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Plasma protein profiling of patients with intraductal papillary mucinous neoplasm of the pancreas as potential precursor lesions of pancreatic cancer

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ABSTRACT

Efforts for the early diagnosis of the pancreatic ductal adenocarcinoma (PDAC) have recently been driven to one of the precursor lesions, namely intraductal papillary mucinous neoplasm of the pancreas (IPMN). Only a few studies have focused on IPMN molecular biology and its overall progression to cancer. Therefore, IPMN lacks comprehensive characterization which makes its clinical management controversial. In this study, we characterized plasma proteins in the presence of IPMNs in comparison to healthy controls, chronic pancreatitis, and PDAC by a proteomics approach using data-independent acquisition based mass spectrometry. We describe several protein sets that could aid IPMN diagnosis, but also differentiation of IPMN from healthy controls, as well as from benign and malignant diseases. Among all, high levels of carbonic anhydrases and hemoglobins were characteristic for the IPMN group. By employing ELISA based quantification we validated our results for human tissue inhibitor of metalloproteinase inhibitor 1 (TIMP-1). We consider IPMN management directed towards an early potential cancer development a crucial opportunity before PDAC initiation and thus its early detection and cure.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignant disease. With a constantly increasing mortality rate, PDAC is the fourth most common cause of death in the USA. Patients have a 5 year relative survival rate of merely 8% after diagnosis [1] and by 2030, PDAC could become the second leading cause of cancer-related death [2]. Because of the advanced stage of the non-symptomatic disease at the time of diagnosis, more than half of the cases cannot benefit from surgery, the only curative clinical approach, and the 5 year survival rate dramatically drops down to 2% [1]. Even if considerable efforts have been made for the early detection of PDAC, carbohydrate antigen 19-9 (CA 19-9) is the only marker in clinical use [3], not as a screening marker but only to monitor treatment response. Considering all of this, efforts for a better early detection marker have driven attention to its precursor lesions, especially intraductal papillary mucinous neoplasm of the pancreas (IPMN) [4–6]. Prevalence and risk factors were emphasized in several studies and an association between IPMN and PDAC was reported [7–10].

IPMN are mucin producing cystic lesions arising from the main pancreatic duct or its side branches exclusively diagnosed by imaging techniques where they exhibit several morphological characteristics [10–12]. Reports on IPMN prevalence show high variation [13], and also IPMNs heterogeneous biology and malignant potential to progress

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into cancer are poorly understood [12]. Altogether, IPMN clinical management [14,15] is fraught with uncertainties, and overtreatment as well as undertreatment can put patients at risk [16]. Previously, cyst size was used as an indication for resection [14,15] but resulted in overtreatment and thus Choi et al. [17] investigated the cumulative cancer development risk for unresected IPMN. Alarmingly, the 10 years incidence of cancer development ranged from 8 to 25%. Of concern, high level malignancy was also detected in small size cysts. Cancer was present in 34% of resected IPMN cyst size smaller than 3 cm and in 48% of cyst size between 2 and 3 cm [18]. Even more discouraging, overall morbidity after cyst resection ranges from 30 to 50%, which is similar to cancer resection [16]. In contrast, in a large meta-analysis, the risk of patients with branch duct IPMN to develop malignant pancreatic disease was estimated to be low [9].

Besides being controversial and raising debates, current IPMN management lacks biofluid based markers. In this regard, based on a multi reaction monitoring (MRM) proteomics study Kim et al. [19] proposed a six plasma protein multimarker panel to assist the overall diagnosis of IPMN in comparison to healthy controls. Furthermore, first efforts to classify IPMN as benign or malignant were directed towards already established cancer biomarkers. CA19-9, carcinoembryonic antigen (CEA), as well as pancreatic amylase and lipase failed to predict IPMN progression to cancer [20,21]. However, fibrinogen blood levels were found to discriminate between low- and high-risk IPMN [22]. By employing tissue immunohistochemistry, three proteins, namely thrombospondin-1 [23], plectin-1 [24], and periostin [25] were reported as malignancy indicators. Still, these three molecules were highly expressed only in samples where cancer invasiveness was already present and their potential as early indicators remains unconfirmed. As such, there is still need for a better characterization of IPMN by global profiling of blood as a source of biomarkers.

Therefore, we profiled plasma protein composition of IPMN patients with small cyst size in comparison to healthy controls, benign, and malignant pancreatic disease patients by a mass spectrometry data-independent acquisition based approach.

2. Material and methods

2.1. Study participants and plasma collection

Four groups of Caucasian participants were included in this study: 16 healthy blood donors (healthy controls, HC) (aged 44–63 years), 17 patients with intraductal papillary mucinous neoplasms (IPMN) (aged 34–80 years), 15 chronic pancreatitis patients (CP) (aged 35–85 years), and 15 pancreatic ductal adenocarcinoma patients (PDAC) (aged 48–89 years). Out of 17, four IPMNs showed high risk stigmata according to the International Consensus Guidelines of 2012 [14], (3 main duct involvement, 1 main duct dilatation $>$ 10 mm), and two others presented with worrisome features (one non-enhancing nodules, one pancreatic duct stenosis). Three main duct IPMNs underwent resection, but neither showed high grade dysplasia or invasive growth. All others were followed up and did not progress during the observation period. IPMN diagnosis was made if cyst fluid analysis revealed increased CEA levels above a cut-off of 192 ng/mL or a duct connection between the cystic lesion and the main duct was visualized either on endoscopic ultrasound or magnetic resonance imaging in the absence of pancreatitis. Patient's samples were collected at the diagnosis visit during 2010–2015, before any pharmacological or surgical intervention took place. Blood was collected at the clinic in tubes containing lithium heparin as anticoagulant and plasma was prepared following the tube manufacturer's protocol and stored at − 80 °C until analysis. The study was performed in accordance with the WMA Declaration of Helsinki and written informed consent was sought from all participants prior to the sample collection. The study was approved by the ethics committee of the University of Greifswald.

2.2. Depletion of highly abundant plasma proteins

Six highly abundant plasma proteins (HAP) namely serum albumin, immunoglobulin gamma, immunoglobulin alpha, serotransferrin, haptoglobin, and alpha-1-antitrypsin, were depleted by using multi-affinity chromatography (MARS6-human, Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol. After trichloroacetic acid precipitation (final concentration 15%) of the residual protein fraction, the pellet was resuspended in 80 µL urea/thiourea solution (8/2 M) (Merck Millipore, Darmstadt, Germany) solution and samples were stored at − 80 °C until use.

2.3. Sample preparation for mass spectrometric analysis

Protein concentration was determined using a Bradford Assay (BioRad Laboratories, Munich, Germany) [26] with bovine serum albumin as standard protein. Four µg of each protein sample were subjected to reduction with dithiothreitol (2.5 mM, 1 h at 60 °C), alkylation with iodoacetamide (10 mM, 15 min at 37 °C) and proteolytic digestion by trypsin (Promega, Madison, WI, USA) at 1: 25 protease to protein ratio (overnight at 37 °C). 1% acetic acid was used to stop digestion and desalting of peptides was done using ZipTip µC18 tips (Millipore Cooperation, Billerica, MA, USA) following the manufacturer's protocol. Lyophilized peptides were dissolved in 0.1% acetic acid: acetonitrile (98:2 v/v) to a final concentration of 0.1 µg/µL.

For the generation of a spectral library, four pools of samples were prepared by mixing individual samples from each group. Peptides were generated by tryptic digestion as described above, synthetic iRT peptides (Biognosys, Schlieren, Switzerland) were spiked in and mixtures were analyzed in data-dependent acquisition mode (DDA). Individual plasma samples to be analyzed in data-independent acquisition mode (DIA) were spiked with hyper reaction monitoring (HRM) calibration peptides (Biognosys). All synthetic peptides were spiked according to manufacturer's instructions.

2.4. LC-MS/MS setup for data-dependent-acquisition (DDA) analyses

All sample analyses were performed with an UltiMate3000 RSLC system coupled on-line to a Q Exactive Plus™ Orbitrap-MS (Thermo Electron, Bremen, Germany). Reversed-phase chromatography was performed on an Accucore 150-C18 column (25 cm × 2.6 µm) (Thermo Fisher Scientific Inc., Idstein, Germany) using a binary buffer system consisting of 0.1% acetic acid: acetonitrile (95:5 v/v) (buffer A) and acetonitrile: acetic acid (99.9:0.1 v/v) (buffer B). The peptides were separated by applying a linear gradient from 2 to 25% buffer B over a time of 95 min. The flow rate was 300 nL/min and the column temperature was 40 °C.

For data-dependent acquisition (DDA) full-scan MS was recorded at a mass range of m/z 300 to 1650 and paralleled by sequential isolation of the top ten most intense ions ($z = +2$ to $+6$) for fragmentation using high-energy collision dissociation (HCD) with dynamic exclusion for 30 s and disabled early expiration.

2.5. Building the ion library for DIA data analysis

The final spectral library to be used for DIA data analysis was generated from data acquired from triplicate measurements of the four pooled samples described above and an existing in-house plasma spectral library based on 93 DDA acquisitions on Q Exactive instruments (Thermo Electron, Bremen, Germany). As a starting point, two separate spectral libraries were built by running two different software packages. Spectral library 1 was generated from the search results of Proteome Discoverer 2.0 (Thermo Fischer Scientific) using a combination of search engines that included Mascot, Sequest HT and MS Amanda [27]. Spectral library 2 was generated from the MaxQuant search results [28]. Common search parameters for all the search