

Proteome-based biomarkers in pancreatic cancer

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Abstract

Pancreatic cancer, as a highly malignant cancer and the fourth cause of cancer-related death in world, is characterized by dismal prognosis, due to rapid disease progression, highly invasive tumour phenotype, and resistance to chemotherapy. Despite significant advances in treatment of the disease during the past decade, the survival rate is little improved. A contributory factor to the poor outcome is the lack of appropriate sensitive and specific biomarkers for early diagnosis. Furthermore, biomarkers for targeting, directing and assessing therapeutic intervention, as well as for detection of residual or recurrent cancer are also needed. Thus, the identification of adequate biomarkers in pancreatic cancer is of extreme importance. Recently, accompanying the development of proteomic technology and devices, more and more potential biomarkers have appeared and are being reported. In this review, we provide an overview of the role of proteome-based biomarkers in pancreatic cancer, including tissue, serum, juice, urine and cell lines. We also discuss the possible mechanism and prospects in the future. That information hopefully might be helpful for further research in the field.

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INTRODUCTION

Pancreatic cancer is a highly lethal disease and despite continuous research efforts, results have only marginally improved patient outcome with minor overall changes in death rate over the last four decades. Pancreatic cancer is the fourth cause of cancer-related death and 36 800 pancreatic cancer-related deaths were reported in the United States in 2010, corresponding to 6.5% of all deaths from cancer^[1]. Similar overall observations are reported from the other parts of the Western world^[2-5].

Surgical resectable pancreatic cancer is associated with an improved outcome, especially if the diagnosis is obtained in an early phase. Regrettably, most symptoms, including e.g., profound weight loss, abdominal pain, new onset type 2 diabetes mellitus, jaundice and nausea, are usually vague and occur late during the course of disease. Only 20% of patients with pancreatic cancer are candidates for a potentially curative resection^[6]. Efficient tumor markers for population screening are absent. Current markers used for pancreatic cancer, especially carcinoembryonic antigen and cancer antigen 19-9 (CA19-9), lack appropriate sensitivity and specificity. Biomarkers for therapeutic assessment, detection of residual or recurrent cancer and even for targeted therapy in pancreatic cancer in a more customized fashion are needed. The identification of biomarkers in pancreatic cancer is thus essential for improving outcome.

The development of proteomic techniques has increased the interest for clinical applications of biomarkers in pancreatic cancer. However, the identification of suitable biomarkers with good sensitivity and specificity for clinical use in pancreatic cancer has been sparse. In this review, we focus on potential proteome-based biomarkers to be used in pancreatic cancer (Table 1), hopefully indicative for further research within the field.

PROTEOMIC-BASED BIOMARKERS IN PANCREATIC CANCER TISSUE

Pancreatic cancer tissue is the most direct source of proteomic biomarkers for cancer detection, as it is likely to have the highest concentrations of cancer-specific markers. However, there are two major reasons that make it less available for cancer screening. Invasive biopsy material for screening is usually not readily available, and percutaneous biopsies might even result in seeding of cancer cells. Pathological evaluation renders the final diagnosis and is the best choice when pancreatic cancer tissue is available. Ongoing biomarker research obtained from pancreatic cancer tissue is not only done for diagnostic purposes, but also for the development of potential future targeted therapies.

When comparing pancreatic cancer tissue with normal pancreatic tissue by MALDI-TOF-MS, the levels of galectin-3 and calgizzarin (S100A11) protein were found to be 3-fold higher in cancer patients^[7]. Galectin-3 is a member of a family of β -galactoside-binding animal lectins, and has been found helpful in diagnosing e.g., thyroid cancer^[7], but is also up-regulated in liver, stomach, and tongue cancers. In the same family, galectin-1 has also been identified as a potential biomarker^[8,9]. S100A11, a calcium-binding protein and a member of the S100 protein family is expressed in the nucleus and cytoplasm. S100 proteins regulate a number of cellular processes like cell growth, cell cycle, differentiation, transcription and secretion. S100 proteins have been reported over-expressed in different cancers, like breast and thyroid cancer^[10]. Other studies have shown up-regulation of S100A6, S100A8, S100A9 and S100A10 in pancreatic cancer^[11-13]. Using laser capture microdissection and 2-DE to analyze protein expression in stromal components of pancreatic cancer, it was demonstrated that high levels of S100A8 and S100A9 were present in the tumor-associated stroma but not in benign or malignant epithelium^[12]. Immunohistochemistry confirmed high levels of both S100A8 and S100A9 in specific stromal cells, which were later identified as monocytes or immature macrophages (CD14⁺/CD68). In a subset of these cells, S100A8 and S100A9 were co-expressed, and this relationship appeared to be influenced by the Smad4 status of the corresponding tumor cells. This study provides further evidence of the complex tumor-stroma interaction and demonstrates that stromal tissue can become a novel and highly promising source of biomarkers.

The differential diagnosis between pancreatic cancer and mass-forming chronic pancreatitis is clinically challenging. A large-scale immunoblotting analysis with more than 900 primary antibodies was performed on cancer tissue, chronic pancreatitis and normal pancreas^[14]. A total of 30 proteins were found to be differentially expressed between chronic pancreatitis and normal pancreas, while 102 proteins were different between pancreatic cancer and normal pancreas. Several proteins, such as UHRF1, ATP7A and AOX1, differed in their expression between chronic pancreatitis and pancreatic cancer, suggesting their importance in pancreatic carcinogenesis. The combination of these proteins can become a useful diagnostic tool for endoscopic ultrasonography-guided fine needle aspiration specimens obtained before surgery or treatment.

Pancreatic cancer (PDAC) develops through several phases of pancreatic intraepithelial neoplasia (PanINs) lesions from benign to fully malignant. Pancreatic cancer pathology may be helpful for diagnosis and treatment by providing knowledge on which phases the patient is in. Despite research showing different genetic alternations during different phases, such as *K-ras* in the early PanIN-1A/B, *p16* in the intermediate PanIN-2 and *p53* in the late PanIN-3 phases^[15], biological mechanisms still remain largely unclear. One reason is the difficulty in studying early molecular changes in pancreatic cancer, due to lack of suitable tissue specimens, as patients in the early phase often are without existing symptoms, and thus tissue samples are not available. Plectin-1 has been shown to be up-regulated in PanINs and in PDAC in genetic defect mouse models and early-stage pancreatic cancer cell lines^[16]. Plectin-1 is a large 500 kDa protein associated with filamentous-actin, microtubules and intermediate filaments. Plectin-1 was found to be exclusively associated with mitochondria and may thus provide an important link of this organelle with the intermediate filament system^[17]. Plectin-1 can also bind specific peptides, which may be helpful in detecting precursor lesions and PDAC, when conjugated to magnetofluorescent nanoparticles.

Proteome changes of pancreatic cancer tissue during different stages have been identified by 2-DE. Five candidate protein biomarkers were selected from a total of 31 identified nonredundant proteins, including 14-3-3 sigma, major vault protein (MVP), anterior gradient 2 (AGR2) and Annexin A4^[13]. AGR2 is increased early on during tumor progression, and is also present in pancreatic juice^[18]. MVP expression, associated with the PI3K pathway, and 14-3-3 sigma were found to be increased in PanIN-2 and -3^[19,20]. On the other hand, annexin A4 was down-regulated. Annexin A4 is a Ca²⁺- and phospholipid-binding protein like annexin A2, which previously has been reported in PDAC^[21].

To decrease the complexity and large dynamic range of proteins found in pancreatic tissue samples, subcellular fractionation with mass spectrometric techniques has been used to identify potential biomarkers associated with pancreatic cancer. McKinney *et al.*^[22] identified 2393

Table 1 A selection of potential biomarkers for pancreatic cancer

Potential biomarkers for pancreatic cancer	Expression	Proteomic tools	Ref.
Tissue			
14-3-3 sigma	+	2-D SDS PAGE, MS	[13]
Annexin A4	+	2-D SDS PAGE, MS	[13]
Anterior gradient 2	+	2-D SDS PAGE, MS	[13]
AOX1	-	PowerBlot	[14]
ATP7A	+	PowerBlot	[14]
Biglycan	+	SDS-PAGE, LC-MS/MS	[22]
Galectin-1	+	2-D SDS PAGE, MS	[9]
Galectin-3	+	MALDI-TOF-MS	[7]
Gelsolin	-	Proteomic chip	[23]
Major vault protein	+	2-D SDS PAGE, MS	[13]
Pigment epithelium-derived factor	+	SDS-PAGE, LC-MS/MS	[22]
Plectin-1	+	Western blotting	[16]
S100A6	+	2-D SDS PAGE, MS	[11]
S100A8 (stroma)	+	2DE, LC-MS/MS	[12]
S100A9 (stroma)	+	2DE, MALDI-TOF-MS	[12]
S100A10	+	2-D SDS PAGE, MS	[13]
S100A11	+	MALDI-TOF-MS	[7]
Thrombospondin-2	+	SDS-PAGE, LC-MS/MS	[22]
β IGH3	+	SDS-PAGE, LC-MS/MS	[22]
UHRF1	+	PowerBlot	[14]
Serum/plasma			
α -1B-glycoprotein precursor	+	DIGE, MS/MS	[38]
Anterior gradient 2	+	iTRAQ, MS/MS	[18]
Apolipoprotein A-II	-	SELDI-TOF, MS	[24]
Apolipoprotein C- I	+	SELDI-TOF, MS	[24]
Caldecrin	-	ICAT, MS	[43]
CXCL 7	-	LC-MS	[29]
DJ-1	+	DIGE, MS/MS	[38]
Fibrinogen β chain	+	ICAT, MS	[43]
HSP27	+	Protein-chip technology	[33]
Pancreatic juice			
Lithostathine I α	-	2DE, MALDI-TOF-MS	[40]
Matrix metalloproteinase-9	+	DIGE, MS/MS	[38]
Neural cell adhesion molecule L1	+	ICAT, MS	[43]
p-Akt	+	Bio-Plex suspension array	[32]
p-ERK1/2	+	Bio-Plex suspension array	[32]
Phosphor-cAMP response element binding protein	+	Bio-Plex suspension array	[32]
Phosphor-p90 ribosomal S6 kinase	+	Bio-Plex suspension array	[32]
p-I κ B- α	+	Bio-Plex suspension array	[32]
Plasminogen	+	ICAT, MS	[43]
Platelet factor 4	-	MALDI-TOF	[31]
p-MEK1	+	Bio-Plex suspension array	[32]
Transthyretin	+	2DE, MALDI-TOF-MS	[45]
Urine			
Annexin A2	-	2-D SDS PAGE	[46]
CD59	-	2-D SDS PAGE	[46]
Gelsolin	-	2-D SDS PAGE	[46]
Cell lines			
Apoprotein E	+	SILAC	[48]
Cadherin	Not in metastatic tumor cell	LC-MS/MS	[52]
Catenin	Not in metastatic tumor cell	LC-MS/MS	[52]
CD9	+	SILAC	[48]
Fibronectin receptor	+	SILAC	[48]
Galectin	Not in primary tumor cell	LC-MS/MS	[52]
Glucagon	+	Protein array	[53]
Integrin	Not in metastatic tumor cell	LC-MS/MS	[52]
Perlecan	+	LC-MS/MS	[48]
Prolactin	-	Protein array	[53]

SILAC: Stable isotope labelling with amino acids in cell culture; DIGE: Difference gel electrophoresis; MS: Mass spectrometry; iTRAQ: Isobaric tags for relative and absolute quantification; ICAT: Isotope-coded affinity tag; β IGH3: Ig-h3 precursor; A1BG: α -1B-glycoprotein precursor; +: Up-regulated; -: Down-regulated expression in pancreatic cancer as compared with controls.

unique proteins in normal and pancreatic tissue with cancer, and determined 104 proteins that were significantly

changed in pancreatic cancer. Four secreted and up-regulated proteins have been validated as potential biomark-

ers for diagnosing pancreatic cancer, biglycan, pigment epithelium-derived factor, thrombospondin-2 and transforming growth factor β induced protein Ig-h3 precursor, though data for sensitivity and specificity for these markers are not yet available.

Information on lymph node metastasis is very important for the surgical strategy-making and also for deciding other additional treatments (e.g., chemotherapy). Proteome comparison of pancreatic cancer tissue with corresponding non-cancerous normal tissue obtained from the same patients on antibody capture-based proteomic chips^[23] identified gelsolin as a candidate biomarker for detection of lymph node metastasis in pancreatic cancer. Gelsolin is an important actin-binding protein that plays a major role in maintaining an organized actin cytoskeleton. The expression of gelsolin in pancreatic ductal adenocarcinomas with lymph node involvement (71.4%) was reported markedly increased as compared to lymph node negative pancreatic cancers (20%)^[23].

BIOMARKERS IN BODY FLUIDS

Serum and plasma

Blood is the most frequently used source for biomarkers, being minimally invasive, easily accessible, generally inexpensive and reproducible to obtain and analyse. However, some highly abundant proteins, such as albumin or globulin, can affect the detection of less abundant, but for the diagnosis, valuable proteins.

One study aimed to identify biomarkers in a total of 319 serum samples from pancreatic cancer patients and controls. Using SELDI-TOF MS technology, 21 peaks were identified to be differentially expressed between pancreatic cancer and disease controls (DC), and 18 peaks between pancreatic cancer and healthy volunteers (HV)^[24]. Apolipoprotein C-I (ApoC-I) and apolipoprotein A-II (ApoA-II) were significantly increased and decreased, respectively. ApoC-I plays an important role in controlling plasma lipid metabolism, and is expressed in gastric, breast and pancreatic cancer^[25]. ApoA-II is present on the surface of lipid particles and may play a diagnostic role in prostate cancer^[26]. The receiver operating characteristic area under the curve (AUC) of ApoA-II, ApoC-I and CA19-9 was greater than that of CA19-9 alone for pancreatic cancer *vs* DC (0.90 *vs* 0.84) and for pancreatic cancer *vs* HV (0.96 *vs* 0.90), results supported by others^[27].

CXCL-7 is a chemokine member of the angiogenic ELRb CXC chemokine family, expressed within the megakaryocyte lineage^[28]. Using a novel combination of hollow fiber membrane-based low-molecular-weight protein enrichment and LC-MS-based quantitative shotgun proteomics identified a peptide derived from CXCL-7 to be significantly reduced in pancreatic cancer patients^[29]. These authors compared the plasma proteome in a small cohort (24 patients with pancreatic cancer and 21 healthy controls) to get 53 009 MS peaks. They then further validated their CXCL-7 finding in an independent blinded

cohort ($n = 237$) using a high-density reverse-phase protein microarray. Combination with CXCL-7 significantly improved the AUC value of CA19-9 to 0.961. However, in this study, the precise molecular mechanisms explaining the CXCL-7 reduction in patients with pancreatic cancer remained unclear. Platelet factor 4 (PF4) is another member of the CXC chemokine family (CXCL-4), and is present in α -granules of all mammalian platelets, as well as in the granules of mast cells^[30]. PF4 had been identified as a potential marker for pancreatic cancer by MALDI-TOF-MS-based clinical serum profiling in 80 samples^[31]. Validation by ELISA techniques in 40 serum samples showed the AUCs of PF4 concentrations used for the discrimination between healthy controls and pancreatic cancer was 0.833. The discrimination between patients with pancreatic cancer and acute pancreatitis was 0.829.

Protein phosphorylation is one of the most common ways of modifying biological systems, including the carcinogenic progress. Several phosphoprotein levels were significantly increased in serum from pancreatic cancer patients as compared to controls. Six candidate phosphoproteins have been found in serum of pancreatic cancer patients by using a Bio-Plex suspension array; p-ERK1/2, p-MEK1, phospho-p90 ribosomal S6 kinase (p-p90RSK), phospho-cAMP response element binding protein (p-CREB), p-Akt and p-I κ B- α ^[32]. These phosphoproteins are associated with the Ras/Raf/MEK/ERK signalling pathway, which is a dominating growth promoting pathway in pancreatic carcinomas. Further data from the same study showed a simultaneous increase in phospho- and total-ERK1/2 with a positive correlation to pancreatic cancer patients. In detecting pancreatic cancer, a combination of p-ERK1/2 and CA19-9 can potentially avoid false-negatives (87.2%) and improve the discriminatory power.

Heat shock protein 27 (HSP27) is a powerful molecular chaperone that can prevent the aggregation of nascent and stress-induced misfolded proteins^[33]. HSP27 has been identified in serum of pancreatic cancer patients by Protein-Chip technology and 2-DE. HSP27 was found to be up-regulated in pancreatic cancer as compared with normal tissue, with a sensitivity of 100% and a specificity of 84% in the detection of pancreatic cancer, and has further been suggested to play an important role in gemcitabine resistance^[34,35].

Pancreatic juice

Pancreatic juice is rich in proteins directly secreted from pancreatic ductal cancer cells and should therefore constitute a perfect source for specific protein biomarkers for pancreatic cancer detection. However, pancreatic juice is not readily accessible and in addition, the endoscopic retrograde cholangiopancreatography procedure *per se*, in order to obtain pancreatic juice, may induce acute pancreatitis in 4%-7% of patients^[36]. To date approximately 170 proteins have been identified in human pancreatic juice, one third of which are enzymes^[37].

When comparing pancreatic juice from 9 PDAC patients and 9 healthy volunteers by using difference gel electrophoresis and tandem mass spectrometry (MS/MS), three potential biomarkers were identified: matrix metalloproteinase-9 (MMP-9), oncogene DJ1 (DJ-1) and α -1B-glycoprotein precursor (A1BG)^[38]. DJ-1 is a mitogen-dependent protein involved in the Ras signalling pathway, reported to be increased in serum from pancreatic cancer patients^[33,39]. A1BG, a secreted plasma protein from the immunoglobulin superfamily, was also increased in the cytoplasm of malignant epithelia in 86.3% of pancreatic cancer tissue specimens.

Obstruction of the main pancreatic ducts may alter the protein composition of pancreatic juice. Comparing the 2-DE profiles of pancreatic juice from a patient with pancreatic body cancer and a patient with benign pancreatic disease, it was found that blockade of juice secretion strongly affected protein composition^[40]. A subsequent analysis of patients with comparable obstruction of the pancreatic ducts was performed. The isomeric form of lithostathine I α was identified as one of five protein spots that were consistently reduced in pancreatic cancer.

Quantitative proteomic analysis using stable isotope labelling (iTRAQ) and MS/MS were applied to identify proteins abnormalities, elevated in the pancreatic juice from PanIN-3 patients^[18]. Anterior gradient-2 (AGR2) was significantly increased in PanIN-3 juice samples among 20 differently expressed proteins. AGR2 is a secreted protein and over-expressed in many cancers, including pancreatic cancer and influences pancreatic cancer cell proliferation and invasion. Further analyses showed that AGR2 had 67% sensitivity and 90% specificity in predicting PanIN-3 in pancreatic juice samples^[41,42]. Proteomics can also be used to differentiate pancreatic cancer from pancreatitis. In a comparative study between pancreatic cancer and pancreatitis by Isotope-Coded Affinity Tag and MS, 72 variable proteins were identified in pancreatic juice^[43]. Some of the identified proteins, including fibrinogen β -chain, plasminogen, neural cell adhesion molecule L1 and caldecorin, demonstrated at least a 2-fold change in abundance in pancreatic juice. In addition, 9 proteins (hemoglobin, fibrinogen, trypsin I, trypsin II, chymotrypsinogen b, Ig- α 1 chain c region, Ig- μ chain c region, ribonuclease, and human serum albumin) were up-regulated both in the pancreatic juice of pancreatitis and pancreatic cancer patients.

Transthyretin (TTR) was identified as a potential protein biomarker in pancreatic juice for the detection of pancreatic cancer. Using 2-DE and MALDI-TOF-MS, it was shown that TTR in the pancreatic juice increased more than 2-fold in pancreatic cancer as compared with chronic pancreatitis and choledocholithiasis^[44]. However, TTR was only present in islet cells and not expressed in pancreatic cancer cells, in line with what has been reported by others^[45].

Urine

Urine is a potential source of biomarkers, as it is easily and

noninvasively available. However, a limitation of urine is the dilution of the proteins of potential interest. Secondly, the urine is derived from the kidneys, only being “in contact” with the pancreas through blood, and most of the proteomic information exists in circulating blood.

Using proteomic techniques multiple deregulated proteins were detected in urine samples from patients with pancreatic cancer, implicating urine to potentially be a valuable source of biomarkers for pancreatic cancer^[46]. Five potential protein biomarkers (including annexin A2, gelsolin, CD59 and S100A9) from a total of 127 statistically valid and differentially expressed protein spots were identified, most of which have been reported associated with pancreatic cancer in other studies.

BIOMARKERS IN CELL LINES

Cell lines are the most easily obtained proteomic source. This allows analysis of secreted proteins. The most relevant limitation when using data obtained from cell lines is that it may not be representative for primary tissue samples in the clinical setting. Thus, few studies have used cell lines for identifying biomarkers of relevance in pancreatic cancer^[47].

By analysis of secreted proteins between Panc-1 pancreatic cancer cells and immortalized non-neoplastic HPDE cells, 145 differentially secreted proteins were identified. Several proteins were validated by immunohistochemistry, such as CD9, perlecan, apoprotein E (ApoE) and fibronectin receptor^[48]. CD9 is a membrane protein expressed on the surface of human platelets. CD9 plays a role in many cellular functions, like adhesion, migration, signal transduction and differentiation^[49]. Perlecan is involved in angiogenesis and growth, as a receptor for basic fibroblast growth factor^[50]. ApoE is a protein component of lipoproteins that has anti-tumor activity in pancreatic cancer^[51]. Fibronectin receptor is another member of the integrin family.

Development of metastases, as part of the progress of pancreatic cancer, evidently involves a number of important proteins. Proteomic research comparing primary and metastatic PDAC cell lines can reveal functional proteins, which are helpful for the prediction of metastasis and potential therapy against this process. One metastatic PDAC cell line, AsPC-1 and one primary PDAC cell line, BxPC-3, were studied for this purpose^[52]. Using SDS-PAGE and LC-MS/MS, 221 and 208 proteins were identified from AsPC-1 and BxPC-3 cells, respectively, with 109 proteins present in both cell lines. Analysis of other proteins showed different levels in the two cell lines, including catenin, cadherins, integrins, galectins, annexins and collagens. Cadherins are a class of type-1 transmembrane proteins that depend on calcium ions and combined complexes with catenin to mediate cell adhesion. They were all found in primary tumor cells (BxPC-3), but not in metastatic tumor cells (AsPC-1), suggesting a defect in cellular adhesion in metastatic AsPC-1 cells. Integrins are glycoprotein members that form heterodimeric receptors.

Integrin $\alpha 2$ and $\alpha 5$, which represent major adhesion molecules, were only identified in BxPC-3 cells. Conversely, galectins, as carbohydrate-binding proteins on the cell surface and extracellular glycoproteins, were found only in AsPC-1. Most of these proteins play a role in tumor cell adhesion and motility.

Springbio Antibody Microarrays were used to detect different proteins between the pancreatic cancer cell lines SW1990 and SW1990HM, highly liver metastatic-related cell lines^[53]. Increased glucagon and decreased prolactin were selected as potential biomarkers for cancer detection from 40 reproducible, altered proteins. Glucagon induces glucose production and regulates carbohydrate and protein metabolism. Prolactin is a hormone released by the pituitary gland with effects on female breast development and milk production. Both are localized at the plasma membrane, and can influence tumor cell adhesion.

FUTURE ASPECTS

Compared with other types of cancers, pancreatic cancer is probably one of the solid tumors with the highest levels of genetic alterations resulting in aberrant expression of a large number of proteins. A panel of proteomic biomarkers with the appropriate combination of high sensitivity and specificity will likely be better than a single biomarker. Many researchers have focused on proteomic profiling for pancreatic cancer detection using a combined biomarker approach and results so far have gained interest^[54-56]. In addition, some studies have investigated differentially expressed proteins of pancreatic cancer stem cells, where increasing evidence indicates an important role in tumorigenesis, growth and formation of metastasis^[57]. Targeting and eliminating pancreatic cancer stem cells may significantly improve the prognosis and avoid recurrence in pancreatic cancer patients after pancreatic resection^[58].

CONCLUSION

Due to the characteristics of pancreatic cancer, with often vague symptoms, but associated tumor aggressiveness, resistance to standard therapy and a poor prognosis, identification of sensitive and specific biomarkers is essential. Such biomarkers would be of extreme value for disease detection during an earlier phase. Up to now, despite the development of novel techniques and potential markers reported, only a limited number may be of potential use in the clinical situation. Research on pancreatic cancer biomarkers is, however, intensive and the use of proteomic technology may provide a completely novel tool and possibility of potential improvement, achieving early diagnosis, targeted therapy, and discovery of recurrence in patients with pancreatic cancer.

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