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Clinicopathological and prognostic significance of MUC13 and AGR2 expression in intraductal papillary mucinous neoplasms of the pancreas

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ABSTRACT

Background: Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is a primary pancreatic ductal epithelial neoplasm with the potential to develop into an invasive adenocarcinoma. This study aimed to investigate the clinicopathologic and prognostic significance of four potential biomarkers for the preoperative evaluation of patients with IPMN.

Materials and methods: Clinicopathologic materials from 104 patients with IPMN who underwent surgical resection at Jichi Medical University Hospital were analyzed. IPMNs (110 lesions in total) were histologically classified into low-grade IPMN (Group 1; n = 68), high-grade IPMN (Group 2; n = 16), or IPMN with an associated invasive carcinoma (Group 3; n = 26). We evaluated the immunohistochemical expression of MUC13, AGR2, FUT8, and FXYD3, which were previously reported to be overexpressed in pancreatic ductal adenocarcinoma.

Results: The expression of MUC13 was more common in Group 3 compared with groups 1 and 2 ($p < 0.001$) and was associated with poor prognosis ($p = 0.004$). The expression of MUC13 was not associated with age, sex, tumor location, histological subtype, lymphatic or vascular invasion, or neural invasion. In most cases of IPMN, the loss of expression of AGR2 appeared to show an association with tumor recurrence and poorly differentiated histology of invasive carcinoma; however, this association was not statistically significant. The expressions of FUT8 and FXYD3were not associated with the clinicopathological features of IPMNs.

Conclusions: The results suggest that MUC13 overexpression and loss of expression of AGR2 may predict the progression of IPMN and an unfavorable prognosis in patients with IPMN.
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Introduction

Intraductal papillary mucinous neoplasms (IPMNs) are commonly diagnosed as cystic neoplasms of the pancreas and account for approximately 21%–33% of all clinically encountered pancreatic cystic lesions [1]. In recent years, they have been characterized by their clinicopathologic features and the associated risk of malignant transformation [2,3]. IPMNs may arise from the main pancreatic duct (main-duct type, MD-IPMN) or its side branches (branch-duct type, BD-IPMN), or may involve both the main pancreatic duct (MPD) and the side branches (combined-type IPMN) [4,5]. MD-IPMN is associated with a significantly higher mean risk of malignancy than BD-IPMN (61.6% vs. 25.5%) [2]. IPMN with MPD involvement warrants surgical resection due to the high risk of malignancy. However, it has been reported that some MD-IPMN has almost unchanged for a long time [6,7].

International consensus guidelines for the management of IPMN were established in 2006, recommending observation for asymptomatic IPMN through the presence of a cyst with a maximum size of 3 cm, as well as a non-dilated MPD, negative cytology, and the

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absence of intramural nodules [8]. As cases accumulated, questions appeared regarding the role of cyst size [9,10] and symptoms [11]. The guidelines were revised in 2012 in Fukuoka, Japan, to include the classifications: "high-risk stigmata" and "worrisome features" [2]. In the revised 2012 guidelines, the threshold of MPD dilation was lowered to ≥5 mm [1]. According to these guidelines, a main duct diameter of 5–9 mm must be considered a "worrisome feature" [2]. However, in this subgroup, resection is only recommended if additional criteria such as obstructive jaundice or a solid nodule component are observed as these criteria are regarded as "high-risk stigmata" [2]. However, reports show that invasive carcinoma may also be found in patients with MPD of a smaller diameter without nodules or symptoms [4,12,13]. This often causes frustration, as surgery could have been performed.

The guidelines suggest a therapeutic strategy based on imaging studies. In addition, biomarkers to distinguish low-grade and high-grade lesions using pancreatic juice cytology or biopsy specimens would be very useful in decision making. This study aimed to investigate the clinicopathologic and prognostic significance of several potential biomarkers in patients with IPMN. We retrieved previous reports and analyzed data on four biomarkers that were reported to be overexpressed in cases of pancreatic ductal adenocarcinoma (PDAC), including mucin 13 (MUC13), anterior gradient protein 2 (AGR2), fucosyltransferase 8 (FUT8), and FXYD domain containing ion transport regulator 3 (FXYD3). MUC13 is a transmembrane mucin aberrantly expressed in ovarian and gastrointestinal cancers [14], AGR2 is a protein disulfide isomerase overexpressed in several adenocarcinomas [16–21], and FUT8 is α1,6-fucosyltransferase associated with fucosylation, one of the most important types of glycosylation for malignant transformation and metastasis [22]. Watanabe et al. reported that fucosylation, particularly α-1,6-fucosylation as indicated by FUT8 expression, is upregulated in IPMNs and may be associated with malignant transformation [22]. FXYD3, a chloride channel or chloride channel regulator and a member of the FXYD family of single membrane span proteins, was found to be expressed differentially in PDAC [23].

Materials and methods

Patients

We retrospectively evaluated tissue samples from 104 patients (63 men and 41 women; mean age, 66.9 years; range, 32–82 years) who underwent surgical resection of IPMN (110 lesions in total) at Jichi Medical University Hospital (Shimotsuke, Japan) between January 1, 2000 and May 31, 2016. The study protocol was approved by the Ethics Committee of the Jichi Medical University. Medical information, such as age, sex, history of diseases and recurrences, and outcomes of the participants, was obtained from clinical records.

Histopathological examination

The resected pancreas samples were fixed in 15% formalin, and paraffin blocks were prepared. IPMNs were classified into one of the three histological groups based on the Baltimore Consensus Meeting for Neoplastic Precursor Lesions in the Pancreas [24]: low-grade IPMN (Group 1), high-grade IPMN (Group 2), or IPMN with an associated invasive carcinoma (Group 3). Furthermore, based on the immunohistochemical and histologic profiles of the proliferating epithelial cells, IPMNs were classified into one of the four subtypes: gastric, intestinal, pancreatobiliary (PB), or oncocytic. If two or more subtypes coexisted in the same lesion, the dominant subtype was used for analysis. To assess the tumor size, we traced the outline of IPMN and the associated invasive carcinoma on each slide and added together the tumor areas on the gross photographs. Subsequently, we measured the largest dimension of the tumor. One patient, for whom a detailed report on the tumor volume was missing, was excluded from the tumor size analysis. IPMNs were classified into three groups based on distribution predominance: MPD type, branch-duct type, or combined type. In addition, we classified the histology of the invasion as colloid carcinoma or tubular adenocarcinoma. When two different invasive features coexisted in the same lesion, the dominant component of the invasive carcinoma was used for the analysis. In patients with IPMN with an associated invasive carcinoma, we also evaluated the lymphatic invasion, vascular invasion, and neural invasion. Each section was reviewed by two authors (KM and NF), and a consensus was reached in all the cases.

Immunohistochemistry

We evaluated the expression of MUC13, AGR2, FUT8, and FXYD3 by immunohistochemistry because the expressions of these genes were previously reported in cases of PDAC. A representative section of each lesion was selected for the analysis. The immunohistochemical analysis was performed on 4-μm sections of paraffin-embedded, formalin-fixed tissues. All procedures were performed using a BenchMark ULTRA fully automated staining instrument (Ventana Medical Systems Inc., Oro Valley, AZ, USA). Each section was deparaffinized and incubated in Cell Conditioning Solution 1 (pH 8.5; Ventana Medical Systems Inc.) for 64 min: AGR2 and FXYD3 or 36 min: MUC13 and FUT8 at 95 °C for antigen retrieval. Then, the sections were incubated with primary antibodies against the following molecules (all obtained from Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK) for 32 min: AGR2 (rabbit polyclonal, NBP2-27393, 1:100 dilution; Novus Biologicals, Littleton, CO), FUT8 (rabbit polyclonal, HPA043410; 1:500 dilution; SIGMA-ALDRICH, St. Louis, MO), and FXYD3 (rabbit polyclonal, HPA010856; 1:100 dilution; SIGMA-ALDRICH, St. Louis, MO) or 16 min: MUC13 (mouse monoclonal, MABC209, clone 2E11.1; 1:1000 dilution, Darmstadt, DE). Signals were visualized using an iView DAB Universal Kit (Ventana Medical Systems Inc.). Finally, the sections were counterstained with Hematoxylin II (Ventana Medical Systems Inc.) for 8 min and post-stained with Bluing Reagent (Ventana Medical Systems Inc.) for 4 min.

The proportion of positive cancer cell staining was graded as follows: 0 (negative), <25% (1+), 25%–50% (2+), 50%–70% (3+), and >75% (4+) [26]. For simplicity, the cells were considered positive if at least 25% of the cytoplasm was stained based on the results of the immunohistochemistry.

In a previous report the expression of MUC13 in colorectal carcinomas [25] was evaluated by localization: membrane, cytoplasm, and nucleus. Furthermore, MUC13 is expressed weakly in the apical membrane of the normal pancreatic duct. The expression of MUC13 tends to gradually increase toward the basal laminae. Therefore, the expression of MUC13 was classified into three grades (Fig. 1) [23,24]. We defined the pattern seen in Fig. 1-A as negative and the patterns seen in Fig. 1-B and 1-C as positive. The intensity of the expression was also evaluated.

Statistical analysis

All statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [27]. Differences between groups in age were compared using the Kruskal–Wallis test or Mann–Whitney *U* test, while all other features were compared using a chi-square test or Fisher exact test. The difference in the expression of MUC13 among cases of IPMN

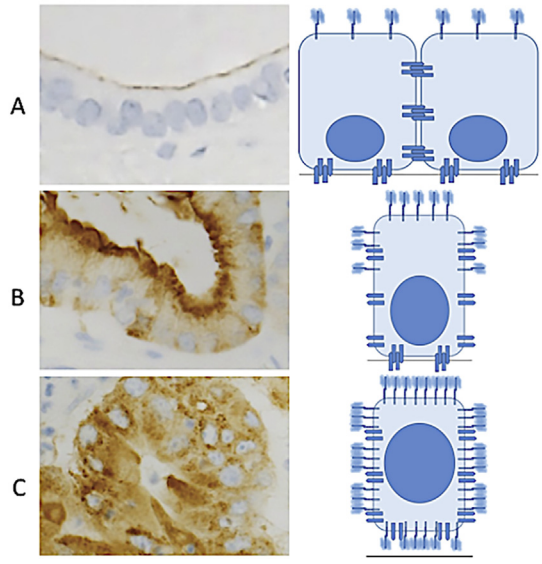

Fig. 1. Normal and atypical cellular expression of MUC13. A: Theoretically, MUC13 is produced in the cytoplasm and is delivered to the apical cell surface. We defined this pattern as MUC 13 expression-negative. B: In low grade neoplastic cells, MUC13 is localized at the lateral and apical cell surface membranes. C: In invasive carcinoma cells, MUC13 is localized at the basal, lateral, and apical cell surface membranes, possibly contributing to the loss of cell–cell and cell–ECM binding. We defined patterns B and C as MUC 13 expression-positive.
These figures were created based on reference [23] and [24].

with an associated invasive carcinoma was assessed by the McNemar's test. Overall patient survival or disease-free survival were estimated using the Kaplan–Meier method; group differences were compared using log-rank test. Univariate and multivariate analyses were performed employing the Cox proportional hazards regression model. The statistically significant variables in the univariate analysis were considered in the multivariate analysis. A p-value of <0.05 was considered statistically significant.

Results

The clinicopathological findings of all cases are summarized in Table 1. First, we evaluated the immunohistochemical expression of MUC13, AGR2, FUT8, and FXYD3 in Group 3. MUC13 was more significantly expressed in invasive carcinoma components than in intraductal components. AGR2 was expressed in almost all lesions. FUT8 overexpression has been reported to be associated with cancerization in cases of IPMN [22]; however, FUT8 expression was low in our study. FXYD3 overexpression has been reported in various carcinomas, including PDAC; however, no FXYD3 overexpression was observed in IPMN [23]. FUT8 and FXYD3 expressions were not associated with the clinicopathological features of IPMNs. Based on these results, we analyzed only MUC13 and AGR2 overexpression in all cases of IPMNs.

Fig. 2 shows MUC13 overexpression based on histological grade. MUC13 overexpression was observed in 23 invasive carcinoma components and in 10 of the 26 intraductal components in Group 3. MUC13 was significantly expressed in the invasive carcinoma components ($p < 0.005$). MUC13 overexpression was not observed in the intraductal components, even in high-grade cases with no expression of MUC13 in invasive carcinoma components. In groups 1 and 2, MUC13 overexpression was observed in only five of the 84 intraductal components. No correlation was observed between MUC13 overexpression and age, sex, tumor location, lymphatic invasion, vascular invasion, neural invasion, histological subtypes, or invasive distance. MUC13 overexpression was not significantly associated with disease-free survival (DFS) ($p = 0.074$) (Fig. 3-A); however, it was significantly associated with overall survival (OS) ($p = 0.004$) (Fig. 3-B). No significant differences were found by multivariate analyses. In Group 3, MUC13 overexpression in

Table 1
Summary of the clinicopathological characteristics in each group.

	Group 1 (IPMN, low-grade)	Group 2 (IPMN, high-grade)	Group 3 (IPMN with an associated invasive carcinoma)	total
n (patients/lesions)	62/68	16/16	26/26	104/110
Age (y)[range]	67.00 [43–82]	69.00 [32–81]	72 [55–82]	67 [32–82]
Sex				
Male	44	10	13	67
Female	24	6	13	43
Tumor size (mm)				
≦20	14	2	2	18
20<, ≦40	26	3	9	38
40<, ≦60	20	5	9	34
>60	8	5	6	19
Location				
Head	39	9	13	61
Body	20	5	11	36
Tail	8	2	2	12
Head to tail	1	0	0	1
Macroscopic type				
Branch	38	2	9	49
Main	7	7	8	22
Combined	23	7	9	39
Histological subtype				
gastric	56	8	6	70
intestinal	9	6	10	25
oncocyte	1	1	0	2
pancreatobiliary	2	1	10	13
Recurrence				
Present	0	5	11	16
Absent	66*	11	14*	11

*2 patients who had no data of the outcome.

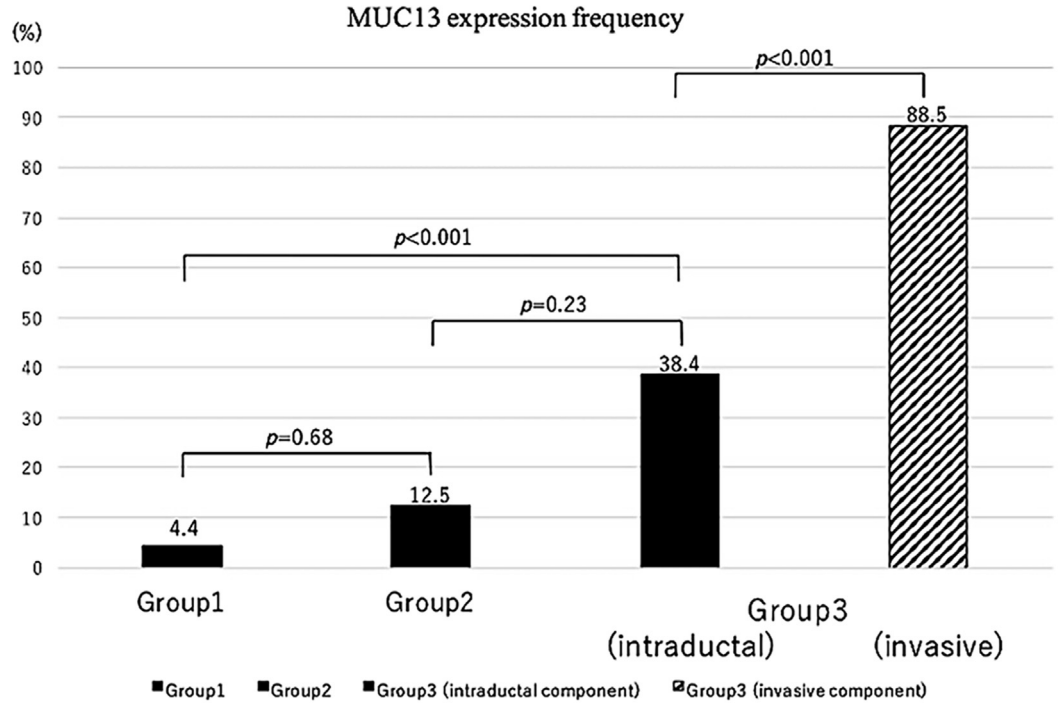

Fig. 2. MUC13 overexpression based on the histological grade. MUC13 overexpression tends to increase according to the histological grade of IPMN. Furthermore, MUC13 overexpression is significantly more common in the invasive carcinoma components than in the intraductal components in Group 3 ($p < 0.001$).

intraductal or invasive components was not associated with OS and DFS. MUC13 was found to have a sensitivity of 38.5% and a specificity of 87.5%.

No differences were observed in AGR2 overexpression between groups. However, a loss of expression of AGR2 was noted in some lesions (Fig. 4). The loss of expression of AGR2 tended to be observed in cases of invasive carcinoma with poorly differentiated features. This was observed in seven patients in Group 3; of these patients, five experienced a recurrence, three died, and three experienced a recurrence and died. In Group 3, 11 patients experienced a recurrence. Five of these patients had a loss of expression of AGR2 whereas six did not. In patients with recurrence, five had positive surgical margins and six had negative surgical margins. Two of the six cases with negative surgical margins showed a loss of expression of AGR2. However, the association between the loss of expression of AGR2 and DFS was not statistically significant. MUC13 overexpression and the loss of expression of AGR2 were not found to be associated.

Discussion

In the present study, we found that MUC13 overexpression was significantly more common in the invasive carcinoma components than in the intraductal components, and it was significantly correlated with OS in patients with IPMN. The loss of expression of AGR2 was observed almost exclusively in cases of invasive carcinomas with poorly differentiated features, suggesting a high recurrence rate.

MUC13 is a recently identified transmembrane mucin that is normally expressed in the large intestine, trachea, kidneys, small intestine, and gastric epithelium [28,29]. In recent studies, MUC13 was shown to be aberrantly expressed in ovarian and gastrointestinal cancers [14]. MUC13 has a large 151-amino acid tandem repeat domain, three epidermal growth factor (EGF)-like domains, and a sea urchin sperm protein enterokinase arginine (SEA) domain within the extracellular component, followed by a short 23-amino acid transmembrane domain and a 69-amino acid cytoplasmic domain (Fig. 5) [23,28]. Subhash et al. first reported that MUC13 was associated with pancreatic cancer [30]. Their data provided novel evidence of the aberrant expression of MUC13 in pancreatic tumors, suggesting a role of MUC13 in pancreatic tumorigenesis and progression [30]. They proposed that MUC13 is overexpressed in pancreatic cancer and induces cellular motility, proliferation, and invasion through the modulation of HER2, PAK1, Akt, S100A4, and p53 expression/activation [14]. In this study, we clarified that MUC13 overexpression in the intraductal neoplastic components increased according to the histological grade. In particular, we found that MUC13 overexpression in the intraductal components was more frequently observed in Group 3 than in Group 2. Maher et al. reported that the aberrant subcellular localization of MUC13 may alter cell signaling due to interactions with the epidermal growth factor receptors, leading to increased tumorigenesis, cell invasion, and metastasis [29]. The results of our study confirmed these findings. MUC13 overexpression may induce weakening and loss of cell-to-cell and cell-to-extracellular matrix binding. During this process, it was proposed that cancer cells acquire the ability to invade and/or spread to other organs.

We also evaluated the sensitivity and specificity of MUC13 in groups 2 and intraductal component of group 3. MUC13 was found to have a low sensitivity and a high specificity. These findings could help physicians with preoperative diagnoses. In lesions with worrisome features, surgical treatment could be recommended in patients with high expression of MUC13 in the biopsy and/or pancreatic juice cytology specimens. In cases with expression of MUC13 in whole cytoplasm, surgery would also be the most appropriate approach. However, if MUC13 is only slightly or not expressed, observation may be recommended due to the low malignant potential. MUC13 has been noted as a therapeutic target in various carcinomas [26]. The results of previous studies and the present study indicate that MUC13 overexpression may be

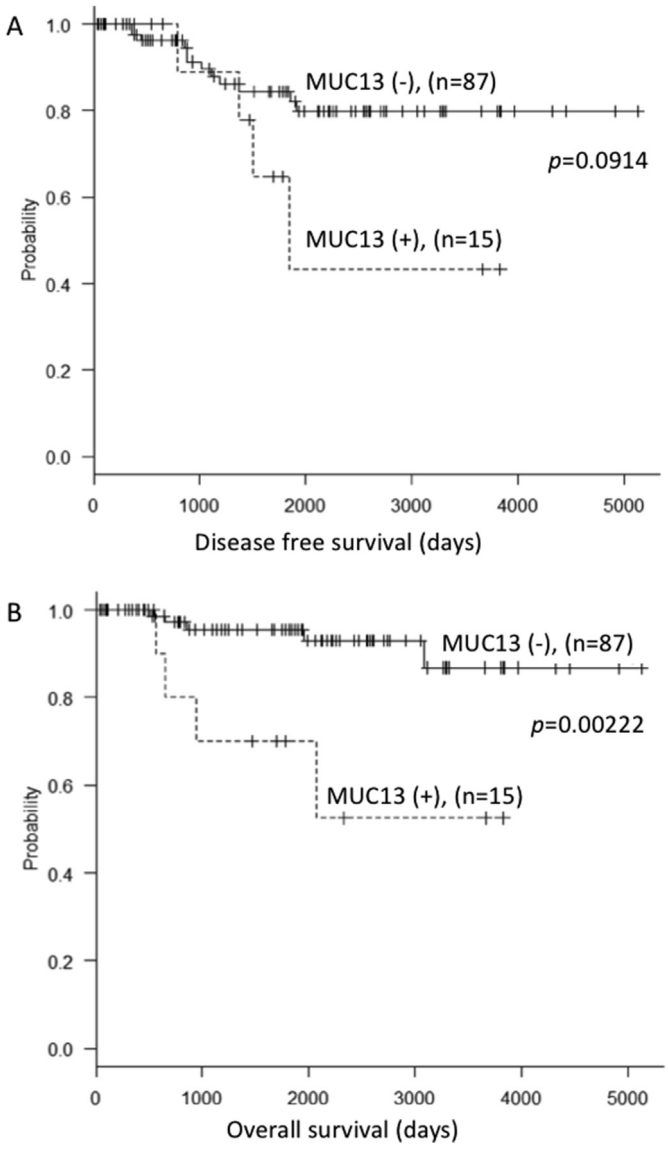

Fig. 3. Kaplan–Meier analysis of disease-free survival (DFS) and overall survival (OS) in patients with IPMN based on MUC13 staining. Patients with MUC13-positive IPMN show a shorter DFS (A) and OS (B) than those with MUC13-negative IPMN (log-rank test).

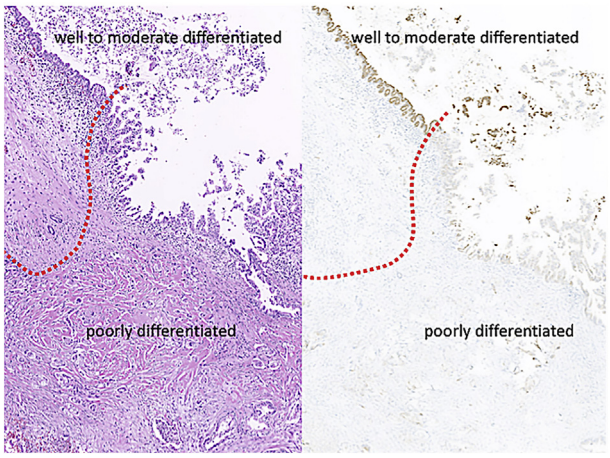

Fig. 4. AGR2 overexpression did not differ between groups. However, the loss of expression of AGR2 tended to be observed in invasive carcinomas with poorly differentiated features.

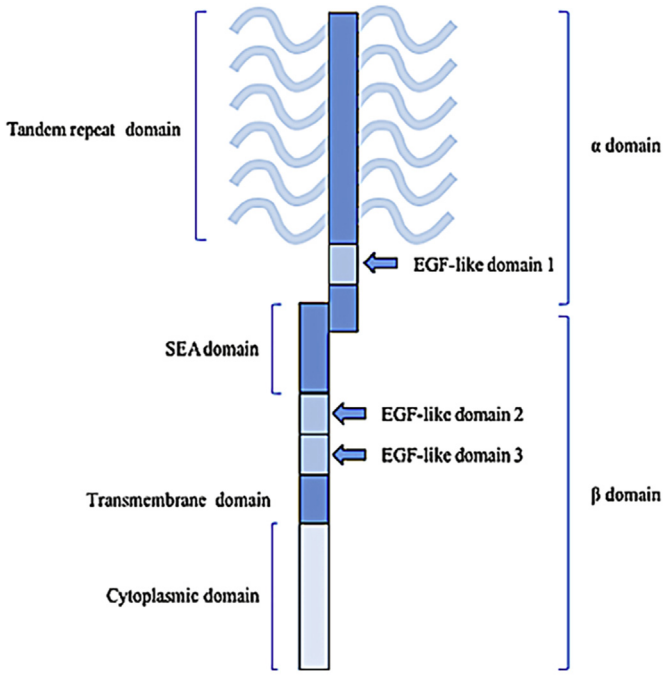

Fig. 5. Schematic representation of the putative domain organization of the human MUC13 protein. This figure was created based on reference [23].

observed in cases of pancreatic malignant tumors. If MUC13 is useful as a therapeutic target, it could serve both as a diagnostic biomarker and a new target for treatment.

In this study, MUC13 was found to be significantly correlated with OS, suggesting that the expression of MUC13 can prove to be useful in preoperative diagnoses. Further research on MUC13 is needed in the future due to its potential as a prognostic factor, diagnostic biomarker, and therapeutic target.

AGR2 is a protein disulfide isomerase [15]. AGR2 overexpression was first noted in the *Xenopus laevis* cement glands. ARG2 sculpts the dorsoanterior ectoderm forming the cement glands and maintains forebrain integrity in this organism [31,32]. It has been reported that AGR2 is associated with cell adhesion [33], cell growth promotion [34], and maintenance of the epithelial barrier [35]. These properties of AGR2 may suggest its potential role in human cancer progression, invasion, and metastasis. AGR2 overexpression has been reported in several adenocarcinomas, including breast [16], colorectal [17], esophageal [18], lung [19], pancreatic [20], and prostate cancers [21]. Mizuuchi et al. reported that AGR2 downregulation is a useful prognostic marker induced by EMT [36]. In the present study, the loss of expression of AGR2 was associated with recurrence; however, it was not statistically associated with DFS. We also evaluated the expression of AGR2 in the surgical specimens and found that the loss of expression of AGR2 may help determine the frequency of follow-up appointments.

Our study's main limitations were the single-center design and the small sample size. It is desirable to analyze a larger number of cases.

In conclusion, MUC13 overexpression and the loss of AGR2 expression may predict the progression of IPMN and confer an unfavorable prognosis in patients with IPMN. These biomarkers, when used in combination, may become useful to determine

disease grade and for prognostic evaluation. MUC13, in particular, may prove to be a useful therapeutic biomarker in patients with IPMN.

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<Typo correction>

p.408, right column

"Immunohistochemistry" 2nd section

- The proportion of positive cancer cell staining was graded as follows: 0 (negative), <25% (1+), 25%-50% (2+), 50%-**70%** (3+), and >75% (4+).

→**75%**

p.409, right column

"Results" 2nd section

- MUC13 was significantly expressed in the invasive carcinoma components ($p <$ **0.005**).

→$p<$**0.001**

- MUC13 overexpression was not significantly associated with disease-free survival (DFS) **($p = 0.074$)** (Fig. 3-A);

→$p=$**0.091**

- however, it was significantly associated with overall survival (OS) **($p = 0.004$)** (Fig. 3-B).

→$p=$**0.002**