# Overexpression of p8 Is Inversely Correlated with Apoptosis in Pancreatic Cancer[1]

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## ABSTRACT

**A recently identified gene, *p8*, has cell growth-promoting activity and is strongly induced in acute pancreatitis. In this study, we detected p8 and single-stranded DNA (ssDNA) for apoptosis by immunohistochemistry in human pancreatic cancer. The p8 was overexpressed (>30% per 1000 cancer cells) in 26 of 44 (59%) pancreatic cancers, and apoptosis (ssDNA-positive cells >10% per 1000 cancer cells) was recognized in 18 of 44 (41%) pancreatic cancers. There was a significant inverse correlation between the p8 overexpression and apoptosis ($P < 0.05$). Moreover, the expression pattern of high p8 and low ssDNA was seen significantly more often in lower age (<65 years), in moderately or poorly differentiated cancers, and in node-positive cases ($P < 0.05$). The p8 expression and apoptosis were not significantly correlated with survival. These results suggest that p8 overexpression is involved in antiapoptotic activity and the biological characteristics of pancreatic cancer.**

## INTRODUCTION

Apoptosis is programmed cell death, characterized by nuclear condensation, membrane convolution, and nucleosomal fragmentation (1). Apoptosis may affect the growth, development, and differentiation of cancer (2). Previous studies have shown an association between spontaneous apoptosis and the biological characteristics of gastrointestinal cancers such as esophageal (3), gastric (4), colorectal (5), and pancreatic (6, 7) cancers.

Recently, *p8*, a cell growth-related gene, has been identified (8–10). The p8 protein is 82 amino acids long, and the *p8* gene comprises three exons separated by two introns and is mapped to chromosome 16 at position p11.2 in humans (10). The *p8* gene is activated in pancreas during the acute phase of pancreatitis, pancreatic development, and regeneration (8). p8 expression is induced by various proapoptotic stimuli, and p8 might have an antiapoptotic function (8, 9). We have revealed p8 expression in human PCs,[3] suggesting that p8 is involved in the pathogenesis of PC (11). However, the relationship between p8 expression and apoptosis in PC has not been reported.

The aims of this study were to investigate p8 expression and spontaneous apoptosis and their mutual relationship in human PCs and to elucidate the clinicopathological significance of p8.

## MATERIALS AND METHODS

**Patients and Tissues Samples.** Pancreatic tissue samples were obtained from 54 patients (44 PCs and 10 normal pancreas) at surgery in 46 cases and at autopsy in 8 cases. Surgical treatments for PC included Whipple's operation in 22 cases, distal pancreatectomy in 8 cases, and bypass operation in 6 cases. Surgical complication was pseudocyst formation in one case. A final pathological diagnosis of 44 PCs was ductal adenocarcinoma. Twelve metastatic lesions (5 liver and 7 lymph node) were also examined. The PC patients were 26 males and 18 females with a median age of 64.8 (range, 50–89) years. The nutritional status of the patients with PC was good in 8 cases, moderate in 23 cases, and poor in 13 cases. Ten normal pancreatic tissues were included in this study as control. These normal pancreata were obtained at operation from patients with advanced gastric cancer who underwent distal pancreatectomy and splenectomy with total gastrectomy.

**Reagents.** Polyclonal antihuman p8 antibody was produced by immunizing New Zealand White rabbits with an oligopeptide corresponding to amino acids 62–82 of human p8 as an immunogen (9). Polyclonal anti-ssDNA antibody (12) was purchased from Dako Co. (Carpinteria, CA). Hoechst 33258 (2′[4-hydroxyphenyl]-5-[4-methyl-piperazinyl]-2,5′-bi-benzimidazole) was purchased from Sigma Chemical Co. (St. Louis, MO).

**Immunohistochemistry.** All pancreatic specimens were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and cut into 3-μm-thick sections. The streptavidin-biotin complex method with an LSAB kit (Dako) was used for

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[3] The abbreviations used are: PC, pancreatic cancer; ssDNA, single-stranded DNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

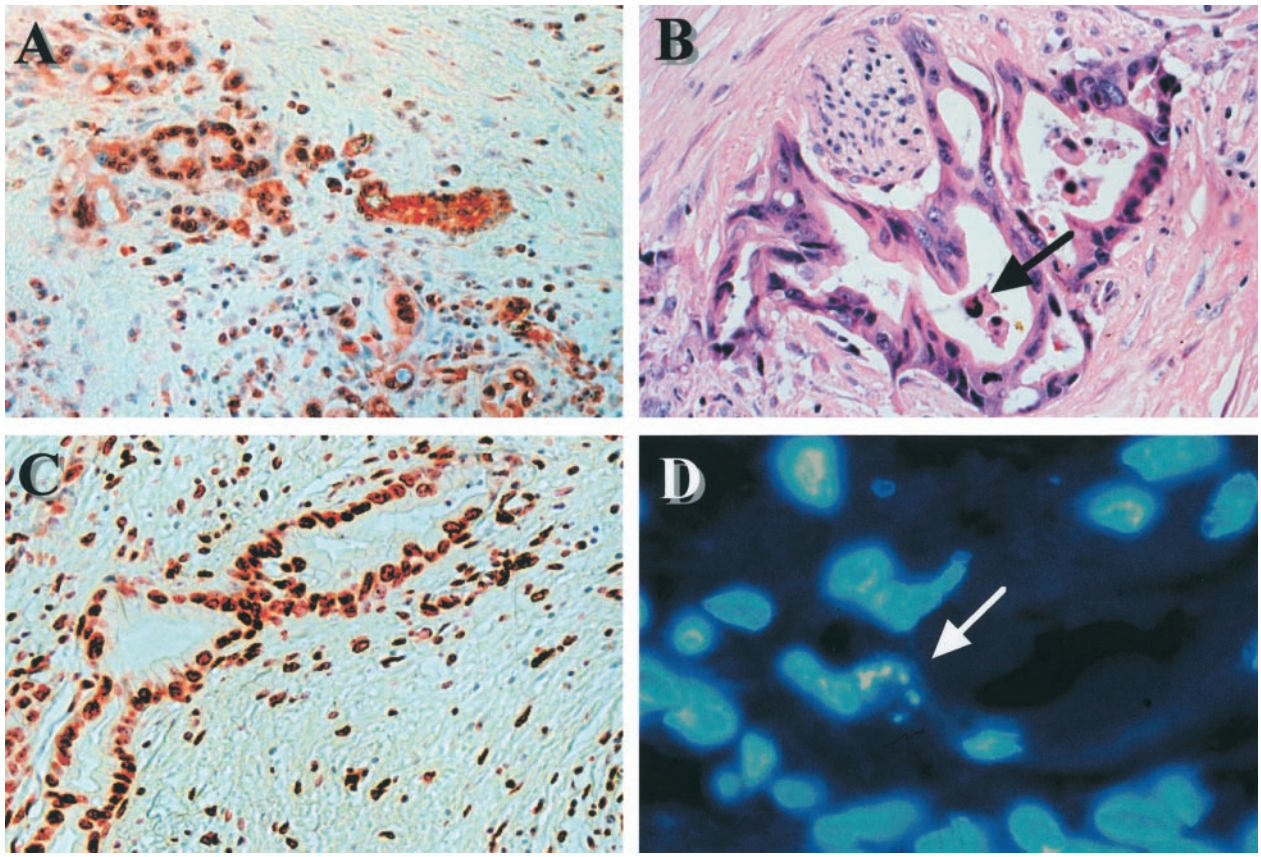


*Fig. 1* The expression of p8 and the detection of apoptosis in PC tissues. *A*, immunostaining for p8. Poorly differentiated adenocarcinoma cells display a strong nuclear and/or cytoplasmic staining. *B*, H&E staining. *Arrow*, apoptotic cells. *C*, immunohistochemistry for ssDNA. Note the strongly positive staining in cancer cell nuclei. *D*, Hoechst 33258 staining. *Arrow*, an apoptotic cell nucleus with chromatin condensation and fragmentation. *A–C*, ×300; *D*, ×750.

immunohistochemistry as described previously (11). The dilutions of the primary antibodies were 1:250 for p8 and 1:100 for ssDNA. Normal rabbit IgG (Dako) was used for negative control experiments. Immunostaining results were divided as −, 1+, 2+, and 3+, defined as follows: −, 0%; 1+, >0% and ≤30%; 2+, >30% and ≤70%; 3+, >70% per 1000 cancer cells. Overexpression of p8 was defined as p8 expression of 2+ or 3+. Apoptosis was recognized as ssDNA and positive (1+, 2+, or 3+).

**Hoechst 33258 Staining.** For inspection of chromatin condensation, the sections were stained as described previously (13), using 8 μg/ml of Hoechst 33258 in $H_2O$ for 10 min at room temperature. An Olympus AX-80U-P2 fluorescence microscope (Olympus Optical Industry Co., Tokyo, Japan) was used for observations and photographs. The excitation wavelength was 490 nm.

**Statistical Analysis.** The $\chi^2$ and Fisher's exact tests were used to examine the significant association of p8 expression and clinicopathological parameters. The multivariate survival analysis was performed by the Statistical Analysis System (SAS Institute, Inc., Cary, NC). All tests were two-sided, and $P < 0.05$ was considered significant.

## RESULTS

**Clinicopathological Findings.** Histopathological differentiation grading and clinical staging were evaluated according to the criteria proposed by Klöppel (14) and Hermreck *et al.* (15), respectively. The histological differentiation grade was determined as well differentiated in 13 cases, moderately differentiated in 15 cases, and poorly differentiated in 16 cases. There were 6 cases in stage I, 4 cases in stage II, 14 cases in stage III, and 20 cases in stage IV. The tumor size was ≤2 cm in 7 cases, ≤4 cm in 22 cases, ≤6 cm in 8 cases, and >6 cm in 7 cases. Twenty-eight tumors were located at the pancreatic head, 9 at the body, and 7 at the tail. Lymph node metastasis was seen in 25 cases, liver metastasis in 11 cases, liver and lymph node metastasis in 1 case, and peritoneal dissemination in 3 cases.

**p8 Expression.** p8 was expressed in the nuclei as well as in the cytoplasm of PC cells (Fig. 1*A*). The p8 expression was 1+ in 9, 2+ in 14, and 3+ in 12 cases; therefore, the overexpression (+2 or +3) rate was 59% (26 of 44) in PC. Expression of p8 in metastatic lesions was 1+ in 8 and 2+ in 4. There was no metastatic lesion that showed 3+ expression. p8 was not expressed in normal pancreas. There was a significant relationship between p8 overexpression and nodal involvement ($P < 0.005$; Table 1).

*Table 1* Correlation between p8 overexpression, apoptosis, and clinicopathological parameters in human PCs

| Clinicopathological parameters | Cases (*n*) | p8: 2+ or 3+ (%) | *P* | ssDNA 1+ or 2+ (%) | *P* | Expression type[a] I | II | III | IV | $P^b$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Age (yr) | | | | | | | | | | |
| ≥65 | 29 | 16 (55) | | 15 (52) | | 7 | 9 | 8 | 5 | |
| <65 | 15 | 10 (67) | 0.462 | 3 (20) | 0.042 | 0 | 10 | 3 | 2 | 0.026 |
| Gender | | | | | | | | | | |
| Male | 26 | 14 (54) | | 7 (27) | | 1 | 13 | 6 | 6 | |
| Female | 18 | 12 (67) | 0.395 | 11 (61) | 0.023 | 6 | 6 | 5 | 1 | 0.216 |
| Differentiation | | | | | | | | | | |
| Well | 13 | 8 (62) | | 5 (38) | | 2 | 6 | 3 | 2 | |
| Moderate | 15 | 9 (60) | | 6 (40) | | 3 | 6 | 3 | 3 | |
| Poor | 16 | 9 (56) | 0.831[c] | 7 (44) | 0.552[c] | 2 | 7 | 5 | 2 | 0.038[c] |
| Stage | | | | | | | | | | |
| I | 6 | 3 (50) | | 3 (50) | | 1 | 2 | 2 | 1 | |
| II | 4 | 2 (50) | | 2 (50) | | 1 | 1 | 1 | 1 | |
| III | 14 | 10 (71) | | 4 (29) | | 2 | 8 | 2 | 2 | |
| IV | 20 | 11 (55) | 0.506[d] | 9 (45) | 0.379[d] | 3 | 8 | 6 | 3 | 0.279[d] |
| Tumor size (cm) | | | | | | | | | | |
| ≤4 (TS1 or TS2) | 29 | 19 (66) | | 11 (38) | | 6 | 13 | 5 | 5 | |
| >4 (TS3 or TS4) | 15 | 7 (47) | 0.414 | 7 (47) | 0.811 | 1 | 6 | 6 | 2 | 0.230 |
| Tumor location | | | | | | | | | | |
| Head | 28 | 17 (61) | | 11 (39) | | 5 | 12 | 6 | 5 | |
| Body/tail | 16 | 9 (56) | 0.772 | 7 (44) | 0.196 | 2 | 7 | 5 | 2 | 0.647 |
| Nodal involvement | | | | | | | | | | |
| Positive | 26 | 20 (77) | | 9 (35) | | 5 | 15 | 4 | 2 | |
| Negative | 18 | 6 (33) | 0.003 | 9 (50) | 0.239 | 2 | 4 | 7 | 5 | 0.020 |
| Liver metastasis | | | | | | | | | | |
| Positive | 12 | 6 (50) | | 6 (50) | | 1 | 3 | 5 | 3 | |
| Negative | 32 | 20 (63) | 0.340 | 12 (38) | 0.340 | 6 | 16 | 6 | 4 | 0.125 |
| Total | 44 | 26 (59) | | 18 (41) | | 7 | 19 | 11 | 7 | |

[a] A detailed description is given in "Results."
[b] Expression pattern type II *versus* I, III, and IV.
[c] Well *versus* moderate or poor.
[d] I and II *versus* III and IV.

**Apoptosis Detected by ssDNA Staining.** A careful observation of H&E-stained sections enabled us to detect apoptotic cells (Fig. 1*B*). For more sensitive and quantitative analysis, ssDNA immunohistochemistry was performed to detect apoptosis in PC tissues. Nuclear staining was clearly seen in cancer cells (Fig. 1*C*). The ssDNA was positive in 41% (18 of 44) of PCs. The expression intensity was 1+ in 13 cases and 2+ in 5 cases. All of the metastatic lesions were negative for ssDNA. There was no case of ssDNA 3+. ssDNA was not expressed in normal pancreas. As shown in Table 1, the ssDNA expression was significantly higher in the elderly ($P < 0.05$) and in females ($P < 0.05$).

**Hoechst 33258 Staining.** To confirm apoptotic changes in PC tissues, Hoechst 33258 staining was performed. Fig. 1*D* shows chromatin condensation and fragmentation in cancer cells, but these findings were not observed in normal cell nuclei.

**Relationship between p8 and Apoptosis.** Nineteen of 26 tumors (73%) with p8 overexpression (p8, +2 or +3) did not show apoptosis (ssDNA, 1+ or 2+). On the other hand, 11 of 18 tumors (61%) with p8 nonoverexpression (p8, − or +1) showed apoptosis. There was a significant inverse correlation between the overexpression of p8 and ssDNA ($P < 0.05$; Table 2). Immunohistochemical results of p8 and ssDNA in the tumor were classified into four expression types, where p8+ meant p8 expression was 2+ or 3+ (overexpression) and ssDNA+ meant ssDNA expression was 1+ or 2+: type I, p8+/ssDNA+ ($n = 7$); type II, p8+/ssDNA− ($n = 19$); type III, p8−/ssDNA+ ($n = 11$); and type IV, p8−/ssDNA− ($n = 7$). As shown in Table 1, the type II expression was seen significantly often in lower age (<65 years), in moderately or poorly differentiated cancers, and in node-positive cases ($P < 0.05$). In metastatic lesions, type II expression was seen in 33% (4 of 12), and type IV was seen in 67% (8 of 12).

*Table 2* Inverse correlation between p8 overexpression and apoptosis (ssDNA expression) in human PCs[a]

| | Apoptosis (ssDNA: 1+ or 2+) Positive | Negative | Total |
|---|---|---|---|
| p8 overexpression (p8: 2+ or 3+) | | | |
| Positive | 7 | 19 | 26 |
| Negative | 11 | 7 | 18 |
| Total | 18 | 26 | 44 |

[a] $P < 0.05$.

**Survival.** Multivariate survival analysis showed that the size ($P < 0.002$), stage ($P < 0.0.0005$), and metastasis ($P < 0.002$) of PCs and nutritional state ($P < 0.05$) were significantly correlated with survival. However, there was no significant correlation of survival with p8, ssDNA, or their expression patterns (Table 3).

*Table 3* Multivariate survival analysis using the Cox regression model

| Factor | Reference | Odds Ratio | *P* |
|---|---|---|---|
| p8 | 2+, 3+ *vs.* −, 1+ | 0.8408 | 0.3592 |
| ssDNA | 1+, 2+ *vs.* − | 1.3707 | 0.2417 |
| Liver metastasis | + *vs.* − | 5.0440 | 0.0073 |
| Lymph node metastasis | + *vs.* − | 2.4695 | 0.0775 |
| Histological type | Mod/poor *vs.* well[a] | 2.530?? | 0.0335 |
| Age | <65 *vs.* <65 | 2.529 | 0.0388 |
| Gender | Male *vs.* female | 1.5182 | 0.3282 |
| Clinical stage | III, IV *vs.* I, II | 6.657 | 0.0010 |
| Tumor size | ≤4 cm *vs.* >4 cm | 1.5179 | 0.3240 |
| Tumor location | Head *vs.* body, tail | 1.1629 | 0.7167 |

[a] Moderately or poorly differentiated type *versus* well differentiated.

## DISCUSSION

In this study, we have revealed an inverse correlation between p8 overexpression and apoptosis in human PC. p8 is strongly induced in acute pancreatitis, pancreatic development, and regeneration (8), indicating its overexpression in active cellular growth. Previous studies suggest that p8 has cell growth-promoting activity (8, 9). These characteristics of p8 prompted us to examine whether p8 is expressed in cancer. There has been no report on p8 expression in human cancer. Recently, we have found an overexpression of p8 in human PC (11). Because p8 might have antiapoptotic activity and there is spontaneous apoptosis in PC (6, 7), we attempted to examine the relationship between p8 expression and apoptosis in human PC.

Overexpression of p8 and spontaneous apoptosis were recognized in 59 and 41% of PC cases examined, respectively. Overexpression of p8 was significantly correlated with lymph node metastasis. ssDNA expression (apoptosis) was significantly lower in males. The type II expression pattern (high p8 and low apoptosis) was seen significantly often in younger age (<65 years), undifferentiated types, and node-positive cases. Although there was no correlation of p8 and ssDNA with survival, expressions of these factors might affect the biological characters of PC cells.

To detect spontaneous apoptosis, we analyzed the expression of ssDNA, because the ssDNA modification in the nucleosomal linker region might constitute an early step in apoptosis (16). The antibody against ssDNA might differentiate apoptosis from necrosis (5). The ssDNA detection is reported to be more specific and sensitive than the TUNEL method (5, 16). The immunohistochemical detection of ssDNA has demonstrated to be of great value for identification of cells that undergo apoptosis during tumorigenesis (5, 16). Although TUNEL is most widely used to detect apoptosis, it stains both apoptotic and necrotic cells and detects only late apoptotic events (17). In our results, the apoptotic indices obtained by the TUNEL method tended to be higher than those obtained by the ssDNA detection (data not shown). Our results with ssDNA are considered to be suitable for apoptosis evaluation. Furthermore, we used Hoechst 33258 staining to confirm apoptosis in cancer cells in this study.

Previous reports have shown that spontaneous apoptosis are involved in an unfavorable prognosis in patients with various cancers such as breast (12), colon (5), and pancreas (7). It has been speculated that tumors with active apoptosis show aggressive behavior (7, 12). However, the relationship between spontaneous apoptosis and malignant grade of cancer is still controversial. Frequent expression of Fas and Fas ligand in human PCs might be important for escaping of cancer cells from immune surveillance through the induction of apoptosis in tumor-attacking lymphocytes (18, 19). The bcl-2-negative, p53-positive cases of PC have shown more aggressive characteristics (20). On the contrary, bax expression seems to enhance apoptosis in tumors (6, 21), and bax-positive PC patients with abundant apoptosis show better prognosis (21). Moreover, enhanced expression of the antiapoptotic gene *bcl-xL* in PC is associated with shorter survival (22).

Vasseur *et al.* (10) have reported that *p8* mRNA is strongly activated in brain after transient ischemic injury. Apoptosis has been observed in experimental hepatic ischemia/reperfusion injury (23). Angiogenesis is induced in cancers in response to ischemia inside of the tumor (24). The growth of human PC in an ischemic condition might require p8 up-regulation, which would suppress ischemia-induced apoptosis of cancer cells.

Neither p8 overexpression nor ssDNA expression was significantly correlated with survival in this study. However, because the overexpression of p8 and the loss of apoptosis were significantly related to poor differentiation and lymph node metastasis, this expression pattern might reflect not only the growth activity and differentiation grade of cancer cells but also invasion and metastasis of human PC.

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