Regular Article

Decreased Gastric Gland Mucin-specific *O*-glycans Are Involved in the Progression of Ovarian Primary Mucinous Tumours

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Ovarian primary mucinous tumours (OPMTs) show an adenoma–borderline–carcinoma sequence with gastrointestinal metaplasia. Gastric gland mucin-specific *O*-glycans are unique with an α1,4-linked *N*-acetylglucosamine (αGlcNAc) residue attached to mucin 6 (MUC6). Although αGlcNAc is expected to be expressed in OPMTs, the relationship between αGlcNAc expression and OPMT progression remains unknown. Here, we analysed 104 areas of benign mucinous tumours (benign), 55 areas of borderline mucinous tumours (borderline), and 18 areas of malignant mucinous tumours (malignant) to investigate the expression patterns of αGlcNAc, mucin 2 (MUC2), mucin 5AC (MUC5AC), and MUC6 during the progression of OPMT from benign to malignant. MUC5AC expression was observed in all areas. The frequencies of MUC6- and αGlcNAc-positive areas were decreased with tumour progression. In particular, the decrease in αGlcNAc-positive areas was remarkable. Furthermore, αGlcNAc expression was lower than MUC6 expression at all grades (benign, $p < 0.0001$; borderline, $p = 0.0014$; malignant, $p = 0.0039$). Conversely, there was no difference in the expression frequency or level of MUC2 among the three grades. These results suggest that decreased expression of αGlcNAc relative to MUC6 occurs early in tumour development and marks the initiation of OPMT progression.

Key words: ovarian primary mucinous tumour, αGlcNAc, MUC6, MUC5AC, MUC2

I. Introduction

Ovarian primary mucinous tumours (OPMTs) are classified into three grades: benign mucinous tumours (benign), borderline mucinous tumours (borderline), and malignant mucinous tumours (malignant). In mucinous malignant tumours, there is often a continuum of architectural and cytological atypia that includes cystadenoma, borderline tumours, and obvious malignant areas [6]. Hence, OPMT has been thought to exhibit an adenoma–borderline–carcinoma sequence [2]. The prognosis of borderline mucinous tumours is excellent with most mucinous carcinomas confined to stage I. Although the exact recurrence rate of mucinous carcinomas is unknown, they recur within 3 years of surgery, and chemotherapy and radiotherapy have no effect on recurrent lesions or metastases [6]. OPMTs exhibit a gastrointestinal phenotype. Therefore, tumour cells similar to goblet cells, surface gastric epithelial cells, and pyloric gland cells can be confirmed. Moreover, both the morphology and expression of mucin core proteins including mucin 2 (MUC2), a representative intestinal-type mucin core protein, mucin 5AC (MUC5AC), and mucin 6 (MUC6), representative gastric-type mucin core proteins, are observed at various rates that depend on the OPMT grade [3, 15].

O-glycans with a terminal α1,4-linked *N*-acetylglucosamine (αGlcNAc) residue are unique to gastric gland mucins secreted from the gastroduodenal mucosa, which are largely attached to a MUC6 scaffold [4, 20]. In normal stomach tissue, MUC6 and αGlcNAc are coexpressed in the pyloric gland and mucous neck

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cells [16, 20]. αGlcNAc biosynthesis is catalysed by α1,4-*N*-acetylglucosaminyltransferase [9], and *A4gnt*-deficient mice spontaneously develop differentiated type gastric adenocarcinoma, which indicates that αGlcNAc is a tumour suppressor in differentiated type gastric adenocarcinoma [5]. In fact, αGlcNAc expression is frequently lost in MUC6-positive human differentiated type gastric carcinoma [14]. Additionally, αGlcNAc expression is reduced with the progression of tumours that express the gastric-type phenotype, which include pyloric gland adenoma of the stomach, pancreatic lesions [pancreatic intraepithelial neoplasia and invasive ductal adenocarcinoma (PanIN–IDCA) sequence, and intraductal papillary mucinous neoplasms and those with associated invasive carcinoma (IPMN–IPMNAIC) sequence], bile duct lesions [biliary intraepithelial neoplasia and invasive adenocarcinomas (BilIN–IAC) sequence], and uterus cervical lesions [lobular endocervical glandular hyperplasia and gastric-type mucinous carcinoma (LEGH–GAS) sequence] [10, 11, 16, 19].

OPMTs have been reported to express αGlcNAc [8, 12]. However, to our knowledge, no report has systematically analysed the relationship between αGlcNAc expression and mucin core proteins (MUC2, MUC5AC, and MUC6) and the progression of OPMT. Therefore, we clarified the relationship between αGlcNAc expression and these mucin core proteins in OPMT progression by immunohistochemistry.

II. Materials and Methods

Patient samples

Between January 2009 and December 2018, 105 cases of OPMTs (mucinous cystadenoma, n = 49; mucinous borderline tumour, n = 38; mucinous carcinoma, n = 18), who underwent surgery at the Department of Gynecology and Obstetrics, Shinshu University Hospital (Matsumoto, Japan), were retrieved from the pathology files of the Department of Laboratory Medicine at the same hospital. In carcinoma cases, upper or lower gastrointestinal endoscopy confirmed that no carcinoma was present. No history of gastrointestinal carcinoma was also confirmed in all cases. Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin (H&E) for histopathological analysis. All H&E-stained tissue specimens of each case were screened for benign, borderline, and malignant areas within lesions in accordance with World Health Organization classification criteria (2014) [6]. Consequently, we analysed 104 benign areas, 55 borderline areas, and 18 malignant areas. Seromucinous tumours were excluded. The areas of each grade are summarised in Supplementary Table S1. This study was approved by the Institutional Ethics Committee of Shinshu University School of Medicine, Matsumoto, Japan (approval no. 4357). This study was performed in line with the principles of the Declaration of Helsinki.

Immunohistochemistry

From among the specimens containing each grade thus histologically identified, one or more specimens showing typical findings were arbitrarily selected and immunostained as follows. Primary antibodies used were anti-MUC2 (1:100, clone CCP58, mouse IgG; Leica Biosystems, Vista, CA, USA), anti-MUC5AC (1:100, clone CLH2, mouse IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MUC6 (1:100, clone CLH5, mouse IgG; Santa Cruz Biotechnology), and anti-αGlcNAc (1:20, clone HIK1083, mouse IgM; Kantokagaku, Tokyo, Japan). The production and specificity of these primary antibodies were described as elsewhere [1, 4, 13]; i.e, the synthetic peptides used in the development of anti-MUC2 (CCP58), anti-MUC6 (CLH5) and anti-MUC5AC (CLH2) antibodies were KYPTTTPISTTTMVTPTPTPTGTQTQTPTTT, SFQTTT TYPTPSHPQTTLPC and SAPTTSTTSAPT, respectively [1, 13]. On the other hand, the immunogen used in the development of HIK1083 antibody was a purified rat gastric mucin, and it specifically recognizes αGlcNAc located at the non-reducing end of the sugar chain [4]. Immunohistochemistry was performed using the EnVision system (DakoCytomation, Carpinteria, CA, USA). Three-micrometre-thick tissue sections were deparaffined in xylene and rehydrated in ethanol. Except for αGlcNAc, antigens were retrieved by boiling in 10 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA for 25 min. Endogenous peroxidase activity was quenched by incubation in absolute methanol containing 0.3% hydrogen peroxide for 30 min. After blocking with 1% bovine serum albumin (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 horseradish peroxidase-conjugated anti-mouse immunoglobulins for 60 min. The sections were developed with 3,3'-diaminobenzidine (Dojindo, Kumamoto, Japan). Negative controls omitted primary antibodies and no specific staining was observed (data not shown). Immunohistochemical evaluation was undertaken by two approaches. First, areas in which > 1% of the total number of tumour cells in each area, which were positively stained, were judged as positive. Second, the expression levels of MUC2, MUC5AC, MUC6, and αGlcNAc were further scored semi-quantitatively from 0 to 4 as follows: 0 (< 1% positive cells), 1 (2%–10% positive cells), 2 (11%–40% positive cells), 3 (41%–70% positive cells), and 4 (≥ 71% positive cells) as described elsewhere with modifications [3, 15].

Statistical analysis

Correlations between each OPMT grade and the number of positive areas were analysed by the Kruskal–Wallis test and Dunn's multiple comparison test. Correlations between each OPMT grade and semiquantitative immunoreactivity scores in MUC2-, MUC6-, and αGlcNAc-stained sections were also analysed by the Kruskal–Wallis test and Dunn's multiple comparison test.

Table 1. *Expression of MUC proteins and αGlcNAc in OPMT*

Tumor grade	Benign	Borderline	Malignant
Number of areas	104	55	18
MUC2			
Positive rate, n (%)	67 (64.42%)	38 (69.09%)	10 (55.56%)
Average expression level score	1.346	1.436	0.8889
MUC5AC			
Positive rate, n (%)	104 (100%)	55 (100%)	18 (100%)
Average expression level score	3.846	3.927	3.667
MUC6			
Positive rate, n (%)	83 (79.81%)	34 (61.82%)	10 (55.56%)
Average expression level score	1.769*	1.164*	1.222
αGlcNAc			
Positive rate, n (%)	68 (65.38%)**	20 (36.36%)**	3 (16.67%)**
Average expression level score	1.212**	0.6182**	0.2222**

* Significant difference in MUC6 expression level score between benign and borderline ($p < 0.01$).

** Significant difference in αGlcNAc-positive rate and expression level score between benign and borderline ($p < 0.01$) and between benign and malignant ($p < 0.01$).

αGlcNAc, α1,4-linked N-acetylglucosamine; OPMT, ovarian primary mucinous tumor.

Differences between semiquantitative immunoreactivity scores in MUC6- and αGlcNAc-stained sections were analysed using the Wilcoxon matched pairs test. As a sub-analysis, the difference in the number of positive areas and semiquantitative immunoreactivity scores between benign areas with borderline or malignant areas and benign areas without borderline or malignant areas in the lesion was analysed using the Mann–Whitney test. Furthermore, the difference in semiquantitative MUC2 immunoreactivity scores in benign areas with or without borderline or malignant areas was determined by Fisher's exact test, and the sensitivity, specificity, positive predictive value, and negative predictive value in the presence of borderline or malignant areas were determined. $p < 0.05$ was considered statistically significant. GraphPad Prism ver. 8.3.4 for Mac OS (GraphPad Software, San Diego, CA, USA) was used for statistical analyses.

III.   Results

MUC5AC, MUC6, and αGlcNAc expression in OPMTs

The positivity rates and expression levels of MUC5AC, MUC6, and αGlcNAc are summarised in Table 1. MUC5AC was positive in all areas of each OPMT grade (Fig. 1). The average scores of MUC5AC expression in benign, borderline, and malignant areas were 3.846, 3.927, and 3.667, respectively. There were no differences among the grades ($p = 0.1822$). The expression rate of MUC6 was 79.81% in benign, 61.82% in borderline, and 55.56% in malignant areas, which decreased with tumour progression. There was a statistically significant difference between the grades ($p = 0.0158$), but no difference was observed in multiple comparisons between each grade (benign vs borderline, $p = 0.0506$; benign vs malignant, $p = 0.1061$; borderline vs malignant, $p > 0.9999$). Conversely, the average scores of MUC6 expression levels in benign, border-

line, and malignant areas were 1.769, 1.164, and 1.222, respectively. There was a statistically significant difference between the grades ($p = 0.0038$). Furthermore, the average score of MUC6 expression in benign areas was significantly higher than that in borderline areas ($p = 0.0047$). However, there was no difference between benign and malignant ($p = 0.2377$) or between borderline and malignant ($p > 0.9999$). The positivity rate of αGlcNAc was 65.38% in benign, 36.36% in borderline, and 16.67% in malignant areas, which decreased with tumour progression (Fig. 1). The difference between the grades was statistically significant ($p < 0.0001$). Furthermore, the αGlcNAc positivity rate in benign areas was significantly higher than that in borderline ($p = 0.0015$) and malignant ($p = 0.0004$) areas, but there was no difference between borderline and malignant ($p = 0.4435$). Additionally, the average scores of αGlcNAc expression in benign, borderline, and malignant areas were 1.212, 0.6182, and 0.2222, respectively with a statistically significant difference between the grades ($p < 0.0001$). By comparing each grade, the average score of αGlcNAc expression in benign areas was significantly higher than that in borderline ($p = 0.0011$) and malignant ($p = 0.0003$) areas, but there was no difference between borderline and malignant areas ($p = 0.4113$).

In 95 benign, 49 borderline, and 18 malignant areas, αGlcNAc was expressed in regions in which MUC6 was expressed or neither MUC6 nor αGlcNAc was expressed regardless of the tumour grade. Differences in the expression levels of MUC6 and αGlcNAc in individual areas at each grade are shown in Fig. 2. At all grades, the expression level of αGlcNAc tended to be significantly lower than that of MUC6 ($p < 0.0001$ for benign, $p = 0.0014$ for borderline, and $p = 0.0039$ for malignant). However, in 8.654% of benign and 10.91% of borderline areas, the expression levels of αGlcNAc were higher than those of MUC6 or αGlcNAc expression was observed where
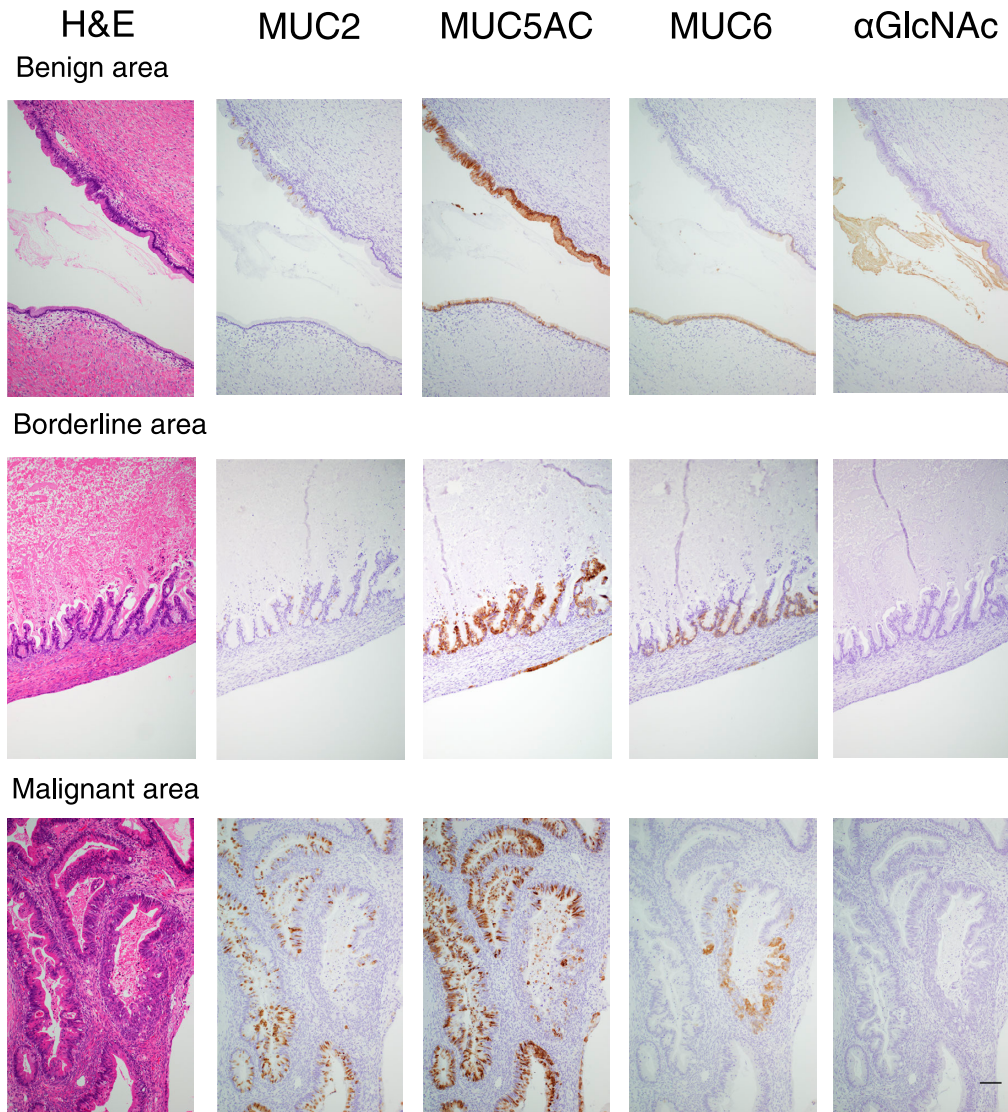
**Fig. 1.**   Immunohistochemistry of MUC2, MUC5AC, MUC6, and αGlcNAc in OPMTs. MUC2 and MUC5AC are expressed in tumour cells irrespective
of the tumour grade. MUC6 tends to be expressed in tumour cells with the pyloric gland phenotype in benign and borderline areas, but is also expressed
in flat columnar epithelium. In many benign areas, tumour cells coexpress αGlcNAc and MUC6. Conversely, in borderline and malignant areas,
αGlcNAc is not expressed in MUC6-positive cells. Bar = 100 μm.

MUC6 expression could not be confirmed (Table 2 and
Fig. 3). In these cases, αGlcNAc was expressed in areas
thar expressed MUC5AC (Fig. 3). In cases with areas
showing predominant expression of αGlcNAc compared
with MUC6, no malignant areas were identified (Table
2). This phenomenon was not observed in malignant areas
(Table 2).

MUC2 expression in OPMTs

The positivity rate and expression levels of MUC2
are summarised in Table 1. No difference was observed
between the grades in terms of the positivity rate (p =
0.5097) and expression level (p = 0.1880) (Fig. 1). How-
ever, focusing on benign areas with or without borderline
or malignant areas, the positive rate of MUC2 was 76.36%

in areas with borderline or malignant areas and 51.02% in
areas without borderline or malignant areas, which showed
a statistically significant difference (p = 0.0083) (Table
3). Furthermore, the average scores of MUC2 expression
were 1.709 for benign areas with borderline or malignant
areas and 0.9388 for benign areas without borderline or
malignant areas, which showed a statistically significant
difference (p = 0.0015) (Table 3). When a series of benign
areas was divided into ≥ 3 and ≤ 2 in accordance with the
expression score of MUC2, the incidence of borderline or
malignant areas was significantly higher in benign areas
that showed ≥ 3 than ≤ 2 (p = 0.0357) (Table 3 and Fig.
4). Of 55 benign areas with borderline or malignant areas,
14 had an expression score of ≥ 3, whereas only four of
49 benign areas without borderline or malignant areas had
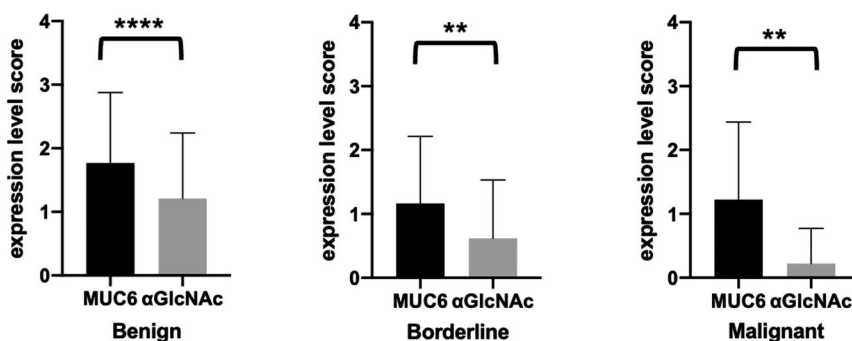
Fig. 2. Expression scores of MUC6 and αGlcNAc at each grade of OPMTs. The expression score of αGlcNAc is significantly lower than that of MUC6 at all tumour grades. $**p < 0.01$, $****p < 0.0001$.
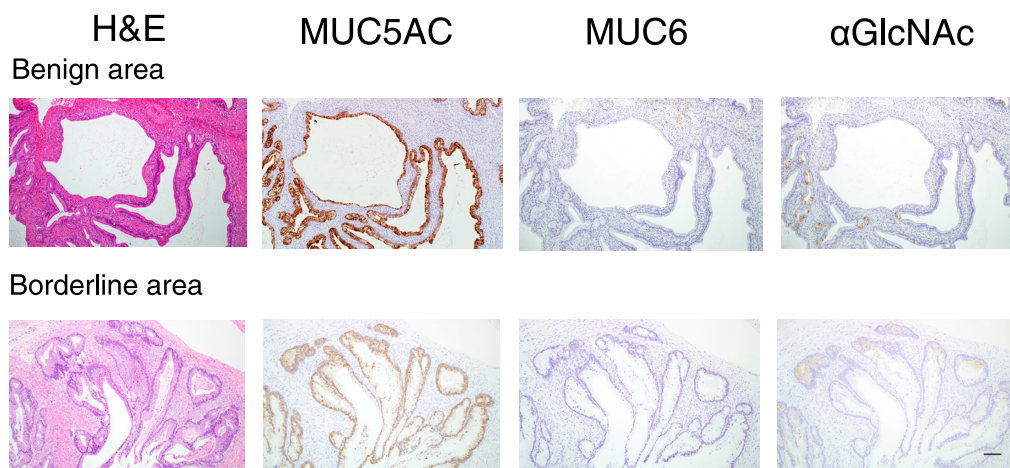
Fig. 3. Predominant expression of αGlcNAc compared with MUC6 in benign and borderline malignancy areas of OPMTs. Benign and borderline areas of OPMTs that expressed αGlcNAc, but not MUC6, are shown. In these cases, MUC5AC is diffusely positive, which suggested that αGlcNAc is attached to MUC5AC. The expression score of αGlcNAc is higher than that of MUC6 in approximately 10% of the benign and borderline malignancy areas, but this is not observed in any of the cases in carcinoma areas. Bar = 100 μm.

**Table 2.** *Frequency of predominant αGlcNAc expression compared with MUC6 in OPMT*

Tumor grade	Number of areas	αGlcNAc overexpression rate n (%)
Benign	104	9 (8.654%)
Without borderline or malignant areas	49	5 (10.20%)
With borderline areas	38	4 (10.53%)
With malignant areas	17	0 (0%)
Borderline	55	6 (10.91%)
Without malignant areas	38	6 (15.79%)
With malignant areas	17	0 (0%)
Malignant	18	0 (0%)

αGlcNAc, α1,4-linked N-acetylglucosamine; OPMT, ovarian primary mucinous tumor.

an expression score of ≥ 3 (Table 3). When the expression score of MUC2 was greater than 3 in benign areas with borderline or malignant areas, the sensitivity, specificity, positive predictive value, negative predictive value, and odds ratio were 25.45%, 91.84%, 77.78%, 52.33%, and 3.841, respectively.

IV.　Discussion

MUC6 and αGlcNAc expression was reduced with OPMT progression. The phenomenon was also observed in IPMN–IPMNAIC and PanIN–IDCA sequences in pancreatic tumours [10], the BilIN–IAC sequence in biliary tract tumours [11], and the LEGH–GAS sequence in uter-

H&E   MUC2

Benign area of a tumor without borderline or malignant areas

Benign area of a tumor with malignant areas
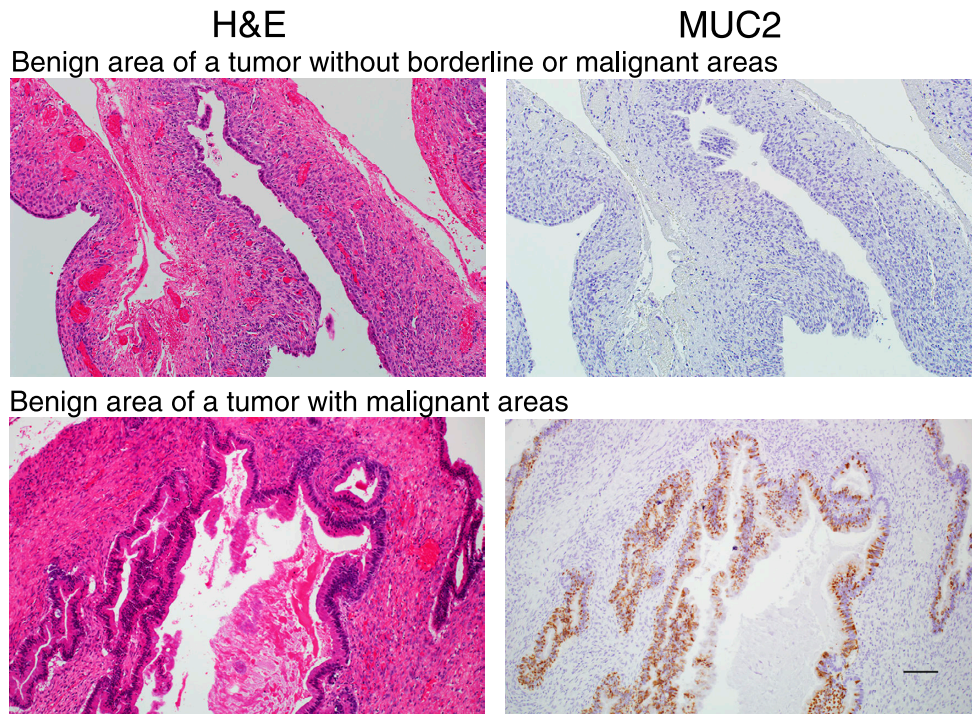
Fig. 4.   Expression of MUC2 in benign areas of malignant OPMTs. In benign OPMTs, MUC2 expression is usually negative or slightly positive. Conversely, in borderline and malignant cases of OPMT, MUC2 is diffusely expressed in benign areas. Bar = 100 μm.

Table 3.   *Expression of MUC2 in benign areas with or without borderline or malignant areas in OPMT*

With or without borderline or malignant area	Number of areas	MUC2 expression Positive rate n (%)	Expression level score					Average score
			0	1	2	3	4	
With borderline or malignant areas	55	42 (76.36%)*	13	10	18	8	6	1.709*
Without borderline or malignant areas	49	25 (51.02%)*	24	10	11	2	2	0.9388*

* Significant difference in MUC2 positive rate and expression level score between with and without borderline or malignant areas ($p < 0.01$).
OPMT, ovarian primary mucinous tumor.

ine cervix tumours [17], which is a common feature of tumours with the gastric phenotype [18]. It is also known that OPMT progresses in a stepwise manner from mucinous cystadenoma to mucinous borderline tumor to mucinous carcinoma [2]. In the present study, we confirmed that all 18 mucinous carcinomas examined contained benign and/or borderline malignant lesions (see Supplementary Table S1). The fact that the expression of αGlcNAc was already decreased in benign and borderline lesions compared to MUC6 suggests that these lesions have the potential to progress to malignancy (see Fig. 2).

Similarly, αGlcNAc expression was also decreased depending on the grade. Moreover, the decreased expression of αGlcNAc was more remarkable than that of MUC6. A decrease in αGlcNAc has been observed in IPMN–IPMNAIC, PanIN–IDAC, BilIN–IAC, and LEGH–GAS sequences [10, 11, 17]. Semiquantitative analysis of αGlcNAc and MUC6 immunoreactivity showed that αGlcNAc expression relative to MUC6 expression

was significantly decreased at all grades. These results suggest that the decreased αGlcNAc expression did not result from the decreased expression of MUC6. Because decreased αGlcNAc expression was also observed in benign lesions, such a decrease may occur during the very early stage of tumour progression. This phenomenon was also observed in IPMN–IPMNAIC, PanIN–IDAC, BilIN–IAC, and LEGH–GAS sequences [10, 11, 17]. We have previously reported that gastric adenocarcinoma develops spontaneously in *A4gnt*-deficient mice [5]. Therefore, αGlcNAc may inhibit the progression of various tumours with the gastric-type phenotype and it can be assumed that loss of αGlcNAc also triggers carcinogenesis in OPMTs.

The present study revealed that αGlcNAc was more highly expressed than MUC6 in approximately 10% of benign and borderline areas (Fig. 3). In human normal gastric mucosa, αGlcNAc is mostly bound to MUC6, but a small amount of αGlcNAc is also bound to MUC5AC [20]. In some cases, αGlcNAc expression was observed in

areas without MUC6 expression, whereas MUC5AC was expressed in these areas (Fig. 3). Therefore, in cases with a higher expression level of αGlcNAc than MUC6, αGlcNAc was bound to MUC5AC. In the LEGH–GAS sequence, αGlcNAc is overexpressed compared with MUC6 and αGlcNAc is expressed even without MUC6 expression [7, 17]. In this study, the carcinoma area was not included in cases with αGlcNAc overexpression. These results suggested that αGlcNAc attached to mucin core proteins other than MUC6 might serve as a tumour suppressor.

MUC5AC was expressed in all OPMT cases. This result is consistent with the findings of Wang *et al.* [15]. Additionally, MUC5AC expression has been observed in most pancreatic tumour lesions with IPMN–IPMNAIC and PanIN–IDCA sequences, and in biliary duct tumours with the BilIN–IAC sequence [10, 11]. Although MUC5AC is normally expressed in gastric surface mucous cells, MUC5AC may be a marker to distinguish tumours with the gastric phenotype from their normal counterpart.

Wang *et al.* and Hirabayashi *et al.* reported that MUC2 expression increases with OPMT progression [3, 15], but these results are different from ours. This difference might be due to the number of cases and the evaluation method for each grade, because their reports included significantly fewer benign cases than our study. Furthermore, even in malignant areas including borderline and malignant, if there was a benign area in the lesion, we also evaluated this area. MUC2 expression did not differ among OPMT grades in terms of both the positivity rate and expression level (see Table 1). However, the positivity rate and expression level of MUC2 were significantly higher in benign areas with borderline or malignant areas than in benign areas without borderline or malignant areas (Table 3). This suggests that MUC2 expression is involved in promoting OPMT progression. Furthermore, assuming that an expression level of ≥ 40% in benign areas is associated with borderline or malignant areas, the sensitivity was low, but the specificity was very high. Hence, in OPMT, MUC2 is a marker for the presence of borderline or malignant areas.

In conclusion, MUC6 and αGlcNAc expression levels were decreased with OPMT progression. In particular, the decrease in αGlcNAc was remarkable. Decreased expression of αGlcNAc relative to MUC6 was an initial event that marked the early phase of OPMT progression. Further studies are needed to determine the molecular mechanisms that regulate αGlcNAc expression to better understand OPMT progression.

V.   Conflicts of Interest

The authors have no conflict of interest.

VI.   Acknowledgments

VII.   References

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