Reduced gland mucin-specific *O*-glycan in gastric atrophy is a possible risk factor for differentiated-type adenocarcinoma of the stomach

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Abstract

Backgrounds and Aims: *O*-glycans exhibiting terminal α 1,4-linked *N*-acetylglucosamine (α GlcNAc) are attached to MUC6 in gastric gland mucins and serve as a tumor suppressor for gastric adenocarcinoma. Gastric atrophy is associated with risk for gastric cancer. However, the significance of α GlcNAc expression in pyloric glands of chronic atrophic gastritis remains unknown. Here, we asked whether reduced α GlcNAc expression in chronic atrophic gastritis is associated with risk for gastric cancer.

Methods: We quantitatively analyzed expression of α GlcNAc relative to MUC6 in pyloric glands by immunohistochemistry in 67 patients with normal mucosa, 70 with chronic atrophic gastritis, 68 with intramucosal differentiated-type adenocarcinoma, and 11 with intramucosal undifferentiated-type adenocarcinoma. We also compared the Ki-67 labeling index in gastric epithelial cells between chronic atrophic gastritis and normal gastric mucosa with respect to α GlcNAc reduction.

Results: In normal pyloric mucosa, α GlcNAc was co-expressed with MUC6. By contrast, in chronic atrophic gastritis, pyloric gland α GlcNAc expression was significantly reduced relative to MUC6. In intramucosal gastric cancer, α GlcNAc expression in pyloric glands found just beneath differentiated-type adenocarcinoma was also reduced relative to MUC6. However, pyloric glands present beneath undifferentiated-type adenocarcinoma exhibited no α GlcNAc decrease. The Ki-67 labeling index in chronic atrophic gastritis showing α GlcNAc reduction was significantly increased relative to that in normal gastric mucosa.

Conclusions: Because α GlcNAc prevents the gastric cancer development, reduced α GlcNAc expression in chronic atrophic gastritis is a possible risk factor for

differentiated-type adenocarcinoma of the stomach.

Keywords

chronic atrophic gastritis, immunohistochemistry, mucin, *O*-glycan

Introduction

Since the discovery of *Helicobacter pylori* (*H. pylori*) by Warren and Marshall in 1983,¹ it has been accepted that this bacterium causes peptic ulcer² and chronic gastritis.³ In 1994, the International Agency for Research on Cancer identified *H. pylori* as the causative microbe for gastric cancer,⁴ and the relationship between gastric cancer and *H. pylori* has since been extensively analyzed.^{5,6} Notably, clinicopathological and epidemiological studies have revealed that gastric atrophy is closely associated with risk of gastric cancer.⁷⁻⁹ Nonetheless, mechanisms underlying this activity remain largely unknown.

To protect the gastric mucosa from the external environment, the stomach secretes heavily glycosylated gel-forming mucins, which are divided into surface and gland mucins;¹⁰ i.e, the former are secreted from mucous cells lining the surface layer of the gastric mucosa, while gland mucin is secreted from gland mucous cells such as mucous neck cells and pyloric gland cells located in a lower mucosal layer. Gland mucins contain unique *O*-linked oligosaccharides (*O*-glycans) exhibiting terminal α 1,4-linked *N*-acetylglucosamine residues (α GlcNAc)^{11,12} that are largely presented on the scaffold protein MUC6, which is secreted from the stomach and duodenum of mammals, including humans.^{13,14}

Previously, we used expression cloning to isolate cDNA encoding human α 1,4-*N*-acetylglucosaminyltransferase (α 4GnT), the enzyme responsible for α GlcNAc biosynthesis¹⁵ and then revealed that α GlcNAc suppresses motility and growth of *H. pylori*.¹⁶ More recently, we established *A4gnt*-deficient mice and reported that they lack

α GlcNAc and develop gastric cancer through a hyperplasia/dysplasia/carcinoma sequence in the absence of *H. pylori* infection.¹⁷ These findings indicate that α GlcNAc plays an inhibitory role in gastric carcinogenesis.¹⁸ In fact, loss of α GlcNAc expression on MUC6-positive tumor cells was associated with progression and poor outcome of gastric differentiated-type adenocarcinoma.¹⁹ Interestingly, expression of α GlcNAc relative to MUC6 in Barrett's esophagus adjacent to Barrett's adenocarcinoma was reduced significantly compared to Barrett's esophagus without Barrett's adenocarcinoma,²⁰ indicating that α GlcNAc loss occurs not only in cancer cells but also in adjacent non-cancerous cells.

Recent advances in gastroenterological endoscopy have allowed detection of gastric cancer at extremely early stages when tumor cells are limited to the upper layer of gastric mucosa. Although α GlcNAc is co-expressed with MUC6 in gland mucin in normal human gastric mucosa,^{13,17} we observed that α GlcNAc often disappears in non-neoplastic pyloric glands found just beneath intramucosal adenocarcinoma of the stomach (Yamada and Nakayama, unpublished findings), suggesting that loss of α GlcNAc in pyloric glands signals a risk for gastric adenocarcinoma.

In the present study, we compared expression levels of α GlcNAc in pyloric glands of normal gastric mucosa without *H. pylori* infection, in pyloric glands with chronic atrophic gastritis in the presence of *H. pylori* infection, and in non-tumorous pyloric glands located just beneath intramucosal gastric adenocarcinoma. Mitotic activity of epithelial cells in normal gastric mucosa and chronic atrophic gastritis with or without α GlcNAc expression was also assessed by Ki-67 labeling index. Our results suggest

that reduced expression of the gastric gland mucin-specific *O*-glycan α GlcNAc is a possible risk factor for development of differentiated-type gastric cancer.

Patients and methods

Patients

We retrieved tissue blocks of gastric biopsies from the pathology file of Shinshu University Hospital, Matsumoto, Japan, including 70 patients with normal gastric mucosa without *H. pylori* infection and 83 with *H. pylori*-infected chronic atrophic gastritis. Samples were obtained from the lesser curvature of antral mucosa using a 2.8 mm forceps. The presence of *H. pylori* was confirmed by immunohistochemical staining with an anti-*H. pylori* antibody, as described.²¹ Among patients without *H. pylori* infection, we excluded those with a history of malignant tumors, eradication of *H. pylori*, or previous infection with *H. pylori* as indicated by intestinal metaplasia. On the other hand, among *H. pylori*-infected patients, we excluded those with a history of malignant tumors including lymphoma. In addition, 89 tissue blocks of endoscopically-resected gastric intramucosal adenocarcinoma were retrieved from the pathology files of Shinshu University Hospital, Matsumoto, Japan, and from Iiyama Red Cross Hospital, Iiyama, Japan. These gastric adenocarcinomas composed of 78 patients with differentiated-type adenocarcinoma and 11 with undifferentiated-type adenocarcinoma, according to the classification of Nakamura et al.²² The Ethics Committee of Shinshu University School of Medicine, Matsumoto, Japan, approved the study plan (no. 2693) and also granted a waiver of informed consent to use formalin-fixed and paraffin-embedded tissue specimens retrieved from pathology files of all hospitals enrolled, since diagnostic use of samples was complete before the study

and there was no risk to patients involved. Samples were also coded to protect patient anonymity.

Immunohistochemistry

Expression of MUC6, α GlcNAc, and Ki-67 antigen was evaluated by immunohistochemistry. Three- μ m thick consecutive sections were deparaffinized and hydrated through a graded alcohol series. With the exception of samples immunostained for α GlcNAc, after inhibition of endogenous peroxidase by treatment with a 3% H_2O_2 /methanol solution, antigen retrieval was carried out in 10mM Tris-HCl buffer (pH8.0) containing 1mM EDTA in a microwave oven for 30 min. Following washing in phosphate-buffered solution, sections were incubated with the following primary antibodies: MUC6 (CLH5, diluted 1:200; Novocastra, Newcastle Upon Tyne, UK), α GlcNAc (HIK1083, diluted 1:30; Kanto Chemical, Tokyo, Japan), and Ki-67 (MIB-1, diluted 1:200; Dako, Glostrup, Denmark). Sections were then incubated with the secondary antibody anti-mouse IgG conjugated with horseradish peroxidase (Histofine, Simple Stain MAX-PO (M), Nichirei Biosciences, Tokyo, Japan) and developed using 3,3'-diaminobenzidine tetrachloride as a chromogen. Haematoxylin served as a nuclear counterstain. Negative controls were carried out omitting the primary antibodies, and no specific staining was seen.

Evaluation of α GlcNAc expression in pyloric glands

In samples of either normal gastric mucosa without *H. pylori* infection or *H. pylori*-infected chronic atrophic gastritis, we evaluated expression of α GlcNAc relative to MUC6 in pyloric glands by counting the number of pyloric glands expressing MUC6

and those expressing α GlcNAc in serial tissue sections from specimens biopsied and then calculating the ratio of α GlcNAc- to MUC6-positive glands. We excluded 3 tissue specimens of normal mucosa and 13 specimens of chronic atrophic gastritis from the analysis because MUC6 was not detected in pyloric glands. We also excluded 10 specimens of differentiated-type adenocarcinoma because non-neoplastic pyloric glands were absent beneath intramucosal adenocarcinoma cells. We also selected representative tissue specimens containing an entire carcinoma lesion, counted α GlcNAc-positive and MUC6-positive non-neoplastic pyloric glands remaining beneath intramucosal adenocarcinoma cells, and calculated the ratio of α GlcNAc-positive to MUC6-positive glands. Throughout the analysis, the numbers of pyloric glands positive for MUC6 and α GlcNAc were counted under a microscope BX51 (Olympus, Tokyo, Japan) using a hand tally counter by one investigator (YS) of the authors.

Determination of the Ki-67 labeling index

The Ki-67 labeling index in gastric epithelial cells was compared between chronic atrophic gastritis and normal gastric mucosa with respect to α GlcNAc reduction. Six cases of *H. pylori*-infected chronic atrophic gastritis showing a complete lack of α GlcNAc in MUC6-positive pyloric glands, 6 cases of *H. pylori*-infected chronic atrophic gastritis with normal levels of α GlcNAc in MUC6-positive pyloric glands, and 6 cases of normal pyloric mucosa that showed no reduction in α GlcNAc were selected for immunostaining using Ki-67 antigen. Ki-67-positive cells in gastric mucosa were counted using the so-called “hot spot” method, as described.²³ Briefly, one investigator (YS) of the authors identified 3 optical fields with the greatest number of Ki-67-expressing epithelial cells in each sample at x100 magnification under a

microscope AX80 PROVIS equipped with a CCD camera (Olympus). Then, the numbers of Ki-67-positive epithelial cells and all epithelial cells contained in a given area (0.037 mm²) were counted on the printed pictures taken at x400 magnification. Finally, the percentage of the total number of Ki-67-positive epithelial cells relative to the total number of all epithelial cells found in 3 given areas was calculated to obtain the Ki-67 labeling index.

Statistical analysis

Data from different tissue types were compared using the Chi-square test. Expression of α GlcNAc and MUC6 was compared using the Wilcoxon *t*-test. α GlcNAc/MUC6 ratios in groups were compared using the Kruskal-Wallis H-test. Ki-67 labeling indices for Ki-67 antigen were compared using the Mann-Whitney U-test. P-values < 0.05 were considered significant. Statistical analysis was carried out using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

Results

The number of MUC6-positive pyloric glands decreases in chronic atrophic gastritis

Gastric atrophy is characterized by a marked decrease in the number of glandular cells, such as those from pyloric or fundic glands. Because MUC6 is expressed in pyloric glands,^{12,13} we first compared the number of MUC6-positive pyloric glands in *H. pylori*-infected chronic atrophic gastritis with those seen in normal pyloric mucosa. In chronic atrophic gastritis, the number of MUC6-positive glands contained in each biopsied specimen significantly decreased ($P < 0.001$) relative to normal pyloric

mucosa (Fig. 1), indicating that the reduction of MUC6-positive pyloric glands was characteristic for chronic atrophic gastritis.

α GlcNAc expression decreases in MUC6-positive pyloric glands in chronic atrophic gastritis

We next asked whether α GlcNAc expression decreased in MUC6-positive pyloric glands in chronic atrophic gastritis. To this end, we first analyzed 67 patients with normal gastric mucosa. As reported previously^{13,17} and confirmed here, α GlcNAc and MUC6 were generally co-expressed in pyloric glands of normal gastric mucosa (Fig. 2), and we observed no significant differences in numbers of α GlcNAc-positive and MUC6-positive pyloric glands ($P = 0.19$) (Fig. 3A), suggesting that α GlcNAc is attached to MUC6 in normal glands. However, α GlcNAc staining in pyloric glands relative to that of MUC6 decreased or disappeared in chronic atrophic gastritis specimens (Fig. 2), suggesting that the number of α GlcNAc-positive glands decreases in that condition. Statistical analysis of 70 patients with chronic atrophic gastritis confirmed that there were significantly fewer α GlcNAc-positive pyloric glands than MUC6-positive glands in chronic atrophic gastritis ($P < 0.001$) (Fig. 3B).

The number of α GlcNAc-positive non-neoplastic pyloric glands decreases in differentiated-type but not undifferentiated-type intramucosal adenocarcinoma

To determine whether the number of α GlcNAc-positive pyloric glands also decreases in non-neoplastic pyloric glands remaining beneath intramucosal adenocarcinoma cells, we assessed specimens from 68 patients with differentiated-type intramucosal gastric adenocarcinoma. Similar to chronic atrophic gastritis, α GlcNAc staining in

non-neoplastic pyloric glands located just beneath the adenocarcinoma decreased or disappeared relative to that of MUC6 (Fig. 2), suggesting that the number of α GlcNAc-positive glands decreases compared to MUC6-positive glands. Statistically, the number of α GlcNAc-positive pyloric glands significantly decreased compared with MUC6-positive pyloric glands in differentiated-type adenocarcinoma ($P < 0.001$) (Fig. 3C). By contrast, in 11 patients with undifferentiated-type intramucosal adenocarcinoma, α GlcNAc and MUC6 generally co-localized in the same pyloric glands (Fig. 2), and there was no significant difference in the number of α GlcNAc-positive and MUC6-positive pyloric glands remaining just beneath undifferentiated-type adenocarcinoma cells ($P = 0.08$) (Fig. 3D).

The α GlcNAc/MUC6 ratio varies among tissues from normal gastric mucosa, chronic atrophic gastritis, and adenocarcinoma

To compare apparent reduction in the number of α GlcNAc-positive pyloric glands relative to MUC6-positive glands in normal gastric mucosa, chronic atrophic gastritis, and non-neoplastic pyloric glands in intramucosal differentiated-type adenocarcinoma and undifferentiated-type adenocarcinoma, we compared the α GlcNAc/MUC6 ratio in each category. α GlcNAc/MUC6 ratios in both chronic atrophic gastritis and differentiated-type adenocarcinoma were significantly smaller than those in normal gastric mucosa ($P < 0.001$) (Fig. 4), confirming that relative expression α GlcNAc to MUC6 in pyloric glands decreases in both of those conditions.

Assessment of the Ki-67 labeling index in chronic atrophic gastritis with or without α GlcNAc reduction

A4gnt-deficient mice that lack α GlcNAc in the gastroduodenal mucosa show significantly increased gastric epithelial cell proliferation in the antrum.¹⁷ Thus, we asked whether the number of Ki-67-positive cells in chronic atrophic gastritis showing reduced α GlcNAc expression also increased. To this end, we compared the Ki-67 labeling index in MUC6-positive cells among chronic atrophic gastritis lacking α GlcNAc expression, chronic atrophic gastritis in which α GlcNAc expression was present in MUC6-positive glands, and normal gastric mucosa that showed physiological α GlcNAc levels in MUC6-positive pyloric glands. Statistical analysis revealed that the Ki-67-labeling index of chronic atrophic gastritis lacking α GlcNAc expression was significantly higher than that in normal gastric mucosa ($P < 0.05$) (Fig. 5). Although a significant difference was not observed, the Ki-67 labeling index of chronic atrophic gastritis that showed normal α GlcNAc levels had a low tendency than that of chronic atrophic gastritis lacking α GlcNAc expression.

Discussion

In this study, we clearly demonstrate that pyloric glands expressing MUC6 are significantly decreased in number in chronic atrophic gastritis compared to those in normal gastric mucosa. In addition, we also showed that the relative expression of α GlcNAc to MUC6 in pyloric glands in chronic atrophic gastritis or in non-neoplastic pyloric glands remaining just beneath intramucosal differentiated-type adenocarcinoma cells was significantly decreased compared to pyloric glands in normal gastric mucosa and undifferentiated-type adenocarcinoma. These observations indicate that decreased α GlcNAc expression in pyloric glands is a phenomenon common to both chronic atrophic gastritis and differentiated type adenocarcinoma.

Previously, we showed that α GlcNAc plays dual roles in antagonizing gastric cancer.¹⁸ First, α GlcNAc serves as a natural antibiotic against *H. pylori* infection by suppressing biosynthesis of cholesteryl α -D-glucopyranoside, which is important for *H. pylori* survival.¹⁶ In addition, α GlcNAc acts as tumor suppressor for differentiated-type adenocarcinoma, as demonstrated by phenotypes seen in *A4gnt*-deficient mice, which completely lack of α GlcNAc: i.e., null mutants spontaneously develop differentiated-type adenocarcinoma in the gastric mucosa through a hyperplasia/dysplasia/carcinoma sequence.¹⁷ The result revealed by the present study that no relative reduction of α GlcNAc to MUC6 in the non-neoplastic pyloric glands remaining just beneath intramucosal undifferentiated-type adenocarcinoma is well concordant with the fact that *A4gnt*-deficient mice do not develop undifferentiated-type adenocarcinoma.¹⁷ In humans, we reported that α GlcNAc loss in MUC6-positive gastric adenocarcinoma cells was significantly correlated with depth of invasion, stage, venous invasion, and poor prognosis in differentiated-type but not undifferentiated-type adenocarcinoma.¹⁹ Furthermore, we demonstrated that α GlcNAc expression was more significantly attenuated in Barrett's esophagus adjacent to early stage Barrett's adenocarcinoma than in Barrett's esophagus without Barrett's adenocarcinoma.²⁰ These results collectively suggest that decreased α GlcNAc expression in pyloric glands in chronic atrophic gastritis is a risk factor for development of differentiated-type gastric adenocarcinoma.

It is at issue whether or not the intestinal metaplasia is a preneoplastic lesion of gastric adenocarcinoma.^{24,25} As we reported previously²⁶ and further confirmed here, α GlcNAc

was barely detected in intestinal metaplasia (data not shown). However, the absence of α GlcNAc in intestinal metaplasia does not necessary mean that the intestinal metaplasia is a preneoplastic lesion of gastric adenocarcinoma, because *A4gnt*-deficient mice rarely exhibit intestinal metaplasia.¹⁷ Future study will be needed to determine the significance of α GlcNAc loss in the intestinal metaplasia.

Lipkin et al. (1985) demonstrated that epithelial cell proliferation in chronic atrophic gastritis increases two-fold compared to normal gastric mucosa.²⁷ In the present study, we found that proliferation of epithelial cells in pyloric mucosa lacking α GlcNAc expression significantly increased relative to those in normal gastric mucosa expressing α GlcNAc. This result agrees with our previous study of *A4gnt*-deficient mice;¹⁷ in it, we reported that epithelial cell proliferation in the gastric mucosa increased as mice aged. In the same study, we also found that genes encoding inflammatory chemokine ligands, proinflammatory cytokines, and growth factors, such as chemokine (C-C motif) ligand 2, interleukin-11, and hepatocyte growth factor, were also upregulated, even as early as 10 weeks of age, a stage in which animals show low-grade dysplasia. It is possible that tumor-promoting inflammation occurs in pyloric glands that exhibit decreased α GlcNAc expression. Further study is required to determine whether inflammatory cell infiltrates in pyloric glands are associated with lack of α GlcNAc expression in these cells.

In conclusion, our present study reveals that expression of α GlcNAc relative to MUC6 in the pyloric glands significantly decreases in chronic atrophic gastritis and differentiated-type adenocarcinoma but not undifferentiated-type adenocarcinoma. In

addition, reduced α GlcNAc expression seen in epithelial cells of chronic atrophic gastritis was significantly associated with high mitotic activity. These results collectively suggest that reduced expression of the gastric gland mucin-specific *O*-glycan α GlcNAc is a potential risk factor for development of differentiated-type adenocarcinoma of the stomach, and immunohistochemistry for α GlcNAc and MUC6 on gastric biopsies from patients with chronic atrophic gastritis could be helpful to assess the potential risk of gastric atrophy in the patients.

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Competing interests

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1 Comparison of the number of MUC6-positive pyloric glands in normal mucosa versus chronic atrophic gastritis. The number of MUC6-positive glands contained in each biopsied specimen significantly decreased in chronic atrophic gastritis compared with normal pyloric mucosa. Boxes represent 25-75% of gland numbers, and the line shows the median value. The whiskers indicate the lowest datum included in the 1.5 x interquartile range (IQR) of the lower quartile and the highest datum included in the 1.5 x IQR of the upper quartile. **, $P < 0.001$.

Figure 2 α GlcNAc and MUC6 expression in pyloric glands of the gastric mucosa. In normal gastric mucosa, α GlcNAc and MUC6 are co-expressed in pyloric glands. In chronic atrophic gastritis α GlcNAc expression is lost in pyloric glands expressing MUC6. In intramucosal gastric differentiated-type adenocarcinoma, α GlcNAc expression disappears in MUC6-positive pyloric glands remaining just beneath adenocarcinoma cells, whereas both α GlcNAc and MUC6 are co-localized in pyloric glands remaining in undifferentiated-type adenocarcinoma. HE, Hematoxylin and eosin staining. Immunohistochemistry with monoclonal antibodies HIK1083 for α GlcNAc and CLH5 for MUC6. Scale bar = 200 μ m.

Figure 3 Comparison of the number of MUC6- and α GlcNAc-positive pyloric glands present in normal gastric mucosa, chronic atrophic gastritis, and intramucosal adenocarcinoma. No significant difference was observed between the number of MUC6- and α GlcNAc-positive pyloric glands in normal gastric mucosa ($P = 0.19$) (A), while there was a significant reduction of the number of α GlcNAc-positive pyloric

glands compared with the number of MUC6-positive pyloric glands in chronic atrophic gastritis (B). In pyloric glands remaining just beneath intramucosal differentiated-type adenocarcinoma, the number of α GlcNAc-positive pyloric glands significantly decreased compared with the number of MUC6-positive pyloric glands (C). However, these differences were not observed in pyloric glands remaining just beneath intramucosal undifferentiated-type adenocarcinoma (D). Boxes represent 25-75% of gland numbers, and the line shows the median value. The whiskers indicate the lowest datum included in the 1.5 x IQR of the lower quartile and the highest datum included in the 1.5 x IQR of the upper quartile. **** $P < 0.001$.**

Figure 4 Comparison of the α GlcNAc/MUC6 ratio in pyloric glands in normal gastric mucosa, chronic atrophic gastritis, and intramucosal adenocarcinoma. The α GlcNAc/MUC6 ratio in both chronic atrophic gastritis and differentiated-type adenocarcinoma was significantly less than that in normal gastric mucosa ($P < 0.001$). Boxes represent 25-75% of α GlcNAc/MUC6 ratio, and the line shows the median value. The whiskers indicate the lowest datum included in the 1.5 x IQR of the lower quartile and the highest datum included in the 1.5 x IQR of the upper quartile. **** $P < 0.001$.**

Figure 5 Comparison of the Ki-67 labeling index in gastric epithelial cells from normal gastric mucosa, chronic atrophic gastritis with relatively normal α GlcNAc expression, and chronic atrophic gastritis lacking α GlcNAc expression. A Mann-Whitney *U*-test revealed that the Ki-67-labeling index of chronic atrophic gastritis lacking α GlcNAc expression was significantly higher than that seen in normal gastric mucosa. Boxes

represent 25-75% of Ki-67 labeling index, and the line shows the median value. The whiskers indicate the lowest datum included in the 1.5 x IQR of the lower quartile and the highest datum included in the 1.5 x IQR of the upper quartile. * $P < 0.05$.

Fig. 1

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Fig. 3

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