Loss of Peroxiredoxin Expression Is Associated with an Aggressive Phenotype in Pancreatic Adenocarcinoma

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Abstract. *Background: The role of the redox-regulating peroxiredoxin (Prx) enzymes I-VI in pancreatic carcinoma is poorly characterized. Materials and Methods: The expression of Prxs I, II, III, V and VI was immunohistochemically evaluated in benign pancreas and in 69 pancreatic adenocarcinoma samples. Results: Cytoplasmic Prx I expression was significantly greater in cancer cells than in benign pancreas (p=0.002) and Prx I expression in adenocarcinoma cells was associated with a larger tumour (p=0.005). Stronger cytoplasmic Prx III expression was associated with node negativity (p=0.007) and better tumor differentiation (p=0.033). Greater cytoplasmic Prx V expression was associated with smaller tumours (p=0.029) and negative nodal status (p=0.003). Among patients with T3-4 tumours, stronger intensity of cytoplasmic Prx I was associated with longer relapse-free survival (p=0.041). In patients with tumours of T3-4 class only, cytoplasmic Prx VI expression was associated with longer disease-free survival (p=0.0037). Conclusion: Peroxiredoxins appear to be promising prognostic factors in cases of pancreatic adenocarcinoma, and this may be related to their potential as tumour suppressors.*

Pancreatic andenocarcinoma is still one of the most lethal types of cancer worldwide, reflected by a 5-year survival rate of less than 5% (1). Multiple agents, such as reactive oxygen species (ROS), cytokines, growth factors and extracellular matrix proteins, have been identified in the pathogenesis of pancreatic cancer (2). During oxidative phosphorylation, a vital process in all aerobic organisms, ROS, including oxygen radicals and non-radical derivatives of oxygen, are produced as by-products (2, 3). Under oxidative stress, the effects of growth factor stimulation may result in harmful changes in DNA, proteins and lipids. Signalling by ROS, especially hydrogen peroxide (H_2O_2), plays an essential role in several cellular events such as growth, proliferation, and carcinogenesis (4, 5). Peroxiredoxins (Prxs) are a ubiquitous family of peroxidases with antioxidant capacity and roles in modulation of intracellular signalling and regulation of cell proliferation. They are involved in a variety of activities where H_2O_2 is generated, including cellular metabolism, growth, differentiation, inflammation and proliferation (6).

By using reversibly oxidized cysteines, Prxs catalytically reduce H_2O_2 to reduce the level of oxidative stress (4, 7, 8). By doing so, Prxs themselves are oxidized at their cysteine sites, giving sulfinic acid. Sulfiredoxin and thioredoxins catalyze the enzymatic reversal of inactivated Prxs by reduction of sulfinic acid formation, thus saving Prxs from destruction (9, 10). Kelch-like ECH-associated protein 1 (KEAP1) regulates the activity of nuclear factor E2-related factor 2 (NRF2), which binds to antioxidant-responsive elements and substantially up-regulates the expression of sulfiredoxin and Prxs during oxidative stress (4). We recently demonstrated that NRF2 and KEAP1 are promising prognostic factors in cases of pancreatic adenocarcinoma (10, 11).

Peroxiredoxins are classified into three sub-groups (2-cysteine, atypical 2-cysteine and 1-cysteine) based on the number and structure of cysteines at their active catalytic site. Ubiquitously expressed Prxs are widely distributed subcellularly in various tissues and organs. For example, Prxs I, II, III, V and VI are located in the cytoplasm, Prx I in the nucleus, Prxs III, V and VI in mitochondria, and Prx V in peroxisomes and mitochondria (6, 7, 12).

Because of the diversity of Prx expression at subcellular locations, Prx family isomers may have distinct functions besides being involved in oxidative stress (7). In addition, besides activation of antioxidant-responsive element-dependent genes, H_2O_2 signaling also contributes to essential processes,

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including cell division, differentiation and migration. H_2O_2 signaling mechanisms are still somewhat unclear and poorly defined. Peroxiredoxins may act either as tumour suppressors or as pro-oncogenic factors, depending on cellular context, which is also thus far poorly understood.

Many previous studies have shown that certain Prxs are overexpressed in several human cancer types including of the breast (Prxs I, II and III) (9, 13), lung (Prxs I and III) (1, 4), bladder (Prxs I and VI) (14), thyroid (Prx I) (15) and in malignant mesothelioma (Prxs I, II, III, V and VI) (16). The contribution of Prxs to tumourigenesis and progression of pancreatic adenocarcinoma is poorly understood, but two previous studies revealed increased expression of Prxs I and VI in pancreatic cancer compared with benign pancreatic conditions (17, 18). In the present study, we immunohistochemically examined the expression of Prx isoforms I, II, III, V and VI in 69 pancreatic adenocarcinoma cases in order to evaluate their possible prognostic value and association with traditional prognostic factors.

Materials and Methods

Samples. The material evaluated consisted of pre-treatment samples from 69 patients with histologically confirmed pancreatic adenocarcinoma. The specimens had been fixed in neutral formalin and embedded in paraffin blocks. All carcinomas were diagnosed and treated at the Oulu University Hospital between 1993 and 2011. The median follow-up time was 19 months and during follow-up, 61 patients (88.4%) died of pancreatic cancer. Evaluation of immunostaining was performed by an experienced histopathologist (KMH). Diagnoses were reviewed by a specialist pathologist and accurate and updated patient information was obtained in each case from patient records. Pathological TNM staging was available in 94.2% (n=65) of the cases and clinical TNM staging was available in 4.3% (n=3). In one of the cases, reliable TNM staging was absent. Patients who had undergone only palliative surgery were also included to enable a sufficient number of histological samples for reliable immunostaining and accurate TNM staging. Procedures with curative intent (mainly pancreaticoduodenectomy) were carried out for 64 patients and palliative surgery for five patients (7.2%).

Immunohistochemistry. The specimens were sectioned at 4 μm, deparaffinized in xylene and rehydrated in a descending series of ethanolic solutions. They were then covered in 10 mM citric acid monohydrate (pH 6) and heated in a microwave oven for 15 min. Next, endogenous peroxide was removed by placing the slides in 3% H_2O_2 in methanol for 10 minutes. The sections were incubated for 1 h at 37˚C with primary antibodies (Ab Frontier, Seoul, Korea) against Prxs I, II and V and for 30 min with anti-Prx III and anti-Prx VI. The dilutions were 1:750 for Prx I, 1:5000 for Prx II, 1:500 for Prx III, 1:500 for Prx V and 1:3000 for Prx VI. Immunostaining was carried out using a Novolink Polymer Detection Systems Kit (Leica Biosystems, Newcastle-upon-Tyne, UK) for Prxs I, III and V according to the supplier's instructions, a Histostain-Plus kit (Invitrogen, Camarillo, CA, USA) for Prx II and an Envision + System-HRP kit (Dako North America, Carpinteria, CA, USA) for Prx VI. For negative controls, we used phosphate-buffered saline instead of primary antibodies. Colour was generated with 3,3-diaminobenzidine and the sections were counterstained with haematoxylin, immersed in 0.75% ammonia and mounted with Immu-Mount (Shandon, Pittsburgh, PA, USA).

Immunostaining intensity was graded as negative, low (visible at ×40 magnification), strong (visible at ×20 magnification) or very strong (visible at ×10 magnification). It was separately assessed in nuclei, cytoplasm, perimembranously and in benign pancreas if it was reliably evaluable. Benign pancreas consisted mainly of exocrine tissue. Immunostaining results were compared according to grading, tumour size, nodal status, the presence of distant metastases and lymphovascular invasion.

For statistical analyses, tumour size was divided into T1-2 and T3-4, nodal status into either node-negative and node-positive, and grade to either I or II–III. For survival analyses, Prx immunostaining results were divided into a two-classed variable as negative to low intensity or moderate to strong intensity.

Statistical analyses. Statistical analyses were performed by using IBM SPSS Statistics software, v. 22.0.0.0 (IBM Corporation, Armonk, NY, USA). The significance of associations was defined by using two-sided Chi-square tests. Kaplan–Meier curves with the log-rank test were applied in survival analysis. Disease-free (DFS), relapse-free (RFS) and pancreatic cancer-specific (PCSS) survival were calculated from the time of diagnosis to disease recurrence at any site (DFS), in the pancreas (RFS), or to the time of confirmed pancreatic cancer-related death (PCSS). Values of p of less than 0.05 were considered significant.

The Ethics Committee of the Northern Ostrobothnia Hospital District approved the study design (114/2011).

Results

Staining patterns. Malignant tissue: Peroxiredoxin I was identified in all pancreatic adenocarcinoma lesions (n=67) (Figure 1). Two samples were not evaluable because of exhaustion of the blocks. Cellular staining intensity ranged from weak (+) to strong (+++), but only two samples showed strong immunopositivity. Membrane-associated Prx I staining was seen in 16 cases. Dotted paranuclear staining was also observed in some samples. Cytoplasmic Prx II was detected in all but one (98.5%) of the pancreatic adenocarinoma lesions (65 evaluable samples). Strong cytoplasmic staining was identified in 21 samples. Membrane-associated Prx II was observed 21 cases. The intensity ranged from weak to moderate. Prx III expression was recorded in all pancreatic adenocarcinoma lesions (n=66). Three of the samples were not evaluable because of exhaustion of the blocks or the occurrence of non-representative areas. Cytoplasmic Prx III was weakly to moderately positive in malignant pancreas tissue in all cases. Strong cytoplasmic staining was seen in 19 cases. Membrane-associated Prx III was expressed in several samples. Prx V was positive in all pancreatic adenocarcinoma lesions (n=68). Cytoplasmic Prx V was seen in all evaluable samples. Strong cytoplasmic Prx V staining was identified in 12 pancreatic adenocarcinoma lesions. Membrane-associated Prx V was observed in almost half of the specimens (n=30). Prx VI was also identified in all pancreatic adenocarcinoma lesions (n=66).

Three samples were not evaluable because of exhaustion of the blocks or the occurrence of non-representative areas. Strong cytoplasmic Prx VI staining was identified in six samples. Only a few samples showed membrane-associated positivity.

Most of the nuclei were negative with regard to Prxs I, II and III in cancerous areas. Prx V staining in nuclei in tumour tissue was totally negative, but nuclear Prx VI staining was positive in 64 samples (97.0%).

Benign tissue: In benign pancreatic tissue, cytoplasmic Prx I staining was weak in most cases. Cytoplasmic Prxs II and III were identified in every sample. Cytoplasmic Prx II intensity was comparable to that in malignant tissue. Cytoplasmic Prx III intensity appeared weaker in benign than in malignant samples in 14 cases. Cytoplasmic Prx V was weakly to very strongly positive in all benign pancreas samples. Staining of Prxs I, II, III and V in the nuclei of benign samples was negative. In benign pancreatic tissue, both cytoplasm and nuclei were positive for Prx VI.

When comparing Prx expression in benign and malignant pancreatic tissue, cytoplasmic Prx I expression was significantly greater in cancer cells than in benign endocrine and exocrine pancreas (p=0.002) (Figure 2). No other statistically significant differences in Prx expression in benign *vs*. malignant tissue were observed.

Greater membrane-associated Prx I expression in adenocarcinoma cells was associated with increasing tumour size (T1-2 *vs*. T3-4; p=0.005). Greater Prx II expression in cellular membranes was observed more frequently in node-positive than in node-negative cases (p=0.025). Greater cytoplasmic Prx III expression in cancer cells was related to node negativity (p=0.007) and better tumour differentiation (p=0.033). Greater cytoplasmic Prx V expression was associated with smaller tumour size (p=0.029) and negative nodal status (p=0.003).

No associations between PCSS, RFS or DFS and Prx expression were found when analyses were performed on the whole study cohort. However, when considering only patients with T3-4 tumours, stronger intensity of cytoplasmic Prx I was associated with longer RFS (median survival with negative to low Prx I cytoplasmic intensity was 18 months *vs*. 71 months for those with strong to very strong intensity; p=0.041). In addition, only in tumours of T3-4 class was cytoplasmic Prx VI expression associated with longer DFS (median survival for patients with negative to low Prx VI cytoplasmic intensity was 8 months *vs*. 18 months for those with strong to very strong intensity; p=0.0037) (Figure 3).

Discussion

The current results suggest an association between low-intensity cytoplasmic expression of Prxs I, III and V and an aggressive pancreatic cancer phenotype, such as larger tumour size, nodal involvement and poor differentiation. The data also show that patients with high Prx I expression in the sub-group of patients with T3-4 tumours may particularly have very greatly lengthened RFS periods of up to 71 months. As a limitation, the sample size was rather too small to consider reliable multivariate analysis. However, the material was homogeneous, since only patients treated with at least palliative surgery at a single institution were included in this study. We also focused on careful assessment of immunohistochemical staining, both in benign and malignant samples.

A recent study by Cai *et al*. revealed Prx I overexpression in pancreatic cancer tissues compared to para-cancerous tissues and the investigators also suggested Prx I to be a marker of poor prognosis (17). We observed a similar increase of Prx I expression in malignant tissue but in our material, Prx I expression in cancer cells was associated with longer RFS in patients with the most invasive T3-4 tumours. In addition, in the study by Cai *et al*., tumour diameter was not related to Prx I expression, whereas in our patients high Prx I expression was associated with smaller tumour size. Since Prx expression is easily induced under oxidative conditions, higher Prx I expression in cancer cells may reflect enhanced local metabolism and ROS production in malignant but not in benign tissue. The partial contradiction between our results and those of Cai *et al*. cannot be fully explained, but at least the use of different primary antibodies may have contributed to the results. Prxs have molecular chaperone functions with different oligomerization (19). These modifications in enzyme structure lead not only to functional changes but may also change the immunoreactivity of protein complexes (20).

Proteomics analysis has revealed increased Prx I expression in pancreatic adenocarcinomas compared to normal tissues and pancreatitis (21). As far as we know, there are no studies other than that by Cai *et al*. concerning Prx protein expression in relation to clinical data in pancreatic carcinoma. However, some data exist in relation to other types of carcinoma. For example, we and others have recorded up-regulation of Prxs II-VI in borderline compared to benign ovarian tumours (22), increased expression of Prxs I-III in breast cancer compared with benign breast tissue (13), up-regulation of Prxs I and III in lung cancer compared to benign lung tissue (23), and increased expression of Prxs I-VI in pleural mesothelioma compared with healthy pleural tissue (16). On the other hand, malignant melanomas exhibited a decreased expression of Prxs I and II compared to dysplastic and benign naevi (24), and of Prx II in endometriosis-associated ovarian cancer compared to benign endometriosis (25).

The association of Prxs I, III and V with smaller tumour size, reduced invasion and negative nodal status, and increased DFS and RFS in cases with the largest tumours, suggest that these Prx isoforms may have tumour-suppressive properties in pancreatic adenocarcinoma. Despite the lack of data on pancreatic cancer, Prx I-deficient mice display elevated tumourigenesis in various tissues *via* enhanced ROS production

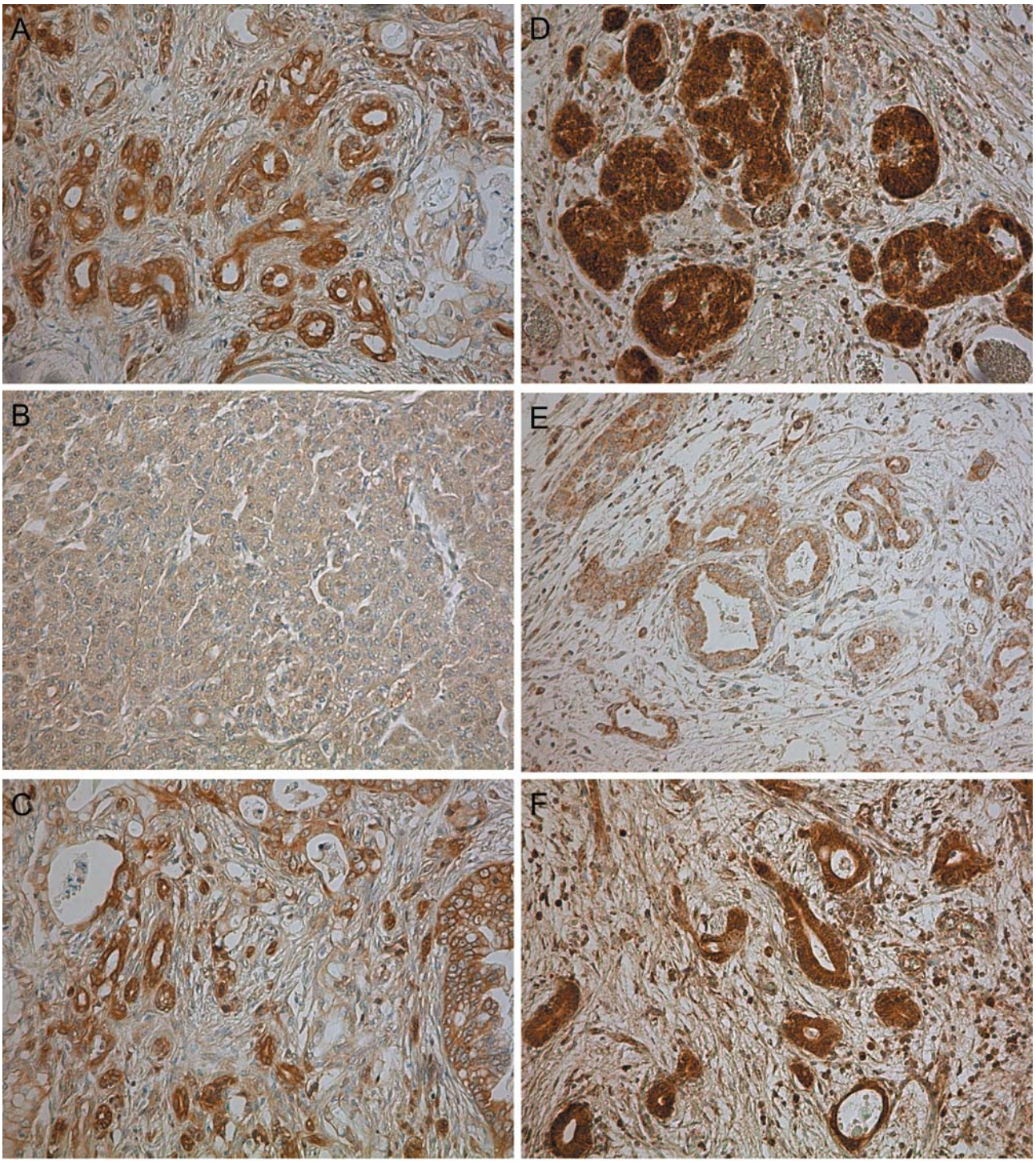

Figure 1. *Continued*

and Prx I appeared to have cancer-preventative properties in a mouse model (26, 27). Protein expression of Prx I also seems to have substantial tumour-suppressive properties in oesophageal squamous cell carcinoma, higher expression being associated with smaller tumour size, negative nodal status and earlier stage (28). In breast cancer, immunohistochemical expression of Prxs III and IV was associated with better prognosis (9). Nevertheless, most published studies with

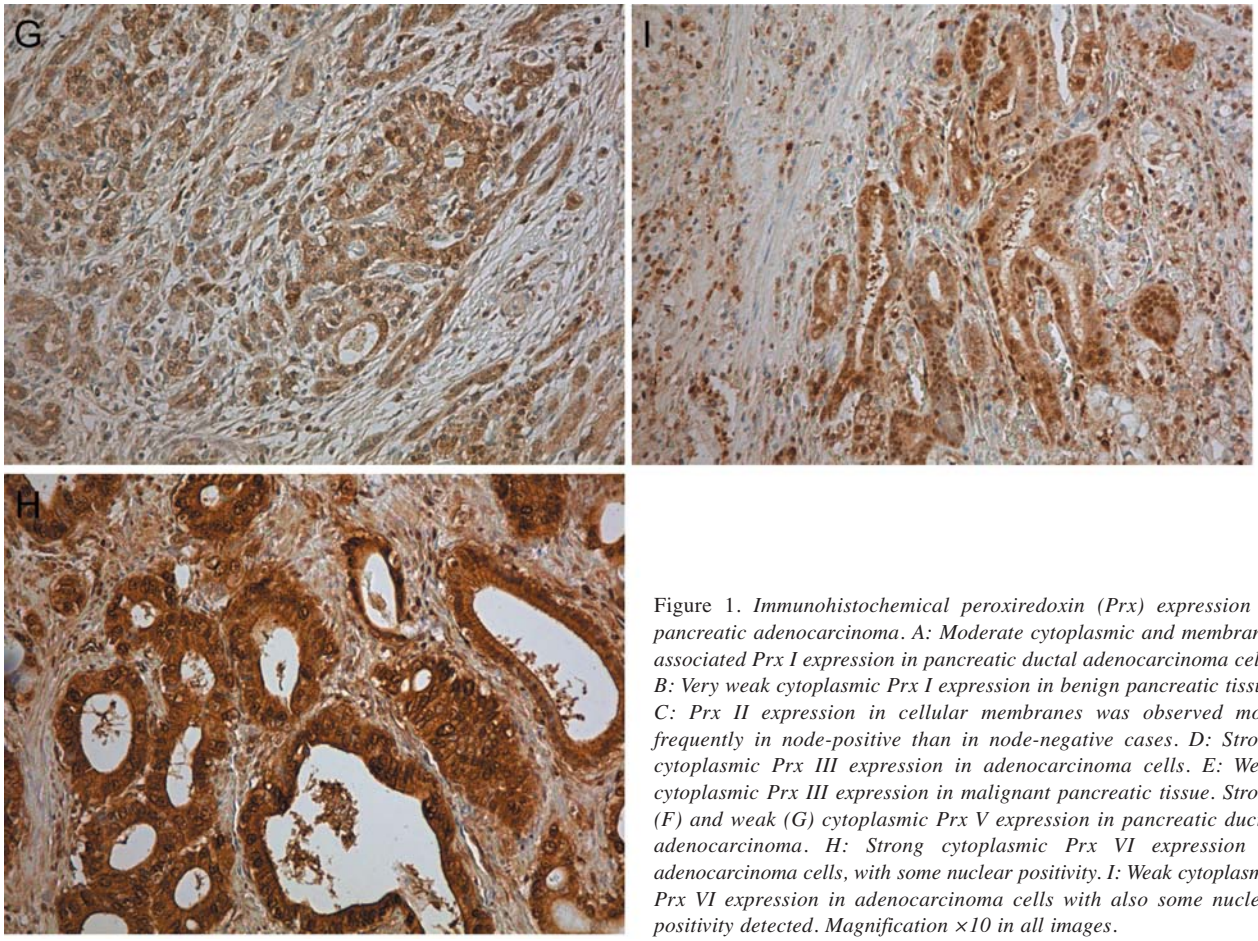

Figure 1. *Immunohistochemical peroxiredoxin (Prx) expression in pancreatic adenocarcinoma. A: Moderate cytoplasmic and membrane-associated Prx I expression in pancreatic ductal adenocarcinoma cells. B: Very weak cytoplasmic Prx I expression in benign pancreatic tissue. C: Prx II expression in cellular membranes was observed more frequently in node-positive than in node-negative cases. D: Strong cytoplasmic Prx III expression in adenocarcinoma cells. E: Weak cytoplasmic Prx III expression in malignant pancreatic tissue. Strong (F) and weak (G) cytoplasmic Prx V expression in pancreatic ductal adenocarcinoma. H: Strong cytoplasmic Prx VI expression in adenocarcinoma cells, with some nuclear positivity. I: Weak cytoplasmic Prx VI expression in adenocarcinoma cells with also some nuclear positivity detected. Magnification ×10 in all images.*

clinical material have reported an association between Prx expression and either aggressive tumour features or poor prognosis. In the above-mentioned breast cancer study, Prx V expression was associated with lengthened survival (9). Similar observations of overexpression of Prxs or their association with reduced survival rate have been reported in connection with many solid tumour types, including of the gastrointestinal tract, such as colorectal carcinoma (Prx IV) (29), hepatocellular carcinomas (Prx I) (30) and oesophageal cancer (Prx I) (31). Therefore, it seems that Prxs may have different roles according to the carcinoma type. Different Prx isoforms may also have diverse roles in progression of carcinomas of specific types, as reflected in stronger Prx II expression and positive nodal status observed in this study.

The staining patterns of Prx isoforms investigated herein are in line with those in previous reports concerning other types of carcinoma. Although cytoplasmic expression appeared to be the most interesting in terms of association with the clinical course of the disease, widespread nuclear and membrane-associated expression were also found. The expression of Prxs III and VI in membranes has been reported, although its biological significance is poorly defined (32, 33). Interestingly, perimembranous Prx II expression was associated with the presence of nodal metastases, whereas expression of other Prxs (with mainly cytoplasmic expression) was associated with less aggressive disease. It may be hypothesized that Prx II increases nodal involvement *via* activation of epithelial–mesenchymal transition, as does Prx I in lung cancer cells (34). The role of Prxs in chemoresistance and radioresistance has mostly concerned Prx II (35, 36). There is emerging evidence that Prx II might also be associated with resistance to gemcitabine, the standard drug in adjuvant treatment of pancreatic adenocarcinoma (37-39).

In conclusion, the Prx isoforms studied here appear to be expressed in almost all pancreatic adenocarcinomas at some level. Low intensity staining of cytoplasmic Prxs I, II and V was associated with more aggressive primary tumour behaviour, nodal involvement and poor tumour differentiation. In addition, lack of expression of Prxs I and VI was associated with dismal RFS and RFS in patients with T3-4 pancreatic

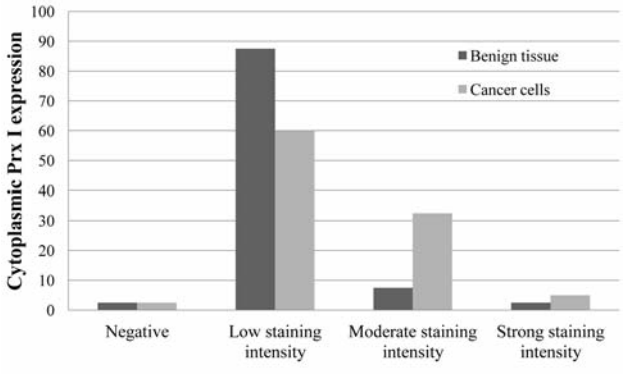

Figure 2. *Cytoplasmic peroxiredoxin (Prx) I expression in benign pancreas compared to cancer cells. p=0.002. Y-axis represents the percentage of cases in each staining intensity class.*

adenocarcinomas. Although specific putative mechanisms of pancreatic cancer-suppressive roles of Prxs I, III and V cannot be determined by the current methods, their ROS-suppressing properties, roles as essential H_2O_2-regulating transcription factors or chaperone functions may at least partly explain the results (5, 20). To resolve whether or not these Prx isoforms themselves are tumour suppressors or if they are subsidiarily down-regulated in the most aggressive tumours, requires more mechanistic studies.

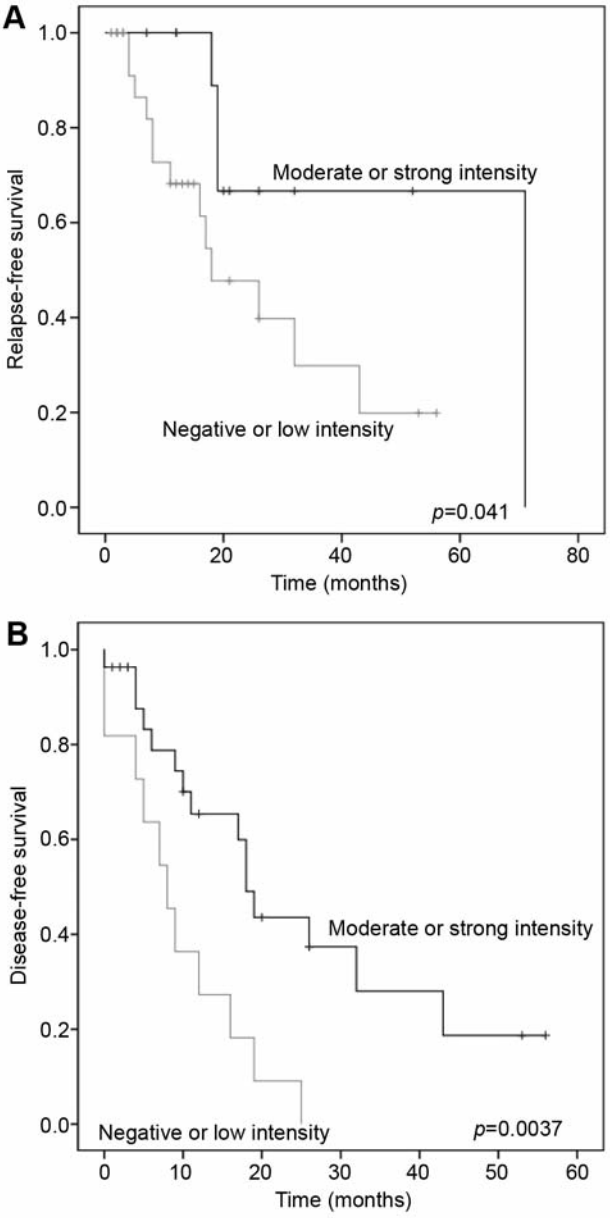

Figure 3. *Kaplan–Meier curves of survival for patients with T3-4 tumours by cytoplasmic Prx I (A) and cytoplasmic Prx VI (B) expression.*

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