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Localization of the Most Severely Dysplastic/Invasive Lesions and Mucin Phenotypes in Intraductal Papillary Mucinous Neoplasm of the Pancreas

Karasaki, Hidenori ; Mizukami, Yusuke ; Tokusashi, Yoshihiko ; Koizumi, Kazuya ; Ishizaki, Akira ; Imai, Kouji ; Yoshikawa, Daitaro ; Kino, Shuichi ; Sasajima, Junpei ; Tanno, Satoshi ; Matsumoto, Kakuya ; Miyokawa, Naoyuki ; Kono, Toru ; Kohgo, Yutaka ; Furukawa, Hiroyuki

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Corresponding Author: Dr. Karasaki Hidenori, M.D., Ph.D.

Corresponding Author's Institution: Asahikawa Medical College

First Author: Karasaki Hidenori, M.D., Ph.D.

Order of Authors: Karasaki Hidenori, M.D., Ph.D.; Yusuke Mizukami, MD, PhD; Yoshihiko Tokusashi, MD, PhD; Kazuya Koizumi, MD, PhD; Akira Ishizaki, MD, PhD; Kouji Imai, MD, PhD; Daitaro Yoshikawa, MD, PhD; Shuichi Kino, MD, PhD; Junpei Sasajima, MD; Satoshi Tanno, MD, PhD; Kakuya Matsumoto, MD; Naoyuki Miyokawa, MD, PhD; Toru Kono, MD, PhD; Yutaka Kohgo, MD, PhD

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Methods: Thirty-nine surgically resected IPMNs excluding obviously invasive carcinomas were examined. The distribution of the most severely dysplastic lesions were mapped on specimens. Immunohistochemical analysis for MUC1 and MUC2 was performed on sections containing the histologically predominant lesions and the most severely dysplastic areas.
Results: The presence of MNs correlated well with the histologic grade of IPMN ($p < 0.01$); however, the most severely dysplastic lesions were associated with a flat/non-elevated area rather than MNs (78.9 %). In the MUC1-positive subgroup, minimally invasive carcinoma (MI) was co-localized to MNs, whereas most severely dysplastic foci including MI with components of mucinous and tubular adenocarcinoma were observed in the areas apart from MNs in the MUC2-positive and MUC1, 2-negative subgroups, respectively.
Conclusions: Although our data support the concept that MNs represent areas of higher grade dysplasia within IPMN, development of invasive lesions from MNs may be limited to cases that are MUC1-positive. Careful attention should be paid to the emergence of invasive IPMN from flat foci in MUC2-positive and MUC1, 2-negative cases.

Original article

Localization of the most severely dysplastic/invasive lesions and mucin phenotypes in intraductal papillary mucinous neoplasm (IPMN) of the pancreas

Hidenori Karasaki, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Hokkaido, Japan

Yusuke Mizukami, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical University, Hokkaido, Japan

Yoshihiko Tokusashi, MD, PhD

Department of Surgical Pathology, Asahikawa Medical University, Hokkaido, Japan

Kazuya Koizumi, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical University, Hokkaido, Japan

Akira Ishizaki, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Hokkaido, Japan

Kouji Imai, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Hokkaido, Japan

Daitaro Yoshikawa, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Hokkaido, Japan

Shuichi Kino, MD, PhD

Department of Clinical Laboratory and Blood Center, Asahikawa Medical University, Hokkaido, Japan

Junpei Sasajima, MD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical University, Hokkaido, Japan

Satoshi Tanno, MD, PhD

Department of Gastroenterology, Kotoni Royal Hospital, Hokkaido, Japan

Kakuya Matsumoto, MD

Division of Metabolism and Biosystemic Science, Asahikawa Medical University, Hokkaido, Japan

Naoyuki Miyokawa, MD, PhD

Department of Surgical Pathology, Asahikawa Medical University, Hokkaido, Japan

Toru Kono, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Hokkaido, Japan

Yutaka Kohgo, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical University, Hokkaido, Japan

Hiroyuki Furukawa, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Hokkaido, Japan

Correspondence to: Hidenori Karasaki, MD, PhD

Division of Gastroenterologic and General Surgery, Department of Surgery, Asahikawa Medical University.

2-1 Midorigaoka Higashi, Asahikawa, Hokkaido 078-8510, Japan

Phone: 81-166-68-2503; Fax; 81-166-68-2193; E-mail: karasaki@asahikawa-med.ac.jp

Running title: Mural nodule and malignant focus in IPMN

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Structured Abstract

Objective: The aim of this study was to define the relevance of mural nodules (MNs) as a 'direct' indicator of malignancy of intraductal papillary mucinous neoplasm (IPMN) of the pancreas.

Methods: Thirty-nine surgically resected IPMNs excluding obviously invasive carcinomas were examined. The distribution of the most severely dysplastic lesions were mapped on specimens. Immunohistochemical analysis for MUC1 and MUC2 was performed on sections containing the histologically predominant lesions and the most severely dysplastic areas.

Results: The presence of MNs correlated well with the histologic grade of IPMN ($p < 0.01$); however, the most severely dysplastic lesions were associated with a flat/non-elevated area rather than MNs (78.9 %). In the MUC1-positive subgroup, minimally invasive carcinoma (MI) was co-localized to MNs, whereas most severely dysplastic foci including MI with components of mucinous and tubular adenocarcinoma were observed in the areas apart from MNs in the MUC2-positive and MUC1, 2-negative subgroups, respectively.

Conclusions: Although our data support the concept that MNs represent areas of higher grade dysplasia within IPMN, development of invasive lesions from MNs may be limited to cases that are MUC1-positive. Careful attention should be paid to the emergence of invasive IPMN from flat foci in MUC2-positive and MUC1, 2-negative cases.

Key Words: intraductal papillary-mucinous neoplasm of the pancreas, mucin phenotype, severely dysplastic lesion, minimally invasive carcinoma

Introduction

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas have a histological spectrum ranging from benign adenoma to invasive cancer[1]. The diameters of cysts and the main pancreatic duct as well as the presence of mural nodules (MNs) correlate with histologically malignant grades of IPMN[2-4], and these criteria are widely utilized to exclude benign lesions from surgical intervention. Among factors assessed via preoperative imaging, the presence of MNs is recognized as the most important factor associated with malignant epithelial lesions[3-5]. The postoperative prognosis of IPMN is, in general, favorable according to published guidelines[6]; however, a current clinical dilemma is that large numbers of benign lesions undergo surgical resection. Since accurate preoperative prediction of malignancy is not thus far feasible by imaging modalities, identification of pre-invasive lesions and establishment of a novel molecular based management strategy is of interest. Ultimately, appropriate criteria that can identify IPMN cases with rapidly invasive adenocarcinoma are needed. This would allow follow-up of less aggressive lesions and obviate unnecessary surgery.

Early detection of invasive components(s) of IPMN lesions is important, as recent studies demonstrated that a large number of branch duct type IPMN without MN could be safely followed-up without surgery[7-8]. Since IPMN is generally thought to be a slow-growing tumor and in most cases develops in elderly people, follow-up alone could be recommended for large number of branch duct cases if MNs are not identified. Careful attention is however required to

detect the emergence of invasive lesions[9], since it has been reported that the presence of an obviously invasive lesion in IPMN signifies poor outcome[10-11]. Thus it is desirable to resect IPMN at the stage of minimally invasive carcinoma (MI). Nevertheless, current imaging modalities are limited in making an accurate diagnosis of IPMN with MI[10]. More importantly, it is not formally validated whether an invasive lesion in IPMN develops within MNs or not.
It is challenging to postpone surgical intervention until the lesions develop high-grade dysplasia without losing the current level of safety. One potential approach includes a classification of IPMN into subtypes based on mucin expression phenotypes, as previously demonstrated[12-14]. In addition, specification of "pre-invasive" lesion by precise histologic assessment and grading of epithelial lesions is also required. In the current study, we sought to reevaluate the clinical significance of MNs, which are believed to be the finding most suggestive of malignancy in IPMN[15]. We specifically focused on the distribution of the most dysplastic lesions within IPMNs and their mucin subtypes in order to predict IPMN patients likely to develop high-grade dysplasia with potential invasiveness.

Materials and Methods

Pancreatic tissues from IPMN patients were obtained as surgical discards as part of an IRB and Ethical Committee approved protocol at the Asahikawa Medical College Hospital. The subjects included 39 cases of IPMN resected at the Division of Gastroenterologic and General Surgery,

Department of Surgery, Asahikawa Medical College between January 1994 and December 2008 and diagnosed pathologically as IPMN. Cases with evident invasive cancer were excluded. The resected pancreas was fixed in 10% formaldehyde, and the whole specimen was sliced at a thickness of 5-mm and stained with hematoxylin-eosin (H-E). Mapping based on the grades of dysplasia on H-E staining was then performed by three gastroenterologists/pathologists to identify the distribution of the most severely dysplastic lesions and minimally invasive lesions.

According to the diagnosis and grade criteria of Armed Forces Institute of Pathology (AFIP)[1], dysplastic lesions were categorized into low-, moderate-, and high-grade dysplasia. Adenocarcinoma lesions that had infiltrated into the stroma microscopically, but not macroscopically, were designated as MI. When a resected pancreas contained different grades of dysplasia, the section with the highest grade was designated as the most severely dysplastic area. MN was defined as a lesion of greater than or equal to 3mm in height histologically, and the lesions were categorized into those in which the highest dysplasia was localized to MNs and those in which the highest dysplasia was found in areas apart from MNs, *i.e.*, flat or low papillary lesions. Each case was categorized as main duct type or branch duct type according to the macroscopic quantitative predominance(**Table 1**).

Areas of the predominant histological grade and the most severely dysplastic areas were subjected to immunohistochemical staining for MUC1/2. Serial sections 4 μ m in thickness were sliced from paraffin blocks and deparaffinized. After intrinsic peroxidase was blocked, antigens

in slices were retrieved in 10 mM TRIS buffer (pH 10.0) at 110 ºC for 10 minutes by microwaving. Then, they were incubated for 30 minutes at room temperature with anti-MUC1 antibody (clone, Ma695) and anti-MUC2 antibody (clone, Ccp58). The samples that were negative for MUC1 and diffusely positive for MUC2 were defined as the MUC2-positive type, while those that were negative or partly positive for MUC1 and negative for MUC2 were defined as the MUC1/2-negative type, and those that were diffusely positive for MUC1 and negative for MUC2 were defined as the MUC1-positive type.

Statview 5.0 software (SAS Institute Inc., Cary, NC) was employed for statistical analysis. Differences between categorical variables were evaluated using χ^2 test. A P value less than 0.05 was considered statistically significant.

Results

Correlation between presence of MNs and grades of pancreatic intraductal dysplasia

A total of 19 IPMNs with MNs included 6 with moderate dysplasia, 7 with high grade dysplasia, and 6 with MI. The 20 cases without MNs included 1 with low grade dysplasia, 15 with moderate dysplasia, 3 with high grade dysplasia, and 1 with MI. Therefore, high grade dysplasia and MI were significantly more common when MNs were present ($p < 0.01$) ~~(**Table 2**)~~.

Distribution of the most severely dysplastic intraepithelial lesions

We next tested the hypothesis that MNs correspond to the most severe lesions histologically. However, to our surprise, the most severely dysplastic lesions were observed in the MNs of 4 out of 19 IPMN with MNs (21.1%) (**Figure 1**). Indeed, the most severely dysplastic epithelial lesions were localized in areas apart from MNs in 15 cases (78.9%) (**Figure 2,** **Table 2**). There were 7 cases with MI (3 main duct type and 4 branch duct type IPMN). MNs were present in 6 of 7 cases with MI lesions. However, an invasive front was observed within MNs in only 2 cases (28.6 %). In a larger numbers of cases (5 of 7 cases, 71.4 %), MI lesions were localized in the flat or low papillary area, distant from MNs (**Figure 3,** **Table 3**).

Distributions of the most dysplastic lesions and macroscopic types

Eleven cases were categorized as main duct type, while 28 were branch duct type (**Table 1**). MNs were evident in 9 of 11 cases with main duct type (81.8 %), and in 10 of 28 (35.7 %) with branch duct type IPMNs. Thus, MNs were more frequently observed in the main duct type IPMN ($p < 0.05$) ~~(**Table 2**)~~.

The most dysplastic lesions were localized in the MNs in 3 of 9 (33.3 %) with main duct types and in 1 of 10 (10.0 %) with branch duct IPMNs. Therefore, the most severely dysplastic lesions were more frequently observed in locations apart from MNs in both macroscopic types, and there was no significant difference in the frequency between the two subtypes ($p = 0.3034$) (**Table 2**).

Relationship between mucin phenotypes and the most severely dysplastic lesions

We next sought to evaluate mucin phenotype by performing MUC1/2 immunohistochemistry. The predominant mucin phenotypes included 19 MUC2-positive cases (48.7 %), 16 MUC1/2-negative cases (41.0 %), and 4 MUC1-positive cases (10.3 %) (**Figure 4**). The relationship between mucin phenotype-based subtypes and the most severe dysplastic grade in cases with and without MNs is shown in **Table 4**. In MUC2-positive cases, high grade dysplasia or MI was found in 7 of 11 cases with MNs (63.6 %), whereas only 1 case revealed high grade dysplasia in subjects without MNs (11.1 %), demonstrating a correlation between the presence of MN and grade of dysplasia in MUC2-positive IPMN (P = 0.06). In addition, MI was also evident in all three MUC1-positive cases with MNs, and one other case without MNs revealed moderate dysplasia. However, in the MUC1/2-negative group, MI was identified in patients without MNs and a low to moderate grade as the most severe dysplasia was found in cases with MNs (2 cases; 40.0 %), implying no apparent correlation between the presence of MNs and dysplastic grade in this subtype.

Mucin phenotypes and location of the most severely dysplastic lesions

We next sought to define the relationship between mucin phenotypes and location of the most severely dysplastic lesions in 19 IPMN subjects with MNs. The most severely dysplastic lesion was present in the areas apart from MNs in 10 of 11 (90.9 %) MUC2-positive cases, 4 of 5 MUC1/2-negative (80.0 %) cases, and 1 of 3 (33.3 %) MUC1-positive case. Therefore, although the severely dysplastic epithelial lesions could be frequently found in the MNs in MUC1-positive

cases, the majority appeared to develop in the areas outside of MNs in MUC2-positive and MUC1/2-negative cases (**Table 2**).

Mucin phenotypes and location of the minimally invasive lesions

Invasive phenotype is one of the hallmarks of malignant tumors, and MNs in IPMN have generally been considered to be the lesions that initiate invasion into pancreatic parenchyma. We thus investigated the relationship between the invasive area of MI and mucin phenotypes. Consistent with the observation that the most severely dysplastic lesions were recognized in areas apart from MNs in MUC2-positive cases, invasive lesions were also observed outside of MNs (**Table 3**). However, this was not found in the MUC1-positive subtype, since MIs were co-localized with MNs in 2 of 3 MUC1-positive IPMN (66.7 %). In the MUC1/2-negative subtype with MI, including a case without MN, invasive lesions were observed in the low papillary area.

Discussion

A number of reports highlight the presence of MNs in IPMN as a reliable sign indicative of malignancy[2, 4-5]. However, previous studies have not specifically addressed the localization of the most severely dysplastic lesions, and it remains to be determined whether or not invasive lesions generally develop from MNs. Therefore, it is important to identify the distribution of

"pre-invasive" lesions with potential to invade in order to determine appropriate clinical management: the decision as to follow-up versus surgical intervention.

In the current study, the presence of MNs strongly correlated with histological grades of malignancy, and high grade dysplasia and MI were frequently observed in cases with MNs ($p = 0.0036$) ~~(Table 1)~~, validating the current strategy for the management of IPMN patients based on the consensus guidelines[6]. However, to our surprise MNs were found co-localized with the most severely dysplastic lesions in only 21.1 % cases of IPMN (33.3 % in the main duct and 10.0 % in branch duct type) **(Table 2)**. These results imply that the most severely dysplastic area is occasionally localized in flat or low papillary lesions apart from MNs, and that MNs are not always co-localized to the most severely dysplastic area. Therefore, the appearance of MNs is an indirect marker for the potential development of high-grade dysplastic lesions in the pancreas, rather than direct evidence of severely dysplastic lesions.

We also found the invasive front localized in the flat or low-papillary areas in 5 of 7 cases with MI **(Table 3)**, indicating that invasive IPMN can frequently develop outside of MNs. Consequently, careful attention should also be paid to lesions outside of MNs, since our data show that flat or low-papillary areas can also be pre-invasive lesions in patients with IPMN. Therefore, post surgical survey will be required as dysplastic lesions may be left as remnants subsequent to limited surgical resection of the IPMN lesion containing MNs and could potentially develop into invasive carcinoma.

Currently, IPMN is categorized into four subtypes: intestinal, gastric, pancreatobiliary, and oncocytic, and each type displays a specific mucin phenotype[12-14]. However, the clinical relevance of this classification is not yet apparent. It has been demonstrated that the prognosis of intestinal type IPMN was favorable[14], while other reports have argued that it was poor because of a frequent concurrence of carcinoma *in situ* and invasive cancer[12]; thus far no consensus has been reached. In the current study, 7 of 8 MUC2-positive cases without MNs were histologically diagnosed as moderate dysplasia and only 1 as high-grade dysplasia. In contrast, in cases with MNs, high-grade dysplasia or MI was observed in 7 of 11 cases, implying that the presence of MNs in MUC2-positive IPMN is indicative of a higher grade of dysplasia (**Table 4**). Hence, a careful clinical follow-up could be feasible for MUC2-positive IPMN until there is evidence of MNs, and in principal MUC2-positive IPMN with MNs should be resected. However, it should be noted that the most severely dysplastic areas and MI were occasionally found in areas outside of MNs in this category. Therefore, early invasive adenocarcinoma may be missed if follow-up is solely focused on MNs.

The prognosis of pancreatobiliary type IPMNs remains unclear; however, an argument against surgical intervention is limited due to frequent incidence of malignant lesions[14, 16-18]. In the present study, MNs in MUC1-positive type corresponded to MI lesions, suggesting that IPMN lesions in this type remain localized in epithelia for a very short time and are likely to invade rapidly. Even in a case in which invasive lesions were observed outside of MNs, high grade dysplasia was also evident in MNs *per se*. Thus, considering the fact that development of MNs in

MUC1-positive cases directly reflects the appearance of severely dysplastic lesions and that such cases are likely to develop invasive cancer rapidly, surgical intervention should be considered at an early time point after diagnosis.

It has been reported that mildly dysplastic and benign lesions are often observed in gastric type IPMN, and a number of studies have demonstrated a good prognosis[12, 14]. MUC1/2-negative cases could be categorized as gastric type IPMN[13]. However, in the current study, both MUC1/2-negative cases composed of severe dysplasia and MI in flat lesions are included in cases with MNs. It should be noted that in the current criteria, based on the morphology of epithelial lesions, gastric type IPMN is histologically defined as lesions with "basally located nuclei". In other words, the criteria *per se* could be biased by the grades of dysplasia[12-14]. On the other hand, "complex papillae" and "enlarged hyperchromatic nuclei" are included in the criteria for the pancreatobiliary type IPMN, restricting this subtype to severely dysplastic lesion. Although some of the MUC1/2-negative cases in our study may be categorized as a pancreatobiliary type by other studies, we carried out classifications based solely on mucin phenotypes without considering morphology and grade of dysplasia because mucin expression phenotype was used as a parameter independent of macroscopic type, presence of MN, and histological grade of dysplasia in the current study.

Although gastric type IPMN has been shown to include benign branch duct type[12], there is no significant difference in the incidence of high-grade dysplasia or MI between MUC1/2-negative

cases (6 of 16 cases; 37.5 %) and MUC2-positive cases (8 of 19 cases; 42.1 %). The common site of the most severely dysplastic areas and MI was outside of MN in both MUC1/2-negative and MUC2-positive cases. However, all MUC1/2-negative cases exhibited tubular adenocarcinoma at the invasion front within the MI lesion. Further investigation into the natural history and prognosis is necessary for the MUC1/2-negative type.

Mucin phenotype-based preoperative diagnosis can allow a rational decision on the management of IPMN patients. Hibi, *et al.* reported that in 79 % of cases, cytological assessment during endoscopic retrograde pancreatography (ERP) and histological subtype of resected specimens were consistent in terms of mucin subtype[19]. In the present study, the relationship between cytology and subtype was not examined; however, in 89.5 % of cases with MNs intraepithelial lesions extended to the main pancreatic duct. Therefore, in most cases, mucin-based classification could be achieved by immunostaining for MUC1 and MUC2, utilizing either pancreatic fluid or a biopsied specimen obtained by ERP. Classification based solely on mucin phenotypes may offer important additive information to conventional image-based macroscopic types and morphological classification such as presence of MNs, even if histological information regarding structural atypia is not obtained.

Discrepancy during pathological assessment of IPMNs is a clinical issue that needs to be resolved. Multicenter prospective analysis based on a more objective rule will be required to overcome the confusion. To date, it has been elucidated that IPMN is one of the risk factors for

concurrently developing invasive ductal carcinoma of the pancreas[9, 20]. If an invasive lesion is localized apart from IPMN, it is considered to be a synchronous cancer. However, the invasive lesion may be expected to arise from IPMN when the invasive compartment is located close to IPMN or the invasive front is evident within the IPMN. When two tumors are adjacently located and the invasive front is unclear it is hard to determine whether they are synchronous carcinoma (collision cancer) or IPMN-derived invasive carcinoma. MI lesions in this study were considered IPMN-derived invasive carcinoma because of continuity between intraductal and invasive lesions. However, the possibility that *de novo* carcinoma is included in the current study is not fully excluded. Identification of reliable molecular markers allows us to appropriately understand the process of progression of IPMN.

In conclusion, the presence of MNs strongly correlated with grades of dysplasia of IPMN, but severely dysplastic lesions or an invasive front can develop from the flat or low papillary regions rather than MNs. In terms of mucin phenotype-based subtypes and presence of MNs, an invasive lesion appeared to arise from MN in the MUC1-positive type based on our analysis in limited number of cases. However, this is not the case with the MUC2-positive and MUC1/2-negative group, in which mucinous and tubular adenocarcinoma potentially develop outside of MNs. Collectively, the current study proposes a requirement of careful survey by close inspection of flat/non-elevated lesions as foci with potential invasiveness.

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Figure legends

Figure 1

A case with the most severely dysplastic lesion in the mural nodule (MN). Most MNs in the dilated branches showed moderate dysplasia, but high-grade dysplasia was observed at the root of the MNs (A, C). Macroscopic appearance of H-E staining. Arrow indicates the area of histologically predominant moderate dysplasia in MNs, as shown in magnified view (B). Moderate dysplasia was predominant as indicated by the arrow in the panel (A). An arrowhead indicates the most severely dysplastic lesion at the root of MNs, as shown in magnified view (D). High-grade dysplasia was observed in the area indicated by the arrowhead in panel (C). Scale bars, 500 μm.

Figure 2

A case with the most severely dysplastic lesion present outside of MNs. The whole MN showed moderate dysplasia, and the most severely dysplastic lesion was found at the low papillary region of the main pancreatic duct as shown in macroscopic view (A, B). The arrow in (A) indicates histologically predominant moderate dysplasia in the MNs, which is magnified in (C). The

arrowhead in (B) indicates the most severely dysplastic lesion in the low papillary region. Non-invasive, but highly dysplastic lesion is shown in (D) with a magnified view. Scale bars, 500 μm.

Figure 3

A case with minimally invasive cancer that had the most severely dysplastic lesion and an invasion front at the flat area outside of MNs (A, C). A representative macroscopic view of a slice of the resected specimen including a MN 3cm in diameter recognized within the dilated branch. The largest part of the lesion was of moderate dysplasia (arrow), while a minute high grade dysplastic area and minimally invasive cancer were present at the flat part of the wall of the cyst (arrowhead). A magnified view of the histologically predominant moderate dysplasia (B). Invasive front developed from a high grade dysplastic area at the flat part of the wall of the cyst into the stroma was observed (C). Scale bars, 500 μm.

Figure 4

A representative case of subtypes based on mucin expression. H-E staining (A, D, G). immunostaining for MUC1(B, E, H). Immunostaining for MUC2(C, F, I). The MUC2-positive

type (*upper panel*) was negative for MUC1 and positive for MUC2 (B, C). The MUC1/2-negative type (*middle panel*) was negative for MUC1 and MUC2 (E, F). The MUC1-positive type (*lower panel*) was positive for MUC1 and negative for MUC2 (H, I). Each subtype represents a unique histology corresponding to intestinal, gastric, and pancreatobiliary type, respectively. Scale bars, 500 μm.

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Signature Akira Ishizaki Printed Name Akira Ishizaki,MD,PhD Date 2010/4/30
Government Financial Disclosure Attached

Signature Koji Imai Printed Name Kouji Imai,MD,PhD Date 2010/4/30
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Signature Daitaro Yoshikawa Printed Name Daitaro Yoshikawa,MD,PhD Date 2010/4/30
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Signature Shuichi Kino Printed Name Shuichi Kino,MD,PhD Date 5/14/2010.
Government Financial Disclosure Attached

Signature Junpei Sasajima Printed Name Junpei Sasajima,MD Date 5/17/2010.
Government Financial Disclosure Attached

Signature Kakuya Matsumoto Printed Name Kakuya Matsumoto,MD Date 5/14/2010
Government Financial Disclosure Attached

Signature Naoyuki Miyokawa Printed Name Naoyuki Miyokawa,MD,PhD Date 5/14/2010
Government Financial Disclosure Attached

Signature Toru Kono Printed Name Toru Kono,MD,PhD Date 5/13/2010
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Signature _____ Printed Name Yutaka Kohgo,MD,PhD Date 5/14/10
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Signature _____ Printed Name Hiroyuki Furukawa,MD,PhD Date 4/30/10
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Signature _____ Printed Name _____ Date _____
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Table

Table 1. Patients Characteristics

	Main duct type	Branch duct type
age; mean±sd	68.6 ± 8.51	67.8 ± 7.61
gender; male/female	5/6	22/6
mural nodule; +/–	9/2	10/18
dysplastic grade; low/mod/high/MI	0/4/4/3	1/17/6/4
mucin phenotype; $M1^+M2^-/M1^-M2^+/M1^-M2^-$	2/6/3	2/13/13

Low, low grade dysplasia; mod, moderate dysplasia; high, high grade dysplasia; MI, minimally invasive carcinoma; $M1^+M2^-$, $MUC1^+MUC2^-$; $M1^-M2^+$, $MUC1^-MUC2^+$; $M1^-M2^-$, $MUC1^-MUC2^-$.

Table 2. Location of the highest atypia.

Subtype	Location of the highest atypia	
	Mural nodule	flat or low papillary
Branch-duct type	1(10.0)	9(90.0)
Main-duct type	3(33.3)	6(66.7)
total	4(21.1)	15(78.9)
MUC2+	1(9.1)	10(90.9)
MUC1,2-	1(20.0)	4(80.0)
MUC1+	2(66.7)	1(33.3)

Parentheses denote percentages

Table 3 Location of invasion of minimally invasive carcinoma.

Case	Subtype	Invasive type	MN	Location of invasion
1	MUC1+	Tub	+	MN
2	MUC2+	Muc	+	low papillary
3	MUC1+	Muc, Tub	+	low papillary
4	MUC1+	Tub	+	MN
5	MUC1/2-	Tub	–	flat
6	MUC1/2-	Tub	+	low papillary
7	MUC2+	Muc, Tub	+	flat

Tub, tubular adenocarcinoma; Muc, mucinous carcinoma; MN, mural nodule.

Table 4. Relationship among mucin phenotype-based subtypes, mural nodules, and atypia.

Subtype	Atypia	Mural nodule	
		+	–
MUC2+ (MUC1–)	low-mod	4	7
	high	5	1
	MI	2	0
		11 (57.9)	8 (42.1)
MUC1,2–	low-mod	2	8
	high	2	2
	MI	1	1
		5 (31.3)	11 (68.7)
MUC1+ (MUC2–)	low-mod	0	1
	high	0	0
	MI	3	0
		3 (75.0)	1 (25.0)

Low, low-grade dysplasia; mod, moderate dysplasia; high, high-grade dysplasia; MI, minimally invasive carcinoma. Parentheses denote percentages.

***Cover Letter**

Re: Pancreas journal - PANCREAS 10325

Dear Dr. Vay Liang W. (Bill) Go:

We have received your review of our manuscript "Localization of the most severely dysplastic/invasive lesions and mucin phenotypes in intraductal papillary mucinous neoplasm (IPMN) of the pancreas." We are grateful for the thoughtful comments and have addressed each of the reviewers' concerns as outlined below.

As you suggested the abstract was sectioned and included in the manuscript. The revised abstract satisfied the limit of length, just 200 words. In addition, Key words are described after the revised abstract and original image files for Figures in an EPS format have uploaded. We have edited references based on the journal style for Refs 2, 11, 15, 16, and 20. Finally, we apologize about CTAF. CTAF designated by Dr. Satoshi Tanno has been uploaded onto Editorial Manager. We would appreciate and reconsider if you have an additional suggestions and corrections needed to make for publication.

The specific changes in the manuscript have been highlighted by red text.

Thank you for your consideration of our revised manuscript. Please do not hesitate to contact me if you have any questions.

Sincerely yours,

Hidenori Karasaki, MD, PhD

*Response to Reviewers

Response to Editorial Comments:

1. Include an abstract in the manuscript. Format your abstract to include sections labeled, Objectives, Methods, Results, and Conclusions. Limit the abstract to 200 words or less. The abstract length should not be reduced by eliminating fully spelled out terms in favor of using only abbreviations.

>>> As you suggested the abstract was sectioned and included in the manuscript. The revised abstract satisfied the limit of length, just 200 words.

2. Provide up to 6 key words/phrases in a section after the abstract.

>>> Key words are described after the revised abstract.

3. Upload figures as separate TIFF or EPS format files in at least 300 dpi resolution in CMYK color or Grayscale. Submit line art at 1200 dpi. Your figures were submitted in RGB color mode, 300 dpi. -Check the quality and clarity of all images before uploading because our office cannot fix pixelated or blurry images. Please see our publisher's PDF guide to formatting pictures by visiting http://edmgr.ovid.com/lww-final/accounts/5StepsforArt.pdf. If you cannot make the resolution and/or color changes yourself or if picture quality cannot be retained after increasing resolution, submit the figures as PowerPoint files.

>>> We have uploaded original image files in an EPS format.

4. Format references according to our journal's Instruction for Authors. (http://edmgr.ovid.com/pancreas/accounts/ifauth.htm) Note only the first three authors are listed followed by et al. (Please check Refs 2, 11, 15, 16, 20).

>>> We have edited references based on the journal style.

5. Please download a Copyright Transfer Agreement form at http://edmgr.ovid.com/pancreas/accounts/copyrightTransfer.pdf and return it to us with all authors' signatures along with your revision. Satoshi Tanno needs to sign the CTAF. The CTAF needs to include pages 1 and 2 of the original document. Please upload the signed CTA onto Editorial Manager, fax your signed CTA to the Pancreas Editorial Office (fax number 310-824-5990), mail to Pancreas Editorial Office; 900 Veteran Avenue; Warren Hall 13-146; Los Angeles, CA 90095, OR scan and email to pancreasofc@ucla.edu.

>>> We apologize for this. CTAF designated by Dr. Satoshi Tanno has been uploaded onto Editorial Manager.

Response to Reviewer #1's Comments:

Although the author insists that the occurrence of invasive lesions in MNs may be limited to MUC1-positive IPMNs, the number of MUC1$^+$ IPMNs is only four. Nothing conclusive may be retrieved from such a small number of cases, if there is no convincing explanation about specific characteristics of MUC1$^+$ IPMNs. Thus, firstly, the authors should mention about the fundamental limitation of study due to small number of cases in the 'Discussion'.

>>> We are grateful for the reviewer's suggestions with respect to the limitation of our retrospective study. We have revised our overstatement in the last paragraph of Discussion and mentioned as "In terms of mucin phenotype-based subtypes and presence of MNs, an invasive lesion appeared to arise from MN in MUC1-positive type based on our analysis in limited number of cases" (p.17, line 14-16) .

Second, reasonable explanation (or speculation) should be presented about why highly atypical or invasive lesions are frequently associated with MNs in MUC1$^+$ IPMNs, compared with other MUC phenotypes. Otherwise, authors should explain (or discuss) why lesions of the highest atypia or minimal invasion are likely to occur in the flat or low-papillary lesion among MUC2$^+$ or MUC1/2$^-$ IPMNs.

>>> We agree that this is one of the most important issues needed to be resolved but so far we are not able to make reasonable explanations. It is generally believed that invasive IPMN may arise from MN, but in our cases invasive front could be found in MN only in MUC1+ cases. MUC1 is known to act as cell adherent molecule, however, the precise role of MUC1 on the progression of tumor has not been fully understood. On the other hand, MUC2 is considered to be a differentiation marker expressed by intestinal type epithelium, and in colorectal cancer MUC2 positive tumor exhibit polypoid growth pattern before invade. Therefore, it is unlikely that MUC2 expression directly influent development of invasive lesion. Although our data strongly suggested that in MUC2+ and MUC1–/MUC2– subtype highest atypia or minimal invasion could arise from flat or low-papillary lesion, those two subtype represent distinct phenotype; i.e. there is no association between the presence of mural nodule and grade of atypia in MUC1–/MUC2– cases. Further studies are required to identify difference in molecular signature in each IPMN subtype based on MUC1/2 expression.

The numbers of tables are too much. For examples, the description of macroscopic type in Table 2 has been already presented in Table 1, and the description of dysplastic grade in Table 2 is essentially the same as Table 5. Such a redundancy should be omitted. In addition, Table 3 and Table 6 can be combined. For the explanation of short tables such as Table 2, 3, 6, a description in the text is enough.

>>> As the reviewer suggested, the Tables are combined together and total number of revised Tables are now 4. Old Table 2 was deleted, and Table 3 and 6 was combined as new Table 2. Table 4 and 5 has been labeled as new Table 3 and 4.

P.7 line 18, the numbers of main duct, and branch duct type IPMNs should be written in the 'Result' section.

>>> We appreciate the reviewer's comments and the patients characteristics has been moved to "Results" section, P.10 line 12.

Response to Reviewer #2's Comments:

1. How were mural nodules initially located (were they seen radiographically? Were they grossly evident?). These lesions may be easily overlooked in specimen grossing and this problem might be addressed in the paper. It is also quite confusing how on retrospective review accurate mapping of the mural nodules and dysplasia in surrounding tissue could be made. This should be clarified in the materials and methods.

>>> We appreciate the reviewer's thoughtful comments. In the current study, we defined MNs as mucosal elevated lesions higher than 3-mm histologically as described in *Materials and Methods*. In other words, those lesions could be macroscopically recognized on the resected specimens, and most of them were preoperatively pointed out by imaging modalities including EUS. I agree that retrospective review accurate mapping of the MN is not very easy process. However, we routinely handle resected specimen by 5-mm in width after a formalin fixation in the presence 2 gastroenterologists involved in preoperative imaging diagnosis in addition to 2 surgeons. We carefully performed imaging-histology correspondence for every case enrolled in this manuscript in order to discover "true bud" for invasive lesions. Indeed, 18 of 19 MNs (average size was ## ± ##) assessed in this study were what demonstrated preoperatively.

2. Appreciated discussion of problems stratifying risk based on morphologic subtypes and mucin subtypes. However, the attempt to correlate mucin staining pattern with MN was unconvincing in this small sample, and it is difficult to see how the findings justify pursuing preoperative evaluation of mucin phenotype, as seems to be the recommendation of the authors. If this is a conclusion of the paper the case could be stated more convincingly.

>>> We agree with this point. As we mentioned in Response to Reviewer #1's Comment, we have stated about limitation of our retrospective study in Discussion section (P.17 line 14). However, we are optimist regarding the "preoperative evaluation of mucin phenotype". As described in Discussion, Hibi *et al.* reported that immunostaining of the cytology sample obtained via pancreatic duct could be corresponded in 79 % cases. We also have similar preliminary data showing that "immunological" cytodiagnosis could be successfully performed by pancreatic juice collection, and the content will be sent for another submission.

3. The most important contribution of this paper is in helping to establish the relationship of mural nodules to dysplasia and invasive carcinoma. It is to be hoped that further evaluation of the significance/etiology of MN, including possible clonal relationships to dysplastic epithelium will be pursued in the future.

>>> We are grateful for the reviewer's suggestions with respect to our future plan. We have previously performed Kras mutation analysis using IPMN samples and reported that there are clonal variations even in a single dilated duct (Izawa T, *et al.* Cancer 2001). We are currently

extended this approach to clarify whether highly dysplastic (or invasive) compartment of IPMN possess same Kras mutation pattern or not.

4. A sentence in the next-to-last paragraph ("Identification of reliable molecular markers allows us to appropriately understand the process of progression of IPMN") alludes to molecular markers but should either be somewhat expanded on or removed, since no other reference is made to molecular markers in the body of the paper.

>>> As the reviewer suggested, the selected part was deleted. We agree the statement is a general perspective and could not be concluded based on our results.

Cover Letter

Dear,

We would like to share with you our manuscript entitled “Localization of the most severely dysplastic/invasive lesions and mucin phenotypes in intraductal papillary mucinous neoplasm (IPMN) of the pancreas” for a submission review.

IPMN of the pancreas have a histological spectrum ranging from benign adenoma to invasive cancer. Although IPMN is generally thought to be a slow-growing tumor, the invasive lesion signifies poor outcome. Number of reports highlighted the relationship between the presence of mural nodules (MNs) and the histological malignancy grades of IPMN. However, thus far, previous studies have not specifically addressed the localization of the most severely dysplastic lesions, and it is not formally validated whether an invasive lesion in IPMN develops within MNs. The current study demonstrated for the first time that most severely dysplastic or invasive lesions *per se* more frequently localized in the flat or low papillary regions rather than MNs. Therefore, we’d like to emphasize the requirement of careful survey by paying attention to flat/non-elevated lesions as foci with potential invasiveness, and requirement of surgical intervention could be considered based on their biological phenotype such as MUC1/2 expression.

The content of this manuscript has not been published or submitted for publication elsewhere except as a brief abstract in the proceedings of a scientific meeting or symposium. All authors contributed to this work are in agreement with the content of the manuscript, and the investigators have no financial conflicts of interest to disclose.
The attached paper titled “Localization of the most severely dysplastic/invasive lesions and mucin phenotypes in intraductal papillary mucinous neoplasm (IPMN) of the pancreas” has been carefully reviewed by an experienced medical editor whose first language is English and who is specialized in the editing of papers written by physicians and scientists whose native language is not English.

Thank you for your consideration, and please do not hesitate to contact me if any questions arise.

Best regards,

Hidenori Karasaki, MD, PhD
Division of Gastroenterologic and General Surgery, Department of Surgery, Asahikawa Medical College.
2-1 Midorigaoka Higashi, Asahikawa, Hokkaido 078-8510, Japan
Phone: 81-166-68-2503; Fax; 81-166-68-2193; E-mail: karasaki@asahikawa-med.ac.jp

Table

Table 1. Patients Characteristics

	Main duct type	Branch duct type
age; mean±sd	68.6 ± 8.51	67.8 ± 7.61
gender; male/female	5/6	22/6
mural nodule; +/–	9/2	10/18
dysplastic grade; low/mod/high/MI	0/4/4/3	1/17/6/4
mucin phenotype; $M1^+M2^-/M1^-M2^+/M1^-M2^-$	2/6/3	2/13/13

Low, low grade dysplasia; mod, moderate dysplasia; high, high grade dysplasia; MI, minimally invasive carcinoma; $M1^+M2^-$, $MUC1^+MUC2^-$; $M1^-M2^+$, $MUC1^-MUC2^+$; $M1^-M2^-$, $MUC1^-MUC2^-$.

Table 2. Relationship between presence of mural nodules and dysplastic grades of intraductal lesions of the pancreas.

	Mural nodule		
	+	–	
Dysplastic grade			
low-mod	6	16	
high-MI	13	4	p<0.01
Macroscopic type			
Main duct type	9	2	
Branch duct type	10	18	p<0.05

Low, low-grade dysplasia; mod, moderate dysplasia; high, high-grade dysplasia; MI, minimally invasive carcinoma.

Table 3. Location of the highest atypia.

	Location of the highest atypia	
	Mural nodule	Flat or low papillary
Branch duct type	1 (10.0)	9 (90.0)
Main duct type	3 (33.3)	6 (66.7)
Total	4 (21.1)	15 (78.9)

Parentheses denote percentages.

Table 4. Location of invasion of minimally invasive carcinoma.

Case	Subtype	Invasive type	MN	Location of invasion
1	MUC1+	Tub	+	MN
2	MUC2+	Muc	+	low papillary
3	MUC1+	Muc, Tub	+	low papillary
4	MUC1+	Tub	+	MN
5	MUC1/2-	Tub	–	flat
6	MUC1/2-	Tub	+	low papillary
7	MUC2+	Muc, Tub	+	flat

Tub, tubular adenocarcinoma; Muc, mucinous carcinoma; MN, mural nodule.

Table 5. Relationship among mucin phenotype-based subtypes, mural nodules, and atypia.

Subtype	Atypia	Mural nodule	
		+	–
MUC2+ (MUC1–)	low-mod	4	7
	high	5	1
	MI	2	0
		11 (57.9)	8 (42.1)
MUC1,2–	low-mod	2	8
	high	2	2
	MI	1	1
		5 (31.3)	11 (68.7)
MUC1+ (MUC2–)	low-mod	0	1
	high	0	0
	MI	3	0
		3 (75.0)	1 (25.0)

Low, low-grade dysplasia; mod, moderate dysplasia; high, high-grade dysplasia; MI, minimally invasive carcinoma. Parentheses denote percentages.

Table 6. Location of the highest atypia in relation to mucin phenotype-based subtypes.

Subtype	Location of the highest atypia	
	Mural nodule	Flat or low papillary
MUC2+	1 (9.1)	10 (90.9)
MUC1,2–	1 (20.0)	4 (80.0)
MUC1+	2 (66.7)	1 (33.3)

Parentheses denote percentages.

Original article

Localization of the most severely dysplastic/invasive lesions and mucin phenotypes in intraductal papillary mucinous neoplasm (IPMN) of the pancreas

Hidenori Karasaki, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical College, Hokkaido, Japan

Yusuke Mizukami, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Hokkaido, Japan

Yoshihiko Tokusashi, MD, PhD

Department of Surgical Pathology, Asahikawa Medical College, Hokkaido, Japan

Kazuya Koizumi, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Hokkaido, Japan

Akira Ishizaki, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical College, Hokkaido, Japan

Kouji Imai, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical College, Hokkaido, Japan

Daitaro Yoshikawa, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical College, Hokkaido, Japan

Shuichi Kino, MD, PhD

Department of Clinical Laboratory and Blood Center, Asahikawa Medical College, Hokkaido, Japan

Junpei Sasajima, MD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Hokkaido, Japan

Satoshi Tanno, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Hokkaido, Japan

Kakuya Matsumoto, MD

Division of Metabolism and Biosystemic Science, Asahikawa Medical College, Hokkaido, Japan

Naoyuki Miyokawa, MD, PhD

Department of Surgical Pathology, Asahikawa Medical College, Hokkaido, Japan

Toru Kono, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical College, Hokkaido, Japan

Yutaka Kohgo, MD, PhD

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Hokkaido, Japan

Hiroyuki Furukawa, MD, PhD

Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical College, Hokkaido, Japan

Correspondence to: Hidenori Karasaki, MD, PhD

Division of Gastroenterologic and General Surgery, Department of Surgery, Asahikawa Medical College.

2-1 Midorigaoka Higashi, Asahikawa, Hokkaido 078-8510, Japan

Phone: 81-166-68-2503; Fax; 81-166-68-2193; E-mail: karasaki@asahikawa-med.ac.jp

Running title: Mural nodule and malignant focus in IPMN

Grant Support: This work was supported by grant to H.K. from Pancreas Research Foundation of Japan.

Introduction

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas have a histological spectrum ranging from benign adenoma to invasive cancer[1]. The diameters of cysts and the main pancreatic duct as well as the presence of mural nodules (MNs) correlate with histologically malignant grades of IPMN[2-4], and these criteria are widely utilized to exclude benign lesions from surgical intervention. Among factors assessed via preoperative imaging, the presence of MNs is recognized as the most important factor associated with malignant epithelial lesions[3-5]. The postoperative prognosis of IPMN is, in general, favorable according to published guidelines[6]; however, a current clinical dilemma is that large numbers of benign lesions undergo surgical resection. Since accurate preoperative prediction of malignancy is not thus far feasible by imaging modalities, identification of pre-invasive lesions and establishment of a novel molecular based management strategy is of interest. Ultimately, appropriate criteria that can identify IPMN cases with rapidly invasive adenocarcinoma are needed. This would allow follow-up of less aggressive lesions and obviate unnecessary surgery.

Early detection of invasive components(s) of IPMN lesions is important, as recent studies demonstrated that a large number of branch duct type IPMN without MN could be safely followed-up without surgery[7-8]. Since IPMN is generally thought to be a slow-growing tumor and in most cases develops in elderly people, follow-up alone could be recommended for large

number of branch duct cases if MNs are not identified. Careful attention is however required to detect the emergence of invasive lesions[9], since it has been reported that the presence of an obviously invasive lesion in IPMN signifies poor outcome[10-11]. Thus it is desirable to resect IPMN at the stage of minimally invasive carcinoma (MI). Nevertheless, current imaging modalities are limited in making an accurate diagnosis of IPMN with MI[10]. More importantly, it is not formally validated whether an invasive lesion in IPMN develops within MNs or not.
It is challenging to postpone surgical intervention until the lesions develop high-grade dysplasia without losing the current level of safety. One potential approach includes a classification of IPMN into subtypes based on mucin expression phenotypes, as previously demonstrated[12-14]. In addition, specification of "pre-invasive" lesion by precise histologic assessment and grading of epithelial lesions is also required. In the current study, we sought to reevaluate the clinical significance of MNs, which are believed to be the finding most suggestive of malignancy in IPMN[15]. We specifically focused on the distribution of the most dysplastic lesions within IPMNs and their mucin subtypes in order to predict IPMN patients likely to develop high-grade dysplasia with potential invasiveness.

Materials and Methods

Pancreatic tissues from IPMN patients were obtained as surgical discards as part of an IRB and Ethical Committee approved protocol at the Asahikawa Medical College Hospital. The subjects

included 39 cases of IPMN resected at the Division of Gastroenterologic and General Surgery, Department of Surgery, Asahikawa Medical College between January 1994 and December 2008 and diagnosed pathologically as IPMN. Cases with evident invasive cancer were excluded. The resected pancreas was fixed in 10% formaldehyde, and the whole specimen was sliced at a thickness of 5-mm and stained with hematoxylin-eosin (H-E). Mapping based on the grades of dysplasia on H-E staining was then performed by three gastroenterologists/pathologists to identify the distribution of the most severely dysplastic lesions and minimally invasive lesions.

According to the diagnosis and grade criteria of Armed Forces Institute of Pathology (AFIP)[1], dysplastic lesions were categorized into low-, moderate-, and high-grade dysplasia. Adenocarcinoma lesions that had infiltrated into the stroma microscopically, but not macroscopically, were designated as MI. When a resected pancreas contained different grades of dysplasia, the section with the highest grade was designated as the most severely dysplastic area. MN was defined as a lesion of greater than or equal to 3mm in height histologically, and the lesions were categorized into those in which the highest dysplasia was localized to MNs and those in which the highest dysplasia was found in areas apart from MNs, *i.e.*, flat or low papillary lesions. Each case was categorized as main duct type or branch duct type according to the macroscopic quantitative predominance; 11 cases were categorized as main duct type, while 28 were branch duct type (**Table 1**).

Areas of the predominant histological grade and the most severely dysplastic areas were subjected to immunohistochemical staining for MUC1/2. Serial sections 4 μ m in thickness were sliced from paraffin blocks and deparaffinized. After intrinsic peroxidase was blocked, antigens in slices were retrieved in 10 mM TRIS buffer (pH 10.0) at 110 ºC for 10 minutes by microwaving. Then, they were incubated for 30 minutes at room temperature with anti-MUC1 antibody (clone, Ma695) and anti-MUC2 antibody (clone, Ccp58). The samples that were negative for MUC1 and diffusely positive for MUC2 were defined as the MUC2-positive type, while those that were negative or partly positive for MUC1 and negative for MUC2 were defined as the MUC1/2-negative type, and those that were diffusely positive for MUC1 and negative for MUC2 were defined as the MUC1-positive type.

Statview 5.0 software (SAS Institute Inc., Cary, NC) was employed for statistical analysis. Differences between categorical variables were evaluated using χ^2 test. A P value less than 0.05 was considered statistically significant.

Results

Correlation between presence of MNs and grades of pancreatic intraductal dysplasia

A total of 19 IPMNs with MNs included 6 with moderate dysplasia, 7 with high grade dysplasia, and 6 with MI. The 20 cases without MNs included 1 with low grade dysplasia, 15 with

moderate dysplasia, 3 with high grade dysplasia, and 1 with MI. Therefore, high grade dysplasia and MI were significantly more common when MNs were present ($p < 0.01$) (**Table 2**).

Distribution of the most severely dysplastic intraepithelial lesions

We next tested the hypothesis that MNs correspond to the most severe lesions histologically. However, to our surprise, the most severely dysplastic lesions were observed in the MNs of 4 out of 19 IPMN with MNs (21.1%) (**Figure 1**). Indeed, the most severely dysplastic epithelial lesions were localized in areas apart from MNs in 15 cases (78.9%) (**Figure 2, Table 3**). There were 7 cases with MI (3 main duct type and 4 branch duct type IPMN). MNs were present in 6 of 7 cases with MI lesions. However, an invasive front was observed within MNs in only 2 cases (28.6 %). In a larger numbers of cases (5 of 7 cases, 71.4 %), MI lesions were localized in the flat or low papillary area, distant from MNs (**Figure 3, Table 4**).

Distributions of the most dysplastic lesions and macroscopic types

MNs were evident in 9 of 11 cases with main duct type (81.8 %), and in 10 of 28 (35.7 %) with branch duct type IPMNs. Thus, MNs were more frequently observed in the main duct type IPMN ($p < 0.05$) (**Table 2**).

The most dysplastic lesions were localized in the MNs in 3 of 9 (33.3 %) with main duct types and in 1 of 10 (10.0 %) with branch duct IPMNs. Therefore, the most severely dysplastic lesions

were more frequently observed in locations apart from MNs in both macroscopic types, and there was no significant difference in the frequency between the two subtypes (p = 0.3034) (**Table 3**).

Relationship between mucin phenotypes and the most severely dysplastic lesions

We next sought to evaluate mucin phenotype by performing MUC1/2 immunohistochemistry. The predominant mucin phenotypes included 19 MUC2-positive cases (48.7 %), 16 MUC1/2-negative cases (41.0 %), and 4 MUC1-positive cases (10.3 %) (**Figure 4**). The relationship between mucin phenotype-based subtypes and the most severe dysplastic grade in cases with and without MNs is shown in **Table 5**. In MUC2-positive cases, high grade dysplasia or MI was found in 7 of 11 cases with MNs (63.6 %), whereas only 1 case revealed high grade dysplasia in subjects without MNs (11.1 %), demonstrating a correlation between the presence of MN and grade of dysplasia in MUC2-positive IPMN (P = 0.06). In addition, MI was also evident in all three MUC1-positive cases with MNs, and one other case without MNs revealed moderate dysplasia. However, in the MUC1/2-negative group, MI was identified in patients without MNs and a low to moderate grade as the most severe dysplasia was found in cases with MNs (2 cases; 40.0 %), implying no apparent correlation between the presence of MNs and dysplastic grade in this subtype.

Mucin phenotypes and location of the most severely dysplastic lesions

We next sought to define the relationship between mucin phenotypes and location of the most severely dysplastic lesions in 19 IPMN subjects with MNs. The most severely dysplastic lesion

was present in the areas apart from MNs in 10 of 11 (90.9 %) MUC2-positive cases, 4 of 5 MUC1/2-negative (80.0 %) cases, and 1 of 3 (33.3 %) MUC1-positive case. Therefore, although the severely dysplastic epithelial lesions could be frequently found in the MNs in MUC1-positive cases, the majority appeared to develop in the areas outside of MNs in MUC2-positive and MUC1/2-negative cases (**Table 6**).

Mucin phenotypes and location of the minimally invasive lesions

Invasive phenotype is one of the hallmarks of malignant tumors, and MNs in IPMN have generally been considered to be the lesions that initiate invasion into pancreatic parenchyma. We thus investigated the relationship between the invasive area of MI and mucin phenotypes. Consistent with the observation that the most severely dysplastic lesions were recognized in areas apart from MNs in MUC2-positive cases, invasive lesions were also observed outside of MNs (**Table 4**). However, this was not found in the MUC1-positive subtype, since MIs were co-localized with MNs in 2 of 3 MUC1-positive IPMN (66.7 %). In the MUC1/2-negative subtype with MI, including a case without MN, invasive lesions were observed in the low papillary area.

Discussion

A number of reports highlight the presence of MNs in IPMN as a reliable sign indicative of malignancy[2, 4-5]. However, previous studies have not specifically addressed the localization of

the most severely dysplastic lesions, and it remains to be determined whether or not invasive lesions generally develop from MNs. Therefore, it is important to identify the distribution of "pre-invasive" lesions with potential to invade in order to determine appropriate clinical management: the decision as to follow-up versus surgical intervention.

In the current study, the presence of MNs strongly correlated with histological grades of malignancy, and high grade dysplasia and MI were frequently observed in cases with MNs ($p = 0.0036$) (**Table 1**), validating the current strategy for the management of IPMN patients based on the consensus guidelines[6]. However, to our surprise MNs were found co-localized with the most severely dysplastic lesions in only 21.1 % cases of IPMN (33.3 % in the main duct and 10.0 % in branch duct type) (**Table 2**). These results imply that the most severely dysplastic area is occasionally localized in flat or low papillary lesions apart from MNs, and that MNs are not always co-localized to the most severely dysplastic area. Therefore, the appearance of MNs is an indirect marker for the potential development of high-grade dysplastic lesions in the pancreas, rather than direct evidence of severely dysplastic lesions.

We also found the invasive front localized in the flat or low-papillary areas in 5 of 7 cases with MI (**Table 3**), indicating that invasive IPMN can frequently develop outside of MNs. Consequently, careful attention should also be paid to lesions outside of MNs, since our data show that flat or low-papillary areas can also be pre-invasive lesions in patients with IPMN. Therefore, post surgical survey will be required as dysplastic lesions may be left as remnants

subsequent to limited surgical resection of the IPMN lesion containing MNs and could potentially develop into invasive carcinoma.

Currently, IPMN is categorized into four subtypes: intestinal, gastric, pancreatobiliary, and oncocytic, and each type displays a specific mucin phenotype[12-14]. However, the clinical relevance of this classification is not yet apparent. It has been demonstrated that the prognosis of intestinal type IPMN was favorable[14], while other reports have argued that it was poor because of a frequent concurrence of carcinoma *in situ* and invasive cancer[12]; thus far no consensus has been reached. In the current study, 8 of 9 MUC2-positive cases without MNs were histologically diagnosed as moderate dysplasia and only 1 as high-grade dysplasia. In contrast, in cases with MNs, high-grade dysplasia or MI was observed in 7 of 11 cases, implying that the presence of MNs in MUC2-positive IPMN is indicative of a higher grade of dysplasia (**Table 5**). Hence, a careful clinical follow-up could be feasible for MUC2-positive IPMN until there is evidence of MNs, and in principal MUC2-positive IPMN with MNs should be resected. However, it should be noted that the most severely dysplastic areas and MI were occasionally found in areas outside of MNs in this category. Therefore, early invasive adenocarcinoma may be missed if follow-up is solely focused on MNs.

The prognosis of pancreatobiliary type IPMNs remains unclear; however, an argument against surgical intervention is limited due to frequent incidence of malignant lesions[14, 16-18]. In the present study, MNs in MUC1-positive type corresponded to MI lesions, suggesting that IPMN lesions in this type remain localized in epithelia for a very short time and are likely to invade

rapidly. Even in a case in which invasive lesions were observed outside of MNs, high grade dysplasia was also evident in MNs *per se*. Thus, considering the fact that development of MNs in MUC1-positive cases directly reflects the appearance of severely dysplastic lesions and that such cases are likely to develop invasive cancer rapidly, surgical intervention should be considered at an early time point after diagnosis.

It has been reported that mildly dysplastic and benign lesions are often observed in gastric type IPMN, and a number of studies have demonstrated a good prognosis[12, 14]. MUC1/2-negative cases could be categorized as gastric type IPMN[13]. However, in the current study, both MUC1/2-negative cases composed of severe dysplasia and MI in flat lesions are included in cases with MNs. It should be noted that in the current criteria, based on the morphology of epithelial lesions, gastric type IPMN is histologically defined as lesions with "basally located nuclei". In other words, the criteria *per se* could be biased by the grades of dysplasia[12-14]. On the other hand, "complex papillae" and "enlarged hyperchromatic nuclei" are included in the criteria for the pancreatobiliary type IPMN, restricting this subtype to severely dysplastic lesion. Although some of the MUC1/2-negative cases in our study may be categorized as a pancreatobiliary type by other studies, we carried out classifications based solely on mucin phenotypes without considering morphology and grade of dysplasia because mucin expression phenotype was used as a parameter independent of macroscopic type, presence of MN, and histological grade of dysplasia in the current study.

Although gastric type IPMN has been shown to include benign branch duct type[12], there is no significant difference in the incidence of high-grade dysplasia or MI between MUC1/2-negative cases (6 of 15 cases; 40.0 %) and MUC2-positive cases (8 of 20 cases; 40.0 %). The common site of the most severely dysplastic areas and MI was outside of MN in both MUC1/2-negative and MUC2-positive cases. However, all MUC1/2-negative cases exhibited tubular adenocarcinoma at the invasion front within the MI lesion. Further investigation into the natural history and prognosis is necessary for the MUC1/2-negative type.

Mucin phenotype-based preoperative diagnosis can allow a rational decision on the management of IPMN patients. Hibi, *et al.* reported that in 79 % of cases, cytological assessment during endoscopic retrograde pancreatography (ERP) and histological subtype of resected specimens were consistent in terms of mucin subtype[19]. In the present study, the relationship between cytology and subtype was not examined; however, in 89.5 % of cases with MNs intraepithelial lesions extended to the main pancreatic duct. Therefore, in most cases, mucin-based classification could be achieved by immunostaining for MUC1 and MUC2, utilizing either pancreatic fluid or a biopsied specimen obtained by ERP. Classification based solely on mucin phenotypes may offer important additive information to conventional image-based macroscopic types and morphological classification such as presence of MNs, even if histological information regarding structural atypia is not obtained.

Discrepancy during pathological assessment of IPMNs is a clinical issue that needs to be resolved. Multicenter prospective analysis based on a more objective rule will be required to overcome the confusion. To date, it has been elucidated that IPMN is one of the risk factors for concurrently developing invasive ductal carcinoma of the pancreas[9, 20]. If an invasive lesion is localized apart from IPMN, it is considered to be a synchronous cancer. However, the invasive lesion may be expected to arise from IPMN when the invasive compartment is located close to IPMN or the invasive front is evident within the IPMN. When two tumors are adjacently located and the invasive front is unclear it is hard to determine whether they are synchronous carcinoma (collision cancer) or IPMN-derived invasive carcinoma. MI lesions in this study were considered IPMN-derived invasive carcinoma because of continuity between intraductal and invasive lesions. However, the possibility that *de novo* carcinoma is included in the current study is not fully excluded. Identification of reliable molecular markers allows us to appropriately understand the process of progression of IPMN.

In conclusion, the presence of MNs strongly correlated with grades of dysplasia of IPMN, but severely dysplastic lesions or an invasive front can develop from the flat or low papillary regions rather than MNs. In terms of mucin phenotype-based subtypes and presence of MNs, an invasive lesion may arise from MN in the MUC1-positive type. However, this is not the case with the MUC2-positive and MUC1/2-negative group, in which mucinous and tubular adenocarcinoma potentially develop outside of MNs. Collectively, the current study proposes a requirement of careful survey by close inspection of flat/non-elevated lesions as foci with potential invasiveness.

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Figure legends

Figure 1

A case with the most severely dysplastic lesion in the mural nodule (MN). Most MNs in the dilated branches showed moderate dysplasia, but high-grade dysplasia was observed at the root of the MNs (A, C). Macroscopic appearance of H-E staining. Arrow indicates the area of histologically predominant moderate dysplasia in MNs, as shown in magnified view (B). Moderate dysplasia was predominant as indicated by the arrow in the panel (A). An arrowhead indicates the most severely dysplastic lesion at the root of MNs, as shown in magnified view (D). High-grade dysplasia was observed in the area indicated by the arrowhead in panel (C). Scale bars, 500 μm.

Figure 2

A case with the most severely dysplastic lesion present outside of MNs. The whole MN showed moderate dysplasia, and the most severely dysplastic lesion was found at the low papillary region of the main pancreatic duct as shown in macroscopic view (A, B). The arrow in (A) indicates histologically predominant moderate dysplasia in the MNs, which is magnified in (C). The arrowhead in (B) indicates the most severely dysplastic lesion in the low papillary region. Non-invasive, but highly dysplastic lesion is shown in (D) with a magnified view. Scale bars, 500 μm.

Figure 3

A case with minimally invasive cancer that had the most severely dysplastic lesion and an invasion front at the flat area outside of MNs (A, C). A representative macroscopic view of a slice of the resected specimen including a MN 3cm in diameter recognized within the dilated branch. The largest part of the lesion was of moderate dysplasia (arrow), while a minute high grade dysplastic area and minimally invasive cancer were present at the flat part of the wall of the cyst (arrowhead). A magnified view of the histologically predominant moderate dysplasia (B). Invasive front developed from a high grade dysplastic area at the flat part of the wall of the cyst into the stroma was observed (C). Scale bars, 500 μm.

Figure 4

A representative case of subtypes based on mucin expression. H-E staining (A, D, G). immunostaining for MUC1(B, E, H). Immunostaining for MUC2(C, F, I). The MUC2-positive type (*upper panel*) was negative for MUC1 and positive for MUC2 (B, C). The MUC1/2-negative type (*middle panel*) was negative for MUC1 and MUC2 (E, F). The MUC1-positive type (*lower panel*) was positive for MUC1 and negative for MUC2 (H, I). Each subtype represents a unique histology corresponding to intestinal, gastric, and pancreatobiliary type, respectively. Scale bars, 500 μm.

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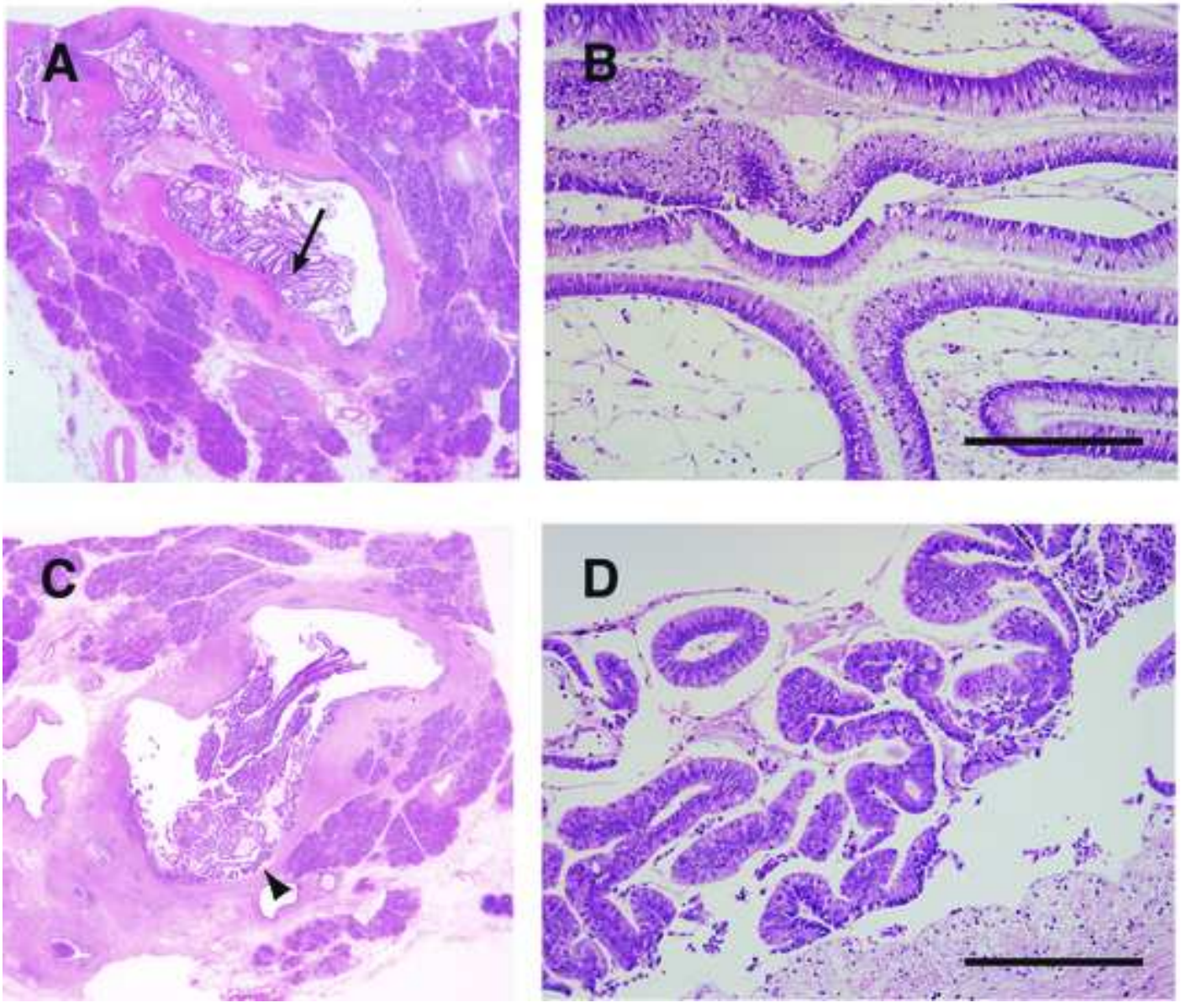

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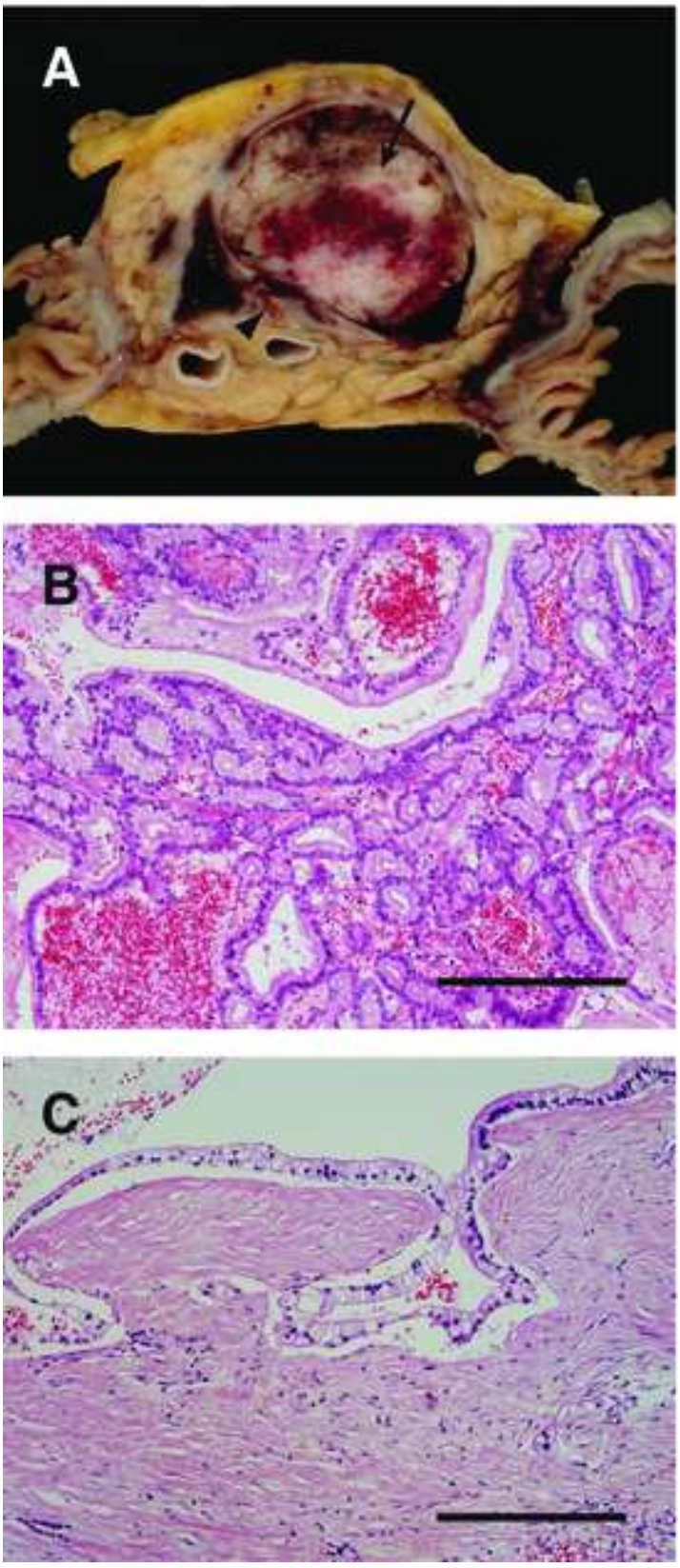

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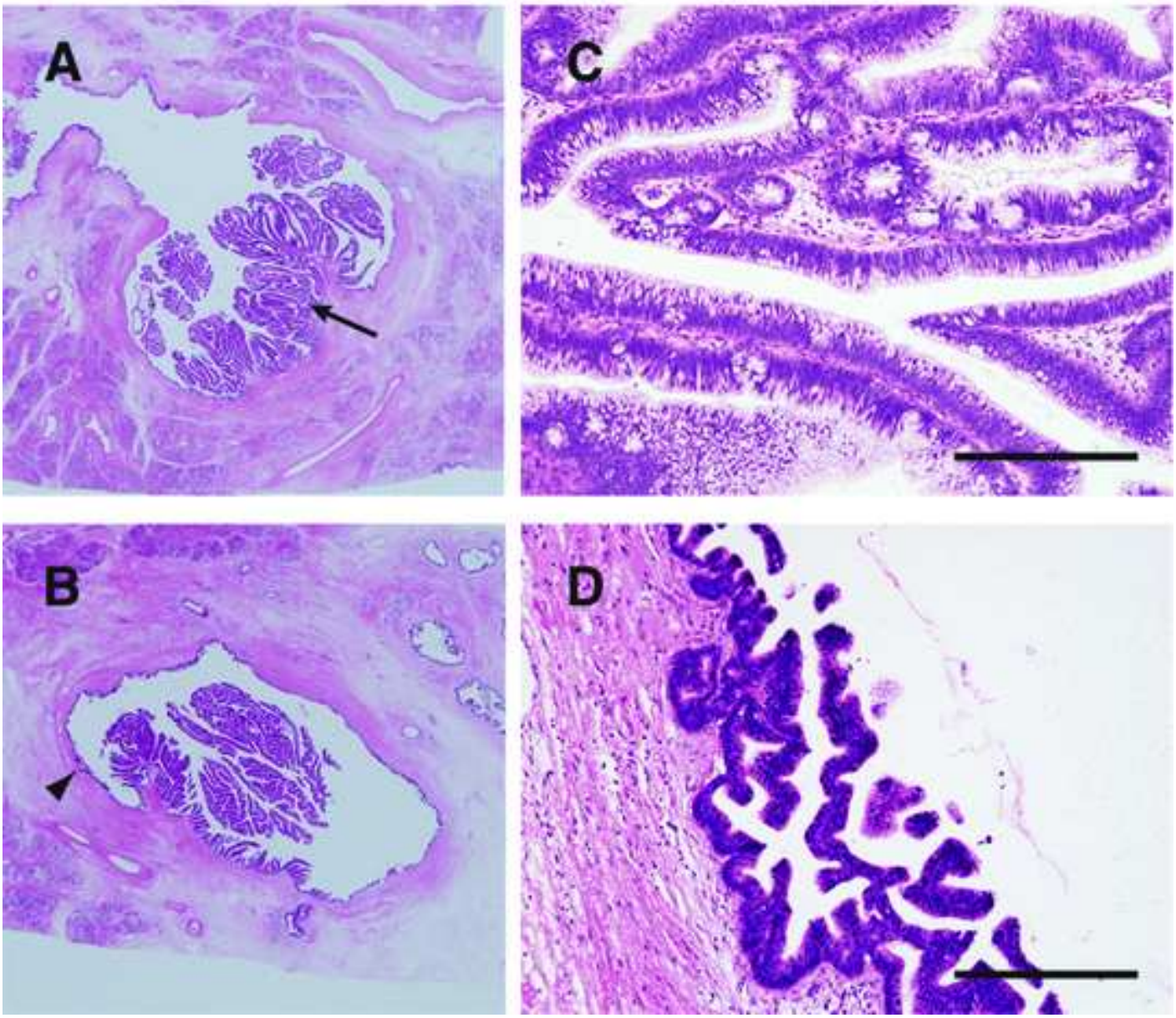

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Figure 4