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Identification of tissue biomarkers of prognostic significance in pancreatic cancer

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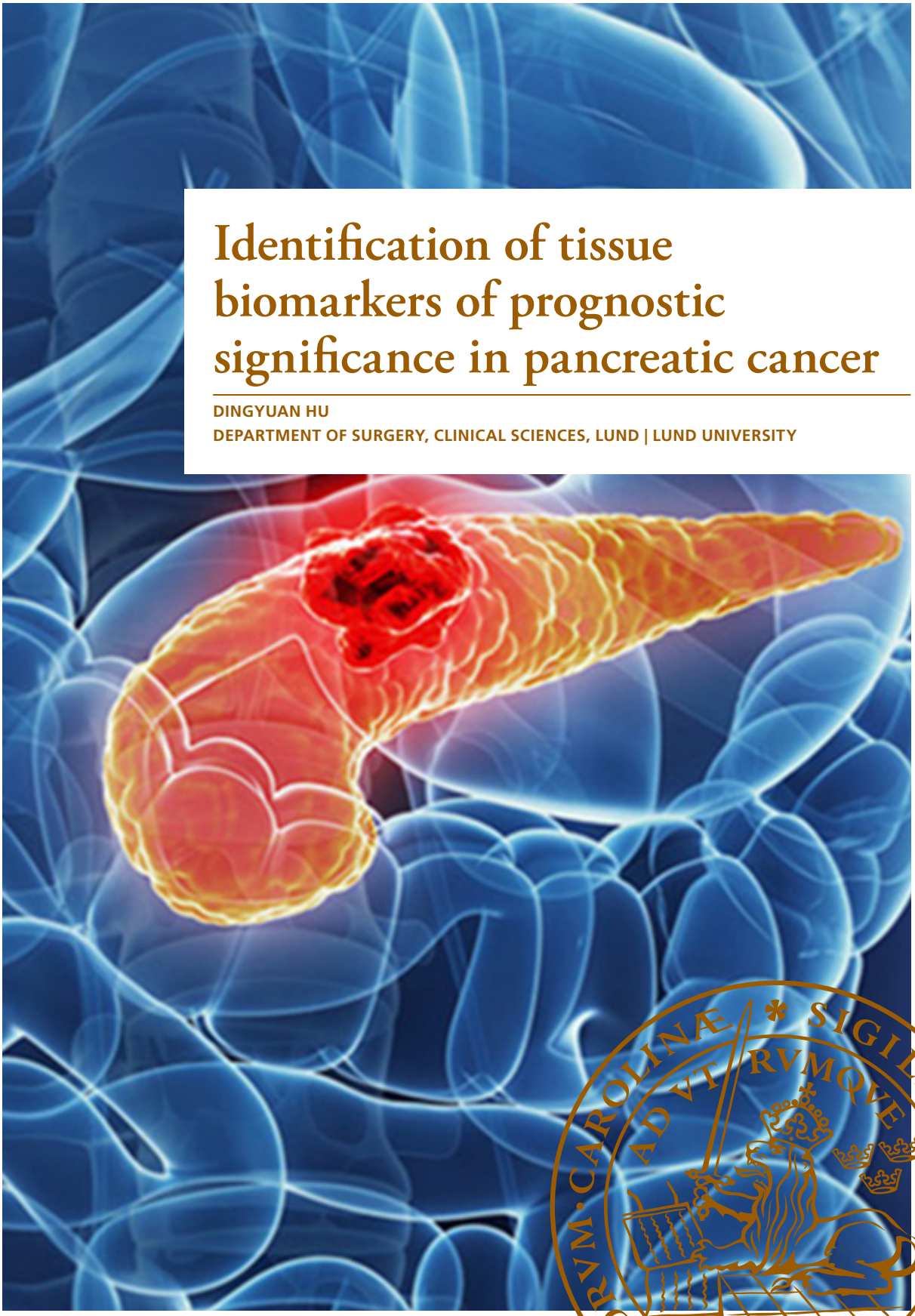

Identification of tissue biomarkers of prognostic significance in pancreatic cancer

Dingyuan Hu, MD

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Abstract

Background: Pancreatic cancer is the third leading cause of cancer-related mortality. Lack of early detection strategies and therapeutic resistance are main contributors to the poor prognosis. Unfortunately, there are no tissue biomarkers available for the prognosis of pancreatic cancer in routine clinical use.

Aim: To identify and validate novel tissue biomarkers for the prognosis of pancreatic cancer.

Methods: A mass spectrometry-based proteomic approach was applied to formalin-fixed paraffin-embedded specimens from surgically resected pancreatic cancer in 9 patients with short survival (<12 months) and 10 patients with long survival (>45 months). The dysregulated biomarkers were further verified by targeted proteomics, parallel reaction monitoring. Finally, we evaluated prognostic candidates (CLCA1, galectin 4, P4HA2, PRTN3 and fibronectin) by tissue microarray and immunohistochemistry in a larger cohort of patients with pancreatic cancer who underwent surgical resection (n=144). Bioinformatic analysis was exploited to assess pathways and networks linked to the prognosis. Kaplan-Meier and Cox proportional hazards modeling were used to explore the association between biomarkers and survival.

Results/Conclusion: A total of 24 and 147 proteins were significantly upregulated in patients with short survival and long survival, respectively. Bioinformatic analysis linked proteins representing “activated stroma factors” and “basal tumor factors” to poor prognosis and highlighted TCF1 and CTNNB1 as possible upstream regulators. By targeted proteomics, seven proteins were verified to be upregulated in patients with short survival (MMP9, CLIC3, MMP8, PRTN3, P4HA2, THBS1 and FN1), while 18 proteins were upregulated in patients with long survival, including EPCAM, galectin 4, VIL1, CLCA1 and TPPP3 (I). By immunohistochemical validation, we found that low CLCA1 expression correlated significantly with shorter disease-free survival (II). Furthermore, galectin 4 expression significantly correlated with disease recurrence within 1 year of surgery and with overall survival at 1- and 3-year (III). Besides, a low P4HA2 and high PRTN3 expression pattern correlated with shorter disease-free survival and overall survival (IV). Finally, high stromal FN1 expression was associated with aggressive tumor characteristics in patients with resected pancreatic cancer, although it was not associated with survival (V).

Key words: pancreatic cancer, proteomics, mass spectrometry, biomarkers, prognosis, CLCA1

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Dingyuan Hu, MD

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Dedicated to my family

"Hit it without hope but with resolution"

Ernest Hemingway

"只求耕耘，不问收获"

曾国藩

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I. Hu D, Ansari D, Pawlowski K, Zhou Q, Sasor A, Welinder C, Kristl T, Bauden M, Rezeli M, Jiang Y, Marko-Varga G, Andersson R. Proteomic analyses identify prognostic biomarkers for pancreatic ductal adenocarcinoma. Oncotarget. 2018; 9:9789-9807.

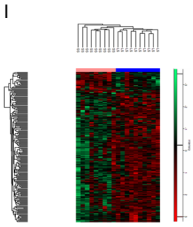
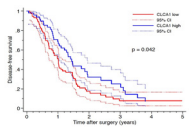
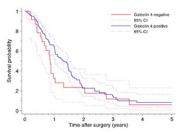
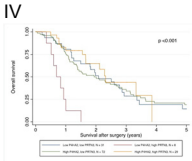
II. Hu D, Ansari D, Zhou Q, Sasor A, Hilmersson KS, Bauden M, Jiang Y, Andersson R. Calcium-activated chloride channel regulator 1 as a prognostic biomarker in pancreatic ductal adenocarcinoma. BMC cancer. 2018; 18:1096.

III. Hu D, Ansari D, Zhou Q, Sasor A, Said Hilmersson K, Andersson R. Galectin 4 is a biomarker for early recurrence and death after surgical resection for pancreatic ductal adenocarcinoma. Scandinavian Journal of Gastroenterology. 2019; 54:95-100.

IV. Hu D, Ansari D, Zhou Q, Sasor A, Said Hilmersson K, Andersson R. Low P4HA2 and high PRTN3 expression predicts poor survival in patients with pancreatic cancer. Scandinavian Journal of Gastroenterology. 2019 Mar:1-6; doi: 10.1080/00365521. 2019.1574360.

V. Hu D, Ansari D, Zhou Q, Sasor A, Said Hilmersson K, Andersson R. Stromal fibronectin expression in patients with resected pancreatic ductal adenocarcinoma. World Journal of Surgical Oncology. 2019; 17:29.

Thesis résumé

Study	Aim of the study	Methods	Results & Conclusions
I 	To profile the proteins in pancreatic cancer tissues and discover prognostic biomarkers of the disease.	Mass spectrometry-based proteomics approach was applied to FFPE primary tumor samples from patients with resected pancreatic cancer with short survival (<12 months, n=9) or with long survival (>45 months, n=10).	By in-depth proteome sequencing and targeted proteomics, we found 25 protein candidates of prognostic significance for pancreatic cancer. Besides, the activated stroma status, involvement of Wnt signaling pathway, as well as TP53 associated proteins, were revealed to be associated with a worse prognosis of pancreatic cancer.
II 	To validate the prognostic significance of CLCA1 in pancreatic cancer.	Tissue microarray and immunohistochemical analysis of CLCA1 in a retrospectively cohort of 140 patients with resected pancreatic cancer with well annotated clinical information.	Low CLCA1 expression was found to be an independent factor of shorter disease-free survival.
III 	To validate the prognostic significance of galectin 4 in pancreatic cancer.	Galectin 4 expression was detected by Immunohistochemistry in 140 patients with resected pancreatic cancer and was linked to the survival.	Galectin 4 expression may serve as a novel biomarker for early recurrence and mortality after surgical resection for pancreatic cancer.
IV 	To validate the prognostic significance of P4HA2 and PRTN3 in pancreatic cancer.	In patients with resected pancreatic cancer (n=140), the expressions of P4HA2 and PRTN3 were evaluated by tissue microarray and immunohistochemistry and were linked to the survival.	A low P4HA2 together with high PRTN3 expression status was significantly associated with poor survival in retrospectively collected patients with resected pancreatic cancer.
V	To validate the prognostic significance of FN1 in pancreatic cancer.	Expression of FN1 on tumor tissues from 138 patients with resected pancreatic cancer was assessed by tissue microarray and Immunohistochemical analysis and was linked to the survival.	FN1 was not likely to serve as a prognostic biomarker for pancreatic cancer.

Abstract

Background: Pancreatic cancer is the third leading cause of cancer-related mortality. Lack of early detection strategies and therapeutic resistance are main contributors to the poor prognosis. Unfortunately, there are no tissue biomarkers available for the prognosis of pancreatic cancer in routine clinical use.

Aim: To identify and validate novel tissue biomarkers for the prognosis of pancreatic cancer.

Methods: A mass spectrometry-based proteomic approach was applied to formalin-fixed paraffin-embedded specimens from surgically resected pancreatic cancer in 9 patients with short survival (<12 months) and 10 patients with long survival (>45 months). The dysregulated biomarkers were further verified by targeted proteomics, parallel reaction monitoring. Finally, we evaluated prognostic candidates (CLCA1, galectin 4, P4HA2, PRTN3 and fibronectin) by tissue microarray and immunohistochemistry in a larger cohort of patients with pancreatic cancer who underwent surgical resection (n=144). Bioinformatic analysis was exploited to assess pathways and networks linked to the prognosis. Kaplan-Meier and Cox proportional hazards modeling were used to explore the association between biomarkers and survival.

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Populärvetenskaplig sammanfattning

Pankreascancer, dvs cancer i bukspottkörteln, utgör nu den tredje vanligaste orsaken till död i cancer och kommer om inga genombrott görs att utgöra andra orsak till cancerrelaterad död inom några år (efter lungcancer). Avsaknad av tidiga symtom och en uttalad terapisvikt är bidragande faktorer till den dåliga prognosen. Femårsöverlevnaden ligger på endast maximalt 6 % och pankreascancer orsakar, förutom den höga dödligheten, också betydande kostnader för samhället.

Det finns idag ett mycket begränsat antal diagnostiska biomarkörer för såväl blodprov som vävnadsdiagnostik. I serum har sedan decennier tumörmarkören CA 19-9 använts medan man i vävnad inte haft några möjligheter att identifiera biomarkörer som skulle ge en mera individbaserad behandling och därmed potentiellt kunna öka överlevnad.

Syftet var att identifiera och validera nya biomarkörer från pankreascancervävnad för en förbättrad prognostisk information.

Med hjälp av s k proteomik och en teknik benämnd masspektrometri studerades initialt formalin-fixerad paraffininbäddad cancervävnad från nio patienter som genomgått pankreaskirurgi med en efterföljande kort överlevnad (< 12 månader), respektive tio patienter med en förhållandevis lång överlevnad (> 45 månader). Slutligen definierades 25 proteiner, varav 18 var uppreglerade vid lång överlevnad och sju uppreglerade vid kort överlevnad.

I de åtföljande fyra delarbetena utförs ett valideringsarbete på cirka 140 patienter som genomgått kirurgisk resektion av sin pankreascancer. Teknikerna som genomgående använts var konstruktion av en s k tissue microarray, dvs ett mycket litet vävnadsblock, som sedan snittas och infärgas med immunhistokemi riktat mot de olika markörer som identifierats i den initiala proteomikstudien.

I delarbete 2 studeras calcium activate chloride channel regulator-1 (CLCA1) där en låg nivå visar sig korrelera med en kortare sjukdomsfri överlevnad.

I delarbete 3 befinnes markören galectin-4 korrelera till återfall av pankreascancer inom ett år och korrelerar också i sin nivå med den totala överlevnaden både ett och tre år efter kirurgi.

I delarbete 4 kommer vi in på de betydelsefulla fynd man har vid pankreascancer, dvs att enbart 10-20 % av tumören utgörs av cancerceller medan kringliggande bindväv med såväl specifika celler och kollagen dominerar och har en koppling till tumörens tillväxt och metastaseringspotential.

I det fjärde arbetet visade sig lågt uttryck av P4HA2, som bildar kollagen, och ett högt uttryck av PRTN3, som bryter ner kollagen, där denna kombination korrelerar till en mycket kort både sjukdomsfri som total överlevnad.

I det femte delarbetet studeras fibronectin-1-uttrycket i stroma där ett högt uttryck korrelerar med aggressiva tumörkaraktäristika på opererade patienter, men någon koppling till överlevnad kan dock inte ses.

Sammanfattningsvis finns här nu en “panel” med nya pankreascancerbaserade biomarkörer som ger prediktiv och prognostisk information. Ytterligare valideringsarbete kommer att göras.

摘要

背景：胰腺癌是导致肿瘤相关性死亡的第三大原因。其预后差的原因主要包括缺乏早期诊断策略和治疗抵抗。此外，在临床实践中，尚无能判断胰腺癌预后的组织标记物。

目的：寻找并验证新的能判断胰腺癌预后的组织标记物。

方法：在接受胰腺切除术的 9 例短生存期(<12 月)和 10 例长生存期(>45 月)胰腺癌患者中，收集福尔马林固定石蜡包埋的组织，并进行基于质谱仪的蛋白质组学研究。再通过靶向蛋白质组学方法，即平行反应监测，进一步证实差异性表达的蛋白质标记物。最后，在一个较大的接受胰腺切除术的胰腺癌患者队列(n=144)中，我们通过组织微阵列和免疫组织化学方法，进一步评估备选预后标记物，包括氯离子通道辅助蛋白 1 (CLCA1)， 半乳糖凝集素 4 (galectin 4)， 脯氨酰 4-羟化酶 2 (P4HA2)， 蛋白酶 3 (PRTN3)和纤维连接蛋白(FN1)。最后，采用生物信息学分析来评估和预后相关的生物学通路和网络，采用 Kaplan-Meier 和 Cox 比例风险模型研究标记物和生存期的关系。

结果/结论：在短生存期患者和长生存期患者中，分别有 24 个和 147 个蛋白质的表达显著升高。生物信息学分析发现，代表“激活的基质因子”和“基础肿瘤因子”的蛋白质和预后差有关，而转录因子 1 (TCF1)和 β-连环蛋白(CTNNB1)可能是造成两组蛋白质差异表达的上游调节因子。通过靶向蛋白质组学研究，我们证实 7 个蛋白质在短生存期患者中上调，包括基质金属蛋白酶 9 (MMP9)，胞内氯离子通道蛋白 3 (CLIC3)，基质金属蛋白酶 8 (MMP8)，PRTN3，P4HA2，凝血栓蛋白 1 (THBS1)和 FN1；而 18 个蛋白质在长生存期患者中上调，包括上皮细胞粘附分子 (EPCAM)， galectin 4，绒毛蛋白 1 (VIL1)， CLCA1 和促微管聚合蛋白 3(TPPP3)(论文 I)。通过免疫组织化学验证分析，我们发现低 CLCA1 表达和缩短的无病生存期有关(论文 II)。此外，galectin 4 表达和术后一年内复发、1 年和 3 年总体生存期有关(论文 III)。低 P4HA2 和高 PRTN3 表达和缩短的无病生存期和总体生存期有关(论文 IV)。最后，FN1 高表达和胰腺癌患者的侵袭性肿瘤特征有关，但和其生存期无关(论文 V)。

Abbreviations

ADEX	aberrantly differentiated endocrine exocrine
AJCC	American joint committee on cancer
AMBIC	ammonium bicarbonate
ASCO	American Society of Clinical Oncology
BRPC	borderline resectable pancreatic cancer
CLCA1	calcium-activated chloride channel regulator 1
CT	computed tomography
CTCs	circulating tumor cells
ctDNA	circulating tumor DNA
DDA	data-dependent acquisition
DFS	disease-free survival
ECM	extracellular matrix
ECOG	Eastern Cooperative Oncology Group
EMT	epithelial-mesenchymal transition
ERCP	endoscopic retrograde cholangiopancreatography
EUS	endoscopic ultrasound
FDR	false discovery rate
FFPE	formalin-fixed paraffin-embedded
FN1	fibronectin
FNA	fine-needle aspiration
HPP	Human Proteome Project
HRs	hazard ratios
HUPO	Human Proteome Organization
IPA	Ingenuity Pathway Analysis
IPMNs	intraductal papillary mucinous neoplasms
LAPC	locally advanced pancreatic cancer
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LS	long survival
m/z	mass/charge
MCN	mucinous cystic neoplasms
MMPs	matrix metalloproteases
MRCP	magnetic resonance cholangiopancreatography
MRI	magnetic resonance imaging
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
nab	nanoparticle albumin-bound
NLR	neutrophil-to-lymphocyte ratio
OS	overall survival
P4HA2	prolyl 4-hydroxylase subunit alpha 2
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate-buffered saline

PC	pancreatic cancer
PCA	principal component analysis
PDAC	pancreatic ductal adenocarcinoma
PET	positron emission tomography
PRM	parallel reaction monitoring
PRTN3	proteinase 3
PSC	pancreatic stellate cell
PSMs	peptide-spectrum matches
QM-PDA	quasi-mesenchymal
R1	resection margin positive
RT	room temperature
SPARC	secreted protein acidic and rich in cysteine
SS	short survival
TMA	tissue microarray
TME	tumor microenvironment
US	ultrasound

1 Introduction

1.1 Pancreas: anatomy, physiology and function

The pancreas is a soft and elongated glandular organ located in the retroperitoneal cavity and behind the stomach. It anatomically comprises the head, neck, body and tail (Figure 1). The head of the pancreas is encircled by the duodenum, while the tail is adjacent to the spleen. Pancreas is also adjacent to essential large vessels in the abdomen, including the portal vein, superior mesenteric vein, superior mesenteric artery and celiac axis, which may make surgery on the pancreas difficult and technique-demanding.

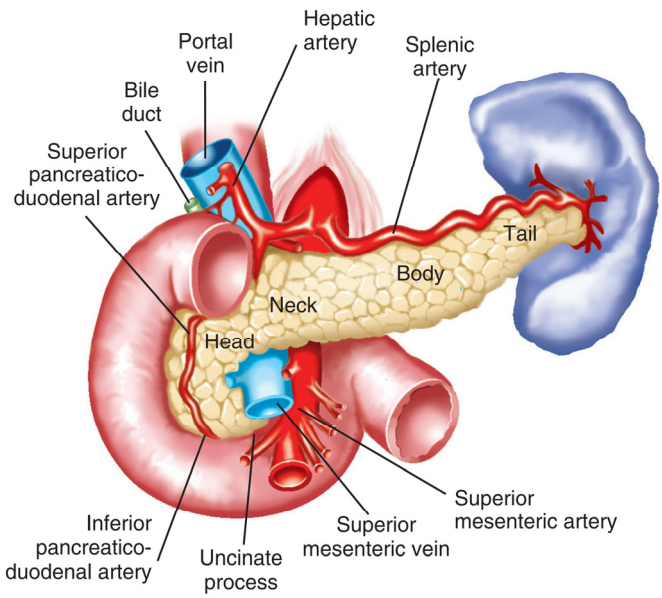

Figure 1. Anatomy of pancreas.
(Sleisenger and Fordtran's Gastrointestinal and Liver Disease, 10th edition. Elsevier Inc).

Pancreas has two functions, i.e., exocrine and endocrine functions. The basic subunit of the exocrine portion is the acinus composed of secretory acinar cells, which is connected to ductal cells. The acinar cells are responsible for secretion of inactive precursors of enzymes, such as amylase and lipase, whereas the ductal cells absorb chloride and actively secrete bicarbonate and water to sustain sufficient liquid volume and a suitable

pH of pancreatic juice. The inner lumen of these epithelial cells forms the duct system, in which zymogens and liquid are secreted and delivered to the duodenum through pancreatic duct. The zymogens are activated in the small intestine and essentially involved in the digestion of food.

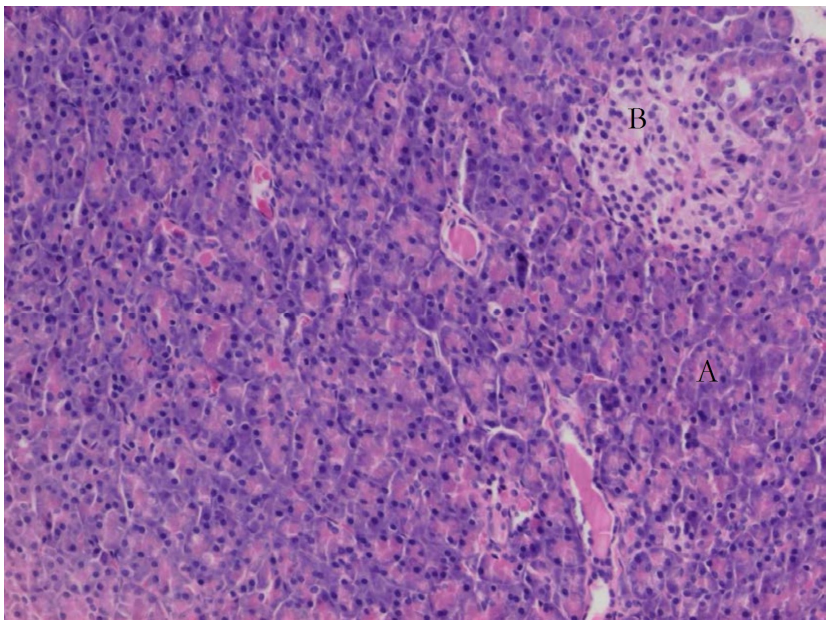

Figure 2. Hematoxylin and eosin staining of normal pancreas.
A: spherical acinus composed of dark-staining secretory acinar cells; B: the islets of Langerhans, which are light-staining and form in spherical clusters.

While exocrine acinar and ductal structures dominate the pancreas, the endocrine glands account for only 5% of the pancreas and consist of the islets of Langerhans (Figure 2). They mainly comprise 5 major cell types: beta cells for secretion of insulin, alpha cells for glucagon, PP cells for pancreatic polypeptide and adrenomedullin, delta cells for somatostatin, and epsilon cells for ghrelin. These hormones are secreted into the bloodstream and play key roles in the body, especially the role of maintaining a stable blood glucose level.

1.2 Pancreatic cancer

Pancreatic cancer almost always originates from epithelial cells, including ductal cells, acinar cells and endocrine (islet) cells. Pancreatic ductal adenocarcinoma is the most common form, accounting for over 85% of pancreatic tumors. Endocrine neoplasms represent less than 5% of pancreatic tumors. Nonepithelial pancreatic malignancies are exceedingly rare.

1.3 Epidemiology

Pancreatic cancer is a disease of aging, rarely diagnosed in those below the age of 45 years, whereas its occurrence rises sharply after 50 years of age. The median age at diagnosis is 68-70 years. The incidence is slightly higher in men than women, being 7.76 and 6.75 in 100,000 in Nordic countries, respectively (2015) [1]. The incidence of pancreatic cancer is also higher in African Americans than in Caucasians. In China, the incidence rate is lower than in western countries, but is increasing in recent years [2].

Pancreatic cancer has surpassed breast cancer and presently represents the third leading cause of cancer-related mortality both in EU [3] and the United States [4]. It has been projected that pancreatic cancer will rise to the second cause of death in cancer by the year of 2030, following lung cancer [5]. In 2018, approximately 458,918 new cases and 432,242 deaths have been estimated worldwide [6]. Pancreatic cancer has a dismal prognosis (Figure 3). Although achievements on safety of surgical resection and the addition of adjuvant chemotherapy have been made, especially in recent years, pancreatic cancer still has the worst survival among all cancers (6%) for all stages combined.

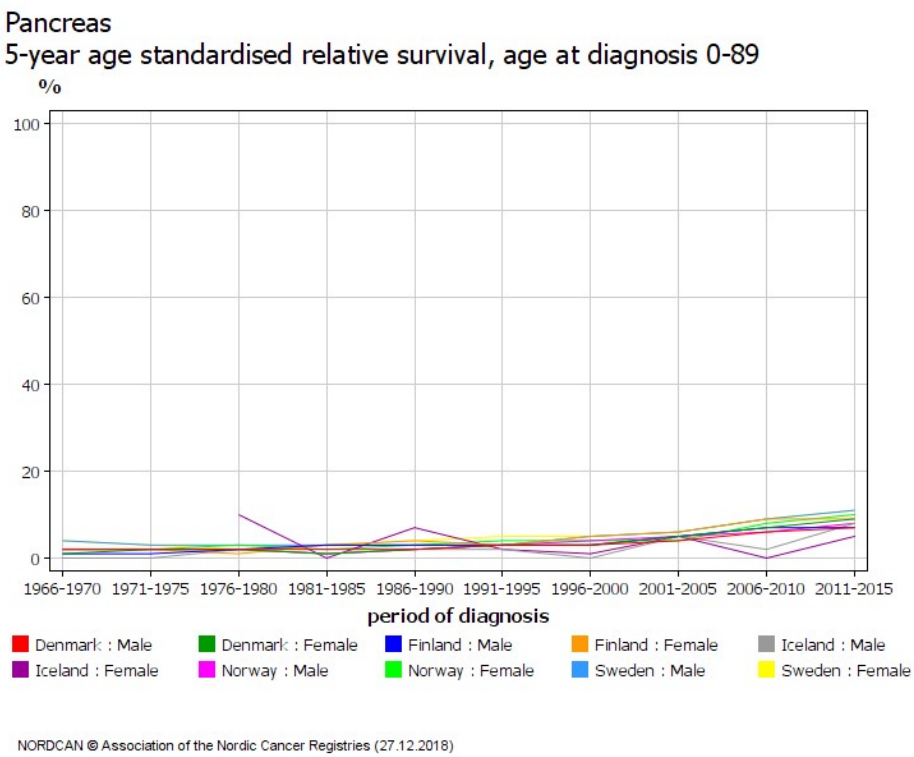

Figure 3. The 5-year survival of pancreatic cancer with all stages in Nordic country.
Data were taken from NORDCAN [1].

1.4 Risk factors

An epidemiologic association of diabetes mellitus with an increased risk of pancreatic cancer has been demonstrated [7]. Long-term diabetes type 2 approximately doubles the risk of pancreatic cancer, presented in 15% of patients with pancreatic cancer and 8% in the controls [8]. Moreover, around 1% of new-onset diabetes (within 3 years) subjects with ages of 50 years and over are diagnosed with pancreatic cancer [9, 10]. Studies have shown that 75% of patients referred to operations for pancreatic cancer had either impaired glucose tolerance or diagnosed diabetes [11, 12]. Therefore, new-onset diabetes type 2 may be essential for the diagnosis and is an indicator for screening of pancreatic cancer [13]. Chronic pancreatitis is also an established risk factor relating to pancreatic cancer with an odds ratio of nearly three folds [14].

Cigarette smoking is a well-known risk factor for pancreatic cancer, reportedly contributing to about 20% of the occurrences [15]. The risk of smoking appears to be pack-years dependent. Recent evidence has suggested that smoking contributes to not only the initiation, but also the promotion of pancreatic carcinogenesis [15]. Although early investigations could not prove the association between alcohol consumption and the risk of pancreatic cancer, recent studies have stressed that heavy drinkers are associated with an increased risk of pancreatic cancer [15, 16]. Other risk factors include obesity, reduced physical activity, and helicobacter pylori infection [15, 17].

1.5 Hereditary and genetic factors

Most pancreatic cancers occur sporadically. However, less than 10% of patients with pancreatic cancer have a familial/hereditary pancreatic disease history. Hereditary pancreatitis is one of the most prominent diseases, which is caused by mutation of the PRSS1 gene on chromosome 7. Other genetic syndromes that increase the risk of pancreatic cancer include familial pancreatic cancer, familial atypical multiple mole and melanoma syndrome (p16), hereditary breast and ovarian cancer syndromes (BRCA1, BRCA2, PALB2), Peutz-Jeghers syndrome (STK11), hereditary nonpolyposis colon cancer (Lynch syndrome) (MLH1, MSH2, MSH6), familial adenomatous polyposis and ataxia telangiectasia [18]. Non-O blood group is also reported to be associated with an increased susceptibility of pancreatic cancer [19].

1.6 Etiology

1.6.1 Molecular pathology and genetic alterations

Pancreatic tumorigenesis is the result from a complex series of events, involving the effects of multiple intracellular genetic mutations, a hypovascular and hypoxic extracellular microenvironment, reprogramming of cellular metabolism, and evasion of tumor immunity [20, 21]. Figure 4 depicts the typical stepwise progression of pancreatic cancer from normal pancreas and mechanisms involved. Pancreatic cancers mainly arise from pancreatic intraepithelial neoplasia (PanIN), but also from intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCN).

Activating mutations in oncogene KRAS and inactivating mutations of suppressor genes TP53, CDKN2A (p16) and SMAD4 (DPC4), are considered to be the four most important genetic events in pancreatic tumorigenesis. KRAS mutations are presented in >90% of tumors, while the other three mutations occur in 50-80% of pancreatic cancers [13]. Moreover, KRAS mutations are also detected in PanIN of all grades

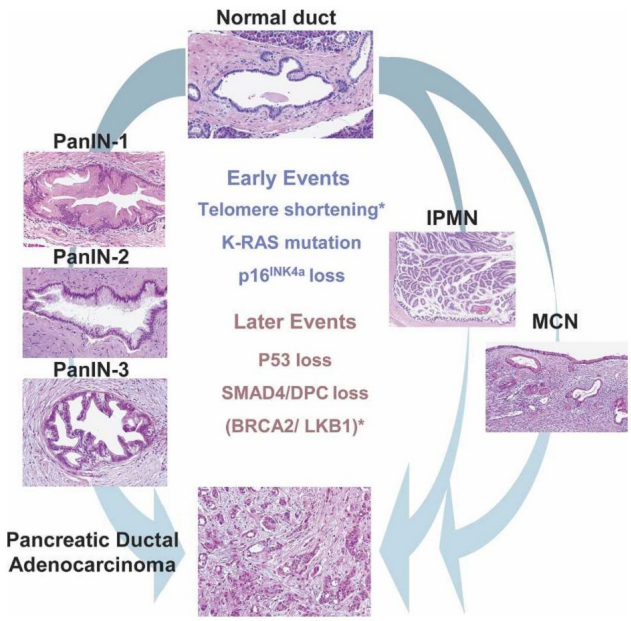

Figure 4. Pancreatic precursor lesions and genetic events involved in pancreatic adenocarcinoma progression. Pictured are three known human PDAC precursor lesions: PanIN, MCN, and IPMN. The PanIN grading scheme is shown on the left; increasing grade (1-3) reflects increasing atypia, eventually leading to adenocarcinoma. The right side illustrates the potential progression of MCNs and IPMNs to PDAC. The genetic alterations documented in adenocarcinomas also occur in PanIN, and to a lesser extent in MCNs and IPMNs, by an apparent temporal sequence, although these alterations have not been correlated with the acquisition of specific histopathological features. The various genetic events are listed and divided into those that predominantly occur early or late in PDAC progression. Asterisks indicate events that are not known to be common to all precursors (telomere shortening and BRCA2 loss are documented in PanIN and LKB1 loss is documented in a subset of PDACs and IPMNs). IPMN, intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm; PanIN, pancreatic intraepithelial neoplasia. (From Hezel AF, Kimmelman AC, Stanger BZ, et al. Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev 2006; 20:1218-49) [22]

(including grade 1) mutations are also detected in PanIN of all grades (including grade 1) in more than 90% of cases [23], indicating that virtually all PanINs harbor KRAS mutations. CDKN2A is found mutated in, respectively, 30%, 50%, and 70% of PanIN-1, PanIN-2, and PanIN-3 lesions [24]. Mutations in TP53 and SMAD4 are mainly observed in PanIN-3 lesions, suggesting these mutations as late events in PDAC development [25, 26]. Genetically engineered animal models have successfully reinforced the conception that activated KRAS serves to initiate PanIN lesions, while tumor suppressor CDKN2A functions to constrain the malignant conversion of these PanIN lesions into potentially lethal ductal adenocarcinoma [27]. There are also a number of mutations with less frequency, which may also contribute to the processes of pancreatic tumorigenesis, such as BRCA2, ARID1A, MLL3 and TGFBR2 [28, 29].

Recent application of comprehensive whole-genome sequencing has unveiled a more informative mutational landscape on patients with pancreatic cancer, including thousands of point mutations, small insertions and deletions as well as chromosomal structural variants [30].

The mutations occurring in the pancreas drive a cascade of events towards tumorigenesis. Jones et al. applied global genomic sequencing to human pancreatic adenocarcinoma and revealed that, although tumor genetic alterations were extremely complex, these alterations were defined as a core set of only 12 cellular signaling pathways and processes, as shown in Table 1 [31]. Five of the pathways describe specific cellular functions: apoptosis, DNA damage repair, G1/S phase cell cycle progression, cell-cell adhesion and invasion [32].

Table 1. Twelve commonly involved pathways in pancreatic cancer and representative altered gene from these pathways.

Regulatory pathway or process	Representative altered genes
Apoptosis	
DNA damage control	**TP53**
Regulation of G1/S phase transition	**CDKN2A**, APC2
Hedgehog signaling	
Homophilic cell adhesion	CDH1
Integrin signaling	
c-Jun N-terminal kinase signaling	TNF
KRAS signaling	**KRAS**
Regulation of invasion	
Small GTPase-dependent signaling (other than KRAS)	
TGF-β signaling	**SMAD4**, SMAD3, **TGFBR2**
Wnt/Notch signaling	MYC, TCF4

1.6.2 Tumor microenvironment

One characteristic of PDAC is that tumor cells are surrounded by as much as 90% stroma (Figure 5), consisting of proliferating myofibroblast-like cells (pancreatic stellate cells), immune cells and inflammatory cells, and components of extracellular

matrix (ECM), such as collagen, fibronectin, proteoglycans, hyaluronic acid, and secreted protein acidic and rich in cysteine (SPARC) [33, 34]. Activated pancreatic stellate cells are the main source of ECM components. Collagens, the most abundant ECM components, can bind to the integrin receptor in tumor cells and activate intracellular signaling that induce pro-tumorigenic programs. Proteoglycans consist of core proteins that undergo post-translational glycosylation, which affects cell signaling function [35]. Expression of SPARC has been found to be a strong prognostic factor in patients with PDAC [36, 37]. Due to its overexpression in PDAC and albumin-binding properties, SPARC has been postulated to enhance peritumoral drug delivery of nanoparticle albumin-bound (nab)-paclitaxel [35].

The microenvironment of pancreatic adenocarcinoma has a complex role in tumor growth and therapeutic response. Precursor lesions of PDAC, such as pancreatic intraepithelial neoplasms, have already presented stromal activation and ECM deposition [38]. Initially, the existence of a dense stroma is thought to promote tumor progression and metastasis [39-41]. For example, in-vitro studies have suggested that various stromal elements can enhance cancer cell proliferation and invasion [42]. However, this concept has been challenged by recent experimental evidence, which showed that stromal depletion approaches may favor tumor aggressiveness and spread [43-45]. The pancreatic tumor microenvironment (TME) contributes to drug resistance through the rigidity of the ECM, which compresses blood vessels and reduces perfusion, and ultimately impedes the delivery of drugs directed at neoplastic cells [20, 46].

Suppression of the immune response, either directly by tumor cells or via other cells in the TME, has been regarded as a key step for tumor establishment and survival [41]. Some constituents of the tumor stroma act to restrain tumor growth by participating in immune surveillance and restraint of tumor angiogenesis [43, 47, 48]. Because current chemotherapy regimens for pancreatic cancer are relatively inefficient, a better understanding of the mechanisms how pancreatic cancer cells interact with their TME might render new possibilities to enhance chemotherapeutic drug delivery and response in PDAC.

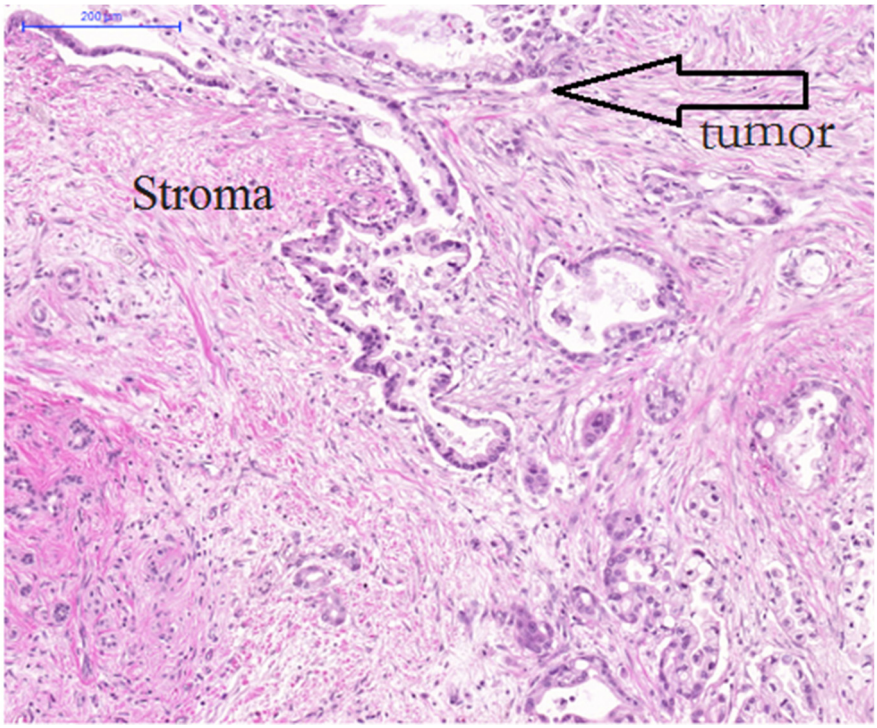

Figure 5. Hematoxylin and eosin staining of pancreatic cancer tissues.
Tumor cells are surrounded by abundant fibrotic stroma.

1.7 Diagnosis

1.7.1 Clinical presentation

Most patients with pancreatic cancer have vague symptom at least during the early stages, leading to a significant delay in diagnosis. Tumors located in the head of the pancreas tend to produce symptoms (e.g. jaundice) earlier during the course of disease than those located in the distal part of pancreas. Direct compression of the common bile duct, mostly from tumors in the head of the pancreas, leads to obstructive jaundice, characterized by yellowing of skin and eyes, pruritus, dark urine and pale-colored stools. Abdominal pain is another common symptom and is primarily due to invasion of the celiac or superior mesenteric arterial plexus and nerves [49]. Other nonspecific symptoms include nausea, fatigue, anorexia, and weight loss. New-onset diabetes and glucose intolerance may be linked to an underlying pancreatic cancer, particularly in patients over 50 years of age. Physical examination is usually inconclusive, but may reveal signs for differential diagnosis of pancreatic cancer, such as presence of jaundice, tenderness in the upper abdomen. A palpable nontender gallbladder (Courvoisier's sign) can be occasionally found in patients with pancreatic cancer.

1.7.2 Imaging

Since there are no specific early warning signs of pancreatic cancer, the diagnosis of pancreatic cancer mainly depends on imaging. Imaging modalities include transabdominal ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde cholangiopancreatography (ERCP), endoscopic ultrasound (EUS) and PET/CT (positron emission tomography/CT). Transabdominal US is often the first screening check-up modality for patients with abdominal pain or jaundice, which can be possibly caused by pancreatic cancer. It's sensitivity to identify pancreatic cancer is reported to be comparable with CT [50], but it highly depends on the operator's experience and presence of obesity and bowel gas in the patients. Pancreatic protocol CT with contrast enhancement allows precise delineation of a hypodense tumor mass in the pancreas and assessment of vascular invasion by the tumor, making this modality usually the golden standard to evaluate the resectability of any lesion (Figure 6). High-quality CT imaging should be carried out no more than 4 weeks before surgery [51].

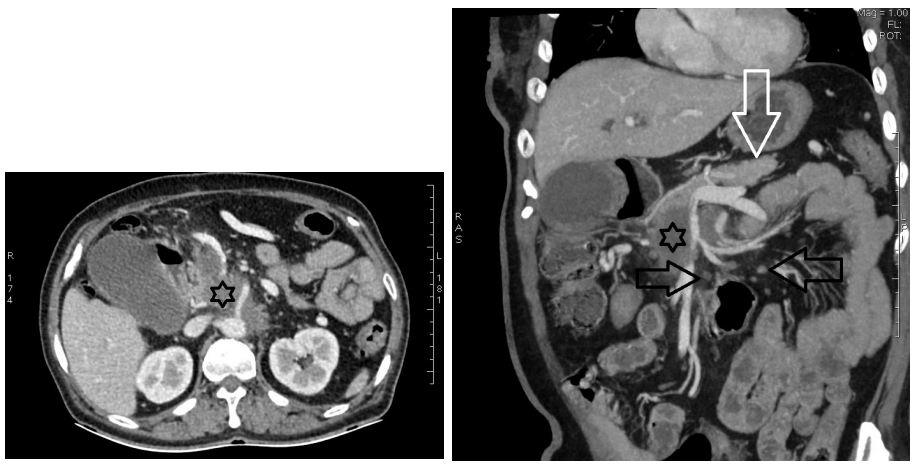

Figure 6. Enhanced CT scanning of a patient with advanced pancreatic cancer.
Left: a star marks the location of the tumor with low density. Right: three-dimension reconstruction of the pancreas, the star marks the location of the tumor, the black arrows show the lymph node metastases, the white arrow shows the tail of the pancreas. Images were provided by the First Affiliated Hospital of Wenzhou Medical University.

Compared to CT, MRI is advantageous to evaluate suspected liver metastases, while PET/CT is advantageous to detect metastases of the liver, other organs and lymph nodes. Therefore, additional evaluation by MRI or PT/CT has been suggested to those patients with suspected metastasis, or when the tumor is difficult to be delineated [52, 53]. EUS is presently not included as a routine staging tool. However, it may provide additional information for diagnosis and differential diagnosis [54], and allows guided biopsy sampling when preoperative tissue-based diagnosis is needed. Application of ERCP is generally limited to those patients in need of therapeutic interventions, through which a biliary stent can be placed to relieve the obstructive jaundice. However, routine stenting

before surgery for pancreatic cancer patients with endurable jaundice, and a short interval to planned surgical resection, provide no improvement of the outcomes and can be avoided [55].

1.7.3 Biopsy sampling

A histopathological diagnosis is not required before surgery in clearly resectable or borderline resectable pancreatic cancer (BRPC). However, cytological diagnosis is necessary before administration of adjuvant therapy, in patients staged with locally advanced pancreatic cancer (LAPC), or in metastatic disease [56]. It is also necessary in patients with BRPC if neoadjuvant/downstaging chemotherapy is planned before surgery [56]. The biopsy of primary tumors is usually conducted through fine-needle aspiration (FNA) biopsy with either EUS guidance (preferred) or CT. A meta-analysis showed that sensitivity and specificity of EUS-FNA for diagnosis of solid pancreatic lesions is 90.8% and 96.5%, respectively [57]. The presence of a cytologist immediately evaluating the puncture result and sample yield is helpful. Biopsies that fail to confirm a suspected malignancy should be repeated.

1.7.4 Serum markers

CA 19-9, a sialylated Lewis A blood group antigen, is the best-validated serum marker with clinical usefulness in the diagnosis and, importantly, surveillance of pancreatic cancer. Unfortunately, CA19-9 lacks sufficient sensitivity and specificity for early detection of pancreatic cancer. The sensitivity and specificity to diagnose the disease vary with its cutoff values, among which 37 U/mL achieves a balanced sensitivity and specificity of 86% and 87%, respectively [58]. False-positive results may occur in individuals with biliary infection, or biliary obstruction, while false-negative results occur in pancreatic cancer patients with a negative Lewis blood group phenotype, which constitutes around 10% of the population [59].

1.7.5 Screening of pancreatic cancer

A successful screening of pancreatic cancer could lead to early detection of the disease, thus avoiding late diagnosis in an advanced stage where curative surgery is no longer an option. Due to the relatively low incidence of pancreatic cancer, routine screening for the disease is generally not recommended for asymptomatic individuals. However, the International Cancer of the Pancreas Screening Consortium has developed consensus guidelines of screening in high-risk individuals [60], defined as first-degree relatives of patients with pancreatic cancer from familial kindreds; carriers of p16 or BRCA2 mutations with an affected first-degree relative; patients with Peutz-Jeghers syndrome; and patients with Lynch syndrome and an affected first-degree relative with pancreatic cancer. EUS and/or MRI/magnetic resonance cholangiopancreatography

(MRCP) are recommended for the screening, both of which outperform CT [60, 61]. Serum CA 19-9 is in general thought not suitable to serve as a marker for screening due to its limited sensitivity. However, recent evidence showed that CA19-9 levels may be elevated in patients up to 2 years before a pancreatic cancer diagnosis, indicating that CA 19-9 has some potential as a biomarker for screening of high-risk patients [62].

1.8 Treatment

There have been some improvements in treatment of pancreatic cancer during the past years [34, 63]. At diagnosis, around 10-20% of patients present with resectable tumors, 30-40% present with BRPC or LAPC, and 50-60% present with metastatic or systemic disease. For patients with resectable pancreatic cancer, surgery followed by adjuvant chemotherapy remains the only potentially curative option for pancreatic cancer. For cancers of the pancreatic head, a partial pancreaticoduodenectomy (Whipple's procedure) is usually required, with or without partial resection of the distal stomach. For tumors of the pancreatic tail, distal pancreatectomy with splenectomy is carried out. Perioperative mortality has been reduced substantially. Pancreatectomy performed in high volume centers is also a favorable factor for the outcome of the operation [63, 64].

Gemcitabine has been the standard drug for chemotherapy. In Japan, a randomized phase 3 trial (JASPAC-01) has demonstrated that adjuvant oral S-1, a fluoropyrimidine derivative, is superior to gemcitabine (5-year survival 44.1% versus 24%) [65]. Therefore, oral S-1 is the recommended drug for adjuvant chemotherapy in Japan. This finding remains to be confirmed in non-Asian populations. The most recent ESPAC-4 clinical trial, published in 2017, showed that the gemcitabine-capecitabine combination therapy outperformed gemcitabine alone in patients with resected PDAC, with a 5-year survival approaching 30% [66]. Since then, gemcitabine-capecitabine is recommended over other adjuvant therapy regimens for potentially curable pancreatic cancers by the American Society of Clinical Oncology (ASCO) in 2017 [67]. Furthermore, an abstract in 2018 at the ASCO annual meeting reported an unprecedented median survival of modified FOLFIRINOX adjuvant chemotherapy (54.4 months) as compared to gemcitabine (34.8months) in patients with resected pancreatic cancer [68]. Besides, the application of neoadjuvant therapy before surgery in resectable patients has rendered interest to physicians and researchers. Although there might be difficulties in patient enrollment given the fear of losing opportunity of surgery, some clinical trials are still addressing this issue [69, 70]. Preliminary results of the PREOPANC-1 trial in 2018 showed that preoperative chemoradiotherapy significantly improved the outcome in (borderline) resectable pancreatic cancer compared to upfront surgery [71].

In patients with BRPC, neoadjuvant therapy, i.e., administration of FOLFIRINOX (a combination of folinic acid, 5- fluorouracil, irinotecan and oxaliplatin) or gemcitabine plus albumin-bound paclitaxel before surgical resection, with or without chemoradiation, might be beneficial [72]. Evidence has suggested that approximately

one-third of initially BRPCs and selected LAPCs may become resectable after neoadjuvant therapy [73]. However, more studies are needed to reach a consensus regarding the benefit of upfront surgery or neoadjuvant therapy [63].

The indications for surgery have been extended from resectable pancreatic cancer (stages I and II) to locally advanced disease (stage III). This is due to advances in both surgery and systemic chemotherapy. Firstly, although companied with high risks of morbidity, arterial resections in locally advanced disease are now technically feasible and might contribute to long-term survival in strictly selected patients [74]. Secondly, neoadjuvant FOLFIRINOX has been reported to enable more than half of tumors to be resected even if they are initially unresectable [75, 76]. For patients with locally advanced or metastatic pancreatic cancer, chemotherapy is given based on the performance of patients to balance the efficacy and toxicity. FOLFIRINOX, or gemcitabine plus albumin-bound paclitaxel is recommended for patients with good ECOG performance status, where ECOG is short for Eastern Cooperative Oncology Group. If ECOG performance status scores more than 2, gemcitabine monotherapy or best supportive care is more beneficial [13].

Palliative support is also essential for a better survival and quality of life. Patients may present with pain, biliary obstruction, gastric outlet obstruction, cachexia and anorexia, exocrine insufficiency or depression. Management of these symptoms involves positive assessment and medical intervention, both physically and mentally.

1.9 Staging and resectability

Table 2 shows the 8th version of The American Joint Committee on Cancer (AJCC) Staging Manual, released from 2017, which is currently the standard for classifying patients with pancreatic cancer, predicting prognosis, and guiding treatment decisions [77]. Compared to the 7th version, there are two major changes in the new staging [77, 78]. Firstly, category of T is based on tumor size and not on extra-pancreatic invasion, which is more objective and correlated with survival [79]. Secondly, subdivision of lymph node positive group into N1 and N2 is proposed based on the number of metastatic regional lymph nodes. N2 without metastasis is staged as III. AJCC staging is most applicable to surgically treated patients. Before surgery, the staging information is obtained by imaging or laparoscopy.

Resectability of pancreatic cancer is mainly based on the anatomical extent of the tumor and the suspected involvement of neighboring vessels in preoperative cross-sectional imaging using CT and/or MRI [63]. Pancreatic cancer is generally deemed to be resectable if it does not involve major blood vessels. Owing to advances in both surgery and systemic chemotherapy, the indications for surgical resections have been extended from stage I and II to locally advanced, previously unresectable pancreatic cancer [63]. Tumors involving the portal vein and/or the superior mesenteric vein are considered to be borderline resectable (staging III). Apart from this anatomic factor, biological factor

(CA 19-9 level) and conditional factor (performance status) have also been incorporated into the definition of borderline resectability by the International Association of Pancreatology [80].

Table 2. American Joint Committee on Cancer (AJCC) TNM Staging of Pancreatic Cancer (8th edition, 2017).

Category/Staging	Definition
Category T (primary tumor)	
Tis	Carcinoma in situ
T1	Tumor ≤2 cm in greatest dimension
T2	Tumor >2 and ≤4 cm in greatest dimension
T3	Tumor >4 cm in greatest dimension
T4	Tumor involves the coeliac axis, superior mesenteric artery, and/or common hepatic artery, regardless of size
Category N (lymph nodes)	
N0	No regional lymph node metastasis
N1	1-3 metastatic regional lymph nodes
N2	≥4 metastatic regional lymph nodes
Category M (distant metastasis)	
M0	No distant metastasis
M1	Presence of one or more distant metastasis(es)
TNM Staging	
IA	T1N0M0
IB	T2N0M0
IIA	T3N0M0
IIB	T1-T3, N1M0
III	T1-T3, N2M0; or
	T4, any N, M0
IV	Any T, any N, M1

1.10 Prognosis

Pancreatic cancer is characterized by early metastasis and an extremely dismal prognosis. Its survival for all stages combined is the worst (6%) among all cancers. Most patients are diagnosed when the tumor has distant spread, with a median survival less than one year and 5-year survival of 3% [4, 34]. For resectable tumors, median survival and 5-year survival are approaching 30 months and 30%, respectively, after resection and adjuvant chemotherapy [66].

Surgery is the only potentially curative treatment and its indication has also been expanded given the improvements of surgery and chemotherapy over the past decades. On the other hand, metastases still occur within three years in most patients undergoing curative surgery and adjuvant chemotherapy. It has been suggested that systemic micrometastases may exist already at the time of resection for an apparently localized disease [13, 63]. This conception is supported by an animal model study, in which pancreatic epithelial cells invaded and entered the bloodstream unexpectedly early, before frank malignancy could be detected by rigorous histologic analysis [81].

Epithelial-mesenchymal transition (EMT) is a key step in metastasis formation. Recently, a ‘‘partial EMT’’ phenotype has been revealed in metastasis of pancreatic cancer, which allows tumors to migrate as clusters, contrasting with single-cell migration pattern associated with traditionally defined EMT mechanisms [82].

Effort should be made to optimize treatment strategies. For example, a subgroup of patients with resectable tumors might not benefit from surgery as micro-metastases already exist, but are exposed to a considerable risk of morbidity and mortality following surgery. For these patients, neoadjuvant chemotherapy followed by surgery may be a better therapeutic option. This conception has been reinforced by a recent clinical study involving 1184 patients with pancreatic cancer from three independent cohorts, in which approximately 50% of patients with high expression of S100A2 and S100A4 died within one year of surgical resection [83]. The investigators constructed and confirmed a pre-operative prognostic algorithm incorporating S100A2 and S100A4 expressions, which may guide the selection of surgery or neoadjuvant therapy, and avoid surgery in aggressive disease [83]. As a proof-of-concept, they have also found that biomarker expression status from pre-operative EUS-FNA samples was in high accordance with those from surgically resected samples [83].

Therefore, a good prediction of tumor behavior and prognosis, consequently stratified treatment choices, may improve survival and quality of life of patients [84]. Currently, clinical characteristics such as staging are most commonly used to predict the prognosis, while other markers, such as blood and tissue biomarkers, are emerging.

1.10.1 Prognostic clinical characteristics

In advanced stage disease, host-related factors such as patient performance status (ECOG score) and nutritional status, play a major prognostic role [85]. In contrast, the prognosis of patients with resectable pancreatic cancer is mainly driven by tumor-related factors and the administration of adjuvant chemotherapy [78].

The 8th AJCC TNM staging for resectable tumors is based on objective tumor size and number of lymph node invasion, both of which have been shown to predict the prognosis better than the 7th version [86]. Although one study showed that tumor size was associated with prognosis in a dependent fashion [87], tumor diameter is generally seen as a profound and independent prognostic factor. A threshold of 2 cm in tumor diameter is commonly accepted and has been adopted by the AJCC for T staging [88-91]. An investigation of nearly 60,000 patients concluded that in patients with resected tumors, larger tumor size was associated with worse tumor-specific survival [92]. However, tumor size was not associated with survival in patients with unresected tumors and there was a high rate of distant metastasis (30.6%) even in those with a tumor size ≤ 0.5 cm [92]. A population-based study showed that out of 5036 pancreatic cancer patients with at least 12 lymph nodes resected, 70.6% carried positive lymph nodes [93]. In that study, the number of positive lymph nodes in the resected specimen was a prognostic factor in patients with pancreatic cancer [93]. The association between the number of positive

lymph nodes and survival has also been supported by other studies [88, 94]. This has led to the revision of 8th AJCC TNM staging for pancreatic cancer by introducing two categories for node positive cancers according to the number of involved lymph nodes [77]. Positive lymph node ratio has also been proposed as a significant prognostic factor [95].

Tumor grade has also been reported as an independent prognostic factor in pancreatic cancer [96-98]. Incorporation of tumor grade into the AJCC staging for pancreatic cancer has showed better prediction of the outcome of pancreatic cancer [97], which was confirmed by other studies [96, 98].

Resection margin status is also independently associated with post-resectional survival for patients with tumors in all locations of the pancreas [99-101]. Due to different definitions of margins and R1, ways of sample slicing and rigidity of assessment, the reported R1 resection rates of patients undergoing pancreatic cancer surgery range from 16% to >75% [102]. In pancreatoduodenectomy specimens, circumferential margins should be assessed comprehensively and rigorously, including the anterior surface, which is not a true surgical resection margin [102]. Although international controversy remains regarding the definition of R0/R1, the definition of R1 resection as presence of tumor within 1 mm from the margin has been recognized in Europe [99, 103]. There are three ways of preparing of tissue sections (axial slicing, bivalving slicing and bread-loaf slicing), with no method showing a superiority [104]. A worldwide consensus toward a standard assessment of resection margin of pancreatic cancer is warranted.

1.10.2 Prognostic blood biomakers

Apart from its clinical utility for diagnosis, serum CA 19-9 is also a prognostic biomarker for pancreatic cancer [105-108]. In patients with resectable pancreatic cancer, low or declining levels of serum CA 19-9 after surgery have been shown to correlate with longer survival [106, 107]. One study has also indicated that patients with resected pancreatic cancer with low CA 19-9 levels tend to response better to chemotherapy than those with high CA 19-9 levels [106]. Besides, post-treatment CA 19-9 levels have been proposed as a prognostic marker in patients receiving neoadjuvant therapy with or without subsequent surgical resection [109]. In patients with advanced pancreatic cancer, baseline serum CA 19-9 levels were suggested to be an independent prognostic factor for survival [110, 111], while the levels after chemotherapy may also predict the benefits of treatment [110, 112].

The prognostic role of blood inflammatory profiles, especially neutrophil-to-lymphocyte ratio (NLR), have been frequently investigated. Although few studies failed to confirm the association between NLR and survival of pancreatic cancer [113], most studies and recent meta-analyses suggested that low NLR is associated with overall survival (OS) [114-118], both in resectable and advanced disease settings. However, NLR cut-off values vary among studies, partly contributing to the difficulty of its clinical application. Other blood inflammatory biomarkers such as C-reactive protein

[119, 120], C-reactive protein/albumin ratio [121] and platelet-lymphocyte ratio [118, 122, 123], have also been reported to play a prognostic role in pancreatic cancer.

One emerging interest of blood markers for pancreatic cancer is liquid biopsy [124, 125]. Through liquid biopsy, blood or other liquid samples are collected to analyze objects released from tumors, which include circulating tumor cells (CTCs), cell-free circulating nucleic acids, and extracellular vesicles (e.g. exosomes) containing nucleic acids and proteins [124]. The advantage of a liquid biopsy over EUS guided biopsies is that the former is non-invasive, and can be conducted repetitively for monitoring, which will reflect the entire tumor mass. Although the liquid biopsy technique is being improved, the lack of standardized technology platforms is still one of the factors limiting its clinical application [125]. CTCs are rare in blood from patients with cancer and usually need enrichment, in order to be detected. The hypovascular tumor mass in pancreas and sequestration of CTCs in the portal circulation may account for the low number of CTCs in pancreatic cancer [78, 124]. A meta-analysis including 16 publications suggested that high baseline CTCs in pancreatic cancer is associated with a worsened outcome [126]. Circulating tumor DNA (ctDNA) has also been reported to be an independent prognostic marker in advanced pancreatic cancer [127]. In a recent prospective study, KRAS mutations detected in ctDNA and exosome DNA, were in significant concordance to those detected in surgically resected tissue (>95%) [128]. Moreover, this liquid biopsy approach provides meaningfully predictive and prognostic information for both localized and metastatic pancreatic cancer. In conclusion, the liquid biopsy, as a diagnostic sample, is promising, and is expected to be applied in the clinical setting for pancreatic cancer. More prospective studies and a standardization of these techniques are needed.

1.10.3 Tissue biomarkers

A successful stratification of patients based on prognostic factors can guide a more individualized treatment strategy. Thus, there is an unmet need of a better prediction of the prognosis within pancreatic cancer. In clinical practice, the prognosis of pancreatic cancer is mainly predicted by tumor anatomy, patient performance status, and serum CA 19-9 levels. In the past decades, there have been enormous investigations of tissue molecular biomarkers in pancreatic cancer for prognostic purposes. Most of these biomarkers were assessed by immunohistochemistry in resected tissues from pancreatic cancer and were related to the patient outcome [129, 130]. A systematic review reported that from 2004 to 2014, a total of 230 tissue biomarkers had been suggested to play prognostic roles [129]. Besides, more prognostic tissue biomarkers are being discovered. Although many of them are promising, none has been translated into clinical practice.

Two meta-analyses published in 2011 have showed that vascular endothelial growth factor, Bcl-2, bax, p16, p21, survivin, Ki-67, COX-2, E-cadherin, S100A2 and PD-ECGF were significantly associated with the OS of pancreatic cancer, while well-documented biomarkers, p53, SMAD4 and EGFR failed to ascertain the associations in

pooled analyses [131]. CD24, S100A4, urokinase-type plasminogen activator receptor, atypical protein kinase C, and heat shock protein 27 were reported to be prognostic biomarkers of pancreatic cancer with highest ranking of REMARK criteria [129, 132].

1.11 Molecular subgrouping of pancreatic cancer

Employing omics studies on genome, transcriptome, and epigenomic profiling, several molecular classifications of pancreatic cancer have recently been proposed for better molecular understanding of the disease and individualized clinical care (Table 3) [30, 133-142]. Collisson et al. have classified pancreatic cancer into three subtypes: quasi-mesenchymal (QM-PDA), classical, and exocrine-like, with QM-PDA having the worst prognosis [135]. Subsequently, Moffitt et al. revealed two stromal subtypes (activated and normal) and two tumor subtypes (basal-like and classical) [138]. Bailey et al. also found four subtypes for pancreatic cancer: squamous, aberrantly differentiated endocrine exocrine (ADEX), pancreatic progenitor, and immunogenic [133]. All these three classifications have delineated molecular subtypes with different clinical outcomes in the studied cohort. More recent evidence has indicated that the exocrine-like and ADEX tumor subtypes might be the result from contamination with pancreatic acinar cells [138, 141]. Moreover, the squamous, QM-PDA and basal-like subtypes based on the three different classifications were highly overlapped in independent cohorts and so did the classical and pancreatic progenitor subtypes [133, 134, 138, 141]. Importantly, the classical/pancreatic progenitor and the basal-like/squamous subtypes have been successfully validated by a series of studies and continued to reflect the clinical outcomes [134, 141, 143, 144].

Omics-based subtyping of pancreatic cancer relies on large numbers of markers with relative expression in the whole studied cohort, which is currently difficult for clinical application. However, single or combined markers were also proposed to reflect these subtypes. For example, in Puleo's study [141], there was a significant association between MET and nuclear GLI1 expressions with stroma activated and pure basal-like subtypes, while human equilibrative transporter 1 (hENT1) is relatively more expressed in classical subtypes (i.e., pure and immune). Other studies have also managed to classify pancreatic cancer by immunohistochemistry [39, 145-147], a more clinically applicable method. Recently, deep mining of proteins in pancreatic cancer has also been presented, which leads to a reservoir of protein markers for subtyping of the disease [148, 149].

Table 3. Summary of molecular subtypes of pancreatic cancer.

References	Samples	Subtyping basement	Subtypes	Significance/comments
Erkan 2008 [39]	233 patient tumors	immunohistochemistry (a-SMA and collagen-I)	4 subtypes of stroma: fibrogenic, inert, dormant, and fibrolytic	activated stroma correlated with worse survival
Collisson 2011 [135]	63 patient tumors	transcriptome microdissection	quasi-mesenchymal (QM-PDA); classical; exocrine-like	QM-PDA has the worst prognosis
Moffitt 2015 [138]	145 primary/ 61 metastases	transcriptome; virtual microdissection	stromal subtypes: activated and normal; tumor subtypes: basal-like and classical	activated and basal-like subtypes have worse prognosis
Waddell 2015 [30]	100 patient tumors	genome	4 subtypes based on variation in chromosomal structure: stable; locally rearranged; scattered; unstable	unstable subtype has sensitivity to DNA-damaging agents
Bailey 2016 [133]	456 patient tumors	transcriptome	squamous; aberrantly differentiated endocrine exocrine (ADEX); pancreatic progenitor; immunogenic	squamous subtype has more frequent TP53-mutation and worse prognosis
Raphael 2017 [134]	150 patient tumors and blood samples	integrated genomic, transcriptomic, and proteomic profiling	a subtype harbored multiple KRAS mutations; a subtype with low epithelial-mesenchymal transition and high MTOR pathway scores; non-coding RNA based subtypes concordant with the basal-like and classical subtypes	neoplastic cellularity in tumor samples also influences the molecular characterization and subtyping
Connor 2017 [136]	160+95 patient tumors	genome, transcriptome immunohistochemistry	mutational signatures define 4 subtypes: age related, double-strand break repair (DSBR), mismatch repair (MMR), and 1 with unknown etiology	DSBR and MMR subtypes have elevated local antitumor immunity.
Nicolle 2017 [140]	29 patient-derived xenograft tumors	multiomic profiles	two major subtypes with extensive similarities to the basal and classical human PDAC subtypes.	potential therapeutic targets were revealed by tumor-stroma cross-talk
Knudsen 2017 [145]	109 patient tumors	immunohistochemistry histology, transcriptome	two Immunologic subtypes; three stromal subtypes; composite analysis defines four subtypes	may select patients for immunotherapy in the treatment
Tiriac 2018 [142]	44 patient-derived organoid culture	genome; transcriptome	cluster C1: enriched for TGFβ signaling; cluster C2: enriched for xenobiotic metabolism	this subtyping was similar with basal-like and classic subtyping
Tiriac 2018 [142]	66 patient-derived organoid culture	pharmacotyping and transcriptome	the least responsive; the most responsive; intermediate responsive	drug testing in patient-derived organoid cultures may guide treatment selection
Puleo 2018 [141]	309 formalin-fixed patient tumors	transcriptome	5 subtypes: pure basal like; stroma activated; desmoplastic; pure classical; immune classical	tumor subtypes my select therapies and predict patient outcomes
Lomberk 2018 [137]	23 patient-derived xenografts	histone modification, DNA methylation, transcriptome	epigenomic landscapes define two subtypes	can predict their relative aggressiveness and survival
Mueller 2018 [139]	38 mice tumor cell cultures	transcriptome	C1 characterized by mesenchymal cell differentiation; and C2 characterized by epithelial cell differentiation	C1 represents a pronounced EMT signature and undifferentiated histology
Mahajan 2018 [146]	385+93 patient tumors	immunohistochemistry on 7 markers	7 subtypes characterizing a prognostic signature	reflects immune cell and stromal signature
Wartenberg 2018 [147]	110 patient tumors	immunohistochemistry on 5 markers	the "immune escape"; the "immune rich"; the "immune exhausted"	immune rich subtype has the best outcome

1.12 Proteomics - a high resolution workhorse to identify disease regulating proteins

Clinical protein science is governed by the proteome, i.e., all proteins coded by the genome that has been identified to a major extent, reaching 90% of the whole coverage (https://hupo.org/), corresponding to 17,562 proteins annotated. This achievement is being made by a global science initiative driven by the Human Proteome Project (HPP) [150-152], an international project organized by the Human Proteome Organization (HUPO), whose aim is to revolutionize our understanding of the human proteome. HUPO is mapping the entire human proteome in a systematic effort using currently available and emerging technology platform. The key understanding of protein expression and function will enhance the clinical understanding of human medicine, and cancer disease mechanisms at the cellular level.

Two-dimensional gel electrophoresis has been the traditional way to profile the proteome and extensively used in proteomic studies in the past. By this method, proteins are separated in the gel medium based on the net charge and molecular weight of proteins [153]. However, it carries limitations of low reproducibility and low accuracy. Thus, its application has gradually been replaced by gel-free mass spectrometric techniques. For example, bottom-up or shotgun mass spectrometric technique measures the masses of proteolytic peptide fragments with extremely high resolution, which is also referred as peptide mass fingerprinting. The protein then is identified by matching the measured peptide masses to corresponding peptide masses from protein or nucleotide sequence databases.

1.13 Mass spectrometry-based proteomics for discovery of tissue biomarkers in pancreatic cancer

While genomic and transcriptomic studies have led to molecular characterization and classification of pancreatic cancer, a better understanding of pancreatic cancer at the protein level is also highly needed to identify key drivers of tumorigenesis, biomarkers for diagnosis and prognosis, and therapeutic targets. After all, drugs and medicines mostly target proteins, coded by the genome. Proteomic methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most commonly used approach for high-throughput protein identification and quantification in given samples. The advances of the methodology have made deep mining of proteomes from individual samples possible, including low-abundance proteins, thereby broadening the possibility to discover potential biomarkers [154].

Owing to this technique, together with validation in larger cohorts with different methods (e.g. immunohistochemistry), the pool of potential diagnostic and prognostic

biomarkers in PDAC have been greatly expanded [155-169]. Table 4 summarizes biomarker candidates for pancreatic cancer discovered by LC-MS/MS.

The most commonly applied proteomics approach is the “shotgun” or “bottom-up” approach, in which proteins are digested into peptides, followed by identification of the digests in the tandem MS platform, and their matching to peptides and proteins based on the existing protein database. By this method, proteins are also quantified and compared between groups of samples. There are two approaches of quantification, labeled or label-free quantifications [149]. Label-free quantification is based on signal intensity of the peptides (mass/charge) in the mass spectrometer. Labeled quantification introduces internal standard before MS analysis, by labelling peptides in one group distinguishable from other group(s).

Formalin-fixed paraffin-embedded (FFPE) tissues are the most accessible form of tissue specimens, which are commonly used in clinically histological evaluation and retrospective studies. Fortunately, the MS approach is now applicable and robust for biomarker discovery in these FFPE samples [159]. FFPE samples also allow tumor cell dissection (e.g. laser capture microdissection) for those interested in the tumoral proteome alone, as well as the direct correlation of histological observations with proteomic analysis [149].

Although previous proteomics work on pancreatic cancer have to some extent revealed proteome characteristics and biomarker candidates on a tissue level, the sample size involved in these studies are mostly small (as shown in Table 4), which limits a more detailed biological annotation in pancreatic cancer. Moreover, integration of proteomic, transcriptomic and genomic data will enhance biomarker translation, as well as a thorough molecular characterization of pancreatic cancer [149]. Recently, the Cancer Genome Atlas Research Network has provided such integrated analyses on 150 patients with pancreatic cancer, which revealed a complex molecular landscape of pancreatic cancer and provided a roadmap for precision medicine [134]. Given the increasingly improved performance of MS, in-depth proteome sequencing in pancreatic cancer tissues is a good approach to meet the urgent need of novel biomarkers of prognostic and diagnostic significance.

Table 4. Quantitative proteomics for biomarker discovery of pancreatic cancer.

Tissue type	TCD	Methods/ MS platforms	Groups	Dysregulated/ total proteins	IHC validated biomarker	References
frozen	no	Labelled; LCQ-Deca XP	2 PC; 2 N	151/656	ANXA2, ITGB1	Chen 2005 [155]
frozen	no	2 labelled methods; Q-STAR	10 PC; 10 N; 10 CP; 4 PanIN3	203/770; 38/402 in PanIN3	LAMB1, LGALS1, ACTN4	Pan 2009 [156]
frozen	no	Esquire HCT ultra	3 PC; 3 NPC	422/1009	TGFBI, LTBP2, ASPN	Turtoi 2011 [158]
frozen	no	subcellular; LTQ-XL Orbitrap	5 PC; 5 NPC	104/2393	BGN, TGFBI, SERPINF1, THBS2	McKinney 2011 [157]
FFPE	no	LTQ XL ion trap	11 PC; 8 NPC	47/214	--	Kojima 2012 [159]
FFPE	yes	LTQ Orbitrap XL	7 PC; 7 M	115/1504	S100P, SFN	Naidoo 2012 [170]
FFPE	yes	LTQ FT Ultra	3 PC; 3 CP; 3 N	21/525	--	Paulo 2012 [160]
FFPE	yes	LTQ-Orbitrap hybrid	4 PC-b; 4 PC-p	6/1099	Nm23/NDPK-A	Takadate 2012 [161]
FFPE	yes	LTQ-Orbitrap hybrid	4 PC-b; 4 PC-p; 5 N	170/1229	ECH1, GLUT1, OLFM4, STML2	Takadate 2013 [162]
frozen	no	LTQ-Orbitrap Velos	9 PC; 9 NPC	99/488	VTDB, PRELP	Iuga 2014 [163]
frozen	no	LTQ-Orbitrap hybrid	6 PC; 6 CP; 5 N	N-Glycosylation	--	Pan 2014 [164]
FFPE	yes	QSTAR Elite	10 PC; 10 NPC	247/805	PNMAL1 (prognostic)	Kuwae 2015 [166]
FFPE	yes	LTQ-Orbitrap hybrid	5 PC-b; 5 PC-p	332/1050	RPS8, PRELP, LGALS1	Chen 2015 [165]
FFPE	no	membrane-enriched; LTQ Orbitrap XL	10 PC; 9 NPC	238/~2500	--	Coleman 2018 [167]
frozen	no	HDMS	19 PC; 10 CP; 8 N	519/3192	--	Ger 2018 [168]
frozen	no	LTQ Orbitrap Elite	3 PC; 3 NPC	40/3000+	CPB1	Song 2018 [169]

TCD: tumor cell dissection; MS: mass spectrometry; FFPE: formalin-fixed paraffin-embedded; PC: pancreatic cancer; PC-b/p: PC with better/poor outcome; N: normal tissue; NPC: normal tissue adjacent to PC; M: metastasis.

2 Aim of the Thesis

The general aim of this thesis was to discover and then validate novel tissue biomarkers of prognostic significance in pancreatic cancer.

The specific aims were:

I. to identify tissue prognostic biomarkers in pancreatic cancer patients with different survival by proteomics approach. Based on these biomarker candidates:

II. to further validate the prognostic significance of CLCA1 in patients with pancreatic cancer;

III. to validate the prognostic candidate, galectin 4, in patients with pancreatic cancer;

IV. to validate the prognostic significance of P4HA2 and PRTN3 in patients with pancreatic cancer;

V. to investigate the prognostic value of stromal fibronectin in patients with pancreatic cancer.

3 Material and Methods

3.1 Study designs

A summary of the study designs in each paper included in this thesis is described in Table 5.

Table 5. Overview of design and participants in the papers of the thesis.

	I	II	III	IV	V
Study design	Retrospective CS	Retrospective CS	Retrospective CS	Retrospective CS	Retrospective CS
Subjects	tumor tissues	tumor tissues	tumor tissues	tumor tissues	tumor/normal tissues
Collection period	1995-2011	1996-2017	1996-2017	1996-2017	1996-2017
Last follow-up			April 27th, 2018		
Numbers	19	140	140	140	138+4
Methods	LC-MS/MS	TMA; IHC	TMA; IHC	TMA; IHC	TMA; IHC
Aim	discovery	validation	validation	validation	validation

CS: cohort study; IHC: immunohistochemistry; LC-MS/MS: Liquid chromatography tandem mass spectrometry; TMA: tissue microarray.

3.2 Study population

Patients with PDAC were diagnosed at the Department of Surgery, Skåne University Hospital, Lund and Malmö, Sweden, between 1995 and 2017. All the patients included have undergone surgical pancreatic resection. All tissues with hematoxylin & eosin staining have been re-evaluated and confirmed by our pathologist (A.S.). Positive resection margin (R1) was defined as presence of tumor within 1 mm from the margin. In the validation study (II-V), the staging information was updated according to the 8th version of AJCC staging. Clinical information was recorded carefully as all patients were followed up until death or the last follow-up date, April 27th, 2018. In study V, patients with serous (n=3) or mucinous (n=1) cystadenoma were included as a control group.

The clinical characteristics of all patients, including survival status, have been well annotated with thorough investigation and follow up. Information such as age, gender, administration of adjuvant chemotherapy and tumor size was obtained from the electronic medical records. Overall survival (OS) was defined as the time length from operation to death. Disease-free survival (DFS) was defined as the time length from operation to locoregional recurrence or distant metastasis, or death from a cause other

than PDAC. Recurrence of PDAC after surgery, either locoregional recurrence or metastasis, should be confirmed with undoubtable and well-established evidence by imaging and clinical presentations. For those patients who died due to PDAC, but without any information of when recurrence occurred, the dates of death were assigned as the dates of recurrence.

3.3 Samples

All tissue samples were obtained from the primary pancreas tumors and preserved in FFPE blocks. In study V, disease-free pancreas tissues from patients with serous (n=3) or mucinous (n=1) cystadenoma were also included.

In the discovery study (I), the tissue blocks were sectioned at a thickness of 10 µm. In each patient, two continuous sections of the tumor block were collected in one tube, which were barcoded and stored in the South Swedish Biobank, located in the Center of Excellence in Biological and Medical Mass Spectrometry (CEBMMS), D13, BMC, Lund, Sweden. Two groups of samples from patients with two extremes of survival, i.e., 10 patients with long survival (LS, >45 months) and 9 patients with short survival (SS, <12 months), were included. Of each tumor block, the fore and back continuous sections were stained by hematoxylin-eosin staining and carefully reviewed by our pathologist (Figure 7). Table 6 shows the clinical characteristics of the nineteen patients with PDAC involved in this study.

In validation studies (II-V), entire FFPE tissue blocks, rather than sectioned slides, were preserved for tissue microarrays (TMA) construction and immunohistochemistry. A total of 144 samples from patients with resected PDAC were collected. Notably, of the 19 samples from the discovery study (I), all were overlapped with samples from the validation studies (II-V), except for three samples, including two with SS (PL 15970-95 and PL 6442-99) and one with LS (PL 15365-05) because there were not enough tissue materials. The clinical information of these patients is summarized in Table 7. Ethical approval was obtained from the local human ethics committee at Lund University (Ref 2017/320).

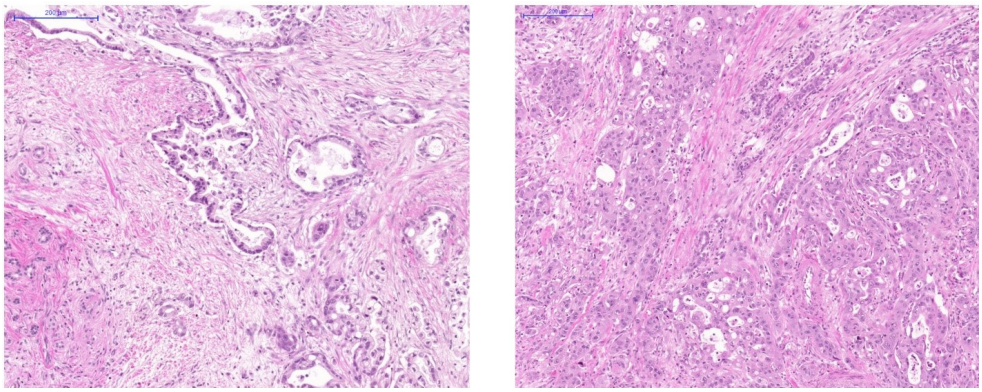

Figure 7. Representative pathological staining of FFPE tissues from patients with pancreatic cancer.
H&E, 10x magnification. Left: A patient with long survival with grade 2 tumor differentiation. Irregular gland formation exists in the dense stroma. Adenocarcinoma is located at the upper right quarter and infiltrates the atrophied pancreas parenchyma. Right: A patient with short survival with grade 3 tumor differentiation. Tumor cells with nuclear pleomorphy and relative scanty cytoplasma are popular in the slide.

Table 6. Clinical characteristics of patients with pancreatic ductal adenocarcinoma for proteomics study.

Characteristics	PDAC (SS)	PDAC (LS)
Diagnosis	PDAC	PDAC
Sex (female/male)	3/6	7/3
Age (median (range), year)	64(48-74)	71(43-77)
Current smoking	5	1
Diabetes mellitus	5	4
Tumor location pancreas head	9	10
Tumor diameter (cm)	2.5(1-6)	3(2-7)
Lymph node metastases	4	7
Staging		
IIA	6	3
IIB	3	7
R1 resection	4	3
Treatment		
Surgery	9	10
Adjuvant chemotherapy	5	9
Gemcitabine	3	5
5-FU	1	1
Capecitabine	0	2
Gemcitabine,5-FU	1	0
Gemcitabine, Capecitabine	0	1
Radiotherapy	1	0
Survival (mean (SD), month)	7.3(1.9-11.5)	59.1(47.0-120.9)

PDAC: Pancreatic ductal adenocarcinoma; SS: short survival; LS: long survival

Table 7. Clinical characteristics overview of 144 patients with pancreatic ductal adenocarcinoma for validation study.

Factors	N (%)	Median (IQR)	Missing
Age at diagnosis (years)		68.5 (63.0-73.0)	
Gender (female)	70 (48.6)		
Size of primary tumor (cm)		3.0 (2.5-4.0)	
T-stage			0.7%
- T1	19 (13.2)		
- T2	97 (67.4)		
- T3	26 (18.1)		
- T4	1 (0.7)		
N-stage			1.4%
- N0	34 (23.6)		
- N1	55 (38.2)		
- N2	53 (36.8)		
AJCC-stage, 8th edition			1.4%
- IA	6 (4.2)		
- IB	20 (13.9)		
- IIA	7 (4.9)		
- IIB	55 (38.2)		
- III	54 (37.5)		
Tumor differentiation			1.4%
- Well	7 (4.9)		
- Moderate	51 (35.4)		
- Poor	80 (55.6)		
- Anaplastic	4 (2.8)		
Positive resection margin (≥R1)	56 (38.9)		0.7%
Adjuvant chemotherapy	117 (81.3)		3.5%

AJCC, American Joint Committee on Cancer.

3.4 Sample preparation for MS analysis

For each of the 19 patients involved in the discovery study (I), two sections of FFPE tissues stored in the South Swedish Biobank were obtained and underwent two times of de-paraffinization, in which samples were immersed in 1mL of EnVision™ FLEX Target Retrieval Solution, High pH (1:50 dilution, Dako, Copenhagen, Denmark) for 10 min at 97 °C. Thereafter, spinning of samples was performed at 14,000g for 3 min to remove the supernatant. Next, resuspension of the pellets with 1mL 500 mM Tris-HCl pH 8.0 at 90 °C for 1.5 hours and subsequent spinning at 14,000g at 4 °C for 15 min, was performed. After removing the supernatant, we added 250 µL 6 M guanidine-HCl in 50 mM ammonium bicarbonate (AMBIC) to the samples, which were subjected to sonication through a sonication probe (Branson SLPe, Emerson Electric, MO, USA). The amplitude of sonication was set to twenty percent and applied for repeated 5 min with 20 seconds rest in-between on ice, in order to avoid increasing temperature. After spinning at 14,000g for 10 min, the supernatant was saved, followed by measurement

of protein concentration using Micro BCA Protein Assay Kit (Thermo Fisher Scientific, CA, USA). One hundred and fifty micrograms of sample proteins in final 180 µL AMBIC solvent, together with 7.5 µL of chicken lysozyme (0.02 µg/µL), were incubated with 3 mM DTT for 1 h at 56 °C, followed by incubation with 15 mM iodoacetamide for 30 min at 24 °C in dark. Next, nine times of volume of absolute alcohol was added to the denatured proteins and stored at -20 °C overnight, which was aimed to precipitate proteins and get rid of other chemicals. On the next day, the supernatant was removed with caution after a spinning step at 14,000g at 4 °C for 15 min. The remains were resuspended into 200 µL AMBIC. Subsequently, 1.25 micrograms of trypsin (Promega, WI, USA) was added to each sample to digest proteins into peptides at 37 °C for 18 h. Measurement of peptide concentrations were also performed after digestion by Micro BCA kit.

MicroSpin column (10-100 µg capacity, SEM HIL-SCX, The Nest group, MA, USA) was used for off-line separation of peptides, which is based on strong cation exchange. Thirty micrograms of digests were carefully added to this MicroSpin column, resulting in five fractions by sequential washing with 20 mM, 40mM, 60mM, 100mM and 500mM KCl in 10 mM KH_2PO4 containing 20% acetonitrile (pH=2.8). Next, each fraction was added into Ultra MicroSpin Silica C18 column (3-30 µg capacity, SUM SS18V, The Nest group) to remove KCl and KH_2PO4. Finally, samples were placed into SpeedVac vacuum concentrator to further purify peptide samples, followed by resuspension with thirty microliters of solvent A (0.1% formic acid). All the five fractions from each sample will be measured independently in the LC-MS/MS system.

3.5 nanoLC-MS/MS analysis

The digests were measured by liquid chromatography system with Easy-nLC 1000 pump, which was directly linked to Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific). Triplicate analyses in LC-MS/MS platform were applied to each fraction sample. For each measurement, one microgram of digests were obtained from the samples and injected into the C18 trap column (Acclaim PepMap 100 pre-column, 3 µm particles, 100 Å pore size, 2 cm x 75 µm ID, PN: 164705, Thermo Fisher Scientific), which was connected to a C18 analytical column (EASY-Spray column, 2 µm particles, 100 Å pore size, 25 cm x 75 µm ID, PN: ES802, Thermo Fisher Scientific). The adjustable high pressure allowed the liquid samples to move forward in the columns with a stable flow rate of 300 nL/min. The concentration of solvent B (0.1% formic acid in acetonitrile) in solvent A (0.1% formic acid) started with 7% and gradually increased to 26% in 70 min. In the next 20 min, the concentration reached 35% with a constant increase, which then was increased to 90% in 5 min and maintained for 15 min. A blank injection of solvent A was executed after each sample injection.

The peptides already separated by liquid chromatography were then analyzed on the mass spectrometer based on mass/charge (m/z) with a data-dependent acquisition (DDA) approach, in which the most abundant ten peptides with charge state no less than

two were picked up for fragmentation. Firstly, a spray voltage with 1.8-2.0 kV and capillary temperature of 280°C were applied to ionize peptides from the liquid chromatography system. Then, the Orbitrap mass analyzer archived the full MS scans with a m/z range of 350-1800, a resolution of 70,000 (at m/z 200), target AGC value of 1e6, and injection time of 100 ms at maximum. The selected peptides underwent fragmentation in the HCD collision cell with normalized collision energy of 30%. Tandem mass spectra were recorded with resolution of 35,000 (at m/z 200), target AGC value of 1e6, and injection time of 120 ms at maximum.

3.6 Verification by parallel reaction monitoring

To confirm the differentially expressed proteins between the two groups discovered by the above-mentioned DDA approach, a targeted proteomic approach based on the same LC-MS/MS platform, parallel reaction monitoring (PRM), was used. In this analysis, one microgram of unfractionated, instead of fractionated, protein digests were injected, while the mass spectrometry aimed at detecting one or two unique peptides of each protein of interest with higher sensitivity. A total of 110 peptides from 73 proteins, together with 5 peptides from chicken lysozyme (internal standard) were planned for detection. The information needed for targeted detection, including retention time, precursor m/z, and charge state of peptides, was obtained from the DDA approach and trained by preliminary PRM. Time-scheduled acquisition of peptides of interest was applied to targeted MS^2 mode, by which acquisition of each peptide occurred in 10 min windows centering on the pre-assigned retention time. MS1 scanning was carried out with a resolution of 17,500 resolution (AGC target 1×10^5, 50 ms maximum injection time), while MS/MS scans were obtained with a resolution of 70,000 at m/z 200.

3.7 Tissue microarray

Tissue microarray (TMA) is a well-recognized method for immunohistochemistry studies, which allows tissue samples to be handled more efficiently and with minimized experimental variability, and saves tissue material [171]. A tumoral area with diameter of two micrometers from FFPE tissue was marked by our pathologist (A.S.). Four tumoral areas were selected in each sample. TMA construction was performed by fixing the selected tissue cylinders into paraffin blocks with an automated tissue array device (Minicore® 3, Alphelys, Plaisir, France). In each TMA-block, there were approximately 120 tissue cylinders, corresponding to four replicate samples from 30 patients (Figure 8). The TMA blocks were then sliced into sections with a thickness of three micrometer and stored in room temperature (RT), pending for immunohistochemical studies.

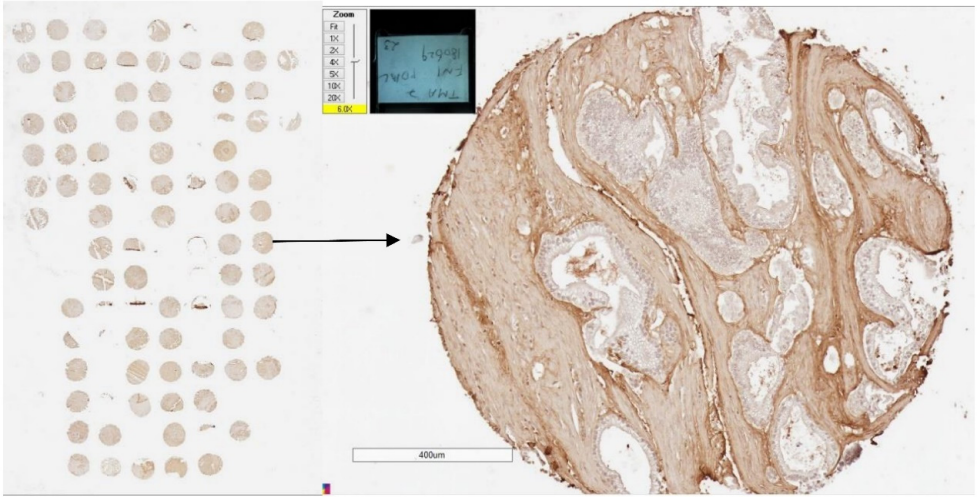

Figure 8. Representative slide of tissue microarray.
Left: a digitally scanned tissue microarray slide, which was stained with fibronectin; Right: one sample from the slide with higher magnification.

3.8 Immunohistochemistry

The immunohistochemical studies of the five proteins involved in this thesis were performed in sequence, including calcium-activated chloride channel regulator 1 (CLCA1), galectin 4, prolyl 4-hydroxylase subunit alpha 2 (P4HA2), proteinase 3 (PRTN3) and fibronectin (FN1). TMA slides were placed into the holder for heat treatment in 60°C for 1 hour, followed by cooling down in RT. Commercial EnVision FLEX Target Retrieval Solutions with high or low pH (Dako) was exploited to remove paraffin and retrieve antigen from the slides and rehydrate the tissue synchronously. After optimization of the experimental protocols, the high pH solution was finally applied to CLCA1 (K800421-2, Dako), while low pH solution was applied to galectin 4, P4HA2, PRTN3 and FN1 (K800521-2, Dako). After pre-heating of the retrieval solution to 60°C in the heating platform, an automated PT Link (Dako, Glostrup, Denmark), TMA slides were placed into the solution, which was subsequently heated up to 96°C and maintained at this temperature for 20 min. Next, the slides were removed to a container for 5 min to be immersed by phosphate-buffered saline (PBS, pH 7.6), which was composed of NaCl (137 mmol/L), KCl (2.7 mmol/L), Na_2HPO4 (10 mmol/L) and KH_2PO4 (1.8 mmol/L). This step was repeated for additional two times. Next, slides were sunken in fresh PBS containing 0.3% hydrogen peroxide and 1% methanol for 10 min, in order to block against endogenous peroxidase activity. After three times of rinse by PBS, the remaining buffer was removed from the slides, while the tissues were encircled with a liquid blocker pen. Subsequently, the slides were incubated with 5% goat serum in PBS with 1% BSA and 0.2% triton, for 1 hour at RT. Thereafter, the slides were sequentially immersed by avidin and biotin blocking kit (SP-

2001, Vector Laboratories, Burlingame, CA, USA) for 15 min at RT, respectively, according to the instructions made by the manufacturer. The slides were covered with primary antibodies, respectively, with individualized dilutions at 4°C overnight. Solvent without antibody was applied to one slide, which was regarded as the negative control. Antibody information and dilution is listed in Table 8.

Table 8. Primary and secondary antibodies.

Antibodies	Property	Cat. No.	Manufacturers	Dilution
CLCA1	rabbit monoclonal	ab180851	Abcam	1:2000
Galectin 4	rabbit polyclonal	HPA031184	Atlas Antibodies	1:100
P4HA2	rabbit polyclonal	HPA016997	Atlas Antibodies	1:300
PRTN3	rabbit polyclonal	HPA005938	Atlas Antibodies	1:300
FN1	rabbit monoclonal	ab2413	Abcam	1:4000
Secondary antibody	biotinylated goat anti-rabbit	BA-1000	Vector Laboratories	1:200

On the next day, after removal of the primary antibody and three times of rinse steps by PBS, the slides were immersed in the biotinylated second antibody (shown in Table 8) for 1 hour at RT. After three times of rinse by PBS, avidin-biotin-peroxidase complex (PK-6100, Vectastain Elite ABC-HRP Kit, Vector Laboratories) was prepared according to the instructions made by the manufacturer, to immerse the slides for 30 min at RT. Then, the slides were covered in chromogen diaminobenzidine (SK-4100, Vector Laboratories) for 5 min. After rinse in deionized water for 5 min, slides were removed to another container with Mayer's hematoxylin (Histolab, Gothenburg, Sweden) for 30 s, which was immediately followed by rinse in running tap water for 5 min. Then the slides underwent dehydration sequentially in distilled water, 70% ethanol, 90% ethanol, 95% ethanol, twice of absolute ethanol, and twice of xylen for 5 min. Microscope cover glasses were placed on the slides after applying the mounting medium Pertex (Histolab). The slides were then scanned by an Aperio scanscope scanner (Leica Biosystems, Wetzlar, Germany).

3.9 Scoring procedure

The evaluation of the antibody reactivity with the proteins in question was conducted by a senior pathologist (A.S) specialized in pancreatic histology, but not aware of the clinical information corresponding to the samples. The expression of the proteins, assessed in a semi-quantitative manner, was classified into negative if the reactivity was found in less than ten percent of the tumors, and into mild, moderate or strong according to the intensity, if the reactivity was presented in more than ten percent of the tumors. Negative or mild expressions were defined as low expression, whereas moderate and strong expressions were defined as high expression. For galectin 4 exclusively, negative and positive expressions were subtyped, with the latter representing mild, moderate or

strong expressions. The classification of galectin 4 was referred to the study from Hayashi et al. [172]

3.10 Bioinformatics and statistics

A series of softwares and databases were used for bioinformatics and statistics in the thesis (shown in Table 9). Original files from five fractions of each sample were integrated into one data file through multidimensional protein identification technology (MudPIT), which was subjected to peptide annotation in Proteome Discoverer 1.4 by matching mass spectra to a peptide from a searching database, Uniprot Human Reviewed (released 2013/09). Sequest HT served as the matching algorithm for protein identification and quantification. To trace the false discovery rate (FDR), a decoy database, including all proteins from the searching database, but with reversed sequence order, was also added to a searching engine. The precursor and fragment mass tolerances were set as 10 ppm and 0.02 Da, respectively. The searching engine was also endowed with the ability to identify oxidation and carbamidomethylation, which was introduced intentionally during the sample preparation. Peptides with two or more missed cleavages were not assigned for identification. Protein identifications relied on no less than two peptides with high confidence (FDR<1%). To quantify peptides, precursor ions area detector was used. Protein intensities were represented by mean values of the top three abundant peptides.

Proteins detected in more than five samples in either group were included for further analysis. Normalization of intensities of proteins to the median intensity in one sample (an integration of 5 fractions) was conducted, followed by log 2 transformation in order to obtain a normal distribution of the data, which was suitable for the subsequent comparison. Missing value imputation of intensities was employed by Perseus software. After averaging the values from three replicates of each sample, which has undergone normalization and log 2 transformation, protein expressions were compared between the two groups by Student's t-test. Notably, if proteins detected in one group were more frequent (≥5 samples) than those detected in the other group, these proteins were also regarded as differentially expressed. Hierarchical clustering and principal component analysis (PCA) were also shown by Perseus software for classifying samples and visualization of differentially expressed proteins.

In targeted proteomic studies by PRM, Skyline software was exploited for MS1 filtering and MS1 quantitation. The intensities of detected peptides from proteins of interest were log 2 transformed, followed by comparison in two groups using Student's t-test. The peptide with higher intensity was compared if two peptides were scheduled and quantified.

The bioinformatics analyses of pathways, protein-protein networks and upstream regulators of the differentially expressed proteins were conducted through the STRING database, Reactome, and a commercial platform, Ingenuity Pathway Analysis (IPA, Qiagen, Inc. Redwood City, CA, USA). Overrepresentation of the differentially

expressed proteins, as well as their functional annotations, were assessed by Gene Ontology resources, Panther, Reactome, David and IPA. In David, Panther and IPA, all the proteins identified from the DDA approach were resigned as the analysis backgrounds.

In the validation studies, Chi-square test, Fisher's exact test and Mann Whitney U test were employed to compare categorical and continuous clinical data between groups classified by expression of proteins. Kaplan-Meier analysis, log rank and Breslow tests were exploited to visualize and estimate the significance of difference in DFS and OS. Hazard ratios (HRs) were estimated by Cox regression proportional hazards models. A *P*-value less than 0.05 was defined as statistically significant. These statistics were done by STATA MP 14.1 (StataCorp LLC, Texas, USA) or SPSS (IBM. SPSS Statistics for Windows. Version 24.0. Armonk, NY, USA).

Table 9. Softwares and databases used for bioinformatics and statistics.

Softwares / databases	Description
Proteome Discoverer 1.4	protein identification and quantification
Perseus [173]	Data comparison, hierarchical clustering and principal component analysis
STRING [174]	Investigation of protein-protein networks
Ingenuity Pathway Analysis	Comprehensive bioinformatics including pathways, upstream regulators
Gene Ontology resources [175]	overrepresented functional annotations
Panther [176]	Protein classification and annotation
Reactome [177]	visualization, interpretation and analysis of pathways
David [178]	functional annotation of proteins
Skyline	MS1 filtering and MS1 quantitation in PRM
SPSS version 24.0	Data description, comparison, survival analysis
STATA MP 14.1	Data description, comparison, survival analysis

4 Results

4.1 Overview of proteome data

A preliminary study was conducted on one protein stock from a sample, in order to assess the stability of the LC-MS/MS platform and the technical reproducibility. The protein stock underwent three independent sample preparation and analyses in the LC-MS/MS platform. As a result, the protein intensities in the three replicates were in good correlation with each other (r^2=0.973, 0.920 and 0.931, respectively).

Of all the 19 samples with three replicates, the internal standard (spiked-in chick lysozyme) showed a coefficient of variations of 6.6% for its log 2 transformed and normalized intensities. As much as 3,000,000 peptide-spectrum matches (PSMs) corresponding to 58,505 peptides with high confidence have been identified, which can be mapped to 4,942 proteins. The large number of protein identifications were partly contributed by the fractionation procedure, an off-line separation of peptides (Figure 9). The protein intensities from the fractionation protocol were closely correlated with those obtained from the unfractionation protocol applying to an identical protein sample (r^2=0.9373).

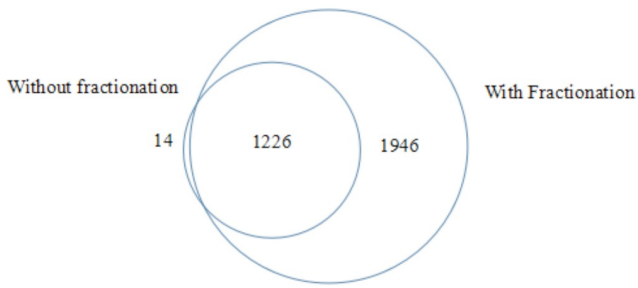

Figure 9. Venn diagram of the number of protein identifications by two approach of sample preparation.
The approach with factionation identified more than two times of proteins compared to the approach without fractionation.

Of the 4,942 proteins, 3,103 were presented in at least five samples in one or two group, which were qualified for further analyses. By cellular component analysis, 640 proteins were found to be plasma membrane proteins, while 108 were cell surface proteins and 163 were ECM proteins, all of which may be released to the bloodstream and potentially serve as blood biomarkers.

4.2 Potential prognostic protein candidates for pancreatic cancer

A total of 304 proteins showed statistical difference of intensities between LS group and the SS group ($P<0.05$). Of these, 171 proteins were considered to be significantly dysregulated in the LS or SS groups, which followed the criteria below: 1) folds change ≥2 or ≤0.5, and $P<0.05$; or 2) proteins that were more frequently detected (≥5) in one group than the other. Of these dysregulated proteins, 24 were upregulated in the SS group while 147 were upregulated in the LS group (see Figure 10).

The 171 differentially expressed proteins in 19 tissue samples were subjected to the two-way unsupervised hierarchical clustering and were visualized in the heat map (Figure 11). The clustering of 19 tissue samples was in good agreement with the clinical classification. PCA analysis based on the dysregulated proteins also classified patients into two groups that also stood for patients with LS and SS (Figure 12). Concerning the subcellular localization of the dysregulated proteins, a significant overrepresentation of mitochondrial proteins (34 proteins, $P=0.017$) was found by David analysis, especially mitochondrial large ribosomal subunit (6 proteins, $P=0.002$) and mitochondrial respiratory chain complex I (5 proteins, $P=0.033$). Besides, Panther pathways analysis of dysregulated proteins revealed an overrepresentation of Wnt signaling pathway (CDH1, CSNK2A2, GNA11, CTBP2, CDH17, SMARCE1, $P=0.02$).

STRING analysis of these dysregulated proteins, together with KRAS and TP53, showed enriched protein-protein interactions among these dysregulated proteins (Figure 13). Seven proteins were clustered in a tight interaction network centered on TP53, including CDH1, THBS1, MMP9, EPCAM, WDR5, CSNK2A2 and PADI4.

Top canonical pathways, which were revealed by IPA analysis on the dysregulated proteins between the SS and the LS groups, included oxidative phosphorylation and mitochondrial dysfunction. The differentially expressed proteins amounted to 7 out of 22 oxidative phosphorylation pathway proteins ($P=0.002$). IPA analysis revealed subnetworks with closely connected interaction. These subnetworks tend to center on certain protein hubs, whose roles in PDAC have been demonstrated previously. These protein hubs included Akt kinase and mitochondrial complex 1 proteins, NF-kB and TCF transcription factors, ERK kinases, collagens and matrix metalloproteases (MMPs), and HNF4A and mitochondrial ribosomal proteins.

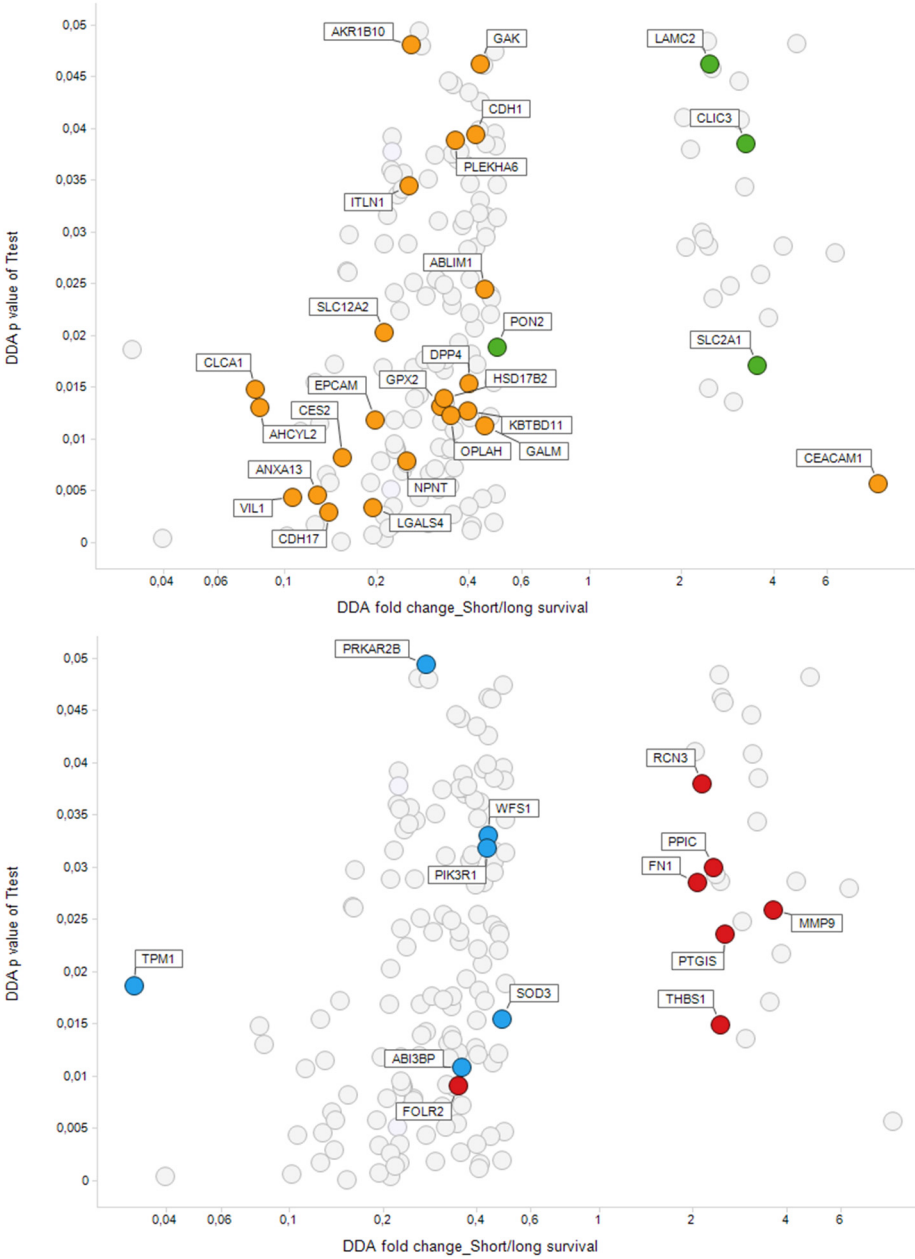

Figure 10. Volcano plot dysregulated proteins in patients with short or long survival.
The upper highlights dysregulated proteins that have been overlapped with the two subtypes of tumor-related factors by Moffitt et al, including basal tumor (green) and classic tumor (orange). The lower highlights proteins belonging to two subtypes of stroma related-factors by Moffitt et al, including normal stroma (blue) and activated stroma (red).

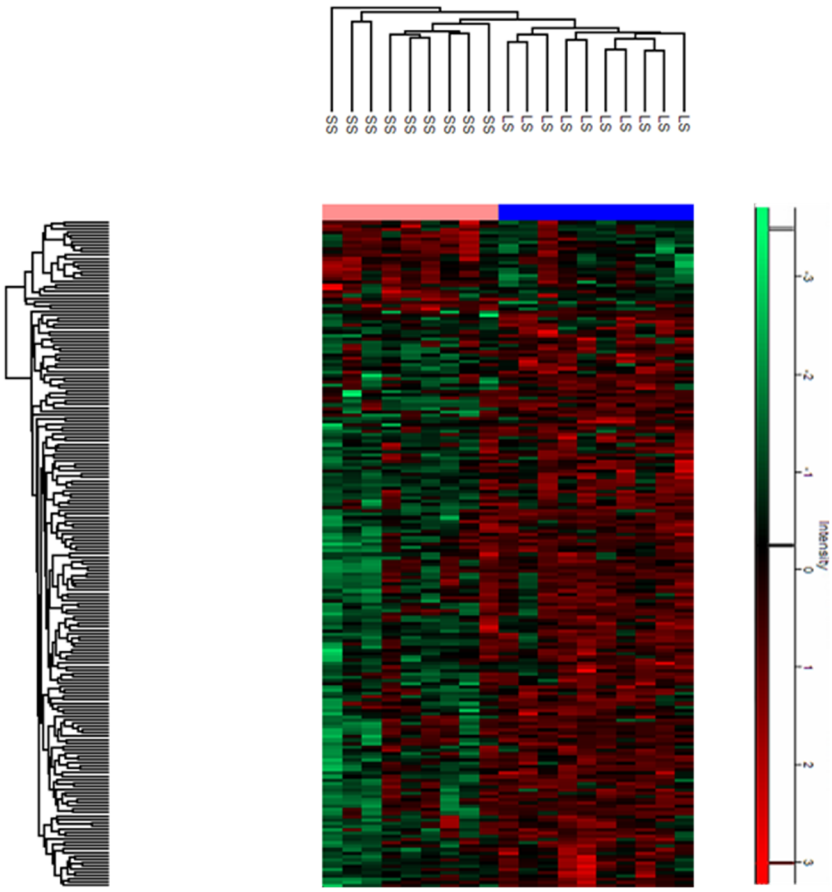

Figure 11. Hierarchical clustering of dysregulated proteins in patients with short survival (SS) and long survival (LS).
Two-way unsupervised hierarchical clustering of the 171 dysregulated proteins in pancreatic cancer patients with SS and LS ($P < 0.01$, fold change ≤ 0.5 or ≥ 2) shows well separation of the SS and LS groups.

IPA analysis enabled identification of possible upstream regulators that were potentially responsible for the dysregulation of the proteins. As a result, HNF1A and CTNNB1, a well-known cancer regulatory hub important for the Wnt signaling pathway, were proposed as upstream regulators. Eight dysregulated proteins (ALDH3A2, CEACAM1, CRAT, EPCAM, GPX2, HSD17B2, MUC6 and PCCA) are shown in the HNF1A mechanistic network (Figure 14).

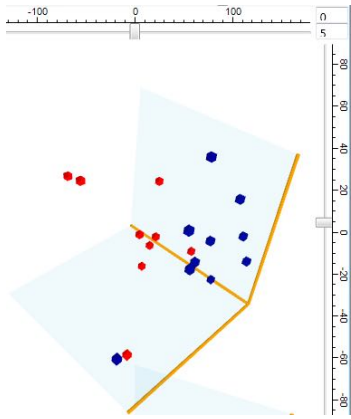

Figure 12. Global principal component analysis based on dysregulated proteins in patients with short or long survival.
Red dots stand for samples from pancreatic cancer patients with short survival, while blue dots stand for samples from patients with long survival.

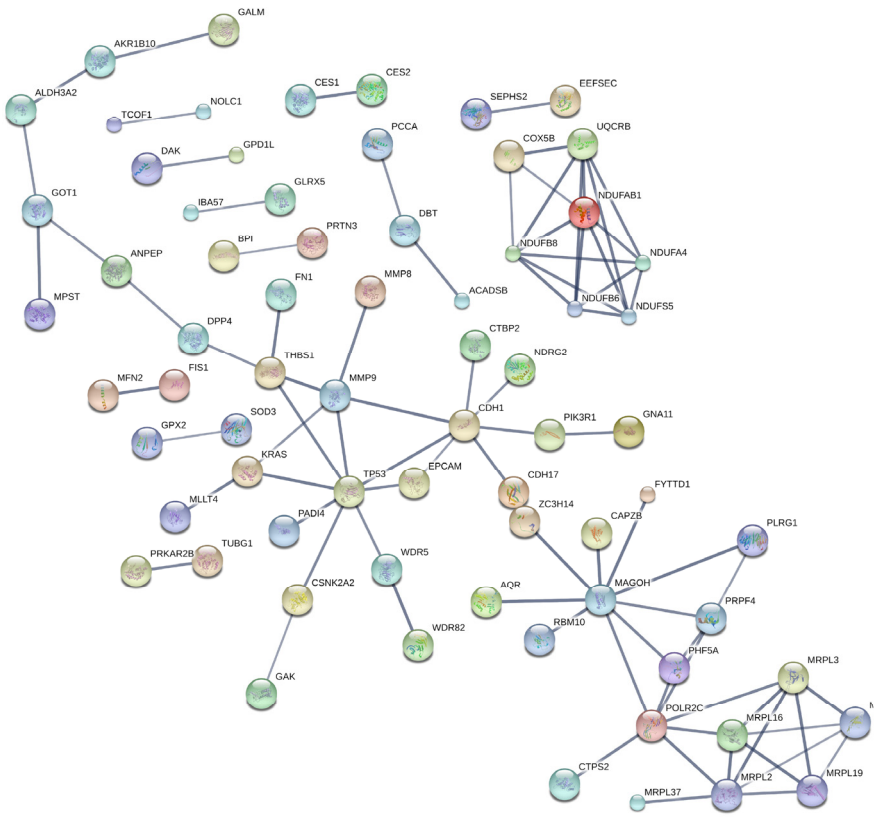

Figure 13. Protein-protein networks of dysregulated proteins in patients with short or long survival.
Protein-protein networks of 171 differentially expressed proteins analyzed by the STRING database. TP53 and KRAS, which were not detected in this study, were also included for analysis. TP53 was interacted by 7 dysregulated proteins (CDH1, THBS1, MMP9, EPCAM, WDR5, CSNK2A2, PADI4).

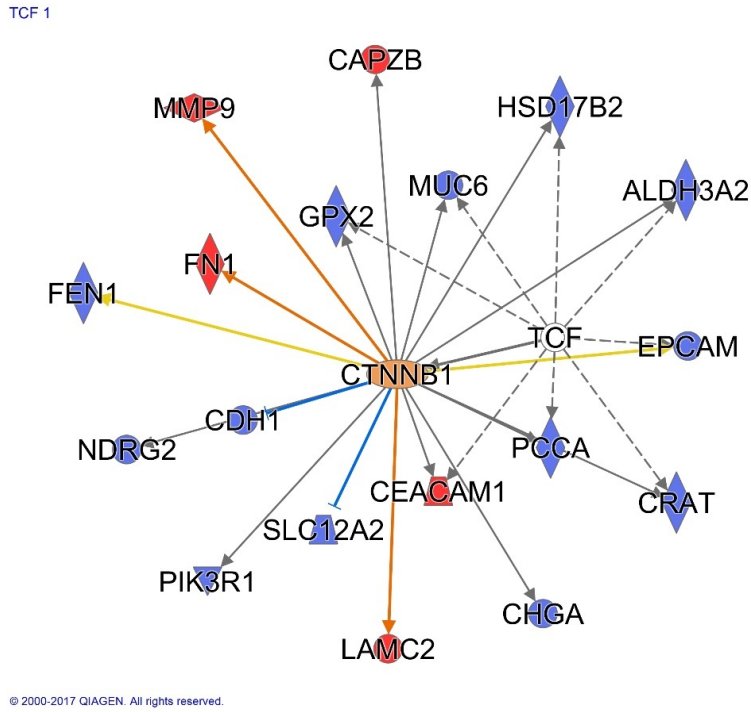

Figure 14. Ingenuity Pathway Analysis identified two hubs of upstream regulatory network (TCF1 and CTNNB1). Red and blue colors highlight proteins upregulated in patients with short survival and long survival, respectively. Red and blue lines indicate the activating and inhibitory roles of the hubs to downstream proteins, respectively. Yellow line indicates that the role of the hubs was inconsistent with the change of downstream protein levels.

4.3 Verification of candidate prognostic proteins by targeted MS/MS

To confirm the result of comparison of protein expressions between the LS and the SS groups, 171 dysregulated proteins discovered by DDA proteomics approach were subjected to verification by targeted proteomics, PRM. A total of 73 proteins were able to be scheduled in one PRM analysis. Consequently, there were 36 statistically dysregulated proteins between the LS and the SS groups ($P<0.05$), of which 25 showed significant fold changes (>1.5 or <0.5). Of these 25 prognostic protein candidates, 7 were upregulated in the SS group (MMP9, CLIC3, MMP8, PRTN3, P4HA2, THBS1, FN1), while 18 proteins were upregulated in the LS group (TMED4, GPD1L, SOD3, NPNT, ABHD14B, ACADSB, DHRS1, EPCAM, WDR82, HDHD2, TPPP3, CHGA, LGALS4, TTC38, COQ9, CES2, VIL1, CLCA1) (Figure 15 and Table 10).

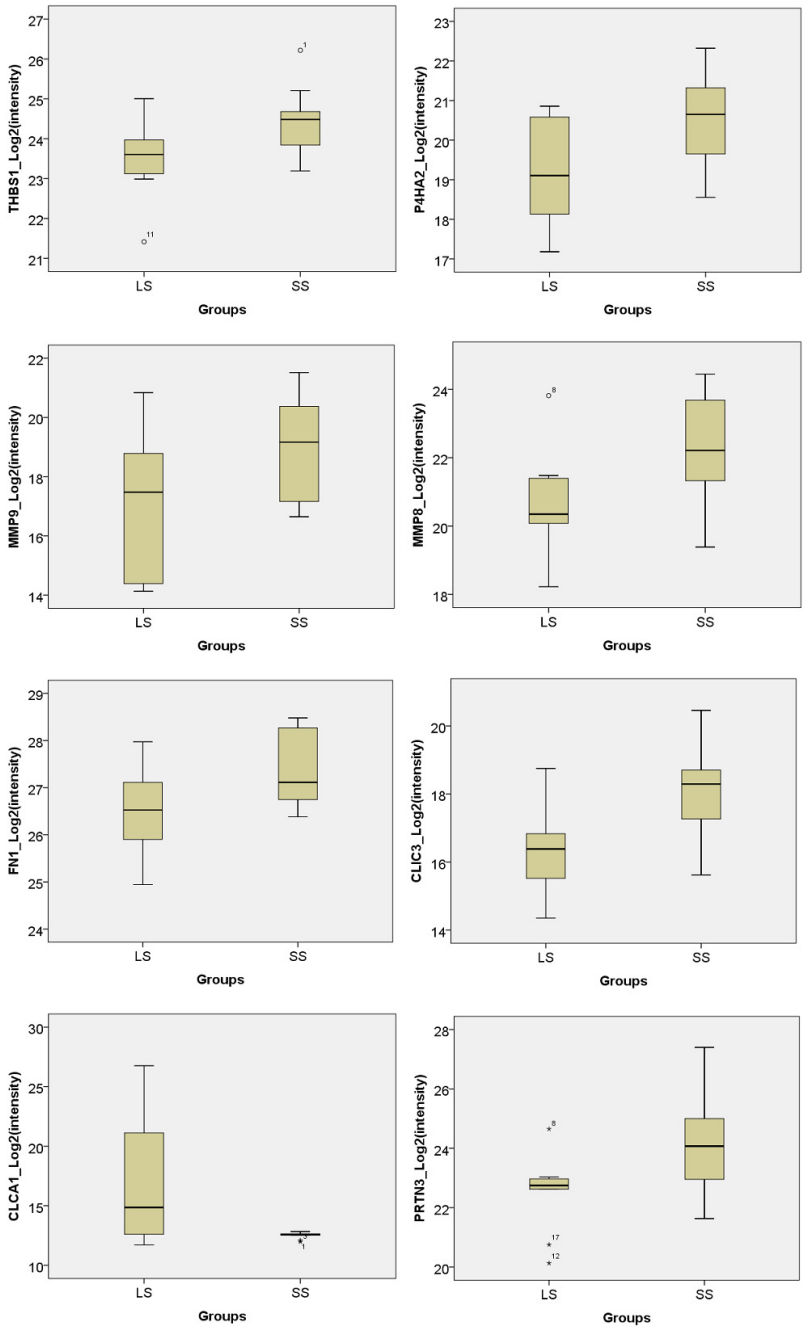

Figure 15. Comparison of intensities of dysregulated proteins in pancreatic cancer patients with short survival (SS) and long survival (LS).
Intensities of proteins in the two groups by targeted proteomics approach is shown in boxplot. Intensities of 7 proteins (THBS1, P4HA2, MMP9, MMP8, FN1, CLIC3, PRTN3) were increased in the SS group (SS/LS fold change > 1.5, $P<0.05$), whereas CLCA1 was significantly increased in the LS group (SS/LS fold change < 0.5, $P<0.05$).

Table 10. Potential biomarkers of prognostic significance in pancreatic cancer.

Entry	Gene	DDA				PRM			Description
		LS (n)	SS (n)	P value	SS/LS Fold change	Pep. no.	P value	SS/LS Fold change	
P14780	MMP9	10	9	0.026	3.62	2	0.045	4.44	Matrix metalloproteinase-9
O95833	CLIC3	1	6	0.039	3.25	2	0.010	3.42	Chloride intracellular channel protein 3
P22894	MMP8	1	6	0.029	4.28	1	0.046	3.06	Neutrophil collagenase
P24158	PRTN3	7	9	0.022	3.85	2	0.031	2.98	Myeloblastin
O15460-2	P4HA2	6	9	0.025	2.88	2	0.029	2.66	Isoform IIa of Prolyl 4-hydroxylase subunit alpha-2
P07996	THBS1	10	9	0.015	2.46	2	0.028	2.01	Thrombospondin-1
P02751	FN1	10	9	0.029	2.07	2	0.034	1.92	Fibronectin
A8K7I4	CLCA1	5	0	0.015	0.08	1	0.029	0.05	Calcium-activated chloride channel regulator 1
P09327	VIL1	10	5	0.004	0.11	2	0.008	0.12	Villin-1
O00748	CES2	5	0	0.008	0.15	2	0.029	0.16	Cocaine esterase
O75208	COQ9	8	2	0.004	0.27	2	0.004	0.19	Ubiquinone biosynthesis protein COQ9, mitochondrial
Q5R3I4	TTC38	10	5	0.017	0.21	1	0.035	0.20	Tetratricopeptide repeat protein 38
P56470	LGALS4	10	8	0.003	0.19	2	0.005	0.23	Galectin-4
P10645	CHGA	7	2	0.038	0.23	2	0.025	0.27	Chromogranin-A
Q9BW30	TPPP3	9	1	0.001	0.10	1	0.000	0.28	Tubulin polymerization-promoting protein family member 3
Q9H0R4	HDHD2	10	5	0.005	0.22	2	0.026	0.30	Isoform 2 of Haloacid dehalogenase-like hydrolase domain-containing protein 2
Q6UXN9	WDR82	7	2	0.039	0.22	1	0.023	0.31	WD repeat-containing protein 82
P16422	EPCAM	8	2	0.012	0.20	1	0.021	0.33	Epithelial cell adhesion molecule
Q96LJ7	DHRS1	7	1	0.007	0.24	1	0.017	0.37	Dehydrogenase/reductase SDR family member 1
P45954	ACADSB	9	3	0.017	0.15	2	0.026	0.38	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial
Q96IU4	ABHD14B	10	9	0.007	0.31	2	0.013	0.46	Alpha/beta hydrolase domain-containing protein 14B
Q6UXI9-6	NPNT	10	3	0.008	0.25	1	0.016	0.46	Isoform 6 of Nephronectin
P08294	SOD3	10	9	0.015	0.48	2	0.003	0.47	Extracellular superoxide dismutase (Cu-Zn)
Q8N335	GPD1L	9	4	0.030	0.16	2	0.010	0.47	Glycerol-3-phosphate dehydrogenase 1-like protein
Q7Z7H5	TMED4	10	4	0.002	0.13	2	0.040	0.48	Transmembrane emp24 domain-containing protein 4

DDA: data-dependent acquisition; LS: long survival group; Pep. No: number of peptides for proteins in the PRM panel; PRM: parallel reaction monitoring; SS: short survival group. (n): numbers of samples with positive detections in the group by DDA.

4.4 Validation studies by immunohistochemistry

4.4.1 CLCA1

CLCA1 expression in pancreatic cancer

Table 11 presents the baseline characteristics of 140 patients with pancreatic cancer, which is stratified by CLCA1 expression. Most tumors presented positive expressions of CLCA1 (90/140, 64.3%). CLCA1 reactivity was only shown in tumor cells. Figure 16 shows representative immunostaining of CLCA1 in pancreatic cancer.

Table 11. Clinicopathological features of low and high CLCA1 expression subgroups of patients with pancreatic cancer.

Factors		All patients N = 140	Low CLCA1 N = 87	High CLCA1 N = 53	*P* value	Missing
Age, years		69 (63-73)	69 (62-75)	68 (64-72)	0.258	
Female gender		67 (47.9)	43 (49.4)	24 (45.3)	0.634	
T-stage					0.649	0.7%
-	T1	19 (13.6)	12 (13.8)	7 (13.2)		
-	T2	93 (66.4)	58 (66.7)	35 (66.0)		
-	T3	26 (18.6)	16 (18.4)	10 (18.9)		
-	T4	1 (0.7)	0	1 (1.9)		
N-stage					0.485	1.4%
-	N0	33 (23.6)	21 (24.1)	12 (22.6)		
-	N1	54 (38.6)	30 (34.5)	24 (45.3)		
-	N2	51 (36.4)	34 (39.1)	17 (32.1)		
AJCC stage, 8th edition					0.835	1.4%
-	IA	6 (4.3)	4 (4.6)	2 (3.8)		
-	IB	19 (13.6)	12 (13.8)	7 (13.2)		
-	IIA	7 (5.0)	4 (4.6)	3 (5.7)		
-	IIB	54 (38.6)	31 (35.6)	23 (43.4)		
-	III	52 (37.1)	34 (39.1)	18 (34.0)		
Tumor differentiation					0.879	1.4%
-	Well	7 (5.0)	4 (4.6)	3 (5.7)		
-	Moderate	48 (34.3)	28 (32.2)	20 (37.7)		
-	Poor	79 (56.4)	50 (57.5)	29 (54.7)		
-	Undifferentiated	4 (2.9)	3 (3.4)	1 (1.9)		
R1 resection margin		55 (39.3)	35 (40.2)	20 (37.7)	0.552	0.7%
Adjuvant chemotherapy		113 (80.7)	68 (78.2)	45 (84.9)	0.129	3.6%

Qualitative data is expressed as N (%) and quantitative data as median (interquartile range). AJCC, American Joint Committee on Cancer.

Association of CLCA1 expression with the clinical features

There was no statistical difference of CLCA1 expression between subgroups of patients stratified by clinical factors, including age, gender, TNM stage, histological grade, resection margin status and presence of adjuvant chemotherapy.

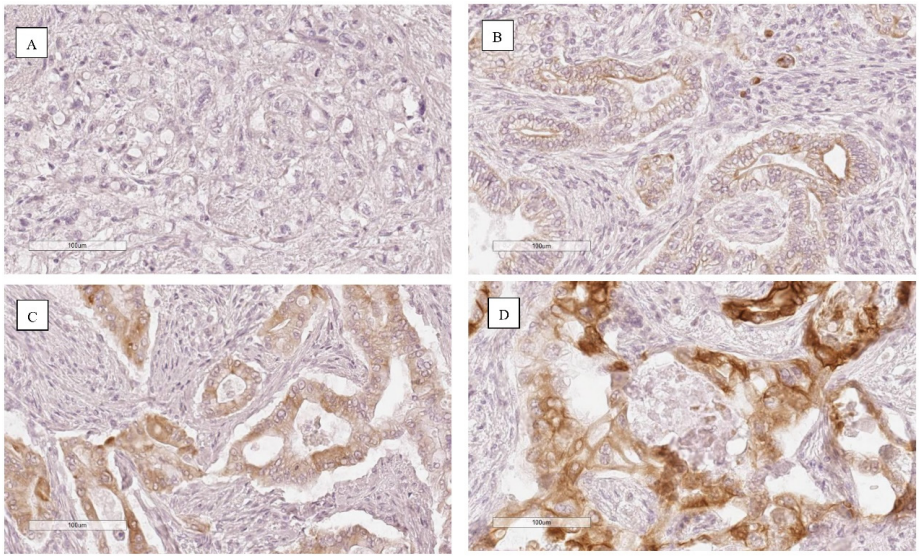

Figure 16. Representative microscopic immunostaining of CLCA1 expression in pancreatic cancer.
A: negative staining, B: weak staining, C: moderate staining, D: strong staining.

Association between CLCA1 expression and survival

Low CLCA1 expression was found to be associated with a shorter DFS by Kaplan-Meier analysis (median DFS 11.9 vs. 17.5 months, *P*=0.042, Figure 17). Similar results were achieved by univariable Cox regression analysis (HR 0.66, 95% CI: 0.44-0.99, *P*=0.044). The association remained significant in the multivariable Cox regression model (HR 0.61, 95% CI: 0.40-0.92, *P*=0.019), adjusted for differentiation grade and resection margin status (Table 12). The estimated median OS in the low CLCA1 expression group was slightly shorter than in the high CLCA1 expression group (23.5 and 27.8 months, respectively). However, there was no statistical difference of OS in the two groups (*P*>0.05) (Figure 18).

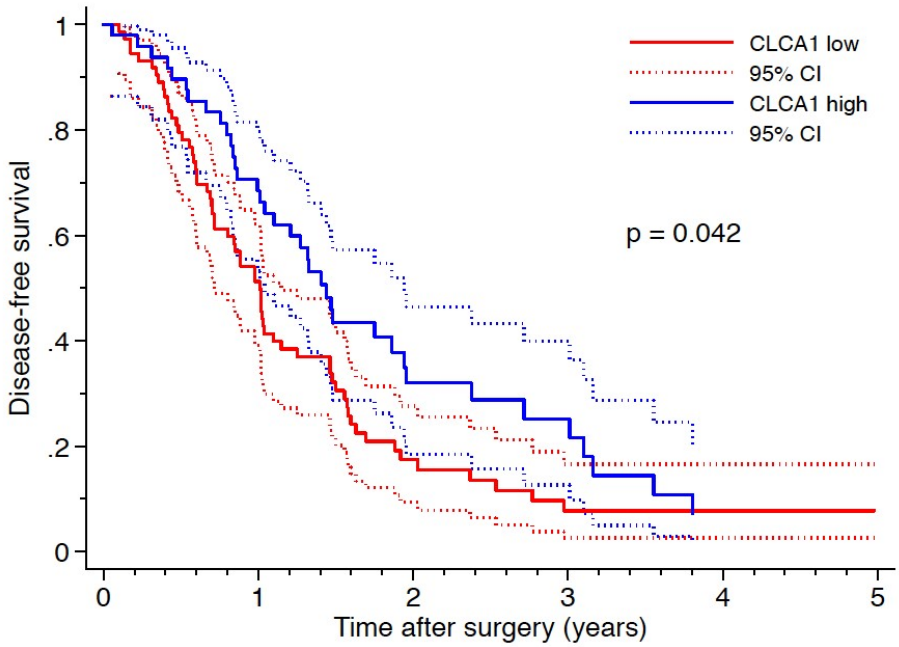

Figure 17. Low CLCA1 expression on tumors from patients with pancreatic cancer is associated with a worsened disease-free survival.

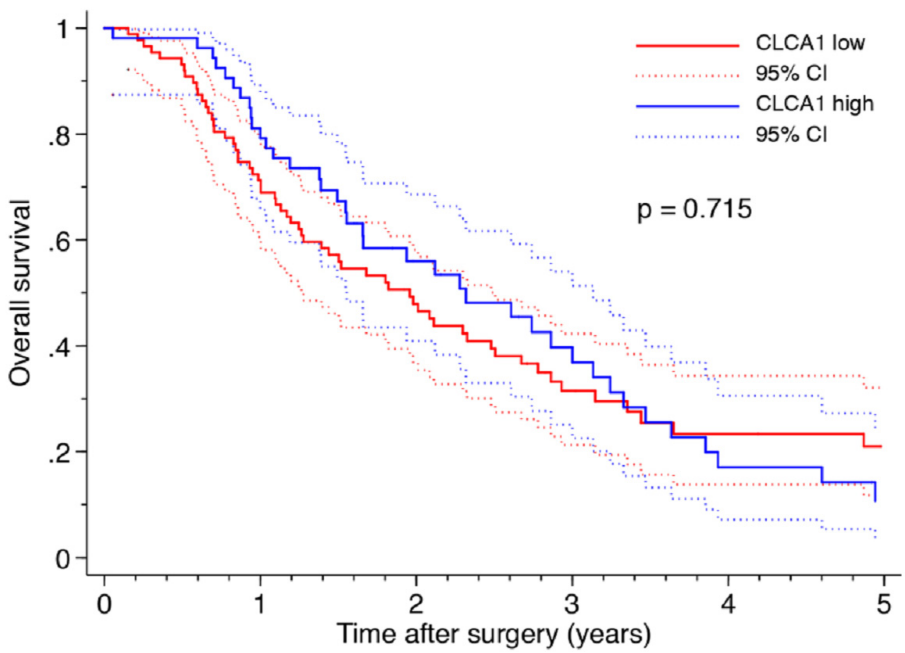

Figure 18. Overall survival curve of low and high CLCA1 expression and in patients with resected pancreatic cancer.

Table 12. Cox regression analyses of disease-free survival in patients with resected pancreatic cancer.

Variables	Univariable analysis			Multivariable analysis		
	HR	95%CI	P value	HR	95% CI	P value
Age	0.98	0.96-1.00	0.076			
Female gender	0.66	0.45-0.98	0.039			
T-stage	1.14	0.82-1.58	0.441			
N-stage	1.14	0.89-1.45	0.311			
Differentiation grade	1.48	1.06-2.07	0.023	1.55	1.09-2.18	0.014
Resection margin (R1)	1.55	1.02-2.33	0.036	1.63	1.07-2.49	0.023
Adjuvant chemotherapy	1.57	0.86-2.89	0.144			
CLCA1 expression, high vs low	0.66	0.44-0.99	0.044	0.61	0.40-0.92	0.019

4.4.2 Galectin 4

Expression of Galectin 4 in pancreatic cancer

The clinicopathological features of patients with pancreatic cancer stratified by galectin 4 expression are shown in Table 13. Staining of galectin 4 was found positive in cytoplasmic/membranous and nuclear part of tumor cells in most patients (n=111, 79.3%), which was categorized into weak (n=32, 22.9%), moderate (n=51, 36.4%) and strong (n=28, 20.0%). Figure 19 shows representative reactivity of galectin 4 in tumor tissues from patients with pancreatic cancer. There was a significant association of galectin 4 expression with tumor differentiation (*P*=0.001), tumor size (*P*=0.008) or administration of adjuvant chemotherapy (*P*=0.019).

Association between galectin 4 expression and DFS

There was a significant association between galectin 4 expression and disease recurrence within the first year of surgery (*P*=0.014). The finding was confirmed by multivariable analysis (adjusted HR 0.485, *P*=0.027). Expression of galectin 4 was not associated with 3- or 5-year DFS (Table 14). Kaplan-Meier analysis estimated that the median DFS in patients with galectin 4-negativity and those with galectin 4-positivity were 10.4 and 15.9 months, respectively (log-rank *P*=0.224, Breslow *P*=0.087), Figure 20.

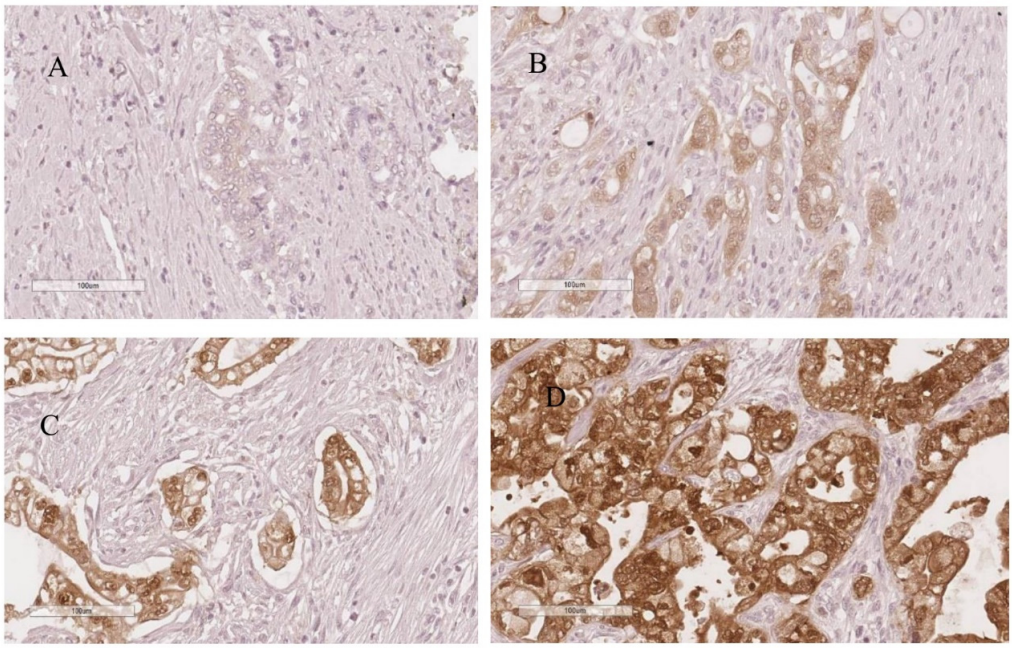

Figure 19. Representative immunostaining of galectin 4 in pancreatic cancer.
A) negative staining, B) weak staining, C) moderate staining, D) strong staining.

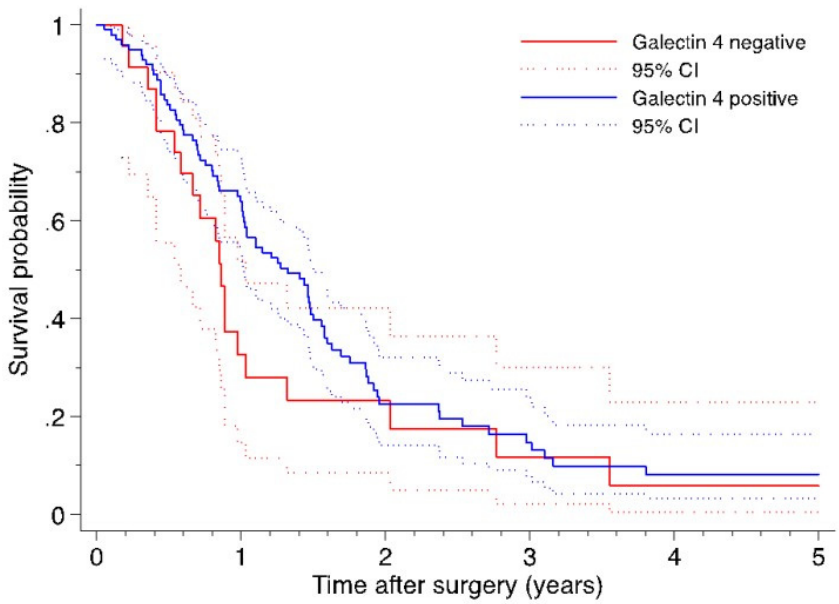

Figure 20. The association of galectin 4 expression with disease-free survival in patients with resected pancreatic cancer.
Log-rank *P*=0.224, Breslow *P*=0.087.

Association between galectin 4 expression and OS

The expression of galectin 4 was significantly associated with 1-year OS (*P*=0.036), which was confirmed in multivariable analysis (adjusted HR 0.482, *P*=0.047). Galectin 4 expression was also associated with 3-year OS in univariable analysis (*P*=0.031) and multivariable analysis (adjusted HR 0.550, *P*=0.025). However, there was no association between galectin 4 expression and the 5-year OS. The median OS was 14.0 months and 27.6 months in galectin 4-negative and galectin 4-positive groups, respectively (log-rank *P*=0.118, Breslow *P*=0.021), Figure 21.

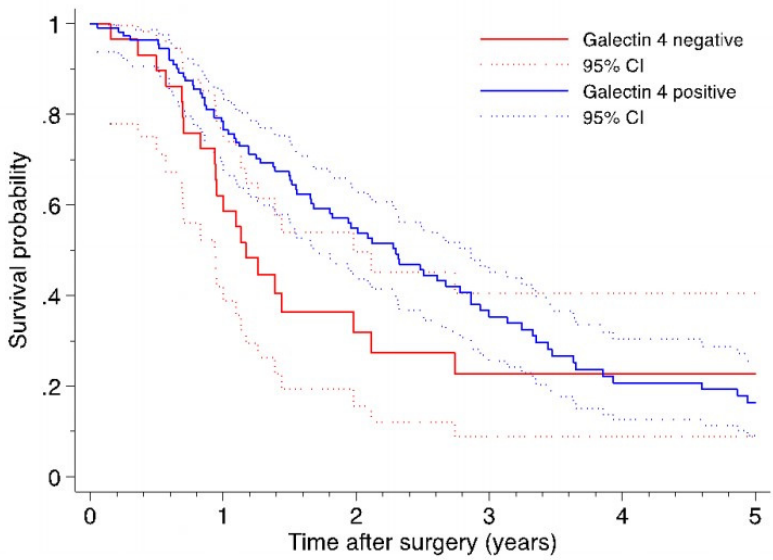

Figure 21. Overall survival curves by galectin 4 expression in patients with surgically resected pancreatic cancer. Log-rank *P*=0.118, Breslow *P* =0.021.

Table 13. Clinicopathological features of negative and positive galectin 4 expression subgroups of patients with pancreatic cancer.

Factors	All patients	Galectin 4, Negative Median (iqr) or N (%)	Galectin 4, Positive Median (iqr) or N (%)	*P* value	Missing
Age, years	69 (63-73)	70 (64-76)	68 (63-73)	0.133	
Female gender	66 (47.1)	13 (44.8)	53 (47.7)	0.779	
Tumor size ≥2 cm	118 (84.3)	29 (100)	89 (80.2)	**0.008**	
T-stage				0.389	0.7%
T1	18 (12.9)	0	18 (16.4)		
T2	94 (67.6)	25 (86.2)	69 (62.7)		
T3	26 (18.7)	4 (13.8)	22 (20.0)		
T4	1 (0.7)	0	1 (0.9)		
N-stage				0.519	1.4%
N0	34 (24.6)	5 (17.2)	29 (26.6)		
N1	52 (37.7)	11 (37.9)	41 (37.6)		
N2	52 (37.7)	13 (44.8)	39 (35.8)		
AJCC stage				0.464	1.4%
IA	6 (4.3)	0	6 (5.5)		
IB	20 (14.5)	5 (17.2)	15 (13.8)		
IIA	7 (5.1)	0	7 (6.4)		
IIB	52 (37.7)	11 (37.9)	41 (37.6)		
III	53 (38.4)	13 (44.8)	40 (36.7)		
Tumor differentiation				**0.001**	1.4%
Well	7 (2.9)	0	7 (6.4)		
Moderate	48 (57.2)	3 (10.3)	45 (41.3)		
Poor	79 (34.8)	26 (89.7)	53 (48.6)		
Undifferentiated	4 (5.1)	0	4 (3.7)		
R1 resection	54 (38.8)	11 (37.9)	43 (39.1)	0.909	0.7%
Adjuvant chemotherapy	113 (83.7)	19 (67.9)	94 (87.9)	**0.019**	3.6%

AJCC, American Joint Committee on Cancer, 8th edition; iqr, interquartile range.

Table 14. Cox regression analyses of galectin 4 expression with disease-free survival and overall survival.

Survival	Unadjusted			Adjusted*		
	HR	95% CI	*P* value	HR	95% CI	*P* value
1-year DFS	0.465	0.253-0.855	**0.014**	0.485	0.256-0.920	**0.027**
3-year DFS	0.714	0.430-1.186	0.193	0.624	0.360-1.081	0.093
5-year DFS	0.737	0.450-1.208	0.226	0.638	0.371-1.095	0.103
1-year OS	0.475	0.238-0.951	**0.036**	0.482	0.235-0.989	**0.047**
3-year OS	0.579	0.353-0.951	**0.031**	0.550	0.327-0.928	**0.025**
5-year OS	0.676	0.416-1.098	0.114	0.636	0.380-1.063	0.084

DFS, disease-free survival; OS, overall survival. *Adjusted for age, gender, AJCC stage, and resection margin status.

4.4.3 P4HA2 and PRTN3

P4HA2 expression in pancreatic cancer

The staining of P4HA2 was positive in the cytoplasm and cell membrane of tumor cells in 133 patients (95%), which was further denoted as weak staining (n=32, 22.9%), moderate staining (n=63, 45.0%) and strong staining (n=38, 27.1%). Myofibroblasts were positive for P4HA2 in tumors from all patients. Figure 22 shows representative immunohistochemical images of P4HA2 expression in pancreatic cancer.

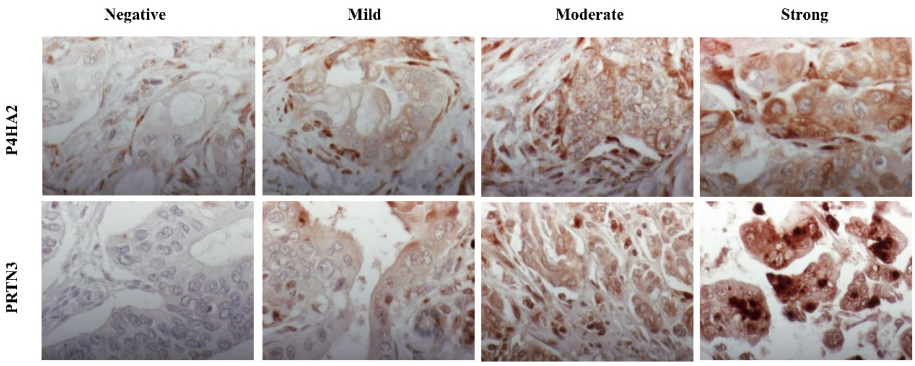

Figure 22. Representative immunostaining of P4HA2 and PRTN3 in pancreatic cancer.

Association between P4HA2 and clinicopathological characteristics and survival

There was no association between P4HA2 expression and any clinicopathological characteristics. P4HA2 expression was not significantly associated with either DFS or OS, in either uni- or multivariable analyses (Table 15). The median DFS and OS were 17.8 and 21.9 months, respectively, in the low P4HA2 expression group, as compared to 12.7 and 27.7 months in the high P4HA2 group.

PRTN3 expression in pancreatic cancer

The staining of PRTN3 was detected in the nuclei, cytoplasm and cell membrane of tumor cells and lymphocytes (Figure 22). PRTN3 expression in tumor cells was considered positive in 77 cases (55%). Weak, moderate and strong staining of PRTN3 accounted for 40 (28.6%), 26 (18.6%) and 11 (7.9%) cases.

Association between PRTN3 and clinicopathological characteristics and survival

PRTN3 expression was not associated with any clinicopathological parameters, except for presence of adjuvant chemotherapy, which was found to be more frequent in the high PRTN3 expression group (94.6% vs. 79.6%, P=0.035). The median DFS and OS were 12.4 and 24.5 months in the low PRTN3 group, while they were 15.5 and 25.8

months in the high PRTN3 group. PRTN3 expression did not correlate with DFS or OS (Table 15).

Table 15. Cox regression analyses of P4HA2 and PRTN3 expression with survival in pancreatic cancer.

Variables	Disease-free survival			Overall survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
P4HA2, unadjusted	0.98	0.64-1.51	0.929	0.74	0.49-1.13	0.165
P4HA2, adjusted*	0.88	0.56-1.40	0.598	0.72	0.45-1.14	0.157
PRTN3, unadjusted	0.95	0.59-1.53	0.837	1.14	0.70-1.85	0.592
PRTN3, adjusted*	0.92	0.56-1.50	0.724	1.13	0.68-1.90	0.634
Low P4HA2 high PRTN3, unadjusted	4.12	1.46-11.63	**0.008**	5.97	2.77-12.85	**<0.001**
Low P4HA2 high PRTN3, adjusted*	3.24	1.13-9.25	**0.028**	8.14	3.41-19.44	**<0.001**

Low P4HA2 and high PRTN3 expression was associated with poor survival

A low P4HA2 and high PRTN3 expression pattern was associated with significantly shorter DFS and OS by Kaplan-Meier analysis (Figure 23-24 and Table 15). The median DFS was 7.0 months in patients with low P4HA2 and high PRTN3 expression compared to 13.4 months in patients with other expression patterns (P=0.004). This association with DFS was also revealed by univariable Cox regression analysis (HR 4.12, 95% CI: 1.46-11.63, P=0.008), and remained significant in multivariable analysis (HR 3.24, 95% CI: 1.13-9.25, P=0.028), adjusted for age, gender, TNM status, differentiation grade, resection margin status and adjuvant chemotherapy (Table 15). The subgroup of patients with a low P4HA2 and high PRTN3 expression pattern also showed shorter OS than those with other expression patterns (median OS: 8.5 vs. 25.8 months, P<0.001). The association between the OS and the combined expression pattern, low P4HA2 and high PRTN3, was also confirmed in univariable Cox regression analysis (HR 5.97, 95% CI: 2.77-12.85, P<0.001), and remained significant in multivariable analysis after adjustment (HR 8.14, 95% CI: 3.41-19.44, P<0.001) (Table 15).

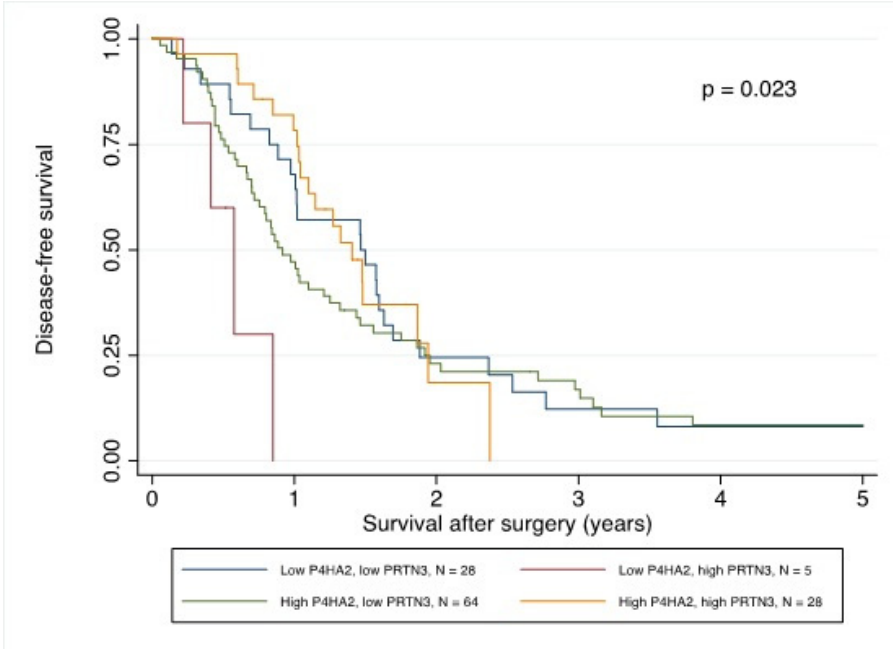

Figure 23. Kaplan-Meier analysis of disease-free survival with P4HA2 and PRTN3 expression patterns in patients with pancreatic cancer.

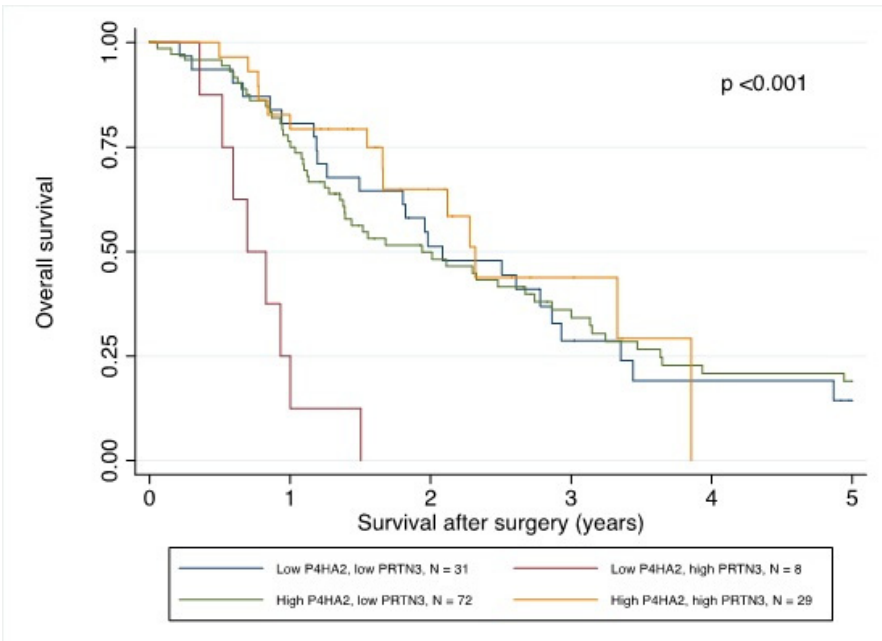

Figure 24. Kaplan-Meier analysis of overall survival with P4HA2 and PRTN3 expression patterns in patients with pancreatic cancer.

4.4.4 Fibronectin (FN1)

FN1 expression in pancreas tissues

The epithelial tumor component was negative of FN1 expression. FN1 expression was localized in non-malignant fibroblasts and ECM of tumors. The categories of FN1 expression were based on the staining in the tumor stroma component. Stromal FN1 expression was negative in 21 (15.3%) tumors, while 66 (47.8%) tumors had mild FN1 expression, 44 (31.9%) had moderate expression and 7 (5.1%) had strong FN1 expression. Representative immunostaining of FN1 in pancreatic cancer is shown in Figure 25. There was no reactivity of FN1 in acinar cells and islets of Langerhans in the four normal control tissues. The absent or minimal expression of FN1 in the normal pancreas is also shown in a public database, the Human Protein Atlas (https://www.proteinatlas.org/ENSG00000115414-FN1/tissue/pancreas).

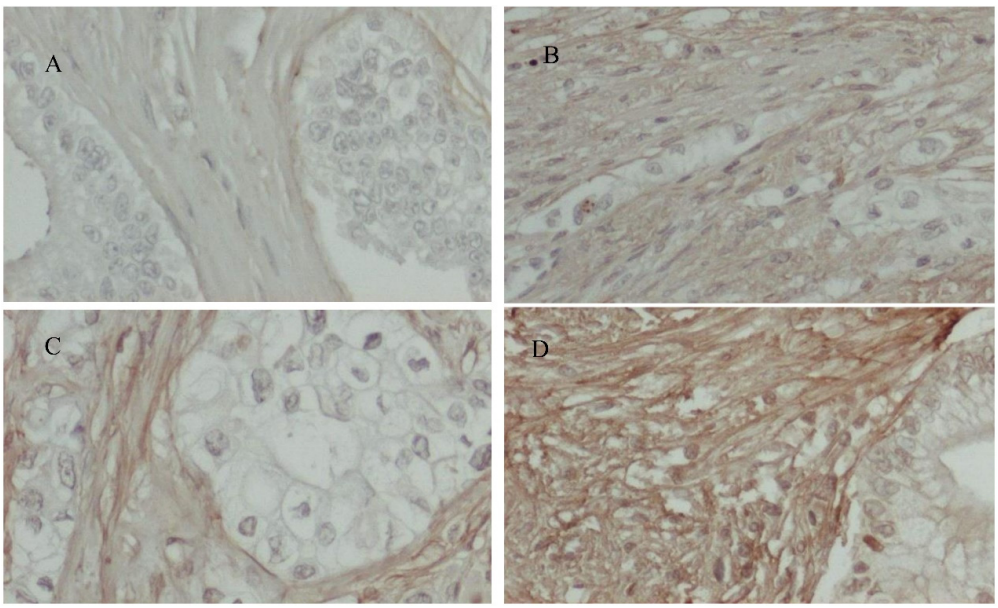

Figure 25. Representative immunostaining of stromal fibronectin in pancreatic cancer.
A: negative; B: mild; C: moderate; D: strong.

Associations of FN1 expression with clinical features in patients with pancreatic cancer

Table 16 shows the clinical features in the low FN1 expression and the high FN1 expression groups of patients with resected pancreatic cancer. The high expression of FN1 was associated with a significantly larger tumor size (96.1% vs 75.9%, *P*=0.002), more advanced T-stage and N-stage (P=0.039 and 0.009) and a worsened AJCC-stage (54.0% vs 28.7% with stage III, *P*=0.003). Furthermore, administration of adjuvant chemotherapy was more frequent in the high FN1 expression group than in the low FN1

expression group (92.0% vs 78.3% *P*=0.040). There were no associations between FN1 expression and other clinical features, including age, gender, tumor location, tumor differentiation and resection margin status (all *P*>0.05).

Association of FN1 expression with DFS and OS

The median DFS in the low FN1 expression group and the high FN1 expression group was estimated to be 17. 9 and 12.3 months, while the median OS was 23.8 and 24.5 months, respectively. Kaplan-Meier analysis showed that there was no statistical difference of DFS or OS in the two groups (both *P*>0.05). Similar results were found in univariable Cox regression analysis (*P*>0.05, Tables 17). By multivariable Cox regression analysis, FN1 was still not linked to the DFS and OS, while histological grade and resection margin status were significantly associated with DFS or OS (Table 17).

Table 16. Clinical features of low and high FN1 expression subgroups of patients with pancreatic cancer.

Clinical Characteristics	Categories	All patients (n, %)	FN1 low expression (n, %)	FN1 high expression (n, %)	*P* value
Age (years)	-	68.5 (63-73)	69 (63-73)	68 (63-73)	0.618
Gender	female	65 (47.1)	41 (47.1)	24 (47.1)	0.994
Tumor size	≦2cm	23 (16.7)	21 (24.1)	2 (3.9)	**0.002**
	>2cm	115 (83.3)	66 (75.9)	49 (96.1)	
T-stage	T1	19 (13.8)	17 (19.5)	2 (3.9)	**0.020**
	T2	92 (66.7)	56 (64.4)	36 (70.6)	
	T3	26 (18.8)	13 (14.9)	13 (25.5)	
	T4	1 (0.7)	1 (1.1)	0 (0)	
N-stage	N0	34 (24.8)	25 (28.7)	9 (18.0)	**0.009**
	N1	52 (38.0)	38 (43.7)	14 (28.0)	
	N2	51 (37.2)	24 (27.6)	27 (54.0)	
Tumor differentiation	poor/anaplastic	82 (59.8)	51 (58.6)	31 (62.0)	0.698
AJCC-stage, 8th edition	I-II	85 (62.3)	62 (71.3)	23 (46.0)	**0.003**
	III	52 (37.7)	25 (28.7)	27 (54.0)	
Resection margin	R1	53 (38.4)	33 (37.9)	20 (39.2)	0.881
Adjuvant chemotherapy	yes	111 (83.5)	65 (78.3)	46 (92.0)	**0.040**

Table 17. Cox regression analyses of FN1 expression with survival in pancreatic cancer by univariate and multivariate methods.

Variables	Disease-free survival						Overall survival					
	Univariable analysis			Multivariable analysis			Univariable analysis			Multivariable analysis		
	HR	95%CI	*P* value	HR	95% CI	*P* value	HR	95%CI	*P* value	HR	95% CI	*P* value
Age	0.99	0.96-1.01	0.202				1.00	0.97-1.02	0.737			
Female gender	0.66	0.45-0.99	0.042				0.78	0.53-1.16	0.784			
Tumor size	1.15	0.68-1.94	0.598				1.08	0.66-1.79	0.758			
T-stage	1.12	0.81-1.55	0.481				1.13	0.82-1.56	0.472			
N-stage	1.14	0.89-1.45	0.307				1.14	0.89-1.46	0.294			
Differentiation grade	1.50	1.07-2.10	0.018	1.54	1.10-2.17	0.012	1.43	1.03-1.97	0.033	1.42	1.02-1.97	0.038
AJCC-stage	1.17	0.78-1.74	0.446				1.04	0.69-1.56	0.859			
Resection margin (R1)	1.73	1.14-2.62	0.010	1.84	1.20-2.80	0.005	1.48	0.99-2.22	0.059	1.70	1.12-2.58	0.012
Adjuvant chemotherapy	1.36	0.77-2.41	0.286				0.70	0.43-1.15	0.161			
FN1 expression, high vs low	0.83	0.55-1.24	0.357				0.82	0.54-1.25	0.366			

Table 18. Expression data not included in the cohort of patients with pancreatic cancer.

Patient ID	CLCA1	LGALS4	PRTN3	P4HA2	FN1	OS (m)	DFS (m)	Age (y)	Gender	Tumor size (cm)	Stage	R1
1			+	+		51	NA	78	M	3.8	1B	0
2	+				+	17.2	17.2	72	M	3.2	2B	0
3	+					13.6	5.4	65	F	3.5	3	1
4			+	+		8.6	NA	69	F	4	2B	1
5					+	38.1	36.6	70	F	3.7	3	1
6			+	+		56.0	NA	68	M	2.5	2B	0
7	+					31.4	22.7	65	F	3	1B	0
8					+	18.3	NA	58	F	NA	NA	NA
9		+				38.3	7.3	58	F	2	2B	0
10	+	+			+	16.5	11.2	46	F	2.9	3	0
11		+			+	33.0	12.4	48	F	3.5	2B	1
12		+	+		+	15.2	15.2	73	F	2.8	2B	1

"+" stands for the failed detection of the protein expression in that column; F, female; M, male; NA, data not available.

4.4.5 Consideration of missing values

In all the validation studies, a total of 144 patients were included in the beginning of the experiment. A small proportion of the samples failed to be detected by the TMA-based immunohistochemistry. For each biomarker, the samples with missing values accounted for less than 5% of the cohort (Table 18).

5 Discussion

A good prediction of tumor behavior and prognosis, and consequently stratified treatment choices, may improve survival and quality of life of patients [84]. Currently, clinical characteristics such as ECOG performance and staging, as well as serum CA 19-9, are the main factors used to predict the prognosis in clinical practice. As there is a growing interest to stratify patients with pancreatic cancer based on clinical outcomes and to predict the prognosis, hundreds of potential prognostic tissue biomarkers have been reported [129]. However, few biomarkers have been validated and none has been translated into clinical practice. Thus, there is still an unmet clinical need for a better prediction of the prognosis in pancreatic cancer.

In this thesis, firstly, we discovered 171 differentially expressed proteins in patients with two extremes of survival by in-depth proteome sequencing. Then, using targeted proteomics approach with higher sensitivity, we confirmed that 25 proteins were dysregulated in patients with LS or with SS. Finally, we selected potentially prognostic proteins to be validated in more than 100 corresponding patient tumor tissues by immunohistochemistry. In these validation studies, we found that CLCA1, galectin 4 and combination of P4HA2 and PRTN3 may play prognostic roles in pancreatic cancer, while FN1 was not an indicator of prognosis.

5.1 Proteome profiling in pancreatic cancer

The molecular understanding of pancreatic cancer has been largely expanded in recent years. This accumulating knowledge has led to molecular classification of pancreatic cancer into subtypes, which may be linked to corresponding clinical characteristics, such as survival and chemotherapy response. However, most of the studies were based on genomic and trancriptomic analysis, while the proteome profiles in pancreatic cancer remain less understood. Studies on the global protein expressions are helpful to gain an integrated molecular view on the pancreatic cancer from gene, mRNA to protein. Moreover, by utilizing high throughput proteomics methodology, proteome profiling in pancreatic cancer provides an efficient way to develop novel prognostic protein biomarkers, whose function in the disease is not necessarily to be understood in advance.

In study I, a retrospective cohort study was performed to identify prognostic biomarkers on FFPE primary tumor samples from 19 patients with resected pancreatic cancer with SS (<12 months) or with LS (>45 months). Employing the LC-MS/MS platform, we

were able to conduct proteome deep mining based on formalin-fixed paraffin-embedded PDAC tissues. Consequently, around 5000 proteins have been mapped in samples from 19 surgically isolated patient tumors. The number of protein identification was among the highest in the proteomics studies on pancreatic cancer tissues, most of which have been shown in Table 4. One contributor of the robust protein identification was the high performance of LC-MS/MS, which has enabled the detection of low abundance proteins and hydrophobic membrane proteins.

A total of 171 proteins were dysregulated in the SS group or the LS group. Further targeted proteomics approach included 73 of these dysregulated proteins and confirmed that 7 and 18 proteins, were upregulated in the SS and the LS patients, respectively. Some of the verified proteins have been shown to be associated with the prognosis of pancreatic cancer, for instance, CLIC3 [179], SOD3 [180], THBS1 [181] and MMP9 [182].

Apart from the discovery of dysregulated proteins, comprehensive bioinformatics has also added novel value to the understanding of potential pathways or regulators linked to the prognosis. Moffitt et al. have performed a large-scale genomics analysis on PDAC and characterized the transcriptional profiles of both tumoral and stromal part inside the tumor [138]. They linked the poor prognosis to sets of proteins classified as “basal tumor factors” as well as “activated stroma factors”, as compared to “classic tumor factors” and “normal stroma factors” subtypes corresponding to the better prognosis. Strikingly, our data was in good agreement with their results. Proteins classified by Moffitt et al. as “basal tumor factors” and “activated stroma factors” were upregulated in short survival samples, while proteins classified as “classic tumor factors” and “normal stroma factors” were upregulated in long survival samples. The protein characteristics for these tumor features made up as much as approximately 20% of differentially expressed proteins. Thus, our results supported the classification defined by Moffitt et al. on protein level.

The histological hallmark in PDAC is that tumor cells are surrounded by as much as 90% stroma. This study has unveiled a group of dysregulated proteins that are related to the TME. For example, FN1, one of main components of TME, has been found to be upregulated in the SS group. Reactome pathway analysis showed that nine of them were involved in extracellular matrix organization (THBS1, PLOD1, LAMC2, P4HA2, MMP9, MMP8, FN1, CDH1 and CEACAM1). Four of these proteins take part in collagen formation (PLOD1, LAMC2, P4HA2 and MMP9), while five proteins play a role in degradation of the extracellular matrix (LAMC2, FN1, MMP8, MMP9 and CDH1). The dysregulated proteins involved in the formation and degradation of the extracellular matrix were mainly found to be upregulated in the SS group, indicating a more activated stroma status in this group with worse prognosis. Notably, some dysregulated proteins, which were engaged in the process mentioned above, were not shown in the Reactome pathway analysis. Specifically, P4HA2 participates in the biosynthesis of collagens [183], while PRTN3 and CLCA1 function as extracellular proteases [184, 185].

Two upstream regulators, HNF1A and CTNNB1, have been estimated by IPA analysis as "drivers" of the differentially expressed proteins. HNF1A and CTNNB1 belong to a well-known cancer regulatory hub important for the Wnt signaling pathway. Additionally, by STRING protein-protein analysis, a set of dysregulated proteins (CDH1, THBS1, MMP9, EPCAM, WDR5, CSNK2A2 and PADI4) showed close interplay with TP53 [133, 182], whose loss-of-function mutation is progressively involved in pancreatic cancer.

5.2 Validation of prognostic biomarkers

CLCA1

Calcium-activated chloride channel regulators (CLCAs), also named "chloride channel accessory proteins", are a family of secreted self-cleaving proteins whose main functions are to activate calcium-dependent chloride currents. As one type of ion channels, Ca^{2+}-activated chloride channels are involved in regulation of cell proliferation, cell migration and metastasis and have been proposed as potential therapeutic targets in cancer [186-188]. It has been reported that TMEM16A, a calcium-dependent chloride channel, was involved in tumor growth and invasion of prostate cancer, lung cancer, and head and neck squamous cell carcinomas [189-192]. As a direct modulator of TMEM16A, CLCA1 has also drawn attention to its role in tumorigenesis. Recent studies have suggested that CLCA1 might take part in tumorigenesis of colorectal cancer and that low expression of CLCA1 was associated with a worsened prognosis in patients with colorectal cancer [193].

Moreover, a metalloprotease activity in CLCA1 has been predicted and demonstrated by Pawlowski et al. in 2006 [185], which was confirmed by other subsequent studies [186, 194]. In contrast with other members of CLCAs, CLCA1 plays a unique role in mucus homeostasis [195]. Recent study has demonstrated that CLCA1 was responsible for an increased mucus thickness and penetrability in the intestine, through its proteolytic activity, which was independent of the ion conductance or mucus secretion [194].

In a previous study, knockdown of CLCA1 in colorectal cancer cell line (Caco-2) led to inhibition of cell differentiation and promotion of cell proliferation [196]. Further *in-vitro* investigations indicated that CLCA1 may act as a tumor suppressor in colorectal cancer by inhibiting the Wnt/beta-catenin signaling pathway and epithelial-mesenchymal transition, while *in-vivo* overexpression of CLCA1 resulted in inhibition of proliferation and metastasis [197].

In study II, the prognostic significance of CLCA1 was validated by immunohistochemistry in a retrospectively cohort of 140 patients with resected pancreatic cancer. Consequently, low CLCA1 expression was found to be an independent factor of shorter DFS.

CLCA1 has previously been proposed as a supportive marker to distinguish between cystic precursor lesions and pancreatic cancer using cyst fluid samples [198]. The role of CLCA1 in mucus homeostasis leads us to suspect whether it is also involved in the progression from mucinous cystic neoplasms and intraductal papillary mucinous neoplasms, two precursor forms of pancreatic cancer, to pancreatic cancer.

Pancreatic cancer is characterized by the abundant amount of tumor microenvironment, which participates in tumor progression and resistance to chemotherapy [45, 199]. Studies have shown that some matrix metalloproteases were involved in pancreatic cancer and may serve as therapeutic targets [200-203]. The metalloprotease activity of CLCA1 might play a similar role in pancreatic cancer. While the role of CLCA1 in pancreatic cancer remains unclear, its target modulator, TMEM16A, has been reported to be overexpressed in pancreatic cancer cells and promote the cell migration [204].

Re-validation in other cohorts of patients will be needed to ascertain the prognostic significance of CLCA1 in pancreatic cancer. In the future, CLCA1 may be integrated into an immunohistochemical panel to predict prognosis and treatment response in patients who undergo surgical resection.

Galectin 4

Galectins are a family of animal lectins with affinity for β-galactosides. They are both intra- and extra-cellular components and bind to a various glycoproteins and glycolipids on the cell surface or in ECM [205]. By cell-to-cell and cell-to-ECM adhesion, galectins activate intracellular signaling pathways and are involved in cell proliferation, apoptosis, adhesion and immune response, thus functioning as a modulator in cancer [205]. Galectins-4 is categorized into the tandem repeat subtype of the galectin family [206].

There have been several studies on the involvement of galectin 4 in tumors. However, the conclusions are dependent on specific tumor types. Galectin-4 expression was reported to be decreased in colorectal cancer while the expression is raised in pancreatic cancer and liver cancer [205]. It has been revealed that galectin-4 acted as a tumor suppressor in colorectal cancer [207-209], PDAC [210, 211], hepatocellular carcinoma [212], and prostate cancer [213]. On the contrary, galectin-4 may play a role as a tumor promoter in lung and gastric cancer [172].

Low galectin-3 expression and high galectin-1 expression have been suggested to be linked to a poor survival in patients with PDAC, respectively [214-216]. While the expressions of galectin-1 and galectin-3 are on stromal cells, galectin 4 expression is mainly presented in tumor cells. Based on a relatively small size of patients, galectin 4 expression has shown a tendency to be associated with a better outcome in PDAC [210].

In study III, a similar retrospective study has been designed to evaluate the prognostic role of galectin 4 in pancreatic cancer. Galectin 4 expression may serve as a novel biomarker for early recurrence and mortality after surgical resection for pancreatic cancer. We defined early recurrence as locoregional recurrence or metastasis within one

year from surgery, as has been suggested [217]. Thus, the galectin 4 expression may be tested to select patients with early recurrence, by mean of pre-operative guided biopsy. Those without galectin 4 expression may potentially benefit from alternative treatment, such as neoadjuvant chemotherapy before operation. However, more studies are needed to confirm this predictive role of galectin 4. Functional studies should also be performed in the future to elucidate the underlying mechanisms.

P4HA2 and PRTN3

The implication of TME in tumor progression and resistance of chemotherapy has been investigated extensively during the past decade [38]. The TME in pancreatic cancer is a large amount of fibrotic stroma surrounding the tumor cells, which is composed of various cellular and molecular components. Cellular components mainly include pancreatic stellate cells (PSCs) and immune cells. Molecular components include ECM, such as collagen, fibronectin and hyaluronic acid. The TME in the tumor is in a dynamic status, which involves synthesis and break down of components, such as collagen. Activated PSCs are the main source for deposition of collagen, which can also be degraded by MMPs from PSCs, cancer cells and inflammatory cells [39]. Studies have revealed that an activated stroma status was associated with the progression of pancreatic cancer and a worsened survival in these patients [218]. Proteome profiling in study I also supported an activated stroma status in patients with poor outcome.

In study IV, two prognostic candidates that closely interact with TME, P4HA2 and PRTN3, were investigated in a retrospective cohort of 140 patients with resected PDAC. Although P4HA2 and PRTN3 expression did not separately correlate with disease-free survival or overall survival, we found that a combined expression status of low P4HA2 with high PRTN3 correlated with poor survival in patients with PDAC.

Strikingly, the role of P4HA2 in cancer in this study seemed opposite to previous studies, in which high expression of P4HA2 conferred an worsened prognosis in breast cancer [183, 219]. Overexpression of P4HA2 expression has been reported in breast cancer [183], oral cavity squamous cell carcinoma [220] and papillary thyroid cancer [221].

P4HA2 has been implicated in the formation of collagen [222], whereas PRTN3 takes part in the degradation of the ECM component [223]. PRTN3 has also been suggested to activate MMPs, which might facilitate the tumor invasion and metastasis [224, 225]. Moreover, recent studies showed that breast cancer and melanoma cells could uptake PRTN3 in the TME, which was secreted by neutrophils, thereby increasing the susceptibility to PR1-targeting therapies [226, 227]. These findings shed light on the tumoral PRTN3 as a potential therapeutic target.

As the main constitute of ECM, collagen plays a key role in tumor progression [228]. Erkan and colleagues have proposed that the fibrolytic stroma, characterized by high α-SMA and low collagen, was independently associated with a worse prognosis of pancreatic cancer [38, 39]. This has been further confirmed by a recent study [146].

Our study delineated a subgroup of patients (around 5%) with low P4HA2 and high PRTN3 expression, who had poor survivals and might not benefit from upfront surgery. However, we have to stress that these results were based on combined protein expression in retrospectively collected samples, which may not stand for the entire population of patients with PDAC. Thus, it is highly recommended to re-validate the results in other cohorts. Besides, the underlying rationale behind the link between the poor prognosis and the low P4HA2 and high PRTN3 expression pattern, either through the collagen dynamics in TME or through other unknown mechanisms, are to be studied.

FN1

Fibronectin (FN1) is a main constituent of the extracellular matrix in TME and is produced mainly by fibroblasts, but also by tumor cells themselves [229]. Normally, FN1 supports cell-ECM interactions and is essential for wound healing, development, and maintaining tissue homeostasis [230]. In PDAC, its binding to receptors in tumor cells, typically cell surface integrins, triggers FN1 signaling pathways, which may promote tumor cell survival and chemoresistance, cell invasion, metastasis and angiogenesis [229]. Abrogating FN1-integrin interactions has produced strikingly positive pre-clinical results in various animal models of cancer by impeding angiogenesis and inhibiting tumor growth [231-233]. However, these drugs, such as PF-04605412, have failed in the treatment of tumors in clinical trials [234]. A recent study has uncovered an anti-metastatic role of fibronectin from tumor cells responding to immunological surveillance of natural killer cells [235].

In study V, the potential prognostic role of FN1 in PDAC was explored. As a result, high tumoral FN1 expression was associated with aggressive tumor characteristics in patients with resected PDAC. However, no correlation between FN1 expression and survival was found. This indicated that FN1 is not likely to serve as a prognostic biomarker in PDAC.

6 Conclusions

The main conclusions of studies from the thesis is listed as below:

I. By in-depth proteome sequencing and targeted proteomics, we found 25 protein candidates of prognostic significance for pancreatic cancer. Besides, the activated stroma status, involvement of Wnt signaling pathway, as well as TP53 associated proteins, were revealed to be associated with a worse prognosis of pancreatic cancer.

II. Low CLCA1 expression was found to be an independent factor of shorter disease-free survival.

III. Galectin 4 expression may serve as a novel biomarker for early recurrence and mortality after surgical resection for pancreatic cancer.

IV. A low P4HA2 together with high PRTN3 expression status was significantly associated with poor survival in retrospectively collected patients with resected pancreatic cancer.

V. FN1 is not likely to serve as a prognostic biomarker for pancreatic cancer.

7 Future perspectives

Although several prognostic biomarkers on tissue level for pancreatic cancer have been identified and validated in a larger retrospective cohort, there are still many steps before the potential clinical significance is achieved. Five aspects need to be further explored.

Firstly, the prognostic candidates need to be re-evaluated in other cohorts. There are already hundreds of tissue biomarkers proposed for pancreatic cancer. One reason why they are not translated into clinical practice is the lack of robust validation. High volume centers managing pancreatic cancer allow accessible biobanking and registries with complete clinical follow-up data that are needed. Cooperation with these centers may accelerate validation of the prognostic value of our biomarker candidates.

Secondly, the biomarkers need to be tested in tissue samples collected before surgery in a prospective cohort study. This is an approach one-step forward to the clinical application. The main purpose of prognostic biomarkers is to guide stratified treatment based on the expression of the biomarkers. Guided biopsy, such as through EUS, can be obtained before surgery. Besides, it can also be obtained in patients with advanced stage of pancreatic cancer, which actually account for most of the patients. Therefore, the prognostic value of the biomarkers can be investigated in patients with all stage and before any treatment. There is an overlap of diagnostic, prognostic and predictive biomarkers in one condition. A prospective cohort of patients with detailed clinical information will also allow us to investigate whether the biomarkers can predict the response to certain chemotherapy.

Thirdly, some other prognostic candidates discovered by the proteomic study need to be validated. CLIC3, SOD3 and MMP8 are the candidates of most interest. We have used the tissue microarray method to handle samples in a more efficient and standardized way. The recent concept of digital pathology, which can analyze several biomarkers together in one sample, may allow us to evaluate a panel of bimarkers on one slide and at one time. This will efficiently expand tissue expressional information of each patient, and help to guide an individualized treatment. We are currently in contact with Lomito AB, a company dedicated for digital pathology in Lund, for potential application of this new method to validate other biomarkers of prognostic interest.

Fourthly, our tissue biomarkers may serve as candidate pool for blood biomarkers. Blood biomarkers are more accessible from individuals. Those proteins that can be released into the bloodstream from the tumors but have limited concentration in healthy blood, may play a diagnostic or prognostic role in pancreatic cancer. We have collected

more than 100 serum samples from patients with pancreatic cancer. We are going to investigate the diagnostic and prognostic role of biomarkers of interest (e.g. CLCA1 and galectin 4) in serum.

Lastly, functional studies of the biomarkers in tumorigenesis are also warranted. For example, the underlying rationale behind the link between the poor prognosis and a low P4HA2 and high PRTN3 expression pattern, either through the collagen dynamics in TME or through other unknown mechanisms, are to be studied. These studies may not only explain the prognostic role of the biomarkers, but also potentially provide therapeutic targets for the disease.

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Paper I

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Research Paper

Proteomic analyses identify prognostic biomarkers for pancreatic ductal adenocarcinoma

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy. Here we show that shotgun and targeted protein sequencing can be used to identify potential prognostic biomarkers in formalin-fixed paraffin-embedded specimens from 9 patients with PDAC with "short" survival (<12 months) and 10 patients with "long" survival (>45 months) undergoing surgical resection. A total of 24 and 147 proteins were significantly upregulated [fold change ≥2 or ≤0.5 and P<0.05; or different detection frequencies (≥5 samples)] in patients with "short" survival (including GLUT1) and "long" survival (including C9orf64, FAM96A, CDH1 and CDH17), respectively. STRING analysis of these proteins indicated a tight protein-protein interaction network centered on TP53. Ingenuity pathway analysis linked proteins representing "activated stroma factors" and "basal tumor factors" to poor prognosis of PDAC. It also highlighted TCF1 and CTNNB1 as possible upstream regulators. Further parallel reaction monitoring verified that seven proteins were upregulated in patients with "short" survival (MMP9, CLIC3, MMP8, PRTN3, P4HA2, THBS1 and FN1), while 18 proteins were upregulated in patients with "long" survival, including EPCAM, LGALS4, VIL1, CLCA1 and TPPP3. Thus, we verified 25 protein biomarker candidates for PDAC prognosis at the tissue level. Furthermore, an activated stroma status and protein-protein interactions with TP53 might be linked to poor prognosis of PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has recently surpassed breast cancer to become the third leading cause of cancer-related mortality according to the American Cancer Society, with a 5-year survival in the single digits [1]. Despite improvements in surgical techniques and adjuvant chemoradiotherapy, the survival from the disease has not changed substantially over the past four decades. It is estimated that PDAC will surpass colorectal cancer to become the second leading cause of cancer-related mortality following lung cancer by the year 2020 [2]. The main reason underlying the low survival rate of PDAC is that most patients are diagnosed at an advanced stage, at which curatively intended surgery, no longer represents an option. Currently, CA19-9 is the only

serum tumor marker used in the clinical management of PDAC. However, the sensitivity for CA19-9 is 79% with a specificity of 82%, limiting its use for screening purposes [3].

Traditionally, PDAC has been looked upon as a gradual process associated with the sequential accumulation of genetic changes during a comparably long period of time [4]. Novel data has though implied that the development of PDAC may not be a slow and gradual process. Using whole genome sequencing, it was reported that genomic instability from mitotic errors might occur simultaneously resulting in rapid tumor development and metastases in a subset of patients [5]. These findings have been supported by a recent publication on approximately 60,000 patients with histopathologically verified PDAC where survival and metastatic spread were correlated to tumor size [6]. It was reported that already at a small tumor size up to 5 mm, as much 30% of patients had remote cancer growth. This implies the predominant role of molecular tumor biology in determining outcome for the individual patient. It also emphasizes the need for better tools for staging, for example with novel biomarkers in order to render the necessary prognostic and predictive information and support choice of therapy in a more precision-medicine fashion.

While large scale genomics studies have provided understanding of mutational processes underlying the development of PDAC [7, 8], and helped to define molecular subtypes of PDAC [9, 10], proteomics technology has accelerated our understanding of PDAC at the protein level by identifying key drivers of disease progression and biomarkers for diagnosis and targeted intervention [11, 12]. Recent proteomic studies and further validation studies have greatly expanded the pool of potential diagnostic and prognostic biomarkers in PDAC. For instance, at the tissue level, Turtoi et al. found that ASPN, LTBP2, TGFBI were overexpressed in PDAC [13], while Takadate and colleagues suggested that ECH1, GLUT1, OLFM4 and STML2 were potentially diagnostic biomarkers of PDAC [14]. Furthermore, Chen and colleagues found that PRELP, LGALS1 and RPS8 might be significant prognostic factors for pancreatic cancer [15], while another study showed that PNMA1 was associated with prolonged overall survival and might serve as a prognostic biomarker for pancreatic cancer [16]. At the plasma level, ICAM1 and TIMP1 have been proposed as biomarkers for the detection of pancreatic cancer [17]. However, these biomarkers were mostly studied in small population cohorts and thus further validation is warranted prior to clinical use.

Formalin-fixed paraffin-embedded (FFPE) tissues are used routinely in hospitals for histopathological diagnosis and staging of diseases like cancer. FFPE samples with associated clinical and histological characterization represent a valuable source of biomarker investigation. The application of mass spectrometry technology to FFPE samples has been shown to be technically feasible and highly robust for biomarker discovery and validation [18]. Specifically, deep mining of proteomes from individual samples, including membrane proteins and low-abundance proteins, broadens the possibility to discover potential biomarkers. Using proteome bioinformatic tools, the many functional partnerships and interactions that occur between proteins are revealed and put into context for molecular systems biology. In our study, we selected tissue samples from PDAC patients with divergent survival, aiming to identify prognostic biomarker panels correlating with outcome.

RESULTS

Quality control and overview of proteome profiles

To evaluate the technical reproducibility of sample handling including reduction, alkylation, precipitation and fractionation and instrument performance, we performed three independent sample preparations using an identical protein stock extracted from one sample. The intensities of proteins in the three experiments showed good correlations (r^2 = 0.973, 0.920 and 0.931). Besides, sample preparations with and without fractionation were applied to an identical sample to compare the consistency of protein intensities from these two methods. In result, the protein intensities were in good correlation between the two methods (r^2 = 0.9373). Moreover, the fractionation step achieved a remarkable enlargement of protein number being identified, which enabled a deep mining of proteome in pancreatic tissue in this study (Figure 1). Among 57 replicates from 19 samples, coefficient of variations (CV) of Log 2 transformed intensities of spiked-in chick lysozyme before and after normalization was 34.0% and 6.6%, respectively. Around 3,000,000 peptide-spectrum matches (PSMs) and 58,505 peptides with high confidence were identified, which were mapped to 4942 proteins (minimum 2 peptides per protein). Among them, 3103 proteins were identified in more than half (≥ 5) of the samples in at least one group. Gene Ontology analysis was conducted based on the 3103 proteins. Cellular component analysis showed that there were 640 plasma membrane proteins, 108 cell surface proteins and 163 extracellular matrix proteins, which were considered as potential proteins for potential serum detection and also candidate therapeutic targets. Notably, PANTHER pathway analysis indicates that Integrin signaling pathway is significantly enriched (3.23 fold, P = 6.58E-19).

Candidate prognostic proteins for PDAC

A total of 304 proteins were differentially expressed between the "long" survival (LS) and "short" survival (SS) groups ($P < 0.05$), including 33 proteins and 271

proteins statistically upregulated in "short" survival group and "long" survival group, respectively. Among them, 171 proteins were significantly differentially expressed between the two groups which meet the criteria: 1) SS/LS fold change ≥ 2 or ≤ 0.5 and $P < 0.05$; or 2) different detection frequencies (≥ 5 samples), namely, 83 proteins that were more frequently detected (≥ 5) in one group than the other one. Of these 171 proteins, 24 and 147 proteins were upregulated in "short" survival group and "long" survival group, respectively (see Figure 2).

The 171 differentially expressed proteins from 19 tissue samples were submitted to two-way unsupervised hierarchical clustering and visualized in the heat map (Figure 3A). The clustering of 19 tissue samples was in good agreement with the clinical classification. The principal component analysis further confirmed that patients with "long" survival and "short" survival were well stratified by group of differentially expressed proteins (Figure 3B). The set of differentially expressed proteins exhibited striking trend in terms of subcellular localization. David analysis showed significant overrepresentation of mitochondrial proteins (34 proteins, *P*-value 0.017), and specifically mitochondrial large ribosomal subunit (6 proteins, *P*-value 0.002) and mitochondrial respiratory chain complex I (5 proteins, *P*-value 0.033). PANTHER pathways analysis of differentially expressed proteins revealed overrepresentation of Wnt signaling pathway (CDH1, CSNK2A2, GNA11, CTBP2, CDH17, SMARCE1, *p*-value 0.02), followed by Alzheimer disease-presenilin pathway (MMP8, MMP9, MLLT4, CDH1, *P*-value 0.04).

In order to better assess proteins upregulated in "short" and "long" survival groups, these two sets (24 and 147 proteins, respectively), were separately submitted to

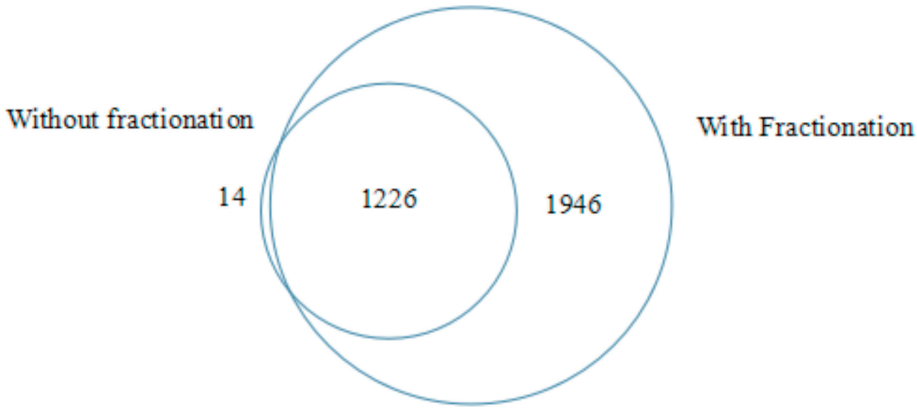

Figure 1: Venn diagram of protein numbers being identified in one identical sample by methods with and without fractionation.

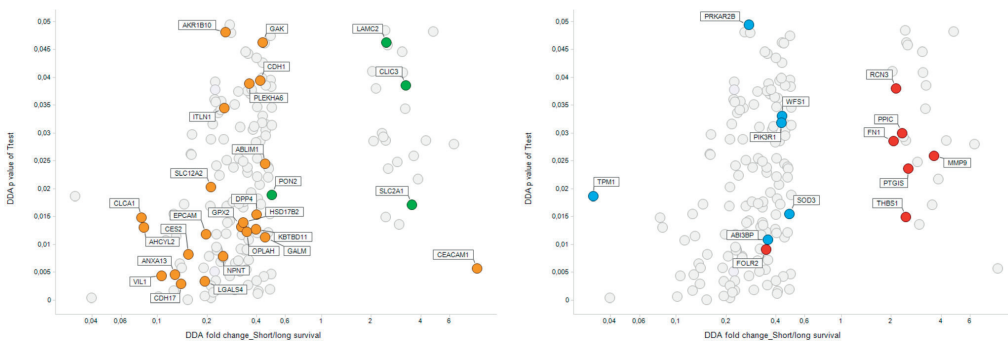

Figure 2: Volcano plot. Comparison of protein expression in short survival tumors (SS) vs long survival ones (LS). Vertical axis: *t*-test *p*-value, horizontal axis: SS/LS fold change. Colouring by proteins characteristic for PDAC subtype factors according to Moffitt et al. Left: Tumor-related factors: Green: Basal tumor, Orange: Classic tumor. Right: Stroma related-factors. Blue: Normal stroma, Red: Activated stroma. (see text).

Panther functional analysis. Among proteins upregulated in the "long" survival group, remarkably overrepresented were mitochondrial proteins (*P*-value 3e-5), which translated into overrepresentation of oxidoreductase activity (*P*-value 1.8e-3). Among proteins upregulated in the "short" survival group, overrepresented were secretory vesicle proteins (*P*-value 5e-6) and extracellular proteins (*P*-value 4e-4). This was related to overrepresentation of activities such as peptidase activity (*P*-value 2.5e-2), collagen binding (*P*-value 4.8e-4), heparin binding (*P*-value 7e-6) and lipid binding (*P*-value 3e-2).

STRING database [19] was employed to investigate the functional and physical protein interactions among the 171 differentially expressed proteins (Figure 4). Since TP53 and KRAS were essential in the pathogenesis of pancreatic cancer, these two proteins were manually added to identify potentially related pathways. With high confidence (minimum required interaction score 0.700), a total of 86 protein-protein interactions were observed and they were significantly enriched based on the given protein nodes (P-value < 0.001), indicating that these differentially expressed proteins are at least partially biologically connected. Seven proteins clustered in a tight interaction network centered on TP53, including CDH1, THBS1, MMP9, EPCAM, WDR5, CSNK2A2, PADI4. Of this protein cluster, CDH1 also closely interacts with CDH17, PIK3R1, NDRG2, CTBP2, MMP9 and EPCAM while THBS1 is centered by FN1, DPP4 and MMP9. Besides, intensive interactions were also observed in the other three clusters of proteins, which were related to respiratory electron transport (COX5B, UQCRB, NDUFS5, NDUFA4, NDUFAB1, NDUFB6 and NDUFB8), mitochondrial translation (MRPL37, MRPL2, MRPL3, MRPL16, MRPL19 and MRPL23) and mRNA Splicing (PRPF4, POLR2C, MAGOH, PLRG1, CWC15 and PHF5A).

A complementary Ingenuity Pathway Analysis (IPA), using curated, literature-derived relationships, showed a picture similar to the STRING analysis (Figure 5). Top canonical pathways, which were significantly enriched among proteins differing between the "short" survival group and "long" survival group, included Oxidative Phosphorylation and Mitochondrial Dysfunction. For example, the differentially expressed proteins amounted to 7 out of 22 Oxidative Phosphorylation pathway proteins (*P*-value 0.002). Similarly to the non-curated networks generated by STRING, also Ingenuity analysis yielded tightly connected relationship subnetworks, built around protein hubs, which are known PDAC actors, even if these hub proteins were not themselves differentially expressed. These subnetworks are constructed automatically as dense subsets of global network of literature-derived

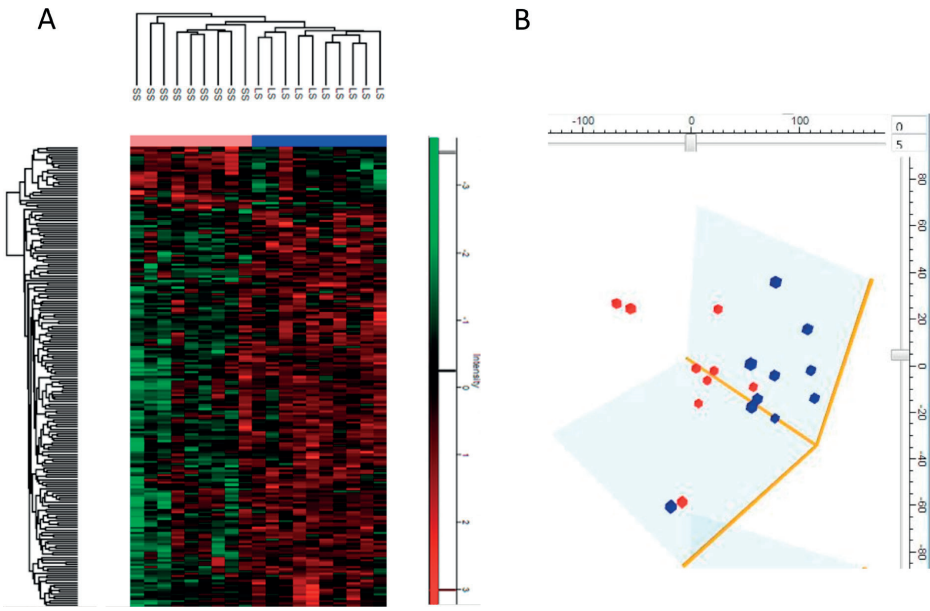

Figure 3: (**A**) Heat map of differentially expressed proteins in pancreatic cancer with long survival (LS) and short survival (SS). The heat map visualized two-way unsupervised hierarchical clustering of 171 differentially expressed proteins in pancreatic cancer patients with short survival (SS) compared to those with long survival (LS) ($P < 0.01$, SS/LS fold Change ≥ 2). (**B**) Global principal component analysis of protein profiles in 19 samples. Dots representing pancreatic cancer (PC) patient samples with long survival (blue) and short survival (red) were well clustered, which was in good agreement with the clinical classification.

relationships between proteins and genes. First such subnetwork was centered on Akt kinase and mitochondrial complex 1 proteins. The second subnetwork was centered on NFkB and TCF transcription factors. The hubs of the third subnetwork were the ERK kinases, collagens and matrix metalloproteases (MMPs). The fourth subnetwork was focused on HNF4A and mitochondrial ribosomal proteins.

Additionally, an IPA analysis of possible upstream regulators of the differentially expressed proteins yielded a mechanistic network regulated by HNF1A (TCF1) and CTNNB1, a well-known cancer regulatory hub important for the Wnt signaling pathway. The HNF1A mechanistic network was significant, with *p*-value 4.2E-05, and included 8 proteins from the differentially expressed list: ALDH3A2, CEACAM1, CRAT, EPCAM, GPX2, HSD17B2, MUC6 and PCCA, see Figure 6.

Verification of candidate prognostic proteins by targeted MS/MS

To evaluate the potential candidate proteins, 171 differentially expressed proteins from the discovery phase were selected for targeted proteomics study. Unfortunately, 98 proteins of them failed in the PRM approach. Finally, 73 proteins were successfully detected and scheduled in one assay panel. The proteins were detectable in all samples. Thirty-six proteins were differentially expressed between the two groups, including 7 proteins and 29 proteins statistically upregulated in "short" survival group and "long" survival group, respectively ($P < 0.05$). Of them, seven proteins were significantly upregulated (SS/LS fold change > 1.5) in patients with "short" survival (MMP9, CLIC3, MMP8, PRTN3, P4HA2, THBS1, FN1), while 18 proteins were significantly upregulated (SS/

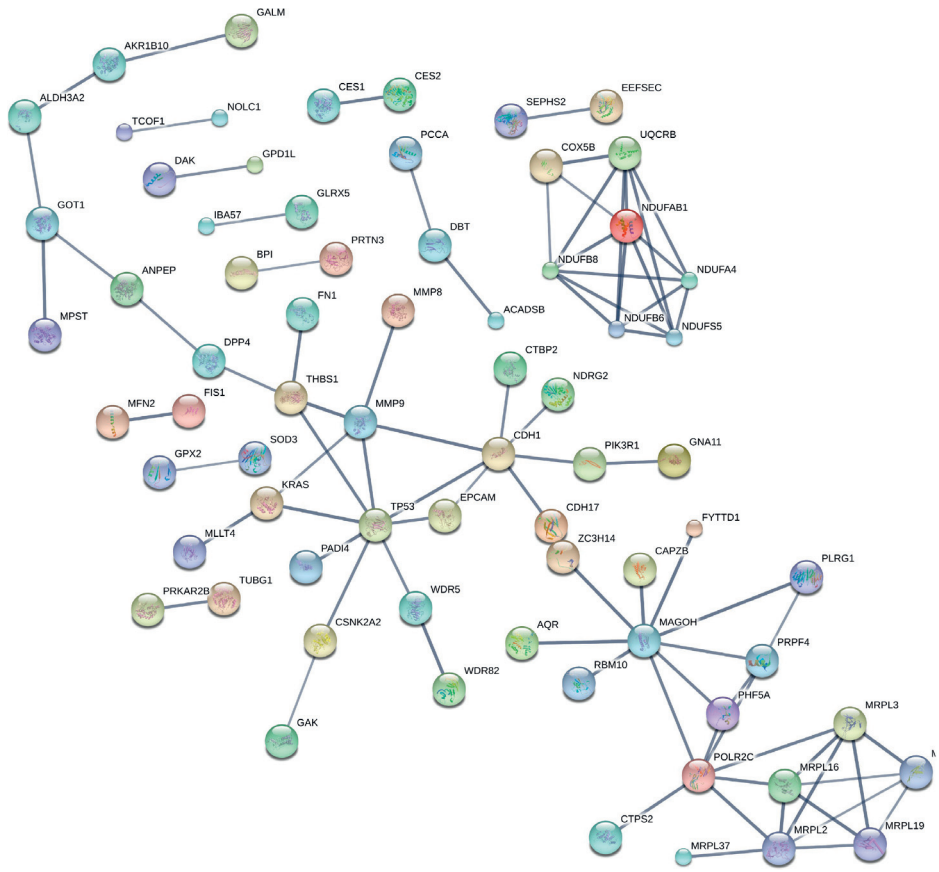

Figure 4: Protein-protein interactions among prognostic candidate proteins. Protein-protein interactions of the 171 dysregulated proteins extracted from the STRING database. TP53, KRAS were manually added to identify potentially related pathways. Notably, seven proteins were centered on TP53, including CDH1, THBS1, MMP9, EPCAM, WDR5, CSNK2A2, PADI4.

LS fold change <0.5) in patients with "long" survival (TMED4, GPD1L, SOD3, NPNT, ABHD14B, ACADSB, DHRS1, EPCAM, WDR82, HDHD2, TPPP3, CHGA, LGALS4, TTC38, COQ9, CES2, VIL1, CLCA1) (Figure 7 and Table 1). After the expression values of each protein were divided into two groups: lower expression (9 cases) and higher expression (10 cases), Kaplan-Meier analysis showed that four proteins were significantly negatively correlated to the survival months (TPPP3, WDR82, LGALS4 and EPCAM, P values were < 0.001, 0.008, 0.020 and 0.010, respectively) (Figure 8).

DISCUSSION

PDAC is considered one of the most aggressive and lethal forms of human cancer. However, there exists a small proportion of patients that actually reach a comparably "long" survival after surgical resection and adjuvant chemotherapy, even when they have "advanced stage" disease (size) or other markers of poor prognosis [20–22]. There have been very few studies relating global protein expression to survival in PDAC [14, 23]. The characterization of protein profiles at the tissue level might help to understand better the molecular basis of PDAC progression and identify potential biomarkers for diagnosis and prognosis of the disease. In this study, we have established a comprehensive method for proteome deep mining based on formalin-fixed paraffin-embedded PDAC tissues, which led to discovery of around 5000 proteins, making it possible to detect low abundance proteins and hydrophobic membrane proteins. A total of 171 proteins were dysregulated in patients with "short" survival compared to those with "long" survival. A further validation panel, targeting 73 of the differentially expressed proteins confirmed that 7 and 18 proteins, were upregulated in the "short" survival and the "long" survival patients, respectively.

In this study, several aspects accounting for the aggressiveness of PDAC have been highlighted. Among well-known hallmarks of cancer metabolism, shift from

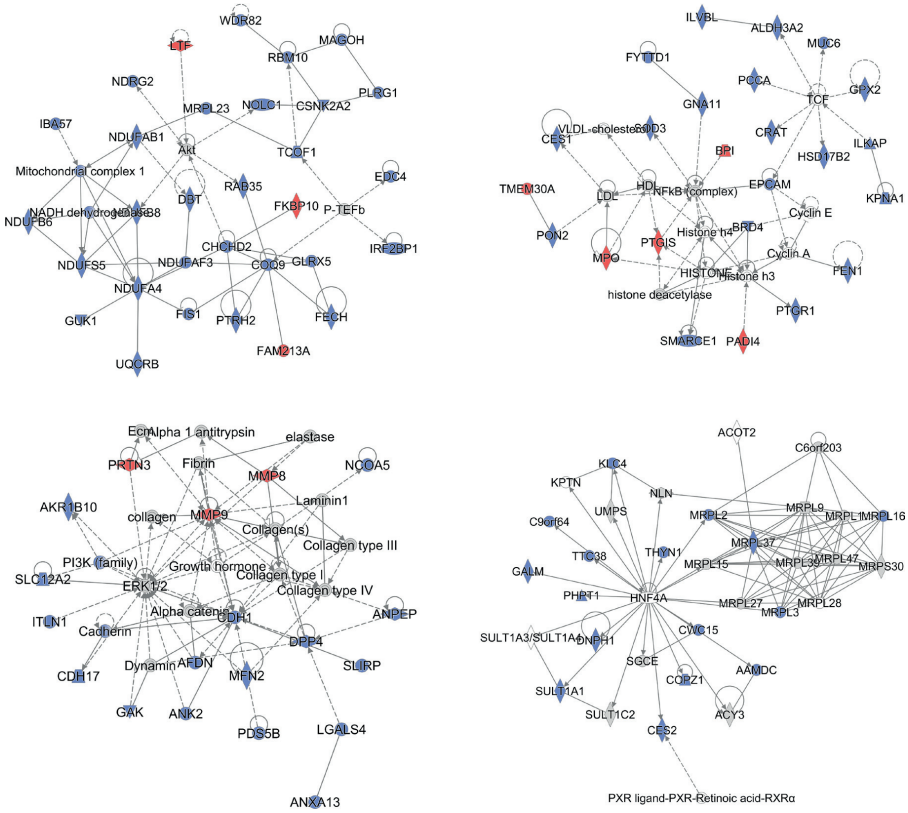

Figure 5: Protein-protein relationships among prognostic candidate proteins extracted by the Ingenuity IPA analysis. Top four subnetworks shown. Red: proteins upregulated in short survival patients. Blue: proteins upregulated in long survival patients.

oxidative phosphorylation towards glycolysis is well-known [24], and specific glucose metabolic phenotype was proposed for pancreatic cancer [25]. Strikingly, in the current study, this metabolic shift was seen as generally lower expression of mitochondrial proteins in "short" survivors. Most notably, this affected a set of mitochondrial respiratory chain complex I proteins (likely resulting in lowered oxidative phosphorylation) and a set of mitochondrial ribosomal proteins (likely resulting in lowered mitochondrial translation rates).

Another cancer metabolism hallmark is the deregulation of glucose intake [24]. Glucose transporter 1 (GLUT1), also known as facilitated glucose transporter member 1 (SLC2A1), is a pivotal rate-limiting element in the transport of glucose in malignant cells. GLUT1 has also been implicated in the pathogenesis of PDAC. Nagarajan et al. found that by stimulating GLUT1-mediated glucose transport, paraoxonase 2 favored the tumor growth and metastasis of PDAC [26]. It has also been reported that HMGB2 predicts poor prognosis in PDAC by facilitating HIF1-α-mediated glycolysis through the expression of GLUT1 [27]. NDRG1, a tumor suppressor, was also shown to inhibit cancer metabolism in PDAC partly through the regulation of GLUT1 gene [28]. High levels of GLUT1 have been previously correlated to poor outcome in PDAC [29, 30]. Accordingly, in our results, GLUT1 was significantly upregulated in "short" survivors. Recently, GLUT1 was shown to be a promising target in pancreatic cancer stem cells in mice [31].

Several differentially expressed proteins (CDH1, THBS1, MMP9, EPCAM, WDR5, CSNK2A2 and PADI4) have a close interplay with TP53, which is frequently mutated and progressively involved in pancreatic cancer [32–35]. THBS1, MMP9 and PADI4 were upregulated in patients with "short" survival, while the other four proteins were upregulated in patients with "long" survival.

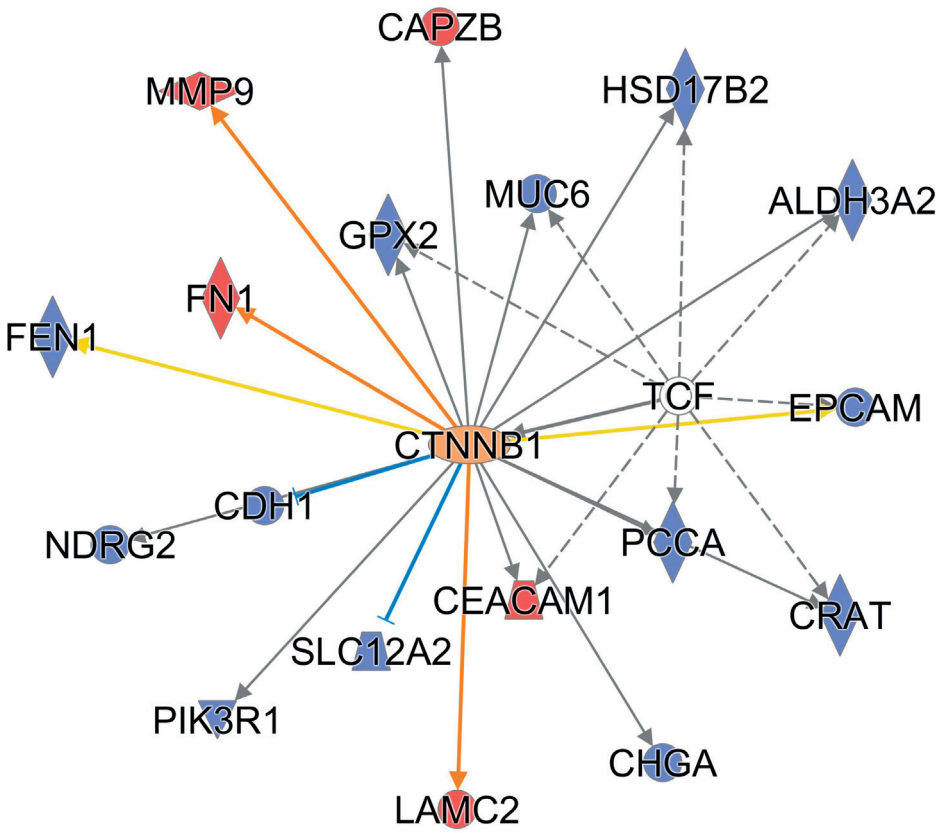

Figure 6: TCF1 and CTNNB1 are hubs of a mechanistic upstream regulatory network (IPA). Red symbols: proteins upregulated in short survival patients. Blue symbols: proteins upregulated in long survival patients. Orange edges: relationships predicted as activating. Blue edges: relationships predicted as inhibitory. Yellow edges: relationships inconsistent with downstream protein state.

Table 1: List of candidate prognostic biomarkers for pancreatic cancer

Entry	Gene	DDA				PRM			Description
		LS Freq.	SS Freq.	*P* value	SS/LS Fold change	Pep. no.	*P* value	SS/LS Fold change	
P14780	MMP9	10	9	0.026	3.62	2	0.045	4.44	Matrix metalloproteinase-9
O95833	CLIC3	1	6	0.039	3.25	2	0.010	3.42	Chloride intracellular channel protein 3
P22894	MMP8	1	6	0.029	4.28	1	0.046	3.06	Neutrophil collagenase
P24158	PRTN3	7	9	0.022	3.85	2	0.031	2.98	Myeloblastin
O15460-2	P4HA2	6	9	0.025	2.88	2	0.029	2.66	Isoform IIa of Prolyl 4-hydroxylase subunit alpha-2
P07996	THBS1	10	9	0.015	2.46	2	0.028	2.01	Thrombospondin-1
P02751	FN1	10	9	0.029	2.07	2	0.034	1.92	Fibronectin
A8K7I4	CLCA1	5	0	0.015	0.08	1	0.029	0.05	Calcium-activated chloride channel regulator 1
P09327	VIL1	10	5	0.004	0.11	2	0.008	0.12	Villin-1
O00748	CES2	5	0	0.008	0.15	2	0.029	0.16	Cocaine esterase
O75208	COQ9	8	2	0.004	0.27	2	0.004	0.19	Ubiquinone biosynthesis protein COQ9, mitochondrial
Q5R3I4	TTC38	10	5	0.017	0.21	1	0.035	0.20	Tetratricopeptide repeat protein 38
P56470	LGALS4	10	8	0.003	0.19	2	0.005	0.23	Galectin-4
P10645	CHGA	7	2	0.038	0.23	2	0.025	0.27	Chromogranin-A
Q9BW30	TPPP3	9	1	0.001	0.10	1	0.000	0.28	Tubulin polymerization-promoting protein family member 3
Q9H0R4	HDHD2	10	5	0.005	0.22	2	0.026	0.30	Isoform 2 of Haloacid dehalogenase-like hydrolase domain-containing protein 2
Q6UXN9	WDR82	7	2	0.039	0.22	1	0.023	0.31	WD repeat-containing protein 82
P16422	EPCAM	8	2	0.012	0.20	1	0.021	0.33	Epithelial cell adhesion molecule
Q96LJ7	DHRS1	7	1	0.007	0.24	1	0.017	0.37	Dehydrogenase/reductase SDR family member 1
P45954	ACADSB	9	3	0.017	0.15	2	0.026	0.38	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial
Q96IU4	ABHD14B	10	9	0.007	0.31	2	0.013	0.46	Alpha/beta hydrolase domain-containing protein 14B
Q6UXI9-6	NPNT	10	3	0.008	0.25	1	0.016	0.46	Isoform 6 of Nephronectin
P08294	SOD3	10	9	0.015	0.48	2	0.003	0.47	Extracellular superoxide dismutase [Cu-Zn]
Q8N335	GPD1L	9	4	0.030	0.16	2	0.010	0.47	Glycerol-3-phosphate dehydrogenase 1-like protein
Q7Z7H5	TMED4	10	4	0.002	0.13	2	0.040	0.48	Transmembrane emp24 domain-containing protein 4

Abbreviations: DDA:data-dependent acquisition; PRM:parallel reaction monitoring; LS: long survival; SS: short survival; Pep. No: number of peptides for proteins in the PRM panel. Freq.: number (frequency) of cases in which the protein was detected.

The predictive potential of THBS1 and MMP9 for the prognosis of pancreatic cancer has been reported in a few previous studies [14, 36, 37]. It has been suggested that TP53 inhibits angiogenesis by the regulation of THBS1 synthesis [38], while MMP9 degrades the extracellular matrix component and facilitates the invasion of tumors. PADI4 acts as a transcriptional corepressor for TP53 [39]. A study revealed that the TP53-PADI4 pathway participated in the response to DNA damage, nuclear fragmentation and TP53-mediated cell death

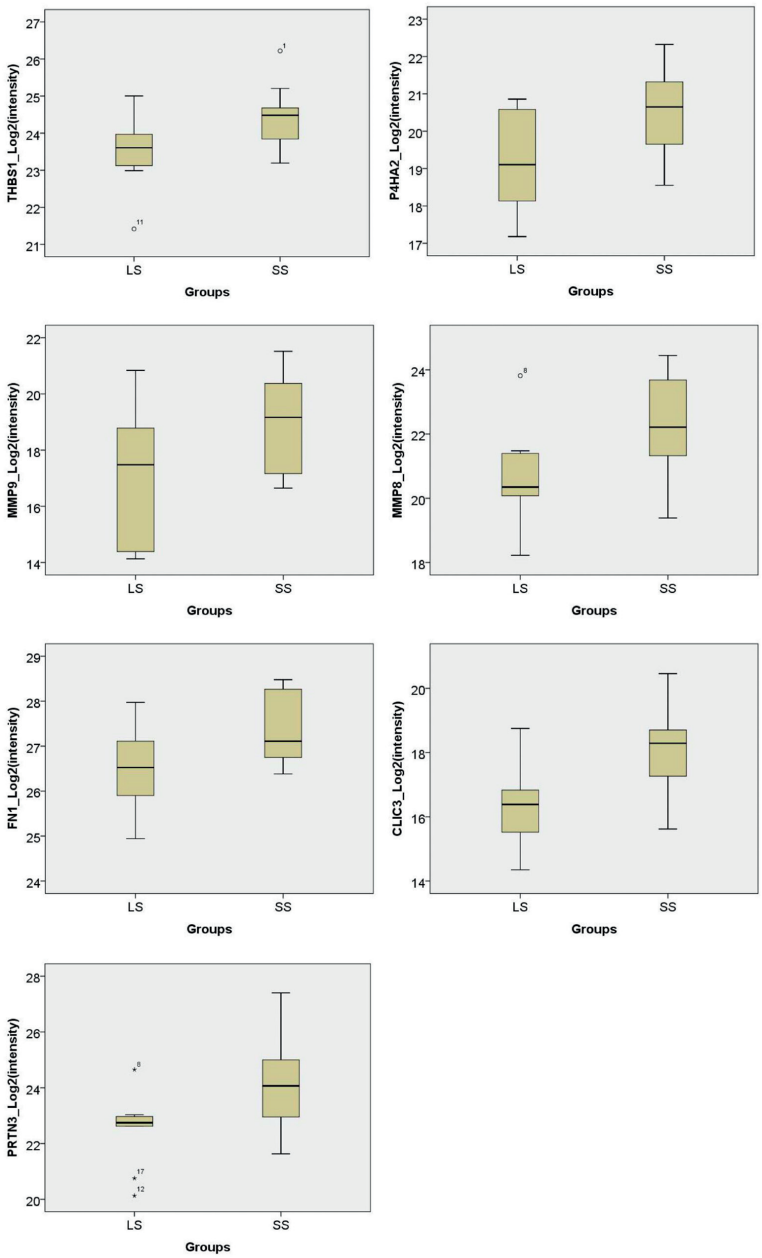

Figure 7: Boxplot of intensities of prognostic proteins from PRM phase in PDAC patients with "short" survival (SS) compared to "long" survival (LS) (all $P < 0.05$). Seven proteins (THBS1, P4HA2, MMP9, MMP8, FN1, CLIC3, PRTN3) were significantly upregulated (SS/LS fold change > 1.5) in patients with SS while CLCA1 were significantly upregulated (SS/LS fold change < 0.5) in patients with LS.

[40]. Inhibition of TP53 was also implicated in the downregulation of CDH1 and cell invasion in invasive carcinoma [41]. Notably, CDH1 has functional protein associations with differentially expressed proteins in our study including CDH17, PIK3R1, NDRG2, CTBP2, MMP9 and EPCAM according to the STRING database. Kaplan-Meier analysis showed that the expression of EPCAM was inversely correlated to the survival (months) of pancreatic cancer. It has been found that the TP53 protein negatively regulates EPCAM expression by binding to a response element within the EPCAM gene [42]. Higher expression of EPCAM is associated with an improved outcome in pancreatic cancer by suppressing cell activity [43, 44].

A histological hallmark of PDAC is that tumor cells are surrounded by as much as 90% stroma consisting of proliferating myofibroblast-like cells (pancreatic stellate cells), immune cells and inflammatory cells and extracellular matrix components such as collagen, fibrinogen, hyaluronan, and fibrin [45]. The microenvironment of pancreatic adenocarcinoma has a complex role in tumor growth and therapeutic response. While the existence of a dense stroma is thought to promote tumor progression and metastasis [46, 47], this concept has been challenged by recent experimental evidence showing that some elements of the stroma may actually restrain the tumor arguing for stromal re-shaping rather than pure depletion [48–50]. A number of clinical trials targeting the tumor-stroma interactions in PDAC are ongoing, however, the results seem to be inconclusive. Therefore, a further understanding of the tumor microenvironment is needed. A recent large-scale genomics analysis of PDAC by Moffitt et al. employed so-called virtual microdissection to elucidate tumor subtypes and to account for cellular heterogeneity in tumor samples, typically containing a large amount of stroma alongside the tumor itself [10]. They linked poor prognosis to sets of proteins named "activated stroma factors" as well as "basal tumor factors". Strikingly, our data parallels closely to their results. As seen in Figure 2, proteins classified by Moffitt as "activated stroma factors" and "basal tumor factors" were upregulated in short survival patients while proteins classified as "normal stroma factors" and "classic tumor factors" were upregulated in long survival patients. The proteins characteristic for these tumor features made up as much as approximately 20% of differentially

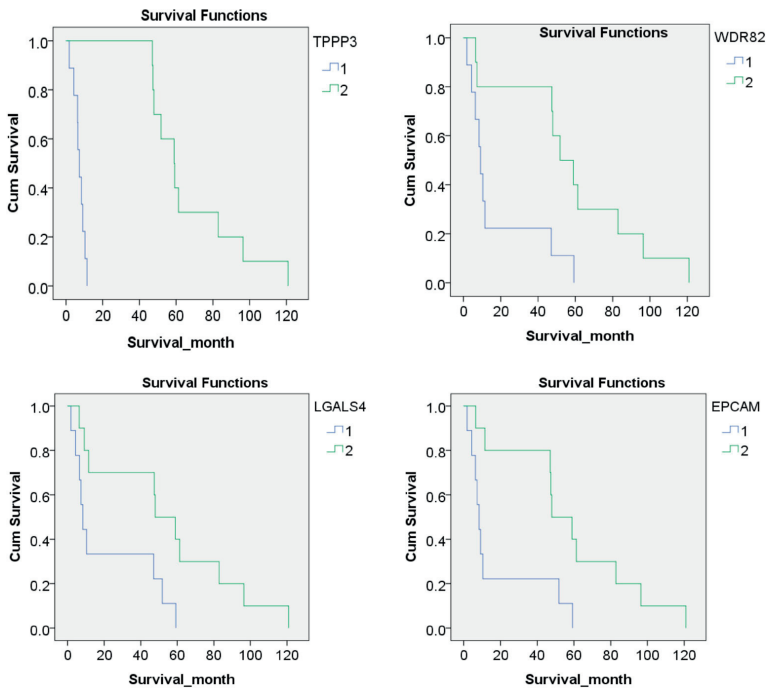

Figure 8: Kaplan-Meier analysis of protein expression. According to the expression of each protein, patients were divided into two groups: lower expression (9 cases, Blue line, marked by 1) and higher expression (10 cases, green line, marked by 2), Kaplan-Meier analysis showed that four proteins were significantly correlated to the survival months (TPPP3, WDR82, LGALS4 and EPCAM, *P* values were < 0.001, 0.008, 0.020 and 0.010, respectively).

expressed proteins. Our results are in accordance with the findings of Moffitt et al. and support the idea that an activated stroma state may be linked to poor prognosis [10].

In our study, many potentially prognostic proteins are related to the microenvironment of pancreatic cancer. Reactome pathway analysis revealed that 9 of the differentially expressed proteins were involved in extracellular matrix organization, including THBS1, PLOD1, LAMC2, P4HA2, MMP9, MMP8, FN1, CDH1 and CEACAM1. Four proteins participating in collagen formation, PLOD1, LAMC2, P4HA2 and MMP9, were all upregulated in patients with "short" survival compared to those with "long" survival. In comparison, out of five proteins participating in degradation of the extracellular matrix, four (LAMC2, FN1, MMP8 and MMP9) were upregulated and one (CDH1) was downregulated in the poor outcome group. This to some extent again suggests that the microenvironment in "short" survival patients was more activated, both in the formation and degradation of the extracellular matrix, which is believed to provide support to the surrounding tissues and serve as a physical barrier to drug delivery in PDAC [51]. Our study also revealed several collagen associated proteins as potential prognostic biomarkers, including P4HA2, THBS1 and FN1. P4HA2 participates in the biosynthesis of collagens by catalyzing the post-translational formation of 4-hydroxyproline in -Xaa-Pro-Gly- sequences in collagens. Studies have shown that the expression of P4HA2 were upregulated in the oral cavity in squamous cell carcinoma, papillary thyroid cancer, and breast cancer [52]. Furthermore, silencing P4HA2 or treatment with the P4HA inhibitor suppresses breast cancer progression by reducing tumor growth and a metastasis, which is accompanied by reduced collagen deposition, indicating its potential role as therapeutic target. FN1 has been suggested as a prognostic biomarker for pancreatic cancer in a proteomics study [14]. FN1 binds to its receptors such as integrins, inducing distinct signals to promote tumor angiogenesis and migration of PDAC cells [53]. A related molecule, regulator of integrin recycling, the CLIC3 intracellular chloride channel which drives invasiveness of pancreatic cancer is also upregulated in "short" survivors in the current study [54].

Two upstream regulators identified in our prognostic study, TCF1 and CTNNB1, emphasized the potential role of Wnt signaling pathway whose improper activation is responsible for establishment of cancer stem cells [55]. It has been recently reported that the disruption of nuclear complexes of CTNNB1 and HNF1A suppressed pancreatic tumor growth [56]. Wnt signaling has been widely implicated in cancer, especially colorectal cancer, in which mutation of key regulatory factors of the Wnt pathway (mainly APC and CTNNB1), was found in ninety percent of tumors, resulting in activation of the Wnt pathway [57–58]. However, the impact of Wnt signaling in PDAC is less clear. Although mutations of key Wnt pathway components are uncommon in PDAC, DNA methylation and expression status of multiple genes are involved in the regulation of Wnt pathway [59]. Nuclear localization of β-catenin is also regularly found in PDAC [60]. Inhibition of Wnt signaling using either a Wnt antagonist or a therapeutic monoclonal antibody in mice has been found to delay PDAC formation [61].

We have also noticed that some proteins mainly derived from polymorphonuclear neutrophils (PMNs), including MMP8, MMP9, MPO and PRTN3, were significantly upregulated in "short" survival patients. PMNs have received attention in the context of inflammation-driven tumorigenesis [62]. More neutrophils were found to be infiltrated in tumor cells in PDAC patients with poor survival [63, 64]. It is suggested that neutrophil-derived matrix-degrading proteases such as MMP8 and MMP9, might modulate the composition of the extracellular matrix and facilitate metastasis [65]. However, the expression of MMP8 and MMP9 can also be detected in tumor cells in patients with PDAC [66]. MMPs are also part of the apoptotic process: they cleave CDH5, PECAM1 and CDH1 during apoptosis of endothelial or epithelial cells [67]. PRTN3, also known as Myeloblastin and c-ANCA, is implicated in degradation of elastin, fibronectin, laminin, vitronectin, and collagen types I, III, and IV in *in-vitro* studies. Furthermore, PRTN3 has been shown to be involved in the degradation of extracellular matrix (ECM) proteins [68]. G12C mutation in the KRAS gene is associated significantly with an altered activity of PRTN3 in pulmonary adenocarcinomas [69]. Downregulation of PRTN3 has also been reported to inhibit proliferation and induces differentiation of promyelocyte-like leukemia cells [70].

In the light of the differential expression of several extracellular proteases, MMP8, MMP9, PRTN3 and DPP4, another protein, CLCA1, merits special mention. It is a novel self-cleaving extracellular metalloprotease [71, 72] and is a homologue of likely tumor suppressors, CLCA2 and CLCA4 [73, 74]. Low expression level of CLCA1 was observed to be linked to poor prognosis in colorectal cancer and CLCA1 itself has been proposed as a prognostic marker [75, 76]. Thus, it is an attractive hypothesis that CLCA1 has a role in tumor suppression in PDAC, either by interaction with tumor microenvironment or by proteolytic activation of yet undiscovered substrates. Another explanation of the link between CLCA1 expression and survival is the confirmed role of this protein in modulating the TMEM16A/ANO1 Ca^{2+}-activated chloride channel [72, 77]. Ion channels in general, and Ca^{2+}-activated chloride channels in particular are known to be involved in regulating cell proliferation, cell migration and metastasis and are believed to be important emerging cancer drug targets in cancer [78, 79], particularly in pancreatic cancer where they may be mediating interactions with the tumor microenvironment [80].

Apart from dysregulated pathways and processes, several of the proteins differentiating "long" and "short" survivors were previously noted as potential tumor markers. FAM96A, upregulated in "long" survivors, has been previously shown to regulate the iron-sulphur cluster assembly [81] and was reported to be a tumor suppressor [82]. CDH1 and CDH17 are also upregulated in "long" survivors. CDH17 is a known gastric cancer marker [83] while upregulation of CDH1 inhibits pancreatic cancer metastasis [84]. Another potential prognostic biomarker upregulated in "long" survivors in this study, LGALS4, was proposed as exocrine-like subtype PDAC marker [85]. Its homologue, LGALS1, has been previously reported to be associated with long-term survival in PDAC [15].

Another novel observation notable among proteins significantly correlated to survival is the UPF0553 protein C9orf64. This is a typical example of an interesting protein whose obscure gene symbol makes it likely to be ignored in large-scale studies [86]. In fact, C9orf64 is a protein of Q_salvage family in the Pfam database (PF10343, previously called DUF2419). Similar to DNA glycosidases and ribonucleoside hydrolases, it is involved in salvaging the micronutrient queuosine [87]. The importance of queuosine, which is involved in tRNA covalent modifications [88] is starting to be appreciated, as its roles in modulating cell proliferation are elucidated and correlation of queuosine deficiency of tRNA to severity of malignancy is revealed [89]. Thus, our results provide the first hypothesis that a link may exist between queuosine modifications and PDAC.

In conclusion, we have identified several tumor-expressed proteins that offer prognostic information in PDAC. Of note, TP53 related proteins and neutrophil-derived proteins were upregulated in PDAC patients with poor survival, supporting their potential role in tumor progression. Our results indicate that the tumor microenvironment, with an activated stroma state, is closely related to disease progression. The findings also highlight the importance of the Wnt signaling pathway. Nevertheless, there are some limitations of the present study that deserve to be mentioned. Firstly, by employing a label-free quantification, only a relative quantification was possible and the absolute upregulation or downregulation of proteins in each survival group remains unknown. Incorporating corresponding normal tissues would also be of value. Secondly, the prognostic significance of the biomarker candidates needs to be validated in larger cohorts with alternative approaches, which are more accessible in the clinic, such as immunohistochemistry and tissue microarray technology. Finally, we recommend further in-depth analysis into the mechanistic role of identified biomarker candidates in order to better understand the pathophysiological events in PDAC.

MATERIALS AND METHODS

Patients and samples

Patients with surgically resectable PDAC were diagnosed and underwent surgery at the Department of Surgery, Skåne University Hospital, Lund, Sweden, between the year of 1995 and 2011.Archival FFPE tissue samples were obtained from the primary tumor and the tissue blocks were sectioned at a thickness of 10 µm. The hematoxylin-eosin staining FFPE slides from each patient were carefully reviewed by our pathologist (Figure 9). For each patient, two sections were collected in one tube, which were barcoded to be traceable and referred

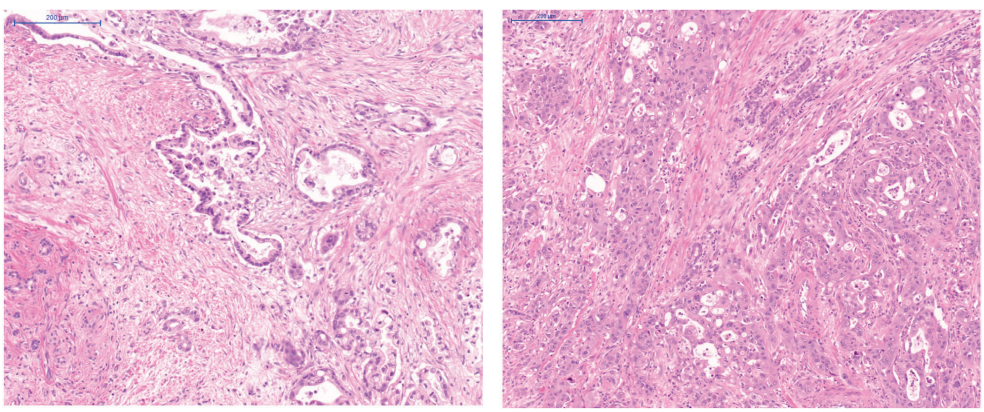

Figure 9: Histology images of FFPE slides from two representative cases. H&E, 10x objective magnification. Left: A long survival PDAC case with histological grade 2. Notice irregular gland formation located in rich stroma. Abnormal epithel imitating normal duct epithel. Adenocarcinoma is situated in upper right part of the picture and infiltrate an atrophied pancreas parenchyma. Right: A short survival PDAC case with histological grade 3. Notice solid area of cancer structures of cells with nuclear pleomorphy and relative scanty cytoplasma. Stroma is not dominant in this picture.

to their patient identities. These tubes were stored in the South Swedish Biobank, which is located in the Center of Excellence in Biological and Medical Mass Spectrometry (CEBMMS), at the Biomedical Center (BMC), Lund, Sweden. From the biobank, we retrospectively selected patients with PDAC who met the following criteria: 1) "short" survival (< 12 months) or "long" survival (> 45 months); 2) resectable disease; 3) tumors located in the head of the pancreas. Accordingly, 9 patients with PDAC with "short" survival and 10 patients with "long" survival were selected for further study. There were no significant differences in terms of pathologically confirmed lymph node metastasis, R1 resection status and use of chemotherapy between "short" and "long" survival groups. The clinical characteristics of the patients are summarized in Table 2. Ethical approval for this study was granted by the institutional review board at Lund University.

Sample preparation

Two sections of FFPE tissues (10 µm) from each patient were obtained and incubated in 1mL of 1:50 diluted EnVision™ FLEX Target Retrieval Solution, High pH (Dako, Glostrup, Copenhagen, Denmark) for 10 min at 97°C, followed by centrifugation at 14,000g for 3 min and removal of the supernatant. After a repeated de-paraffinization step, the pellets were incubated in 1mL 500 mM Tris-HCl pH 8.0 at 90°C for 1.5 hours to break down cross-linking between proteins and other molecules. This was followed by centrifugation at 14,000g at 4°C for 15 min, and the supernatant was removed. For denaturation and extraction of proteins, 250 µL 6 M Guanidine-HCl in 50 mM Ammonium bicarbonate (AMBIC) was added and sonication was applied by sonication probe (Branson SLPe, Emerson Electric Co., St. Louis, MO, USA), operating with 20% amplitude, 5 min for 2 times and 20 seconds cool down period in-between on ice. After centrifugation at 14,000g for 10 min, the supernatant was stored. Protein concentration was determined by Micro BCA Protein Assay Kit (Thermo Fisher Scientific, San José, CA, USA). For each sample 150 µg proteins were diluted by AMBIC in a final volume of 180 µL and 7.5 µL of chicken lysozyme (0.02 µg/µL) was added to evaluate the variance from sample handling and instrument performance among samples. Following reduction with 3 mM DTT (1 h at 56°C) and alkylation with 15 mM iodoacetamide (30 min at 24°C in dark), the samples underwent precipitation with 1:9 volume ratio of samples to pure ethanol overnight. This was followed by centrifugation at 14,000g at 4°C for 15 min and carefully removal of the supernatant. The pellets were dissolved in 200 µL AMBIC, followed by adding 1.25 µg trypsin (Promega, Madison, WI, USA) for digestion at 37°C for 18 h. Peptide concentrations were determined by Micro BCA kit.

Exploiting strong cation exchange by Microspin column (MA SEM HIL-SCX, 10–100 µg capacity, The Nest group Inc., South Borough, MA, USA), 30 µg peptides from each sample were separated into 5 fractions by applying step-wise gradient of 20 mM, 40 mM, 60 mM, 100 mM and 500 mM KCl in 10 mM KH_2PO4 containing 20% ACN (pH = 2.8). Each fraction underwent desalting by Ultra Microspin Silica C18 column (SUM SS18V, 3–30 µg capacity, The Nest group Inc.). Fractions were dried by centrifugal evaporator and each fraction was resuspended with 30 µL of solvent A (0.1% formic acid).

nanoLC-MS/MS analysis (Discovery phase)

The digested peptides were loaded onto a C18 trap column (Acclaim PepMap 100 pre-column, 2 cm x 75 µm ID, 3 µm particles, 100 Å pore size, PN: 164705, Thermo Fisher Scientific) and then separated on a C18 analytical column (EASY-Spray column, 25 cm x 75 µm ID, 2 µm particles, 100 Å pore size, PN: ES802, Thermo Fisher Scientific). A flow rate of 300 nL/min and a column temperature of 35°C were applied. A nonlinear gradient was exploited using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile). The gradient went from 7% to 26% B during the first 70 min, then increasing to 35% B during the next 20 min, followed by a raise to 90% B in 5 min, which was maintained for 15 min. The total amount of fractionated protein digest injected onto the column was estimated to be 1 µg. Fractionated samples were injected in the order of increasing salt concentrations used for elution of the peptides. To avoid carryover, each sample injection was followed by a blank injection with solvent A. Each fraction was measured for three times.

The fractionated protein digests were analysed on a Q-Exactive Plus mass spectrometer connected to an Easy-nLC 1000 pump (Thermo Fisher Scientific) with a top 10 data-dependent acquisition (DDA) method. For ionization, 1.8–2.0 kV of spray voltage and 280°C capillary temperature were used. Full MS scans were acquired with the Orbitrap mass analyser over m/z 350–1800 range with resolution of 70,000 (at m/z 200), target AGC value of 1e6 and maximum injection time of 100 ms. The ten most intense peaks with charge state >= 2 were fragmented in the HCD collision cell with normalized collision energy of 30%, and tandem mass spectra were acquired in the Orbitrap mass analyzer with resolution of 35,000 (at m/z 200), target AGC value of 1e6 and maximum injection time of 120 ms. The ion selection threshold was set to 4.2e4 and dynamic exclusion was 20 s.

Verification by parallel reaction monitoring

Using unfractionated protein digests from each sample, a targeted proteomic method, parallel reaction monitoring (PRM) was employed to verify the

Table 2: Patient characteristics

	PDAC (SS)	PDAC (LS)
Sex (female/male)	3/6	7/3
Age [median (range), year]	64 (48–74)	71 (43–77)
Diabetes mellitus	5	4
Tumor location		
pancreas head	9	10
Tumor diameter (cm)	2.5 (1–6)	3 (2–7)
Lymph node metastasis	4	7
Staging		
IIA	5	3
IIB	4	7
R1 resection	4	3
Surgery	9	10
Adjuvant chemotherapy	5	9
Gemcitabine	3	5
5-FU	1	1
Capecitabine	0	2
Gemcitabine, 5-FU	1	0
Gemcitabine, Capecitabine	0	1
Radiotherapy	1	0
Survival (mean (SD), month)	7.3 (1.9–11.5)	59.1 (47.0–120.9)

Abbreviations: PDAC: Pancreatic ductal adenocarcinoma; SS: short survival; LS: long survival.

differentially expressed proteins. One or two unique peptides of each protein of interest were selected. A panel of 110 peptides from 73 proteins was finally scheduled in one run to verify potentially prognostic proteins in 10 patients with "long" survival and 9 patients with "short" survival. Five peptides from chicken lysozyme and five PRTC peptides (Product no 88320, Pierce, Rockford, IL, USA) were added to the PRM panel to evaluate the experimental process. The samples were prepared in the same way as it was described previously but without SCX fractionation. The retention time, precursor m/z and charge state of peptides was referred to the prior DDA experiments. The retention times and transitions were further modified and confirmed in several preliminary PRM runs. The same LC-MS platform was applied for the PRM study. A total of 1 µg peptide was injected and the same LC parameters were used for the separation. Targeted MS^2 mode was operated with time-scheduled acquisition of the selected peptides in +/− 5 min retention time windows. PRM scanning was performed at 17,500 resolution (AGC target 1×10^5, 50 ms maximum injection time) as triggered by a scheduled inclusion list. The chromatographic peak width is 30 s. Fragmentation was performed with normalized collision energy of 27 and MS/MS scans were acquired with a resolution of 70,000 at m/z 200.

Statistics and bioinformatics

Exploiting multidimensional protein identification technology (MudPIT), the data from 5 fractions of each sample were submitted together to Sequest HT search engine in Proteome Discoverer 1.4, being processed as one continuous input file for protein identification and quantification. The quantification of protein intensities is based on the averaged intensities of their three most abundant peptides. Uniprot Human Reviewed (released 2013/09) was referred as search database. Decoy database containing reversed version of all protein sequences were added for the monitoring of false discovery rate (FDR). For the identification of peptides, precursor and fragment mass tolerances were 10 ppm and 0.02 Da respectively. Oxidation and carbamidomethylation were taken into consideration as variable and static modifications, respectively, and one maximum missed cleavage site was allowed. Proteins were identified based on at least two peptides with high confidence (FDR < 1%). Precursor ions area detector was applied in the search engine for the quantification of peptides. Redundant proteins were automatically grouped by default. Perseus software [90] was used for the statistics. Those proteins that were detected in less than half (<5) of the samples in both groups were excluded from further analysis. To minimize

the technical variance introduced by sample handling and instrument, each sample was run for three times (replicates) whereas the intensities of proteins in each replicate were normalized to its median intensity. Log 2 transformation was applied to the normalized intensities to make the data normally distributed and suitable for further statistics. Missing values were replaced from random numbers drawn from a normal distribution, which represents low abundance measurements (default setting). Using Student's *t*-test, protein intensities were compared between two groups based on the average of log 2 transformed normalized protein intensities in each sample. Proteins were also defined as differentially expressed if detected more frequently (≥ 5 samples) in one group than in the other group. Hierarchical clustering and principal component analysis were also performed to visualize any significant differences between two groups. Skyline software was used for MS1 filtering and MS1 quantitation in the PRM study. The intensities of targeted peptide of each protein were log 2 transformed and then compared between groups by Student's *t*-test. For those proteins having two targeted peptides, the peptide with higher intensity will be compared. The bioinformatics analysis of relationship networks between differentially expressed proteins used STRING [19] and Ingenuity Pathway Analysis (IPA, Qiagen, Inc. Redwood City, CA, USA). Assessment of overrepresented functional annotations and pathways was performed using Gene Ontology resources [91], Panther [92], Reactome [93], David [94] and IPA. In David, Panther and IPA, the whole sets of proteins detected in the study were used as analysis backgrounds.

Abbreviations

CV: coefficient of variation; DDA: data-dependent acquisition; ECM: extracellular matrix; FDR: false discovery rate; FFPE: formalin-fixed paraffin-embedded; IPA: Ingenuity Pathway Analysis; MMPs: matrix metalloproteases; MudPIT: multidimensional protein identification technology; PC: Pancreatic cancer; PDAC: pancreatic ductal adenocarcinoma; PMNs: polymorphonuclear neutrophils; PRM: parallel reaction monitoring; PSMs: peptide-spectrum matches.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Paper II

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Calcium-activated chloride channel regulator 1 as a prognostic biomarker in pancreatic ductal adenocarcinoma

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Abstract

Background: In a previous study utilizing mass spectrometry-based proteomics, we identified calcium-activated chloride channel regulator 1 (CLCA1) as a potential tumor suppressor in pancreatic cancer and the expression was inversely correlated with patient survival. The aim of the study was to further validate the prognostic significance of CLCA1 in pancreatic cancer.

Methods: CLCA1 expression was evaluated with tissue microarrays and immunohistochemistry in 140 patients with pancreatic ductal adenocarcinoma that underwent surgical resection at Skåne University Hospital, Sweden. Kaplan-Meier and Cox proportional hazards modeling were used to explore the association between CLCA1 and clinicopathological factors and survival.

Results: CLCA1 expression was denoted as positive in 90 tumors (64.3%), with positive staining being limited to the tumor cells. There were no significant association between CLCA1 expression and established clinicopathological parameters. Low CLCA1 expression correlated significantly with shorter disease-free survival (11.9 vs 17.5 months, $P = 0.042$). Multivariable Cox regression analysis confirmed the results (HR 0.61, 95% CI-0.40-0.92, $P = 0.019$).

Conclusions: Low CLCA1 expression is an independent factor of poor disease-free survival in pancreatic cancer.

Keywords: Pancreatic ductal adenocarcinoma, CLCA1, Calcium-activated chloride channel regulators, Survival

Background

Pancreatic ductal adenocarcinoma (PDAC) is currently the third leading cause of cancer-related mortality [1]. Although achievements have been made to improve the diagnosis and treatment of PDAC, the five-year survival rate remains as low as 6% [2]. Due to the silent progression of the disease and lack of early screening techniques, most patients are diagnosed at an advanced stage, precluding potentially curative surgery. Moreover, tumor heterogeneity is strongly implicated in the biological behavior of PDAC, as well as the response to therapy [3]. More information is needed concerning molecular factors that can contribute to an earlier diagnosis and a better prediction of prognosis and treatment response.

Calcium-activated chloride channel regulators (CLCAs), also called "chloride channel accessory proteins", are a family of secreted self-cleaving proteins which activate calcium-dependent chloride currents. The human genome encodes 3 functional CLCA proteins, including CLCA1, CLCA2, and CLCA4. As one form of ion channels, Ca^{2+}-activated chloride channels have been implicated in regulation of cell proliferation, cell migration and metastasis and are believed to be emerging therapeutic targets in cancer [4–6]. CLCA1 is mainly expressed in the large and small intestine and appendix, especially in crypt cells, and can be shed into the blood stream. It has been reported that CLCA1 can regulate the differentiation of colorectal cancer cells and function as a prognostic marker in colorectal cancer [7].

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Recent studies also supported that CLCA2 and CLCA4 may serve as tumor suppressors in breast cancer [8].

Our previous mass spectrometry-based proteomics study showed for the first time that CLCA1 is a biomarker for PDAC, with protein expression being 20 fold down-regulated in poor outcome PDAC patients [9]. The aim of the present study was to investigate the prognostic impact of CLCA1 in a large and well annotated clinical cohort of resectable PDAC patients.

Methods

Patients and samples

The REMARK guidelines were followed where possible throughout the whole study [10]. Formalin-fixed paraffin-embedded tissue samples were collected from 140 patients with PDAC who underwent pancreatic resection at the Department of Surgery, Skåne University Hospital, Lund and Malmö, Sweden, between 1996 and 2017. All tissue specimens were re-evaluated by a senior pancreas pathologist (A.S.) to ensure correct diagnosis and histopathological characterization. Disease-free survival (DFS) was defined as the time from pancreatectomy to the first evidence of clinical recurrence (locoregional or distant) or death from any cause. Overall survival (OS) was defined as the time from pancreatectomy to death from any cause or the last date the patient was seen alive. Ethical approval was obtained from the local human ethics committee at Lund University (Ref 2017/320).

Tissue microarray

Tumors with sufficient amount of material were deemed suitable for tissue microarray (TMA) construction. Compared with usage of whole sections, TMA has the advantage of a reduced consumption of both tissue and time which enables studies of a larger scale with reduced experimental variability [11]. Using an automated tissue arraying device (Minicore® 3, Alphelys, Plaisir, France), 4 cores 2 mm of cancer tissues (marked by pathologist A.S) from each specimen were stabilized into paraffin blocks. After a fine quality was assured, the TMA-blocks were sectioned for immunohistochemical analysis.

Immunohistochemistry

TMA-sections (3 μm thick) were heated in 60 °C for 1 h and then cooled in room temperature (RT). Next, using automated PT Link (Dako, Glostrup, Denmark), deparaffinization, rehydration and antigen-retrieval were performed in EnVision FLEX Target Retrieval Solution high pH (K800421–2, Dako) heated to 96 °C for 20 min. After three times of wash in phosphate-buffered saline for 5 min, sections were blocked against endogenous peroxidase activity with 0.3% H_20_2 and 1% methanol in phosphate-buffered saline for 10 min. The specimens were then blocked with 5% goat serum for 1 h at RT to reduce non-specific background staining, followed by avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA) for 15 min at RT, which reduces endogenous avidin and biotin activity. Subsequently, the sections were incubated with rabbit recombinant monoclonal CLCA1 antibody (Abcam, Cambridge, UK; cat no ab180851; dilution 1:2000) at 4 °C overnight. Next, sections were incubated with biotinylated secondary goat anti-rabbit antibodies (BA-1000, dilution 1:200, Vector Laboratories) for 1 h at RT. Following incubation with avidin-biotin-peroxidase complex (Vectastain Elite ABC-HRP Kit, PK-6100, Vector Laboratories) for 30 min at RT, the sections were incubated with chromogen diaminobenzidine (SK-4100, Vector Laboratories) for 5 min. After washing in deionized water for 5 min, nuclei were counterstained with Mayer's hematoxylin (Histolab, Gothenburg, Sweden) for 30 s, and washed in tap water for another 5 min. Finally, the specimens were dehydrated in graded alcohols and mounted using Pertex (Histolab). Negative controls were produced by omitting the primary antibodies. Slides were scanned for evaluation using an Aperio scanscope scanner (Leica Biosystems, Wetzlar, Germany). Variability between individuals was reduced by limiting sample preparation and analysis of expression to one professional respectively.

Scoring procedure

The immunostaining of CLCA1 was assessed semi-quantitatively by an experienced pancreas pathologist (A.S.) blinded to the clinical outcome. Staining below 10% was denoted as negative (0). When > 10% of tumor cells were stained, expression was considered positive and denoted as mild (1), moderate (2) or strong (3) depending on the intensity. Samples with negative staining (0) and mild staining (1) were categorized as low expression group, while those with moderate (2) and strong (3) were categorized as high expression group.

Statistical analysis

Comparisons of categorical data were performed using Chi-square test or Fisher's exact test. Continuous data were compared by the Mann Whitney U test. Kaplan–Meier analysis and log rank test were used to illustrate differences in DFS and OS according to CLCA1 expression. Cox regression proportional hazards models were used for estimation of hazard ratios (HRs) for recurrence and death according to CLCA1 expression. Any variable with a *P*-value less than 0.25 was selected as a candidate for the mulvariable Cox regression analysis. In the iterative process of variable selection using forward, backward and stepwise selection covariates were removed from the model if they were non-significant and not a confounder as described by Hosmer-Lemeshow,

resulting in the main effect model [12]. A *P*-value less than 0.05 was considered statistically significant. All the statistics were performed using STATA MP 14.1.

Results

Patient cohort

Baseline characteristics of patients with PDAC are presented in Table 1. The median age was 69 years (interquartile range 63–73 years) and 73 (52.1%) were male. The estimated median DFS was 13.2 months and the estimated median OS was 25.0 months, respectively. One hundred thirteen (80.7%) of patients received adjuvant chemotherapy.

CLCA1 expression in PDAC

CLCA1 expression was considered positive in 90 (64.3%) of the 140 tumors. The expression of CLCA1 was limited to the tumor cells. Mild, moderate and strong staining of CLCA1 were present in 37 (26.4%), 41 (29.3%) and 12 (8.6%) cases respectively. Figure 1 shows representative immunohistochemical images of CLCA1 expression in PDAC.

Table 1 Clinicopathological characteristics of patients with pancreatic ductal adenocarcinoma stratified by CLCA1 expression

Factors	Low CLCA1 *N* = 87	High CLCA1 *N* = 53	*P* value	Missing
Age, years	69 (62–75)	68 (64–72)	0.258	
male gender	44 (50.6)	29 (54.7)	0.634	
T-stage			0.649	0.7%
- T1	12 (13.8)	7 (13.2)		
- T2	58 (66.7)	35 (66.0)		
- T3	16 (18.4)	10 (18.9)		
- T4	0	1 (1.9)		
N-stage			0.485	1.4%
- N0	21 (24.1)	12 (22.6)		
- N1	30 (34.5)	24 (45.3)		
- N2	34 (39.1)	17 (32.1)		
AJCC stage, 8th edition			0.835	1.4%
- IA	4 (4.6)	2 (3.8)		
- IB	12 (13.8)	7 (13.2)		
- IIA	4 (4.6)	3 (5.7)		
- IIB	31 (35.6)	23 (43.4)		
- III	34 (39.1)	18 (34.0)		
Tumor differentiation			0.879	1.4%
- Well	4 (4.6)	3 (5.7)		
- Moderate	28 (32.2)	20 (37.7)		
- Poor	50 (57.5)	29 (54.7)		
- Undifferentiated	3 (3.4)	1 (1.9)		
R1 resection margin	35 (40.2)	20 (37.7)	0.552	0.7%
Adjuvant chemotherapy	68 (78.2)	45 (84.9)	0.129	3.6%

Qualitative data are expressed as N (%) and quantitative data as median (interquartile range). *AJCC* American joint committee on cancer

Association between CLCA1 expression and clinicopathological characteristics

The expression of CLCA1 was not associated with any traditional clinical parameters, including age, gender, TNM stage, histological grade, resection margin status and adjuvant chemotherapy.

Association between CLCA1 expression and survival

Kaplan–Meier analysis revealed that CLCA1 expression correlated with a significantly shorter DFS, with the worst outcome for tumors with low CLCA1 expression (Fig. 2). Median DFS was 11.9 months in patients with low CLCA1 expression and 17.5 months in patients with high CLCA1 expression, $P = 0.042$. These findings were confirmed in univariable Cox regression analysis (HR 0.66, 95% CI-0.44-0.99, $P = 0.044$), and remained significant in multivariable analysis (HR 0.61, 95% CI-0.40-0.92, $P = 0.019$), adjusted for differentiation grade and resection margin status (Table 2). The OS was also reduced in patients with low CLCA1, but the association did not reach statistical significance. The median OS was 23.5 months in patients with low CLCA1 expression and 27.8 months in patients with high CLCA1 expression ($P > 0.05$) (Fig. 3).

Discussion

This study demonstrated that low CLCA1 expression is an independent factor of shorter DFS. This is in line with our previous proteomics work utilizing mass spectrometry [9], validating our findings with an orthogonal technique in a larger cohort.

Ion channels, in general, and Ca^{2+}-activated chloride channels in particular, are known to be involved in the regulation of cell proliferation, cell migration and metastasis and are considered emerging cancer drug targets [5, 6]. Several studies have reported that the CLCA1 expression is down-regulated in colorectal cancer tissues compared with adjacent normal tissues [13–16], with low CLCA1 expression predicting worse outcomes [7, 17]. Knockdown of CLCA1 in Caco-2 cell lines have been shown to inhibit cell differentiation and promote cell proliferation [15]. Further in-vitro experiments suggested that CLCA1 may function as a tumor suppressor in colorectal cancer by inhibiting the Wnt/beta-catenin signaling pathway and epithelial-mesenchymal transition, while in-vivo overexpression of CLCA1 led to inhibition of proliferation and metastasis [14].

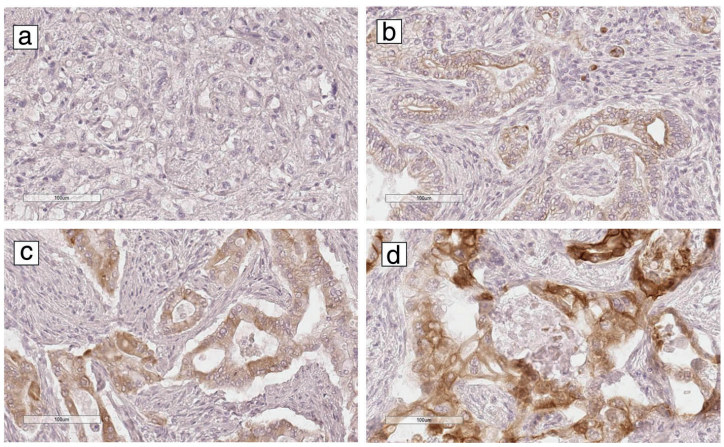

Fig. 1 Representative immunohistochemical images of CLCA1 expression in pancreatic ductal adenocarcinoma. **a** negative, **b** weak, **c** moderate, **d** strong staining

However, there is scant literature on the expression pattern and underlying function of CLCA1 in PDAC. Protein expression data in the Human Protein Atlas indicate that CLCA1 is mainly expressed in small and large intestines and appendix, while absent in both normal and cancerous pancreas tissues [18]. However, we noted that CLCA1 was present in more than half of pancreatic cancer tissues in our study. It is worth mentioning that CLCA1 can be secreted into pancreatic cyst fluid and the blood stream, which makes the CLCA1 a possible serum and fluid biomarker for PDAC [19]. Most recent evidence supported that CLCA1 mediates metalloprotease activity and is involved in intestinal mucus homeostasis by facilitating processing and removal of mucus [20]. This arises interest to address whether similar mechanisms are implicated in intraductal papillary mucinous neoplasm, a precursor of PDAC manifested with mucus contained cyst. Indeed, CLCA1 has been proposed as a supportive marker for high-grade dysplasia and malignant transformation using cyst fluid samples [19].

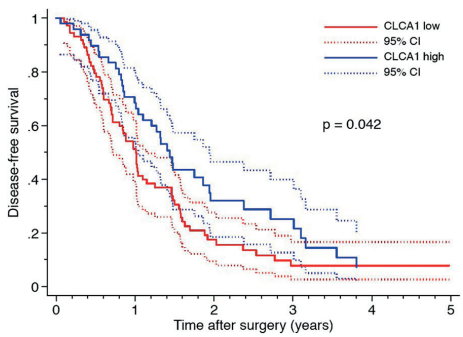

Fig. 2 Low expression of CLCA1 is associated with a poor DFS in pancreatic cancer patients undergoing surgical resection

PDAC is characterized by a dense and heterogenous tumor microenvironment (TME), which drives tumor progression and resistance to therapy. While the past four decades have seen no decline in death rates of this devastating malignancy [1], a better understanding of the mechanisms how pancreatic cancer cell interactions with their TME might open new avenues of research in effective treatments of PDAC. Ion channels are involved in intracellular signaling events and activate specific cellular responses, including cancer-related proliferation, apoptosis, migration and angiogenesis [21]. Ion channels and their interactions with integrin in TME can contribute to tumor development and emerging drug targets [22]. For example, neutrophils in the TME release Cl^- to accomplish their antimicrobial activity [23]. Furthermore, activated vascular endothelial cells are required for angiogenesis, in which Ca^{2+} permeable channels and Ca^{2+}-dependent signaling play crucial roles [21]. Abdel-Gany et al. also confirmed that CLCAs facilitated vascular arrest of cancer cells via interacting with β_4 integrin and promote metastatic growth [23].

Secreted CLCA1 has been demonstrated to be a direct modulator of another calcium-dependent chloride channel, TMEM16A [24, 25]. CLCA1 can stabilize TMEM16A on the cell surface and prevent its internalization, thus

Table 2 Univariable and multivariable Cox survival analyses for DFS

Variables	Univariable analysis			Multivariable analysis		
	HR	95%CI	*P* value	HR	95% CI	*P* value
Age	0.98	0.96–1.00	0.076			
Female gender	0.66	0.45–0.98	0.039			
T-stage	1.14	0.82–1.58	0.441			
N-stage	1.14	0.89–1.45	0.311			
Differentiation grade	1.48	1.06–2.07	0.023	1.55	1.09–2.18	0.014
Resection margin (R1)	1.55	1.02–2.33	0.036	1.63	1.07–2.49	0.023
Adjuvant chemotherapy	1.57	0.86–2.89	0.144			
CLCA1 expression, high vs low	0.66	0.44–0.99	0.044	0.61	0.40–0.92	0.019

activating chloride currents [24, 25]. While the role of CLCA1 in PDAC remain unclear, TMEM16A was found to be overexpressed in PDAC cells and promote the cell migration [26]. TMEM16A has also been proposed to contribute to tumor growth and invasion of lung cancer, prostate cancer and head and neck squamous cell carcinomas [27–29].

In this study, CLCA1 predicted DFS, but not OS. Although DFS and OS are partly related, there are differences. In DFS, any type of recurrence or spread is counted as an event, including isolated local recurrences. Patients with low CLCA1 expression seemed to have more early recurrences, i.e. occurring within 1 year of surgery. Multimodal treatment of recurrent pancreatic cancer has been found to prolong survival [30, 31]. However, after 5 years of median follow-up, the number at risk in the patient cohort was only 11 patients due to the high mortality rate. Therefore, our sample size might be underpowered to show a statistically meaningful result in terms of OS.

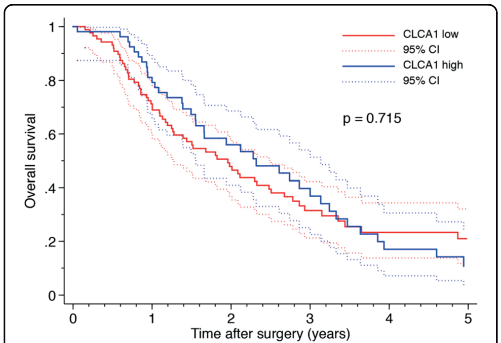

Fig. 3 Association between CLCA1 expression and OS in pancreatic cancer patients undergoing surgical resection

Conclusion

This study shows that low CLCA1 expression is a predictor of worse DFS in PDAC. CLCA1 may in the future be integrated into an immunohistochemistry panel to predict prognosis and treatment response in patients who undergo surgical resection. As ion channels have been suggested as emerging cancer drug targets, further investigation into the molecular mechanisms of CLCA1 in PDAC is needed.

Abbreviations

CLCA1: Calcium-activated chloride channel regulator 1; DFS: Disease-free survival; HRs: Hazard ratios; OS: Overall survival; PDAC: Pancreatic ductal adenocarcinoma; RT: Room temperature; TMA: Tissue microarray; TME: Tumor microenvironment

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Availability of data and materials

All relevant data and materials are included in the manuscript. For the full detailed data, please contact the corresponding author.

Authors' contributions

D.H. performed the experiments, analyzed the data and wrote the initial draft of the manuscript; D.A. collected the clinical data and follow-up of the patients, analyzed the data and assisted in manuscript writing and study conception; A.S. performed the histopathological evaluation of the tissue specimens; Q.Z., K.S.H., M.B., Y.J and R.A. critically revised the manuscript for important intellectual content; R.A. conceived the study; all authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the local human ethics committee at Lund University (Ref 2017/320). Written informed consent was given by participants in this study.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Galectin 4 is a biomarker for early recurrence and death after surgical resection for pancreatic ductal adenocarcinoma

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ABSTRACT

Background: Galectins are a group of carbohydrate-binding proteins that are involved in neoplastic development and progression. In a previous mass spectrometry-based study, we identified galectin 4 as a down-regulated protein in short-term survivors of pancreatic cancer. This study was performed to validate the prognostic value of galectin 4 in a larger cohort of pancreatic cancer patients undergoing surgical resection.

Methods: Galectin 4 expression was evaluated by tissue microarrays and immunohistochemistry in 140 patients with surgically resected pancreatic cancer. Kaplan-Meier and Cox proportional hazards modeling were used to explore the association between galectin 4 and survival.

Results: Galectin 4 staining expression was positive in 111 cases (79.3%). The expression of galectin 4 was significantly associated with tumor size ($p = .008$) and differentiation ($p = .001$). Galectin 4 expression was significantly correlated with disease recurrence within 1 year of surgery (adjusted HR 0.485, $p = .027$). There was also a significant association between galectin 4 and overall survival at 1 year (adjusted HR 0.482, $p = .047$) and at 3 years (adjusted HR 0.550, $p = .025$).

Conclusion: Galectin 4 expression is a novel biomarker for early recurrence and mortality after surgical resection for pancreatic cancer.

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Pancreatic cancer; galectin 4; LGALS4; early recurrence; survival

Introduction

Pancreatic ductal adenocarcinoma is currently the third leading cause of cancer-related mortality and the most lethal cancer in the digestive system [1]. Survival rates remain poor, with <7% of patients surviving beyond 5-years after diagnosis [2]. The main contributors to this poor prognosis are lack of early detection strategies and effective-targeted treatments, as well as the aggressive tumor biology per se.

Surgical resection is the only potentially curative treatment option for pancreatic cancer. However, even patients that are staged with a locally, resectable tumor, may already have micro-metastasis at the time of diagnosis and recurrence within the first year of surgery is common [3]. Tumor size, lymph node involvement, grade and margin status are important prognostic factors, but are insufficient to predict early disease recurrence and survival after surgical resection [4]. No biomarker is yet available to help guide prognosis and treatment selection in pancreatic cancer patients. A search for novel biomarkers at tissue level can thus lead to the development of tumor-derived serum biomarkers that can facilitate clinical decision making.

Galectins, localized both intra- and extra-cellularly, are a family of lectins that have affinity for β-galactosides. Based on their structure and carbohydrate-recognition domains, they are classified into three groups, including prototype galectins (-1, -2, -5, -7, -10, -11, -13, -14), chimera type galectin (-3), and tandem repeat type galectins (-4, -6, -8, -9, -12) [5]. Galectins bind to a wide array of glycoproteins and glycolipids both on the cell surface and in extracellular matrices [6]. Through cell-to-cell and cell-to-extracellular matrix adhesion and triggering signals intracellularly, galectins participate in cell proliferation, apoptosis, adhesion and immune response, thus acting as modulators in cancer [6]. The most well-studied galectins are galectin-1 and galectin-3. Galectin 1 has been found to be implicated in pancreatic cancer pathophysiology, including tumor cell proliferation, invasion, angiogenesis, inflammation, and metastasis [7]. Galectin 3 has recently been demonstrated to interact with KRAS and mediate tumor cell-stroma interactions in pancreatic cancer [8,9].

In a previous mass spectrometry-based study, we identified galectin 4 as a down-regulated protein in short-term

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survivors of pancreatic cancer [10]. The present study aimed to elucidate the prognostic role of galectin 4 in a large cohort of patients with resectable pancreatic cancer.

Methods

Patients and samples

We collected formalin-fixed, paraffin-embedded tissue samples from 140 patients with pancreatic cancer who underwent surgical resection at the Department of Surgery, Skåne University Hospital, Lund and Malmö, Sweden, between 1996 and 2017. Ethical approval was obtained from the local human ethics committee at Lund University (Ref 2017/320). The REMARK guidelines were followed when possible throughout the whole study period [11]. Disease-free survival (DFS) was defined as the time from surgery to the first event of either disease recurrence or death due to any cause. Overall survival (OS) was defined as the time from surgery to death due to any cause.

Tissue microarray

Exploiting an automated tissue arraying device (Minicore® 3, Alphelys, Plaisir, France), tissue microarray (TMA) construction was applied to tumors by stabilizing 4 cores ø 2 mm of cancerous tissues (marked by pathologist A.S.) from each tumor into paraffin blocks. The TMA-blocks were then sectioned to 3-μm-thick slides.

Immunohistochemistry

After incubation in 60 °C for 1 h and cool down in room temperature (RT), TMA-sections underwent a deparaffinization, rehydration and antigen-retrieval procedure in EnVision FLEX Target Retrieval Solution pH = 6 (K800521-2, Dako) heated to 96 °C for 20 min, using automated PT Link (Dako, Glostrup, Denmark). After three times of washing in phosphate-buffered saline for 5 min, slides were blocked against endogenous peroxidase activity with 0.3% H_2O_2 and 1% methanol in phosphate-buffered saline for 10 min. Next, the specimens were blocked with 5% goat serum at RT for 1 h, followed by application of avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA) at RT for 15 min. The sections were then incubated with rabbit polyclonal antibody against human galectin 4 (Atlas Antibodies AB, Bromma, Sweden, cat no HPA031184, dilution 1:100) at 4 °C overnight. Next, sections were incubated with biotinylated secondary goat anti-rabbit antibody (BA-1000, dilution 1:200, Vector Laboratories) at RT for 1 h. After a 30-min incubation with avidin-biotin-peroxidase complex (Vectastain Elite ABC-HRP Kit, PK-6100, Vector Laboratories) at RT, the sections were incubated with chromogen diaminobenzidine (DAB) (SK-4100, Vector Laboratories) for 5 min. After washing in deionized water for 5 min, nuclei were counterstained with Mayer's hematoxylin (Histolab, Gothenburg, Sweden) for 30 s, and washed in tap water for another 5 min. Finally, the specimens were dehydrated in graded alcohol and mounted by Pertex (Histolab). Negative controls were produced by omitting the primary antibodies. Slides were scanned for evaluation using an Aperio scanscope scanner (Leica Biosystems, Wetzlar, Germany).

Scoring procedure

The immunostainings of galectin 4 were assessed semi-quantitatively by an experienced pancreas pathologist (A.S.) blinded to the clinical outcome. The determination of galectin positivity was based on the definition by Hayashi et al. [12]. If more than 10% of tumor cells were stained, expression was considered positive and denoted as weak (1), moderate (2) or strong (3) depending on the intensity. Staining below 10% was denoted as negative (0).

Statistical analysis

Comparisons of categorical and continuous data were performed using Chi-square test or Mann Whitney U test. Kaplan–Meier analysis and log rank and Breslow tests were used to illustrate differences in DFS and OS. Cox regression proportional hazards models were used for estimation of hazard ratios (HRs) for recurrence and death according to galectin 4 expression in both uni- and multivariable analysis, adjusted for age, gender, AJCC stage and resection margin status. Tumor size, differentiation and adjuvant chemotherapy were not included in the multivariable model due to significant correlations to galectin 4 expression. A p-value of $<.05$ was considered statistically significant. All the statistics were performed with STATA MP 14.1.

Results

Patient cohort

The clinical characteristics of patients with pancreatic cancer are presented in Table 1. The median age was 69 years (interquartile range 63–73 years) and 66 patients (47.1%) were female. The estimated median DFS was 13.2 months while the estimated median OS was 24.1 months. One hundred thirteen (80.7%) of the patients received adjuvant chemotherapy.

Galectin 4 expression in pancreatic cancer

Galectin 4 was positively labeled in cytoplasmic/membranous and nuclear compartments of tumor cells in 111 cases (79.3%). Staining was classified as weak in 32 patients (22.9%), moderate in 51 patients (36.4%) and strong in 28 patients (20.0%). Figure 1 shows representative immunohistochemical images of galectin 4 expression in pancreatic cancer. Expression of galectin 4 was significantly associated with tumor differentiation ($p = .001$), tumor size ($p = .008$) and adjuvant chemotherapy ($p = .019$).

Table 1. Baseline clinical characteristics stratified according to galectin 4 expression.

Factors	All patients	Galectin 4, negative median (iqr) or *N* (%)	Galectin 4, positive median (iqr) or *N* (%)	*p*-Value	Missing
Age, years	69 (63–73)	70 (64–76)	68 (63–73)	.133	
Female gender	66 (47.1)	13 (44.8)	53 (47.7)	.779	
Tumor size ≥2 cm	118 (84.3)	29 (100)	89 (80.2)	**.008**	
T-stage				.389	0.7%
T1	18 (12.9)	0	18 (16.4)		
T2	94 (67.6)	25 (86.2)	69 (62.7)		
T3	26 (18.7)	4 (13.8)	22 (20.0)		
T4	1 (0.7)	0	1 (0.9)		
N-stage				.519	1.4%
N0	34 (24.6)	5 (17.2)	29 (26.6)		
N1	52 (37.7)	11 (37.9)	41 (37.6)		
N2	52 (37.7)	13 (44.8)	39 (35.8)		
AJCC stage				.464	1.4%
IA	6 (4.3)	0	6 (5.5)		
IB	20 (14.5)	5 (17.2)	15 (13.8)		
IIA	7 (5.1)	0	7 (6.4)		
IIB	52 (37.7)	11 (37.9)	41 (37.6)		
III	53 (38.4)	13 (44.8)	40 (36.7)		
Tumor differentiation				**.001**	1.4%
Well	7 (2.9)	0	7 (6.4)		
Moderate	48 (57.2)	3 (10.3)	45 (41.3)		
Poor	79 (34.8)	26 (89.7)	53 (48.6)		
Undifferentiated	4 (5.1)	0	4 (3.7)		
R1 resection	54 (38.8)	11 (37.9)	43 (39.1)	.909	0.7%
Adjuvant chemotherapy	113 (83.7)	19 (67.9)	94 (87.9)	**.019**	3.6%

AJCC: American joint committee on cancer, 8th edition; iqr: interquartile range. *p*-value with statistical significance (<.05) is highlighted in bold.

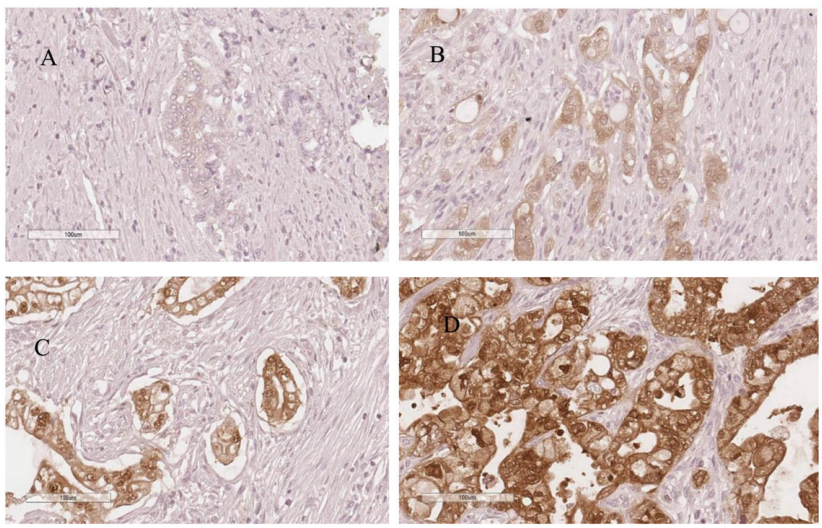

Figure 1. Representative immunohistochemical images of galectin 4 expression in pancreatic cancer, (A) negative expression, (B) weak expression, (C) moderate expression, (D) strong expression.

Association between galectin 4 expression and disease-free survival

Galectin 4 expression was significantly correlated with disease recurrence within the first year of surgery ($p = .014$). Multivariable analysis confirmed the results (adjusted HR 0.485, $p = .027$). Galectin 4 expression was not correlated to 3- or 5-year DFS (Table 2). The median DFS was 10.4 months in patients with galectin 4-negativity and 15.9 months in patients with galectin 4-positivity, as estimated by the Kaplan–Meier method (log-rank $p = .224$, Breslow $p = .087$), Figure 2.

Association between galectin 4 expression and overall survival

Galectin 4 significantly correlated to 1-year OS ($p = .036$), which was confirmed in multivariable analysis (adjusted HR 0.482, $p = .047$). Expression of galectin 4 also correlated to 3-year OS in univariable analysis ($p = .031$) and multivariable analysis (adjusted HR 0.550, $p = .025$). Galectin 4 did not correlate to 5-year OS. The median OS was 14.0 months in patients with lack of galectin 4 expression and 27.6 months in patients with tumors that

expressed galectin 4 (log-rank $p = .118$, Breslow $p = .021$), Figure 3.

Discussion

To our knowledge, this is the first study to report the prognostic utility of galectin 4 in a large cohort of patients with pancreatic cancer. We found that lack of galectin 4 expression is an independent marker for early recurrence and death, defined as occurring within 12 months after curatively aimed surgery. The ability to identify resectable patients that are subjected to early recurrence is a worthy objective, as these selected patients may benefit from other treatment plans, such as neoadjuvant chemotherapy before surgery.

The data in our study are in line with previous experimental studies on the tumor suppressor properties of galectin 4 in pancreatic cancer. It has been demonstrated that galectin 4 inhibits migration and metastasis formation in pancreatic cancer cells both *in vitro* and *in vivo* [13]. Further studies have revealed that galectin 4 markedly reduced cytoplasmic β-catenin levels, counteracted with the function of Wnt signaling, and sensitized pancreatic cancer cells to Wnt inhibitors [14]. Interestingly, β-catenin was also highlighted as an upstream regulator in pancreatic cancer patients with poor survival in our previous study [10]. As Wnt/β-catenin signaling is crucial for the development of pancreatic cancer [15], galectin 4 expression might provide additional information to Wnt/β-catenin signaling targeted treatment in pancreatic cancer.

Our results demonstrated that galectin 4 was labeled in the tumor cells, rather than the stromal cells. In contrast, galectin 3 and galectin 1, which have been reported to correlate with survival in pancreatic cancer, are mainly expressed in stromal cells [16–18]. Global gene expression profiling has proved useful for subtype identification in many human tumor types, including pancreatic cancer. Collisson and colleagues have classified pancreatic cancer into three molecular subtypes, that is, classical, exocrine-like and quasi-mesenchymal, with each subtype presenting different response rates to therapy and survival [19]. Notably, by a recent proteomics approach, galectin 4 was identified as a biomarker for the exocrine-like subtype, characterized by resistance to tyrosine kinase inhibitors and paclitaxel [20]. This information has high clinical relevance as it indicates that galectin 4 expression may be used to predict the response to chemotherapy.

In the present study, galectin 4 expression was associated with tumor size and histopathological differentiation. This indicated that galectin 4 expression may be related to pancreatic tumor biology. Galectin 4 has been regarded as a differentiation biomarker in colon cancer [21]. The loss of galectin 4 expression is also linked to increased tumor size and tumor differentiation in hepatocellular carcinoma and lung cancer [12,22]. Most patients received adjuvant chemotherapy in the present study. There are many limitations associated with the retrospective analysis of chemotherapy data and the association between galectin 4 expression and adjuvant chemotherapy receipt found in our study remains speculative.

Table 2. Univariable and multivariable Cox survival analyses.

	Unadjusted			Adjusted*		
Survival	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
1-year DFS	0.465	0.253–0.855	**0.014**	0.485	0.256–0.920	**.027**
3-year DFS	0.714	0.430–1.186	0.193	0.624	0.360–1.081	.093
5-year DFS	0.737	0.450–1.208	0.226	0.638	0.371–1.095	.103
1-year OS	0.475	0.238–0.951	**0.036**	0.482	0.235–0.989	**.047**
3-year OS	0.579	0.353–0.951	**0.031**	0.550	0.327–0.928	**.025**
5-year OS	0.676	0.416–1.098	0.114	0.636	0.380–1.063	.084

DFS: disease-free survival; OS: overall survival. *Adjusted for age, gender, AJCC stage, and resection margin status. *p*-value with statistical significance (<.05) is highlighted in bold.

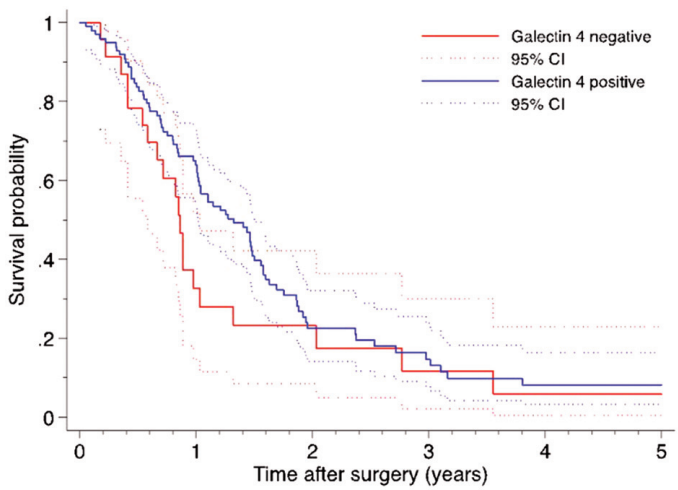

Figure 2. Disease-free survival curves by galectin 4 expression in patients with surgically resected pancreatic cancer (log-rank $p = .224$, Breslow $p = .087$).

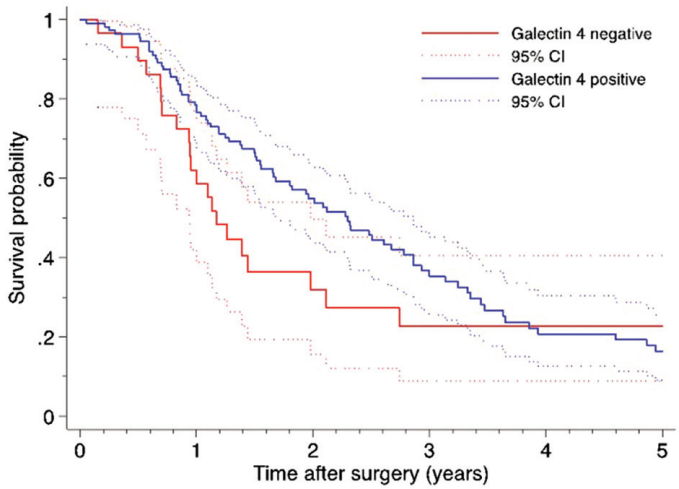

Figure 3. Overall survival curves by galectin 4 expression in patients with surgically resected pancreatic cancer (log-rank $p = .118$, Breslow $p = .021$).

In summary, the results from this study suggest that galectin 4 expression is associated with early recurrence and death after resection for pancreatic cancer. Further studies are needed to determine whether galectin 4 expression can be included in treatment algorithms to stratify patients for neoadjuvant-direct approaches in resectable cases. Additional studies are also needed to elucidate the exact mechanisms by which galectin 4 exerts its tumor suppressive properties.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Paper IV

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ORIGINAL ARTICLE

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Low P4HA2 and high PRTN3 expression predicts poor survival in patients with pancreatic cancer

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ABSTRACT

Background: The tumor microenvironment in pancreatic cancer has a multifaceted role in disease development and progression. Prolyl 4-hydroxylase subunit alpha 2 (P4HA2) and proteinase 3 (PRTN3) are involved in the synthesis and degradation of collagen in the tumor microenvironment and have been identified as prognostic biomarker candidates for pancreatic cancer in our previous mass spectrometric study. This study aimed at validating prognostic performance of P4HA2 and PRTN3 in a larger cohort of patients.

Methods: The expression of P4HA2 and PRTN3 was evaluated with tissue microarrays and immunohistochemistry in 140 patients with pancreatic cancer who underwent surgical resection. Kaplan–Meier and Cox proportional hazards regression modeling were used to explore the association of P4HA2 and PRTN3, either separately or combined, with clinicopathological factors and survival.

Results: Most tumors were positive for P4HA2 (133/140, 95%), whereas 77 tumors (55%) were positive for PRTN3. Expression levels of P4HA2 and PRTN3 did not separately correlate with disease-free or overall survival, in either uni- or multivariable analysis. However, a low P4HA2 and high PRTN3 expression correlated with shorter disease-free survival (median 7.0 vs. 13.4 months, adjusted HR 3.24, 95% CI: 1.13–9.25, $p = .028$) and overall survival (median 8.5 vs. 25.8 months, adjusted HR 8.14, 95% CI: 3.41–19.44, $p < .001$).

Conclusion: Our data show that a low P4HA2 and high PRTN3 expression correlates with poor survival in patients with pancreatic cancer, indicating the involvement of collagen deposition in the restraint of the tumor. The tumoral expression of PRTN3 reinforces the therapeutic potential of PR1-targeting immunotherapy in pancreatic cancer.

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Introduction

Pancreatic cancer is a devastating malignancy with a dismal prognosis. With a 5-year survival rate in the single digits, pancreatic cancer has become the third cause of cancer-related mortality, after colorectal and lung cancer [1]. The poor prognosis is mainly due to the lack of early detection tools and the resistance to current treatment modalities, including chemotherapy, radiotherapy and targeted therapies.

Development of new biomarkers may aid in clinical decision making. CA 19-9 is the only serum marker for pancreatic cancer, but lacks the necessary performance to be used as a screening tool. Although many tissue biomarkers have shown potential prognostic utility in pancreatic cancer [2], few have been translated into the clinical setting and none for routine use. To overcome therapeutic resistance in pancreatic cancer, it has been proposed to subgroup patients based on biomarker profiles in tumor tissue [3,4]. Thus far, only hENT1 expression has been suggested by NCCN as a predictive marker in patients undergoing tumor resection and treatment with gemcitabine.

The pancreatic tumor microenvironment (TME) has attracted much interest in the past decade because of its crucial role in tumor progression and chemoresistance [5]. The TME contains an abundant fibrotic stroma, which encompasses a variety of cellular and molecular entities, such as pancreatic stellate cells (PSCs), and extracellular matrix components (ECM), such as collagen, fibronectin and hyaluronic acid. The TME that interacts with tumor cells is dynamic. Activated PSCs are mainly responsible for deposition of collagen, which also undergoes degradation by PSCs, cancer cells and inflammatory cells through secretion of matrix metalloproteinases (MMPs) [6]. It has been suggested that an activated stroma status relates to progression and consequently poor survival of pancreatic cancer [7].

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In a previous study, we discovered and verified several novel tissue biomarkers for pancreatic cancer utilizing mass spectrometry-based proteomics [8]. We identified prolyl 4-hydroxylase subunit alpha 2 (P4HA2) and proteinase 3 (PRTN3) as biomarkers which are related to the TME. P4HA2 is a component of prolyl 4-hydroxylase (P4H), a key enzyme in collagen synthesis composed of two identical alpha subunits and two beta subunits [9]. P4H catalyzes the formation of 4-hydroxyproline that is essential to the proper three-dimensional folding of newly synthesized procollagen chains. P4HA2 is one of the three P4HA isoforms that has been identified in human tissue (P4HA1, P4HA2 and P4HA3). Increased P4HA2 expression has been detected in breast cancer [10], oral cavity squamous cell carcinoma [11] and papillary thyroid cancer [12].

PRTN3, also known as myeloblastin or c-ANCA (cytoplasmic pattern of antineutrophil cytoplasmic autoantibodies) antigen, is a serine protease secreted by cells of myeloid lineage [13] and allocated to the cell surface of neutrophils [14] and endothelial cells [15]. PRTN3 is related to inflammatory processes, but its link to neoplasia is less understood. Sharing structural similarity with elastase, PRTN3 has an elastase-like specificity for small aliphatic residues (Ala, Val, Ser, Met) and degrades a variety of matrix proteins *in vitro* including fibronectin, laminin, vitronectin, and collagen [16]. PRTN3 is also thought to be involved in MMP activation, hence potentially being involved in tumor invasion and metastasis [17,18]. Moreover, it has been found that PRTN3 induces phosphorylation and nuclear translocation of p44/p42 and JNK1, leading to cancer cell motility, through a non-proteolytic way [19]. Notably, recent studies revealed that PRTN3 expressed by neutrophils within the TME can be taken up by breast cancer and melanoma cells, which in turn increase the susceptibility to PR1-targeting therapies [14,20].

Based on previous experience that P4HA2 and PRTN3, respectively, participate in the synthesis and degradation of collagen in ECM, we hypothesized that these two biomarkers may correlate with the survival of patients with pancreatic cancer, possibly by exerting an influence on the dynamics of the TME. The aim of this study was to validate the prognostic potential of P4HA2 and PRTN3 in a large cohort of patients with resectable pancreatic cancer.

Methods

Patients and samples

Formalin-fixed, paraffin-embedded tissue samples were collected from 140 patients with pancreatic cancer who underwent pancreatic resection at the Department of Surgery, Skåne University Hospital, Lund and Malmö, Sweden, between 1996 and 2017. Ethical approval was obtained from the local human ethics committee at Lund University (Ref 2017/320). Written informed consent was given by participants in this study. The REMARK guidelines were followed when possible throughout the whole study period [21].

Tissue microarray

Tissue microarray (TMA) construction was applied to tumors with sufficient amount of material. Compared with whole sections, TMA has the advantage of a reduced consumption of both tissue and time, which enables studies of a larger scale with reduced experimental variability. Using an automated tissue arraying device (Minicore® 3, Alphelys, Plaisir, France), 4 cores ⌀ 2 mm of cancer tissues (marked by pathologist A.S.) from each specimen were stabilized into paraffin blocks. The TMA-blocks were sectioned to 3 μm thick slides for immunohistochemical analysis.

Immunohistochemistry

TMA-sections were heated in 60 °C for 1 hour and then cooled in room temperature (RT). Next, using automated PT Link (Dako, Glostrup, Denmark), deparaffinization, rehydration and antigen-retrieval were performed in EnVision FLEX Target Retrieval Solution pH = 6 (K800521-2, Dako) heated to 96 °C for 20 min. After three times of washing in phosphate-buffered saline for 5 min, sections were blocked against endogenous peroxidase activity with 0.3% H_2O_2 and 1% methanol in phosphate-buffered saline for 10 min. The specimens were then blocked with 5% goat serum for 1 hour at RT, followed by avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA) for 15 min at RT. Subsequently, the sections were incubated with rabbit polyclonal antibody against human P4HA2 (Atlas Antibodies AB, Bromma, Sweden, cat no HPA016997, dilution 1:300) or PRTN3 (cat no HPA005938, dilution 1:300) at 4 °C overnight. Next, sections were incubated with biotinylated secondary goat anti-rabbit antibodies (BA-1000, dilution 1:200, Vector Laboratories) for 1 hour at RT. Following incubation with avidin–biotin–peroxidase complex (Vectastain Elite ABC-HRP Kit, PK-6100, Vector Laboratories) for 30 min at RT, the sections were incubated with chromogen diaminobenzidine (DAB) (SK-4100, Vector Laboratories) for 5 min. After washing in deionized water for 5 min, nuclei were counterstained with Mayer's hematoxylin (Histolab, Gothenburg, Sweden) for 30 sec and washed in tap water for another 5 min. Finally, the specimens were dehydrated in graded alcohol and mounted using Pertex (Histolab). Negative controls were produced by omitting the primary antibodies. Regarding the positive controls, P4HA2 staining by western blot is remarkably reduced in cells with knock-down of P4HA2, while PRTN3 is highly stained in hematopoietic cells of bone marrow, according to the antibody provider and database 'The Human Protein Atlas' (www.proteinatlas.org) [22]. Slides were scanned for evaluation using an Aperio scanscope scanner (Leica Biosystems, Wetzlar, Germany).

Scoring procedure

The immunostaining of P4HA2 and PRTN3 was assessed semi-quantitatively by an experienced pancreas pathologist (A.S.) blinded to the clinical outcome. The scoring algorithm was modified from Norihiro et al. [23] and had taken into

consideration the proportion of stained tumor cells and the intensity of the staining. Staining less than 10% of tumor cells was denoted as negative (0). When >10% of tumor cells were stained, the expression was considered positive and denoted as weak (1), moderate (2) or strong (3) depending on the intensity. Scoring with (0) and (1) was categorized as the low expression, while scoring with (2) and (3) was categorized as the high expression.

Statistical analysis

Comparisons of categorical data were performed using Chi-square test or Fisher's exact test. Kaplan–Meier analysis and log rank test were used to illustrate differences in disease-free survival (DFS) and overall survival (OS). Cox regression proportional hazards regression models were used for estimation of hazard ratios (HRs) for recurrence and death according to P4HA2 and PRTN3 expression in both uni- and multivariable analysis, adjusted for age, gender, TNM status, differentiation grade, resection margin status and adjuvant chemotherapy. A *p*-value of <.05 was considered statistically significant. All the statistical analyses were performed with STAT MP 14.1.

Table 1. Baseline characteristics of patients with pancreatic ductal adenocarcinoma.

Factors	*N* (%) or median (IQR)	Missing
Age, years	68 (63–73)	
Male gender	72 (51.4)	
T-stage		0.7%
T1	19 (13.6)	
T2	93 (66.4)	
T3	26 (18.6)	
T4	1 (0.7)	
N-stage		1.4%
N0	32 (22.9)	
N1	53 (37.9)	
N2	53 (37.9)	
AJCC stage, 8th edition		1.4%
IA	6 (4.3)	
IB	19 (13.6)	
IIA	7 (5.0)	
IIB	52 (37.1)	
III	54 (38.6)	
Tumor differentiation		1.4%
Well	7 (5.0)	
Moderate	49 (35.0)	
Poor	78 (55.7)	
Undifferentiated	4 (2.9)	
Positive resection margin (R1)	54 (38.6)	0.7%
Adjuvant chemotherapy	113 (80.7)	3.6%

AJCC: American joint committee on cancer.

Results

Patient cohort

Baseline characteristics of patients with pancreatic cancer are presented in Table 1. The median age was 68 years (interquartile range 63–73 years) and 72 patients (51.4%) were male. One hundred thirteen (80.7%) patients received adjuvant chemotherapy.

P4HA2 expression in pancreatic cancer

P4HA2 was positively labeled in the cytoplasm and cell membrane of tumor cells in 133 patients (95%). Weak staining was denoted in 32 cases (22.9%), moderate staining in 63 cases (45.0%) and strong staining in 38 cases (27.1%). Myofibroblasts were positive for P4HA2 in all patients. Figure 1 shows representative immunohistochemical images of P4HA2 expression in pancreatic cancer.

Association between P4HA2 and clinicopathological characteristics and survival

P4HA2 expression was not associated with any clinicopathological characteristics. P4HA2 expression did not significantly correlate with either DFS or OS, in either uni- or multivariable analysis (Table 2). The median DFS and OS were 17.8 and 21.9 months, respectively, in the low P4HA2 expression group, while they were 12.7 and 27.7 months in the high P4HA2 group.

PRTN3 expression in pancreatic cancer

The staining of PRTN3 was detected in the nuclei, cytoplasm and cell membrane of tumor cells and lymphocytes (Figure 1). PRTN3 expression in tumor cells was considered

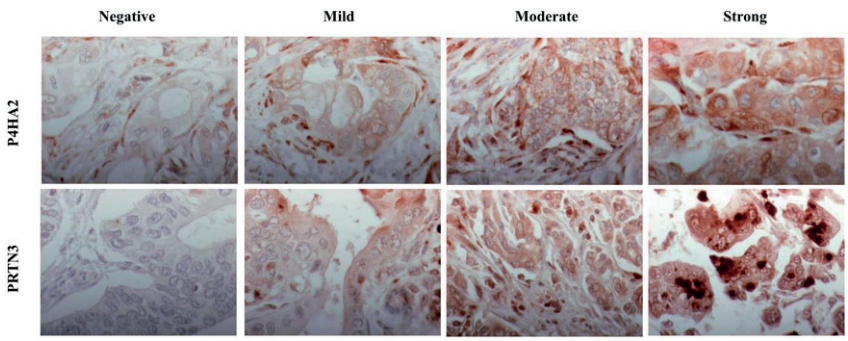

Figure 1. Representative immunohistochemical images of P4HA2 and PRTN3 expressions in pancreatic cancer.

positive in 77 cases (55%). Weak, moderate and strong staining of PRTN3 accounted for 40 (28.6%), 26 (18.6%) and 11 (7.9%) cases.

Association between PRTN3 and clinicopathological characteristics and survival

The high PRTN3 expression group had a higher frequency of adjuvant chemotherapy receipt (94.6% vs. 79.6%, $p = .035$). There were no association of PRTN3 expression with any other clinicopathological parameters, including age, gender, TNM stage, histological grade and resection margin status ($p > .05$). The median DFS and OS were 12.4 and 24.5 months in the low PRTN3 group while they were 15.5 and 25.8 months in the high PRTN3 group. PRTN3 expression did not correlate with DFS or OS (Table 2).

Low P4HA2 and high PRTN3 expression was correlated with poor survival

Kaplan–Meier analysis revealed that a low P4HA2 and high PRTN3 expression correlated with a significantly shorter DFS and OS (Figures 2 and 3). The median DFS was 7.0 months in patients with low P4HA2 and high PRTN3 expression pattern compared to 13.4 months in patients with other expression patterns ($p = .004$). The correlation with DFS was confirmed in univariable Cox regression analysis (HR 4.12, 95% CI: 1.46–11.63, $p = .008$) and remained significant in multivariable analysis (HR 3.24, 95% CI: 1.13–9.25, $p = .028$), adjusted for age, gender, TNM status, differentiation grade, resection margin status and adjuvant chemotherapy (Table 2). The median OS in patients with a low P4HA2 and high PRTN3 expression was shorter than those with other expression patterns (8.5 vs. 25.8 months, $p < .001$). The correlation with OS was also confirmed in univariable Cox regression analysis (HR 5.97, 95% CI: 2.77–12.85, $p < .001$), and remained significant in multivariable analysis after adjustment (HR 8.14, 95% CI: 3.41–19.44, $p < .001$) (Table 2).

Discussion

Our results demonstrate that a combination of low P4HA2 and high PRTN3 expression is associated with shorter DFS and OS. This is to our knowledge, the first report of using P4HA2 and PRTN3 as tissue biomarkers in a large number of patients from a well annotated clinical cohort.

P4HA2 and PRTN3 have been implicated in the formation and degradation of collagen, respectively. As the main constitute of ECM, collagen plays a key role in tumor progression [24]. It has been shown that by regulating collagen deposition, P4HA2 promotes breast cancer progression and metastasis and correlates with a poor prognosis [10]. The dynamics of collagen production and turnover involve various steps, such as activation of PSCs and participation of relavant enzymes. Moreover, heteregenity is strongly implicated in pancreatic cancer both in expressional signatures

Table 2. Cox survival analysis of P4HA2 and PRTN3 expression in pancreatic cancer.

	Disease-free survival			Overall survival		
Variables	HR	95% CI	*p* value	HR	95% CI	*p* value
P4HA2, unadjusted	0.98	0.64–1.51	.929	0.74	0.49–1.13	.165
P4HA2, adjusted*	0.88	0.56–1.40	.598	0.72	0.45–1.14	.157
PRTN3, unadjusted	0.95	0.59–1.53	.837	1.14	0.70–1.85	.592
PRTN3, adjusted*	0.92	0.56–1.50	.724	1.13	0.68–1.90	.634
Low P4HA2 high PRTN3, unadjusted	4.12	1.46–11.63	**.008**	5.97	2.77–12.85	**<.001**
Low P4HA2 high PRTN3, adjusted*	3.24	1.13–9.25	**.028**	8.14	3.41–19.44	**<.001**

*Adjusted for age, gender, T-stage, N-stage, differentiation grade, resection margin status and adjuvant chemotherapy. *P* values in bold signify statistical significance (<.05).

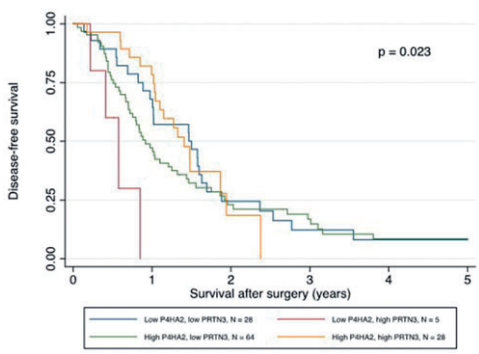

Figure 2. Disease-free survival curves by P4HA2 and PRTN3 expression status in patients with pancreatic cancer.

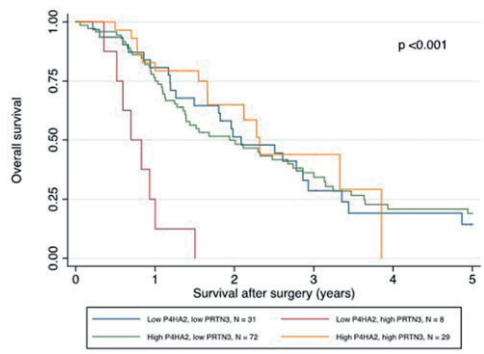

Figure 3. Overall survival curves by P4HA2 and PRTN3 expression status in patients with pancreatic cancer.

and therapeutic response [4,25,26]. It seems reasonable that one biomarker such as P4HA2 or PRTN3 alone fail to capture the characteristics of ECM in the tumor. A combination of biomarkers for the signatures of the TME in pancreatic cancer may better predict the prognosis, as has been shown in several studies [6,27].

Our data indicated that a decrease of collagen in TME may favor progression of pancreatic cancer. Deposition of a collagen-rich TME around the tumor may act as a barrier to confine tumor progression. This is supported by the presence of a capsule around the metastasis correlated with a better prognosis in colorectal liver cancer metastases [28]. Nevertheless, some studies suggested that both increased deposition of collagen in TME and a highly aligned stromal collagen were negative prognostic factors in pancreatic cancer [7,29]. The role of collagen in pancreatic cancer seems to be controversial. Our results were contradictory with previous study in which low mRNA expression of P4HA2 correlated with reduced collagen deposition and better survival of breast cancer [10]. However, Erkan and colleagues have reported that low expression of collagen deposition correlated with poor survival of pancreatic cancer [6]. In their study, using immunohistochemistry of α-SMA and collagen, the stroma in pancreatic cancer was classified according to four patterns of collagen deposition: inert, dormant, fibrogenic and fibrolytic. The fibrolytic stroma, characterized by high α-SMA and low collagen, was independently associated with the worse prognosis [5,6]. This was further confirmed by a recently published larger study [27]. More studies will be needed to elucidate the mechanisms of collagen deposition and the link to the clinical outcome.

Interestingly, more than half of the patients displayed PRTN3 expression in the tumor cells. The absent or minimal RNA-seq expression of PRTN3 in pancreatic cancer cell lines from the Cancer Cell Line Encyclopedia [30], together with the finding that the MIA PaCa-2 cell line was found to display an uptake of PRTN3 [14], led us to the speculate that the tumoral expression of PRTN3 was derived from neutrophils within the TME of pancreatic cancer and then taken up by tumor cells, as has been reported in other tumors [14,20]. Tumor-associated antigen PR1, a peptide derived from PRTN3 and neutrophil elastase, has been shown to be cross-presented in breast cancer and melanoma cells, which in turn increased the susceptibility to PR1-targeting therapies [14,20]. Our results might shed light on the potential of PR1-targeting immunotherapy in patients with pancreatic cancer whose tumor cells cross-present PR1.

In conclusion, our study showed that a low P4HA2 and high PRTN3 expression correlates with poor survival in patients with pancreatic cancer. The finding highlights the involvement of collagen deposition in the restraint of the tumor progression. The tumoral expression of PRTN3 in the majority of pancreatic tumors also reinforces the potential of PR1-targeting immunotherapy. Further studies are needed to confirm this association as well as to investigate the deposition of collagen in TME and the activation status of PSCs in relation to P4HA2 and PRTN3 expression.

Disclosure statement

There are no conflicts of interest.

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Paper V

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RESEARCH Open Access

Stromal fibronectin expression in patients with resected pancreatic ductal adenocarcinoma

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is characterized by an extremely dense stroma, which has a fundamental role in tumor progression. Fibronectin (FN1) is the main constituent of the tumor stroma in pancreatic cancer. This study aimed to explore the association between FN1 and clinicopathological characteristics and disease survival.

Methods: Formalin-fixed paraffin-embedded tissue samples from 138 patients with PDAC were constructed into a tissue microarray, followed by immunohistochemical analysis with a recombinant monoclonal FN1 antibody. Chi-square test or Fisher's exact test were used for comparison of FN1 expression and relevant clinicopathological parameters. Kaplan-Meier survival curves and Cox regression analyses were used to assess the association between FN1 and survival.

Results: FN1 was detected in the stromal compartment in most cases (117/138, 84.8%). Compared to the low FN1 expression group, the high FN1 expression group had significantly larger tumor size ($P = 0.002$), more advanced T stage ($P = 0.039$) and N stage ($P = 0.009$), and also worse AJCC stage ($P = 0.003$). However, stromal FN1 expression was not associated with disease-free survival or overall survival.

Conclusions: This study suggests that high stromal FN1 expression is associated with aggressive tumor characteristics in patients with resected PDAC. However, no association between FN1 expression and survival was found.

Keywords: Fibronectin, Pancreatic ductal adenocarcinoma, Survival, Immunohistochemistry

Background

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer death, characterized by frequent metastases and profound chemoresistance [1]. The median survival for all stages of pancreatic cancer combined is 6 months, with a 5-year survival rate of less than 7% [2]. It is projected that PDAC will become the second cancer-related mortality within the next decade in the Western world [2]. During recent years, a marginal improvement in the treatment of PDAC has been seen, which can be exemplified by the ESPAC-4 clinical trial showing that gemcitabine-capecitabine combination therapy outperformed gemcitabine alone in patients with resected PDAC (median overall survival 28.0 vs 25.5 months) [3]. However, most therapeutic regimens for PDAC have failed, including antiangiogenetic approaches and immunotherapies, which have shown promise in, e.g., renal cell carcinoma and malignant melanoma [4]. It has been speculated that one major contributor to the treatment resistance is the hypovascular and immunosuppressive tumor microenvironment (TME), which is the most prominent histological feature of PDAC [5].

The TME in PDAC accounts for more than half of the tumor mass and has a complex role in tumor growth and the therapeutic response [5]. The high fibrotic stiffness of the TME compresses blood vessels and reduces perfusion that ultimately impedes the delivery of drugs to neoplastic cells. On the other hand, some constituents of tumor stroma act to suppress tumor growth by

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affecting the immune response and restraining tumor angiogenesis [6, 7]. A better characterization of TME is needed for more precise prediction of treatment response and development of new therapies.

Fibronectin (FN1) is a major constituent of the extracellular matrix within the TME and is not only produced mainly by fibroblasts, but also by tumor cells [8]. Normally, FN1 supports cell-ECM interactions and is essential for wound healing, development, and tissue homeostasis [9]. The binding of FN1 to its receptors, typically cell surface integrins, trigger FN1 signaling pathways in pancreatic tumor cells, promoting tumor cell survival and chemoresistance, cell invasion, metastasis, and angiogenesis [8]. Abrogating FN1-integrin interactions have produced strikingly positive pre-clinical results in various animal models of cancer by impeding angiogenesis and inhibiting tumor growth [10–12]. Unfortunately, however, these drugs, such as PF-04605412, have failed in clinical trials [13]. Further understanding of FN1 expression and function in the context of PDAC may potentially help to improve the effectiveness of FN1 inhibition in the clinical setting.

Immunohistochemical studies have confirmed that FN1 mainly is expressed in the stroma of PDAC, while its expression could also be found in neoplastic epithelial cells [6, 14, 15]. Cancer-associated fibroblasts are the main source of FN1 and promote tumor invasion and migration by FN1 assembly [16]. A recent study has also uncovered an anti-metastatic role of fibronectin from tumor cells responding to immunological surveillance of natural killer cells [17]. Moreover, expression of fibronectin in pancreatic tumor cells correlated with poor survival [18]. In a previous proteomic study, we reported that FN1 is an upregulated biomarker in PDAC patients with poor outcome [19]. In this study, we sought to investigate the association of FN1 expression with clinical characteristics and survival of patients with resected PDAC.

Table 1 Baseline characteristics of patients with pancreatic ductal adenocarcinoma ($n = 138$)

Factors	*n* (%)	Median (IQR)	Missing
Age at diagnosis (years)		68.5 (63.0–73.0)	
Gender (male)	73 (52.9)		
Size of primary tumor (cm)		3.0 (2.5–4.0)	
T stage			
- T1	19 (13.8)		
- T2	92 (66.7)		
- T3	26 (18.8)		
- T4	1 (0.7)		
N stage			0.7%
- N0	34 (26.4)		
- N1	52 (37.7)		
- N2	51 (37.0)		
AJCC stage, eighth edition			0.7%
- IA	6 (4.3)		
- IB	20 (14.5)		
- IIA	7 (5.1)		
- IIB	52 (37.7)		
- III	52 (37.7)		
Tumor differentiation			0.7%
- Well	7 (5.1)		
- Moderate	48 (34.8)		
- Poor	78 (56.5)		
- Anaplastic	4 (2.9)		
Positive resection margin (≥R1)	53 (38.4)		
Adjuvant chemotherapy	111 (80.4)		3.6%

Abbreviations: *AJCC* American Joint Committee on Cancer

Methods

Patients and samples

Patients from this study were all diagnosed with PDAC and underwent pancreatectomy at the Department of Surgery, Skåne University Hospital, Lund and Malmö, Sweden. A total of 138 formalin-fixed, paraffin-embedded tissue samples were included. The study period spanned from 1996 to 2017. Hematoxylin and eosin stained tissues from all patients were re-evaluated by our pathologist (A.S.) in accordance with the WHO 2010 classification. As controls, disease-free pancreatic tissues from patients with serous ($n = 3$) or mucinous ($n = 1$) cystadenoma were included. The baseline characteristics of patients with PDAC are presented in Table 1. Ethical approval for this study was granted by the local human ethics committee at Lund University (Ref 2017/320). The study follows the REMARK guidelines where possible [20].

Tissue microarray

To minimize experimental variability and gain reproducibility, tissue microarray (TMA) technology was applied to the formalin-fixed paraffin-embedded specimens [21]. From each specimen, four sites of cancerous tissues with a diameter of 2 mm were obtained, which were marked by our pathologist (A.S.) and stabilized into paraffin blocks by an automated tissue array device (Minicore® 3, Alphelys, Plaisir, France). The established blocks based on TMA were then sliced into sections with a thickness of 3 μm for further immunohistochemical assessment. Each TMA slide contains around 120 cores, corresponding to samples from 30 patients with 4 replicates. Duplicated TMA slides underwent immunohistochemical staining (Fig. 1).

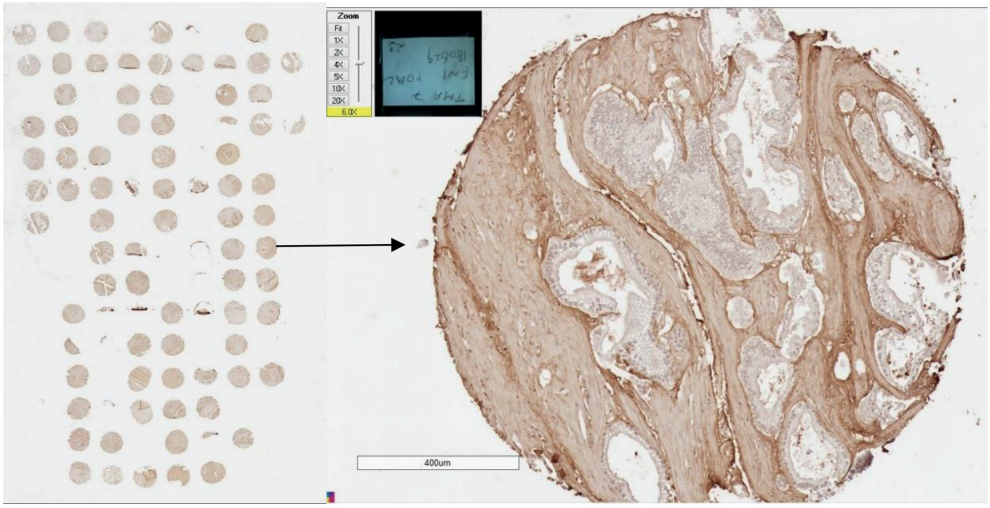

Fig. 1 Overview of a tissue microarray slide and overall fibronectin staining

Immunohistochemistry

Immunohistochemistry was performed as previously described [22]. Briefly, TMA slides were firstly pre-warmed for 1 h at 60 °C. Secondly, slides were added to EnVision FLEX Target Retrieval Solution low pH (K800521–2, Dako, Glostrup, Denmark) heated to 96 °C for 20 min in an automated PT Link (Dako, Glostrup, Denmark). Then, slides were immersed in phosphate-buffered saline for 5 min, which was repeated twice. Subsequently, slides were immersed into phosphate-buffered saline containing hydrogen peroxide (0.3%) and methanol (1%) for 10 min. The sections were then incubated with 5% goat serum for 1 h at room temperature (RT). After careful removal of the liquid on the slides, successive incubation with avidin and biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA) was conducted for 15 min at RT, respectively. Next, the primary antibody, a rabbit recombinant monoclonal FN1 antibody (Abcam, Cambridge, UK; cat no ab2413; dilution 1:4000), was added on the slides. One slide was added with solvent without primary antibody for quality control. After making sure that all tissues were covered by the diluted antibody, samples were preserved in a refrigerator at 4 °C overnight. The next day, biotinylated secondary goat anti-rabbit antibody (BA-1000, dilution 1:200, Vector Laboratories) was applied on the slides at RT for 1 h. To amplify the target antigen signal, an avidin-biotin-peroxidase complex (Vectastain Elite ABC-HRP Kit, PK-6100, Vector Laboratories) was prepared according to the instructions of the manufacturer and used to immerse slides for 30 min at RT. Then, the specimens were covered by chromogen diaminobenzidine (SK-4100, Vector Laboratories) for 5 min, which was followed by deionized water immersion for 5 min. The slides were then immersed in Mayer's hematoxylin (Histolab, Gothenburg, Sweden) for 30 s and quickly replaced in running tap water for 5 min. Lastly, the slides underwent routine dehydration in alcohol and xylen before mounting by Pertex (Histolab).

Scoring procedure

The reactivity of the FN1 antibody in samples was evaluated by our pathologist (A.S.), who was blinded to the survival information. The scoring algorithm was modified from Norihiro et al. [23] and takes the proportion of stained cells into consideration, as well as the intensity of the staining. The reactivity was scored in a semi-quantitative manner, which was categorized as negative if less than 10% staining was observed in the stroma; and mild, moderate, or strong based on the intensity if the percentage was > 10%. Low expression was defined as negative and mild reactivity, whereas high expression represented moderate or strong reactivity.

Statistical analysis

SPSS (IBM. SPSS Statistics for Windows. Version 24.0. Armonk, NY, USA) was used for statistical analysis. Chi-square test or Fisher's exact test were employed to investigate the association of FN1 expression with clinical characteristics. Kaplan-Meier survival curves were drawn and comparisons were made with the

log-rank test. Cox regression proportional hazards models were employed to estimate hazard ratios (HR) according to FN1 expression in both uni- and multivariable analysis, adjusted for age, gender, TNM status, differentiation grade, resection margin, and adjuvant chemotherapy. A two-tailed P value < 0.05 was regarded as statistical significance.

Results

FN1 expression patterns in pancreatic tissues

FN1 expression was evaluated in the tumor stroma component, localized to non-malignant fibroblasts and extracellular matrix. The epithelial tumor component was negative. Stromal FN1 expression was negative in 21 (15.3%) of tumors, while 66 (47.8%) tumors had mild FN1 expression, 44 (31.9%) had moderate expression, and 7 (5.1%) had strong FN1 expression. Figure 2 shows representative immunohistochemical images of FN1 expression in PDAC. FN1 was not stained in acinar cells and islets of Langerhans of disease-free control pancreatic tissues (data not shown). The public Human Protein Atlas database also shows absent or minimal expression of FN1 in normal pancreas (https://www.proteinatlas.org/ENSG00000115414-FN1/tissue/pancreas) [24].

Associations between FN1 expression and clinicopathological characteristics in patients with PDAC

When compared to the low FN1 expression group, the high FN1 expression group had significantly larger tumor size ($P = 0.002$), more advanced T stage ($P = 0.039$) and N stage ($P = 0.009$), and worse AJCC stage (54.0% vs 28.7% with stage III, $P = 0.003$) (Table 2). Furthermore, adjuvant chemotherapy was more common in patients with high FN1 expression as compared to the low expression group (92.0% vs 78.3%, $P = 0.040$). No association was observed between FN1 expression and age, gender, tumor location, tumor differentiation, and resection margin status (all $P > 0.05$).

Association between FN1 expression and survival of patients with PDAC

Kaplan-Meier analysis showed that there were no differences in either disease-free survival (DFS) or overall survival (OS) when comparing high FN1 expression and low FN1 expression (median DFS 17. 9 vs 12.3 months; median OS 23.8 vs 24.5 months; both $P > 0.05$) (Fig. 3). By using Cox analyses, FN1 expression was not found to be associated with OS or DFS ($P > 0.05$, Tables 3 and 4). On multivariable analysis, only histological grade and resection margin status significantly correlated with DFS or OS. Notably, in our study, there were six patients without complete clinical data (Table 1). Re-analysis with exclusion of these six patients resulted in similar results and the same conclusion.

Discussion

PDAC is one of the most stroma-rich cancers. The stroma is composed of non-tumorous cells (such as fibroblasts, pancreatic stellate cells, myofibroblasts, and immune cells), ECM, blood vessels, and soluble proteins including cytokines and growth factors [25]. ECM components are produced by tumor cells and stromal cells and include collagen, FN1, proteoglycans, hyaluronic acid, and SPARC. Collagen, the most abundant ECM

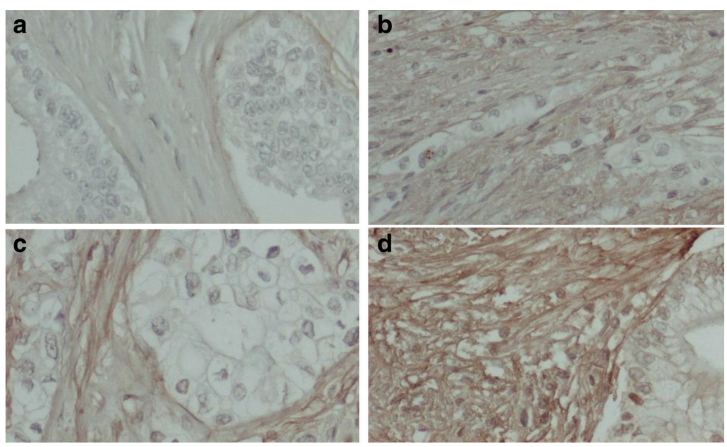

Fig. 2 Representative immunohistochemical images with stromal fibronectin expression in pancreatic ductal adenocarcinoma. **a** Negative. **b** Mild. **c** Moderate. **d** Strong

Table 2 Association between fibronectin expression and clinicopathological characteristics

Clinical characteristics	Categories	FN1 low expression (*n*, %)	FN1 high expression (*n*, %)	*P* value
Age	–	69 (63–73)	68 (63–73)	0.618
Gender	male	46 (52.9)	27 (52.9)	0.994
Tumor size	≦2 cm	21 (24.1)	2 (3.9)	0.002
	> 2 cm	66 (75.9)	49 (96.1)	
T stage	T1	17 (19.5)	2 (3.9)	0.020
	T2	56 (64.4)	36 (70.6)	
	T3	13 (14.9)	13 (25.5)	
	T4	1 (1.1)	0 (0)	
N stage	N0	25 (28.7)	9 (18.0)	0.009
	N1	38 (43.7)	14 (28.0)	
	N2	24 (27.6)	27 (54.0)	
Tumor differentiation	Poor/anaplastic	51 (58.6)	31 (62.0)	0.698
AJCC stage, eighth edition	I-II	62 (71.3)	23 (46.0)	0.003
	III	25 (28.7)	27 (54.0)	
Resection margin	R1	33 (37.9)	20 (39.2)	0.881
Adjuvant chemotherapy	yes	65 (78.3)	46 (92.0)	0.040

Abbreviations: *AJCC* American Joint Committee on Cancer

component, can bind to the integrin receptor in tumor cells and activate intracellular signaling that induce pro-tumorigenic programs. Proteoglycans consist of core proteins that undergo post-translational glycosylation, which affects cell signaling function [26]. Expression of SPARC has been found to be a strong prognostic factor in patients with PDAC [27, 28]. Due to its overexpression in PDAC and albumin-binding properties, SPARC has been postulated to enhance peritumoral drug delivery of nanoparticle albumin-bound

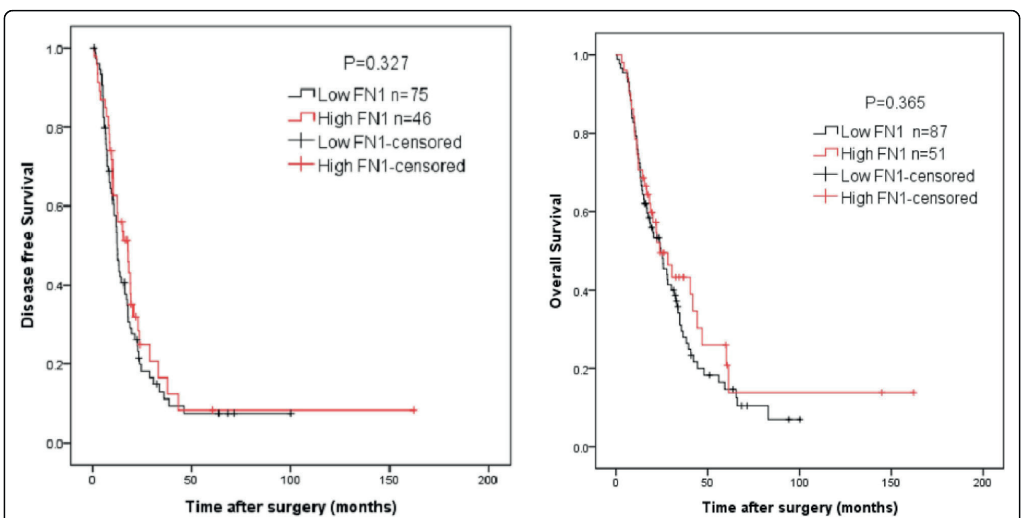

Fig. 3 Association between stromal FN1 expression and overall survival and disease-free survival in patients with pancreatic ductal adenocarcinoma (both $P > 0.05$)

Table 3 Univariable and multivariable Cox survival analyses for disease-free survival

Variables	Univariable analysis			Multivariable analysis		
	HR	95%CI	*P* value	HR	95% CI	*P* value
Age	0.99	0.96–1.01	0.202			
Female gender	0.66	0.45–0.99	0.042			
Tumor size	1.15	0.68–1.94	0.598			
T stage	1.12	0.81–1.55	0.481			
N stage	1.14	0.89–1.45	0.307			
Differentiation grade	1.50	1.07–2.10	0.018	1.54	1.10–2.17	0.012
AJCC stage	1.17	0.78–1.74	0.446			
Resection margin (R1)	1.73	1.14–2.62	0.010	1.84	1.20–2.80	0.005
Adjuvant chemotherapy	1.36	0.77–2.41	0.286			
FN1 expression, high vs low	0.83	0.55–1.24	0.357			

(nab)-paclitaxel [26]. FN1 shares similarities with collagen, as it also preserves binding sites for collagens and supports the role of the latter. In previous experimental studies, it was found that pancreatic cancer cells adhering to FN1 display increased cell proliferation and enhanced chemoresistance [29]. Moreover, cancer-associated fibroblasts assemble FN1 and trigger invasion through integrin-αvβ3 [16].

Our study revealed that expression of FN1 is abundant in the TME of PDAC, while there is a little or minimal expression in normal pancreatic tissue. Stromal FN1 expression was associated with aggressive tumor properties, including larger tumor size, more advanced T stage and N stage, and worse AJCC stage. To our knowledge, this is the first study to report the association of stromal FN1 expression with advanced clinicopathological stage. FN1 is considered to be a biomarker of epithelial–mesenchymal transition (EMT) [18], which has been proposed as a key step for the behavior of tumor metastasis by allowing neoplastic epithelial cells to acquire a more mesenchymal phenotype [30]. It has been reported that EMT status was an important prognostic factor for pancreatic cancer and associated with portal vein invasion and lymph node metastasis, although this study utilized two EMT markers other than FN1 [31].

Previous studies in other malignancies have highlighted the controversial role of FN1 in tumor biology. In glioblastomas, FN1 produced by the tumor cells, facilitate the collective invasion of tumor cell spheroids and significantly enhances tumor growth and angiogenesis [32]. In contrast, using a mouse xenografts model, Liu et al. revealed that silencing of FN1 in human thyroid carcinoma cells exhibited enhanced tumor growth and metastases by upregulation of melanoma-associated antigen [33]. Recently, Glasner and colleagues showed that natural killer

Table 4 Univariable and multivariable Cox survival analyses for overall survival

Variables	Univariable analysis			Multivariable analysis		
	HR	95%CI	*P* value	HR	95% CI	*P* value
Age	1.00	0.97–1.02	0.737			
Female gender	0.78	0.53–1.16	0.784			
Tumor size	1.08	0.66–1.79	0.758			
T stage	1.13	0.82–1.56	0.472			
N stage	1.14	0.89–1.46	0.294			
Differentiation grade	1.43	1.03–1.97	0.033	1.42	1.02–1.97	0.038
AJCC stage	1.04	0.69–1.56	0.859			
Resection margin (R1)	1.48	0.99–2.22	0.059	1.70	1.12–2.58	0.012
Adjuvant chemotherapy	0.70	0.43–1.15	0.161			
FN1 expression, high vs low	0.82	0.54–1.25	0.366			

cell-mediated IFN-γ production led to the increased expression of FN1 and resulted in decreased metastasis formation in melanoma [17].

In the present study, stromal FN1 expression patterns did not predict survival in PDAC. There is only one previous study on the prognostic impact of FN1 expression in PDAC. In a small series with 34 patients, Javle et al. reported that high expression of FN1 correlated with p-ERK and a worsened survival [18]. Differences between studies may be related to discrepancies in patient cohorts, antibodies, scoring procedures, and interpretations. Furthermore, sample selection bias could exist in our retrospective study. Patients with advanced, non-operable pancreatic cancer were not included in this study. Although FN1 expression-associated clinical characteristics, including tumor size and AJCC stage, were not associated with the survival, they may still confound the role of FN1 in the prognosis of pancreatic cancer. Additional larger studies may be needed to ascertain the potential association of stromal FN1 expression with survival in PDAC.

Conclusion

The present study showed that stromal FN1expression is associated with larger tumor size, more advanced T stage and N stage, and worse AJCC stage, but not associated with survival in patients with resected PDAC.

Abbreviations

AJCC: American joint committee on cancer; DFS: Disease-free survival; ECM: Extracellular matrix; EMT: Epithelial–mesenchymal transition; FN1: Fibronectin; OS: Overall survival; PDAC: Pancreatic ductal adenocarcinoma; RT: Room temperature; SPARC: Secreted protein acidic and rich in cysteine; TMA: Tissue microarray; TME: Tumor microenvironment

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Authors' contributions

RA designed the study. DA was responsible for sample collection and patient follow up. DH conducted the experiments and statistical analysis and drafted the manuscript. AS helped with the TMA construction and evaluated the tissue staining. QZ, KSH, and RA revised the manuscript thoroughly with important intellectual input. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The local human ethics committee at Lund University has approved the study protocol (Ref 2017/320). Informed consent was received from included patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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