | 47.2 ± 8.3 (9.0-90.0) | $< 0.001$ |
| Percentage of MUC5AC (mean ± SE [range], %) | 77.5 ± 8.9 (2.0-100.0) | 70.5 ± 9.4 (5.0-100.0) | 0.595 |
| Percentage of MUC6 (mean ± SE [range], %) | 36.4 ± 7.5 (10.0-80.0) | 10.9 ± 2.7 (0.0-30.0) | 0.005 |
| Percentage of CDX2 (mean ± SE [range], %) | 4.2 ± 1.7 (0.0-15.0) | 18.3 ± 3.2 (0.0-50.0) | 0.022 |

$^a$n = 11 in MUC and CDX2 immunohistochemistry.

SIM, specialized intestinal metaplasia.

Between 2 groups, the gender, age at diagnosis, number of surveillance endoscopic exam, duration of follow-up, proportion of erosive reflux disease, state of H. pylori infection, symptom of reflux and frequency of reflux symptom did not differ. Also, the proportion of current smoker, alcohol use and treatment of PPIs after BE diagnosis was not different between the 2 groups. The duration of taking PPIs and total PPI dose were higher in the persistence group compared to the regression group, but there was no significant difference.

Histology and Immunohistochemistry

None of all biopsies showed dysplasia or cancer. The biopsy tissues at the initial diagnose of BE were used for comparative analysis between the 2 groups. In the view of grade of SIM, the lower grade of SIM was more frequent in the regression group than in the persistence group (grade 1: 91%, grade 2: 9% in the regression group vs. grade 1: 8%, grade 2: 42%, grade 3: 50% in the persistence group; $P < 0.001$) (Fig. 1A). Ki67 proliferative index was lower in the regression group with significance than in the persistence group (35.4/gland vs. 19.6/gland, $P = 0.008$). The mean extent of CDX2 was 4.2% in the regression group and 18.3% in the persistence group ($P = 0.022$). While the extent of MUC2 was lower in the regression group (6% vs. 47%, $P < 0.001$), the extent of MUC6 showed higher level in the regression group (36% vs. 11%, $P = 0.005$). That of MUC5AC was similar in 2 groups (78% vs. 71%, $P = 0.595$) (Table 2). All predominant mucin phenotype showed no regression. However, BE regressed in about two-thirds of the predominant mucin phenotype ($P = 0.018$) (Fig. 1B). Figure 2 illustrates examples of the IHC for the detection of the CDX2, MUC2, MUC5AC,
MUC6 and Ki67 expression in both of the regression and persistence group.

Discussion

It is known that the prevalence of BE, particularly the long-segment type, is low in East Asians. In Korea, the prevalence of BE is also very low, such as below 1%, and most of BE is known as the SSBE. Although the influence of the extent of BE on its natural history is controversial, more recent studies have observed a strong relationship between length of BE and development of adenocarcinoma and dysplasia. The reported incidence of dysplasia varies with different publications and is generally around 2-5%. Especially, the rate of dysplasia detection in SSBE was much lower as 0.95%. Likewise in this study all of the BE was short-segment type and dysplasia or cancer was not detected.

In the present study, the regression rate was 44%, much higher than expected. This high regression rate of BE might be explained by PPI treatment after the diagnosis of BE. Some investigators demonstrated that PPI produced a regression of BE and lowered the risk of developing dysplasia. However, the regression rate of 44% of Barrett’s mucosa in the present study is rather higher than 30% in other report. Moreover, there have been controversies regarding the reversibility of BE by PPIs. Amano et al reasoned that the difficulty in judging the length of BE and the diversity of the mucin phenotype contributed to these controversies. In this study, there were no differences in clinical and demographic factors including PPI treatment between the regression and persistence group although...
the number of patients was small. Therefore it is considered that
there would be other factors that affected the regression of BE.

Although there is still argument remained, the presence of
metaplastic or intestinalized columnar epithelium with goblet
cells is necessary for the histological diagnosis. In addition, there
is debate about the degree to which SIM of the columnar-lined
esophagus increases the risk of esophageal adenocarcinoma.1
However, our results clearly showed that the degree of SIM had
a close association with the regression of BE using diverse IHC
methods. With lower grade of SIM, the regression rate of BE
was higher. There could be a possibility of sampling error or limi-
tations by small number of biopsies.1,28 However, biopsy speci-
mens had been taken at the previous biopsy sites, which were
clearly identified by photograph taken during the first endoscopy.
In addition, the circumference and length of BE was rather short
in most cases. Furthermore, only one experienced endoscopist
and one pathologist who was blinded to the sample conducted
each endoscopic and histopathologic exam.

Ki67, a nuclear antigen, has been used as an index for cell
proliferation.29-31 Ki67 is known to be overexpressed in Barrett’s
metaplasia and as a suitable biomarker for progression towards
neoplastic degeneration.32 Recently, one study showed that Ki67
expression was significantly increased in BE in comparison with
columnar mucosa without SIM, but the range of Ki67 expression
overlapped between 2 groups and the difference was minimal on-
ly as 1% even if there was statistical significance.29 That is, Ki67
proliferative index between columnar mucosa with SIM and
without SIM seems to be too similar to separate BE from
non-Barrett’s metaplasia. In the present study, Ki67 immuno-
staining nuclei were counted in glands of columnar cells, which
were selected in order of the degree of immunostain and counted
up to minimum of 300 cells whether there was SIM or not.
Although the mean percentage of Ki67 positively staining nuclei
was lower in the regression group than in the persistence group,
there was no statistical significance. However, the Ki67 pro-
liferation index, which was expressed as the average number of
stained cells per one gland of Barrett’s mucosa, was significantly
lower in the regression group.

As the degree of SIM was shown to be related with re-
gression of BE, we further studied other markers that reflect the
intestinal metaplasia. Cdx2, a kind of homeobox genes, is known
to play role in the gut development such as the transformation of
endoderm to columnar epithelium and also to regulate the ex-
pression of muc2, gene of intestinal mucin-phenotype.33 Therefore,
the CDX2 reflects the degree of intestinal maturation.33,34
Furthermore, one study showed that CDX2 positivity was ob-
erved not only in goblet columnar epithelium but also in
non-goblet columnar epithelium, and even in inflamed esoph-
ageal squamous epithelium. This finding suggests that CDX2 expression precedes the phenotypic change.\textsuperscript{35} Therefore, it could be helpful in early detection of the potency for intestinal metaplasia. In agreement with other studies’ results showing CDX2 expression levels to be higher in BE than in non-BE,\textsuperscript{36,37} CDX2 positivity and percentage of SIM also had positive correlation with statistical significance in this study (the Pearson correlation coefficient was 0.743, \( P < 0.001 \), data is not shown). In addition, the mean extent of CDX2 stain was lower in the regression group.

Muc genes code for the secretion of mucin glycoproteins by epithelial cell. Mucins can be subdivided into membrane-associated and secreted forms. In normal tissues, mucins are expressed in a relatively organ- and cell-specific manner.\textsuperscript{38} To date, 21 human genes have been identified.\textsuperscript{39,40} Among diverse muc gene products, MUC2, MUC5AC and MUC6 are all secretory mucin. While MUC2 is commonly found in goblet cells of the small intestine and colon, MUC5AC and MUC6 are strongly expressed in normal gastric mucosa.\textsuperscript{39,41} It is known that MUC5AC is secreted by surface foveolar cells, whereas MUC6 is secreted by deep neck and gland cells. That is, MUC5AC and MUC6 are considered as markers of gastric epithelial cells, whereas MUC2 is known as typical of the intestinal epithelial cell phenotype. However, the gastric-type markers for MUC5AC and MUC6 and intestinal-type marker for MUC2 can be specifically expressed in aberrant conditions like as BE.\textsuperscript{42}

In this study, the MUC2 expression of BE was lower in the regression group than in the persistence group, whereas MUC6 expression was higher in the regression group. Amano et al\textsuperscript{43} demonstrated that the absence of the intestinal predominant mucin phenotype was a positive predictor for newly developing squamous re-epithelialization at the site of biopsy of Barrett’s mucosa. When the mucin phenotype was classified by the result of MUC2 IHC, we also showed similar results that all of the intestinal predominant mucin phenotype was not regressed, while MUC2 is known as typical of the intestinal epithelial cell phenotype. However, the gastric-type markers for MUC5AC and MUC6 and intestinal-type marker for MUC2 can be specifically expressed in aberrant conditions like as BE.\textsuperscript{42}

In conclusion, the regression of BE by PPI treatment was more frequent than expected in Korea. Not only the grade of SIM but also the immunohistochemical detection of mucin phenotype, Ki67 and CDX2 expression in Barrett’s mucosa could be useful as predictable markers for regression of SIM in BE.

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