Usefulness of endoscopic brushing and magnified endoscopy with narrow band imaging (ME-NBI) to detect intestinal phenotype in columnar-lined esophagus

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Background：Barrett's esophagus (BE) with specialized intestinal metaplasia (SIM), which is at high risk of esophageal adenocarcinoma, has been identified by obtaining biopsy specimens randomly. Magnified endoscopy with narrow band imaging (ME-NBI) is reported to be useful for detecting SIM.

Aim：To evaluate the usefulness of endoscopic brushing followed by ME-NBI for the detection of intestinal phenotype.

Methods： Biopsy and brushing samples were taken following endoscopic observation by ME-NBI. Total RNA was extracted from the whole and microdissected samples, and quantitative reverse transcription (RT)-PCR analysis of *SHH*, *CDX2*, and mucin mRNA expression was performed.

Results：Fifty patients (32 men, 18 women, average age 67.3 years old) with metaplastic columnar epithelium of the lower esophagus were studied. The frequencies of *MUC2* (85% vs. 65%) and *CDX2* (95% vs. 75%) were detected more frequently in the brushing samples compared to the biopsy samples. *MUC2* expression levels were significantly higher in the brushing samples than those in the biopsy samples. *CDX2* and *MUC2* expression levels in the brushing samples were significantly higher in the mucosa with tubular/villous pattern observed by ME-NBI than those in the others.

Conclusion : Endoscopic brushing in the tubular/villous mucosal pattern of columnar epithelium visualized by ME-NBI is useful to detect intestinal phenotype.

Introduction

The majority of esophageal adenocarcinomas are thought to evolve through a multistep process starting with conversion of squamous epithelium to mucinous columnar epithelium and progressing through goblet-cell metaplasia followed by the development of dysplasia and finally carcinoma [1, 2, 3]. The current definition of Barrett's esophagus (BE), widely accepted in North America, is the endoscopic appearance of a columnar epithelium in the tubular esophagus and a biopsy demonstrating specialized intestinal metaplasia (SIM). SIM is recognized histologically by the presence of goblet cells, and is considered as the hallmark lesion of BE because it is known to predispose to the development of dysplasia and, therefore, adenocarcinoma regardless of the location in the esophagus [4]. Because adenocarcinoma has a poor prognosis when detected at an advanced stage, the prevention has focused on detection of patients with BE and the identification of SIM requires biopsy [5, 6]. The surveillance method commonly used in BE involves periodic upper endoscopy with biopsies of suspicious areas and random 4-quadrant biopsies each 2 cm [7]. This biopsy protocol is time-consuming, carries the risk of sampling error, and is hampered by low compliance [8]. Techniques are needed that improve visualization of Barrett's epithelium and distinguish metaplasia from dysplasia and neoplasia to provide for more accurate biopsies and potentially more efficient use of screening endoscopy [9-12].

A number of endoscopic methods including chromoendoscopy have been tried for the optical detection of intestinal metaplasia in the esophagus and stomach [13-16]. Narrow band imaging (NBI) is a novel technique that enhances the diagnostic capability of endoscopes in characterizing tissues by using a narrow-band width filter in a red-green-blue (RGB) sequential illumination system. Magnified endoscopy with narrow band imaging (ME-NBI) has been introduced which negates the need for dye spray providing better details of the mucosa and vascular patterns of minute lesions, including early gastric adenocarcinoma and gastric intestinal metaplasia [17, 18]. Recent reports have indicated that ME-NBI may be helpful in identifying SIM and dysplasia in BE, and several classification systems were developed for BE evaluation using ME-NBI [19].

Cytology, which is safer and minimally invasive method compared to biopsy, as well as histology can be complementary to each other in the evaluation of patients with BE, [20-22] although cytology alone has been shown to be a poor diagnostic tool for dysplasia [23] and has no generally accepted role in establishing the diagnosis of BE. However, there have been no reports about endoscopic brushing followed by ME-NBI in columnar-lined esophagus.

Recent reports have indicated that dedifferentiation of squamous epithelium is one of the mechanisms of conversion to columnar epithelium [25], although the molecular pathogenesis of BE is poorly understood. The CDX proteins are intestine-specific transcription

factors and aberrant expression of CDX2 in the upper gastrointestinal (GI) tract is thought to be a key event in the pathogenesis of Barrett's mucosa in the esophagus as well as in intestinal metaplasia in the stomach [11, 12]. On the other hand, Sonic hedgehog (SHH) is a peptide morphogen [26], and is abundantly expressed in the normal gastric fundus. Ectopic SHH expression has been observed in fundic gland metaplasia [27, 28] including BE, whereas no SHH protein was observed in the normal esophagus.

In the present study, we evaluated the usefulness of endoscopic brushing followed by ME-NBI for the detection of SIM or intestinal phenotype in columnar-lined esophagus. We also examined the gene expression of gastric and intestinal phenotypes including CDX2 and SHH in the brushing samples and biopsy samples as well as microdissected glands taken from the mucosa visualized by ME-NBI.

Subjects and methods

The subjects were outpatients with metaplastic columnar epithelium of the lower esophagus (segment length >1cm) detected by previous endoscopy who were recommended prior to upper GI endoscopy following reflux esophagitis, screening of ulcers or cancer regardless of GI symptoms. The study was performed at Kawasaki Medical School Hospital in Japan and was approved by the Kawasaki Medical School Ethical Committee. Informed consent was obtained from each patient, and the patients were enrolled in the study between April 2010 and July 2011.

All subjects underwent upper GI endoscopy. Exclusion criteria were the taking of non-steroidal anti-inflammatory drugs (NSAIDs) and/or a history of gastrectomy. Patients were also excluded if they had gastric cancer or other malignant lesions, haemorrhagic diseases, cirrhosis or renal failure. Demographic data collected at study entry included age, sex, smoking habits, alcohol consumption and drug treatments including the use of anti-secretory drugs. Drinking and smoking were defined as regular intake when consumption was more than 35g of ethanol or five cigarettes per day, respectively.

Endoscopic examination

All endoscopies were performed with an endoscopic tri-modal imaging (ETMI) system using zoom endoscopy (GIF-Q240Z or GIF-H260Z, Olympus Inc., Tokyo, Japan). The light source

(XCLV-260HP) contains two rotating RGB filters; one conventional for high-resolution white-light endoscopy (WLE) and an additional one for NBI in which the band-pass ranges are narrowed to wavelengths of 530–550nm (green) and 390–445nm (blue). Endoscopies were performed by experienced endoscopists using WLE and ME-NBI after patients had fasted for 12 h, and endoscopic photographs were obtained for subsequent review by two well-trained endoscopists. Magnified observation of the lower esophagus using ME-NBI to determine the pit patterns of columnar epithelium which were classified into three patterns; small round, long oval, and tubular/villous (Fig. 1) [29, 30]. If the pit patterns tend to consist of two or three patterns, the tubular/villous patterns were preferentially classified because those were reported to be characteristics of the presence of goblet cells [29, 30]. After observation by WLE and ME-NBI, brushing and biopsy samples were taken from the targeted columnar epithelium.

At first, brushing samples were taken using endoscopic cytology brush (CELEBRITY™ Endoscopy**;** Boston Scientific**,** Boston, MA, USA**)** from half of the targeted columnar epithelium. Then, two specimens were taken from the remaining half of the targeted part as biopsy samples using endoscopic forceps (FB231K (A) Olympus); one specimen was used as a whole sample and the other was used for laser-captured microdissection. Biopsy samples were immediately frozen using liquid nitrogen and stored at −80 ◦C until use.

Laser-captured microdissection

Frozen samples were embedded in TissueTek OCT medium (VWR Scientific, Torrance, CA). Cryostat sections (8μm) were laser microdissected with a PixCell II laser-microdissection system (Arcturus Engineering, Mountain View, CA).

RNA extraction and quantitative polymerase chain reaction

Total RNA was extracted from brushing and whole biopsy samples using the RNeasy Mini kit (Qiagen, Hilden, Germany) and from microdissected tissue using a Pico Pure RNA Isolation kit (Arcturus Bioscience). Then cDNA synthesis was performed using the SuperScriptIII First Strand Synthesis System (Invitrogen, Carlsbad, CA, US). Quantitative reverse transcription (RT)-PCR analysis of S*HH, CDX2, MUC5AC, MUC6, MUC2* and *β*-actin mRNA expression was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) employing the TaqMan gene expression assay according to the manufacturer's instructions (Applied Biosystems).The RT-PCR was performed with 20 ng RNA for both target genes and the endogenous control using the TaqMan one-step RT-PCR Master Mix Reagent kit (P/N 4309169). Each amplification reaction was performed in triplicate, and the average of the threshold cycles was used. The target amount was obtained by normalization to an endogenous reference (*β*-actin) and relative to a calibrator.

Immunofluorescence

After withdrawing the brush, the material was smeared onto clean, dry, labeled glass slides. Two smears were made with each brushing and cryostat sections (4µm) from embedded frozen samples were used. The fixed samples were permeabilized in 0.1% Triton X-100/ phosphate buffered saline (PBS). Nonspecific binding was prevented by immersion of samples in blocking solutions (PBS containing 0.1% Tween 20; PBST) for one hour at room temperature. The smears were supplemented with primary antibodies: MUC5AC antibody (dilution 1:500, Novocastra, Wetzlar, Germany) for one hour at room temperature. The sections were incubated with MUC5AC (dilution 1:200, Novocastra), MUC2 antibody (dilution 1:100, Novocastra) or mouse monoclonal CDX2 antibody (1:50, Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C. After rinsing with PBST, samples were then supplemented with secondary antibodies: Alexa 488 (dilution 1/1000, Invitrogen, NY, USA), for one hour at room temperature. After rinsing with PBST, samples were staining by Propidium Iodide (PI). After rinsing with PBS, samples were mounted and observed with laser scanning confocal microscope.

Statistical analyses

Values are expressed as the mean ± SD or the median with a 25–75% range, whichever was appropriate depending on whether the data were normally distributed. Statistical analyses for significant differences of parameters were performed using the non-parametric Mann–Whitney U test between the two groups. A two-sided *p* value of less than 0.05 was considered statistically significant. All statistical computations were performed using SPSS (SPSS Inc., Chicago, IL, USA).

Results

The study group consisted of 50 patients (32 men, 18 women, average age 67.3 years) with an endoscopic diagnosis of metaplastic columnar epithelium of the lower esophagus. Demographic and clinical characteristics of the study group are shown in Table 1.

MUC5AC expression including *SHH* was detected in the all samples taken by biopsy and brushing, and the majority of cells taken by brushing and most glands taken by biopsy expressed MUC5AC protein, while MUC2 and, CDX2 expression were relatively limited at the surface of epithelium (Figure 3 and 4). *CDX2* (85% vs. 65%, p=0.04) and *MUC2* (95% vs. 75%, p=0.01) in the brushing samples were detected more frequently in the brushing samples than in the biopsy samples (Table 2). *MUC6* expression levels were significantly lower and *MUC2* expression levels were significantly higher in the brushing samples than those in the biopsy samples (Table 3). There was no significant difference in the expression of any examined genes between the H. pylori-positive and negative patients.

In the classification of columnar epithelium visualized by ME-NBI (Figure 1), the frequencies of small round, long oval and tubular/villous patterns were 2%, 66% and 32% of the subjects, respectively. The goblets cells in the biopsy samples obtained from the tubular/villous pattern lesions were detected more frequently than those from the long oval pattern (Figure 2). In the brushing samples, the median expression levels of *CDX2* (2.9×10^{-3} vs. 0.47×10^{-3}, p=0.03) and

MUC2 (64.7×10^{-3} vs. 2.5×10^{-2}, p=0.01) were significantly higher in the columnar epithelium with the tubular/villous mucosal pattern than in the others (Figure 5). In the microdissected glands, *MUC2* levels (3.13×10^{-3} vs. 0.00×10^{-3}, p=0.006) were significantly higher in the patients with the tubular/villous pattern than in those with the other patterns (Figure 6).

Discussion

Endo et al.[29] classified the fine mucosal patterns (pit patterns) of Barrett's mucosa into five categories (small round, straight, long oval, tubular, and villous) by magnifying observation not using NBI. The tubular and villous pit patterns were characteristics of the presence of goblet cells possessing an intestinal mucin phenotype, which existed in all 10 biopsy specimens taken from the mucosa with tubular/villous pattern. The long oval pit pattern had an intermediate phenotype and goblet cells were observer in 40% (8/20) of the mucosa, whereas the other patterns possessed no goblet cells [29]. In the later ME-NBI studies, the sensitivity, specificity for diagnosis of SIM were 92% and 77% respectively for tubular/villous pattern and 93.5%, 86.7%, respectively for the ridge/villous pattern corresponding to tubular/villous patterns [31, 32] . ME-NBI is effective to detect SIM and the tubular/villous patterns seem to be characteristics of the presence of goblet cells. Therefore, brushing and biopsy samples in our subjects were taken from the targeted columnar epithelium using ME-NBI and tubular/villous pattern lesions were preferentially targeted. In our subjects, long oval and/or tubular/villous pattern were visualized in 98% of subjects, and MUC2 expression was detected in the 95% of brushing samples supporting the previous results of ME or ME-NBI studies [29, 31, 32].

In contrast, the similar study by Goda et al. [30] indicated that the frequency of SIM in the fine mucosal pattern 3 and pattern 4, which appeared to correspond to the tubular/villous

pattern, were 43% and 61%, respectively that were markedly less compared to the other results. Moreover, the recent prospective validation study indicated that the classification systems using high-quality ME-NBI videos of BE have limitations in terms of accuracy for the detection of SIM, identification of dysplastic BE, and concerning inter-observer agreement, irrespective of the endoscopist's expertise [33]. Biopsies were still recommended for a correct evaluation of Barrett's mucosa, although taking biopsy samples is more invasive methods compared to observation using ME-NBI.

In the present study, we found that brushing cytology detected intestinal phenotype more frequently with less bleeding compared to biopsy. *MUC2* expression levels were significantly higher and *MUC6* expression levels were significantly lower in the brushing samples than those in the biopsy samples. The possible problems of cytology and histology include sampling bias because the BE segment is heterogeneous and composed of different clones with variable malignant potential [9, 10], whereas endoscopic brushing in the targeted columnar epithelium using ME-NBI seems to be able to reduce sampling bias and increase the detection rate for SIM and dysplasia in BE. The reason for higher detection rate for intestinal phenotype in the brushing samples compared to the biopsy samples may be because brushing cytology can sample a greater area than biopsy. Moreover, expression of intestinal phenotypes was relatively limited in the surface of epithelium, while MUC6-positive cells tend to exist in

deep layer o. also reported the utility of brushing cytology indicating that a combination of brushing cytology and detection of loss of heterozygosity (LOH) improved diagnostic accuracy of esof mucosa. Therefore, brushing cytology which collected a wide range of superficial mucosal cells can detect higher rate for intestinal phenotype, while the biopsy samples containing deeper mucosal cells such as MUC6-positive cells and stromal cells. Endoscopic brushing in the tubular/villous mucosal pattern of columnar epithelium visualized by ME-NBI seems to be useful to detect intestinal phenotype, and is less invasive and better than the targeted biopsy using ME-NBI. Lin X et alphageal lesions such as BE, dysplasia, and adenocarcinoma [24]. They investigated LOH, which is the loss of normal function of one allele of a gene in which the other allele was already inactivated, of 17 microsatellite repeat markers near tumor suppressor genes in brushing samples and biopsy samples taken from gastroesophageal lesions. All the LOHs detected by biopsy were also detected by brushing and in addition, significantly more LOHs were detected by brushing than in biopsy. They demonstrated that brushing can samples a greater area than biopsy, and some areas may be missed by biopsy.

In summary, ME-NBI is effective to detect SIM in Barrett's mucosa compared to WLE and brushing cytology is more useful and less invasive to detect intestinal phenotype compared to biopsy. Therefore, combination of ME-NBI and targeted brushing in Barrett's mucosa is

practical method to detect intestinal phenotype or SIM which is known to be the hallmark lesion of BE and may predispose to dysplasia and adenocarcinoma.

In conclusion, endoscopic brushing in columnar epithelium with the tubular/villous mucosal pattern visualized by ME-NBI is more useful and less invasive to detect intestinal phenotype compared to targeted biopsy. The further prospective studies including more patients with long segment BE and patients with dysplasia or Barrett's adenocarcinoma are required to determine more specific mucosal pattern and additional capillary patterns observed by ME-NBI as high risk for Barrett's adenocarcinoma.

References

[1] Reid BJ. Barrett’s esophagus and esophageal adenocarcinoma. Gastroenterol Clin North Am. 1991;20:817–34.

[2] Thompson JJ, Zinsser KR, Enterline HT. Barrett’s metaplasia and adenocarcinoma of the esophagus and gastroesophageal junction. Hum Pathol. 1983;14:42–61.

[3] di Pietro M, Fitzgerald RC. Barrett’s oesophagus: an ideal model to study cancer genetics. Hum Genet. 2009;126:233–46.

[4] Wang KK, Sampliner RE. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus. Am J Gastroenterol. 2008;103:788-97.

[5] Sharma P. Recent advances in Barrett’s esophagus: short-segment Bar- rett’s esophagus and cardia intestinal metaplasia. Semin Gastrointest Dis. 1999;10:93–102.

[6] DeMeester SR, DeMeester TR. Columnar mucosa and intestinal metaplasia of the esophagus: fifty years of controversy. Ann Surg. 2000;231:303–21.

[7] Wang KK, Sampliner RE. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett’s esophagus. Am J Gastroenterol. 2008;103:788-97.

[8] Abrams JA, Kapel RC, Lindberg GM, Saboorian MH, Genta RM, Neugut AI, et al. Adherence to biopsy guidelines for Barrett’s esophagus surveillance in the community setting in the United States. Clin Gastroenterol Hepatol. 2009;7:736-42; quiz 710.

[9] Macdonald PM, Struhl G. A molecular gradient in early Drosophila embryos and its role in specifying the body pattern. Nature. 1986;324:537–45.

[10] Moreno E, Morata G. Caudal is the Hox gene that specifies the most posterior Drosophile segment. Nature. 1999;400:873–7.

[11] Phillips RW, Frierson Jr HF, Moskaluk CA. Cdx2 as a marker of epithelial intestinal differentiation in the esophagus. Am J Surg Pathol. 2003;27:1442–7.

[12] Silberg DG, Sullivan J, Kang E, Swain GP, Moffett J, Sund NJ, et al. Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. Gastroenterology. 2002;122:689–96.

[13] Wallace MB, Perelman LT, Backman V, Crawford JM, Fitzmaurice M, Seiler M, et al. Endoscopic detection of dysplasia in patients with Barrett's esophagus using light-scattering spectroscopy. Gastroenterology. 2000;119:677–82.

[14] Panjehpour M, Overholt BF, Vo-Dinh T, Haggitt RC, Edwards DH, Buckley FP 3rd. Endoscopic fluorescence detection of high-grade dysplasia in Barrett's esophagus. Gastroenterology. 1996;111:93–101.

[15] Mayinger B, Horner P, Jordan M, Gerlach C, Horbach T, Hohenberger W, et al. Light-induced autofluorescence spectroscopy for the endoscopic detection of esophageal cancer. Gastrointest Endosc. 2001;54:195–201.

[16] Poneros JM, Brand S, Bouma BE, Tearney GJ, Compton CC, Nishioka NS. Diagnosis of specialized intestinal metaplasia by optical coherence tomography. Gastroenterology. 2001;120:7–12.

[17] Kadowaki S, Tanaka K, Toyoda H, Kosaka R, Imoto I, Hamada Y, et al. Ease of early gastric cancer demarcation recognition: a comparison of four magnifying endoscopy methods. J Gastroenterol Hepatol. 2009;24:1625–1630.

[18] Nakayoshi T, Tajiri H, Matsuda K, Kaise M, Ikegami M, Sasaki H. Magnifying endoscopy combined with narrow band imaging system for early gastric cancer: correlation of vascular pattern with histopathology (including video). Endoscopy. 2004;36:1080–4.

[19] Uedo N, Ishihara R, Iishi H, Yamamoto S, Yamamoto S, Yamada T, et al. A new method of diagnosing gastric intestinal metaplasia: narrow-band imaging with magnifying endoscopy. Endoscopy. 2006;38:819–24.

[20] Geisinger KR, Teot LA, Richter JE. A comparative cytopathologic and histologic study of atypia, dysplasia and adenocarcinoma in Barrett's esophagus. Cancer. 1992;69:8–16.

[21] Fusaroli P, Fedeli P, Manta R, et al. Histology vs. brush cytology (BC) in the diagnosis and follow up of Barrett's esophagus (BE). Gastrointestinal Endosc. 2005;61:AB131.

[22] Hardwick RH, Morgan RJ, Barren BF, Lott M, Alderson D. Brush cytology in the diagnosis of neoplasia in Barrett's esophagus. Dis Esophagus. 1997;10:233–7.

[23] Geboes K, Van Eyken P. The diagnosis of dysplasia and malignancy in Barrett's oesophagus. Histopathology. 2000;37:99-107.

[24] Lin X, Finkelstein SD, Zhu B, Ujevich BJ, Silverman JF. Loss of heterozygosities in Barrett esophagus, dysplasia, and adenocarcinoma detected by esophageal brushing cytology and gastroesophageal biopsy. Cancer. 2009;117:57-66.

[25] Krishnadath KK. Novel findings in the pathogenesis of esophageal columnar metaplasia or Barrett's esophagus. Curr Opin Gastroenterol. 2007;23:440–5.

[26] Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature. 1980;287:795–801.

[27] van den Brink GR, Hardwick JC, Tytgat GN, Brink MA, Ten Kate FJ, Van Deventer SJ, et al. Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. Gastroenterology. 2001;121:317–28.

[28] van den Brink GR, Hardwick JC, Nielsen C, Xu C, ten Kate FJ, Glickman J, et al. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. Gut. 2002;51:628–33.

[29] Endo T, Awakawa T, Takahashi H, Arimura Y, Itoh F, Yamashita K, et al. Classification of Barrett's epithelium by magnifying endoscopy. Gastrointest Endosc. 2002;55:641–7.

[30] Goda K, Tajiri H, Ikegami M, Urashima M, Nakayoshi T, Kaise M. Usefulness of magnifying endoscopy with narrow band imaging for the detection of specialized intestinal metaplasia in columnar-lined esophagus and Barrett's adenocarcinoma. Gastrointest Endosc. 2007;65:36-46.

[31] Norimura D, Isomoto H, Nakayama T, Hayashi T, Suematsu T, Nakashima Y, et al. Magnifying endoscopic observation with narrow band imaging for specialized intestinal metaplasia in barrett's esophagus with special reference to light blue crests. Dig Endosc. 2010;22:101-6.

[32] Sharma P, Bansal A, Mathur S, Wani S, Cherian R, McGregor D, et al. The utility of a novel narrow band imaging endoscopy system in patients with Barrett's esophagus. Gastrointest Endosc. 2006;64:167-75.

[33] Silva FB, Dinis-Ribeiro M, Vieth M, Rabenstein T, Goda K, Kiesslich R, et al. Endoscopic assessment and grading of Barrett's esophagus using magnification endoscopy and narrow-band imaging: accuracy and interobserver agreement of different classification systems (with videos). Gastrointest Endosc. 2011;73:7-14.

Figure legends

Figure 1. Classification of NBI observation.

Figure 2. Histological findings (HE staining) of the biopsy samples obtained from the long oval and tubular/villous pattern lesions.

Figure 3. MUC5AC fluorescent immunostaining in the brushing sample.

Figure 4. CDX2, MUC2, MUC5AC fluorescent immunostaining in the cryostat sections.

Figure 5. Comparison of *CDX2* and *MUC2* expression levels in the brushing samples between those with the tubular/villous pattern and the others. Horizontal bar= median; Box=25th-75th interquartile range; Vertical lines= range of values. *p* Values were calculated using the non-parametric Mann-Whitney U test. Data are expressed relative to the control gene *ß-actin*.

Figure 6. Comparison of *MUC2* expression levels in the microdissected glands between those with the tubular/villous pattern and the others. Horizontal bar= median; Box=25th-75th interquartile range; Vertical lines= range of values. *p* Values were calculated using the non-parametric Mann-Whitney U test. Data are expressed relative to the control gene *ß-actin*.

Table 1. Clinical characteristics of the patients

	Total (n=50)
Age mean (SD)	67.3 (9.5)
Gender men/women	32/18
Current smokers	10 (20%)
Regular alcohol intake	23 (46%)
Anti-acid drug use	23 (46%)
H. pylori-positive*	13 (26%)
H. pylori-negative	37(74%)
Eradication	28 (56%)
Hiatus hernia	30 (60%)

H. pylori-positive patients including 4 patients with suspected past infection and *H. pylori*-negative patients including 28 patients with history of *H. pylori* eradication

Table 2. Comparison of mRNA expression frequency between biopsy and brushing

	Biopsy	Brushing	p values
SHH	100%	100%	1.00
MUC5AC	100%	100%	1.00
MUC6	97.5%	70%	0.001
CDX2	75%	95%	0.01
MUC2	65%	85%	0.04

p values by χ^2 square test

Table 3. Comparisons of mRNA levels between biopsy and brushing

	Biopsy	Brushing	*p*
	median (25%-75%)	median (25%-75%)	
SHH	16.0(5.6-26.5)	15.5(3.6-34.0)	0.95
MUC5AC	878(478-1932)	1023(388-2594)	0.36
MUC6	165(107-270)	0.37(0.06-1.29)	<0.001
CDX2	0.41(0.09-0.88)	0.77(0.30-2.77)	0.06
MUC2	0.29(0.03-7.62)	7.77(0.50-30.4)	0.03

p values by Wilcoxon signed-rank test