ORIGINAL ARTICLE

Detection of pancreatic cancer using serum protein profiling

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Abstract

Background: Currently, no suitable biomarkers for the early detection of pancreatic cancer (PC) are available. Proteins present in the serum could reflect a state of the disease. In this study, these profiles as a diagnostic marker for PC were evaluated.

Methods: Serum samples were obtained from PC patients ($n = 50$ calibration set, $n = 39$ validation set) and healthy volunteers ($n = 110$ and $n = 75$ respectively) according to a uniform standardized collection and processing protocol. For peptide and protein isolation, automated solid-phase extraction (SPE) with Weak Cation Exchange (WCX) magnetic beads (MB) was performed using a 96-channel liquid handling platform. Protein profiles were obtained by mass spectrometry (MS) and evaluated by linear discriminant analysis with double cross-validation.

Results: A discriminating profile for PC has been identified, with a sensitivity of 78% and a specificity of 89% in the calibration set with an area under the curve (AUC) of 90%. These results were validated with a sensitivity of 74% and a specificity of 91% (AUC 90%).

Conclusion: Serum profiles of healthy controls and PC can be discrimated between. Further research is warranted to evaluate specificity and whether this biosignature can be used for early detection in a high risk population.

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Introduction

Although pancreatic cancer (PC) has an annual incidence of only 8.2 cases per 100 000 males and of 5.4 cases per 100 000 females, it is the fifth (male) and fourth (female) leading cause of cancer death in developed countries.[1] Patients with PC have an extremely poor prognosis with an overall 5-year survival rate of less than 5%.[2] When a surgical resection is possible, 5-year survival rates increase to approximately 25%, but unfortunately most tumours are at an advanced stage when diagnosed.[3,4] Delays in diagnosis are often caused by the lack of specific symptoms for early cancers, such as pain, jaundice and weight loss. Biomarkers might be an additional tool for diagnostics next to currently available imaging techniques. The mostly studied available clinical serum biomarker carbohydrate antigen 19-9 (CA19-9) has a sensitivity of 80% and a specificity of 90% but misses the appropriate sensitivity and specificity for small, resectable cancers.[5] Moreover, CA19-9 is often elevated in benign cholangitis, pancreatitis and other cancers, and therefore lacks the specificity for detecting potentially curable lesions. At this moment, the use of CA19-9 is only recommended for follow-up. Currently, only imaging techniques such as ultrasound (transcutaneous or endoscopic), computed tomography (CT) scan, endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging (MRI) and MR cholangiopancreatography (MRCP) are used for the diagnosis and staging of the pancreatic disease.

Chronic pancreatitis could mimic PC at diagnostics and hampers patient selection for a pancreaticoduodenectomy. For these patients a new biomarker that discriminates between pancreatitis and cancer could be of great value.

It has been estimated that 5% to 10% of PC cases are associated with an inherited predisposition. Tumour syndromes associated

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with an increased risk of PC include Peutz–Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM), hereditary breast cancer (BRCA2 mutation carriers) and possibly Lynch syndrome. The lifetime risk of PC varies between 5% in BRCA2 mutation carriers and 36% in patients with Peutz–Jeghers syndrome.[6–9] For this patient group, early detection is of paramount importance as the prognosis is usually poor when diagnosis follows symptoms. No studies for the early detection in this specific high-risk group using CA19-9 have been performed. At this time this is only possible through imaging surveillance.[10] Therefore, there is an urgent need for new and better biomarkers for PC.

A sensitive and specific option could be the use of proteomic serum biomarkers. During transformation of a normal cell into a neoplastic cell, distinct changes occur at the protein level which may affect cellular function.[11] Therefore, proteins are considered promising targets for biomarker discovery. Mass spectrometry (MS) has become the method of choice for protein analysis in serum.[12,13] Provided standardized sample workup, MS measurement, data processing and evaluation, peptide and protein profiles are highly reproducible.[14] With respect to speed and automation strategies needed for high-throughput screening, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS workflows remain unrivalled.[12,15,16] As human body fluids such as serum are highly complex a suitable 'clean-up' procedure is required.[17] Based on the physicochemical properties of protein separation techniques, magnetic beads (MB) have been functionalized accordingly [e.g. Weak Cation Exchange (WCX)]. These beads are not only suited for clean-up, but also enrich subsets of peptides and proteins and can thus contribute to the sensitivity of the assay.[18] The present serum peptide/protein capture procedure has been fully automated with a liquid handling robot, such as a 96-channel Hamilton STARplus® platform (Hamilton, Bonaduz, Switzerland). This ensures reproducibility and allows high-throughput screening which is essential for large-scale disease profiling studies.[19,20] In the last decade, multiple studies have been carried out using a magnetic bead-based method for offline serum peptide/protein capture and MALDI-TOF-MS readout.[14,21–25]

For PC, several proteomic studies have been performed since the introduction of protein profiling in 2002 by Petricoin *et al*,[26] all with different fractionation platforms and type of MS.[27–33] For example, Koopman *et al.*[34] used a surface enhanced laser desorption/ionization (SELDI)-TOF approach combined with WCX and metal affinity protein chips as the solid-phase extraction (SPE) method to discriminate between patient groups with a sensitivity of 78% and a specificity of 97%. No study has been published using a combination of WCX MB and MALDI-TOF.

For this study, a MALDI-TOF serum platform in combination with functionalized WCX MB was used to generate serum protein profiles in a first attempt to differentiate between PC patients and healthy controls in a stringent sample handling and high throughput and automated processing protocol. The obtained discriminating profile was validated in a second case–control group.

Material and methods

Patients

Blood samples were obtained from 50 patients with PC prior to surgery, and from 110 (age-and gender-matched) healthy volunteers at the outpatient clinic of the Leiden University Medical Center (LUMC), the Netherlands from October 2002 until December 2008. Healthy volunteers were partners or accompanying persons of included patients. For the validation set, blood samples were obtained from 39 patients and 75 healthy (age and gender matched) volunteers, included from January 2009 until July 2010. Patients were selected candidates for curative surgery; this meant that no patients with primary irresectable tumours were included. All surgical specimens were examined according to routine histological evaluation and the extent of the tumour spread was assessed by TNM (TNM Classification of Malignant Tumours) classification. Furthermore, the tumour marker CA-19.9 was noted if determined pre-operatively. An Elecsys CA19-9 tumour marker assay based on the monoclonal 116-NS 19-9 antibody (Roche Diagnostics GmbH, Mannheim, Germany) was used. This tumour marker has a normal reference value of 0.0–27.9 U/ml (95th percentile). Informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of the LUMC.

Blood collection

Samples from both the calibration set and the validation set were collected and processed according a standardized protocol:[14] in short, all blood samples were drawn by antecubital venapuncture while the individuals were seated and had not been fasting prior to any invasive procedure. The samples were collected in an 8.5-cc Serum Separator Vacutainer Tube (BD Diagnostics, Plymouth, UK) and maximally within 4 h at room temperature centrifuged at 1000 *g* for 10 min.[14] The samples were then distributed into sterile 500-µl barcode labelled polypropylene aliquots (Thermo Fisher Scientific, Hudson, NH, USA) and stored at −80°C.

Sample processing

Aliquotting and storage

An overview of the processing platform of the serum samples, MALDI-TOF profiles and data is given in Fig. 1. All serum samples were thawed on ice once and randomly placed in barcode labelled racks in an 8-channel Hamilton STAR® pipetting robot (Hamilton) for automated aliquotting in 60-µl daughter tubes. The aliquots were stored at −80°C until further sample processing. The processing steps for the validation set were identical to those for the calibration set.

WCX-MB sample work up

The isolation of proteins from serum was performed using a commercially available kit based on magnetic bead purification (Bruker Daltonics, Bremen, Germany). The WCX MB were applied according to the manufacturer's instructions with further optimization to allow implementation on a 96-channel Hamilton