Reduced expression of αGlcNAc in Barrett's esophagus adjacent to Barrett's adenocarcinoma - Possible biomarker to predict the malignant potential of Barrett's esophagus –

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ABSTRACT

Aims: Gastric gland mucin contains *O*-glycans exhibiting terminal α1,4-linked *N*-acetylglucosamine residues (αGlcNAc). Recently we demonstrated that mice deficient in αGlcNAc in gastric gland mucin spontaneously develop gastric adenocarcinoma, indicating that αGlcNAc is a tumor suppressor for gastric cancer (Karasawa et al. J. Clin. Invest. 2012; 122; 923-934). However, the role of αGlcNAc in Barrett's esophagus (BE) remains unknown. Here, we asked whether reduced αGlcNAc expression in BE is associated with development of Barrett's adenocarcinoma (BAC).

Methods and results: Thirty-five BE lesions adjacent to BAC were examined by immunohistochemistry for αGlcNAc, MUC6, and CDX2. As controls, 35 BE lesions without BAC obtained from patients with esophageal squamous cell carcinoma were also analyzed. Expression of αGlcNAc relative to its scaffold MUC6 in BE adjacent to BAC was significantly reduced compared to control BE. Decreased αGlcNAc expression in BE adjacent to BAC was particularly significant in patients with smaller tumor size (< 20 mm) and minimal invasion of tumor cells to the superficial muscularis mucosae. There was also a significant inverse correlation between αGlcNAc and CDX2 expression in BE adjacent to

BAC.

Conclusions: Decreased expression of αGlcNAc compared with MUC6 in BE is a possible hallmark to predict BAC development.

Introduction

The incidence of esophageal adenocarcinoma has increased in the past several decades in the United States and in other western countries.[1] The same increased trend is also expected in Japan, since the rate of acquisition of *Helicobacter pylori* (*H. pylori*) infection has been decreased.[2] Barrett's esophagus (BE) is the most important risk factor for Barrett's adenocarcinoma (BAC).[3] Originally, the presence of specialized columnar epithelium (SCE) showing goblet cell metaplasia was required for a BE diagnosis.[4] However, in Japan and the United Kingdom the existence of goblet cell metaplasia is not required for a diagnosis of BE.[5] Dysplasia is a promising risk factor for BAC development.[6] It is well known that SCE in BE is a risk factor for BAC development.[7] However, BE without SCE also has a potential to BAC development.[8-10] In addition, BE length is known to be associated with risk for BAC development,[11] but no correlation between BE length and risk for BAC development is also reported.[12,13] Thus, hallmarks for BE without dysplasia predictive of BAC are needed.

O-glycans containing terminal α1,4-linked *N*-acetylglucosamine residues

(αGlcNAc) are unique to the gland mucin secreted from gastric and duodenal mucosae. We previously reported that the expression of αGlcNAc relative to that of its scaffold MUC6 was significantly reduced in differentiated type gastric adenocarcinomas,[14] suggesting that αGlcNAc suppresses development of that condition. Recently, we demonstrated that *A4gnt*-deficent mice, which lack αGlcNAc in the gland mucin, develop gastric adenocarcinoma, indicating a tumor suppressor role for αGlcNAc in gastric cancer.[15] We also found that αGlcNAc was also detectable in BE (personal communications: YI and JN). Altered glycosylation in BE such as increased expression of βGlcNAc and αFuc was demonstrated by lectin histochemistry,[16,17] and more recently, a molecular imaging technique with conventional endoscopy was developed by using WGA lectin, which binds to sialic acid specifically appeared in high-grade dysplasia in BE.[18] However, the expression and role of αGlcNAc in BE remains unknown.

Here, we asked whether reduced αGlcNAc expression is associated with BAC development by analyzing expression profiles of αGlcNAc and MUC6 in BE tissues adjacent to early stage BAC compared with those in control BE without BAC using immunohistochemistry.

Materials and methods

PATIENTS

We examined a consecutive series from March 1996 to August 2011 of 35 specimens from 34 patients with early stage BAC, who exhibited T1 tumors based on the TNM Classification of Malignant Tumours.[19] Twenty-one specimens were endoscopically-resected and 14 were surgically-resected, and all were acquired at Shinshu University Hospital, Nagano Municipal Hospital, Nagano Prefectural Kiso Hospital, Matsumoto City General Hospital, Suwa Red Cross Hospital or Iiyama Red Cross Hospital. All of these hospitals are located in Nagano prefecture, in Japan. Among patients examined in the BAC group, 27 were male and 7 were female. Ages ranged from 50 to 84 years (mean: 67.7 years). All BAC tissues were tubular adenocarcinoma, according to the World Health Organization classification.[20] To compare BE with and without BAC, we examined surgically-resected specimens of esophageal squamous cell carcinoma accompanying BE taken from 35 patients (34 male and 1 female) defined as the control group. Their ages ranged from 52 to 83 years (mean: 65.9 years). The study plan was approved by the Ethical Committee of Shinshu

University School of Medicine, Matsumoto, Japan (No. 1461; 2 March, 2010). That committee also granted a waiver of informed consent to use formalin-fixed and paraffin-embedded tissue specimens retrieved from the pathology files of all hospitals enrolled, since diagnostic use of the samples was completed before the study and there was no risk to the involved patients. Samples were also coded to protect patient anonymity.

ASSESSMENT OF *H. PYLORI* INFECTION AND BE LENGTH

The status of *H. pylori* infection was determined by histology, culture, rapid urease test, a ^{13}C urea breath test, and/or serology. The presence of *H. pylori* infection was judged if one or more indicators were positive. To determine BE length, we used endoscopic findings of the gastric folds as a landmark of the distal esophagus, sometimes using findings of the distal limit of lower esophageal longitudinal or palisade vessels as a reference. Based on the Prague C & M Criteria,[21] we classified BE into long-segment and short-segment BE, depending on whether the circumferential extent of BE was longer than 3 cm.

HISTOPATHOLOGY

Histopathological diagnosis of BE was undertaken when the so-called columnar-lined esophagus (CLE) was identified as the esophagus origin; in other words, the presence of esophageal glands proper, squamous islands, and double muscularis mucosae supported identification of esophagus origin.[22-25] Confirmation of goblet cell metaplasia was not required for BE diagnosis, based on criteria of histological diagnosis recommended in Japan and the United Kingdom.[5] Adenocarcinoma of esophageal origin adjacent to BE was defined as BAC.

IMMUNOHISTOCHEMISTRY

The primary antibodies used in this study were: MUC6 (CLH5, dilution 1:200; Novocastra, Newcastle upon Tyne, UK), αGlcNAc (HIK1083, dilution 1:20; Kanto Chemical, Tokyo, Japan), MUC2 (Ccp58, dilution 1:200; Novocastra), MUC5AC (CLH2, dilution 1:100; Novocastra), CD10 (56C6, dilution 1:100; Novocastra) and CDX2 (CDX2-88, dilution 1:50; BioGenex, San Ramon, CA, USA). Immunohistochemisry was performed using the labeled streptavidin-biotin method on formalin-fixed, paraffin-embedded tissue sections. After

deparaffinization and rehydration, antigen retrieval was carried out by boiling tissue sections in 10 mM citrate buffer (pH 6.0) for 30 min in a microwave except for tissues to be stained for αGlcNAc. After blocking endogenous peroxidase activity with a 0.3% H_2O_2 methanol solution, sections were incubated with 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) for 15 min. After incubation with a primary antibody diluted with BSA/PBS for 60 min, sections were incubated with biotinylated secondary antibody (Histofine, Nichirei, Tokyo, Japan) for 60 min. After washing with PBS, slides were incubated with horseradish peroxidase-conjugated streptavidin for 60 min. The color reaction was developed with 3,3'-diaminobenzidine containing 0.02% H_2O_2. Sections were counterstained with hematoxylin. Negative controls were done omitting the primary antibodies, and these samples showed no specific staining.

EVALUATION

To assess potential reduction of αGlcNAc in BE, we determined the ratio of the area stained by αGlcNAc to the area stained by its scaffold MUC6 in tissue sections. Based on the stained area, we classified the relative expression of

αGlcNAc to MUC6 as four grades ranging from 3 to 0 (3 (no to mild reduction of αGlcNAc): αGlcNAc/MUC6 > 67%, 2 (moderate reduction of αGlcNAc): αGlcNAc/MUC6 = 34-66%, 1 (severe reduction of αGlcNAc): αGlcNAc/MUC6 = 6-33%, and 0 (no expression of αGlcNAc): αGlcNAc/MUC6 = 0-5%) (Figure S1). According to the classification of mucin expression phenotype in gastric cancer,[23] we classified BAC mucin expression phenotypes into four categories: namely, gastric (positive only for MUC5AC and/or MUC6), mixed (which was further classified into predominantly gastric or predominantly intestinal), and intestinal (positive only for MUC2 and/or CD10). For comparison of CDX2 with αGlcNAc expressed in BE with BAC, we measured percentages of CDX2-positive BE cells in total BE cells as well as αGlcNAc-positive BE cells in total BE cells, and then the percentages were ranked into four grades ranging from 0 to 3 (0: 0-5%, 1: 6-33%, 2: 34-66%, and 3: >67%). Immunostained sections were observed under a BX-51 microscope (Olympus, Tokyo, Japan).

STATISTICAL ANALYSIS

All data are expressed as the mean ± SEM. Differences between groups were statistically analyzed by the Mann-Whitney *U*-test, χ^2 test (with Yates' correction),

Fisher’s exact test and Spearman's correlation, all using StatFlex software (Artech Co., Osaka, Japan). *P* values less than 0.05 were considered significant.

Results

CLINICOPATHOLOGICAL FEATURES OF PATIENTS

Clinicopathological characteristics of BAC group patients were compared with those of the control group without BAC. Significant differences between 2 groups were found in gender and *H. pylori* infection (Table 1). However, there were no significant differences in variables such as age and BE length.

For the BAC group, tumor size ranged from 3 to 48 mm (mean 19.0 ± 9.9 mm). In 20 patients, tumor cells invaded up to the lamina propria mucosae or muscularis mucosae, while in 15 patients, tumor cells invaded up to the submucosa. Based on mucin expression, BAC was subclassified into four subtypes: 9 specimens of gastric type, 11 of mixed predominantly gastric type, 8 of mixed predominantly intestinal type, and 7 of intestinal type. In 15 specimens, no goblet cells were seen in BE adjacent to BAC. Dysplasia was not identified in all BE specimens of both BAC and control groups.

BE HISTOPTHOLOGY

Figure 1 shows BE histopathology representative of the control group. The presence of squamous islands located at the proximal side is a BE hallmark (Figure 1A,B). In controls, most mucous glands secreting αGlcNAc also secreted MUC6 (Figure 1C,D), strongly suggesting that αGlcNAc is attached to MUC6, as seen in normal gastric mucosa.[27] By contrast, mucous glands secreting MUC5AC alone were apparently distinct from those secreting both MUC6 and αGlcNAc (Figure 1C,E). Notably, goblet cells secreting MUC2 were rarely observed in BE exhibiting both MUC6 and αGlcNAc in mucous glands (Figure 1F).

Figure 2 shows representative BE with BAC specimens. BE adjacent to BAC often showed goblet cell metaplasia indicative of a specialized columnar epithelium (Figure 2A,B). Interestingly, mucous glands secreting MUC6 were found in the lower layer of BE, but αGlcNAc was not detected in those glands (Figure 2C,D). Furthermore, some mucous glands located in the lower layer of BE secreted both MUC5AC and MUC6, MUC2 and MUC6, or MUC5AC, MUC6

and MUC2, expression patterns rarely seen in the control group (Figure 2C,E,F). Such co-expression patterns were more frequently observed in BE with extensive goblet cell metaplasia.

αGLCNAC AND MUC6 EXPRESSION IN BACKGROUND BE ADJACENT TO BAC

To assess the reduction of αGlcNAc expression in BE adjacent to BAC, we compared the immunostained area of αGlcNAc to that of MUC6 in BE between BAC and control groups. The mean relative expression of αGlcNAc to MUC6 in BE of BAC versus control groups were 1.94 ± 0.16 and 2.69 ± 0.11, respectively (P = 0.0025), indicating significantly reduced αGlcNAc levels in BE of BAC group relative to the control group (Figure 3). These results strongly suggest that reduction of αGlcNAc expression is correlated with Barrett's carcinogenesis.

We next divided the BAC group into 2 subgroups based on the clinicopathological variables of BAC or BE, and then compared the relative expression of αGlcNAc to MUC6 with that of the control group. Relevant to BAC tumor size, the relative expression of αGlcNAc to MUC6 in BE adjacent to BAC

was significantly reduced compared to the control group, irrespective of tumor size; however, reduction of αGlcNAc was more statistically significant in the case of smaller (< 20 mm) versus larger (> 20 mm) tumors (*P* = 0.0083 vs *P* = 0.0251) (Figure 4A). In terms of depth of BAC invasion, the relative expression of αGlcNAc to MUC6 in BE adjacent to minimally invasive BAC (that is, up to the lamina propria mucosae or muscularis mucosae) was significantly reduced compared to control BE (*P* = 0.0013). However, such a significant reduction of αGlcNAc was not observed once tumor cells invaded the submucosa (*P* = 0.14) (Figure 4B). For mucin expression in BAC, the relative expression of αGlcNAc to MUC6 in BE adjacent to intestinal type BAC (containing intestinal type and mixed predominantly intestinal type) was significantly reduced compared with that seen in control BE (*P* = 0.0004) (Figure 4C). However, that reduction was not significant when gastric type BAC (containing gastric type and mixed predominantly gastric type) was compared with control BE (*P* = 0.135). Finally, relevant to goblet cell metaplasia, the relative expression of αGlcNAc to MUC6 in BE with goblet cell metaplasia was significantly reduced compared with control BE (*P* = 0.0007). However, that significant reduction was not seen in comparisons of BE without goblet cell metaplasia to control BE (*P* = 0.099)

(Figure 4D). Overall, these results indicate that αGlcNAc expression in BE is more significantly decreased when BAC adjacent to BE 1) is smaller in size (< 20 mm), 2) invades the superficial muscularis mucosae, 3) shows an intestinal phenotype, or 4) exhibits goblet cell metaplasia.

ASSOCIATION OF αGLCNAC WITH CDX2 IN BE

To examine the relationship between BE intestinalization and αGlcNAc, we performed CDX2 immunostaining of BE adjacent to BAC in the BAC group. CDX2 was not expressed in BE showing high αGlcNAc expression. However, CDX2 was often expressed in BE showing low αGlcNAc expression (Figure 5A). We then scored CDX2 expression in BE areas adjacent to BAC based on four grades, and compared those expression levels with αGlcNAc expression grades. We observed a significant inverse correlation between αGlcNAc and CDX2 expression in BE (r = -0.615, P < 0.001; Figure 5B).

Discussion

Here, we have clearly demonstrated that αGlcNAc expression is more significantly reduced in BE adjacent to early stage BAC than in control BE

without BAC. Because αGlcNAc functions as a tumor suppressor for gastric cancer,[15] the present study suggests that αGlcNAc reduction in BE might be associated with Barrett's carcinogenesis. Functional study will be of great significance to determine whether αGlcNAc also functions as a tumor suppressor for BAC in future. Previously it was demonstrated that reduced MUC6 expression is associated with Barrett's adenocarcinoma progression.[28,29] In the present study, we showed that αGlcNAc was more drastically decreased in BE associated with BAC compared to MUC6. Thus, our study indicates that decreased expression of αGlcNAc compared with MUC6 in BE is a possible hallmark to predict BAC development.

It is well known that *H. pylori* infection is closely associated with pathogenesis of gastric cancer.[30-33] However, it remains a matter of discussion whether *H. pylori* infection is associated with Barrett's carcinogenesis.[34-37] Nonetheless, inflammation brought about by gastric acid or bile acid reflux likely has a major influence on Barrett's carcinogenesis, and a metaplasia-dysplasia-carcinoma sequence most likely has an effect on BAC pathogenesis.[38,39] Recently, we demonstrated that αGlcNAc plays a dual role in gastric cancer development by

not only blocking *H. pylori* infection but also by suppressing tumor-promoting inflammation in the gastric mucosa.[15,40] In particular, we revealed that *A4gnt*-deficient mice show no αGlcNAc expression in gastric mucosa and spontaneously develop gastric differentiated-type adenocarcinoma though a metaplasia-dysplasia-carcinoma sequence. We also showed that genes encoding inflammatory chemokine ligands, proinflammatory cytokines, and growth factors, such as *Grem1*, *Cxcl1*, *Ccl2*, *Cxcl5*, *Il11*, *Hgf*, *Il1b*, and *Fgf7* are upregulated in the gastric mucosa of *A4gnt*-deficient mice.[15] Notably, expression levels of CC chemokine CCL2 is reportedly increased in reflux esophagitis.[41] In addition, HGF, which functions in gastric epithelial proliferation,[42] is significantly upregulated in BE, suggesting a possible role in Barrett's carcinogenesis.[43] Overall, these results suggest that αGlcNAc is protective against tumor-promoting inflammation in BE, thereby decreasing the risk of BAC development.

The present study also indicates that reduction of αGlcNAc in BE was much more evident when the mucin expression pattern of BAC was of the intestinal phenotype or if BE showed goblet cell metaplasia. We also observed an inverse

correlation between αGlcNAc and CDX2 in BE of the BAC group. Because CDX2 functions in the development of goblet cell metaplasia,[44] our findings suggest that reduced αGlcNAc expression might be associated with intestinalization of both BAC and BE. In the stomach, *H. pylori* infection is reportedly associated with intestinal metaplasia and intestinalization of gastric adenocarcinoma.[45] However in the esophagus, factors promoting intestinalization of BE and BAC remain unclear. Recently, it was reported that reflux of both gastric acid and bile acid promotes BE intestinalization.[46-48] More recently, Byrne et al. demonstrated that deoxycholic acid disrupts Golgi structure of cultured esophageal epithelial cells, thus affecting protein secretion and glycosylation processes.[49] Further study is needed to clarify whether αGlcNAc prevents BE intestinalization by protecting BE from bile acid reflux.

CDX2 promotes BE intestinalization by activating NF-κB,[50,51] which is induced by IL-1β.[52] Notably, IL-1β is upregulated in *A4gnt*-deficient mice, suggesting that αGlcNAc blocks BE intestinalization mediated by the IL-1β-NF-κB-CDX2 axis. Although it remains controversial whether goblet cell metaplasia is a risk factor for BAC development, Khor et al. recently reported that BAC carcinogenesis can

occur via gastric or intestinal pathways.[53] Our data indicate that increased CDX2 expression in BAC exhibiting a gastric phenotype is relatively uncommon and that in those cases αGlcNAc expression is not decreased in BE adjacent to BAC. By contrast, CDX2 and αGlcNAc showed an inverse expression profile in BAC of intestinal phenotype. These results suggest that αGlcNAc may function in carcinogenesis in BAC with intestinal rather than gastric phenotype.

In conclusion, our present study reveals that αGlcNAc expression is more significantly reduced in BE adjacent to BAC than in control BE without BAC. This pattern was more apparent at early stages of BAC. Our results suggest that αGlcNAc antagonizes Barrett's carcinogenesis, possibly by preventing tumor-promoting inflammation in BE. In order to establish the reduction of αGlcNAc as a defined hallmark for BAC development, a prospective study will be needed to test whether loss of αGlcNAc expression compared with MUC6 in biopsied tissue specimens from BE without dysplasia could be associated with progression of BAC.

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

1. Pohl H and Welch HG. The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *J. Natl. Cancer Inst.* 2005; **97;** 142-146.

2. Kusano C, Gotoda T, Khor CJ *et al*. Changing trends in the proportion of adenocarcinoma of the esophagogastric junction in a large tertiary referral center in Japan. *J. Gastroenterol. Hepatol.* 2008; **23;** 1662-1665.

3. Hameeteman W, Tytgat GN, Houthoff HJ, van den Tweel JG. Barrett's esophagus: development of dysplasia and adenocarcinoma. *Gastroenterology* 1989; **96;** 1249-1256.

4. Wang KK, Sampliner RE. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus. *Am. J. Gastroenterol.* 2008; **103;** 788-797.

5. Playford RJ. New British Society of Gastroenterology (BSG) guidelines for the diagnosis and management of Barrett's oesophagus. *Gut* 2006; **55;** 442.

6. Reid BJ, Blount PL, Rubin CE *et al.* Flow-cytometric and histological progression to malignancy in Barrett's esophagus: prospective

endoscopic surveillance of a cohort. *Gastroenterology* 1992; **102;** 1212-1019.

7. Ruol A, Parenti A, Zaninotto G *et al*. Intestinal metaplasia is the probable common precursor of adenocarcinoma in barrett esophagus and adenocarcinoma of the gastric cardia. *Cancer* 2000; **88;** 2520-2528.

8. Takubo K, Aida J, Naomoto Y *et al*. Cardiac rather than intestinal-type background in endoscopic resection specimens of minute Barrett adenocarcinoma. *Hum. Pathol.* 2009; **40;** 65-74.

9. Liu W, Hahn H, Odze RD, Goyal RK. Metaplastic esophageal columnar epithelium without goblet cells shows DNA content abnormalities similar to goblet cell-containing epithelium. *Am. J. Gastroenterol.* 2009; **104;** 816-824.

10. Riddell RH, Odze RD. Definition of Barrett's esophagus: time for a rethink - Is intestinal metaplasia dead? *Am. J. Gastroenterol.* 2009; **104;** 2588-2594.

11. Iftikhar SY, James PD, Steele RJ, Hardcastle JD, Atkinson M. Length of Barrett's oesophagus: an important factor in the development of dysplasia and adenocarcinoma. *Gut* 1992; **33;** 1155-1158.

12. Rudolph RE, Vaughan TL, Storer BE *et al*. Effect of segment length on risk for neoplastic progression in patients with Barrett esophagus. *Ann. Intern. Med.* 2000; **132;** 612-620.

13. Yousef F, Cardwell C, Cantwell MM *et al.* The incidence of esophageal cancer and high-grade dysplasia in Barrett's esophagus: a systematic review and meta-analysis. *Am. J. Epidemiol.* 2008; **168;** 237-249.

14. Nakajima K, Ota H, Zhang MX *et al*. Expression of gastric gland mucous cell-type mucin in normal and neoplastic human tissues. *J. Histochem. Cytochem.* 2003; **51;** 1689-1698.

15. Karasawa F, Shiota A, Goso Y *et al*. Essential role of gastric gland mucin in preventing gastric cancer in mice. *J. Clin. Invest.* 2012; **122;** 923-934.

16. Shimamoto C, Weinstein WM, Boland CR. Glycoconjugate expression in normal, metaplastic, and neoplastic human upper gastrointestinal mucosa. *J Clin Invest.* 1987; **80;** 1670-1678.

17. Neumann H, Wex T, Mönkemüller K, Vieth M, Fry LC, Malfertheiner P. Lectin UEA-I-binding proteins are specifically increased in the squamous epithelium of patients with Barrett's esophagus. *Digestion*. 2008; **78:** 201-207.

18. Bird-Lieberman EL, Neves AA, Lao-Sirieix P *et al*. Molecular imaging using fluorescent lectins permits rapid endoscopic identification of dysplasia in Barrett's esophagus. *Nat Med.* 2012; **18;** 315-321.

19. Sobin LH, Gospodarowicz MK, Wittekind C. *TNM Classification of Malignant Tumours.* 7th ed. Wiley-Blackwell. New York, 2009

20. Werner M, Flejou JF, Hainaut P *et al*. Adenocarcinoma of the oesophagus. In: Hamilton SR, Aaltonen LA, editors. *Pathology and Genetics of Tumours of the Digestive System.* Lyons: International Agency for Research on Cancer Press; 2000. pp. 20-26.

21. Sharma P, Dent J, Armstrong D *et al*. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. *Gastroenterology* 2006; **131;** 1392-1399.

22. Takubo K, Sasajima K, Yamashita K, Tanaka Y, Fujita K. Double muscularis mucosae in Barrett's esophagus. *Hum. Pathol.* 1991; **22;** 1158-1161.

23. Takubo K, Vieth M, Aryal G *et al*. Islands of squamous epithelium and their surrounding mucosa in columnar-lined esophagus: a pathognomonic feature of Barrett's esophagus? *Hum. Pathol.* 2005; **36;** 269-274.

24. Coad RA, Woodman AC, Warner PJ *et al.* On the histogenesis of Barrett's oesophagus and its associated squamous islands: a three-dimensional study of their morphological relationship with native oesophageal gland ducts. *J. Pathol.* 2005; **206;** 388-394.

25. Takubo K, Nixon JM, Jass JR. Ducts of esophageal glands proper and paneth cells in Barrett's esophagus: frequency in biopsy specimens. *Pathology* 1995; **27;** 315-317.

26. Tajima Y, Shimoda T, Nakanishi Y *et al*. Gastric and intestinal phenotypic marker expression in gastric carcinomas and its prognostic significance: immunohistochemical analysis of 136 lesions. *Oncology* 2001; **61;** 212-220.

27. Zhang MX, Nakayama J, Hidaka E *et al*. Immunohistochemical demonstration of α1,4-N-acetylglucosaminyltransferase that forms GlcNAcα1,4Galβ residues in human gastrointestinal mucosa. *J. Histochem. Cytochem.* 2001; **49;** 587-596.

28. Glickman JN, Blount PL, Sanchez CA *et al*. Mucin core polypeptide expression in the progression of neoplasia in Barrett's esophagus. *Hum Pathol.* 2006; **37;** 1304-1315.

29. Burjonrappa SC, Reddimasu S, Nawaz Z *et al*. Mucin expression profile in Barrett's, dysplasia, adenocarcinoma sequence in the esophagus. *Indian J Cancer.* 2007; **44;** 1-5.

30. Sugiyama A, Maruta F, Ikeno T *et al*. Helicobacter pylori infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. *Cancer Res.* 1998; **58;** 2067-2069.

31. Parsonnet J, Friedman GD, Vandersteen DP *et al*. Helicobacter pylori infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 1991; **325;** 1127-1131.

32. Nomura A, Stemmermann GN, Chyou PH *et al.* Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. *N. Engl. J. Med.* 1991; **325;** 1132-1136.

33. Forman D, Newell DG, Fullerton F *et al*. Association between infection with Helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. *Brit. Med. J.* 1991; **302;** 1302-1305.

34. Weston AP, Badr AS, Topalovski M *et al.* Prospective evaluation of the prevalence of gastric Helicobacter pylori infection in patients with GERD, Barrett's esophagus, Barrett's dysplasia, and Barrett's adenocarcinoma.

Am. J. Gastroenterol. 2000; **95;** 387-394.

35. Rokkas T, Pistiolas D, Sechopoulos P, Robotis I, Margantinis G. Relationship between Helicobacter pylori infection and esophageal neoplasia: a meta-analysis. *Clin. Gastroenterol. Hepatol.* 2007; **5;** 1413-1417, 7 e1-2.

36. Watari J, Moriichi K, Tanabe H *et al*. Differences in genetic instability and cellular phenotype among Barrett's, cardiac, and gastric intestinal metaplasia in a Japanese population with Helicobacter pylori. *Histopathology* 2009; **55;** 261-269.

37. Liu FX, Wang WH, Wang J, Li J, Gao PP. Effect of Helicobacter pylori infection on Barrett's esophagus and esophageal adenocarcinoma formation in a rat model of chronic gastroesophageal reflux. *Helicobacter* 2011; **16;** 66-77.

38. Wild CP, Hardie LJ. Reflux, Barrett's oesophagus and adenocarcinoma: burning questions. *Nat. Rev. Cancer* 2003; **3;** 676-684.

39. Jankowski JA, Wright NA, Meltzer SJ *et al*. Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am. J. Pathol.* 1999; **154;** 965-973.

40. Kawakubo M, Ito Y, Okimura Y, *et al*. Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. *Science* 2004; **305;** 1003-1006.

41. Isomoto H, Wang A, Mizuta Y *et al*. Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. *Am. J. Gastroenterol.* 2003; **98;** 551-556.

42. Takahashi M, Ota S, Shimada T *et al*. Hepatocyte growth factor is the most potent endogenous stimulant of rabbit gastric epithelial cell proliferation and migration in primary culture. *J. Clin. Invest.* 1995; **95;** 1994-2003.

43. Konturek PC, Nikiforuk A, Kania J *et al.* Activation of NFκB represents the central event in the neoplastic progression associated with Barrett's esophagus: a possible link to the inflammation and overexpression of COX-2, PPARgamma and growth factors. *Dig. Dis. Sci.* 2004; **49;** 1075-1083.

44. Eda A, Osawa H, Satoh K *et al*. Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J. Gastroenterol.* 2003; **38;** 14-22.

45. Mizoshita T, Tsukamoto T, Takenaka Y *et al*. Gastric and intestinal phenotypes and histogenesis of advanced glandular stomach cancers in carcinogen-treated, Helicobacter pylori-infected Mongolian gerbils. *Cancer Sci.* 2006; **97;** 38-44.

46. Wong NA, Wilding J, Bartlett S *et al*. CDX1 is an important molecular mediator of Barrett's metaplasia. *Proc. Natl. Acad. Sci. USA.* 2005; **102;** 7565-7570.

47. Kazumori H, Ishihara S, Rumi MA, Kadowaki Y, Kinoshita Y. Bile acids directly augment caudal related homeobox gene Cdx2 expression in oesophageal keratinocytes in Barrett's epithelium. *Gut* 2006; **55;** 16-25.

48. Kazumori H, Ishihara S, Takahashi Y, Amano Y, Kinoshita Y. Roles of Kruppel-like factor 4 in oesophageal epithelial cells in Barrett's epithelium development. *Gut* 2011; **60;** 608-617.

49. Byrne AM, Sharma R, Duggan G, Kelleher D, Long A. Deoxycholic acid impairs glycosylation and fucosylation processes in esophageal epithelial cells. *Glycobiology* 2012; **22;** 638-648.

50. Aggarwal BB. Nuclear factor-κB: the enemy within. *Cancer Cell* 2004; **6;** 203-208.

51. Debruyne PR, Witek M, Gong L *et al*. Bile acids induce ectopic expression of intestinal guanylyl cyclase C through nuclear factor-κB and Cdx2 in human esophageal cells. *Gastroenterology* 2006; **130;**1191-1206.

52. Renard P, Zachary MD, Bougelet C *et al*. Effects of antioxidant enzyme modulations on interleukin-1-induced nuclear factor kappa B activation. *Biochem. Pharmacol.* 1997; **53;** 149-160.

53. Khor TS, Alfaro EE, Ooi EM *et al*. Divergent expression of MUC5AC, MUC6, MUC2, CD10, and CDX-2 in dysplasia and intramucosal adenocarcinomas with intestinal and foveolar morphology: is this evidence of distinct gastric and intestinal pathways to carcinogenesis in Barrett Esophagus? *Am. J. Surg. Pathol.* 2012; **36;** 331-342.

Figure Legends

Figure 1. Barrett's esophagus without Barrett's adenocarcinoma. (**A**) Seen is a squamous island (arrow) lying the distal side of the columnar epithelium and an esophageal gland proper (EGP) under the columnar epithelium, both characteristics of Barrett's esophagus. No dysplasia is found. (**B**) Higher magnification image of boxed area in (**A**). Expression of (**C**) MUC6, (**D**) αGlcNAc, (**E**) MUC5AC, and (**F**) MUC2. Note that αGlcNAc staining is comparable to that of MUC6. MUC5AC-stained areas differ from MUC6- and αGlcNAc-stained areas. No MUC2-positive cells are seen. Bar, 500 μm in (**A**), and 200 μm in (**B**) - (**F**).

Figure 2. Barrett's esophagus with Barrett's adenocarcinoma (BAC). (**A**) Barrett's esophagus with diffuse goblet cell metaplasia is adjacent to BAC. (**B**) Higher magnification image of boxed area in (**A**). Barrett's esophagus shows no dysplasia. Expression of (**C**) MUC6, (**D**) αGlcNAc, (**E**) MUC5AC, and (**F**) MUC2. MUC6 staining is observed in the lower layer of the mucosa, while αGlcNAc staining is absent. Some mucous cells in the lower layer are positive for MUC5AC and MUC6, or MUC2 and MUC6, or MUC5AC, MUC6, and MUC2. Bar,

500 μm in (**A**), and 200 μm in (**B**) - (**F**).

Figure 3. Relative expression of αGlcNAc to MUC6 in Barrett's esophagus (BE). In BE adjacent to Barrett's adenocarcinoma (BAC), the relative expression αGlcNAc to MUC6 is significantly reduced relative to control BE without BAC. Data are presented as the mean ± SEM. ***P* < 0.01.

Figure 4. Relative expression of αGlcNAc to MUC6 in Barrett's esophagus (BE) with respect to clinicopathological features. (**A**) Relative expression of αGlcNAc to MUC6 in BE adjacent to Barrett's adenocarcinoma (BAC) is significantly reduced compared with control BE without BAC regardless of tumor size. However, the reduction is more significant in smaller (< 20 mm) tumor cases. (**B**) Relative expression of αGlcNAc to MUC6 in BE adjacent to minimal invasive BAC, which invaded to the lamina propria mucosae or muscularis mucosae, is significantly reduced compared to control BE without BAC. The relative expression of αGlcNAc is not significantly reduced in BE adjacent to the submucosal invasion compared to control BE. (**C**) Relative expression of αGlcNAc to MUC6 in BE adjacent to intestinal type BAC is significantly reduced

compared with control BE without BAC. That grade is not reduced significantly in BE adjacent to gastric type BAC compared to control BE. (**D**) Relative expression of αGlcNAc to MUC6 in BE with goblet cell metaplasia is significantly reduced compared with control BE without BAC. The grade is not reduced significantly in BE without goblet cell metaplasia compared to control BE. Data are presented as the mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant. M = in the lamina propria mucosae or muscularis mucosae. SM = in the submucosa.

Figure 5. (**A**) Upper panel. A case of Barrett's esophagus with high αGlcNAc expression. Note that CDX2 is not expressed. Lower panel. A case of Barrett's esophagus showing lower αGlcNAc expression. Note that CDX2 is highly expressed in the MUC6-stained area. Bar, 100 μm. (**B**) A scatter diagram showing the expression levels of CDX2 and αGlcNAc. There is a significant negative correlation between CDX2 and αGlcNAc expression.

Figure Legend for Figure S1.

Figure S1. Representative staining patterns of MUC6 and αGlcNAc in Barrett's esophagus. Relative expression of αGlcNAc to MUC6 is determined depending on the ratio of αGlcNAc to MUC6 staining into four grades ranging from 3 to 0 (3: no to mild reduction of αGlcNAc, 2: moderate reduction of αGlcNAc, 1: severe reduction of αGlcNAc, and 0: no expression of αGlcNAc). BAC: Barrett's adenocarcinoma. Bar, 50 μm.

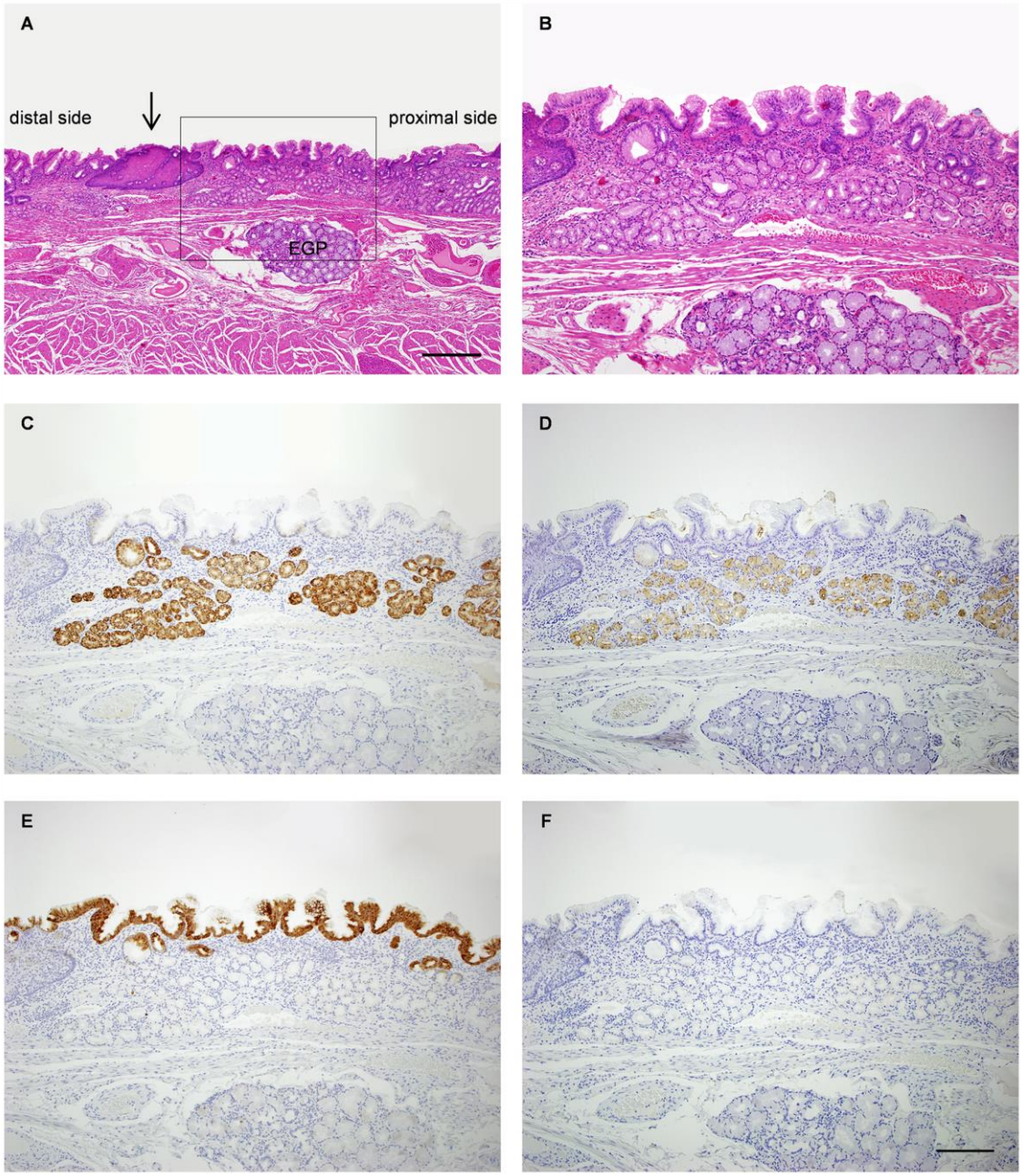

Fig.1

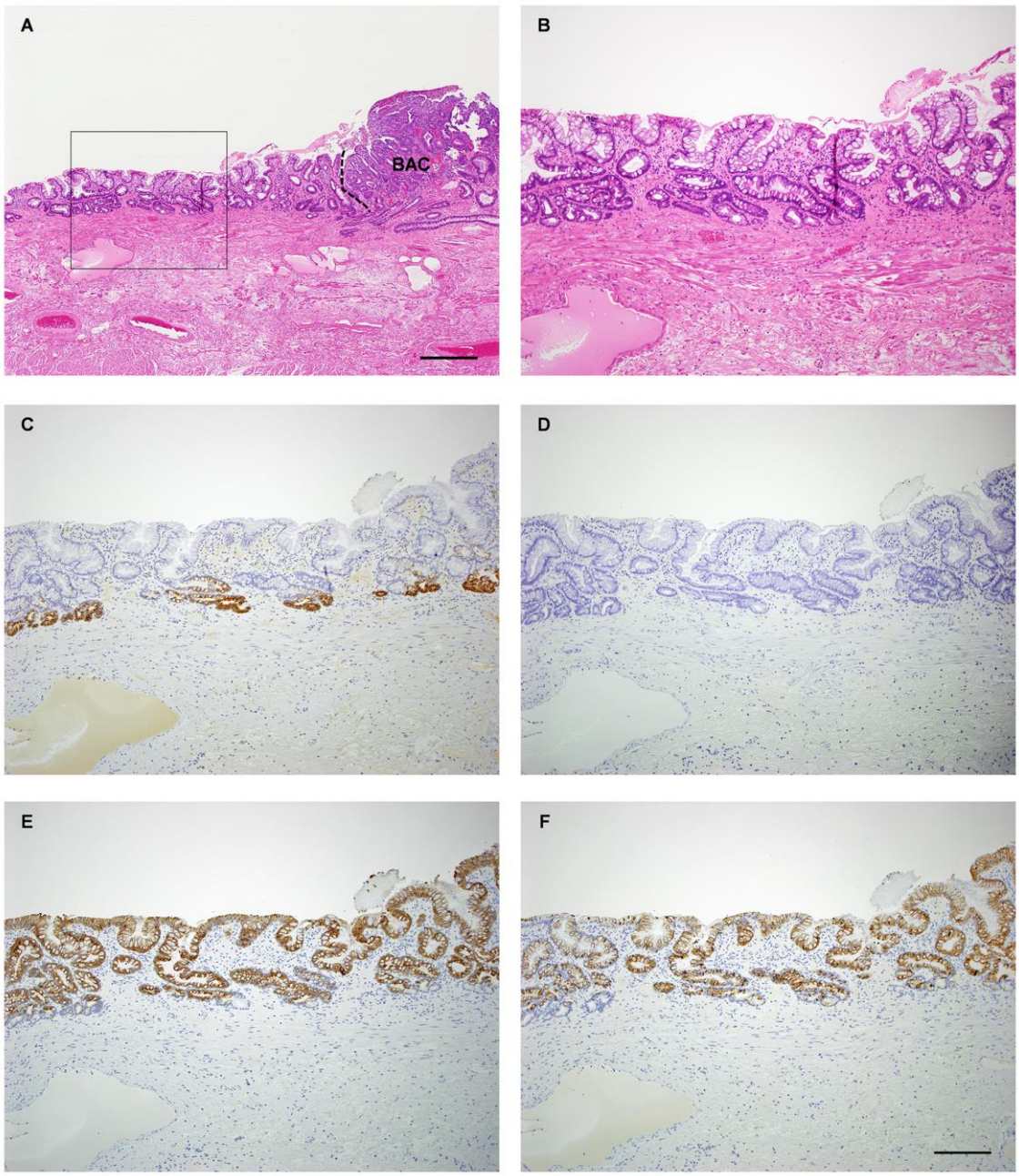

Fig.2

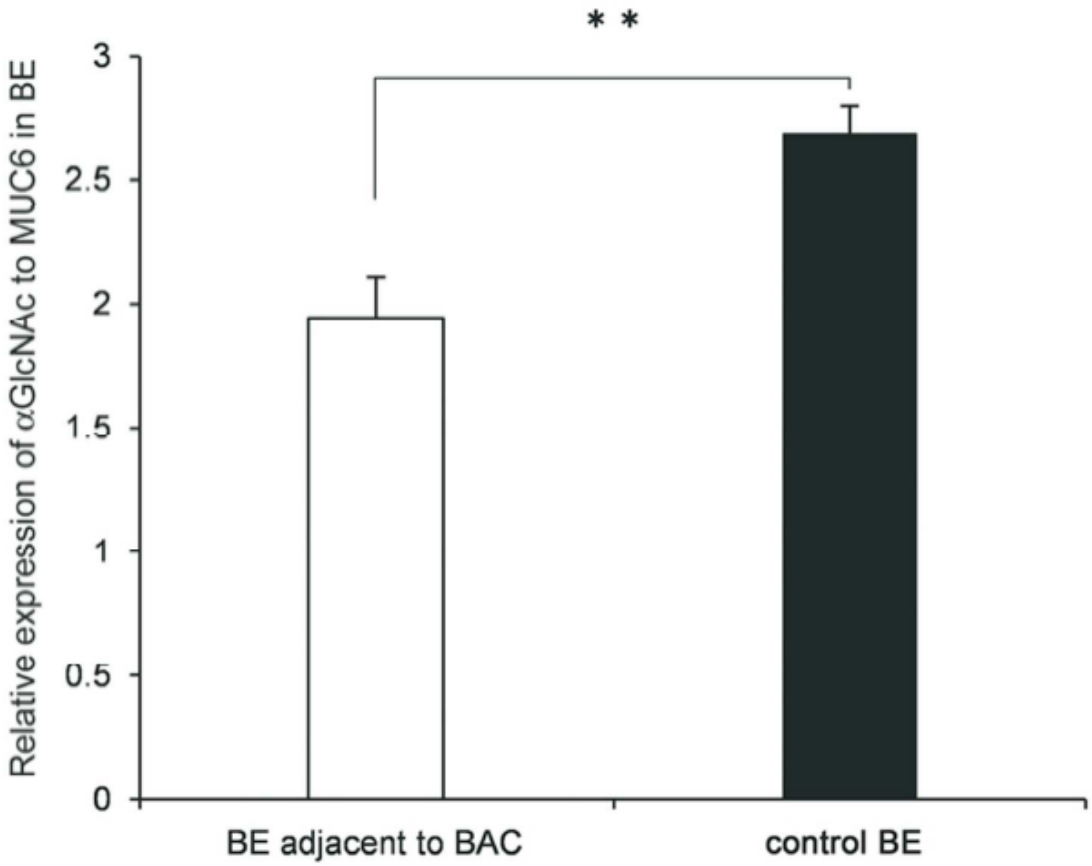
* *
Relative expression of αGlcNAc to MUC6 in BE
3
2.5
2
1.5
1
0.5
0
BE adjacent to BAC
control BE

Fig.3

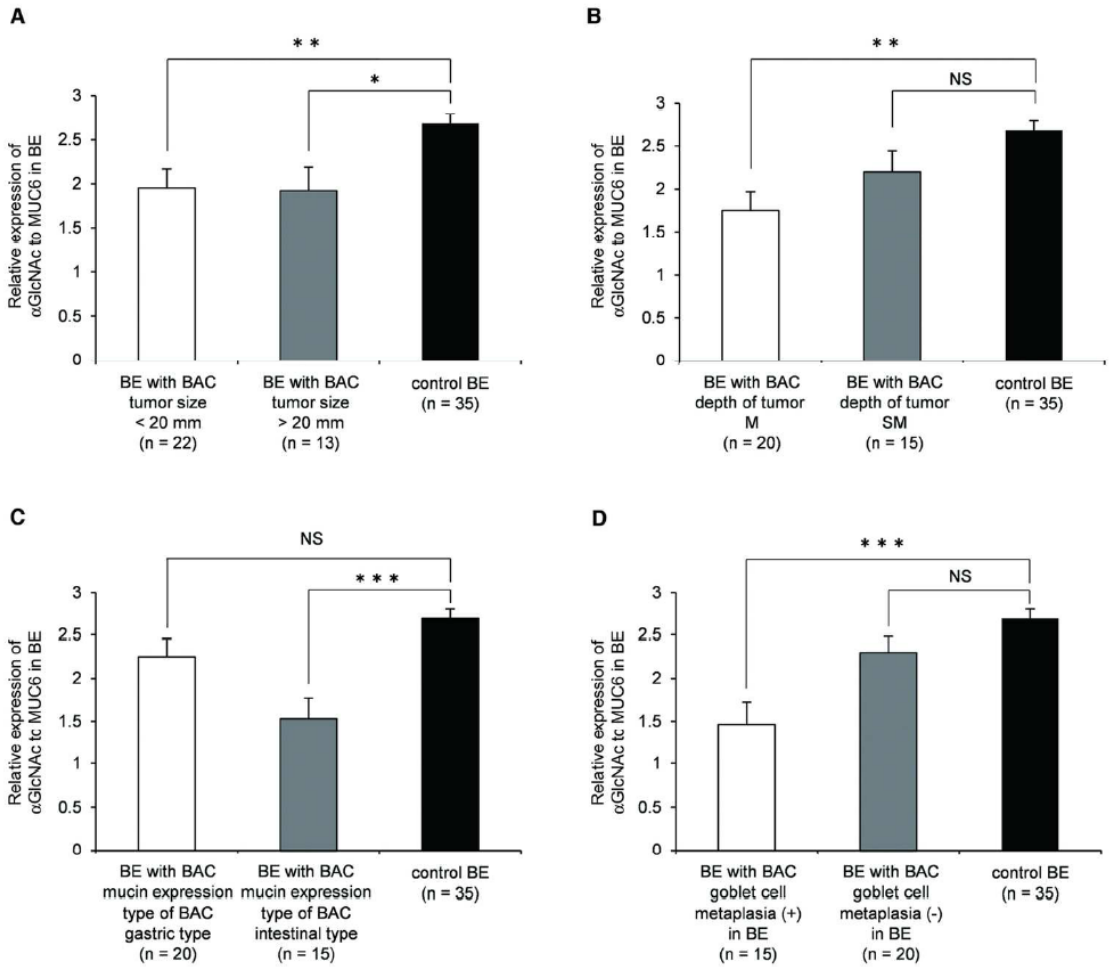
A
* *
*
Relative expression of αGlcNAc to MUC6 in BE
3
2.5
2
1.5
1
0.5
0
BE with BAC tumor size < 20 mm (n = 22)
BE with BAC tumor size > 20 mm (n = 13)
control BE (n = 35)
B
* *
NS
Relative expression of αGlcNAc to MUC6 in BE
BE with BAC depth of tumor M (n = 20)
BE with BAC depth of tumor SM (n = 15)
control BE (n = 35)
C
NS
* * *
Relative expression of αGlcNAc tc MUC6 in BE
BE with BAC mucin expression type of BAC gastric type (n = 20)
BE with BAC mucin expression type of BAC intestinal type (n = 15)
control BE (n = 35)
D
* * *
NS
Relative expression of αGlcNAc tc MUC6 in BE
BE with BAC goblet cell metaplasia (+) in BE (n = 15)
BE with BAC goblet cell metaplasia (-) in BE (n = 20)
control BE (n = 35)

Fig.4

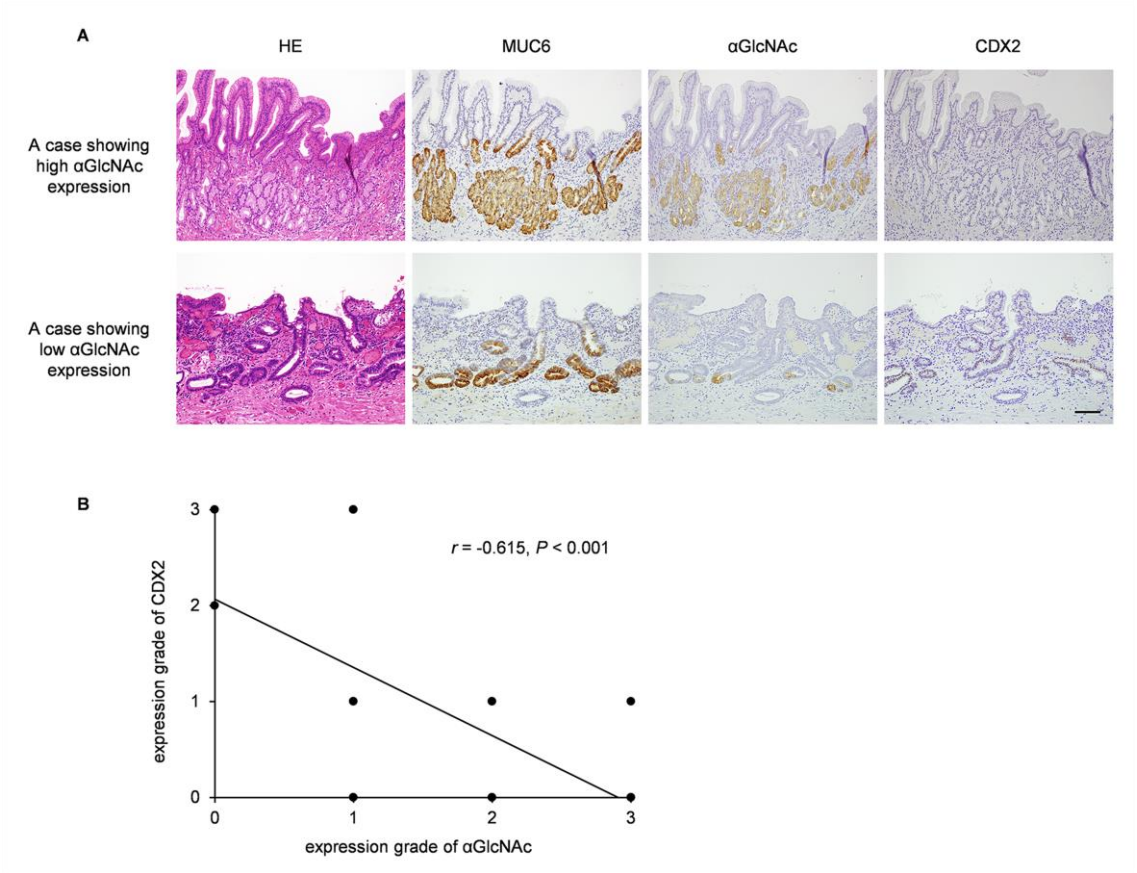
A
HE
MUC6
αGlcNAc
CDX2
A case showing high αGlcNAc expression
A case showing low αGlcNAc expression
B
r = -0.615, P < 0.001
expression grade of CDX2
expression grade of αGlcNAc
0
1
2
3

Fig.5

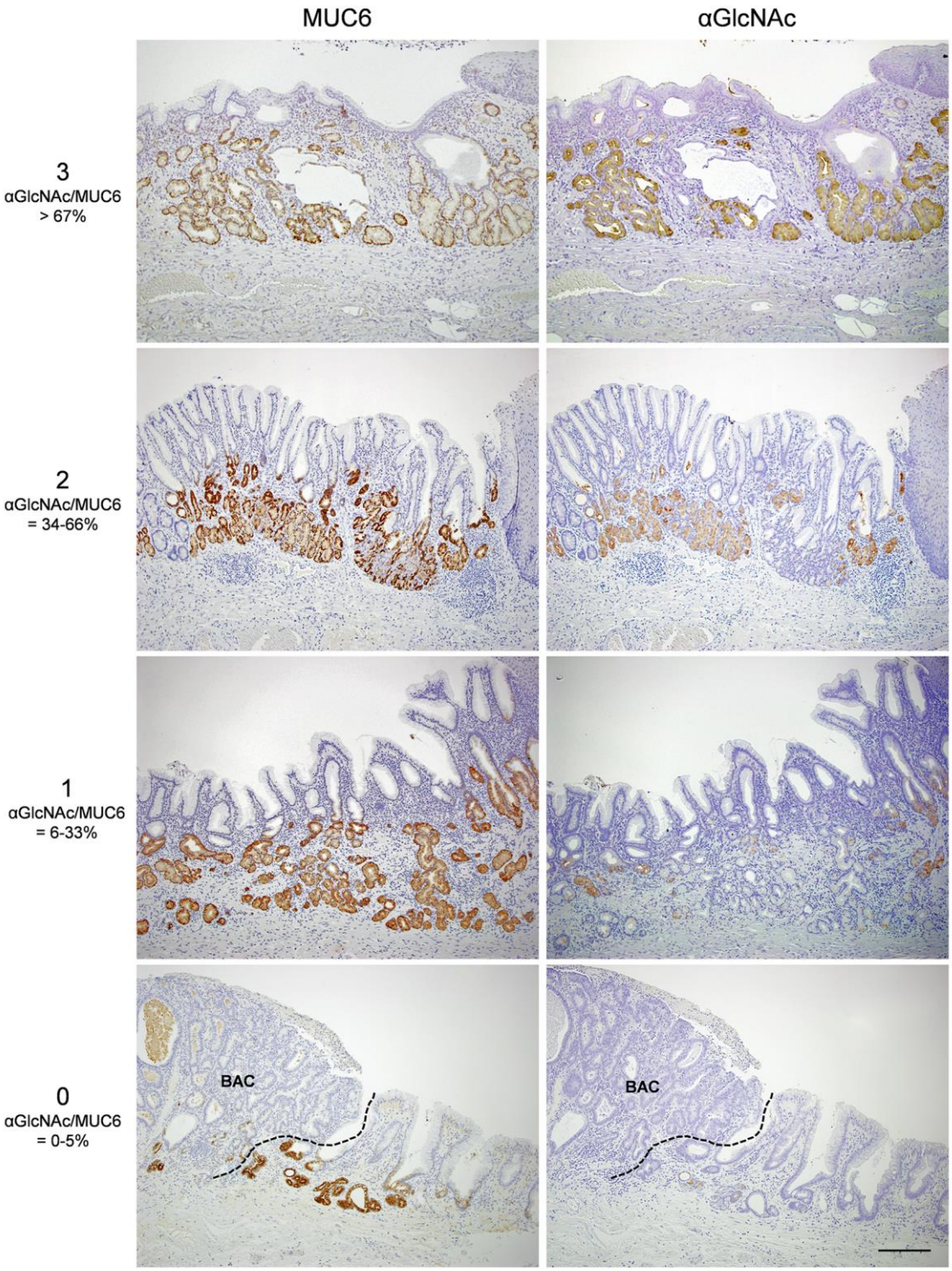
MUC6
αGlcNAc
3
αGlcNAc/MUC6
> 67%
2
αGlcNAc/MUC6
= 34-66%
1
αGlcNAc/MUC6
= 6-33%
0
αGlcNAc/MUC6
= 0-5%
BAC
BAC

Figure S1

Table 1. Clinicopathological features of Barrett's adenocarcinoma and control group

	BAC (patient: n=34, specimen: n=35)	**Control** (n = 35)	***P* value**
Mean age (range)	67.7（50-84）	65.9 （52-83）	0.443
Gender (Male (%))	79.4	97.1	0.028
Helicobacter pylori infection (%)	15.6 (5/32)	54.8 (17/31)	0.003
BE length (SSBE：LSBE)	30 : 4	34 : 1	0.198
Mean tumor size, mm (range)	19.0 (3-48)	—	—
Depth of tumor invasion (M：SM)	20 : 15	—	—
Mucin expression in BAC (gastric: mixed predominantly gastric : mixed predominantly intestinal : intestinal)	9 : 11 : 8 : 7	—	—
Adjacent to goblet cells (yes : no)	15 : 20	—	—

BAC: Barrett's adenocarcinoma, BE: Barrett's esophagus

SSBE: short-segment BE (<3cm), LSBE: long-segment BE (>3cm)

M: in the lamina propria mucosae or muscularis mucosae, SM: in the submucosa