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Original Articles

Discriminating patients with early-stage pancreatic cancer or chronic pancreatitis using serum electrospray mass profiling

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ABSTRACT

Blood tests are needed to aid in the early detection of pancreatic ductal adenocarcinoma (PDAC), and monitoring pancreatitis development into malignancy especially in high risk patients. This study exhibits efforts and progress toward developing such blood tests, using electrospray-mass spectrometry (MS) serum profiling to distinguish patients with early-stage PDAC or pancreatitis from each other and from controls. Identification of significant serum mass peak differences between these individuals was performed using *t* tests and "leave one out" cross validation. Serum mass peak distributions of control individuals were distinguished from those of patients with chronic pancreatitis or early-stage PDAC with *P* values $<10^{-15}$, and patients with chronic pancreatitis were distinguished from those of patients with early-stage PDAC with a *P* value $<10^{-12}$. Sera from 12 out of 12 patients with PDAC stages I, IIA and IIB were blindly validated from controls. Tandem MS/MS identified a cancer phenotype with elements of PDAC involved in early-stage PDAC/control discrimination. These studies indicate electrospray-MS mass profiling can detect serum changes in patients with pancreatitis or early-stage pancreatic cancer. Such technology has the potential to aid in early detection of pancreatic cancer, biomarker development, and in monitoring development of pancreatitis into PDAC.

Introduction

Early detection of pancreatic ductal adenocarcinoma (PDAC) is an important aspect of cancer treatment because early clinical stages (I, II) are easier to cure than later stages (III, IV) [1]. There is a need for robust, accurate and non-invasive detection methodology, e.g. from blood, for the early stages of pancreatic cancer [1,2]. Serum protein CA-19.9 is used to monitor existing pancreatic cancer but is not useful in diagnosis [3]. Multiple micro (mi) RNAs from plasma were shown to be indicators for pancreatic cancer, and mi-155 is possibly predictive for early-stage pancreatic neoplasia [4]. However there are still some discrepancies among micro RNA technologies [5,6]. A variety of serum biomarkers in an antibody-protein microarray format had positive results detecting late-stage pancreatic cancer and chronic pancreatitis (CP) [7]. Chronic pancreatitis is a significant risk factor for the development of pancreatic cancer [8,9]. One of the prominent mechanisms by which PDAC is hypothesized to develop, e.g., from pancreatitis to pancreatic cancer, is through cellular and genetic changes involving pancreatic intraepithelial neoplasias (PanINs) which can be found in chronic pancreatitis [10,11].

The profiling of bodily fluids using all-liquid electrospray ionization (ESI) mass spectrometry (MS) has the potential to distinguish differences between blood/sera of disease-free individuals and individuals with pathological conditions [12–15]. Serum mass profiling is useful in cancer diagnostics including pancreatic cancer, and in therapeutic development [14–17]. The underlying hypothesis is that sera contain ample numbers and kinds of peptides and other biomolecules (e.g., proteins, nucleic acids, glycoconjugates, lipids), and this complexity will vary between disease states [12–15]. The basis for some of this complexity involves exoprotease degradation of proteins [18] and cellular signaling mechanisms [19], and is hypothesized to reflect homeostatic as well as defense/stress mechanisms which change with physiological state [16–19]. Consequently, organs/tissues shed and/or secrete varying amounts and different kinds of biomolecules into the peripheral blood in

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response to different physiological conditions. All-liquid ESI-MS is possibly the simplest biomarker platform available, requiring only a serum dilution and injection into the mass spectrometer. Liquid MS analyzes disease-related phenotypic profiles in sera, as opposed to indirect genotypic/nucleic acid classifications. ESI-MS serum mass profiling examines potentially all biomolecules in sera, whereas other biomarker platforms (DNA, RNA, metabolomics, and various antibody methods) focus on a single component or small groups of similar components and can require a significant amount of preparation prior to analysis. To improve specificity in disease detection, the more biomolecules analyzed at once, the greater disease discriminatory powers of the platform [17,18]. Importantly, MS analysis meets the accuracy, robustness, and reproducibility guidelines for stringent clinical laboratory testing [20–23]. Standard statistical approaches, like those used in this study, are better suited than novel algorithms [20,23].

Previously, we utilized electrospray ionization mass spectrometry (ESI-MS) peaks to distinguish sera from early-stage ovarian, lung, and pancreatic cancer patients from healthy disease-free individuals [15–17,24–26]. In the present study, electrospray serum mass profiling is used to distinguish early-stage PDAC patients (stages I, IIA, IIB) from healthy individuals and from patients with chronic pancreatitis. Leave one out cross validation (LOOCV) of the mass peak data and randomization of cohort sera samples is used to check for and help ameliorate "over-fitting" of the mass peak data. "Hold out" databases are formed and used to validate blinded early-stage PDAC, CP, or control serum sets. Tandem MS/MS [27] is used to help identify peptides/proteins potentially involved in PDAC/control discrimination. Such straight-forward analyses, from an accessible body fluid like serum, holds promise for aiding in the diagnosis and disease monitoring and, in the future, understanding pancreatic carcinogenesis mechanisms as well as aiding in the development and analysis of therapeutic interventions for this deadly disease.

Materials and methods

Patients and clinical samples

Patient-related information concerning individuals with stage I, IIA, or IIB pancreatic cancer, chronic pancreatitis, as well as healthy control individuals, is listed in Table 1. Patient/serum samples are divided into three groups: complete databases, validation databases, and blind validation samples. Tumor pathological staging was according to the TNM staging system (tumor size, node involvement, metastasis presence) [28]. Tumor and pancreatitis pathology was determined at the Surgical Pathology Laboratories of the University of Oklahoma Health Sciences Center Hospital. Sera were obtained from patient peripheral blood at the University of Oklahoma Health Sciences Center, before treatments, according to standard procedures [29]. Blood and serum samples were also collected from healthy volunteers from the University community in identical fashion. Sera aliquots (100 µl) were frozen at –80 °C, and not reused after initial freezing and thawing. Histology and hematoxylin and eosin (H&E) staining of PDAC, CP, and control tissues were performed as described [25].

ESI-MS of sera from PDAC and CP patients and healthy controls

A serum aliquot from patients with PDAC, CP, or control individuals was diluted 1–300 into a solution of 50% methanol and 2% formic acid. The samples were loop injected (20 µl) into the nano source of an LCQ Advantage ion trap mass spectrometer (ThermoScientific), fitted with a 20 micron inner diameter (100 micron outer diameter) fused silica (Polymicro Technologies) tip at a flow rate of 0.5 µl/min using an Eldex MicroPro series 1000 pumping system [24]. High-resolution triplicate mass spectra were collected from disease and disease-free sera in random fashion per day. The spectra were sampled at an m/Z (mass divided by charge) resolution of two hundredths over an m/Z range of 400–2000. Positive ion mode spectra were collected over 30 min for each injection. Raw spectral data from the Advantage LCQ instrument were extracted using the manufacturer's software "Qual Browser" version 1.4SR1. Spectral data were exported in a format providing rounded unit m/Z and intensity values. Data were then normalized to the highest m/Z sum intensity value in segments of 25 m/Z from 400 to 2000. MS spectral peak assignments were calculated as centroid m/Z peak area values (valley to valley) using Mariner Data Explorer 4.0.0.1 software (Applied BioSystems). Centroid area is defined as the area of the peak calculated from its geometrical m/Z center. For tandem MS/MS mass peak identifications [27], 60 m/Z ions (peak range, 700–940 m/Z, identified by LOOCV analysis for discriminating non-cancer controls from patients with stage IIB pancreatic cancer) were screened at 12 m/Z ion intervals in 10 control and 10 PDAC serum samples in the ThermoScientific ion-trap MS instrument used in the cohort discriminations. Samples were diluted 1–390 in 50% methanol, 2% formic acid, 48% water, and analyzed at a flow rate of 0.20 microliters/min. Peak protein identifications were determined using SEQUEST Proteome Discoverer 1.0 (Thermo Scientific) using the "no cleavage" setting on a "*Homo sapien*" database created through the Discoverer software from a non-redundant database downloaded from NCBI on 07/08/2014. Serum samples on average contained 1.95 (range: 0–5) parent ions with significant differences in standard MS spectral data between the pre and post MS/MS scans of the 60 parental ions observed.

Statistical and quantitative analysis

m/Z peak area data were exported into Excel 2010, and triplicate peak areas at each m/Z value were averaged for each serum sample. Using a nested LOOCV protocol [30,31], individual m/Z peak areas of a "left out" serum sample were analyzed for significance against the "left in" database (e.g., remaining control versus PDAC samples) using *t*-tests (one-tailed, two sample unequal variance) [16,26]. LOOCV was performed by removing one patient or control serum m/Z peak area data set at a time from the total m/Z peak database for each class of sera samples, and then reforming the total MS peak database in the absence of that single sample m/Z data set. For each "left out" m/Z LOOCV peak area tested against the database of significant peaks, a value greater than the 50% "cutoff " (see Fig. 1E) was assigned to a patient group descriptor like PDAC, and a value equal to or less than the 'cutoff mean' was assigned to the control or other group descriptor. This procedure was repeated for all sera samples in the control, PDAC, and pancreatitis cohorts. In addition,

Table 1
Patient groups and characteristics.

Databases	Patient groups	Mixed age Mean (range)	Female age Mean (range)	Male age Mean (range)	Patient (*N*) (male/female)
Complete LOOCV databases (all patient samples)	Pancreatitis	55.0 (39–80)	52.8 (46–74)	56.3 (39–80)	14 (9/5)
	Pancreatic cancer stages I & IIA	60.0 (42–73)	66.6 (56–73)	56.6 (42–68)	9 (6/3)
	Pancreatic cancer stage IIB	68.2 (49–84)	65.4 (49–76)	71.3 (60–84)	19 (9/10)
	Pancreatic cancer stage IIA & IIB	68.0 (49–84)	66.5 (49–76)	69.8 (58–84)	23 (11/12)
	Pancreatic cancer I, IIA & IIB	65.5 (42–84)	65.6 (49–76)	65.4 (42–84)	28 (15/13)
	Non-cancer (control)[a]	55.9 (40–69)	54.9 (40–63)	56.8 (47–69)	22 (12/10)
Validation LOOCV databases (blind validation patient samples excluded)	Pancreatitis	51.2 (39–69)	47.6 (46–49)	53.0 (39–69)	9 (6/3)
	Pancreatic cancer stage IIA & IIB	70 (56–84)	70.2 (56–76)	69.9 (58–84)	13 (8/5)
	Pancreatic cancer stage IIB	70.8 (56–84)	69.5 (56–76)	71.5 (60–84)	11 (7/4)
	Pancreatic cancer I, IIA & IIB	65.9 (42–84)	68.2 (56–76)	64.6 (42–84)	20 (13/7)
	Non-cancer (control)	56.1 (40–69)	54.0 (40–63)	57.8 (47–69)	14 (8/6)
Blind validation samples withheld from validation database groups	Pancreatitis	62.0 (47–80)	60.5 (47–74)	63.0 (50–80)	5 (3/2)
	Pancreatic cancer stage IIA & IIB	60.5 (39–76)	60.7 (49–76)	60.0 (39–74)	10 (3/7)
	Pancreatic cancer stage IIB	64.6 (47–80)	62.7 (49–76)	70.5 (67–74)	8 (2/6)
	Pancreatic cancer stage I, IIA & IIB	59.9 (39–76)	59.9 (49–76)	60.0 (39–74)	12 (4/8)
	Non-cancer (control)	55.6 (51–58)	56.3 (51–58)	55.0 (53–56)	8 (4/4)
	Non-cancer (control) group 2[b]	57.7 (41–75)	55.9 (41–65)	59.2 (47–69)	18 (10/8)
Lung cancer	Lung cancer stage I	64.2 (45–84)	62.3 (45–84)	65.0 (50–82)	40 (28/12)

[a] Non-cancer (control) and [b]non-cancer (control) group 2 are unique groups and do not have any members in common.