Expression of p8 in Human Pancreatic Cancer[1]

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ABSTRACT

The *p8* gene is a recently identified gene with mitogenic activity. p8 expression is induced in acute pancreatitis, pancreatic development, and regeneration. However, the expression of p8 in pancreatic cancer is not reported. We investigated p8 expression in 72 human pancreatic tissues, including 38 pancreatic cancers (PCs), by immunohistochemistry. p8 was overexpressed (positive cells >25% in 1,000 cells) in 71% (27 of 38) of PCs, but in only 17% (3 of 18) of chronic pancreatitis cases. There was no overexpression in mucinous cystadenoma or in normal pancreas. The p8 overexpression rate in PC was significantly higher than that in other conditions ($P < 0.05$). Reverse transcription-PCR analysis confirmed *p8* mRNA overexpression (tumor/nontumor ratio >2) in 75% (3 of 4) of PCs. p8 was overexpressed also in human pancreatic cancer cell lines (MIA PaCa-2 and PANC-1). These results suggest that p8 is involved in the development of pancreatic cancer, reflecting its mitogenic activity.

INTRODUCTION

PC[3] is one of the most aggressive gastrointestinal malignancies. It is usually resistant to treatment and shows poor prognosis. Recently, several studies on molecular pathogenesis have shown an association between PC and mutations of cancer-associated genes such as the K-*ras* oncogene (1) and *p53* (1, 2) and *p16* (3) tumor suppressor genes. Identifying genetic markers is of critical importance for early diagnosis of PC.

The *p8* gene was identified in 1997 (4). Rat p8 protein is an 80-amino acid polypeptide that is activated in pancreas tissue during the acute phase of pancreatitis, pancreatic development, and regeneration, and it promotes cellular growth (4). Mouse and human p8 homologues are 80- and 82-amino acid long and showed 91% and 75% identity with their rat counterparts, respectively (5, 6). The human *p8* gene comprises three exons separated by two introns, and it was mapped to chromosome 16 at position p11.2 (5). Interestingly, this region is frequently amplified in breast cancer (7), and it has been shown that *p8* mRNA is expressed in a human hepatoma HepG2 cell line *in vitro* (5). However, the expression of *p8* in human PC has not been reported.

The aim of this study was to investigate the expression of p8 and its clinicopathological significance in human PC.

MATERIALS AND METHODS

Pancreatic Tissue Samples and Cell Lines. Pancreatic tissue samples were obtained from 72 patients after surgery. Thirty-eight PCs consisted of 36 ductal adenocarcinomas and 2 adenosquamous carcinomas. Metastatic lesions of the liver (five cases) and of the lymph nodes (seven cases) were also examined. The subjects were 21 males and 17 females with a median age of 68.3 years of age (range: 50–89 years of age). Six pancreatic MCs, 18 CPs, and 10 NPs were also examined. NP was obtained during operation for gastric cancer that required gastrectomy with pancreato-splenectomy. There was no gastric cancer invasion in the NP used in the present study.

Two pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in MEM supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Reagents. Rabbit polyclonal antibody against human p8 was produced by immunizing New Zealand White rabbits with an oligopeptide corresponding to amino acids 62–82 of human p8 as an immunogen (5). The primers (Biognostik GmbH, Göttingen, Germany) for RT-PCR were: (*a*) sense, 5′-GGCAC-GATGGCCACCTTCCCACC-3′; and (*b*) antisense, 5′-CT-CATCTCCAGCTCTGTCTCAGCG-3′ defining a *p8* fragment of 273 bp.

Immunohistochemistry. All surgical specimens were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin and cut into slices 3 mm thick. Cell lines were fixed in 95% ethanol at room temperature for 10 min. Sections were stained with the streptavidin-biotin complex method using a Dako SLAB kit (Dako Co., Carpinteria, CA) as described previously (8). After deparaffinization and blocking of nonspecific binding, the sections were incubated with the primary antibody

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[3] The abbreviations used are: PC, pancreatic cancer; MC, mucinous cystadenoma; CP, chronic pancreatitis; NP, normal pancreas; RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; T:N, tumor:nontumor.

Table 1 Expression of p8 protein in various types of pancreatic tissues and cells

Pancreatic tissues	Staining grade 0	1	2	3	Overexpression rate %	P[a]
Pancreatic cancer ($n = 38$)	2	9	15	12	71.1 (27/38)	<0.05
Metastasis of pancreatic cancer						
Liver ($n = 5$)	0	1	4	0	80.0 (4/5)	<0.02
Lymph node ($n = 7$)	0	7	0	0	0	ns
MC ($n = 6$)	1	5	0	0	0	ns
CP ($n = 18$)	5	10	3	0	16.7 (3/18)	ns
NP ($n = 10$)	8	2	0	0	0	

[a] The percentage of the p8-positive cells per 1000 cells was calculated. The p8 immunostaining was graded according to the p8-positive percentage as follows: 0, 0%; 1, >0 and <25%; 2, 25–50%; 3, >50%. The p8 was considered to be overexpressed when the staining grade was 2 or 3.

against human p8 (5) at a 1:250 dilution (for pancreatic tissues) or at a 1:800 dilution (for cell lines) at 4°C overnight. Then, the sections were incubated with a biotinylated link antibody for 20 min and with peroxidase-labeled streptavidin at room temperature for 20 min. The chromogen used for the color reaction was 3-amino-9-ethyl-carbazole. Normal liver sections were used as a positive control. Primary antibody was replaced by normal rabbit IgG (Dako Co.) for negative control experiments. An absorption experiment was not done because of the shortage of p8 antigen. The p8 immunostaining of the pancreas was graded as follows: 0, 0%; 1, >0 and <25%; 2, 25–50%; and 3, >50% per 1000 parenchymal cells. Overexpession of p8 was defined as the expression of grades of 2 and/or 3**.**

RT-PCR Analysis. Total RNA was extracted with an Ultraspec-II RNA extraction kit (Biotex Laboratories, Inc., Houston, TX) from pancreatic tissues, and with a Simple Nucleic Acid Prep (S.N.A.P.) RNA extraction kit (Invitrogen BV, Groningen, The Netherlands) from pancreatic cancer cell lines. Total RNA of 10 μg was reverse transcribed into cDNA with the StrataScript RT-PCR kit (Stratagene, Inc., La Jolla, CA). One μl of the cDNA product was used for each PCR in a 25-μl reaction volume containing 200 mM dNTPs, 200 mM sense and antisense primers, 1 × PCR buffer 2.5 ml, and 1.25 units of Amli*Taq* Gold DNA polymerase (Perkin-Elmer Cetus, Inc., Foster City, CA). PCR was performed on a DNA thermal cycler (Perkin-Elmer Cetus, Inc., Norwalk, CT) for 9 min at 94°C, for 1 min at 94°C of hot start, for 1 min at 94°C, for 1 min at 55°C, and for 1 min at 72°C for 30 cycles, and then an extension reaction was done at 72°C for 10 min. Each PCR product of 10 μl was separated on a 2% agarose gel with 50 V for 1 h. The gels were dyed with SYBR Green (Molecular Probes, Inc., Eugene, OR) at a ×10,000 concentration in DMSO, and exposed under UV light (312 nm). G3PDH was used as an internal control. The primers for G3PDH RT-PCR were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The RT-PCR assay was repeated at least three times per each sample to confirm the reproducibility of the results.

Semi-quantitative Analysis of RT-PCR. The electrophoresed PCR products were scanned by densitometry. The relative expression intensity of *p8* band compared with the G3PDH band was calculated in each sample. To estimate the expression of *p8* mRNA in tumor tissues relative to that in the matched nontumor tissues, we calculated the T:N ratio representing the relative expression intensity of *p8* mRNA (*p8*: G3PDH) in tumor tissues compared with that in nontumor tissues. The *p8* mRNA was considered to be overexpressed when the T:N ratio of *p8* mRNA was >2 (9).

Statistical Analysis. Statistical analysis was performed using Statview 4.5 software (Abacus Concepts, Inc. Berkeley, CA). The χ^2 and Fisher's exact tests were used to examine the association of p8 expression and clinicopathological parameters. A $P < 0.05$ was considered as statistically significant.

RESULTS

Histopathological Findings

Histopathological differentiation and clinical staging were graded according to the criteria proposed by Klöppel (10) and Hermreck *et al.* (11), respectively. The grade of PC was identified as well differentiated (G1) in 13 cases, as moderately differentiated (G2) in 11 cases, and as poorly differentiated (G3) in 14 cases. There were 3 cases in stage I, 4 cases in stage II, 12 cases in stage III, and 19 cases in stage IV. Tumor sizes were ≤2 cm in 9 cases, ≤4 cm in 17 cases, ≤6 cm in 9 cases and >6 cm in 3 cases. Twenty-five tumors were located at the pancreatic head, five cases at the body, and eight cases at the tail. Twenty cases had lymph node metastasis and 18 cases did not. Follow-up data were available for 35 patients in which 16 cases survived >6 months and 19 cases survived <6 months.

Immunohistochemistry

p8 Expression in PC, MC, and PC Cell Lines. As shown in Table 1, p8 was overexpressed in 71.1% of PC and in 100% of PC cell lines, whereas it was not overexpressed in MC. p8 overexpression rate in PC was significantly higher than that in MC. p8 was immunolocalized in the nucleus (and cytoplasm) of cancer cells (Fig. 1*A*) and of the lining cells of MC. p8 was overexpressed in four (80.0%) of five liver metastases of PC. p8 was overexpressed in the nuclei of pancreatic cancer cells MIA PaCa-2 (Fig. 1*B*) and PANC-1. There was no signal in the experiment with a rabbit IgG as a negative control (data not shown).

The correlations between the overexpression of p8 protein and various clinicopathological parameters in PC are shown in Table 2. Among clinicopathological parameters, only the tumor size (≤2 cm *versus* >2 cm) showed a significant difference in

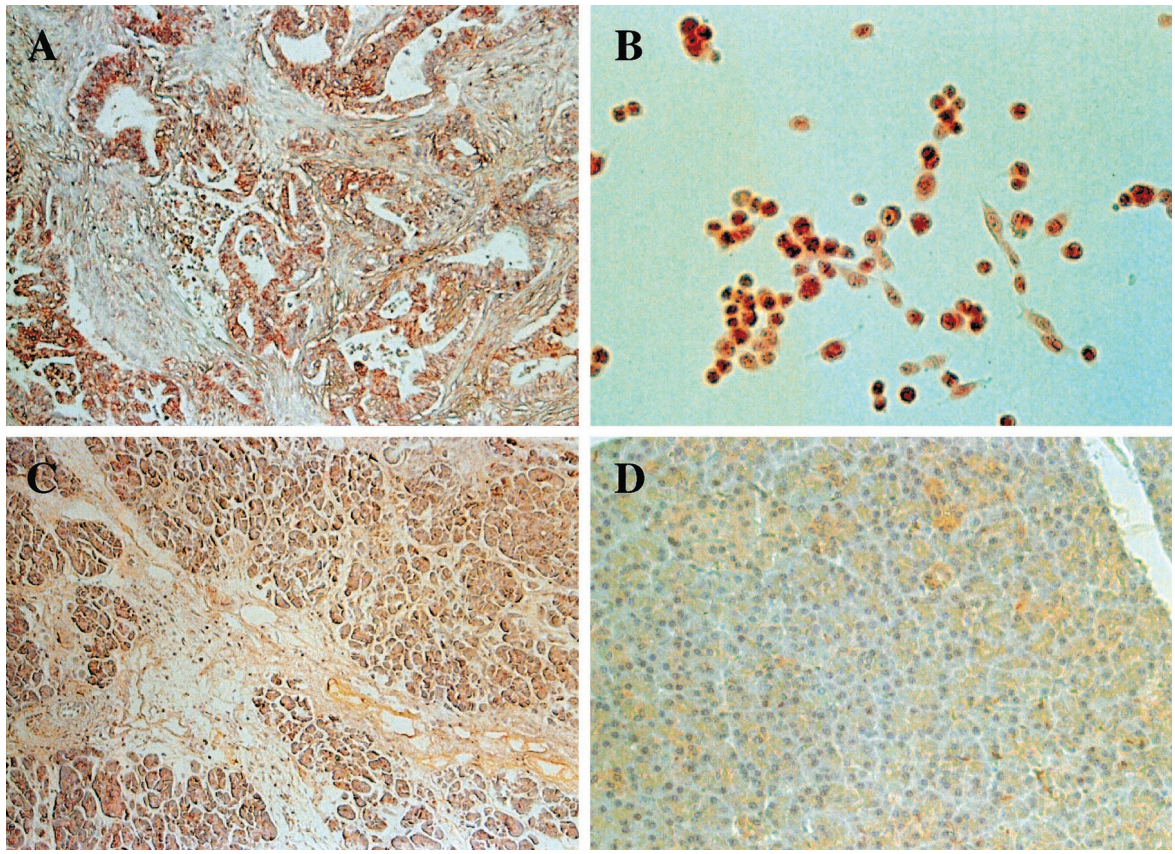

Fig. 1 Immunohistochemistry for p8 expression. *A*, pancreatic ductal adenocarcinoma; *B*, MIA PaCa-2 pancreatic cancer cell line; *C*, chronic pancreatitis; and *D*, normal pancreas. Original magnification: *A* and *D*, ×50; *B*, ×100; and *C*, ×25.

the overexpression of p8 ($P < 0.01$). The p8 overexpression rate tended to be higher in cases characterized by: (*a*) lower age; (*b*) male sex; (*c*) moderate and poor differentiation; (*d*) stages III and IV; (*e*) tumor location at the body or tail; (*f*) nodal involvement; and (*g*) short survival (Table 2).

p8 Expression in CP and NP. As shown in Table 1, p8 was overexpressed in only 3 out 18 cases of CP, and it was expressed (grade 1) in only 2 of 10 NPs. There was no significant difference in the p8 overexpression rate between CP and NP. p8 was expressed in acinar and inflammatory cells in CP (Fig. 1*C*) and in a few acinar cells in NP (Fig. 1*D*). p8 was immunolocalized mainly in the nucleus, but cytoplasmic expression was also recognized.

p8 mRNA Expression Analyzed with RT-PCR

The gene expression of *p8* was examined with RT-PCR in four PCs (tumor and nontumor portions), four CPs, two NP tissues, and two PC cell lines. *p8* mRNA of 273 bp was highly expressed in PC tissues and in PC cell lines but only faintly expressed in CP (Fig. 2). NP was negative for *p8* mRNA (Fig. 2). When overexpression of *p8* mRNA was defined as the T:N ratio of >2, *p8* mRNA was overexpressed in three of four (75%) PC cases but was not overexpressed in four CPs (Table 3). In four PC samples, the T:N ratio ranged from 1.06 to 22.76 (1–2, one case; >2, three cases). *p8* mRNA was overexpressed in

Table 2 Correlation between overexpression of p8 protein and clinicopathological parameters in pancreatic cancer

Parameters	Total cases	p8 overexpression cases (%)	*P*
Age (yr)			
≧65	27	17 (63.0)	
<65	11	10 (91.0)	NS[a]
Gender			
Male	21	17 (81.0)	
Female	17	10 (58.8)	NS
Pathological grade			
G1	12	6 (50.0)	
G2/G3	26	21 (80.8)	NS
Clinical stage			
I/II	7	3 (42.9)	
III/IV	31	24 (81.0)	NS
Tumor size (cm)			
≦2	9	2 (22.2)	
>2	29	25 (96.6)	<0.01
Tumor location			
Head	25	16 (64.0)	
Body/tail	13	11 (84.6)	NS
Nodal involvement			
Positive	20	17 (85.0)	
Negative	18	10 (55.6)	NS
Survival (mo)			
≧6	19	13 (68.4)	
<6	16	12 (75.0)	NS

[a] NS, not significant.

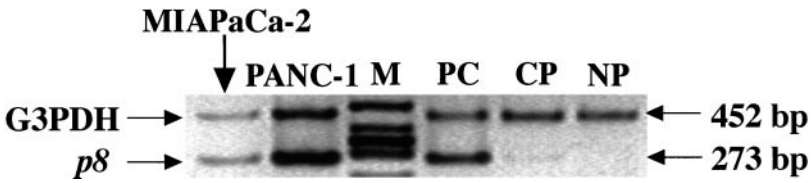

Fig. 2 The *p8* mRNA of 273 bp was expressed in pancreatic cancer (*PC*), chronic pancreatitis (*CP*), and two pancreatic cancer cell lines (*PANC-1 and MIA PaCa-2*), as analyzed by RT-PCR. The *p8* mRNA was not detected in normal pancreas (*NP*). *G3PDH* mRNA of 452 bp was used as an internal control. *M*, molecular size marker.

Table 3 Expression of *p8* mRNA in pancreatic tissues (RT-PCR analysis)

Pancreatic tissues	Relative expression intensity[a]				Overexpression rate (%)[b]
	0	<1	1–2	>2	
PC (n = 4)	0	0	1	3	75
CP (n = 4)	0	2	2	0	0
NP (n = 2)	2	0	0	0	0

[a] The relative expression intensity of *p8* mRNA was graded according to the *p8* mRNA:G3PDH.
[b] Overexpression, the *p8* mRNA/G3PDH ratio >2.

MIA PaCa-2 and in PANC-1 cell lines with T:N ratios of 4.11 and 7.31, respectively. In four CP samples, the *p8*:G3PDH ratios were 0.13, 0.25, 1.19, and 1.90. Although p8 protein was expressed in inflammatory cells in CP by immunohistochemistry, there was no significant correlation between the number of inflammatory cells and *p8* mRNA levels in RT-PCR in CP (data not shown).

DISCUSSION

The *p8* gene is barely expressed in NP but is overexpressed in acute pancreatitis (4, 12). It is also strongly induced in pancreatic development and regeneration (4). We have demonstrated that p8 is overexpressed in PC in the present study. The characteristic expression of p8 is mainly attributable to its mitogenic activity (5). Therefore, p8 expression in PC would not be cancer-specific. However, it should be clarified whether p8 overexpression in PC is simply attributable to the excessive growth activity of cancer cells or to some genetic change(s), such as mutations.

We investigated the correlation between p8 overexpression and various clinicopathological parameters in PC. Larger tumors (>2 cm) showed a significantly higher overexpression rate of p8, and less differentiated types, advanced stages, and cases characterized by shorter survival tended to show p8 overexpression. These results also reflect the mitogenic activity of p8.

Previous reports (4, 5) have shown that p8 expression is induced by various proapoptotic stimuli. It is suggested that p8 has an anti-apoptotic function (4, 5). The significance of apoptosis in cancer cells is controversial. High spontaneous apoptosis is reported to be correlated with poor prognosis in PC (13). If p8 has anti-apoptotic activity, p8 overexpression in PC cells would lead to resistance against apoptosis. Although we have not demonstrated the relationship between p8 and apoptosis in PC, the tendency toward shorter survival in p8-overexpressing cases is not consistent with the previous report (13). It should be investigated whether p8 promotes PC cell growth through its anti-apoptotic activity.

It is suggested that p8 is a DNA-binding protein. As a transcriptional factor, it has a role in some phosphorylation/dephosphorylation signal pathways that involve its translocation to the nucleus and specific binding to DNA (4). Potentially, p8 is phosphorylated by various kinases (4, 5). Recent reports (14) showed that some kinases, such as the phosphatidylinositol 3-kinase or extracellular signal-regulated kinase, lead to inappropriate pancreatic cellular proliferation. Genetic mutations of K-*ras*, *p16*, and *p53* in PC lead to cellular proliferation via the phosphatidylinositol 3-kinase and/or the extracellular signal-regulated kinase pathways (14). It is to be examined whether there is *p8* mutation in PC and how p8 is involved in kinase signaling pathways.

Recently, candidate of metastasis-1, a novel factor in human breast cancer was identified (15). Interestingly, p8 is structurally similar to candidate of metastasis-1 (15). p8 might be involved in cancer metastasis, however, we could not find a significant difference in p8 expression between primary and metastatic lesions in the present study. The relationship between p8 expression and cancer metastasis needs to be studied further.

In summary, we have demonstrated the overexpression of p8 in human pancreatic cancer. The present results suggest that p8 is involved in the development of pancreatic cancer, which reflects its mitogenic activity.

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