Gastric gland mucin-specific O-glycan expression decreases with tumor progression from precursor lesions to pancreatic cancer

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Pancreatic cancer is highly lethal due to difficulty of early diagnosis: most cases of pancreatic cancer are diagnosed at advanced stage, greatly decreasing the chance for a cure. Thus, novel biomarkers of precursor lesions of pancreatic cancer are required. An international consensus meeting held at the Johns Hopkins Hospital, Baltimore, MD, USA in 2003 assessed and reported the current definition and classification of three major precursor lesions to invasive ductal adenocarcinoma (IDAC) of the pancreas; they include pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasm (MCN).11 In 2014, a new international consensus meeting held at the Johns Hopkins Hospital revised the earlier guidelines.12 Specifically, the revised guideline recommends a two-tiered system (i.e. low-grade versus high-grade), instead of a three-tiered system used for former classification of the precursor lesions including PanIN, IPMN and MCN.

Changes in the mucin phenotype of the pancreatic epithelium, particularly acquisition of gastric mucin properties, are crucial events in early stages of pancreatic tumor progression.3–7 Gastric mucins are classified as surface and gland mucins that contain MUC5AC and MUC6, respectively.8 Gland mucin characteristically contains O-linked oligosaccharides (O-glycans) with terminal α1,4-linked N-acetylgalactosamine residues (αGlcNAc) attached largely to a MUC6 scaffold.9,10 In normal gastric mucosa, αGlcNAc and MUC6 are co-expressed in gland mucous cells, such as pyloric gland and mucous neck cells.10,11 Previously, we used expression cloning to isolate cDNA encoding α1,4-N-acetylgalactosaminyltransferase (α4GnT), which catalyzes αGlcNAc biosynthesis.12 We then reported that A4GnT-deficient mice, which show αGlcNAc loss in gland mucin, spontaneously develop gastric adenocarcinoma.13 These findings suggest that αGlcNAc serves as a tumor suppressor.14 In support of this idea, we observed that αGlcNAc expression is frequently lost in human gastric differentiated-type adenocarcinoma expressing MUC6.15 We also showed that reduced αGlcNAc expression relative to MUC6 is associated with malignant potential in pyloric gland adenoma of the human stomach, a precursor of gastric adenocarcinoma.16 These studies suggest overall that αGlcNAc could serve as a critical biomarker of malignant potential in early stages of gastric epithelial neoplasias. In
normal human pancreas, MUC6 and αGlcNAc are co-expressed in periductal mucous gland cells of the main pancreatic duct.\(^{10}\) In addition, we and others reported that αGlcNAc is expressed in PanIN.\(^{17-19}\) However, the relationship between αGlcNAc expression and pancreatic tumor progression remains unknown.

Here, we used immunohistochemistry to examine expression patterns of gastric mucin markers, including MUC5AC, MUC6 and αGlcNAc, in precursor lesions of pancreatic cancer, including PanIN and IPMN, as well as invasive carcinoma. We then compared relative αGlcNAc and MUC6 expression in each lesion.

**Materials and Methods**

**Patient samples.** The present study evaluated pancreatic tissue specimens from 48 surgically resected cases of pancreatic tumors at Shinshu University Hospital, Matsumoto, Japan. Specifically, tissue specimens of IDAC (20 cases) and IPMN (28 cases), which were diagnosed based on World Health Organization classification criteria (2010),\(^{20}\) were retrieved from the pathology files of the Department of Laboratory Medicine of the same hospital. All specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Tissue sections were stained with H&E for histopathological analysis. In 20 cases of IDAC that did not contain IPMN components, we selected lesions exhibiting low-grade PanIN, high-grade PanIN and IDAC classified on a recent consensus.\(^{2}\) Hereafter, we used high-grade PanIN/intraductal spread of IDAC (high-grade PanIN/IDS) for high-grade PanIN, because it is morphologically difficult to distinguish high-grade PanIN from intraductal spreading of IDAC when IDAC exists.\(^{2}\) We eventually selected 17 low-grade PanIN lesions, 12 high-grade PanIN/IDS lesions and 20 IDAC lesions (Table S1). For IPMN, we first excluded 8 cases of intestinal-type IPMN, which is characterized by its MUC2 expression, from 28 cases of IPMN retrieved from the pathology file, because this particular type of IPMN does not express MUC6.\(^{17}\) In fact, all of the excluded cases were negative for MUC6 (Fig. S1). Thus, we classified IPMN lesions into low-grade IPMN, high-grade IPMN, and IPMN with associated invasive carcinoma (IPMN/AIC) lesions based on a recent consensus for histological grade.\(^{2}\) Consequently, 19 lesions of low-grade IPMN, 10 lesions of high-grade IPMN and 8 lesions of IPMN/AIC were selected (Table S2). Furthermore, both low-grade IPMN and high-grade IPMN lesions were morphologically subclassified into gastric type, pancreaticobiliary type and oncocytic type based on World Health Organization classification criteria (2010).\(^{20}\) Because cases with oncocytic-type IPMN were not included in the pathology file, we eventually selected 21 gastric-type IPMN lesions, including 19 lesions of low-grade IPMN and 2 lesions of high-grade IPMN, and 8 lesions of pancreaticobiliary-type IPMN, which were high-grade IPMN (Table S3). This study was approved by the Ethics Committee of the Shinshu University School of Medicine, Matsumoto, Japan (nos. 1338 and 3626) and was in accordance with the Declaration of Helsinki. The Ethics Committee also granted a waiver of informed consent to use formalin-fixed, paraffin-embedded tissue specimens, because diagnostic use of samples was completed before the study and there was no risk to patients involved. Samples were also coded to protect patient anonymity.

**Immunohistochemistry.** Primary antibodies used in this study were: anti-MUC5AC (clone 45M1, mouse IgG; Novocastra, Newcastle, UK) diluted 1:100, anti-MUC6 (clone CLH5, mouse IgG; Novocastra) diluted 1:200, and anti-αGlcNAc (clone HIK1083, mouse IgM; Kantokagaku, Tokyo, Japan) diluted 1:20. Conventional immunohistochemistry for all primary antibodies was carried out using the EnVision system (DakoCytomation, Carpinteria, CA, USA). Tissue sections of 3-μm thickness were deparaffinized in xylene and rehydrated in ethanol. Except for αGlcNAc, antigens were retrieved by boiling sections in 10-mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA for 25 min in a microwave oven. Endogenous peroxidase activity was quenched by soaking sections in absolute methanol containing 0.3% hydrogen peroxide for 30 min. After blocking with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) in TBS (pH 7.6) for 15 min, sections were incubated with each primary antibody at 4°C overnight followed by incubation with HRP-conjugated anti-mouse immunoglobulins for 60 min. The color reaction was developed with 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan). Negative controls were established by omitting primary antibodies from the procedure, and no specific staining was seen. Immunohistochemical evaluation was undertaken in two ways. First, lesions in which >5% of the total number of tumor cells of each lesion were positively-stained were judged positive, as described previously.\(^{15}\) Second, expression levels of MUC6 and αGlcNAc were either scored semi-quantitatively from 0 to 3: 0 (≤5% positive cells), 1 (6%–33% positive cells), 2 (34%–66% positive cells) or 3 (>67% positive cells), as described previously.\(^{16}\)

**Statistical analysis.** Correlations between each grade for PanIN or IPMN and the number of positive lesions were statistically analyzed by Fisher’s exact probability test. Differences between semi-quantitative immunoreactivity scores in MUC6-stained and αGlcNAc-stained sections were statistically analyzed using the Wilcoxon matched pairs test. All analyses were carried out with Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA). P-values <0.05 were considered statistically significant.

**Results**

Expression of mucin core proteins MUC5AC and MUC6 as well as αGlcNAc in pancreatic lesions exhibiting the PanIN-IDAC sequence. MUC5AC was expressed in 45 (91.8%) of 49 lesions associated with the PanIN-IDAC sequence, irrespective of histological grade (Table 1 and Fig. 1a). By contrast, MUC6 was expressed in all 17 low-grade PanIN, 11 (91.7%) of 12 high-grade PanIN/IDS, and 14 (70%) of 20 IDAC lesions. The number of MUC6-positive lesions representing low-grade PanIN was significantly higher than that seen in IDAC (P < 0.05). However, low-grade PanIN and high-grade PanIN/IDS did not show a significant difference (P = 0.41). In contrast, αGlcNAc expression was observed in all 17 low-grade PanIN lesions (100%), 6 (50%) of 12 high-grade PanIN/IDS, and 8 (40%) of 20 IDAC. The frequency of αGlcNAc-positive lesions in both high-grade PanIN/IDS and IDAC was significantly decreased relative to that seen in low-grade PanIN (P < 0.01).

Because αGlcNAc is largely attached to MUC6, and the relatively decreased αGlcNAc expression in MUC6-positive lesions is associated with gastric cancer progression,\(^{10,15}\) we compared αGlcNAc and MUC6 immunoreactivity semi-quantitatively in low-grade PanIN, high-grade PanIN/IDS, and IDAC (Table S1). At any histological grade, αGlcNAc expression levels were significantly decreased relative to those of MUC6.
Expression of MUC5AC and MUC6 as well as αGlcNAc in pancreatic lesions representing the IPMN-IPMNAIC sequence. We next examined expression of MUC5AC, MUC6 and αGlcNAc in lesions exhibiting the IPMN-IPMNAIC sequence. MUC5AC was expressed in all 37 IPMN lesions, irrespective of histological grade (Table 2 and Fig. 2a). MUC6 was expressed in 18 (94.7%) of 19 low-grade IPMN, 7 (70%) of 10 high-grade IPMN, and 3 (37.5%) of 8 IPMNAIC lesions. Statistical analysis revealed that the number of MUC6-positive lesions in low-grade IPMN was significantly greater than that seen in IPMNAIC (P < 0.01). However, the difference in the number of MUC6-positive lesions between low-grade and high-grade IPMN was not significant (P = 0.10). In contrast, αGlcNAc was not detected in any of 8 IPMNAIC lesions. However, αGlcNAc was detected in 18 (94.7%) of 19 low-grade IPMN and 5 (50%) of 10 high-grade IPMN lesions. However, αGlcNAc was not detected in any of 8 IPMNAIC lesions. When we compared the number of αGlcNAc-positive lesions between high-grade IPMN and IPMNAIC or between low-grade IPMN and high-grade IPMN, the frequency of αGlcNAc-positive lesions was significantly decreased in more advanced histological grades (P < 0.05 for high-grade IPMN versus IPMNAIC and P < 0.01 for low-grade IPMN versus high-grade IPMN).

Next, we assessed αGlcNAc and MUC6 immunoreactivity semi-quantitatively in low-grade IPMN, high-grade IPMN, and IPMNAIC (Table S2). In low-grade IPMN, αGlcNAc immunoreactivity was significantly decreased relative to that of MUC6 (P < 0.05) (Fig. 2b). Nonetheless, we did not observe significant differences in αGlcNAc and MUC6 immunoreactivity in either high-grade IPMN or IPMNAIC (P = 0.071 for high-grade IPMN and P = 0.083 for IPMNAIC) (Fig. 2b).

Finally, we semi-quantitatively assessed αGlcNAc and MUC6 immunoreactivity from a standpoint of morphological classifications, gastric-type and pancreatobiliary-type IPMN (Table S3). We found that αGlcNAc immunoreactivity in gastric-type IPMN was significantly decreased compared to MUC6 (P < 0.05) (Fig. 2c). On the other hand, the expression level of αGlcNAc in 8 lesions of pancreatobiliary-type IPMN, all of which belonged to high-grade IPMN, was lower than that of MUC6. However, significant differences were not obtained between them (P = 0.13) (Fig. 2c).

Table 1. Frequency of lesions positive for MUC proteins or αGlcNAc associated with the PanIN-IDAC sequence of pancreatic tumor progression

| Number of lesions | MUC5AC (%) | MUC6 (%) | αGlcNAc (%) |
|-------------------|------------|----------|-------------|
| PanIN-IDAC        |            |          |             |
| Low-grade PanIN   | 17         | 16 (94.1)| 17 (100)*  |
| High-grade PanIN/IDS | 12      | 11 (91.7)| 11 (91.7)  |
| IDAC              | 20         | 18 (90.0)| 14 (70.0)* |
| Total             | 49         | 45 (91.8)| 42 (85.7)  |

*Significant difference in MUC6 positivity between low-grade PanIN and IDAC (P < 0.05). **Significant difference in αGlcNAc positivity between low-grade and high-grade PanIN/IDS (P < 0.01) and between low-grade PanIN and IDAC (P < 0.01).

(P < 0.01 for low-grade PanIN, P < 0.05 for high-grade PanIN/IDS, and P < 0.05 for IDAC) (Fig. 1b).

Fig. 1. Immunohistochemical analysis of MUC5AC, MUC6 and αGlcNAc expression in PanIN and IDAC. (a) MUC5AC is expressed in tumor cells, irrespective of tumor grade. MUC6 is expressed in tumor cells showing pyloric gland phenotypes in low-grade PanIN and high-grade PanIN/IDS. αGlcNAc expression coincides with that of MUC6 in low-grade PanIN. By contrast, in both high-grade PanIN/IDS and IDAC, αGlcNAc is not expressed in MUC6-positive tumor cells. Bar = 100 μm. (b) Semi-quantitation of MUC5 and αGlcNAc expression in low-grade PanIN, high-grade PanIN/IDS, and IDAC. Data are represented as the mean ± SEM. *P < 0.05 and **P < 0.01 by Wilcoxon matched-pair test.
Discussion

The present study revealed that expression levels of αGlcNAc relative to MUC6 begin to decrease early in pancreatic tumor progression in both the PanIN-IDAC and IPMN-IPMNAIC sequences: specifically, lesions positive for αGlcNAc or MUC6 were most frequently detected in low-grade PanIN and low-grade IPMN (Tables 1 and 2). However, semi-quantitative analysis of αGlcNAc and MUC6 immunoreactivities indicated that αGlcNAc expression relative to that of MUC6 had already decreased not only in low-grade PanIN but also in low-grade IPMN (Figs. 1b and 2b). In both high-grade PanIN/IDS and IDAC, the number of αGlcNAc-positive lesions and expression levels of αGlcNAc significantly decreased relative to MUC6 levels. Although we did not observe a significant difference between high-grade IPMN and IPMNAIC, αGlcNAc expression in both lesions was lower than that of MUC6. These results combined together indicate that a decrease in αGlcNAc expression precedes a decrease in MUC6, even in early phases of pancreatic tumor progression. We previously demonstrated that αGlcNAc and MUC6 are largely co-expressed in peri-duodenal accessory glands of the pancreatic duct.\(^{(10)}\) In the present study, we reveal that at the early phase of PanIN-IDAC and of IPMN-IPMNAIC sequence, MUC6 expression significantly predominates over αGlcNAc expression, and as histological grade progresses to pancreatic cancer, expression levels of both decrease. We recently demonstrated that αGlcNAc expression is significantly reduced in pyloric gland adenoma with high-grade dysplasia that is a precancerous lesion of gastric adenocarcinoma.\(^{(16)}\) These results overall suggest that reduced αGlcNAc expression relative to MUC6 occurs at early stages of pancreatic tumor progression. However, molecular mechanism explaining why decreased αGlcNAc expression marks the initiation of tumor progression has yet to be elucidated. Because αGlcNAc functions as a tumor suppressor for differentiated-type gastric adenocarcinoma,\(^{(13)}\) decrement of αGlcNAc might trigger the initiation of tumor progression. Future studies are needed to address this problem.

Morphological subclassification of IPMN into gastric, intestinal, pancreatobiliary and oncocytic type is of significance to predict the malignant potential of tumors and the prognosis of patients; that is, gastric-type IPMN is strongly associated with low histological grade, and other IPMN types are negatively associated with low histological grade.\(^{(22)}\) In fact, all 19 lesions of low-grade IPMN examined in the present study were levels of αGlcNAc significantly decreased relative to MUC6 levels. Although we did not observe a significant difference between high-grade IPMN and IPMNAIC, αGlcNAc expression in both lesions was lower than that of MUC6. These results combined together indicate that a decrease in αGlcNAc expression precedes a decrease in MUC6, even in early phases of pancreatic tumor progression. We previously demonstrated that αGlcNAc and MUC6 are largely co-expressed in peri-duodenal accessory glands of the pancreatic duct.\(^{(10)}\) In the present study, we reveal that at the early phase of PanIN-IDAC and of IPMN-IPMNAIC sequence, MUC6 expression significantly predominates over αGlcNAc expression, and as histological grade progresses to pancreatic cancer, expression levels of both decrease. We recently demonstrated that αGlcNAc expression is significantly reduced in pyloric gland adenoma with high-grade dysplasia that is a precancerous lesion of gastric adenocarcinoma.\(^{(16)}\) These results overall suggest that reduced αGlcNAc expression relative to MUC6 occurs at early stages of pancreatic tumor progression. However, molecular mechanism explaining why decreased αGlcNAc expression marks the initiation of tumor progression has yet to be elucidated. Because αGlcNAc functions as a tumor suppressor for differentiated-type gastric adenocarcinoma,\(^{(13)}\) decrement of αGlcNAc might trigger the initiation of tumor progression. Future studies are needed to address this problem.

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classified as gastric-type IPMN, whereas 8 of 10 lesions of high-grade IPMN were categorized as pancreatobiliary-type IPMN. Table S3. Significant reduction of αGlcNAc relative to MUC6 in gastric-type IPMN shown here supported that αGlcNAc expression already decreased in the early phase of IPMN-IPMNAIC sequence.

Recent studies show that IDAC derived from PanIN frequently exhibits K-RAS mutations but not GNAS mutations, although IPMN typically harbors GNAS mutations.21,23 These findings suggest that PanIN-IDAC and IPMN-IPMNAIC sequences employ different molecular machinery. Here, however, we observed decreased expression of αGlcNAc accompanied by progression of pancreatic neoplasia at both sequences in the tumor progression pathway, suggesting that αGlcNAc expression levels could predict malignant potentials of both PanIN and IPMN. Future studies are needed to define molecular mechanisms underlying regulation of expression of A4GNT gene, which encodes α4GNT.

We also show that MUC5AC and MUC6 are expressed not only in both low-grade PanIN and high-grade PanIN/IDS but also in low-grade and high-grade IPMN, all precursors of pancreatic cancer (Tables 1 and 2). However, MUC6 expression in pancreatic cancer, including IDAC and IPMNAIC, was lower relative to MUC5AC expression. Kim et al. demonstrated that MUC6 expression in PanIN is an early event seen in 74% of PanIN1A lesions, 67% of PanIN1B lesions, 66% of PanIN2 lesions and 56% of PanIN3 lesions, whereas MUC6 is expressed only in 35% of IDAC lesions.15 Our results are consistent with these studies. In terms of other cancer types, Chang et al.24 demonstrate that MUC6 is expressed in metastatic pseudopulmonary glands in the gallbladder and its expression decreases in dysplasia and carcinoma. Matsukita et al.25 also showed a correlation between MUC6 expression and mucinous carcinoma of the breast, suggesting that high MUC6 expression in that context may act as a barrier to cancerous growth and antagonize tumor cell invasivity. All of these studies strongly suggest that MUC6 may play an important role as a tumor suppressor in pancreatic and other tumors, such as the gallbladder and breast.

In conclusion, the present study indicates that decreased expression of αGlcNAc relative to MUC6 is an initial event marking the early phase of pancreatic tumor progression. Further studies are needed to determine molecular mechanisms that regulate αGlcNAc expression to better understand pancreatic tumor progression.

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Disclosure Statement
The authors have no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Immunohistochemical expression of MUC5AC, MUC2 and MUC6 in intestinal-type IPMN. MUC5AC and MUC2 are expressed in tumor cells of intestinal-type IPMN, irrespective of histological grade. By contrast, MUC6 is not detected in the tumor cells. Primary antibody used for MUC2 immunohistochemistry was anti-MUC2 antibody (clone Cp58, mouse IgG, Novocastra). Information about anti-MUC5AC and anti-MUC6 antibodies was provided in the main text. Bar = 100 μm.

**Table S1.** Immunohistochemical scores reflecting MUC6 and αGlcNAc staining in each lesion of 20 cases associated with the PanIN-IDAC sequence.

**Table S2.** Immunohistochemical scores reflecting MUC6 and αGlcNAc staining in each lesion of 20 cases associated with the IPMN-IPMNAIC sequence.

**Table S3.** Morphological subclassification of low-grade IPMN lesions and high-grade IPMN lesions in 20 cases in IPMN-IPMNAIC sequence.