Immunohistochemical Study of Claudin 18 Involvement in Intestinal Differentiation During the Progression of Intraductal Papillary Mucinous Neoplasm

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Abstract. *Background and Aim: A comparison was made between pancreatic ductal adenocarcinoma (PDAC), pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous carcinoma (IPMC) in order to understand the association between several markers and malignant potential. Patients and Methods: Fifteen surgically resected PDACs and four IPMCs were subjected to immunohistochemistry with primary antibodies to Ki-67, p53, MUC2, Gli-1, and Claudin-18 (CLDN18). Results: Ki-67, P53, MUC2, Gli-1 and CLDN18 were positive in 6 (50%), 10 (66.6%), 0 (0%), 4 (26.6%), and 6 (40%) of the 15 PDACs, respectively. Low- to high-grade PanIN complexes were found in 2 out of the 15 PDACs. Gli-1 was continuously expressed in low- and high-grade PanINs. CLDN18 was specifically expressed in high-grade PanINs, whereas the corresponding invasive tubules did not express CLDN18. P53 was positively stained in one of the 4 IPMCs in which minimally invasive tubular type carcinomas were observed. Ki-67 and CLDN18 were positively stained in all 4 IPMCs. CLDN18 was specifically expressed in intestinal-type components of IPMCs. Conclusion: CLDN18 is involved in intestinal-type epithelial differentiation in the progression of IPMCs, contradicting the previous knowledge of its specificity in gastric epithelial differentiation.*

Epithelial differentiation is an important factor in pancreatic carcinogenesis. Intraductal papillary mucinous neoplasm (IPMN) and pancreatic intraepithelial neoplasia (PanIN) are the two major precursors of pancreatic ductal carcinoma (PDAC) (1, 2). Most malignant IPMNs show intestinal-type morphology, characterised by diffuse MUC2 immunolabeling and are localised in the main duct (3, 4), whereas some comprehensive analyses have shown that high-grade PanIN formation is associated with gastric epithelial differentiation (5). In 2005, Prasad *et al*. revealed that the levels of the Hedgehog signaling pathway target genes are up-regulated in high-grade PanINs (5). It was also reported that the gastric epithelial transcription factor SOX2 is involved in PanIN-3 progression (6). In IPMNs, a unique form of carcinoma in the branch ducts was reported showing intestinal-type morphology with a high Ki-67 index, indicating that the malignant potential of IPMN is not sufficiently explained by the localisation in either the main duct or branch ducts (7). To provide a clear estimation of the association between malignant potential and the expression of several molecules including factors involved in epithelial differentiation, this study compared PDACs, PanINs, and IPMCs using immunohistochemistry.

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Key Words: Intraductal papillary mucinous neoplasm, pancreas, claudin-18, Gli-1, p53.

Patients and Methods

Data were retrospectively analysed for a total of 15 cases of surgically resected PDAC and 4 cases of IPMC at the department of Surgical Oncology in Gifu University Hospital between 2005 and 2009. All specimens were sliced consecutively into 5 mm thick sections, and were fixed in 10% formalin and embedded in paraffin. In PDACs, the most predominant histologic subtypes (T and N grade) and vascular invasion stage were determined based on the World Health Organization (WHO) criteria (8). PanIN-rich complexes were identified in 2 out of the 15 cases. In IPMCs, all cases were Stage I. Intraductal papillary components were classified into three subtypes (gastric, intestinal and pancreatobiliary), based on previously established criteria (9). In the four IPMCs, one case showed minimal invasion with tubular growth. Two cases included more than two subtypes. The paraffin sections of the 15 PDACs and the 4 IPMCs were sliced into 3 μm-thick sections for immunohistochemistry (IHC). The methods for IHC have been described previously (6). The primary antibodies

Table I. *Summary of immunohistochemical data.*

Case	T	N	LyorV	Subtype	Stage	p53	Ki-67	MUC2	Gli-1	CLDN18
IDC1	4	0	0	Well	4	2+	0	0	0	1+
IDC2	3	0	1	Well	2	0	0	0	0	1+
IDC3	3	0	1	Well	2	3+	1+	0	1+	2+
IDC4	3	0	1	Poor	2	1+	0	0	0	0
IDC5	2	0	1	Well	1	0	0	0	0	0
IDC6	2	1	0	Poor	3	3+	2+	0	0	1+
IDC7	2	0	0	Mod	2	0	1+	0	0	2+
IDC8	2	0	1	Poor	2	1+	2+	0	0	0
IDC9	2	1	1	Mod	3	2+	0	0	0	0
IDC10	3	1	1	Poor	3	2+	0	0	0	1+
IDC11	2	0	1	Mod	1	0	0	0	2+	0
IDC12	3	1	0	Mod	3	1+	0	0	0	0
IDC13	3	1	0	Mod	3	1+	2+	0	1+	0
IDC14	3	1	0	Well	3	2+	2+	0	0	0
IDC15	2	1	1	Mod	3	0	0	0	2+	0
Rate	*	*	*	*	*	66.6	40%	0%	26.6%	40%
IPMC1	*	*	*	IN	Inv	3+	3+	3+	0	3+
IPMC2	*	*	*	IN+G	*	0	2+	2+	0	1+
IPMC3	*	*	*	IN+PB	*	0	2+	3+	2+	2+
IPMC4	*	*	*	IN	*	0	3+	3+	0	3+
Rate	*	*	*	*	*	25%	100%	100%	25%	100%

Well: well-differentiated; Poor: poorly-differentiated; Mod: moderately differentiated.

used in the present study were: (i) MUC2, 1: 200 dilution; Zymed, CA, USA; (ii) Ki-67, clone MIB-1, 1: 200 dilution; Dakocytomation, Denmark; (iii) p53, clone DO-7, 1: 100 dilution; Dakocytomation; (iv) Gli-1, 1: 200 dilution; H-300 SC-20687, Santacruz, CA, USA; and (v) Claudin-18 (CLDN18), 1: 200 dilution; Zymed. According to the frequency, the expression level was classified into four grades from 0 to 3+: (i) grade 0 corresponded to undetectable staining or staining in fewer than 10% of the components, (ii) grade 1+ corresponded to focal staining in 10 to 30% of the components, (iii) grade 2+ corresponded to diffuse staining in more than 30% of components, and (iv) 3+ corresponded to diffuse staining in more than 50% of the components. Grades 1+, 2+ and 3+ were grouped together and considered as positive expression. Images of representative grading are shown in Figure 1.

Results

The immunohistochemical data are summarised in Table I. Ki-67 was diffusely expressed in all 4 cases of IPMCs, regardless of the histological subtype (Figure 2A and B). In PDACs, invasive components showed positive immunoreactions in 6 (40%) out of the 15 cases; however, only one case showed 3+ staining. P53 was positively stained in 10 (66%) out of the 15 cases of PDACs. In a case of poorly differentiated adenocarcinoma, almost all tumour cells in the closely packed nests showed nuclear expression of p53. In IPMCs, the expression of p53 protein was observed in one out (25%) of the 4 cases. Interestingly, this case showed minimal invasion with tubular morphology (Figure 2C-E). The Hedgehog signaling target protein Gli-1 was expressed in 4 (26.6%) of the 15 PDACs. Localized granular staining was observed in the cytoplasm. In IPMCs, focal cytoplasmic staining was observed in one (25%) case, which was localized in the pancreatobiliary components of the papillary lesions (Figure 2 F and G). MUC2 was specifically stained in IPMCs with intestinal-type components (Figure 2 H and I). CLDN18, a member of the tight junction protein claudin family was identified as a new marker of pancreatic cancer through differential gene expression analyses by Karanjawala *et al*. (10). Of the 15 cases of PDACs, 6 (40%) cases showed positive membranous staining for CLDN18. In IPMCs, intestinal-type components showed diffuse membranous staining in all 4 cases (Figure 2 J and K), whereas gastric- and pancreatobiliary-type components did not express CLDN18 (Figure 3). PanIN-rich complexes adjacent to the invasive components were observed in 2 out of the 15 PDACs. Protein expression in PanINs was assessed in the two cases. Among the five proteins, CLDN18 and Gli-1 were positively expressed in PanINs (Figure 4). Both CLDN18 and Gli-1 were diffusely expressed in high-grade PanINs, showing membranous staining and macular

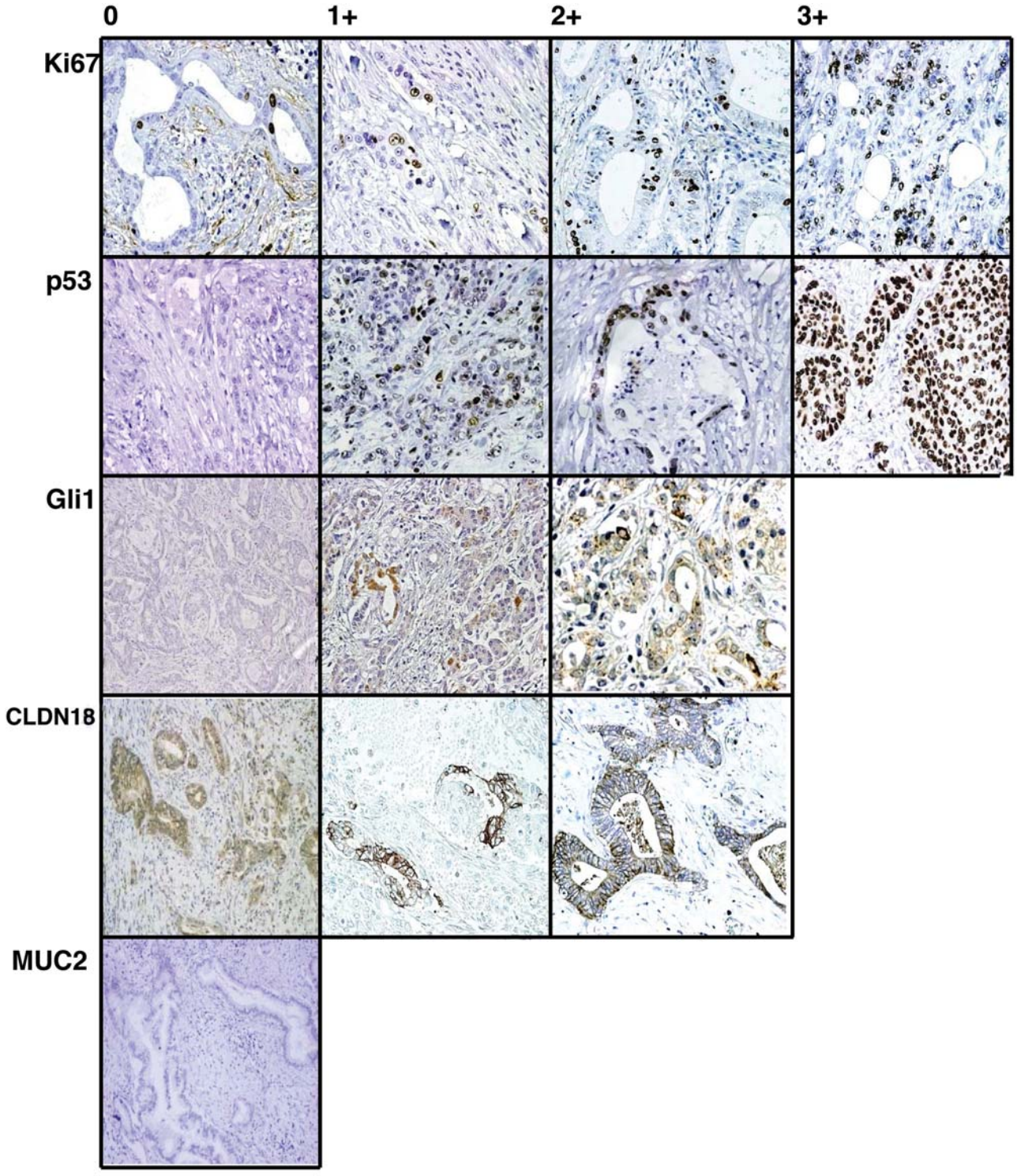

Figure 1. *Representative immunohistochemical images of staining for Ki-67, p53, Gli-1, MUC2, and Claudin-18 (CLDN18) in PDACs.*

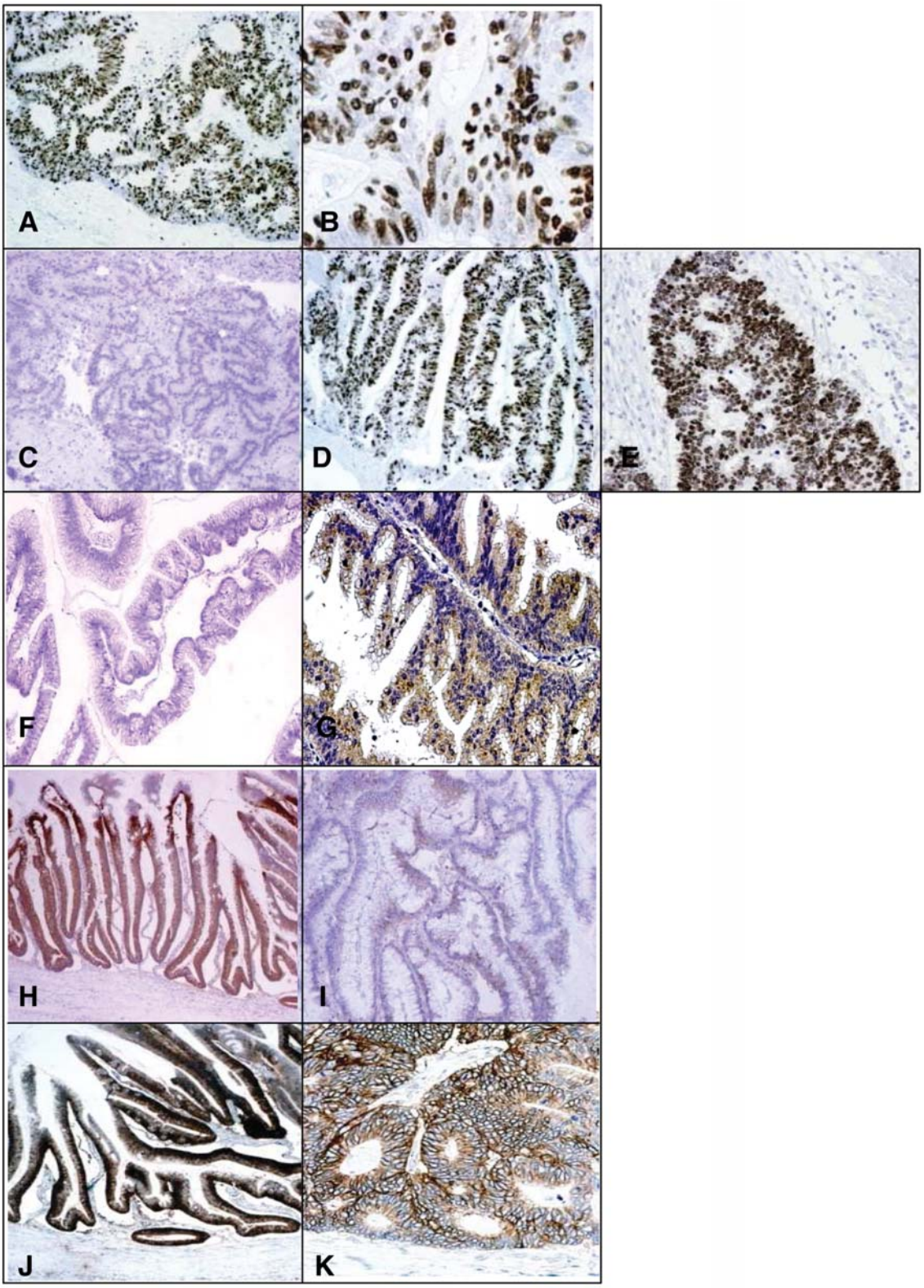

Figure 2. *Representative immunohistochemical images of staining for Ki-67 (A and B), p53 (C-E), Gli-1 (F and G), MUC2 (H and I) and CLDN18 (J and K) in IPMCs. Ki-67 was diffusely expressed regardless of subtype (A and B). P53 was positive in one case (D) with minimal invasion (B). Gli-1 is positively stained only in pancreatobiliary-type components (G), whereas intestinal-type components do not express Gli-1 (F). MUC2 (H) and CLDN18 (J and K) are diffusely stained in intestinal-type components. Gastric-type adenomas do not express MUC2 (I).*

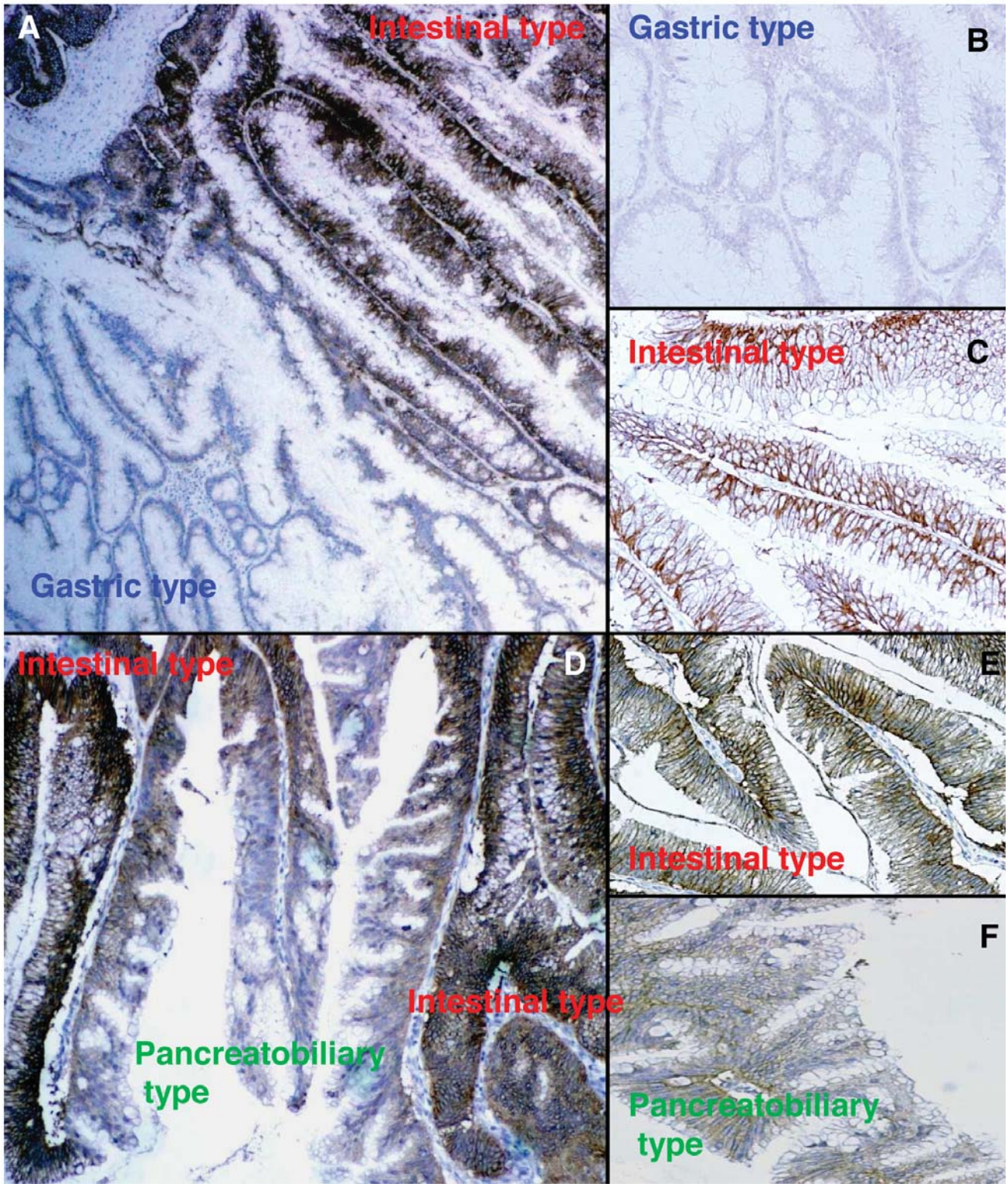

Figure 3. *Expression of CLDN18 in two cases (A-C: case 2; D-F: case 3) of IPMCs composed of a combination of more than one subtype. Diffuse membranous staining can be seen only in the intestinal-type components.*

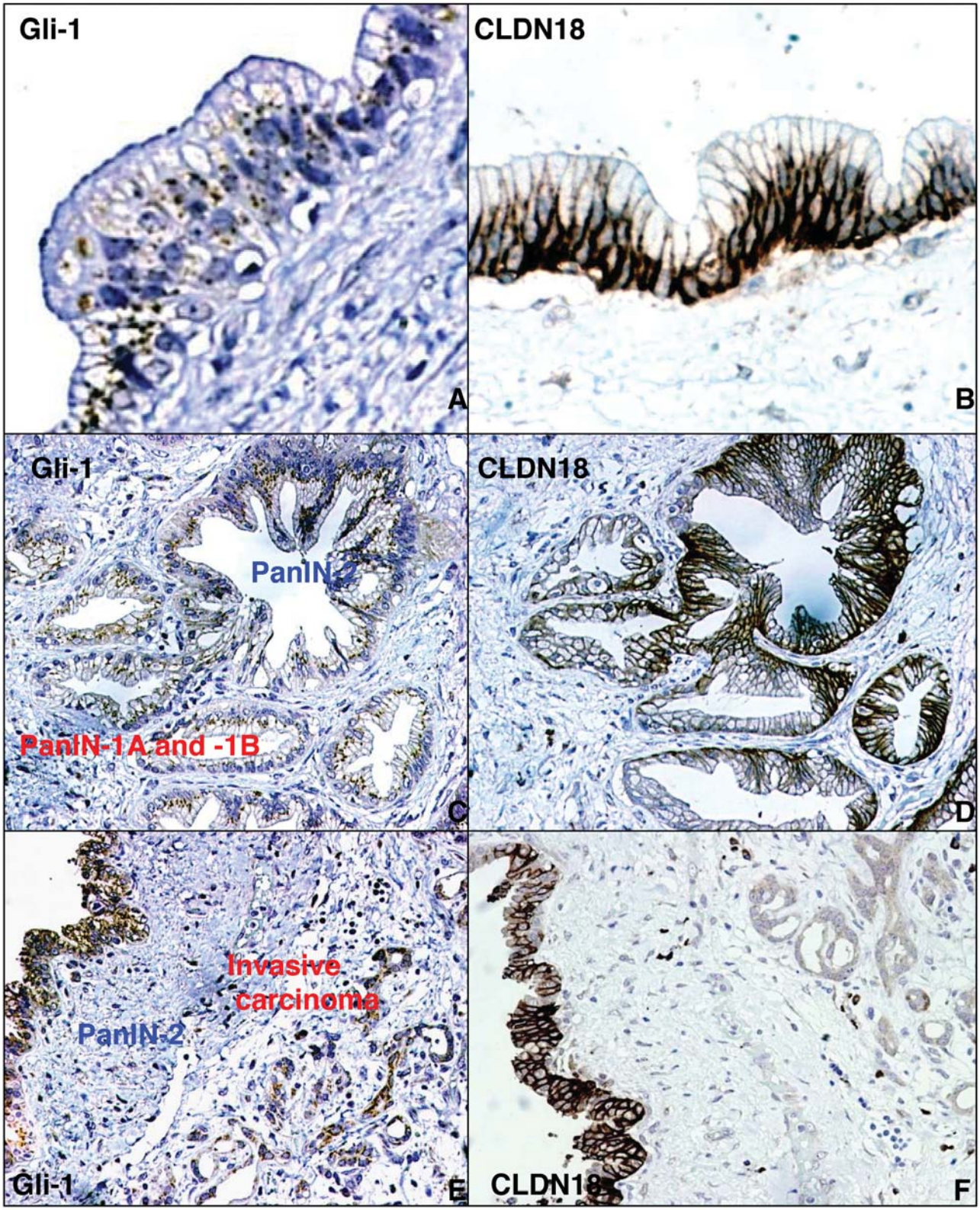

Figure 4. *PanINs showing spotted labeling of Gli-1 in the cytoplasm (A) and membranous labeling of CLDN18 (B). Both PanIN-1 and -2 express Gli-1 evenly (C), whereas positive staining for CLDN18 was seen only in PanIN-2 (D). Expression of Gli-1 was maintained in corresponding invasive components (E), whereas that of CLDN18 disappeared from invasive components adjacent to PanIN-2 (F).*

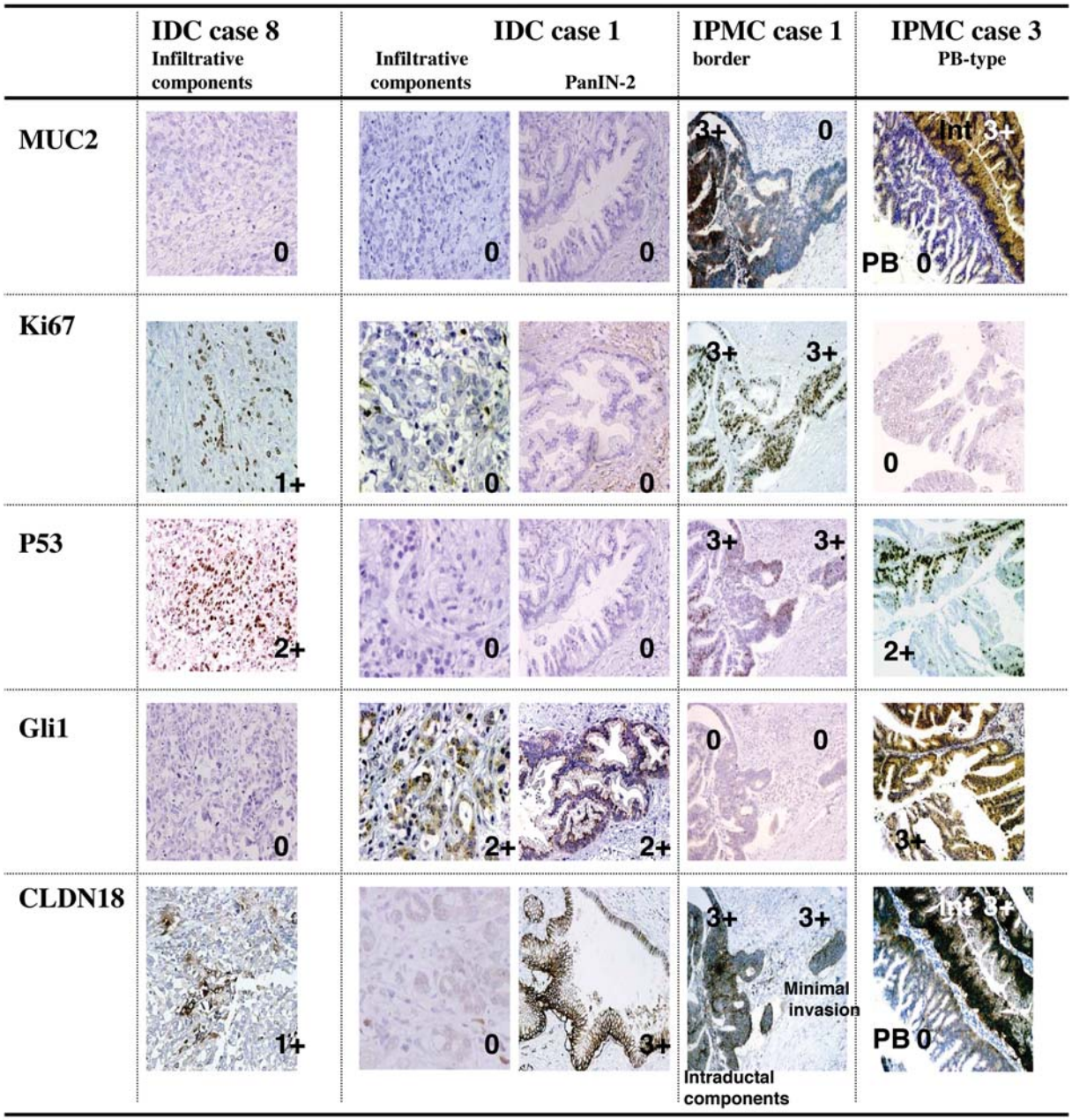

Figure 5. *Comparison of the expression of Ki-67, p53, MUC2, Gli-1, and CLDN18 at the same site. Note the similar expression pattern of CLDN18 and MUC2 in case 3 of IPMC.*

cytoplasmic staining, respectively. Diffuse expression of Gli-1 was maintained during progression from low- to high-grade PanINs through corresponding invasive components. Conversely, CLDN18 was expressed only in high-grade PanINs (PanIN-2), whereas adjacent low grade PanINs and invasive carcinoma did not express CLDN18.

The comparison at the same site clearly showed the similarities and differences in the expression of these five proteins (Figure 5).

Discussion

Because of the small number of cases in the present study, it was not possible to confirm statistically the difference between the expression of the target proteins and the progression of pancreatic carcinoma. However, the IHC performed in this study included a unique aspect: the expression patterns of a range of proteins were assessed by comparison between IPMCs and PDACs, yielding results that challenged the current paradigm.

It has been generally accepted that the Ki-67 labeling index reflects aggressive biological behavior of tumor cells (11). Recently, a case of branch-type IPMC was reported which was made up of intestinal type morphology showing diffuse nuclear staining for Ki-67 (7). Therefore, it was hypothesised that evaluation of Ki-67 may be useful for the detection of malignant potential in IPMNs, regardless of their localization or subtype. However, in the present study, the expression of Ki-67 at >10% was positive in all 4 IPMCs, whereas only 6 (40%) out of the 15 PDACs showed positive staining for Ki-67. Because it is evident that PDAC has much more malignant potential than IPMC, it is concluded that the Ki-67 index cannot be an indicator of highly malignant potential. Diffuse expression of Ki-67 in IPMCs denotes the pathological condition characterized by intraluminal growth of tumor cells.

P53 expression was positive in only one of the 4 IPMCs, which showed minimally invasive tubular-type carcinoma, suggesting that p53 overexpression is involved in the acquirement of invasive activity in IPMCs. It has been generally accepted that mutation of *TP53* occur only in PanIN-3 lesions and PDACs (12), although Real *et al*. proposed that PanIN-2 lesions are the first step in the preneoplastic stage of PDACs (13). In the present study, PanIN-3 could not be detected in two PanIN-rich cases, and p53 expression was not found in PanIN-2 lesions, which is consistent with previous reports. Ruggeri *et al*. analyzed p53 expression immunohistochemically in primary and metastatic pancreatic cancers and found no differences in the ratio of p53 labeling between each group (14). In addition, Embuscado *et al*. evaluated TP53 in a series of metastatic pancreatic cancer tissues obtained at autopsy and found that TP53 showed a similar rate of inactivation to that reported in early stage pancreatic cancers (15). These studies lend support to the hypothesis that genetic alteration of TP53 occurs relatively early in pancreatic carcinogenesis prior to the development of metastatic spread. However, in the present study, all 4 PDACs made up of poorly differentiated adenocarcinoma showed positive staining for p53, including one case in which almost all tumour cells showed positive nuclear staining. In 1998, Ganscauge *et al*. reported that p53 overexpression was more frequent in undifferentiated and locally advanced tumors (16). To determine whether the overexpression of p53 is associated with aggressive biological behavior in PDACs, such as dedifferentiation and metastatic spread, studies using larger number of cases and functional analyses are needed.

CLDN18 belongs to the family of claudins, which are cell surface molecules with four membrane-spanning domains involved in the formation of tight junctions. In normal tissues, CLDN18 plays an important role in gastric epithelial differentiation (16). Recent reports have shown down-regulation of CLDN18 gene in gastric cancer with intestinal phenotype (17, 18).

In 2008, Karanjawala *et al*. reported that diffuse expression of CLDN18 (>80% of the neoplastic cells labeled) was observed in high-grade PanINs and well-differentiated PDACs, suggesting that gastric epithelial morphogenesis is associated with relatively early events in pancreatic carcinogenesis (10). The results of the present study indicated that the specific expression of CLDN18 was observed in PanIN-2s, whereas 6 (40%) out of the 15 PDACs showed positive staining for CLDN18. Interestingly, in the two PDACs with PanIN-complexes, positive staining for CLDN18 was observed only in the PanIN-2s, whereas expression of CLDN18 declined in the corresponding invasive components adjacent to PanIN-2s. Generally, claudins are involved in the preservation of cellular polarity; therefore, the declining expression of CLDN18 is thought to be associated with metastatic spread and dedifferentiation. Given that most IPMCs are intestinal-type, characterized by a MUC2+ pattern, the expression of CLDN18 is expected to be reduced in IPMCs (4). However, opposite results were obtained with the present IHC. Diffuse membranous staining of CLDN18 was observed specifically in the tumor cells showing intestinal-type morphology, whereas combined gastric or pancreatobiliary type components did not show positive staining for CLDN18. In contradiction to previous reports (17, 18), CLDN18 showed an immunolocalization similar to that of MUC2 in the 4 IPMCs. The present results for the CLDN18 expression pattern lead to the following three hypotheses. First, in pancreatic carcinogenesis, a common precursor of PanIN and IPMN progression exists and CLDN18 is involved in the morphogenesis of the common precursor. Second, the mechanism of intestinal differentiation in IPMNs differs from that of other tumors. Third, since the expression pattern of CLDN18 in PanINs and IPMCs were not consistent with Gli-1, signals other than Hedgehog, such as Wnt and Notch signals, may regulate the expression of CLDN18 in pancreatic carcinogenesis. Further functional analyses are in progress in our laboratory.

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Received March 25, 2010
Revised May 13, 2010
Accepted May 18, 2010