Immunohistochemical Localization of P-Glycoprotein and Expression of the Multidrug Resistance-1 Gene in Human Pancreatic Cancer: Relevance to Indicator of Better Prognosis

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We investigated immunohistochemical localization of P-glycoprotein (P-gp) on paraffin-embedded sections from 103 cases of previously untreated pancreatic tumors and also analyzed multidrug resistance-1 (MDR1) gene expression by polymerase chain reaction after reverse transcription in 35 cases. High positive staining for P-gp was observed in 72.8% of pancreatic tumors and in 73.2% of ductal adenocarcinoma. In ductal adenocarcinoma, immunoreactivity of P-gp was inversely correlated with biological aggressiveness of tumors determined by histologic grading ($P < 0.01$), tumor size ($P < 0.01$), retroperitoneal invasion ($P < 0.01$) and portal invasion ($P < 0.05$). Expression of the MDR1 gene was detected in all the pancreatic tumors examined and was significantly higher than that in normal pancreas ($P < 0.05$). The levels of MDR1 mRNA showed a moderate correlation with those of P-gp ($r = 0.62$, $P < 0.0001$). Higher expression levels of MDR1/P-gp significantly correlated with better prognosis of patients with ductal carcinoma ($P < 0.05$). Among patients with ductal carcinoma, the high staining group for P-gp revealed a 3.5-fold better prognosis compared with the low staining group (HR = 3.47, 95% CI = 1.62, 7.45; $P = 0.0016$). In conclusion, MDR1 gene/P-gp expression in pancreatic cancer without chemotherapy inversely correlates with biological aggressiveness and is an independent indicator of favorable prognosis.

Key words: P-Glycoprotein — MDR1 gene — Expression — Prognosis — Pancreatic cancer

Pancreatic adenocarcinoma carries an extremely poor prognosis and is the fifth leading cause of death from malignant diseases in Japan and Western countries.[1] In addition to pancreatectomy, it is often necessary to employ other modalities such as chemotherapy or radiotherapy to prevent recurrence and metastasis. Furthermore, in unresectable cases, palliation of symptoms and improvement of survival for patients must be pursued by these therapies.[2] To date, satisfactory results have not been achieved in anticancer chemotherapy of advanced pancreatic carcinoma.[3] The mechanism of refractoriness to chemotherapy of pancreatic tumors is not fully understood. Multidrug resistance (MDR) is a phenomenon characterized by the development of broad cross-resistance to functionally and structurally unrelated drugs within a single class after patients are treated with a member of the class (MDR drugs), including adriamycin.[4] It has been shown that MDR of cultured cells is closely correlated with overexpression of a plasma membrane glycoprotein termed P-glycoprotein (P-gp), which is encoded by the MDR1 gene.[5] Several studies on childhood sarcoma,[6] neuroblastoma,[7] breast cancer[8] and leukemia[9] have demonstrated a strong association be-

tween detection of P-gp in tumor cells and poor response to chemotherapy. A group of untreated tumors derived from tissues which have relatively high levels of multidrug resistance-1 (MDR1) RNA often express elevated levels of the MDR1 gene and are clinically resistant to chemotherapy.[10] Normal pancreas is one of the organs in which MDR1/P-gp is detectable, and pancreatic cancer is suspected to be one of the tumors with natural MDR.[4] However, although more than 80% of pancreatic cancer arises from pancreatic ducts, only islet cell tumors of the pancreas have been examined for P-gp expression.[11] Confirmation of MDR1/P-gp expression remains to be carried out in exocrine pancreas cancers. In addition, Sugawara et al. failed to demonstrate P-gp expression in pancreatic cancer tissues.[12] In order to evaluate and characterize intrinsic MDR (i.e., MDR in tumors which have not been exposed to chemotherapy), immunohistochemical study of P-gp was performed in 103 cases of pancreatic cancer and MDR1 gene expression was examined by polymerase chain reaction after reverse transcription (RT-PCR) in 35 cases.

MATERIALS AND METHODS

Tissue preparation Tissue specimens of pancreatic tumors were obtained, with the written informed consent

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of patients, by surgical resection or intraoperative biopsy at Kyoto University Hospital. We studied the immuno-histochemistry of P-gp in 103 cases which had not received chemotherapy or radiation therapy. Frozen tissue specimens for the study of MDR1 gene expression were available from 35 cases and the numbers of these cases are shown in parentheses; The series comprised 71 (23) ductal adenocarcinomas, 10 (4) mucinous cystadenocarcinomas, 5 (2) islet cell tumors, 9 (4) metastatic lesions of pancreatic carcinoma, 3 (2) other pancreatic carcinomas and 5 cystadenomas. The islet cell tumors consisted of 2 (2) non-functioning tumors and 1 case each of insulinoma, glucagonoma and gastrinoma. The specimens for immunostaining were fixed in 10(v/v)% formalin, and paraffin-embedded sections were cut at 4 μm thickness. Those for RNA preparation were immediately embedded in Tissue-Tek O.C.T. Compound (Miles Lab. Inc., Elkhart, IN) and stored at $-80°$C after removal of inappropriate tissues. Clinical staging for pancreatic carcinoma was determined according to the classification of the Japan Pancreas Society (JPS)[13] because survival difference is significant among JPS stages, but not significant among UICC stages.[14]

Immunohistochemical staining and assessment Mouse monoclonal antibodies (mAbs) against P-gp, UIC2 and MRK-16 were generously provided by Dr. Roninson and Dr. Tsuruo, respectively. These mAbs are IgG2a and recognize similar, but distinct extracytoplasmic epitopes of P-gp. Non-immunized mouse IgG2a was used as a negative control of staining. Tissue sections were deparaffinized and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 30 min. They were washed in phosphate-buffered saline (PBS) and blocked with horse normal serum for 30 min at room temperature. Then the tissue sections were incubated with primary antibodies overnight at 4°C. They were washed several times in PBS, and the antibody was visualized using a Vectastain ABC Elite kit (Vector Lab. Inc., Burlingame, CA). At least 10 representative high-power fields per case were examined under a light microscope. Reactivity of immunostaining was evaluated in terms of the relative ratio of P-gp-positive cells in tumor tissues and classified into two groups; tumors with less than 50% of positive cells were defined as the low-staining group and those with 50% or more of positive cells were defined as the high-staining group.

RNA extraction and RT-PCR Cryostat sections were confirmed to be pancreatic tumors under a microscope after hematoxylin and eosin staining. After removal of non-tumorous components, total RNA was extracted from the corresponding block with TRIZOL reagent (Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's protocol. As a negative control for MDR1 gene expression, a human chronic myelogenous

leukemia cell line, K562, was used. Its adriamycin-resistant derivative, K562/ADM, was used as control for high MDR1 gene expression. Both the cell lines were kindly provided by Dr. Tsuruo. RT-PCR for determination of MDR1 gene expression was performed according to the method previously described.[15]

Statistical analysis The results of immunostaining were analyzed statistically by means of the χ^2 test and those of RT-PCR were analyzed by use of the Mann-Whitney U test. Postoperative survival was defined as the time from the first operation for ductal adenocarcinoma to the time of death and was analyzed by log-rank test and Cox's proportional hazard model (JMP statistical software, SAS Institute Inc., NC). The correlation coefficient between the levels of MDR1 mRNA and those of P-gp was analyzed by Spearman's test. Patients who died of postoperative complications were excluded from the analysis.

RESULTS

Expression of P-gp in normal tissues and tumors of the pancreas We confirmed the consistency of immunohistochemical stainings by UIC2 and MRK16 including background staining of smooth muscle cells of arteries. As cellular and subcellular distributions of positive staining using both mAbs were similar and UIC2 gave a better signal-to-background ratio in the sections examined (Fig. 1A, 1B), we chose to use UIC2 for immunohistochemistry in the present study.

In normal parts of the exocrine pancreas P-gp was detected intensely on the centroacinar cells and intercalated duct cells, and moderately on the apical plasma membranes of ducts (Fig. 1C). Although the islet cells of Langerhans in the intact portion did not show appreciable staining for P-gp (Fig. 1C), those surrounded by cancer cells or fibrous connective tissues were homogeneously positive (Fig. 1D). In addition to pancreatic parenchymal cells, endothelial cells of capillaries were also positive. The cases without immunoreactivity in ducts or acinar cells of the non-tumorous part of the pancreas were eliminated from further analysis. In total, 75 cases out of 103 (72.8%) pancreatic tumors and 52 out of 71 (73.2%) ductal adenocarcinoma were in the high-staining group. High positive staining was observed in 6 out of 10 (60%) cystadenocarcinomas and in 1 out of 5 (20%) cystadenomas. In ductal adenocarcinoma, individual tubular structures showed a heterogeneous distribution of P-gp-positive cells. However, the intensity and frequency of positive cells were fairly consistent among different tubular structures. Although P-gp was also membrane-associated, polarity was lost and cytoplasm of tumor cells was granularly positive (Fig. 1E). Islet cell tumors except for gastrinoma exhibited strong staining for P-gp in cytoplasm (Fig. 1F, 1G). All of the
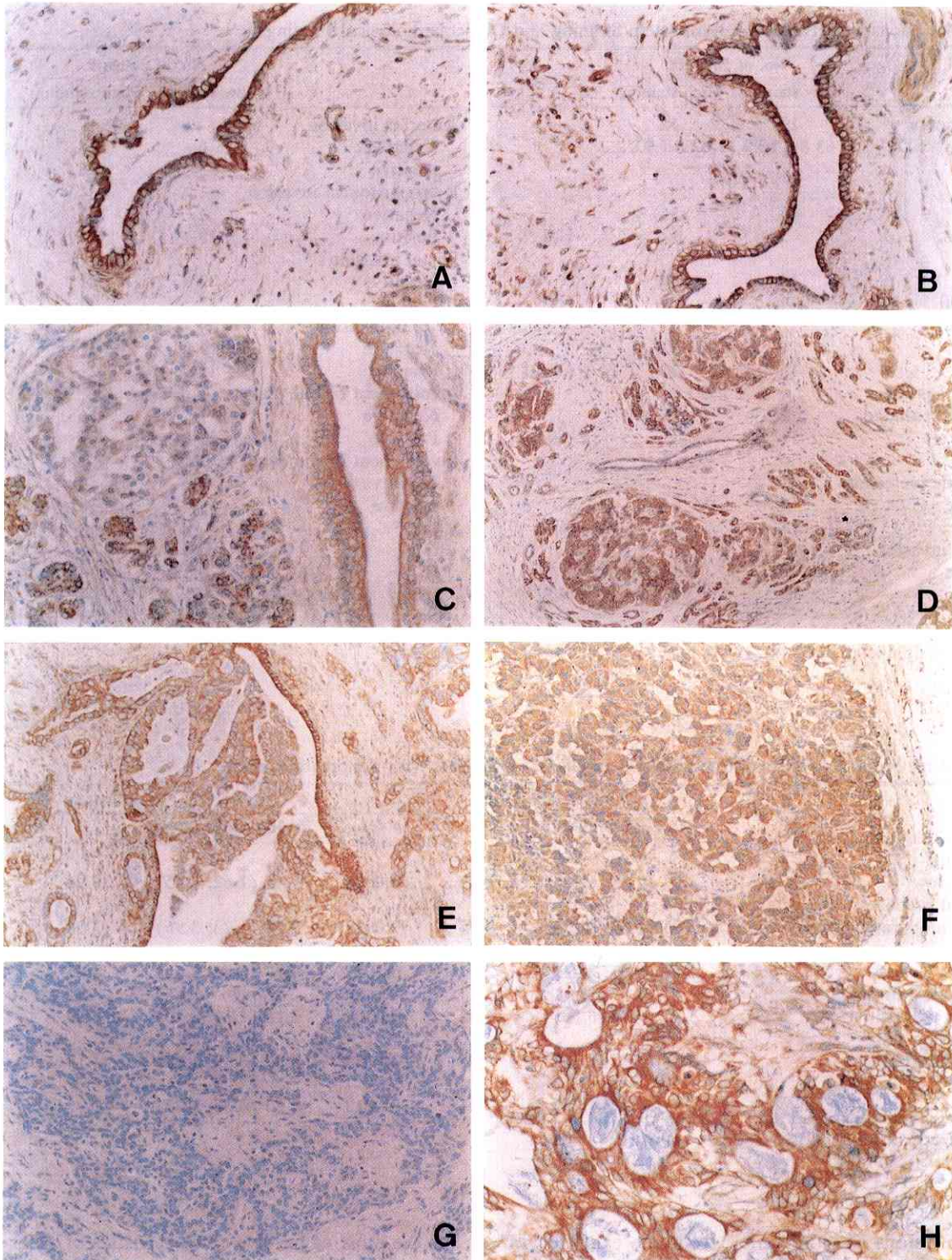
Fig. 1. Immunohistochemistry of P-gp. (A) Detected by UIC2, and (B) by MRK16. (C) Parenchymal cells in the normal part of the exocrine pancreas, especially inner lumens, are positive and the islet cells of Langerhans in the intact portion are negative. (D) Positive staining of residual islet cells surrounded by fibrous connective tissues. (E) Expression is membrane-associated, but polarity is lost in tumor cells. (F) Intensive staining of non-functioning islet cell tumor. (G) Negative staining of gastrinoma. (H) Peritoneal metastatic lesion of pancreatic carcinoma. (Panels A, B, C, and H original magnification ×170; panels D, E, F, and G original magnification ×85)

Table I. Relationship between P-Glycoprotein Staining and Clinicopathological Findings

	No. of patients		χ^2-test		No. of patients		χ^2-test
	Low staining	High staining			Low staining	High staining	
No. of patients	19	52		Anterior invasion			
Age (mean±SD)	63.8±10.9	62.8±9.2		Negative	6	24	
				Positive	13	28	N.S.
Sex				Retroperitoneal invasion			
Male	16	35		Negative	1	22	
Female	3	17	N.S.[a]	Positive	18	30	$P<0.01$
Location				Portal invasion			
Head	15	44		Negative	2	21	
Body/tail	4	8	N.S.	Positive	17	31	$P<0.05$
Tumor size				Arterial invasion			
≤4cm	4	32		Negative	7	31	
4cm<	15	20	$P<0.01$	Positive	12	21	N.S.
Histological grade				Involvement of bile duct			
Well-differentiated	2	18		Negative	7	16	
Moderately	11	31	$P<0.01$	Positive	12	36	N.S.
Poorly	6	3		Involvement of duodenal wall			
Lymph node metastasis				Negative	8	34	
Negative	4	14		Positive	11	18	N.S.
Positive	15	38	N.S.	Stage			
Perineural invasion				I	0	6	
Negative	7	22		II	0	4	
Positive	12	30	N.S.	III	0	7	$P<0.05$
				IV	19	35	

a) N.S., not significant.

Table II. Cox Proportional Hazards Survival Analysis in 63 Patients with Pancreatic Cancer

	Univariate			Multivariate		
	HR[a] (95% CI)[b]	χ^2	P value	HR (95% CI)	χ^2	P value
P-gp staining						
High vs. low	2.97 (1.55; 6.16)	11.32	0.0008	3.47 (1.62; 7.45)	9.94	0.0016
Histological grade						
Well vs. moderately vs. poorly	2.05 (1.32; 3.15)	9.87	0.0017	2.20 (1.23; 3.95)	7.04	0.008
Size						
≤4cm vs. >4cm	1.59 (0.91; 2.81)	2.67	0.102	0.78 (0.38; 1.62)	0.45	0.50
Retroperitoneal invasion						
Negative vs. positive	3.28 (1.76; 6.50)	14.65	0.0001	3.16 (1.20; 9.20)	5.59	0.018
Portal invasion						
Negative vs. positive	2.58 (1.37; 5.22)	8.84	0.0029	0.54 (0.19; 1.70)	1.14	0.286
Stage						
I, II, III vs. IV	2.81 (1.47; 1.76)	10.36	0.0013	1.15 (0.35; 3.72)	0.05	0.82

a) HR, hazard ratio; *b*) CI, confidence interval.

metastatic lesions of pancreatic carcinoma exhibited intense staining for P-gp in cytoplasm, as well as on plasma membranes (Fig. 1H).

Correlation between P-gp expression and clinicopathological data in ductal adenocarcinoma For analysis of various clinicopathological data, we focused on ductal adenocarcinoma because this type is the commonest and

has the most aggressive characteristics of all the pancreatic tumors (Table I). In carcinomas larger than 4 cm in diameter, P-gp immunoreactivity was significantly lower than in those smaller than 4 cm. The expression of P-gp was significantly higher in well-differentiated carcinoma compared with poorly differentiated tumors. Tumors without retroperitoneal or portal invasion showed signifi-
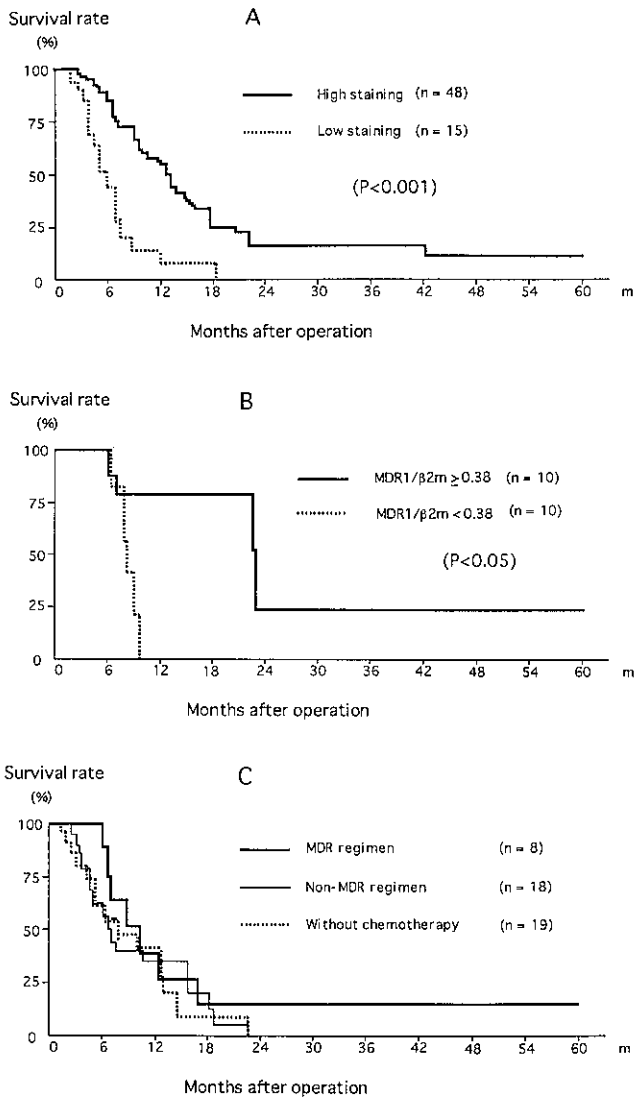
Fig. 2. Survival curves of patients with ductal carcinoma. (A) Effect of P-gp staining. (B) Effect of MDR1 gene expression. (C) Effects of chemotherapeutic treatments on stage IV cases.
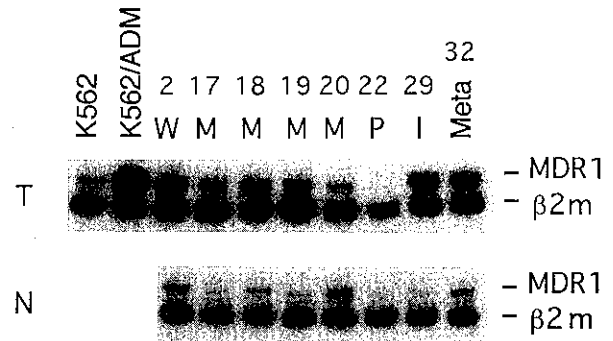

Fig. 3. MDR1 gene expression determined by RT-PCR. Each number corresponds to the case number in Table III. T, pancreatic tumor; N, normal pancreas. W, well; M, moderately; P, poorly differentiated tubular adenocarcinoma. I, nonfunctioning islet cell tumor. Meta, metastatic lesion of pancreatic carcinoma.

cantly higher staining than those with these involvements. There were no significant differences of clinicopathological findings, such as age, sex, tumor location, presence of lymph node involvement or perineural invasion between low- and high-staining groups of P-gp. Cox univariate proportional hazards survival analysis showed that higher histological grading, retroperitoneal invasion, portal invasion, clinically advanced stage (stage IV) and lower P-gp staining were significant indicators for poorer prognosis of patients compared with their counterparts, respectively (Table II). Moreover, multivariate analysis

revealed that lower staining for P-gp was the most significant indicator for poorer prognosis (Table II). Postoperative survival could be followed up in 63 cases of ductal adenocarcinoma (Fig. 2A). The low-staining group for P-gp had a significantly poorer prognosis than the high-staining group.

Expression of the MDR1 gene Expression of the MDR1 gene could be studied in 35 cases and was detected in all the pancreatic carcinomas examined, ranging from 0.14 to 0.80 (0.41 ± 0.17, mean$\pm$SD), which was significantly higher than in normal pancreas tissues (0.22 ± 0.09) (Fig. 3, Table III, $P < 0.05$). The levels of MDR1 gene expression showed a moderate correlation with immunoreactivities of P-gp (Fig. 4). The cases with MDR1 gene expression above 0.38, which was the mean value of moderately differentiated carcinoma, showed significantly better prognosis than those with lower MDR1 gene expression (Fig. 2B). Furthermore, even among the high-staining group of P-gp, the cases with MDR1 gene expression above 0.38 also showed significantly better prognosis than those with lower expression (data not shown). The mean value of MDR1 gene expression in nonfunctioning islet cell tumors was the highest among the tumors studied (Fig. 5).

P-gp expression and chemosensitivity Adjuvant chemotherapy was administered to stage IV cases of ductal carcinoma after pancreatectomy. MDR regimens consisted of adriamycin, solely or in combination with other anticancer drugs. Other than 5-fluorouracil, mitomycin C was regarded as a non-MDR drug in the present study, though this is still controversial.[4] Postoperative survival rates were not significantly different among patients with MDR regimens, non-MDR regimens and without chemotherapy (Fig. 2C).

Table III. Relationship between MDR1 Gene Expression and P-gp Staining, and Clinicopathological Findings

Case	Age/Sex	Stage	Histology[a]	MDR1/β_2m	Reactivity[b] with UIC2 (%)	Case	Age/Sex	Stage	Histology	MDR1/β_2m	Reactivity with UIC2 (%)
1	62/M	III	W	0.43	90	21	54/F	IV	M	0.40	81
2	71/F	I	W	0.61	94	22	66/M	IV	P	0.14	56
3	73/M	IV	M	0.38	82	23	73/M	IV	P	0.27	38
4	68/F	IV	M	0.41	79	24	76/M	II	C	0.19	20
5	61/M	IV	M	0.34	49	25	70/M	II	C	0.28	31
6	63/F	IV	M	0.22	61	26	51/M	I	C	0.74	82
7	79/M	IV	M	0.25	74	27	67/F	III	C	0.44	73
8	63/M	II	M	0.37	64	28	60/F	III	Adsq	0.43	74
9	57/M	IV	M	0.31	58	29	62/F	II	I	0.88	92
10	69/F	IV	M	0.80	93	30	44/F	IV	I	0.58	88
11	54/M	IV	M	0.32	79	31	19/F		SC	0.32	54
12	51/M	IV	M	0.44	79	32	72/M	IV	Meta	0.51	60
13	66/M	IV	M	0.39	49	33	46/F	IV	Meta	0.29	53
14	60/M	IV	M	0.41	75	34	72/M	IV	Meta	0.35	63
15	41/F	IV	M	0.30	74	35	69/M	IV	Meta	0.52	ND
16	70/F	IV	M	0.25	68						
17	55/M	IV	M	0.35	73						
18	52/M	IV	M	0.69	70						
19	55/F	III	M	0.32	87						
20	63/F	IV	M	0.27	74						

a) W, well differentiated tubular adenocarcinoma; M, moderately differentiated tubular adenocarcinoma; P, poorly differentiated tubular adenocarcinoma; C, mucinous cystadenocarcinoma; Adsq, adenosquamous carcinoma; I, non-functioning islet cell tumor; SC, solid and cystic tumor; Meta: metastatic lesion of pancreatic carcinoma.
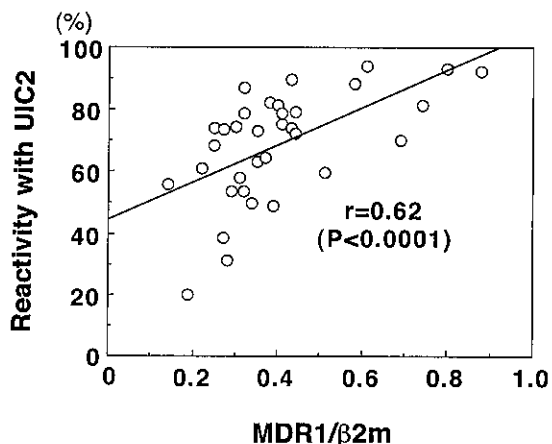*b*) Ratio of P-gp-positive cells in pancreatic tumor; ND, not done.

Fig. 4. Correlation between MDR1 gene expression and P-gp staining.
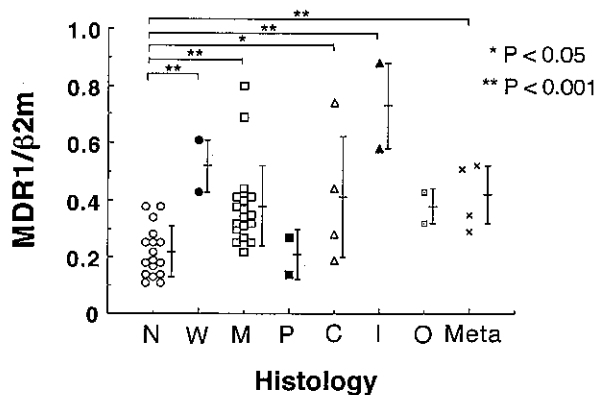

Fig. 5. MDR1 gene expression in relation to histological classification. N, normal pancreas. W, well; M, moderately; P, poorly differentiated tubular adenocarcinoma. C, mucinous cystadenocarcinoma; I, non-functioning islet cell tumor; O, other pancreatic tumor. Meta, metastatic lesion of pancreatic carcinoma.

DISCUSSION

We extensively studied immunohistochemical staining of P-gp in 103 previously untreated human pancreatic

tumors and MDR1 gene expression was also investigated in 35 cases. To our knowledge, expressions of the MDR1 gene and P-gp have not been studied systematically in tumors derived from organs which originally express the

MDR1 gene/P-gp, such as pancreatic cancer. Compared with breast carcinoma ($r=0.865$),[16] the correlation coefficient between MDR1 gene expression and P-gp positivity observed in the present study was moderate, suggesting that the amount of P-gp produced is mainly attributed to the level of MDR1 mRNA. Several rationales for the moderate correlation could be considered; the turnover rate and rate-limiting step of P-gp metabolism between normal tissues and carcinoma cells may be different. RT-PCR and immunohistochemistry cannot quantify gene expressions and amounts of protein in individual cells, respectively. An epitope detected by a mAb could also be present in different proteins from P-gp. As endothelial cells of capillaries are suggested to function as a barrier,[17] it would be interesting to determine whether they contribute to drug resistance in pancreatic tumors.

Although normal pancreas is known to express P-gp, immunohistochemical localization of P-gp in both normal exocrine and endocrine pancreas is controversial, depending on the antibodies used and tissue preparations. Even the distribution of P-gp demonstrated by one of the most reliable mAbs, MRK16, is variable among reports; positive in pancreatic ducts and acini,[18] the apical surface of the epithelial cells of small ductules, but not larger pancreatic ducts,[19] and strongly positive in acini, but negative in islet cells and ducts.[20] Recently zymogen granule membranes were reported to contain a protein which may have homology to P-gp, and which is detected by mAbs against P-gp.[21] Our results in the present study, showing that P-gp is detected strongly on the apical membranes of pancreatic ducts of various sizes and in the cytoplasm of acinar cells, seem to be reasonable. Although P-gp is considered to be a transmembrane protein, it is immunocytochemically detected uniformly in the cytoplasm of tumor cells with low degrees of drug resistance, whereas the plasma membrane is positive in highly resistant cells.[22] The staining pattern in the present study is thought to reflect heterogeneity of P-gp expression among individual tumor cells.

In the present study, the overall expression levels of the MDR1 gene were significantly higher in tumor tissues than in normal tissues and P-gp was highly positive in 73.2% of ductal adenocarcinomas. P-gp expression in tumors not exposed to chemotherapy has been considered to be associated with prognosis. Expression of the MDR1 gene is more often negative in locally advanced gastric carcinomas than in less advanced tumors.[23] Expression of MDR1/P-gp in neuroblastoma is significantly correlated with differentiation and with favorable clinical variables.[24, 25] Although P-gp expression in colon carcinoma is not associated with histologic grade, a high level of P-gp expression is suggested to be a marker of increased tumor aggressiveness and poor prognosis.[26]

Moreover, P-gp is associated with a more advanced malignant phenotype at late stages of rat liver carcinogenesis.[27] In the current study, the more differentiated and less invasive the carcinoma cells were, the higher the levels of the MDR1 gene/P-gp that were expressed. Furthermore, high levels of MDR1 gene/P-gp expression contributed significantly to better prognosis of patients with ductal adenocarcinoma. On the other hand, all the metastatic lesions of pancreatic carcinoma examined exhibited strong staining for P-gp. These results are consistent with those for renal cell carcinoma, in which higher levels of MDR1 gene expression are observed in well differentiated carcinomas compared with poorly differentiated tumors, and in metastatic lesions compared with primary tumors.[28] Both pancreatic cancer and renal cell carcinoma arise from tissues which originally overexpress the MDR1 gene/P-gp. These findings suggest that MDR1/P-gp expression may fluctuate according to the progression stage of carcinogenesis in a manner depending on the origin of the tumors, from the viewpoint of MDR1/P-gp expression. Further study comparing matched pairs of primary and metastatic lesions is needed to clarify the relevance of P-gp expression to local invasion and distant metastasis in pancreatic carcinoma. P-gp has been detected at the surface of the endocrine cells of the islets using mAb C494,[29] and Goldstein et al.[10] demonstrated that the levels of MDR1 mRNA are high in islet cell tumors of the pancreas. In the present study, islet cell tumors except for gastrinoma showed high levels of MDR1 gene/P-gp expression. Interestingly, gastrin is not produced in normal islet cells. Increased staining of P-gp in residual islet cells surrounded by carcinoma suggests that MDR1/P-gp expression is inducible in relation to the endocrine activity of islet cells. In ovarian cancer, even a low level of MDR1 gene expression contributes to chemoresistance.[30] Compared with ovarian cancer[15] expression levels of the MDR1 gene in pancreatic cancer were significantly higher, suggesting that pancreatic cancer has a clinically significant intrinsic MDR phenotype. However, the prognosis of pancreatic cancer at advanced stages was not significantly different, irrespective of chemotherapeutic treatments after surgical operation. There may also be mechanisms other than P-gp which contribute to drug resistance of pancreatic cancer. It is difficult to evaluate the correlation between P-gp expression and response to chemotherapy precisely, because the number of cases was limited and the treatment protocol was not consistent among cases in the current study. In conclusion, MDR1 gene/P-gp expressions in pancreatic cancer without chemotherapy are inversely correlated with parameters of biological aggressiveness, such as high histologic grading, local invasion and poor prognosis.

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