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The expression of several types of mucin is related to the biological behavior of pancreatic neoplasms

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Abstract

Background/Purpose. Mucins are high molecular weight glycoproteins that have oligosaccharides attached to the apomucin protein backbone by O-glycosidic linkages. Here, we report the expression of MUC1 mucin (membrane-bound mucin), MUC2 mucin (intestinal-type secretory mucin), and MUC5AC mucin (gastric-type secretory mucin) in invasive ductal carcinomas (IDCs; $n = 46$) and intraductal papillary-mucinous neoplasms (IPMNs; $n = 33$) of the pancreas, and the relationship of this expression with malignant potential.

Methods. To clarify the precise expression pattern of mucins in IPMNs, we classified IPMNs into three histologic subtypes; IPMN-dark cell type ($n = 19$), IPMN-clear cell type ($n = 10$), and IPMN-compact cell type ($n = 4$).

Results. IDC, with a poor outcome, showed a pattern of MUC1(+), MUC2(−), and MUC5AC(+ or −). In contrast, IPMN-dark cell type tumors, with a fairly favorable outcome, showed a pattern of MUC1(−), MUC2(+), and MUC5AC(+), and IPMN-clear cell type tumors, with a favorable outcome, showed a pattern of MUC1(−), MUC2(−), and MUC5AC(+). On the other hand, IPMN-compact cell type tumors showed a pattern of MUC1(+), MUC2(−), and MUC5AC(+). In IPMN-dark cell type tumors with carcinomatous change showing invasive growth, the invasive areas acquired a characteristic of MUC1 expression that was usually seen in IDC, although their main noninvasive lesions showed no MUC1 expression. The IPMN-compact cell type tumors usually showed high cellular atypia and frequent MUC1 expression, even in the noninvasive areas.

Conclusions. Our study of the mucin expression pattern in IDC and IPMN shows that this pattern may be related to the biological behavior of pancreatic tumors and their malignant potential.

Key words Pancreas · Invasive ductal carcinoma · Intraductal papillary-mucinous neoplasm · Mucin · Biological behavior

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Introduction

Invasive ductal carcinoma of the pancreas (IDC) usually shows markedly invasive growth and has a poor prognosis. In 1980, Ohhashi and Takagi[1] described a different type of pancreatic tumor, which showed dilatation of the pancreatic duct, swelling of the papilla of Vater, and copious mucus secretion. This type of pancreatic tumor, characterized by its comparatively benign biological behavior, is known as intraductal papillary-mucinous neoplasm of the pancreas (IPMN).[2]

Mucins are high molecular weight glycoproteins that have oligosaccharides attached to serine or threonine residues of the mucin core protein backbone by O-glycosidic linkages. Alterations in the glycosylation of mucins have been described in cancer.[3–9] These alterations include aberrant glycosylation, resulting in truncated oligosaccharide side chains such as the sialyl-Tn antigen,[6,7] and incomplete glycosylation, resulting in the accumulation of core oligosaccharide structures such as the Tn antigen.[6,8] We reported that sialyl-Tn antigen was expressed frequently in many adenocarcinomas.[7] We also frequently observed the simultaneous expression of Tn and sialyl-Tn antigens in carcinomas of the pancreas, intrahepatic bile duct, ampulla of Vater, and ovary.[10–14] However, there was no difference in the expression of Tn and sialyl-Tn between IDC and IPMN.[10]

Consequently, we aimed to study mucin core protein (apomucin) expression in pancreatic tumors and the relationship of this expression with malignant potential. During the past several years, core proteins for several human mucins (MUC1-MUC9) have been identified.[15–23] These mucin genes are differentially expressed by different cells and organs. Because the synthesis and secretion of mucin is a common feature of glandular epithelial tissues, the expression of mucin antigens has been investigated mainly in adenocarcinomas.[3,10–14,24–26]

MUC1 is a membrane-associated glycoprotein, consisting of three distinct domains: (1) an amino-terminal region containing a hydrophobic signal sequence and degenerated tandem repeats; (2) around 30 to 90 almost conserved tandem repeats of 20 amino acids; and (3) a carboxyl-terminal region containing degenerate repeats, a membrane spanning region of 31 amino acids, and a cytoplasmic tail of 69 amino acids.[15,27–32] Soluble forms of MUC1 that lack the cytoplasmic tails have been reported. The MUC1 mucin mRNA is detected in most epithelial tissues,[33] and high levels of expression are seen in breast and pancreas. MUC2 is expressed in goblet cells of the colon, small intestine, and airways.[3,34–37] MUC5AC mucin is one of the two main types of mucin that are abundantly present in the stomach, i.e., MUC5AC and MUC6.[38,39]

In order to evaluate the correlation between mucin antigen expression in pancreatic tumors and their malignant potential, we studied the expression of MUC1 mucin (membrane-bound mucin), MUC2 mucin (intestinal-type secretory mucin) and MUC5AC mucin (gastric-type secretory mucin) in IDCs and IPMNs.

Tissue samples

Immunohistochemistry (IHC) and in situ hybridization (ISH) were done on serial sections of formalin-fixed paraffin-embedded tissues of 79 pancreatic tumors (46 IDCs and 33 IPMNs) by the methods described in our previous studies.[10,11,40,41] The samples were collected with the approval of the local Ethics Committee. Normal pancreatic tissue and normal gastric mucosa tissue, obtained from the normal areas of the surgically resected materials, were used as controls.

Classification of IPMN into three subtypes

IPMNs, which usually show expansive growth and have a favorable prognosis, demonstrated distinct patterns of mucin antigen expression compared with the patterns in IDCs.[10,11] To clarify the precise mucin expression patterns, we classified IPMNs into three distinct histologic subtypes, as reported previously.[41] The three types were:

(1) "Dark cell type", composed of dark columnar cells usually showing villous configuration (n = 19). In the 19, IPMN-dark cell type tumors, a carcinomatous component was observed in 17 cases, of which 6 had lesions of apparent invasive mucinous carcinoma, as described later. The other 2 cases had an adenomatous component only.
(2) "Clear cell type", composed of clear columnar cells usually showing a papillary configuration (n = 10). In the 10 IPMN-clear cell type tumors, a carcinomatous component was observed in 3 cases. The other 7 cases had an adenomatous component only.
(3) "Compact cell type", which may be the same tumor as the "intraductal oncocytic papillary neoplasm" reported by Adsay et al.,[42] is composed of stratified oncocytic cells (n = 4). The tumor cells of this type showed high cellular atypia, and all 4 IPMN-compact cell type tumors had a carcinomatous component.

Immunohistochemistry

Antibodies

IHC was carried out with the following antibodies:

(a) Monoclonal antibody (MAb) "DF3" (mouse IgG; Toray-Fuji Bionics, Tokyo, Japan) to detect MUC1 mucin.[15,43] The antigen detected by MAb DF3 is designated "MUC1/DF3".
(b) Polyclonal antibody (PAb) "anti-MUC2" (produced by using a synthetic MUC2 peptide containing the 23-amino-acid tandem repeat peptide of MUC2 apomucin, PTTTPISTTTMVTPTPTPTG TQT) to detect MUC2 mucin.[16,34,44]
(c) PAb "anti-MUC5AC" (produced by using a synthetic MUC5AC peptide containing the 16-amino-acid tandem repeat peptide of MUC5AC apomucin, TTSTTSAPTTSTTSAP) to detect MUC5AC mucin (our unpublished data, 1998).

Biotinylated affinity-purified horse antimouse IgG, goat antirabbit IgG, and avidin-biotinylated peroxidase complex (ABC) were purchased from Vector Laboratories (Burlingame, CA, USA) as the Vectastain ABC Kit.

Staining procedure

Immunohistochemical stainings were done by an immunoperoxidase method, using the ABC complex, as described previously.[10,12–14] Briefly, each section was deparaffinized with xylene. Endogenous peroxidase was blocked by incubating the sections in 0.3% hydrogen peroxide in absolute methanol at room temperature for 30 min. After hydration in decreasing concentrations of ethanol in water, the sections were washed in 0.01 mol/l phosphate-buffered saline (PBS), pH 7.4. Then, 2% horse or goat serum in PBS was applied for 30 min at room temperature to prevent nonspecific staining. In the staining with each antibody, the sections were incubated with dilutions of the primary antibodies (DF3, 1:10; anti-MUC2, 1:40000; anti-MUC5AC, 1:4000) in PBS with 1% bovine serum albumin for 16 h at 4 °C. The

sections were washed three times with PBS and incubated with the biotinylated secondary antibodies, and again washed three times with PBS. All the sections were then treated with ABC for 30 min. After three washes with PBS, the sections were finally reacted with diaminobenzidine substrate for 10 to 30 min for visualization, rinsed with tap water, counterstained with hematoxylin, and mounted. Reaction products were not present when nonimmune serum or PBS was used instead of the primary antibodies.

In situ hybridization

Synthesis of MUC1, MUC2, and MUC5AC complementary DNAs (cDNAs) and labeling procedure

Synthetic oligonucleotide (48-mers) corresponding to each tandem repeat sequence of MUC1, MUC2, and MUC5AC in the antisense direction was used as the antisense probe.[45] Another synthetic oligonucleotide (48-mers) in the sense direction of each MUC1, MUC2, and MUC5AC was used as the sense probe for control staining, as follows:

(a) MUC1 antisense probe, the tandem repeat sequence of MUC1 in the antisense direction (5′ GTC CGG GGC CGA GGT GAC ACC GTG GGC TGG GGG GGC GGT GGA GCC CGG 3′); MUC1 sense probe for the control staining, the tandem repeat sequence of MUC1 in the sense direction (5′ CCG GGC TCC ACC GCC CCC CCA GCC CAC GGT GTC ACC TCG GCC CCG GAC 3′).
(b) MUC2 antisense probe, the tandem repeat sequence of MUC2 in the antisense direction (5′ GGT CTG TGT GCC GGT GGG TGT TGG GGT TGG GGT CAC CGT GGT GGT GGT 3′); MUC2 sense probe for control staining, the tandem repeat sequence of MUC2 in the sense direction (5′ ACC ACC ACC ACG GTG ACC CCA ACC CCA ACA CCC ACC GGC ACA CAG ACC 3′).
(c) MUC5AC antisense probe, the tandem repeat sequence of MUC5AC in the antisense direction (5′ AGG GGC AGA AGT TGT GCT CGT TGT GGG AGC AGA GGT TGT GCT GGT TGT 3′); MUC5AC sense probe for control staining, the tandem repeat sequence of MUC5AC in the sense direction (5′ ACA ACC AGC ACA ACC TCT GCT CCC ACA ACG AGC ACA ACT TCT GCC CCT 3′).

All the oligonucleotide probes were labeled by 3′ digoxigenin tailing, using an oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany). The digoxigenin labeling was confirmed by with a DIG detection kit (Boehringer Mannheim).

The preservation of RNA in the paraffin sections was evaluated with a digoxigenin-labeled 37-meric oligonucleotide that is complementary to the Poly-A tail.

In situ hybridization (ISH) method

The ISH method was adapted from standard protocols, using an ISH kit (Kreatech Diagnostics, Amsterdam, The Netherlands), as previously described.[40,41] Briefly, each section was deparaffinized, dehydrated in ethanol, and air-dried. The sections were digested by proteinase K (10 mg/ml) in 10 mM Tris-HCl, pH 8.0, containing 1 mM ethylene diamine tetraacetic acid (EDTA) for 30 min at 37 °C. After a washing in Tris buffered saline, pH 7.4 (TBS), the sections were dehydrated in ethanol, and air-dried. The sections were then equilibrated in prehybridization solution for 2 h at 37 °C. After being washed in TBS, the sections were dehydrated in ethanol, and air-dried. The digoxigenin-labeled probes were heated for 5 min at 100 °C and chilled on ice, then mixed in prehybridization solution. The mixture was applied to each section, and the sections were incubated overnight at 37 °C. The sections were washed three times in TBS at 37 °C, and then washed two times in TBS at room temperature. The sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin (Fab fragment) for 20 min at room temperature. After three washes in TBS, a mixture of nitroblue tetrazolium solution and 5-bromo, 4-chloro, 3-indolyl-phosphate solution was added, for color development. After the reaction was stopped, the sections were counterstained with nuclear fast red.

Distribution of mucins in normal pancreatic tissue

In the normal pancreatic tissue, MUC1/DF3 was expressed mainly on the luminal surface of the centroacinar cells, intralobular ducts, and interlobular ducts, but not in the main pancreatic ducts, acini, or islets.

MUC2 and MUC5AC were never expressed in the normal pancreatic tissue. MUC2 was always expressed in the perinuclear region of the goblet cells of normal intestinal mucosa. Normal duodenal mucosa adjacent to pancreatic tissue showed MUC2 expression in the perinuclear region of the goblet cells, which seemed to represent an internal positive control for the study of pancreatic tumors. MUC5AC was always detected in the surface mucous cells in the normal gastric mucosa, which was examined as the positive control for MUC5AC expression.

Table 1. Expression of mucins in pancreatic tumors

	MUC1/DF3			MUC2			MUC5AC		
	−	+	++	−	+	++	−	+	++
IDC (n = 46)[a]	2	20	24	46	0	0	23	10	5
IPMN-dark cell type (n = 19)	19	0	0	1	4	14	3	7	9
IPMN-clear cell type (n = 10)	9	0	1	10	0	0	0	0	10
IPMN-compact cell type (n = 4)	1	2	1	3	1	0	0	1	3

IDC, Invasive ductal carcinoma; IPMN, intraductal papillary-mucinous neoplasm
[a] MUC5AC was examined in 38 of the 46 IDCs

Expression of mucins in pancreatic tumors

MUC1 expression was examined mainly by IHC, because the results of ISH for MUC1 mRNA were variable. ISH was appropriate for examining MUC1 mRNA, when specimen fixation was successful. The expression of MUC2 and MUC5AC mRNAs, detected by ISH, and the expression of MUC2 and MUC5AC apomucins, detected by IHC, usually coincided. Table 1 shows a summary of mucin expression, detected by IHC, in pancreatic tumors. The results of the antibody stainings were graded according to the percentages of positively stained neoplastic cells (i.e., −, <5%; +, 5%–50%; and ++, >50% of the neoplastic cells stained).

Mucin expression in IDC

In IDCs that showed invasive growth and a poor prognosis, MUC1/DF3 was expressed in most cases (44 of 46; 96%), whereas MUC2 was not expressed (0 of 46 cases; 0%) (Fig. 1). The MUC1/DF3 expression was observed in the cell apex, cytoplasm, and luminal contents of the carcinoma cells. There was no difference in the MUC1/DF3 expression pattern between well and poorly differentiated components. MUC5AC expression was examined in 38 of the 46 IDCs. Fifteen of the 38 cases (39%) showed expression of MUC5AC in the carcinoma cells.

In summary, IDC showed a pattern of MUC1(+), MUC2(−), and MUC5AC(+ or −). Even in the IDC patients with a good prognosis who survived for more than 3 years (n = 6), this expression pattern was the same.

Mucin expression in IPMN

IPMN-dark cell type

MUC1/DF3 was not expressed in any of the 19 IPMN-dark cell type tumors (0/19; 0%), whereas MUC2 was expressed in 18 of them (18/19; 95%) (Fig. 2). The MUC2 expression was localized exclusively in the cytoplasm. MUC5AC was expressed in 16 of the IPMN-dark cell type tumors (16/19; 84%). The MUC5AC expression was seen in the cytoplasm of the tumor cells.

In the 19 IPMN-dark cell type tumors, 6 had lesions of apparent invasive mucinous carcinoma around the main noninvasive lesions. The invasive mucinous carcinoma lesions in these 6 cases showed MUC1/DF3 expression, although the main noninvasive lesions of the same 6 cases showed no MUC1/DF3 expression (Fig. 3).

IPMN-clear cell type

MUC1/DF3 was not expressed in nine of the ten IPMN-clear cell type tumors, being expressed in only one case (1/10; 10%), and MUC2 was not expressed in any of them (0/10; 0%) (Fig. 4). MUC5AC was expressed in all the cases examined (10/10; 100%). The MUC5AC expression was seen in the cytoplasm of the tumor cells.

IPMN-compact cell type

Three of the four IPMN-compact cell type tumors (3/4; 75%) showed MUC1/DF3, whereas MUC2 expression was expressed in one of them (1/4; 25%) (Fig. 5). MUC5AC was expressed in the cytoplasm of all the cases examined (4/4; 100%).

Different patterns of mucin expression in the IPMN subtypes

We have demonstrated that there are three distinct histological subtypes of IPMN: IPMN-dark cell type, IPMN-clear cell type, and IPMN-compact cell type. Fukushima et al.[46] also published a report outlining a similar morphological classification scheme. We also found that the three histological subtypes showed different patterns of mucin expression. Both the IPMN-dark cell type and the IPMN-clear cell type showed rare MUC1/DF3 expression, unlike IDC, which usually showed MUC1/DF3 expression. On the other hand, there was an apparent difference in MUC2 expression between IPMN-dark cell type and IPMN-clear cell type. The IPMN-dark cell type showed MUC2 expression,

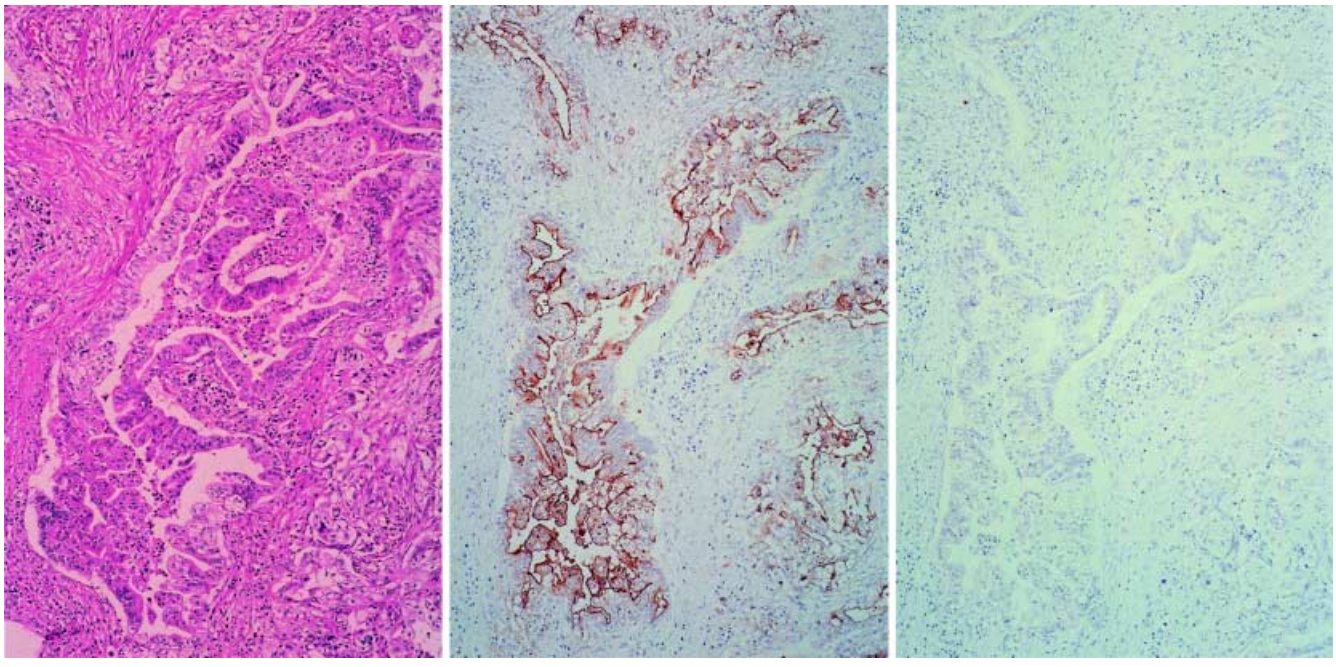

Fig. 1. **A** In invasive ductal carcinoma (IDC), MUC1/DF3 was expressed (**B**), whereas MUC2 was not expressed (**C**). **A**, **B**, **C** ×80

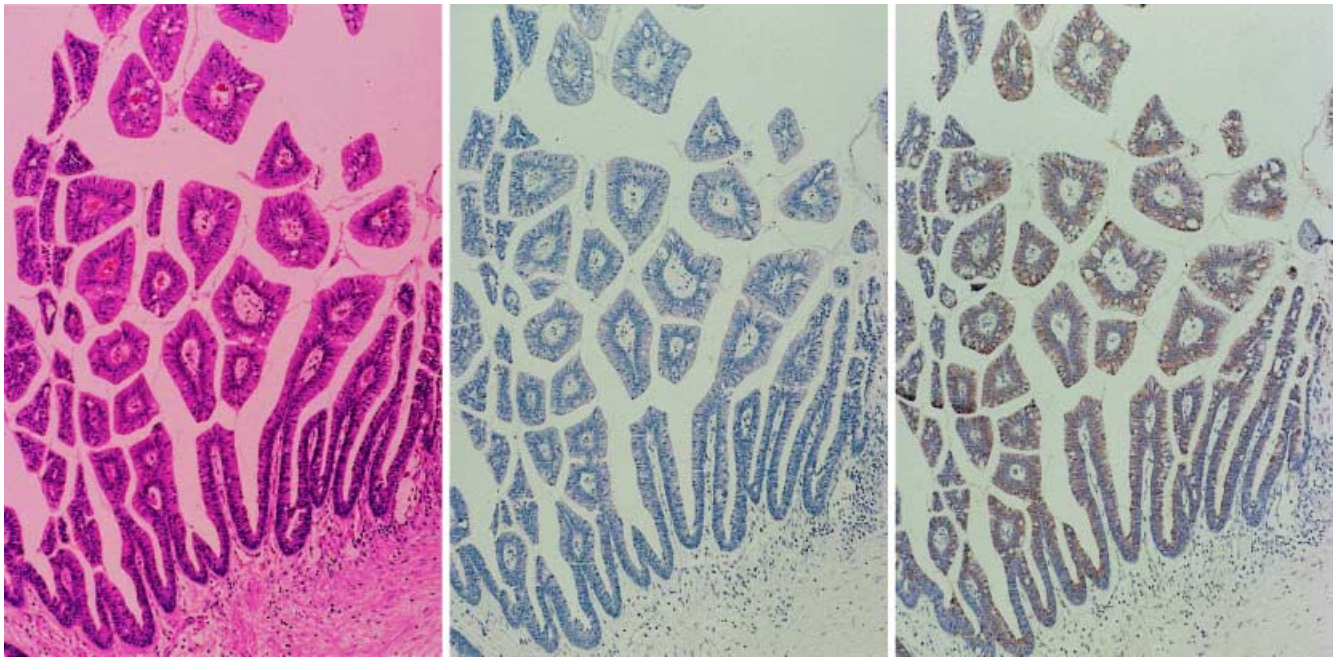

Fig. 2. **A** In intraductal papillary-mucinous neoplasm (IPMN)-dark cell type, MUC1/DF3 was not expressed (**B**), whereas MUC2 was expressed (**C**). **A**, **B**, **C** ×80

whereas the IPMN-clear cell type showed no MUC2 expression.

The tumor cells of the IPMN-dark cell type are morphologically similar to those of colorectal villous adenoma, and they expressed both MUC2 and MUC5AC. This pattern is similar to the MUC2 and MUC5AC expression in the rectosigmoidal villous adenoma reported by Buisine et al.[47] The tumor cells of the IPMN-clear cell type are morphologically similar to the surface mucous cells of gastric mucosa, and they did not express

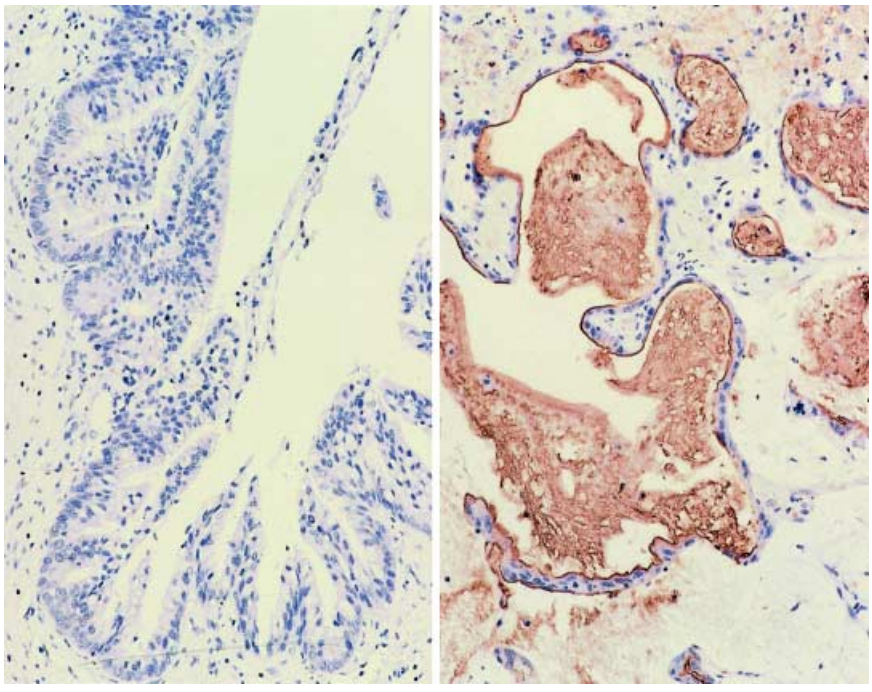

Fig. 3. A The main lesion of IPMN-dark cell type showed no MUC1/DF3 expression; however, the lesions of invasive mucinous carcinoma observed in the same case showed MUC1/DF3 expression (**B**). In the area of the invasive mucinous carcinoma (**B**), positive MUC1/DF3 staining was observed not only in the carcinoma cells themselves but also in the secreted mucinous substance and surrounding stroma. **A**, **B** ×160 (**A** and **B** were reproduced from reference 48, with permission from the publisher)

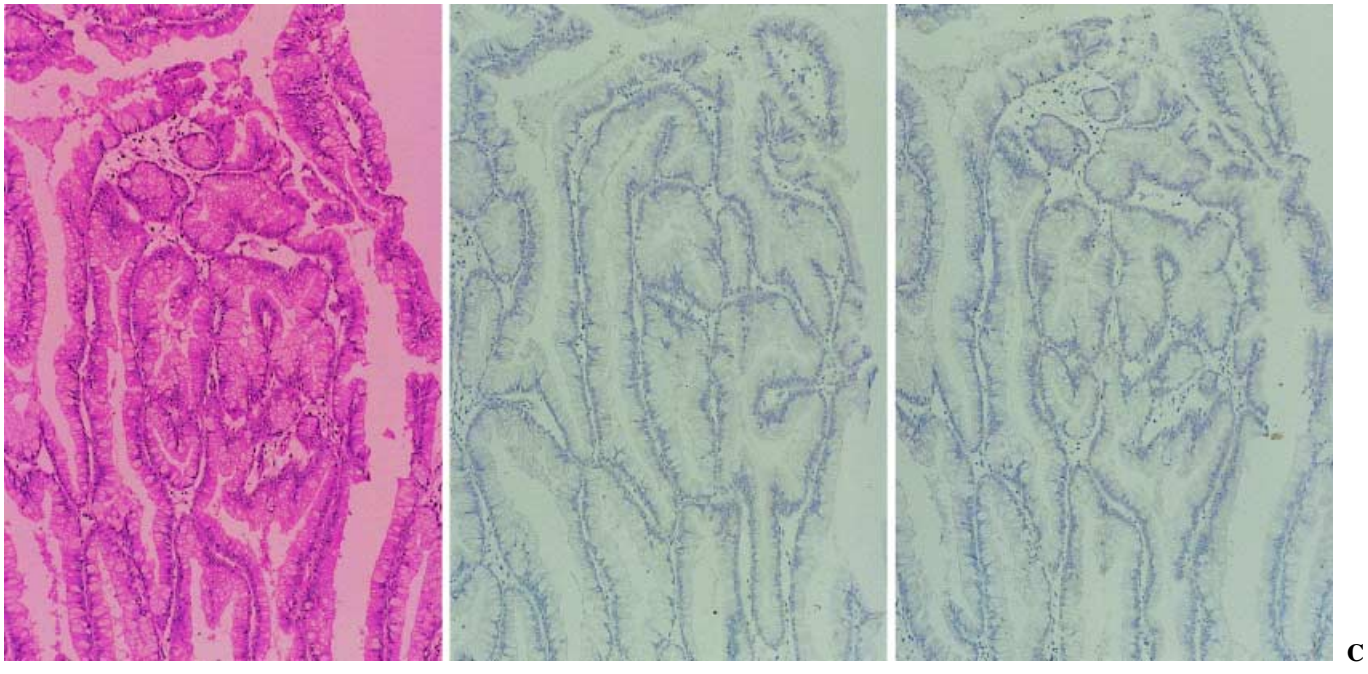

Fig. 4. A In IPMN-clear cell type, neither MUC1/DF3 (**B**) nor MUC2 (**C**) was expressed. **A**, **B**, **C** ×80 (**A** was reproduced from reference 41, with permission from the publisher)

MUC2, but they did express MUC5AC. This pattern of mucin expression is also seen in normal gastric mucosa. From these findings, it seems that IPMN-dark cell type has properties similar to those of colorectal villous adenoma, not only from the morphological aspect but also in terms of mucin gene expression, and IPMN-clear cell type seems to have properties similar to those of gastric surface mucous cells.

Morphologically, the tumor cells of the IPMN-compact cell type differed greatly from the other two

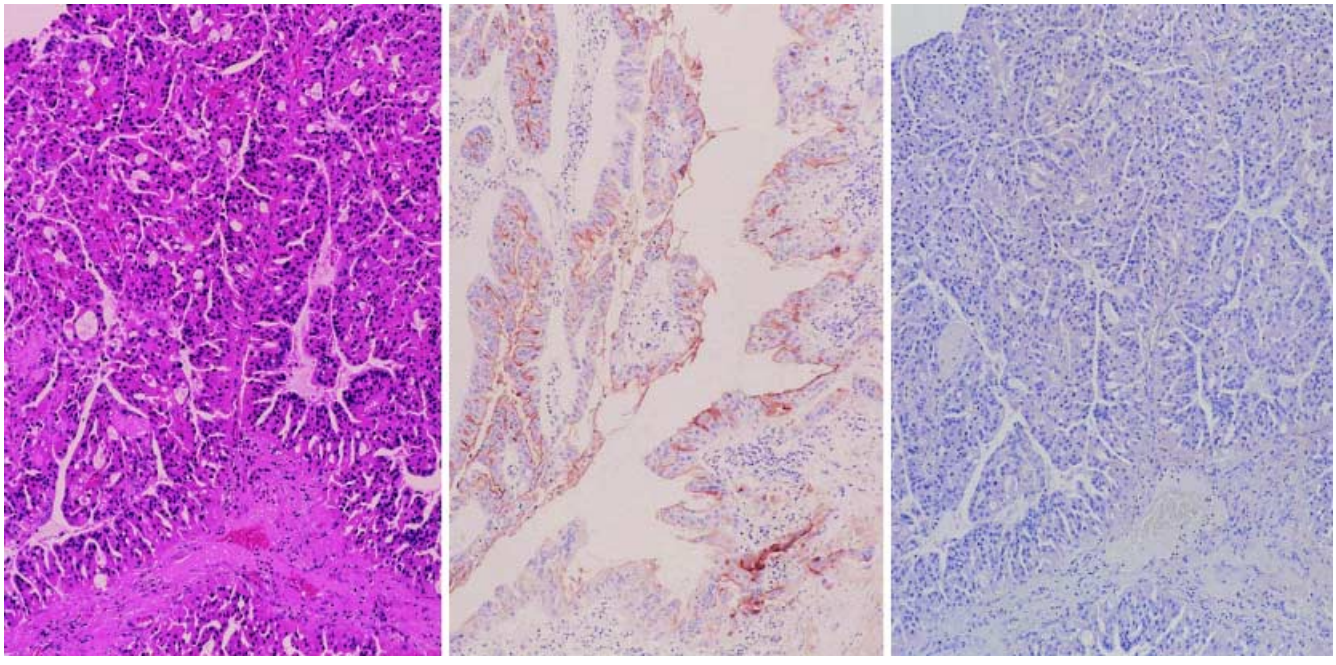

Fig. 5. **A** In IPMN-compact cell type, MUC1/DF3 was expressed (**B**), whereas MUC2 was not expressed in this case (**C**). **A**, **B**, **C** ×80

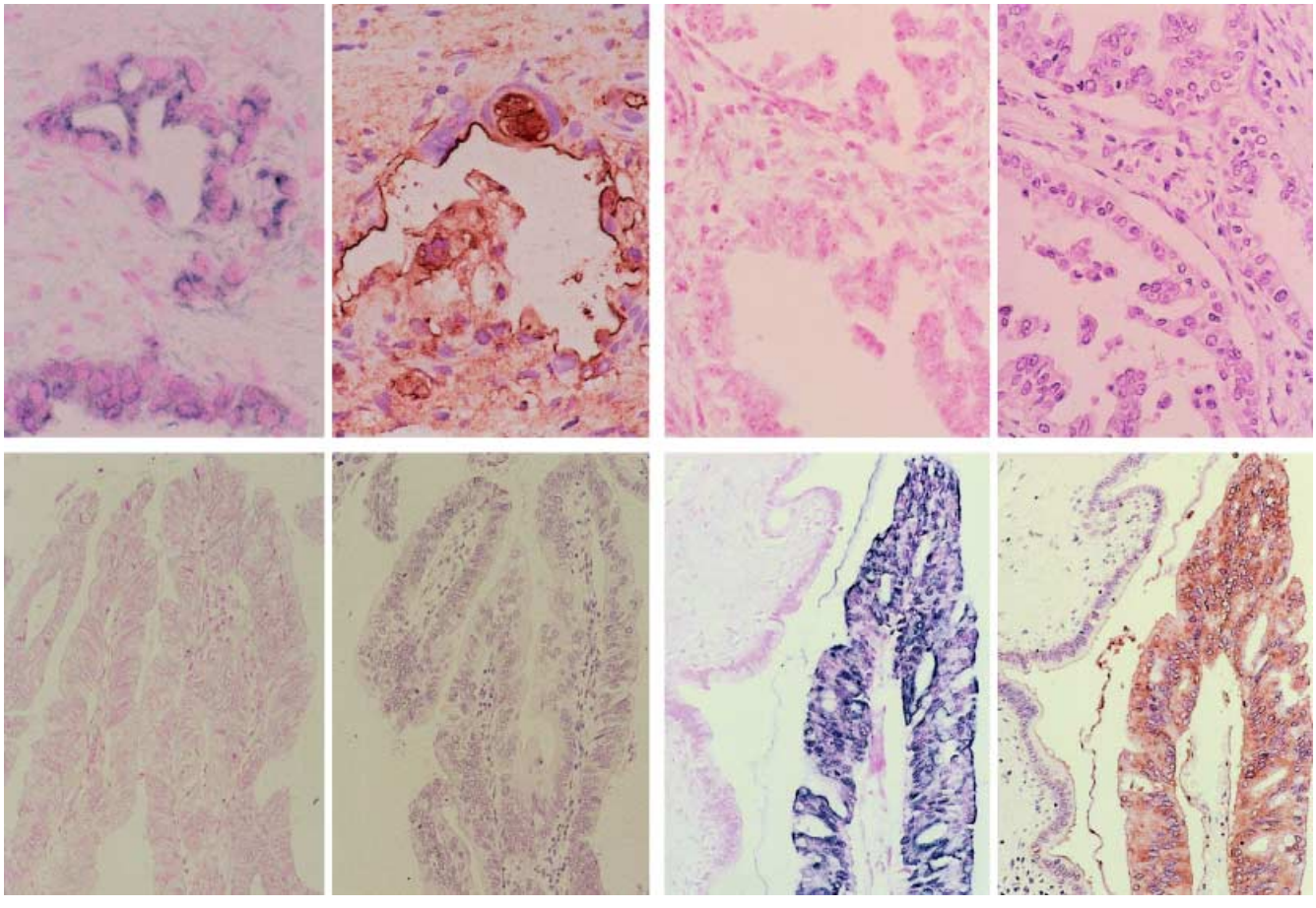

Fig. 6A–D. In situ hybridization (*left side* of **A**, **B**, **C** and **D**) and immunohistochemistry (*right side* of **A**, **B**, **C** and **D**) for MUC1 and MUC2 in IDC (**A**, **B**) and IPMN-dark cell type (**C**, **D**). IDC showed MUC1 mRNA(+) and MUC1/DF3 apomucin(+) expression (**A**), but MUC2 mRNA(−) and MUC2 apomucin(−) expression (**B**). In contrast, IPMN-dark cell type showed MUC1 mRNA(−) and MUC1/DF3 apomucin(−) expression (**C**), but MUC2 mRNA(+) and MUC2 apomucin(+) expression (**D**). **A**, **B** ×160; **C**, **D** ×80 (**D** was reproduced from reference 40, with permission from the publisher)

types and showed MUC1/DF3 expression, like IDC. They showed MUC5AC expression, but did not show MUC2 expression. Further analysis of this IPMN-compact cell type needs to be done, and we are currently looking for more cases.

In our previous studies,[48,49] we reported that MUC1/DF3 was expressed in 4 (25%) of 16 IPMNs examined. In those studies, we analyzed mucin expression without classification of the IPMNs into the three subtypes. The classification of the IPMNs into the three subtypes in the current study clarified that, in the 4 IPMNs with positive MUC1/DF3 expression, 1 case was IPMN-clear cell type, and the other 3 cases were IPMN-compact cell type.

Clinical outcome of patients with IPMN

Two patients with IPMN-dark cell type who died of other diseases were excluded from the outcome analysis in the current study; thus, we analyzed the findings for 17 patients with IPMN-dark cell type, 10 patients with IPMN-clear cell type, and 4 patients with IPMN-compact cell type.

In the 17 patients with IPMN-dark cell type, the tumor lesions of 6 patients showed apparent invasive features of mucinous carcinoma. Three of these 6 patients died of recurrence of carcinoma, although they had been treated by curative tumor resection. One of them died of peritonitis carcinomatosa, and the other 2 died of liver metastasis. The other three patients with mucinous carcinoma were alive during follow-up periods ranging from 1 to 155 months. The remaining 11 patients are all alive and had no recurrence during follow-up periods ranging from 2 to 182 months.

Of the ten patients with IPMN-clear cell type, one patient, who had been treated by segmental resection, died of recurrence of carcinoma in the remnant pancreas. In this patient, the surgical margin of the resected specimen was involved with carcinomatous change at the time of surgery. The other nine patients were all alive and had no recurrence during follow-up periods ranging from 21 to 126 months.

Of the four patients with IPMN-compact cell type, one patient, who had been treated by segmental resection, died of recurrence of carcinoma in the remnant pancreas. In this patient, the surgical margin of the resected specimen was involved with carcinomatous change at the time of surgery. The other three patients were all alive and had no recurrence during follow-up periods ranging from 17 to 79 months.

We found that, on analysis using the Kaplan-Meier method, the outcomes of the patients with IPMN-dark cell type or IPMN-clear cell type were significantly better than the outcomes of those with IDC (unpublished data). The number of patients with IPMN-compact cell type was too small to be analyzed.

Precise discussion of mucin expression in IDC and IPMN-dark cell type tumors

In 1993, we[10] first outlined a clear distinction in the immunohistochemical staining of MUC1/DF3 and MUC2 between IPMN and IDC; IDC showed a MUC1/DF3(+) and MUC2(−) expression pattern, whereas IPMN showed a MUC1/DF3(−) and MUC2(+) expression pattern (Table 1; Figs. 1 and 2). According to the classification mentioned above, all the IPMNs examined in our previous study[10] were "IPMN-dark cell type". Because the precise analysis of mucin expression in IPMN-clear cell type and IPMN-compact cell type has yet to be completed, in the following description, we focus on the comparison of mucin expression in IDC and IPMN-dark cell type.

Mechanism of MUC1 and MUC2 expression in IDC and IPMN-dark cell type tumors

Two hypotheses are put forward to explain the observed patterns of expression of MUC1 and MUC2 in IDC and IPMN-dark cell type. (1) IDC produces MUC1 apomucin, but does not produce MUC2 apomucin, whereas IPMN-dark cell type produces MUC2 apomucin, but does not produce MUC1 apomucin, or (2) both types of tumors produce MUC1 and MUC2 apomucins, but carbohydrate side chains mask the MUC2 apomucin epitope in IDC, resulting in apparently negative MUC2 staining, whereas carbohydrate side chains mask the MUC1 apomucin epitope in IPMN, resulting in negative MUC1 staining.

In order to determine which of these hypotheses explains the mechanism of MUC1 and MUC2 expression in IDC and IPMN-dark cell type tumors, we investigated the expression mechanism of MUC1 and MUC2 mucins in IDCs and IPMN-dark cell type tumors by using a combination of ISH and IHC.

ISH and IHC for MUC1

In the MUC1 ISH, using specimens that were successfully fixed, IDC showed MUC1 mRNA(+) and MUC1/DF3 apomucin(+) (Fig. 6A), whereas IPMN-dark cell type showed MUC1 mRNA(−) and MUC1/DF3 apomucin(−) (Fig. 6C).

ISH and IHC for MUC2

We have demonstrated the expression of MUC2 mRNA by ISH, by using a probe for MUC2 cDNA, pHAM1 (provided by Dr. Basbaum, University of California, San Francisco, CA USA), as well as by using

a synthesized probe for MUC2 cDNA, in IPMNs and IDCs.[40] The two probes showed identical results. The expression of the MUC2 apomucin was also evaluated by IHC in the same cases. Neither MUC2 mRNA nor MUC2 apomucin was detected in normal pancreas. IDC showed MUC2 mRNA(−) and MUC2 apomucin(−) (Fig. 6B), whereas IPMN-dark cell type tumors showed MUC2 mRNA(+) and MUC2 apomucin(+) (Fig. 6D). In the IPMN-dark cell type tumors, the localization of MUC2 mRNA and that of MUC2 apomucin usually coincided. These results indicate that IDCs synthesize neither the mRNA nor the protein of MUC2, whereas IPMN-dark cell type tumors synthesize both the mRNA and the protein of MUC2.[40]

The data showing that neither MUC2 mRNA nor MUC2 apomucin was expressed in pancreatic IDCs are consistent with the results of previous studies of mucin gene expression, using Northern and Western blot analyses in cultured cell lines derived from pancreatic IDCs, in which it was also reported that pancreatic cancer cells did not express detectable MUC2 mRNA or MUC2 apomucin.[50–52] Ho et al.[36] reported that pancreatic carcinomas showed only occasional focal staining with MUC2-related antibodies, although they did not perform RNA analysis in pancreatic cancer specimens. Recently, Balague et al.[45] reported an ISH study in which MUC2 mRNA was not detected in seven of eight ductal adenocarcinomas, and low levels of MUC2 mRNA were detected in one adenocarcinoma. Their study included eight ductal adenocarcinomas that were probably IDCs, using our classification to characterize the histological sections presented in that report. The results in our study were consistent with those reported by Balague et al.[45]

Conclusions reached from the ISH and IHC studies

We found that IDCs, showed MUC1 mRNA(+) and MUC1/DF3(+) expression, but MUC2 mRNA(−) and MUC2(−) expression. In contrast, IPMN-dark cell type tumors showed MUC1 mRNA(−) and MUC1/DF3(−) expression, but MUC2 mRNA(+) and MUC2(+) expression. These results confirmed hypothesis (1) above, indicating that IDCs usually produce MUC1 apomucin but do not produce MUC2 apomucin, whereas IMPN-dark cell type tumors usually do not produce MUC1 apomucin, but do produce MUC2 apomucin.

Invasive proliferation of IPMN-dark cell type tumors, and alteration of mucin expression

Our previous study disclosed that the MUC1 mucin gene product was expressed in the invasive areas of IPMN-dark cell type tumors, when the tumors showed carcinomatous change and began to show invasive growth.[48] IPMN-dark cell type tumors, in contrast to IDCs, which showed MUC1/DF3 expression, usually showed no MUC1/DF3 expression in the main lesions without invasive growth (Fig. 3A). As described in the section above "Clinical outcome of patients with IPMN", of the 19 IPMN-dark cell type tumors, 6 showed apparent invasive features of mucinous carcinoma. The lesions of the invasive mucinous carcinomas acquired the characteristic of MUC1/DF3 expression (Fig. 3B), which is usually seen in IDCs.

Significance of MUC1 expression in IDC and IPMN-dark cell type tumors

Sessa et al.[2] have reported that most IPMNs differ from IDCs in terms of histological structure, marker expression, and behavior. We demonstrated that there was a clear difference in the pattern of mucin expression between IDCs and IPMN-dark cell type tumors. MUC1/DF3 was highly expressed in IDCs, but was not expressed in IPMN-dark cell type tumors.

In our previous studies,[11,53] we analyzed clinicopathological factors such as "lymphatic permeation status" and "lymph node metastasis status" in IDCs and IPMN-dark cell type tumors, according to the *General rules for the study of pancreatic cancer, 4th edition*, of the Japan Pancreas Society (1993). IDCs with frequent MUC1/DF3 expression showed aggressive lymphatic permeation and were positive for lymph node metastases. In contrast, IPMN-dark cell type tumors without MUC1/DF3 expression showed less aggressive lymphatic permeation and were negative for lymph node metastases.

As described previously, MUC1 is a transmembrane glycoprotein with an extracellular domain consisting of a variable number of highly conserved tandem repeats of 20 amino acids. The overexpression of MUC1 by cultured cells inhibits their aggregation, possibly because of its large, extended, and rigid structure.[54] MUC1 expressed in tumors may function as an anti-adhesion molecule that inhibits cell-cell adhesion, inducing the release of cells from the tumor.[54,55] The overexpression of MUC1 on the membranes of cultured cells may also inhibit interaction between cytotoxic lymphocytes and tumor cells.[56] Cells with MUC1 overexpression showed inhibition of integrin-mediated cell adhesion to extracellular matrix.[57] Agrawal et al.[58] showed that cancer-associated MUC1 mucin, and synthetic tandem repeats of MUC1 mucin core peptide, suppressed human T-cell proliferative responses, and they reported that high levels of MUC1 mucin were correlated with immunosupression in adenocarcinoma patients.

Thus, it appears that MUC1 mucin expression may be related to the invasiveness or metastatic behavior of IDC, whereas the lack of MUC1 expression may be related to the less invasive characteristics of IPMN-dark

cell type tumor. Furthermore, the MUC1/DF3 expression in the invasive mucinous carcinoma of IPMN-dark cell type, as shown in Fig. 3B, may be related to the acquisition of invasive properties in noninvasive types of tumor cells.

Significance of MUC2 and MUC5AC expression in IDC and IPMN-dark cell type tumors

In contrast to MUC1, which is a membrane-bound mucin, MUC2 is a secretory mucin that forms long polymers as a result of disulfate bonding, and has a high viscosity in solution.[35,59,60] The expression of MUC2, an abundant extracellular intestinal type secretory mucin with high viscosity, by a majority of IPMN-dark cell type tumors may be correlated with the site-restricted growth of tumors that display lower levels of invasion and metastasis. The cysteine-rich domains of MUC2[35] may play a role in regulating cell proliferation,[4] which may, in turn, contribute to the low malignant potential of IPMN-dark cell type tumors, compared with IDCs. Mucin glycoproteins are heavily glycosylated, with approximately 50%–85% of the total molecular weight accounted for by carbohydrates.[3] MUC2 expression in IPMN-dark cell type tumors was usually intracytoplasmic and was not seen in the secreted mucin fractions. The observed pattern of the intracytoplasmic expression suggests the following possibilities: (a) precursor forms of the MUC2 polypeptide back-bone are not heavily glycosylated in early compartments of the Golgi apparatus, and, therefore, these forms are readily detected by antibodies; (b) heavily glycosylated MUC2 mucin is present in secreted fractions and is difficult to detect with antibodies, owing to the blocking of epitope binding on the polypeptide backbone by carbohydrate side chains. Further investigation of carbohydrate structures linked to the MUC2 polypeptide backbone in the secreted mucin may be important to our understanding of the biological role of the secreted and extracellular mucin in IPMN-dark cell type tumors.

The secretory mucins, MUC2 and MUC5AC, have similar characteristics. Because both form gels of high viscosity,[5,19,35,59,60] the production of MUC5AC by the majority of IPMNs may also be related to a decrease in invasion and metastasis, and a concurrent increase in the size of the primary tumors. Recently, Balague et al.[45] reported the results of an ISH study in which MUC5AC mRNA was detected in 63% (5 of 8) of ductal adenocarcinomas. These eight ductal adenocarcinomas appeared to correspond to IDCs (using our classification to characterize the histological sections presented in that report). The results of our present study showed that 39% (15 of 38) of the IDCs expressed MUC5AC. Further detailed comparative studies that examine histologic classification versus MUC5AC expression need to be done to explain the discrepancy between our data and the data presented by Balague et al.[45]

Expression of MUC1 mucins with different levels of glycosylation in IDC and IPMN — a preliminary study

The MAb DF3, which was used in this study, identifies MUC1 apomucin; however, MAb DF3 binding to protein may be enhanced by the presence of sialic acid.[43] There are differences in the glycosylation of MUC1 in different human tumors and normal cell types. MUC1 expressed by breast carcinomas is reactive with MAb SM-3, which detects poorly glycosylated MUC1 mucin core peptide, whereas normal breast tissue shows little or no reactivity with SM-3.[61–63] This phenomenon is explained, in part, by the finding that MUC1 core epitopes are masked by the carbohydrate side chains produced by normal breast epithelial cells,[62,64] whereas the carbohydrate side chains of MUC1 produced by breast adenocarcinomas are shorter or less densely distributed than those produced by normal cells.[65] On the other hand, the level of expression of glycosylated MUC1 mucin detected by MAb HMFG-1 is high in advanced stages of colorectal carcinomas.[66] We are interested in investigating the relationship between the glycosylation of MUC1 mucin expressed in pancreatic tumors and the malignant potential of the tumors.

Different glycoforms of MUC1 can be identified by different monoclonal antibodies.[15,61,62,67,68] Three MAbs developed by Joyce Taylor-Papadimitriou's group (SM-3, HMFG-2, and HMFG-1)[61,64] have been shown to detect different glycoforms of MUC1. The minimum core peptide epitopes recognized by SM-3, HMFG-2, and HMFG-1 are PDTRP[61,64], DTR,[64] and PDTR,[69] respectively. MAb SM-3 detects poorly glycosylated MUC1 mucin core peptide.[61,62] The binding of MAbs HMFG-2 and HMFG-1 to core-protein epitopes is influenced by the carbohydrate chains;[62] HMFG-2 is able to bind to the epitope when shorter side chains are present, whereas HMFG-1 detects fully glycosylated MUC1 mucin, and its binding is particularly affected by sialic acid.[62,67,68] Yamamoto et al.[70] have developed a new MAb, MY.1E12, which is specific for sialylated-MUC1 mucin.

Our previous preliminary study of the expression of MUC1 glycoforms detected by the four MAbs described above (SM-3, HMFG-2, HMFG-1, and MY.1E12) was performed by using IHC to evaluate pancreas tumors (10 IDCs and 11 IPMNs),[49] although we had not classified the IPMNs into the subtyptes: IPMN-dark cell type, IPMN-clear cell type, and IPMN-compact cell type, in the preliminary study. The positive rates and/or staining densitites of the MUC1 mucin

antigens were higher in the IDCs than in the IPMNs. Among the MUC1 mucins examined by the four MAbs, sialylated-MUC1 mucin, detected by MY.1E12, showed a significant difference between IDCs and IPMNs. The sialylated MUC1 mucin was expressed in all the IDCs, but was not frequently observed in the IPMNs, although the other types of MUC1 glycoforms did not show significant differences between IDCs and IPMNs.

The exposure of cryptic polypeptide epitopes recognized by T and B cells[71–74] may result in carcinoma-specific cytotoxic lymphocytes that recognize MUC1 mucin core peptides,[27] which also serve as antigenic determinants for many anti-MUC1 mucin monoclonal antibodies.[61,62,64,75–80] On the other hand, tumor cells expressing sialomucin were shown to be less sensitive than those not expressing sialomucin to cytolysis by human lymphokine-activated killer lymphocytes.[81–84] Thus, high levels of cell surface sialomucin may be related to the escape from immune killing, resulting in the increased metastatic colonization of carcinoma cells.[84,85] In our study of the tumors of the pancreas, glycosylated MUC1 mucin; in particular, sialylated MUC1 mucin, was seen frequently in IDCs. The sialylation of the MUC1 mucin in IDC may be related to the invasive growth of IDC. A recent study of mucin in cultured pancreatic cancer cells disclosed that both sialyl-Le^a and sialyl-Le^x carbohydrate structures were present on MUC1.[86] However, the expression of sialylated MUC1 mucin detected by MAb MY.1E12 was different from that of other sialylated carbohydrate antigens, such as sialyl-Tn, sialyl-Le^a, and sialyl-Le^x,[49] and this difference should be taken into account in regard to the determination of the epitope of MAb MY.1E12.

A report by Nishimori et al.[87] showed that cell extracts of the human breast adenocarcinoma cell line MCF7 added two O-linked N-acetylgalactosamine glycosylation sites to the tandem repeat domain of the human MUC1 mucin core protein. In multiple copies of tandemly arrayed identical 20 amino-acid repeats having the sequence GVTSAPDTRPAPGSTAPPAH,[28,32] the two threonines underlined are glycosylated. More recent investigations with other cell lines[88] and recombinant enzymes have shown that the S (serine) at GSTAP can also be glycosylated.[89] The T (threonine) of PDTRP, which is included in the minimum peptide epitopes recognized by the monoclonal antibodies SM-3, HMFG-1, and HMFG-2,[62] and the remaining S (serine) in the tandem repeat sequence, are not glycosylated by tumor cell extracts; however, Müller et al.[90] reported that all five glycosylation sites (T and S) in the 20 amino-acid repeats of the human MUC1 mucin core protein were partially glycosylated by normal breast epithelial cells.

Further progress is needed in studies of the structures and mechanisms of the glycosylation of MUC1 mucin to clarify the biological role that carbohydrate side chain structures play in the invasive growth of pancreatic neoplasms. The expression of the different glycosylation levels of MUC1 mucin in each subtype of IPMN would also be an interesting future area of study, because our preliminary study was performed when we had not yet classified the IPMNs into subtypes.

Conclusion

In conclusion, IDC, with a poor prognosis, showed a pattern of MUC1(+), MUC2(−), and MUC5AC(+ or −). In contrast, IPMN-dark cell type tumors, with a fairly favorable prognosis, showed a pattern of MUC1(−), MUC2(+), and MUC5AC(+), and IPMN-clear cell type tumors, with a favorable prognosis, showed a pattern of MUC1(−), MUC2(−), and MUC5AC(+). On the other hand, IPMN-compact cell type, with high cellular atypia, showed a pattern of MUC1(+), MUC2(−), and MUC5AC(+).

Our study of the mucin expression pattern in IDC and IPMN shows that this pattern may be related to the biological behavior of pancreatic tumors and their malignant potential.

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