Feasibility of Identifying Pancreatic Cancer Based on Serum Metabolomics

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Abstract

Background: We postulated that the abundance of various metabolites in blood would facilitate the diagnosis of pancreatic and biliary lesions, which could potentially prevent unnecessary surgery.

Methods: Serum samples from patients with benign hepatobiliary disease (n = 43) and from patients with pancreatic cancer (n = 56) were examined by 1H NMR spectroscopy to quantify 58 unique metabolites. Data were analyzed by “targeted profiling” followed by supervised pattern recognition and orthogonal partial least-squares discriminant analysis (O-PLS-DA) of the most significant metabolites, which enables comparison of the whole sample spectrum between groups.

Results: The metabolomic profile of patients with pancreatic cancer was significantly different from that of patients with benign disease (AUROC, area under the ROC curve, = 0.8372). Overt diabetes mellitus (DM) was identified as a possible confounding factor in the pancreatic cancer group. Thus, diabetics were excluded from further analysis. In this more homogeneous pancreatic cancer group, compared with benign cases, serum concentrations of glutamate and glucose were most elevated on multivariate analysis. In benign cases, creatine and glutamine were most abundant. To examine the usefulness of this test, a comparison was made to age- and gender-matched controls with benign lesions that mimic cancer, controlling also for presence of jaundice and diabetes (n = 14 per group). The metabolic profile in patients with pancreatic cancer remained distinguishable from patients with benign pancreatic lesions (AUROC = 0.8308).

Conclusions: The serum metabolomic profile may be useful for distinguishing benign from malignant pancreatic lesions.

Impact: Further studies will be required to study the effects of jaundice and diabetes. A more comprehensive metabolomic profile will be evaluated using mass spectrometry. Cancer Epidemiol Biomarkers Prev; 20(1); 140-7. ©2010 AACR.

Introduction

Pancreatic cancer is the fourth most common cause of cancer mortalities in North America (1). The 5-year survival rate is only 5.1% (1). The only chance of potential, long-term control of this devastating disease is resection (2). Unfortunately, early diagnosis is difficult. The symptoms most frequently associated with pancreatic cancer (weight loss, malaise, fatigue, and pain) are vague and non-specific. Jaundice is a later manifestation of the disease. This sign is also non-specific, as it may be secondary to a benign biliary stricture or to gallstones. Early pancreatic cancer is invisible on routine radiographic studies. None of the radiographic findings of more advanced disease (including bile duct stricture and pancreatic mass) is pathognomonic (Fig. 1). Benign lesions mimicking pancreatic cancer include pancreatitis and pancreatic cysts. Finally, obtaining a tissue diagnosis is extremely difficult. Bile duct brushings (in the event of a biliary stricture) only have a yield of 23% to 41% (3, 4). The diagnostic rate of EUS (endoscopic ultrasound)-guided biopsies for pancreatic masses is only about 71% (5). Although their sensitivity is about 85%, negative predictive value is only about 64% (6). Therefore, negative biopsies are not particularly informative and do not aid in clinical decision making (2).
A patient underwent a pancreaticoduodenectomy. The findings on CT scan and cholangiogram are indistinguishable between the benign case and the malignant case. In each case, the patient had dilatation secondary to a distal common bile duct stricture (yellow arrow). Cholangiograms demonstrate biliary strictures. The features on each CT scan include a periampullary mass (orange arrow) and dilated extrahepatic bile ducts in association with a distal biliary stricture (yellow arrow). The findings on CT scan and cholangiogram are indistinguishable between the benign case and the malignant case. In each case, the patient underwent a pancreaticoduodenectomy.

Materials and Methods

Sample collection

The study was approved by the Conjoint Health Research Ethics Board at the University of Calgary. Clinically annotated serum samples were collected from patients encountered through a hepatobiliary surgery practice. Samples were obtained from patients with known pancreatic adenocarcinoma or benign pancreatic conditions (including benign masses and chronic pancreatitis). Additional controls consisted of patients undergoing elective cholecystectomy for biliary colic. Patients with any acute inflammation or sepsis were specifically excluded from this latter group. All patients provided consent to participate. Surgical pathology and follow-up data were available for all patients. This enabled more assured classification of patients to benign or malignant categories.

The collection and processing of samples was standardized as much as possible. Samples were collected by venipuncture or through a central line (taking care to exclude any heparin or any other IV infusate) in a plastic gold top Vacutainer tube (BD Biosciences) that contains a clot activator and a gel for serum separation. Samples were processed within 6 hours of collection and then frozen at −20°C until the time of analysis. All samples were stored at room temperature until processing. Samples were processed within 6 hours of collection and then frozen at −20°C until the time of analysis. All samples were processed within 6 hours of collection and then frozen at −20°C until the time of analysis. All samples were processed within 6 hours of collection and then frozen at −20°C until the time of analysis. All samples were processed within 6 hours of collection and then frozen at −20°C until the time of analysis. All samples were processed within 6 hours of collection and then frozen at −20°C until the time of analysis.
NMR spectroscopy

All experiments were performed on a Bruker Avance 600 spectrometer (Bruker Biospin) operating at 600.22 MHz and equipped with a 5-mm TXI probe at 298 K. All 1-dimensional 1H NMR spectra of aqueous samples were acquired using a standard Bruker noe-sypr1d pulse sequence in which the residual water peak was irradiated during the delay of 1.0 second and during the mixing time of 100 millisecond. A total of 1,024 scans were collected into 63,536 data points over a spectral width of 12,195 Hz with a 90-degree pulse width and a 5-second repetition time. A line broadening of 0.1 Hz was applied to the spectra prior to Fourier transformation, phasing, and baseline correction. Additional 2-dimensional NMR experiments were performed for the purpose of confirming chemical shift assignments, including total correlation spectroscopy (2D 1H-13C TOCSY) and heteronuclear single quantum coherence spectroscopy (2D 1H-13C HSQC), using standard Bruker pulse programs.

Metabolites were assigned based on comparison of both 1H and 13C chemical shifts and spin–spin coupling constants with those of model compounds in Human Metabolome Database (HMDB; ref. 18) and Chenomx NMR Suite 5.1 software (Chenomx Inc.). Metabolites were quantified using the targeted profiling approach as implemented in the Chenomx software (19).

Data analysis

Preprocessing of the raw data was carried out for each experiment by normalizing the concentration of each metabolite to a total concentration of all metabolites in the sample. Normalized metabolite concentrations were then log transformed, centered, and scaled. Data were examined for missing or influential values and for their symmetry. Descriptive statistics compared the measured clinical and patient features between the patients with benign disease and those with pancreatic cancer. Two-sample t tests were used for continuous variables and frequent analyses excluded patients with overt DM, so that a supervised O-PLS-DA approach could be considered semi-supervised. Next, a supervised method partitions variance related to the y variable (class separation) from unrelated factors, which are orthogonalized (20). There was a visible separation in the scores plots from patients with benign and malignant disease (Fig. 2). However, further scrutiny of the metabolic profile associated with pancreatic cancer revealed a high degree of abundance of metabolites associated with DM (data not shown). Because incidence of overt DM was significantly greater in the malignant group, all subsequent analyses excluded patients with overt DM, so that a metabolic profile associated with pancreatic cancer could be derived.

In this more defined group (consisting of 43 patients with cancer and 41 patients with benign disease), 22 metabolites had values of $P < 0.3$ threshold and were subsequently analyzed using both unsupervised principal component analysis (PCA) and supervised orthogonal partial O-PLS-DA. This latter method partitions variance related to the y variable (class separation) from unrelated factors, which are orthogonalized (20). There was a visible separation in the scores plots from patients with benign and malignant disease (Fig. 2). However, further scrutiny of the metabolic profile associated with pancreatic cancer revealed a high degree of abundance of metabolites associated with DM (data not shown). Because incidence of overt DM was significantly greater in the malignant group, all subsequent analyses excluded patients with overt DM, so that a metabolic profile associated with pancreatic cancer could be derived.

In this more defined group (consisting of 43 patients with cancer and 41 patients with benign disease), 22 metabolites had values of $P < 0.3$. The scores plot from PCA is depicted in Figure 3A. Because metabolites were selected on the basis of 2-sample t tests, the PCA model could be considered semi-supervised. Next, a supervised classification model was built using disease status, age, and gender as Y-predcv. Cross-validated O-PLS-DA scores indicated that the age and disease status were important ($P = 0.016$ and $P = 1.3 	imes 10^{-5}$, respectively) whereas gender had little influence on the model ($P = 0.99$). Scores plots comparing the metabolic profiles of samples from patients with benign disease were visibly distinguishable from those of patients with pancreatic adenocarcinoma (Fig. 3B).
The degree of differential abundance of individual metabolites is summarized in Figure 3C for the predictive component distinguishing benign from malignant disease. As the effect of age was also considered in the model (Fig. 3D), we were able to distinguish metabolites that are strongly related to disease alone (elevated glutamate, acetone, and 3-hydroxybutyrate) and those related to disease and age (elevated glucose, phenylalanine, formate, and mannose). In the case of benign disease, glutamine, ethanol, and asparagine were related to pathology alone; creatine, proline, and glycerol were related to both pathology and age.

**Model validation and AUROC**

One important aspect of the data-modeling procedures lies in the predictive ability in terms of specificity and sensitivity in distinguishing benign from malignant pancreatic disease. The multivariate O-PLS-DA modeling procedures employed here incorporated a 7-fold cross-validation step. In this case, 7 models were built with exactly one seventh of the data excluded from each model and each sample excluded a single time. The ability of the models to predict those samples not involved in the modeling provided a measure of the overall predictive ability of the metabolite profiling. Using these values (Y-predcv), we were able to generate a receiver-operating characteristic (ROC) curve. The AUROC was 0.8372 (Fig. 4). Values greater than 0.8 indicate excellent predictive ability, and the values obtained here are clearly superior to any current clinical markers.

Ultimately, a diagnostic test for pancreatic cancer should be able to distinguish malignant and benign pancreatic lesions in patients under consideration for surgery. Our control group included patients with conditions that are not typically confused with pancreatic cancer, and there was some imbalance in age and incidence.
dence of jaundice. Therefore, as a final proof of feasibility, we queried whether the metabolomic profile associated with malignancy could discriminate between benign and malignant diagnoses in a group of patients with biliary strictures and pancreatic masses matched by age, jaundice, and incidence of diabetes. This subgroup is described in Supplementary Table 2. The AUROC in this subset of 14 matched pairs (28 samples total) was similar to that of the larger dataset (0.8308).

Discussion

Clinically, distinguishing benign biliary strictures and pancreatic masses from pancreatic cancer and other periampullary malignancies is a challenge. The clinician is often faced with a decision on whether to recommend resection or another treatment directed at a presumed malignancy without a definitive diagnosis. Resection is associated with substantial morbidity and a risk of mortality (12). On the other hand, a delay in diagnosis may result in a missed opportunity to administer definitive treatment. We have demonstrated that it is feasible to distinguish pancreatic cancer from benign hepatobiliary disease using 1H NMR spectroscopy to obtain a metabolomic profile. With further development, it is possible that a diagnostic test can be derived that will aid in clinical decision making.

Presently, a clinician who encounters a patient with a biliary stricture or a pancreatic/periampullary mass must make decisions based on radiographic studies, serum tumor markers, and the availability of any tissue samples. Radiographic findings occur late in the course of pancreatic cancer and findings are not specific (Fig. 1). Serum levels of CA19-9 have a sensitivity of 50% to 80%, although specificity is about 90% (22–25). Serum carcinoembryonic antigen (CEA) is less frequently elevated. Finally, obtaining a reliable tissue diagnosis is extremely difficult. Bile duct brushings only have a yield of 23% to 41% (3, 4). The diagnostic rate of biopsies for pancreatic masses is only about 71% (5). Moreover, although the sensitivity of biopsies is about 85%, negative predictive value is only about 64% (6). Therefore, negative biopsies are not particularly informative and do not aid in clinical decision making (2). Despite the availability of each of these tests, it is extremely difficult to accurately identify patients harboring a malignancy. That is, of all patients subjected to a pancreaticoduodenectomy or a radical pancreatectomy, 7% to 16% (and as high as 25%) are found on final pathology to have benign lesions (7–11). Therefore, there is a need for a more accurate, clinically feasible method of distinguishing patients with benign or malignant disease.

We considered that a metabolomic approach would be useful for a number of reasons. Pancreatic cancer is well known to have associated metabolic changes. The prevalence of DM in pancreatic cancer is reported as 40% to 47%, often preceding the diagnosis within less than 2 years (26–28). Hyperinsulinemia and peripheral insulin resistance are typical in pancreatic cancer, whereas chronic pancreatitis (which also may be associated with a pancreatic mass) is accompanied by islet cell destruction and impaired insulin production (29, 30). Serum lactate levels tend to be higher in patients with periamppillary malignancies than healthy controls and patients with benign periamplillary lesions (31). In animal models of pancreatic cancer (32), metabolic profiles associated with disease progression have been demonstrated. Others have reported that the metabolic profile of bile can discriminate benign and malignant strictures (31, 33). However, bile is generally inconvenient to sample. Recently, it was demonstrated in a small number of patients with pancreatic cancer that the salivary metabolomic profile was significantly different from normal controls (34). Finally, the plasma metabolomic profiles of 5 patients (incidence of diabetes and jaundice unknown) were significantly different from normal controls (35). These early results spurred our interest in more fully exploring the feasibility of using serum metabolomics to improve the diagnosis of pancreatic cancer.

Indeed, we were successful in demonstrating a serum metabolomic profile that reflects the presence of pancreatic cancer. Interestingly, it was observed that 3-hydroxybutyrate and acetone (end products of ketogenesis) were associated with pancreatic cancer (but not other clinical factors). This was particularly intriguing because patients with overt DM were specifically excluded from that analysis. This may reflect the presence of latent diabetogenic changes perhaps resulting from pancreatic cancer.
Although our approach to diagnosing pancreatic cancer is novel and promising, there were a number of limitations to this study. We were successful in demonstrating that it was possible to distinguish benign from malignant pancreatic lesions. A better understanding of the metabolic changes associated with diabetes and hyperbilirubinemia (in patients with benign and malignant lesions) will be required. The specificity of the metabolic profile must also be evaluated by comparing with samples from patients with other periampullary and hepatobiliary malignancies. Thus, although promising results were obtained, the metabolic profiles we have so far obtained cannot yet be considered definitive or diagnostic.

In addition to addressing the above limitations, which will aid in refining the metabolic profile diagnostic for pancreatic cancer, it may be beneficial to make a more comprehensive metabolomic assessment. This could be done using gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS), which may enable the detection of more metabolites (15). A more comprehensive metabolomic profile may improve diagnostic accuracy. In addition, it is possible that we could derive a better understanding of the underlying metabolic processes associated with pancreatic cancer.

Once all discovery phase studies have been completed, validation of the metabolomic profiles will entail testing of samples from an independent patient cohort, preferably consisting of patients undergoing surgery for a pancreatic or periampullary mass, or a biliary stricture. Given the need for further discovery phase studies, it is premature to speculate on the sample size that would be required to validate the metabolomic biomar-

Figure 3. Comparison of the metabolic profiles associated with benign pancreatobiliary disease and pancreatic adenocarcinoma, with diabetic patients excluded. A, cluster scores plot of unsupervised PCA, with benign samples as open circles and malignant samples as black squares. B, cluster scores plot of supervised O-PLS-DA with disease status (colored as in A), and symbols according to age (<50 years, circles; 50–64 years, diamond; >64 years, square). C, histogram demonstrating changes in PLS coefficients of individual metabolites related to disease. Bars to the left (negative) illustrate which metabolites were more abundant in benign disease; those to the right (positive) demonstrate metabolites more abundant in pancreatic cancer. D, histogram demonstrating changes in O-PLS-DA coefficients of individual metabolites related to age. Bars to the left illustrate which metabolites were more abundant in young patients.
kers in this heterogeneous population. Finally, determination of the clinical utility of the diagnostic test will be essential. That is, it will be important to determine how the diagnostic test impacts the clinical management of clinicians. Ideally, this would entail a randomized controlled trial comparing the clinical management in patients who have and have not been tested. To power such a trial, more information will be required to understand the determinants of clinicians’ current management decisions.

In summary, we have demonstrated that it is possible to distinguish the metabolic profile of pancreatic cancer from that associated with benign hepatopancreatobiliary diseases in sera. Our future work will involve the refinement of the metabolic profile obtained, as well as obtaining a more comprehensive profile. Although we have determined internal validity of our findings, further validation in an independent patient cohort will be essential. Ultimately, we will have to determine the clinical utility of the new diagnostic test, determining the effects of the test on clinical decision making.

### Table 2. Features of matched groups—arranged in pairs

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<th>Age, y</th>
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<sup>a</sup>History of jaundice, anicteric at time of collection.

<sup>b</sup>Contained histologic features of mucinous neoplasm.

Figure 4. AUROC distinguishing malignant from benign pancreaticobiliary disease in the data set which excluded diabetic patients.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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