Group II and IV phospholipase A_2 are produced in human pancreatic cancer cells and influence prognosis

M Kashiwagi, H Friess, W Uhl, P Berberat, M Abou-Shady, M Martignoni, S E Anghelacopoulos, A Zimmermann, M W Büchler

Abstract

***Background*—Phospholipase A_2 (PLA_2) is involved in regulating biosynthesis of arachidonic acid and its metabolites. There are three major structurally different forms of PLA_2: group I, also called pancreatic PLA_2 (PLA_2-I); group II, referred to as secretory non-pancreatic or synovial or platelet PLA_2 (PLA_2-II); group IV, referred to as cytosolic PLA_2 (PLA_2-IV).**

***Aims*—To examine PLA_2-I, PLA_2-II, and PLA_2-IV in normal and pancreatic cancer tissues.**

Patients—PLA_2 was studied in 58 pancreatic adenocarcinomas, obtained from 25 women and 33 men undergoing pancreatic resection. Normal organ donor pancreas served as control.

***Methods*—The enzymes were analysed by northern blot, in situ hybridisation, and immunohistochemistry. The molecular findings were correlated with clinical variables of the patients.**

***Results*—Northern blot analysis of total RNA showed enhanced PLA_2 group II and IV mRNA expression in 52% and 55% of the pancreatic cancer samples respectively compared with the normal controls ($p = 0.0013$ and $p = 0.0025$). On immunohistochemical analysis, intense PLA_2-I immunoreactivity was seen in acinar cells, but not in ductal cells, in the normal pancreas. In pancreatic cancer cells, PLA_2-I immunostaining was absent. PLA_2-II immunostaining was visible only in some acinar and ductal cells in the normal pancreas, whereas in pancreatic cancer increased PLA_2-II immunoreactivity was present in 65% of the cancer samples. On in situ hybridisation, weak PLA_2-IV mRNA signals were detected in acinar and ductal cells of normal samples; these signals were present to a much greater extent in pancreatic cancer cells. The presence of PLA_2-II in pancreatic cancer was associated with a higher degree of fibrosis ($p<0.01$). Furthermore, there was a significant correlation between the enhanced expression of PLA_2-II and longer survival after surgery ($p<0.03$), but not of PLA_2-IV and longer postoperative survival.**

***Conclusion*—These data suggest that PLA_2-II and PLA_2-IV are upregulated in human pancreatic cancer, and that up-regulation of PLA_2-II in pancreatic cancer covariates negatively with cancer cell growth.**

(*Gut* 1999;**45**:605–612)

Keywords: pancreas; cancer; phospholipase A_2; survival analysis

Department of Visceral and Transplantation Surgery, University of Bern, Inselspital, Switzerland
M Kashiwagi
H Friess
W Uhl
P Berberat
M Abou-Shady
M Martignoni
S E Anghelacopoulos
M W Büchler

Institute of Pathology, University of Bern, Switzerland
A Zimmermann

Correspondence to:
Dr H Friess, Department of Visceral and Transplantation Surgery, University of Bern, Inselspital, CH-3010 Bern, Switzerland.

Accepted for publication
14 April 1999

Phospholipase A_2 (PLA_2) subtypes make up a family of enzymes that catalyse the hydrolysis of the *sn*-2 fatty acyl chain of many different phospholipid substrates to yield fatty acids and lysophospholipids.[1 2] Their enzymatic activity is a rate-limiting step in the formation of arachidonic acid and subsequently in the synthesis of leukotrienes and prostaglandins. Arachidonic acid and its generated biomediators have been implicated in carcinogenesis acting by immune suppression, stimulation of cell proliferation, invasion, and metastasis.[3–5] In humans, three major genetically different subtypes of PLA_2 with distinct primary structures and molecular masses have now been identified—two 14 kDa low molecular mass forms and a high molecular mass form (85 kDa). The low molecular mass PLA_2s are classified as PLA_2 group I (PLA_2-I) and PLA_2 group II (PLA_2-II). Owing to their apparent cellular localisation, they are also called secretory PLA_2. PLA_2 group IV (PLA_2-IV) is a high molecular mass enzyme mainly located in the cytoplasm.[6 7]

PLA_2-I is mainly synthesised in the exocrine pancreas and was originally thought to serve only as a digestive enzyme, like other pancreatic exocrine enzymes. However, recent research has disclosed its presence in non-digestive organs such as the lung, spleen, and ovary as well,[8–10] suggesting that this enzyme is not only involved in digestive processes, but also possesses other physiological functions that are not readily evident.

PLA_2-II is located in several cells and tissues, as well as extracellularly in human digestive organs, platelets, and synovial fluid. Many studies have shown its involvement in various inflammatory diseases such as sepsis, inflammatory bowel disease, acute pancreatitis, and multiple organ failure.[11–16] Recent studies have reported that the tissue concentration of PLA_2-II in colon and hepatocellular cancer is significantly elevated.[17 18] Furthermore,

Abbreviations used in this paper: PLA_2, phospholipase A_2; DIG, digoxigenin; SDS, sodium dodecyl sulphate.

enhanced PLA_2-II immunoreactivity is observed in gastric and breast cancers in comparison with normal tissue.[19 20]

The 85 kDa PLA_2-IV differs from the 14 kDa enzymes in that after stimulation it translocates from the cytoplasm to the membrane in a micromolar Ca^{2+} dependent manner. It shares no structural homology with 14 kDa PLA_2. PLA_2-IV is transcriptionally regulated by various cytokines as well as by growth factors such as epidermal growth factor and platelet derived growth factor.[7] Growth factors and other mitogens can have long lasting effects on cellular arachidonate release, and this effect may be initiated in part at the level of gene expression. Furthermore, PLA_2-IV is a downstream target of mitogen activated protein kinase, and the phosphorylation of PLA_2-IV by mitogen activated protein kinase is essential for its full activation.[21]

PLA_2s are involved in the generation of a wide range of biomediators, including lysophospholipids, arachidonic acid, and metabolites of arachidonic acid. These lipids can enhance tumour cell growth, invasion, and metastasis, as recently reported.[22]

Pancreatic carcinoma is a devastating neoplasm that is usually associated with a poor prognosis. Although the reasons for the aggressiveness of this disease are not known, enhanced expression of growth factors and growth factor receptors and mutations of tumour suppressor genes and oncogenes have been shown in this malignancy,[23 24] which together may give pancreatic cancer cells a growth advantage. However, it is not known whether PLA_2 isoforms are expressed in human pancreatic cancer and whether PLA_2 isoforms influence the malignant potential of pancreatic cancer cells. Therefore, in this study, we examined the expression and distribution of the three PLA_2 subtypes in pancreatic tumours, using northern blot analysis, in situ hybridisation, and immunohistochemical techniques.

Materials and methods

PATIENTS

Normal human pancreatic tissue samples were obtained from 14 previously healthy subjects (four women, 10 men; median age 41 years (range 16–54)) through an organ donor programme in which no recipients for the pancreas were available. Pancreatic cancer tissues were obtained from 58 patients (25 women and 33 men) undergoing pancreatic resection at the University Hospital of Bern. The median age of the cancer patients was 61 years (range 32–78). Three patients had stage I disease, 20 had stage II disease, 32 had stage III disease, and three had stage IV disease according to the classification of the International Union Against Cancer.[25] Freshly removed tissue samples were immediately fixed in Bouin solution or 4% formaldehyde solution for 18 to 24 hours and embedded in paraffin wax for histological analysis. Concomitantly, tissue samples destined for RNA extraction were immediately frozen in the operating room and maintained at −80°C until use. All studies were approved by the human subjects committee of the University of Bern, Switzerland.

PROBES

The PLA_2-I cDNA probe consisted of a 542 bp *Pst*I/*Pvu*II fragment of rat PLA_2-I cDNA, which cross hybridises with human PLA_2-I (kindly provided by Dr J Ishisaki, Shionogi Research Laboratory, Osaka, Japan).[26] The PLA_2-II cDNA probe consisted of an 830 bp *Xho*I/*Hin*dIII fragment of human PLA_2-II cDNA (also kindly provided by Dr J Ishisaki).[27] Sense and antisense PLA_2-IV cRNA probes were digoxigenin (DIG)-labelled as previously described.[28] Briefly, the cDNA (clone kindly provided by Dr U Tibes, Boehringer Mannheim GmbH, Penzberg, Germany)[29] was linearised and transcribed using the Ribomax System (Promega, Zürich, Switzerland), resulting in DIG-labelled sense and antisense riboprobes.

The 7S cDNA probe consisted of a 190 bp *Bam*HI fragment of the mouse 7S RNA which cross hybridises with human 7S RNA.[28]

NORTHERN BLOT ANALYSIS

After RNA extraction and gel electrophoresis, the RNA was electrotransferred to Nylon membranes (Gene Screen, DuPont, Boston, Massachusetts, USA) and cross linked by UV irradiation.[28 30] For hybridisation, we used a ^{32}P-labelled cDNA probe for PLA_2-I, PLA_2-II, and 7S, and a DIG-labelled cRNA probe for PLA_2-IV. In the case of cDNA probes, the blots were prehybridised overnight, hybridised for 12 hours at 42°C with the ^{32}P-labelled cDNA probe (1×10^6 cpm/ml), and washed at 55°C in $0.2 \times$ SSC (0.15 M NaCl/0.015 M sodium citrate buffer) and 2% sodium dodecyl sulphate (SDS) under conditions appropriate for cDNA probes.[28 30] Membranes were then exposed at −80°C to *x* ray films with intensifying screens for 1 to 12 days. In the case of the DIG-labelled PLA_2-IV cRNA probe, the filters were prehybridised and hybridised overnight at 65°C and washed three times at 68°C for 15 minutes in $0.1 \times$ SSC/0.1% SDS. Afterwards, the filters were incubated in a blocking buffer (1% blocking reagent in 100 mM maleic acid, 150 mM NaCl, pH 7.5) for 30 minutes, and in a blocking buffer containing anti-DIG alkaline phosphatase antibody (1:20 000 dilution; Boehringer Mannheim) for 30 minutes, washed with maleic acid buffer (3×15 minutes), and incubated with 25 mM CDP-Star (Boehringer Mannheim). The membranes were then exposed to *x* ray films.

All the membranes were rehybridised with the 7S cDNA probe in order to assess equivalent RNA loading, as previously reported.[28 30]

The signals obtained by northern blot analysis were quantified by densitometric analysis.[28 30] Because in almost all normal tissue samples the hybridisation signals of PLA_2-II and PLA_2-IV were too low to be adequately measured by densitometry, the increase in the signals of pancreatic cancer samples in comparison with normal could not be calculated. Therefore the expression levels in pancreatic

cancer were divided into four groups according to the densitometric measurement: 0, no expression detectable, equal to normal; 1, weak expression, up to 1 densitometric unit; 2, moderate expression, 1–2 densitometric units; 3, strong expression, ⩾2 densitometric units.

IN SITU HYBRIDISATION OF PLA_2-IV

No antibodies were available for immunohistochemical analysis of PLA_2-IV. Therefore in situ hybridisation was carried out as previously reported.[28 30] Consecutive tissue sections were permeabilised with proteinase K (30 μg/ml) for 15 minutes at 37°C, then fixed with 4% paraformaldehyde in phosphate buffered saline (five minutes). The sections were prehybridised at 60°C for two hours and hybridised at 60°C for 14 hours. After hybridisation, excess labelled cRNA probe was removed by washing in 2 × SSC and by RNase treatment at 37°C for 30 minutes. The sections were washed in 2 × SSC, 0.2 × SSC, and 0.1 × SSC, then incubated with an anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim). 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma, Buchs, Switzerland) were used for colour reaction. No signal was detectable when the sense probe or RNase digested slides were used for in situ hybridisation in control experiments. The in situ results were semiquantitatively evaluated by two independent observers blinded to the patient status, as previously reported.[28 30]

IMMUNOHISTOCHEMISTRY

Consecutive 4 μm tissue sections were subjected to immunostaining using the alkaline phosphatase technique (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA). Tissue sections were deparaffinised, rehydrated, and washed for 10 minutes in TBS buffer (10 mM Tris/HCl, pH 7.4).[30] Then the sections were incubated in 0.4% pepsin in 0.01 M HCl for 10 minutes (PLA_2-I immunostaining) or 25 minutes (PLA_2-II immunostaining). After being washed three times for 10 minutes in TBS buffer, the sections were incubated with 10% normal goat serum (30 minutes) to block non-specific binding, followed by incubation with the specific antibodies (monoclonal antibody for PLA_2-I and polyclonal antibody for PLA_2-II; gift from Dr T Nevalainen, Turku, Finland).[31 32] Bound primary antibody was detected with a biotinylated goat IgG secondary antibody and a streptavidin-alkaline phosphatase complex. This was followed by incubation with PhThaloRED solution (Kirkegaard & Perry Laboratories) as the substrate and counterstaining with haematoxylin.

To ensure specificity of the immunostaining reactions, consecutive tissue sections were incubated either in the absence of the primary antibody or with an irrelevant IgG antibody. In both cases, no immunostaining was detected.

Immunohistochemical results were analysed semiquantitatively as described previously.[33] Briefly, the evaluations were recorded as percentages of positively stained cells in each of four intensity categories, which were denoted as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (intense staining). For each slide, the score was derived by adding the percentages of cell staining at each intensity multiplied by the weighted intensity of staining according to the formula $\sum Pi(i+1)$, where i = 1, 2, or 3 and Pi varies from 0 to 100, 100 meaning that 100% of the cells stained positively.[33]

Analysis of the immunohistochemical findings resulted in an immunohistochemical staining score ranging between 0 and 400. The cut off values for further analysis of patient survival were empirically determined and three cut off values (100, 150, 200) were taken which allowed further statistical testing given the group size.

Histological analysis of the immunohistochemical results was performed by two independent pathologists blinded to patient status, followed by resolution of any differences by joint review and consultation with a third observer.

To assess the degree of fibrosis of the cancer samples, haematoxylin and eosin stained sections were analysed. The degree of fibrosis was determined on at least two tissue sections as previously described.[34]

STATISTICAL ANALYSIS

For statistical analysis, the Mann-Whitney U test, the Spearman test, and multivariate analysis using Cox regression were used. Survival period after surgery was computed using the Kaplan-Meier method and analysed using the Cox non-parametric regression model. Significance was defined as $p<0.05$.

Results

NORTHERN BLOT ANALYSIS (FIG 1)

Strong expression of PLA_2-I mRNA was present in 12 of 14 normal pancreatic tissue samples. In the remaining two, PLA_2-I mRNA was moderately expressed. In contrast, in pancreatic cancer samples, northern blot analysis showed a considerable decrease in PLA_2-I mRNA expression ($p<0.0001$). Based on the immunohistochemical findings using a specific antibody against PLA_2-I, the detectable PLA_2-I mRNA expression in the cancer tissues must be derived from some remaining normal and atrophic pancreatic cells located adjacent to the cancer mass or between the cancer cells.

As determined by northern blot analysis, PLA_2-II and PLA_2-IV mRNA levels were extremely low or undetectable in the normal human pancreas. In most normal samples the signals were too low to be adequately measured by densitometry. Therefore the densitometric results obtained for PLA_2-II and PLA_2-IV mRNA expression in the cancer samples were divided into four groups as described in the Materials and methods section (no, weak, moderate, and strong expression). There was no PLA_2-II mRNA expression in 16% of the pancreatic cancer samples, weak expression in 32%, moderate expression in 13%, and strong expression in 39%. In all pancreatic cancer samples, mRNA expression of PLA_2-IV was detectable. Weak PLA_2-IV mRNA expression was present in 45% of the pancreatic cancer samples, moderate expression in 36%, and

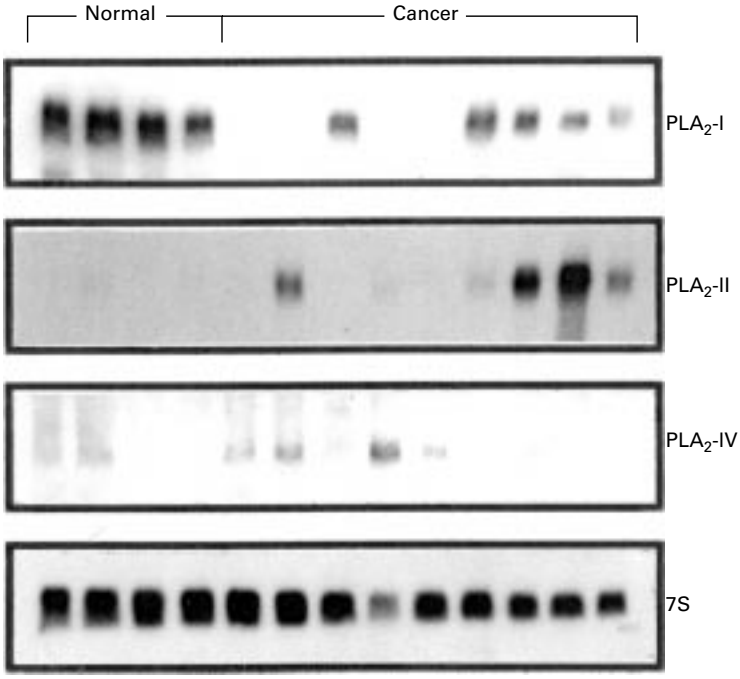

Figure 1 Northern blot analysis of phospholipase A_2 (PLA_2)-I, PLA_2-II, and PLA_2-IV mRNA in the normal pancreas (first four lanes) and in pancreatic tissues obtained from patients with pancreatic cancer (last nine lanes). In cancer samples, levels of PLA_2-I mRNA were reduced compared with the normal controls. In contrast, enhanced levels of PLA_2-II and PLA_2-IV mRNA were found in many cancer samples, whereas low to undetectable PLA_2-II and PLA_2-IV mRNA expression was present in normal controls. 7S mRNA was used to assess equivalent RNA loading.

strong expression in 19%. Statistical analysis using the Spearman test disclosed that these differences between cancerous and normal tissues were highly significant (PLA_2-II, $p = 0.0013$; PLA_2-IV, $p = 0.0025$). However, there was no correlation between PLA_2-II and PLA_2-IV mRNA levels in the pancreatic cancer tissues.

IN SITU HYBRIDISATION OF PLA_2-IV

We carried out in situ hybridisation of PLA_2-IV to detect the exact site of its expression in the pancreas, because no antibodies are at present available for immunohistochemical localisation of the respective protein. In the normal pancreas, faint PLA_2-IV mRNA expression was observed in some acinar, ductal, and islet cells (fig 2A). In contrast, most pancreatic cancer cells exhibited moderate to strong PLA_2-IV mRNA signals (fig 2B). Furthermore, degenerating pancreatic acinar cells, especially areas with ductal metaplasia, showed PLA_2-IV mRNA staining. No mRNA signal was detected when the sense PLA_2-IV probe was applied in in situ hybridisation, either in normal or pancreatic cancer samples (fig 2C).

IMMUNOHISTOCHEMISTRY OF PLA_2-I AND PLA_2-II

In the normal human pancreas, positive PLA_2-I immunostaining was present in almost all acinar cells, whereas ductal cells and islet cells were devoid of any PLA_2-I immunoreactivity (fig 3A). Pancreatic cancer cells did not show any PLA_2-I immunoreactivity (fig 3B). Positive immunoreactivity for PLA_2-I was found in only one cancer sample. However, the intensity of PLA_2-I immunoreaction in the cancer cells was

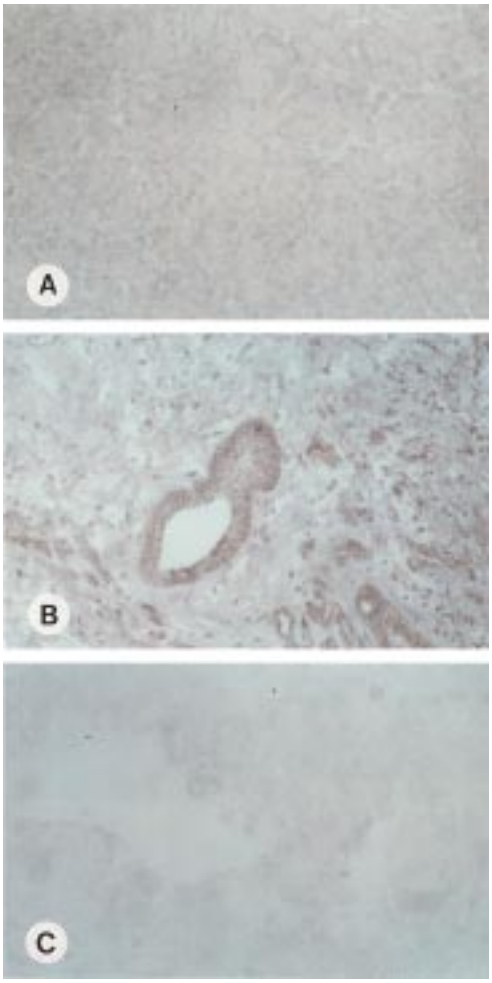

Figure 2 In situ hybridisation of phospholipase A_2 (PLA_2)-IV in the normal pancreas (A) and in pancreatic cancer (B, antisense hybridisation, C, sense hybridisation). In the normal pancreas, faint PLA_2-IV mRNA signals were present in some acinar cells (A). In contrast, intense PLA_2-IV mRNA signals were found in the pancreatic cancer cells (B). (C) Sense hybridisation of a pancreatic cancer sample which exhibited high levels of PLA_2-IV mRNA in northern blot hybridisation. Original magnifications: A, B × 200; C × 400.

very faint in comparison with that found in the normal controls. In areas with chronic pancreatitis-like lesions adjacent to the cancer mass, normal ductal cells and cells of metaplastic ducts often showed a faint granular cytoplasmic pattern of PLA_2-I immunostaining.

Some immunoreactivity for PLA_2-II was visible in the normal pancreatic samples. However, this occurred in only a few acinar and ductal cells, which exhibited weak to moderate cytoplasmic PLA_2-II immunoreactivity in a focal pattern (fig 3C). In contrast, 65% of the cancer samples exhibited weak to strong PLA_2-II immunostaining. It was present on the luminal aspect and/or cytoplasm of the cancer cells (fig 3D). In regions adjacent to the cancer lesion and exhibiting chronic pancreatitis-like changes, the remaining degenerating acinar and ductal cells and acinar cells dedifferentiating into duct-like structures commonly exhibited moderate PLA_2-II immunoreactivity. In the cancer samples, PLA_2-II immunoreactivity was distributed homogeneously throughout the

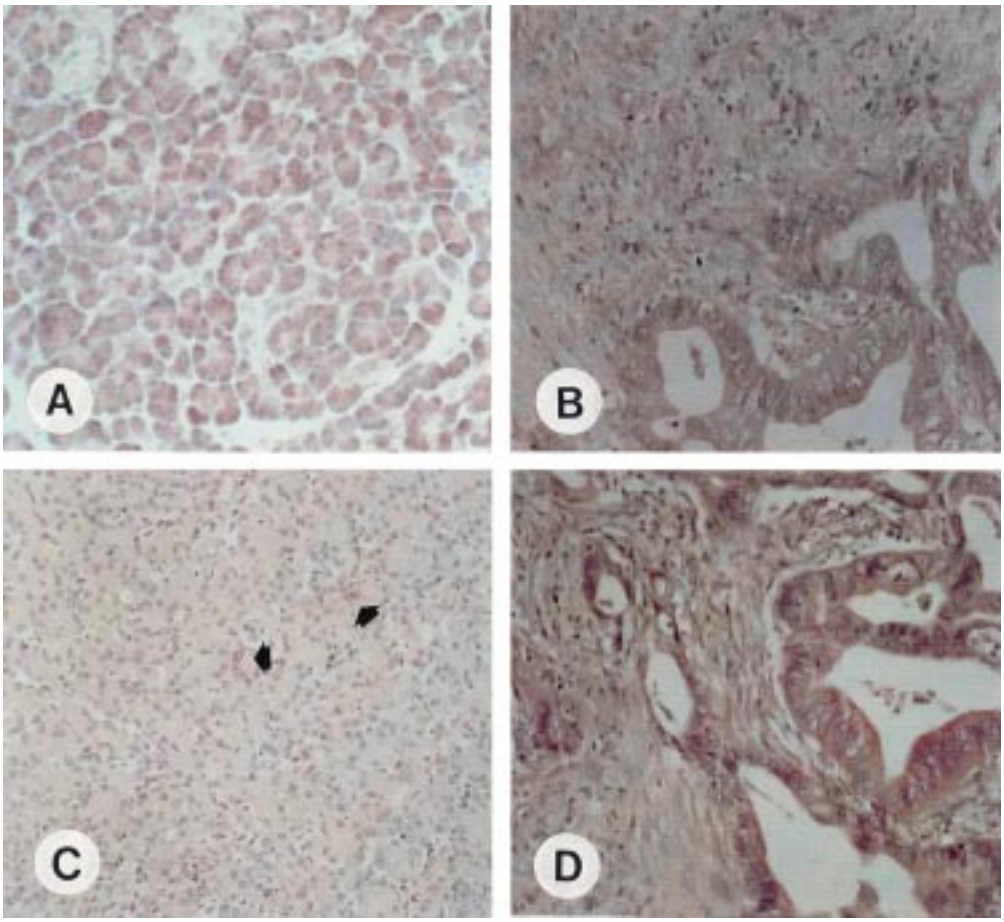

Figure 3 Immunohistochemical analysis of phospholipase A_2 (PLA_2)-I (A, B) and PLA_2-II (C, D) in the normal pancreas (A, C) and in pancreatic cancer (B, D). PLA_2-I immunostaining was abundant in acinar cells of the normal pancreas (A). No PLA_2-I immunostaining was present in the pancreatic cancer cells (B). In the normal pancreas, only a few acinar cells exhibited cytoplasmic PLA_2-II immunoreactivity (black arrows, C), whereas PLA_2-II immunostaining was often present in pancreatic cancer cells (D). The slides are counterstained with haematoxylin. Original magnifications × 200.

cancer lesions. No increase in PLA_2-II immunoreactivity in areas in which cancer cells were invading normal tissue could be observed.

CORRELATION OF PLA_2-II AND PLA_2-IV WITH THE DEGREE OF TUMOUR FIBROSIS

Because of our earlier observation that PLA_2-II and PLA_2-IV influence extracellular matrix generation in chronic pancreatitis, the mRNA expression data obtained by northern blot analysis and in situ hybridisation and the PLA_2-II immunohistochemical data were correlated with the degree of fibrosis present in the cancer samples. There was a significant positive correlation ($r = 0.76$) between PLA_2-II mRNA expression levels and the degree of pancreatic fibrosis ($p<0.01$). In addition, the PLA_2-II immunohistochemical staining score was significantly correlated ($r = 0.68$) with the degree of pancreatic fibrosis ($p<0.01$). When the same analysis was performed with the expression data of PLA_2-IV, no correlation ($r = 0.14$) with the degree of fibrosis was found ($p = 0.28$).

CORRELATION OF MOLECULAR AND IMMUNOHISTOCHEMICAL ANALYSIS WITH SURVIVAL AFTER SURGERY

To evaluate whether the presence of PLA_2-II and PLA_2-IV in pancreatic cancer cells influences their growth behaviour, we analysed clinical and histopathological variables and the survival period after surgery in pancreatic cancer patients.

For PLA_2-II, this analysis was performed using the mRNA expression and immunohistochemical results. For PLA_2-IV, mRNA expression levels were correlated with the patient data. Spearman analysis disclosed no correlation between PLA_2-II mRNA levels and any variable of the tumour, node, metastasis (TNM) classification, the tumour stage, or the histological tumour grading. However, Cox analysis of the Kaplan-Meier survival curves indicated that patients whose tumours exhibited higher PLA_2-II mRNA levels (moderate, strong) lived significantly longer after surgery ($p<0.02$) than those whose tumours exhibited weak PLA_2-II mRNA levels or in whom no signals were detectable (fig 4A). The same analysis for PLA_2-II immunoreactivity was performed using the immunohistochemical staining score described in the Materials and methods section. The median immunoreactivity score for PLA_2-II was 7 (range 0–20) in normal and 101 (range 0–300) in cancer samples. The relation between the immunoreactivity score and the patient's postoperative survival period (fig 4B) was also analysed using the Cox regression test. With a cut off value of

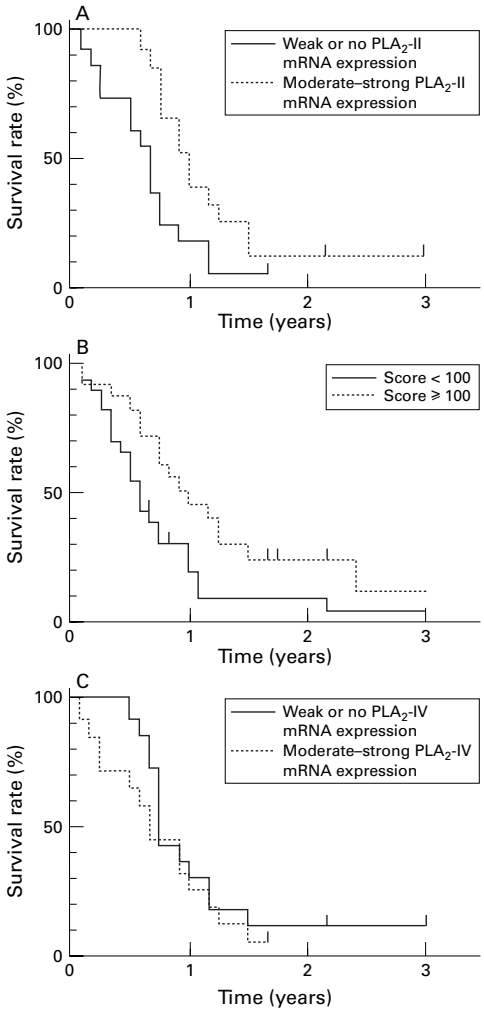

Figure 4 Survival curves. (A) Kaplan-Meier plots of the postoperative survival periods in patients whose tumours were defined as having weak or no phospholipase A_2 (PLA_2)-II mRNA expression versus patients with moderate to strong PLA_2-II mRNA expression (broken line). Cox analysis of the postoperative survival periods showed that patients whose tumours exhibited weak or no PLA_2-II mRNA expression lived a significantly shorter time ($p<0.02$) than those whose tumours exhibited moderate to strong PLA_2-II mRNA levels. (B) Kaplan-Meier plots of the postoperative survival periods in patients whose tumours were defined as having a PLA_2-II immunoreactivity score <100 versus patients with a PLA_2-II immunoreactivity score ⩾100. Cox analysis of the postoperative survival periods showed that patients whose tumours exhibited a low PLA_2-II immunoreactivity score lived a significantly shorter time ($p<0.05$) than those with a higher immunoreactivity score. (C) Kaplan-Meier plots of the postoperative survival periods in patients whose tumours were defined as having weak or no PLA_2-IV mRNA expression versus patients with moderate to strong PLA_2-IV mRNA expression. Cox analysis of the postoperative survival periods indicated no difference in survival between these two patient groups.

100 (fig 4B), 150 or 200 patients whose tumours had higher values lived significantly longer (cut off value 100: $p<0.05$; cut off value 150: $p<0.01$; cut off value 200: $p<0.05$) than patients whose tumours had an immunoreactivity score below these values.

For PLA_2-IV the clinical data could only be correlated with the mRNA expression levels, because no antibodies were available for immunohistochemical analysis. However, in contrast with PLA_2-II mRNA expression, there was no correlation between PLA_2-IV mRNA expression levels in the cancer samples and any variable of the TNM classification, the tumour stage, the histological tumour grading, or the postoperative survival period (fig 4C).

Multivariate analysis using Cox regression and including PLA_2-II, PLA_2-II mRNA expression, PLA_2-IV mRNA expression, gender, age, any variable of the TNM classification, the tumour stage, or the histological tumour grading showed that PLA_2-II mRNA ($p = 0.04$) and PLA_2-IV mRNA ($p = 0.02$) expression are strong independent prognostic indicators of survival in these patients.

In addition to PLA_2-II and PLA_2-IV, the relation between the degree of fibrosis of the cancer samples and the prognosis of the patients was evaluated. However, patients whose tumours had a higher degree of fibrosis showed no difference in survival period compared with those whose tumours had a lower degree of fibrosis.

Discussion

In this study, we determined by comparison with normal pancreatic tissues that human pancreatic cancer tissues show enhanced expression of PLA_2-II and PLA_2-IV mRNA. Increased PLA_2-II mRNA expression levels determined by northern blot analysis were associated with intense PLA_2-II immunoreactivity in the cancer cells. In the case of PLA_2-IV, specific mRNA expression could be located in the pancreatic cancer cells by in situ hybridisation. In contrast, PLA_2-I was abundant in acinar cells of the normal pancreas but absent from the pancreatic cancer cells. However, in the remaining acinar cells surrounding the cancer mass or located between infiltrating cancer cells, PLA_2-I immunostaining was still present. Inasmuch as cultured human pancreatic cancer cells are stimulated to grow by PLA_2-I, infiltrating pancreatic cancer cells may obtain a growth advantage by growing into normal pancreas and by the destruction of acinar cells.[35]

The exact function of PLA_2-II and PLA_2-IV in pancreatic cancer cells is not obvious. PLA_2-II is normally present in a variety of inflammatory cells,[1 12] and in Paneth cells of the intestine,[36] and can be released into the extracellular space and blood in response to inflammatory stimuli such as interleukins 1 and 6 and tumour necrosis factor.[11 12] Elevated levels of PLA_2-II have been detected not only in plasma samples of patients with systemic inflammation,[12 13], but also in those with various malignant tumours,[17–20 36] suggesting that PLA_2-II may play a role in tumour pathogenesis. This hypothesis is supported by several reports indicating that PLA_2-II generated mediators such as prostaglandin E2 stimulate cancer cell growth in vitro.[4 22] Recent studies on gastric cancer and colonic cancer have reported enhanced PLA_2-II immunoreactivity in the cancer cells.[17 20] Interestingly, in both tumour types, intense PLA_2-II immunoreactivity was preferentially located in cancer cells of the invading zones, in which cancer cells were infiltrating non-cancerous tissue. Furthermore, in gastric cancer, high PLA_2-II levels were more

frequently present in poorly differentiated cancer cells.[20] Based on these findings it was concluded that PLA_2-II may be involved in tumour invasion, tumour progression, and tumour dedifferentiation. This hypothesis is also supported by a survival analysis in breast cancer patients showing that women with PLA_2-II positive tumours lived a significantly shorter time than women whose tumours exhibited low PLA_2-II levels.[19] In our study in which 58 pancreatic cancer samples were analysed, we found a different distribution and biological pattern of PLA_2-II from that in gastric or breast cancers.[19 20] On immunohistochemical analysis, PLA_2-II was seen to be homogeneously distributed in the pancreatic cancer cell areas, without any preferential accumulation in cancer cells in the invading areas. Therefore, in pancreatic cancer, PLA_2-II seems not to have a specific function in tumour cell invasion of the adjacent normal tissues. Furthermore, in contrast with breast cancer, enhanced PLA_2-II immunoreactivity was associated with a better prognosis for the patients. These findings suggest that, in pancreatic cancer, PLA_2-II indirectly possesses inhibitory functions rather than potentiating effects on the proliferation and progression of the cancer cells. Interestingly, degenerating pancreatic parenchyma adjacent to the cancer mass and chronic pancreatitis-like lesions exhibited much greater PLA_2-II immunostaining than that observed in the normal control pancreas. These findings are in agreement with our previous findings that chronic pancreatitis is associated with increased expression of PLA_2-II and PLA_2-IV.[37] The presence of PLA_2-II and PLA_2-IV in chronic pancreatitis tissues was correlated with a higher degree of fibrosis, indicating a potential influence of PLA_2 on tissue remodelling and extracellular matrix synthesis.[37] Therefore PLA_2-II may exert an impeding effect on the dissemination of the pancreatic cancer cells by stimulating extracellular matrix production.

A typical morphological characteristic of pancreatic cancer is the increase in extracellular matrix (desmoplastic reaction) which is normally present between the clusters of cancer cells but is also present at the border of the non-cancer areas close to the cancer mass.[38 39] Previous immunohistochemical analysis disclosed a relation between PLA_2-II and the amount of interstitial tissue in pancreatic cancer tissues.[40] It may be possible that the increase in PLA_2-II in the cancer cells and in the areas with remaining pancreatic parenchyma adjacent to the tumour mass contributes to an inflammatory reaction that results in parenchyma destruction and fibrotic replacement of these areas. This hypothesis is in agreement with the previous work of Kurisaki *et al*,[41] who reported that PLA_2-II has direct mitogenic effects on fibroblasts independent of the synthesis of arachidonate products. In addition, PLA_2-II induces prostaglandin synthesis, which subsequently stimulates fibroblasts and induces fibroblast proliferation, thereby also enhancing extracellular matrix synthesis resulting in fibrosis.[41–43] In accordance with a previous report that PLA_2 activation results in induction of an acute inflammatory response, associated with concomitant regression of malignant gliomas,[44] it is possible that the formation of fibrosis in pancreatic cancer—especially in tumour-surrounding areas—negatively influences the growth behaviour and invasion of pancreatic cancer cells. The fibrosis around pancreatic cancer may function as a tissue barrier to aggravate tumour cell invasion. Our data on chronic pancreatitis and pancreatic cancer suggest that PLA_2-II and PLA_2-IV have a similar function in the two pancreatic disorders. They seem to contribute to a chronic inflammatory reaction in the pancreas which results in the destruction of pancreatic acinar and ductal cells and the increase in extracellular matrix. An interesting point is that PLA_2 is present in the cancer cells in pancreatic cancer tissues and in atrophying acinar and ductal cells in both pancreatic cancer and chronic pancreatitis, indicating that at least some of the inflammatory mediators are derived in both diseases directly from the pancreatic parenchyma itself.

The role of PLA_2-IV in cancer pathogenesis is far from clear. PLA_2-IV has been identified in various inflammatory cells and in newer studies also in the kidney, colon, and pancreas.[7 8 29 37 45] On northern blot analysis we found an appreciable increase in PLA_2-IV mRNA levels in pancreatic cancer. In situ hybridisation showed that, in pancreatic cancer, the PLA_2-IV mRNA signals were considerably higher than in the normal pancreas. Therefore PLA_2-IV may play a role in phospholipid metabolism in pancreatic cancer cells. PLA_2-IV has not been studied in human cancers except to some extent in colon cancers,[45] increased PLA_2-IV levels being found in six of 17 cancer samples. It is likely that PLA_2-IV, like PLA_2-II, enhances the synthesis of prostaglandins and leukotrienes, which both stimulate fibroblast proliferation.[42] However, in our study in which 58 pancreatic cancer samples were studied, we found no significant correlation between PLA_2-IV mRNA expression and the degree of fibrosis. It is possible that PLA_2-IV somehow functions synergistically with PLA_2-II.

PLA_2-IV is transcriptionally upregulated by various cytokines, epidermal growth factor, and platelet derived growth factor.[7] Inasmuch as the latter two are increased in many pancreatic cancers, it is possible that their presence in pancreatic cancer cells contributes to the upregulation of PLA_2-IV mRNA in these cells by autocrine, paracrine, and intracrine pathways.[23 24 46–48] Another pathway of PLA_2-IV upregulation may result through *ras* mutations which are often present in pancreatic cancer.[49] Ras induces activation of the PLA_2-IV promoter thereby enhancing PLA_2-IV mRNA expression.[50]

In summary, we found overexpression of both PLA_2-II and PLA_2-IV in pancreatic cancer in comparison with the normal pancreas. Although the exact functions of PLA_2 isoforms are not fully understood, we assume that the presence of PLA_2-II in pancreatic cancer cells does not contribute to tumour cell

growth but rather to an immune cell independent inflammatory reaction which results in the increase in extracellular matrix, thereby preventing tumour cell dissemination.

This study was supported by the Swiss National Fund grant (SNF 32-39529 and SNF 32-049494).

1 Mayer RJ, Marshall LA. New insights on mammalian phospholipase A_2(s): comparison of arachidonoyl-selective and -nonselective enzymes. *FASEB J* 1993;7:339–48.
2 Dennis EA. The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends Biochem Sci* 1997;**22**: 1–2.
3 Wickremasinghe RG. The role of prostaglandins in the regulation of cell proliferation. *Prostaglandins Leukot Essent Fatty Acids* 1988;**31**:171–9.
4 Fulton AM. The role of eicosanoids in tumor metastasis. *Prostaglandins Leukot Essent Fatty Acids* 1988;**34**:229–37.
5 Marnett LJ. Aspirin and the potential role of prostaglandins. *Cancer Res* 1992;**52**:5575–89.
6 Dennis EA. Diversity of group types, regulation, and function of phospholipase A_2. *J Biol Chem* 1994;**269**: 13057–60.
7 Kramer RM, Sharp JD. Recent insights into structure, function and biology of $cPLA_2$. *Agents Actions* 1995;**46**(suppl): 65–76.
8 Seilhamer JJ, Randall TL, Yamanaka M, *et al*. Pancreatic phospholipase A_2: isolation of the human gene and cDNAs from porcine pancreas and human lung. *DNA* 1986;**5**:519–27.
9 Kanda A, Ono T, Yoshida N, *et al*. The primary structure of a membrane-associated phospholipase A_2 from human spleen. *Biochem Biophys Res Commun* 1989;**163**:42–8.
10 Nomura K, Fujita H, Arita H. Gene expression of pancreatic-type phospholipase A_2 in rat ovaries: stimulatory action on progesterone release. *Endocrinology* 1994;**135**: 603–9.
11 Kudo I, Murakami M, Hara S, *et al*. Mammalian non-pancreatic phospholipase A_2. *Biochim Biophys Acta* 1993;**117**:217–31.
12 Vadas P, Browning J, Edelson J, *et al*. Extracellular phospholipase A_2 expression and inflammation: the relationship with associated disease states. *J Lipid Mediat* 1993;**8**:1–30.
13 Büchler M, Malfertheiner P, Schädlich H, *et al*. Role of phospholipase A_2 in human acute pancreatitis. *Gastroenterology* 1989;**97**:1521–6.
14 Uhl W, Beger HG, Hoffmann G, *et al*. A multicenter study of phospholipase A_2 in patients in intensive care units. *J Am Coll Surg* 1995;**180**:323–31.
15 Uhl W, Schrag HJ, Schmitter N, *et al*. Pathophysiological role of secretory type I and II phospholipase A_2 in acute pancreatitis: an experimental study in rats. *Gut* 1997;**40**: 386–92.
16 Uhl W, Schrag HJ, Schmitter N, *et al*. Experimental study of a novel phospholipase A_2 inhibitor in acute pancreatitis. *Br J Surg* 1998;**85**:618–23.
17 Hendrickse CW, Radley S, Donovan IA, *et al*. Activities of phospholipase A_2 and diacylglycerol lipase are increased in human colorectal cancer. *Br J Surg* 1995;**82**:475–8.
18 Ying Z, Tojo H, Komatsubara T, *et al*. Enhanced expression of group II phospholipase A_2 in human hepatocellular carcinoma. *Biochim Biophys Acta* 1994;1226:201–5.
19 Yamashita SI, Yamashita JI, Ogawa M. Overexpression of group II phospholipase A_2 in human breast cancer tissues is closely associated with their malignant potency. *Br J Cancer* 1994;**69**:1166–70.
20 Murata K, Egami H, Kiyohara H, *et al*. Expression of group II phospholipase A_2 in malignant and non-malignant human gastric mucosa. *Br J Cancer* 1993;**68**:103–11.
21 Clark JD, Lin L-L, Kriz RW, *et al*. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* 1991; **65**:1043–51.
22 Steiner MR, Clark MA, Bomalaski JS. Inhibitors of phospholipase A_2 and eicosanoid biosynthesis in cancer. *Drugs News and Perspectives* 1994;**7**:344–51.
23 Friess H, Büchler MW, Korc M. Growth factors and growth factor receptors in pancreatic cancer. In: Neoptolemos JP, Lemoine NR, eds. *Pancreatic cancer.* Oxford: Blackwell Science, 1996:51–60.
24 Friess H, Berberat P, Schilling M, *et al*. Pancreatic cancer: the potential clinical relevance of alteration in growth factors and their receptors. *J Mol Med* 1996;**74**:34–42.
25 Hermanek P, Sobin LH. *TNM classification of malignant tumors*, 4th ed. New York: Springer-Verlag, 1992:71–3.
26 Ishizaki J, Ohara O, Nakamura E, *et al*. cDNA cloning and sequence determination of rat membrane-associated phospholipase A_2. *Biochem Biophys Res Commun* 1989;**162**: 1030–6.
27 Ohara O, Ishizaki J, Nakano T, *et al*. A simple and sensitive method for determining transcription initiation site: identification of two transcription initiation sites in group II phospholipase A_2 gene. *Nucleic Acids Res* 1990;**18**:6997–7002.
28 Guo XZ, Friess H, Graber HU, *et al*. KAI1 expression is up-regulated in early pancreatic cancer and decreased in the presence of metastases. *Cancer Res* 1996;**56**:4876–80.
29 Rodewald E, Tibes U, Maass G, *et al*. Induction of cytosolic phospholipase A_2 in human leukocytes by lipopolysaccharide. *Eur J Biochem* 1994;**223**:743–9.
30 Friess H, Cantero D, Graber H, *et al*. Enhanced urokinase plasminogen activation in chronic pancreatitis suggests a role in its pathogenesis. *Gastroenterology* 1997;**113**:904–13.
31 Santavuori SA, Kortesuo PT, Eskola JU, *et al*. Application of a new monoclonal antibody for time-resolved fluoroimmunoassay of human pancreatic phospholipase A_2. *Eur J Clin Chem Clin Biochem* 1992;**29**:819–26.
32 Nevalainen TJ, Kortesuo PT, Rintala E, *et al*. Immunohistochemical detection of group I and group II phospholipase A_2 in human serum. *Clin Chem* 1992;**38**:1824–9.
33 Bacus S, Flowers JL, Press JW, *et al*. The evaluation of estrogen receptor in primary breast carcinoma by computer-assisted image analysis. *Am J Clin Pathol* 1988;**90**:233–9.
34 Friess H, Malfertheiner P, Isenmann R, *et al*. The risk of the pancreatico-intestinal anastomosis can be predicted preoperatively. *Pancreas* 1996;**13**:202–8.
35 Hanada K, Kinoshita E, Itoh M, *et al*. Human pancreatic phospholipase A_2 stimulates the growth of human pancreatic cancer cell line. *FEBS Lett* 1995;**373**:85–7.
36 Nevalainen TJ, Gronroos JM, Kallajoki M. Expression of group II phospholipase A_2 in the human gastrointestinal tract. *Lab Invest* 1995;**72**:201–8.
37 Kashiwagi M, Friess H, Uhl W, *et al*. Phospholipase A_2 isoforms are altered in chronic pancreatitis. *Ann Surg* 1998;**227**:220–8.
38 Yamanaka Y, Friess H, Büchler MW, *et al*. Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. *Cancer Res* 1993;**53**:5289–96.
39 Gress TM, Müller-Pillasch F, Lerch MM, *et al*. Expression and in situ localization of genes coding for extracellular matrix proteins and extracellular matrix proteases in pancreatic cancer. *Int J Cancer* 1995;**62**:407–13.
40 Kiyohara H, Egami H, Kako H, *et al*. Immunohistochemical localization of group II phospholipase A_2 in human pancreatic carcinomas. *Int J Pancreatol* 1993;**13**:49–57.
41 Kurizaki T, Egami H, Murata K, *et al*. Membrane-associated phospholipase A_2 stimulates DNA synthesis in two murine fibroblasts. *Res Commun Chem Pathol Pharmacol* 1992;**78**:39–45.
42 Nolan RD, Danilowicz RM, Eling TE. Role of arachidonic acid metabolism in the mitogenic response of BALB/c 3T3 fibloblasts to epidermal growth factor. *Mol Pharmacol* 1988;**33**:650–6.
43 Hara S, Kudo I, Inoue K. Augmentation of prostaglandin E2 production by mammalian phospholipase A_2 added exogenously. *J Biochem (Tokyo)* 1991;**110**:163–5.
44 Goddard DH, Bomalaski JS, Lipper S, *et al*. Phospholipase A_2-mediated inflammation induces regression of malignant gliomas. *Cancer Lett* 1996;**102**:1–6.
45 Soydan AS, Tavares IA, Weech PK, *et al*. High molecular weight phospholipase A_2 and fatty acids in human colon tumors and associated normal tissue. *Eur J Cancer* 1996;**32A**:1781–7.
46 Korc M, Chandrasekar B, Yamanaka Y, *et al*. Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor alpha. *J Clin Invest* 1992;**90**:1352–60.
47 Yamanaka Y, Friess H, Büchler MW, *et al*. Coexpression of epidermal growth factor receptor and ligands is associated with enhanced aggressiveness of human pancreatic cancer. *Anticancer Res* 1993;**13**:565–70.
48 Ebert M, Yokoyama M, Friess H, *et al*. Induction of platelet-derived growth factor A and B chains and overexpression of their receptors in human pancreatic cancer. *Int J Cancer* 1995;**62**:529–35.
49 Grünewald K, Lyons J, Fröhlich A, *et al*. High frequency of Ki-ras codon 12 mutations in pancreatic adenocarcinomas. *Int J Cancer* 1989;**43**:1037–41.
50 Heasley LE, Thaler S, Nicks M, *et al*. Induction of cytosolic phospholipase A_2 by oncogenic Ras in human non-small cell lung cancer. *J Biol Chem* 1997;**272**:14501–4.