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Expression of Matrix Metalloproteinases in Intraductal Papillary Mucinous Neoplasm of the Pancreas

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Abstract. *Background/Aim: Intraductal papillary mucinous neoplasm (IPMN) has a variety of histological and morphological appearances. Matrix metalloproteinases (MMPs) have been considered to be associated with tumor progression or poor prognosis. The aim of this study was to elucidate the molecular basis of IPMN variation in different types of lesions. Materials and Methods: The expression of MMP-1,2,7,9 in 51 cases of IPMN were investigated. The MMP score was calculated as the sum of the score of staining distribution and the score of the intensity staining. Results: MMP scores were correlated with histological grade, histological subtype, and type of invasion. MMP high expression groups (MMP score ≥5) had worse prognosis than low-expression groups. Conclusion: MMP expression varied between different types of IPMN, a result supporting differences in molecular basis of malignancies. These considerations may be helpful for optimal management or treatment according to various types of IPMN.*

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is characterized by mucus production, cystic dilation of the pancreatic duct, and papillary proliferation of pancreatic intraductal epithelium (1). Histologically, IPMN includes variable grades of dysplasia. When IPMN is diagnosed at the stage of low-grade to high-grade dysplasia, its treatment by resection offers an extremely good prognosis; however, once it has progressed to IPMN-derived invasive carcinoma (IPMC), its prognosis is poor (2, 3). The classifications according to epithelial morphology and mucus traits have recently been reported to have clinicopathological significance (4, 5). Thus, based on its epithelial morphology and mucus traits, IPMN can be classified as either gastric type, intestinal type, pancreatobiliary type, or oncocytic type, and these four subtypes have different clinicopathological characteristics. IPMC is classified as tubular-type or colloid-type depending on the morphology of invasion, and these types have been found to be associated with different prognoses (5). These findings suggest the existence of molecular differences associated with the growth and invasion of each type of IPMN.

Matrix metalloproteinases (MMPs) are enzymes that specifically degrade structural constituents of the extracellular matrix. They have been implicated in the processing and activation of physiologically active substances, maintenance, repair, and regeneration of tissue architecture, and in tumour invasion, metastasis, growth, progression, and neovascularisation (6, 7). In normal tissues, the levels of MMP expression and activity/inhibition is maintained; however, in tumour tissues this equilibrium is disrupted, causing overexpression of multiple MMPs (7). Studies have identified a correlation between MMP overexpression and malignancy in several types of cancer tissues (8-11), and a number of reports have found that MMP expression is also associated with invasion, metastasis, and poor prognosis in pancreatic cancer (12-14). The small number of studies on MMP expression in IPMN include those that suggest MMP involvement in the progression and invasion of IPMN (15, 16). However, no study has addressed differences in MMP expression between the different histological subtypes of IPMN or different types of invasion. In this study, we compared the levels of expression of MMP1, MMP2, MMP7, and MMP9 in IPMN to investigate the relationship between MMP expression and IPMN and identify the molecular signature of the differences between IPMN types and subtypes.

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Key Words: Intraductal papillary mucinous neoplasm, histological subtype, histological grade, type of invasion, matrix metalloproteinases, immunohistochemistry.

Table I. *Antibodies for immunohistochemistry.*

	Antibody name	Manufacturer	Host animal/Clonality	Pretreatment	Dilution
MMP1	Collagenase-1 Ab-6	Thermo scientific	Rabbit/polyclonal	Autoclave 121˚C 10 min	1:1
MMP2	MMP2 Antibody	Novus biologicals	Rabbit/polyclonal	None	1:500
MMP7	MMP7 Antibody	Gene Tex	Rabbit/polyclonal	None	1:25
MMP9	92kDa Collagenase IV	Thermo scientific	Rabbit/polyclonal	Autoclave 121˚C 10 min	1:2

Materials and Methods

Patients and classifications of IPMN. We investigated 51 IPMN lesions resected from 51 patients in Kurume University Hospital or associated hospitals between 1996 and 2012, and compared these with 30 pancreatic ductal adenocarcinoma (PDAC) lesions resected from 30 patients between 2006 and 2009. Histological grade was classified as low-grade–intermediate dysplasia, high-grade dysplasia, or IPMC, by WHO classification in 2010 (17). Histological subtypes were classified as either gastric type, intestinal type, pancreatobiliary type, or oncocytic type on the basis of epithelial morphology as evidenced by haematoxylin-eosin (HE) staining and MUC staining (4), and the morphology of invasion as tubular or colloid type.

Immunohistochemical staining. Table I shows the names, suppliers, clones, and dilutions of the primary antibodies used. Formalin-fixed, paraffin-embedded slices of each lesion were deparaffinized, and those slices used for MMP1 or MMP9 immunohistochemical staining were subjected to antigen unmasking by autoclaving at 121ºC for 10 min in 0.01-M citric acid buffer solution. Endogenous peroxidase blocking was carried out with 3% hydrogen peroxide at room temperature for 10 min. The samples were incubated with primary antibodies at room temperature for 30 min (90 min for MMP7 immunohistochemical staining). Samples were then incubated with peroxidase-conjugated secondary antibodies coupled with polymer dextran (EnVision-HRP, DAKO, Santa Clara, CA, USA) at room temperature for 30 min, after which the stain was developed with 3, 3'-diaminobenzidine (DAB) and counterstaining with haematoxylin was carried out.

Evaluation of MMP staining results. The most atypical area of each lesion was observed. Following the methods of Shimizu *et al*. (18), both the distribution (the percentage of epithelial cells stained) and the intensity of staining were assessed semi quantitatively. The following system was used to score the distribution of epithelial cells stained: none (0%)=0, focal (1%-30%)=1, multifocal (31%-70%)=2, or diffuse (71%-100%)=3. The intensity of staining was graded as follows: none=0, weak=1, moderate=2, or strong=3. The MMP score was calculated as the sum of these two scores. Staining was evaluated by three investigators. The MMP scores of resected PDAC and IPMN lesions were compared, and the scores were also compared for the different macroscopic types, histological grades, histological subtypes, and types of invasion of IPMN. MMP scores ≥5 were classified as high expression and ≤4 as low expression, and overall survival was compared for these two groups.

Statistical analysis. A Kruskal–Wallis test was used to compare scores between different groups. For the groups classified by MMP score, Kaplan–Meier curves were generated for overall survival in both groups, and assessed using a log-rank test. Analysis was carried out using JMP Pro11.0.0 (SAS Institute Inc., Cary, NC, USA), with $p<0.05$ regarded as significant.

Table II. *Classification and proportion of 51 IPMNs.*

	Total	Histological subtype				
		G	I	PB	O	Unclassified
	51	18	22	8	1	2
Histological grade						
LID	16	14	2	0	0	0
HGD	11	3	7	0	0	1
IPMC	24	1	13	8	1	1
Type of invasion						
TUB	17	1	6	8	1	1
COL	7	0	7	0	0	0

G: Gastric type; I: intestinal type; PB: pancreatobiliary type; O: oncocytic type; LID: low grade-intermediate dysplasia; HGD: high-grade dysplasia; IPMC: IPMN-derived invasive carcinoma; TUB: tubular type; COL: colloid type.

Results

Table II shows the 51 IPMN lesions and their classifications. Nearly all the gastric type lesions were non-invasive IPMN. Nine of the intestinal type lesions were non-invasive IPMN and 13 were IPMC. All the pancreatobiliary type lesions were IPMC. The types of invasion of IPMC lesions were tubular type in 17 cases and colloid type in seven patients. The tubular type lesions comprised 8 pancreatobiliary, 6 intestinal, 1 gastric, 1 oncocytic, and 1 unclassifiable type. All the colloid type lesions were intestinal type.

MMPs were expressed mainly in the cytoplasm of tumor cells in both IPMN and PDAC (Figure 1). There was almost no expression in normal pancreatic duct epithelium.

Table III shows the comparison of mean MMP scores between groups. A comparison of PDAC and IPMN showed that all MMP scores other than MMP9 were significantly higher in PDAC tissues. In terms of histological grades,

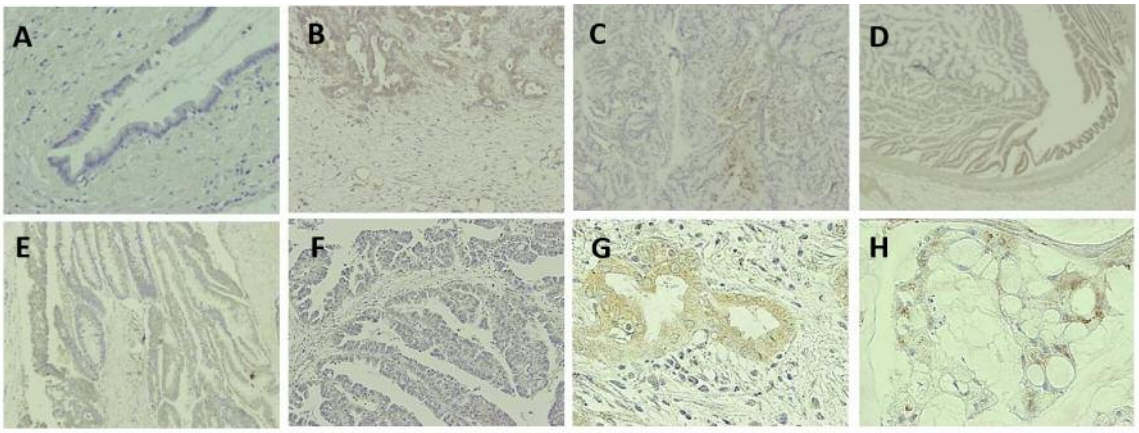

Figure 1. *Representative results of MMP immunohistochemistry. (A) Normal pancreatic duct (×100), (B) pancreatic ductal adenocarcinoma (×100), (C) gastric type IPMN (×100), (D) intestinal type IPMN (×100), (E) pancreatobiliary type IPMN (×100), (F) oncocytic type IPMN (×100), (G) tubular type IPMN-derived invasive carcinoma (×200), (H) colloid type IPMN-derived invasive carcinoma (×200).*

Table III. *Comparison of mean MMP scores in each group.*

		MMP1		MMP2		MMP7		MMP9	
		Mean scores	*p*-Value	Mean scores	*p*-Value	Mean scores	*p*-Value	Mean scores	*p*-Value
IPMN *vs*. PDAC	PDAC (n=30)	4.53	$p=0.036$	4.7	$p=0.0468$	5.33	$p<0.0001$	4.57	ns
	IPMN (n=51)	3.49		3.69		3.55		4.22	
Histological grade	LID (n=16)	2.37	$p=0.0057$	2.44	$p=0.0171$	2.56	$p=0.0080$	2.69	$p<0.0001$
	HGD (n=11)	3.82		3.82		3.55		4.73	
	IPMC (n=24)	4.08		4.46		4.21		4.96	
Histological subtype	G (n=18)	2.33	$p=0.0002$	2.67	$p=0.0010$	2.83	$p=0.0104$	3.06	$p=0.0012$
	I (n=22)	3.68		3.55		3.41		4.68	
	PB (n=8)	5		5.75		4.88		5.13	
Non-invasive IPMN	G (n=17)	2.35	$p=0.0034$	2.59	ns	2.71	ns	2.94	$p=0.0127$
	I n=9)	4		3.67		3.33		4.56	
IPMC	I (n=13)	3.46	$p=0.0292$	3.46	$p=0.0057$	3.46	$p=0.0366$	4.77	ns
	PB (n=8)	5		5.75		4.88		5.13	
Type of invasion	TUB (n=17)	4.82	$p=0.001$	5.41	$p=0.0003$	4.94	$p=0.0003$	5.12	ns
	COL (n=7)	2.29		2.14		2.43		4.71	

PDAC: Pancreatic ductal adenocarcinoma; LID: low grade-intermediate dysplasia; HGD: high-grade dysplasia; IPMC: IPMN-derived invasive carcinoma; G: gastric type; I: intestinal type; PB: pancreatobiliary type; TUB: tubular type; COL: colloid type; ns, not significant.

MMP scores were highest in IPMC, followed by high-grade dysplasia, and low-grade–intermediate dysplasia, and these differences were statistically significant. A comparison of histological subtypes found that MMP scores were highest for pancreatobiliary type, followed by intestinal and then gastric type, and these differences were statistically significant (oncocytic and unclassifiable types were excluded because their numbers were too small). We further divided histological subtypes into non-invasive IPMN and IPMC and compared their MMP scores. The MMP scores of gastric and intestinal types, which accounted for the majority of non-invasive IPMN were compared. Intestinal type tended to have higher MMP scores than gastric type, and this difference was statistically significant for MMP1 and MMP9. We then compared the MMP scores of intestinal and pancreatobiliary types, which accounted for the majority of IPMC. Pancreatobiliary type tended to have higher MMP scores than intestinal type, and this difference was statistically significant for MMP1, MMP2, and MMP7. A comparison of the type of invasion showed that all MMP scores other than MMP9 were significantly higher in tubular type than in colloid type.

Lesions were divided into a high expression group with an MMP score ≥5 and a low expression group with an MMP

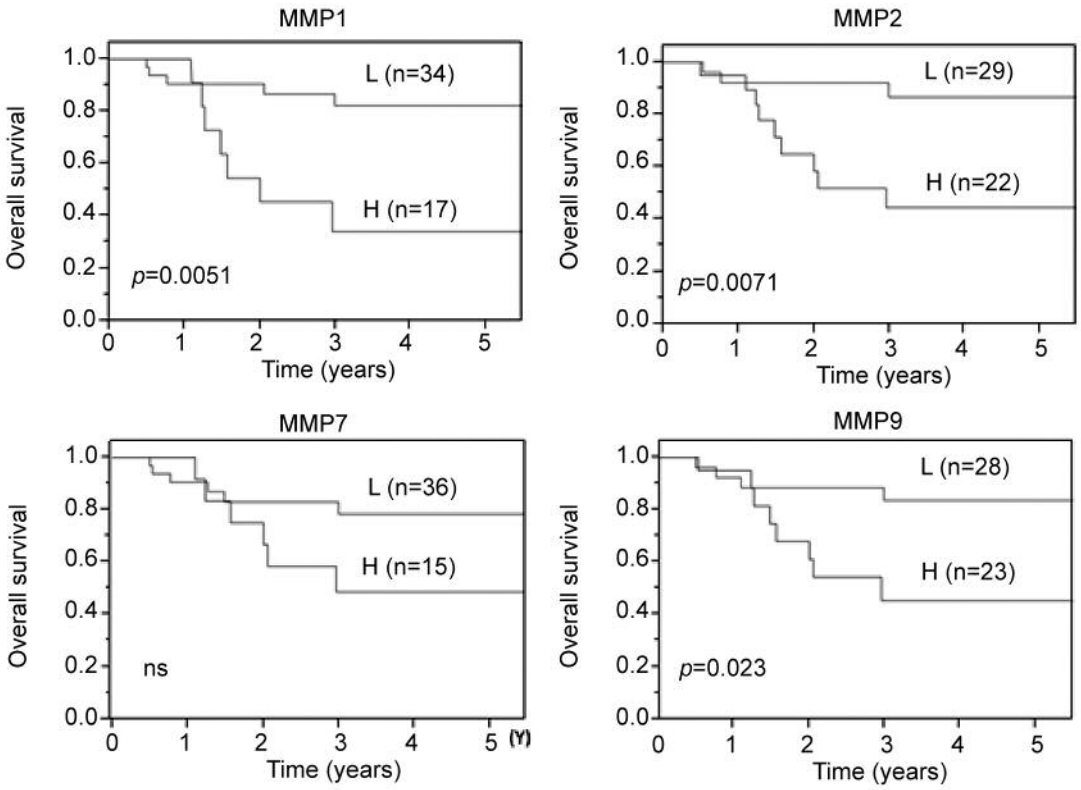

Figure 2. *Overall survival between high MMP expression group and low MMP expression group. L: Low MMP expression group (MMP score ≤4), H: high MMP expression group (MMP score ≥5). High MMP expression groups had worse prognosis than low MMP expression groups. There were significant differences regarding MMP1, 2 and 9 expression.*

score ≤4, and overall survival between the two groups was compared. The high expression group tended to have a poorer prognosis than the low-expression group, and this difference was statistically significant for MMP1, MMP2, and MMP9 (Figure 2).

Discussion

This study was focused on the involvement of MMPs in tumor progression, and MMP expression in IPMN was assessed. We compared histologically and morphologically the expression of MMP1, MMP2, MMP7, and MMP9, well known as secreted MMPs, and found that MMP expression varied in lesions with different histological grade, histological subtype, and type of invasion.

MMPs are reportedly more strongly expressed in pancreatic carcinoma tissue than in normal tissue, and in most cases the levels of expression are associated with malignancy (13, 14). The scores for MMP1, MMP2, and MMP7 were significantly higher in PDAC tissues compared with that of IPMN. Few studies have compared MMP expression in PDAC and IPMN, but there have been reports that MMP expression is more intense in PDAC than in IPMN (15, 16), and our own results support this. These results suggest that differences in MMP expression may underlie differences in these malignancies.

Our comparison between IPMN lesions of different histological grade found that MMP expression increased depending on the degree of dysplasia, with a significant difference for all MMP scores. Nishikawa *et al.* have reported that MMP7 expression levels varied between non-invasive IPMN and invasive IPMC, and hypothesized that MMP7 may contribute to invasion (15). Tamahashi *et al.* have identified a correlation between the rate of MMP7 expression and histological grade of IPMN, and suggested that it may be involved in the progression from adenoma to carcinoma. They have also suggested that MMP1, MMP2, and MMP9 may be involved in tumor progression process in the early stages of IPMN (16). Studies on colorectal and oesophageal carcinoma have also found that MMPs are expressed in the initial stage of tumour growth, and they are involved in tumorigenesis and progression (19, 20). In this study, we found that there was almost no MMP expression in normal pancreatic duct epithelium, but expression began to appear in low-grade dysplasia, the first stage of tumor development, and the level of expression increased according to histological grade, suggesting that MMPs may be involved in tumorigenesis and progression of IPMN.

Clinicohistopathological differences between the histological subtypes of IPMN have been reported (3-5), and their characteristics are broadly understood. However, few studies have addressed differences at the molecular level between subtypes. In this study, MMP scores varied significantly between different subtypes. A comparison between subtypes matched by degree of dysplasia also showed significant differences in several MMP scores. This suggests that the different histological subtypes of IPMN may have different molecular bases.

In this study, we found that tubular type lesions had significantly higher MMP scores for MMP1, MMP2, and MMP7 compared with that of colloid type lesions. These types of invasion may contribute to the difference in prognosis between patients with pancreatobiliary and intestinal type IPMN, because all the pancreatobiliary type IPMC lesions had tubular type invasive morphology, whereas over half of intestinal type IPMC lesions had colloid type invasive morphology.

Previous studies have reported that MMP expression is associated with poor prognosis for patients with a wide variety of tumours (8-11). In this study, overall survival curves found that the prognosis tended to be poor for those in the high expression groups (MMP score ≥5) for all MMPs, and that this difference was significant for MMP1, MMP2, and MMP9. These findings suggested that the evaluation of MMP expression in IPMN may both be helpful for predicting prognosis and for providing a marker for malignancy.

However, the regulatory mechanisms of MMPs are complex and there have been few functional studies of MMPs in IPMN. It remains unknown which specific genes are associated with MMP overexpression in IPMN. Further studies are, therefore, required.

Conclusion

In the present study, MMP expression varied between different histological grade, macroscopic type, histological subtype, and type of invasion of IPMN, a result supporting differences in the molecular level of these malignancies. Furthermore, MMPs may be involved in the development, progression and prognosis of IPMN. Therefore, it was suggested that these evaluations may be helpful for optimal management or treatment according to various types of IPMN.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Masanori Akashi designed the study and wrote the draft of the manuscript. Masanori Akashi, Toru Hisaka, Yoshiki Naito conducted the immunohistochemical analysis. Toru Hisaka contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All other Authors have contributed to data collection and interpretation, and critically reviewed the manuscript.

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