Protein Profiling of Microdissected Pancreas Carcinoma and Identification of HSP27 as a Potential Serum Marker

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Background: Patients with pancreatic adenocarcinomas have a poor prognosis because of late clinical manifestation and the tumor’s aggressive nature. We used proteomic techniques to search for markers of pancreatic carcinoma.

Methods: We performed protein profiling of microdissected cryostat sections of 9 pancreatic adenocarcinomas and 10 healthy pancreatic tissue samples using Protein-Chip technology (surface-enhanced laser desorption/ionization). We identified proteins by use of 2-dimensional gel electrophoresis, peptide fingerprint mapping, and immunodepletion and used immunohistochemistry for in situ localization of the proteins found. We used ELISA to quantify these proteins in preoperative serum samples from 35 patients with pancreatic cancer and 37 healthy individuals.

Results: From among the differentially expressed signals that were detected by ProteinChip technology, we identified 2 proteins, DJ-1 and heat shock protein 27 (HSP27). We then detected HSP27 in sera of patients by use of ELISA, indicating a sensitivity of 100% and a specificity of 84% for the recognition of pancreatic cancer.

Conclusions: The detection of DJ-1 and HSP27 in pure defined tissue and the retrieval of HSP27 in serum by antibody-based methods identifies a potential marker for pancreatic cancer.

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Pancreatic cancer is a formidable challenge in oncology and has the lowest 5-year survival rate (~2%) of any solid cancer (1). Only 10% of patients present with a potentially curable tumor. To gain a chance of combating this cancer type, we must elucidate early tumorigenic processes. In current practice, cancer antigen 19-9 assays and imaging techniques are not optimal for detecting small pancreatic lesions. Improved understanding of DNA/RNA alterations and protein concentrations, in combination with the development of high-throughput, sensitive techniques, could lead to the discovery of a panel of biomarkers that will enable aggressive therapy while tumors are still curable (2).

Surface-enhanced laser desorption/ionization (SELDI)5 is a proteomic high-throughput technique that uses chromatographic surfaces to retain proteins depending on their physicochemical properties, followed by direct analysis via time-of-flight mass spectrometry (MS) (3). This technique requires only a small amount of sample, making it ideal for small biopsies or microdissected tissue (required to produce the homogeneous tissue samples typically used in cancer...
Microdissected tissue material, free of contaminating and unwanted tissue components, is extremely important for producing clean data for biomarker identification in cancer diagnostics and in elucidating clonal heterogeneity of tumors. We were able to show in a previous study that the detection of differentially expressed proteins was possible only in pure microdissected samples (4). In the case of pancreatic cancer, the tumor cells have to be separated from all surrounding tissue constituents. This separation can be done only with an extremely precise technique such as laser-based microdissection. Laser-based microdissection has been combined with ProteinChip technology to identify protein markers in other cancers (5–11).

In this study, we used ProteinChip technology to analyze pure microdissected populations of cells from healthy exocrine pancreatic tissue and the central and peripheral areas of pancreatic adenocarcinomas to detect discriminating specific protein profiles.

### Materials and Methods

#### Laser Microdissection

We obtained 9 pancreatic central tumor areas (pT2/pT3), matched healthy pancreatic samples (n = 10), and 9 pancreatic tumor margins [mean age 61.1 (SD 6.2) years] after surgical resection with informed consent at the Department of General and Visceral Surgery of the Friedrich Schiller University Jena, Germany. The samples were collected fresh, snap-frozen in liquid nitrogen, and stored at −80 °C. We categorized tumor specimens according to their WHO classification.

Laser microdissection was performed with a laser microdissection and pressure catapulting microscope (Palm) as described elsewhere (12). Briefly, we microdissected ~3000 to 5000 cells each on native air-dried, unstained cryostat tissue sections in a maximum of 20 to 30 min. We extracted proteins by incubating with 10 μL lysis buffer (100 mmol/L Na-phosphate, pH 7.5, 5 mmol/L EDTA, 2 mmol/L MgCl2, 3 mmol/L 2-β-mercaptoethanol, 1 mL CHAPS, 500 μmol/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride) for 30 min on ice. After centrifugation (15 min; 13 000g), the supernatant was immediately analyzed or frozen in liquid nitrogen for a maximum of 1 day.

#### Profiling of Tissues

We analyzed the protein lysates from microdissected tissues (central tumor, tumor margin, and healthy tissue) on both strong anion exchange arrays (Q10) and weak cation exchange arrays (CM10; Ciphergen Biosystems) as described (12). We preincubated array spots in a washing/loading buffer containing 100 mmol/L Tris-buffer, pH 8.5, with 0.02% Triton X-100 (for Q10 arrays) and 100 mmol/L Tris-buffer, pH 4.5, with 0.02% Triton X-100 (for CM10 arrays) followed by application of 2 μL sample extract on ProteinChip arrays, which were incubated at room temperature for 90 min in a humidity chamber. After washing 3 times with the same buffer and 2 final washing steps with water, we applied 2 × 0.5 μL sinapinic acid (saturated solution in 0.5% trifluoroacetic acid/50% acetonitrile). We performed mass analysis by use of a ProteinChip Reader (series 4000; Ciphergen) according to an automated data collection protocol. Spectra were normalized with total ion current and cluster analysis of the detected signals. We calculated respective P values for healthy pancreatic tissue and pancreatic carcinoma tissue with CiphergenExpress (version 3.0). We selected normalized spectra with signals between 2.5 and 20 kDa for low range and 20 and 200 kDa for high range, exhibiting a signal-to-noise ratio of at least 10, and analyzed them with the Mann–Whitney U-test for nonparametric data sets.

#### Two-Dimensional Gel Electrophoresis

We prepared samples for 2-dimensional gel electrophoresis (2-DE) directly from surgical material of pancreatic tumor and corresponding healthy pancreatic tissue assessed by a pathologist. Proteins were isolated and 2-DE performed as described (12). In brief, isoelectric focusing was carried out on a Multiphor II (Amersham) using 7-cm immobilized pH gradient strips in a pI interval of 3 to 10. Vertical sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in a Novox MiniGel system (Invitrogen) using 4% to 12% Bis-Tris Zoom™ gel (Invitrogen). The gels were stained with Simply Blue Safe Stain (Enhanced Coomassie; Invitrogen). ProteinChip arrays (Ciphergen). An empty gel piece underwent the same treatment as a control. After addition of 10 μL of a trypsin solution (0.02 g/L; Promega) at 37 °C overnight, we applied supernatants directly on a NP20 ProteinChip array (Ciphergen). An empty gel piece underwent the same treatment as a control. After addition of the matrix (CHCA; Ciphergen), we analyzed peptide fragment masses by use of the ProteinChip reader. The spectra for the peptide-mapping experiments were externally calibrated using 5 proteins including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin β-chain (3495.94 Da). We identified proteins using the fragment masses generated through trypsin digestion by searching in a publicly available database (ProFound; http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

#### In-gel Digestion

We compared protein patterns of the 2-DE gels from healthy pancreatic and tumor tissue and excised consistently differentially expressed proteins as well as ~95 additional spots. In-gel digestion of proteins was performed as described (12). In brief, excised gel pieces were destained and dried. After rehydration and digestion with 10 μL of a trypsin solution (0.02 g/L; Promega) at 37 °C overnight, we applied supernatants directly on a NP20 ProteinChip array (Ciphergen). An empty gel piece underwent the same treatment as a control. After addition of the matrix (CHCA; Ciphergen), we analyzed peptide fragment masses by use of the ProteinChip reader. The spectra for the peptide-mapping experiments were externally calibrated using 5 proteins including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin β-chain (3495.94 Da). We identified proteins using the fragment masses generated through trypsin digestion by searching in a publicly available database (ProFound; http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

#### Immunodepletion Assay

We incubated 2 μL anti-human heat shock protein 27 (HSP27) polyclonal antibody (SPS105P; Acris) or antihuman DJ-1 monoclonal antibody (ab11251; Abcam) with 10 μL protein A-agarose (Sigma-Aldrich) for 15 min on ice. Pellets were generated by centrifugation, and the supernatants were discarded. The pellets were washed...
twice with a buffer containing 20 mmol/L HEPES, pH 7.8, 25 mmol/L KCl, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, and 0.5 mL NP-40. We then incubated 5 μL of a lysate from laser-dissected pancreatic tumor with each pellet for 45 min on ice. We incubated 5 μL of the lysate with protein A-agarose without the specific antibody, as a negative control, for 45 min on ice. After incubation, samples were cleared by centrifugation, and 2 μL of each supernatant was analyzed by use of ProteinChip arrays.

IMMUNOHISTOCHEMISTRY

We placed 8-μm cryostat sections of pancreatic cancer tissue and adjacent healthy tissue on slides, air-dried them for ~60 min at 20 °C, and fixed them in paraformaldehyde as described (11). After fixation, slides were treated in the microwave at 80 watts (3 × 3 min) in 10 mmol/L citric acid, pH 6.0, to inhibit endogenous peroxidase activity. We rinsed them twice with Tris-buffered saline (TBS), pH 7.4, and incubated them overnight at 4 °C in a humidity chamber with a corresponding primary polyclonal antibody against HSP27 (SP5105P; Acris) or a primary antihuman DJ-1 monoclonal antibody (ab11251; Abcam). We rinsed the slides 3 × 10 min in TBS and used the Vectastain Elite ABC reagent set (Vector Laboratories) and the Jenchrom pxbl reagent set (MoBiTec) according to the manufacturer’s instructions to visualize antibody localization. Negative controls were incubated with the labeled secondary antibody only. Sections cut in parallel to the immunohistochemistry (IHC)-treated sections were stained by hematoxylin and eosin for better identification of different tissue areas. IHC staining was evaluated by a pathologist.

QUANTIFICATION OF HSP27 BY ELISA

In addition to the tissue samples, we independently tested a set of serum samples from 35 patients (pancreatic tumor; pT2/pT3), taken before surgery at the Department of General and Visceral Surgery of the Friedrich Schiller University Jena and at the Department of General Surgery, University of Heidelberg. The samples were immediately divided into aliquots and frozen at −80 °C. Serum samples from healthy donors (n = 37) were obtained with the same protocol, divided into aliquots, and frozen at −80 °C. The mean (SD) age for the tumor patients was 61.3 (8.1) years, and for the control volunteer group, 45.6 (15.4) years. The set did not include any sera from patients whose samples were used for ProteinChip array analysis.

We measured serum HSP27 concentrations by use of an appropriate ELISA (Sigma-Aldrich) in duplicate, according to the manufacturer’s instructions. We measured ELISA plates on a microtiter plate reader (MRX II; Dynex Technology) at 450 nm and calculated concentration of the respective protein in serum according to a calibration curve. We calculated P values by 1-sided t-test, constructed ROC curves for HSP27 serum concentration by plotting sensitivity vs 1-specificity, and calculated the areas under the ROC curves.

WESTERN BLOT

Identification of HSP27 and DJ-1 was verified by Western blot. We subjected 30 μg crude extract of pancreatic tumor tissue to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred it to polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with a 1:1000 dilution of anti-HSP27 antibody (SP5105P; Acris) or a 1:1000 dilution of anti-DJ-1 antibody (ab11251; Abcam; diluted in 20 g/L milk powder in TBS containing 05 mL/L Tween 20) and for 3 h with the corresponding secondary antibody. Both HSP27 and DJ-1 were detected by alkaline phosphatase reaction. We estimated band intensities for both proteins by visual inspection.

RESULTS

PROTEIN PROFILING OF CENTRAL PANCREATIC TUMOR, TUMOR MARGIN, AND ADJACENT HEALTHY TISSUE

We excised tissue areas corresponding to ~3000 to 5000 cells per probe by use of laser microdissection and pressure catapulting microscope. In this way, we successfully collected 28 samples in total (10 healthy pancreatic samples, 9 central pancreatic tumors, and 9 tumor margins). All protein lysates from the microdissected tissues were applied to both the Q10 arrays and the CM10 arrays and analyzed on a ProteinChip Reader Series 4000. The SELDI measurements of all tissue samples detected up to 340 peaks in the 2.5- to 200-kDa interval, with normalized intensities. After evaluation with CiphergenExpress, a number of these peaks were found to be significantly different between pancreatic carcinomas and healthy pancreatic tissue samples (Table 1).

IDENTIFICATION OF SIGNALS

To separate protein lysates, we subjected histologically checked pancreatic tumor pieces and biopsies derived

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<th>Table 1. Significantly different signals that separate pancreatic carcinoma and adjacent healthy pancreatic tissue detected on Q10 arrays (anion exchanger) and CM10 arrays (cation exchanger).</th>
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¹ Signals representing DJ-1 and HSP27.
from healthy pancreatic tissue to 2-DE (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4). Numerous protein spots showing differential expression were observed. Approximately 95 protein spots in the interval of 10 to 150 kDa were excised from the gels, and we analyzed peptide fingerprints of the tryptic-digested spots by use of SELDI time-of-flight MS. In this way, we were able to identify 29 proteins by database searching (ProFound; http://prowl.rockefeller.edu/prowl/cgi/profound.exe; see Table 1 in the online Data Supplement).

One of these identified proteins, DJ-1 (see Fig. 1, spot 2, in the online Data Supplement), matched well in molecular mass with a significantly differentially expressed signal detected in prior protein profiling using SELDI. This signal of 20 kDa was detected on Q10 arrays and showed an increased expression in samples derived from pancreatic tumor. Presence of the spot discriminated significantly between central pancreatic carcinoma and tumor margin and healthy adjacent pancreatic tissue ($P = 3.66 \times 10^{-2}$) as well as between pancreatic carcinoma and healthy pancreatic tissue ($2.08 \times 10^{-2}$). Another significantly different signal possessing an $m/z$ of nearly 23 kDa matched well to a protein identified as HSP27 (see Fig. 1, spot 13, in the online Data Supplement). This significantly different signal was up-regulated in pancreatic carcinoma tissue compared with healthy pancreatic tissue ($2.68 \times 10^{-2}$) as detected on CM10 arrays in prior protein profiling.

We double-checked that DJ-1 and HSP27 match the differentially expressed peaks at 19.9 and 22.7 kDa by use of ProteinChip analysis with immunodeplete assays, using microdissected pancreas carcinoma tissue as starting material. Analyses showed that the peaks corresponding to DJ-1 and HSP27 were reduced. In the negative control without the specific antibody, these peaks were clearly detectable (Fig. 1).

CHARACTERIZATION BY IMMUNOHISTOCHEMISTRY AND WESTERN BLOT
To further characterize the identified markers and to localize DJ-1 and HSP27 in tissue sections, we examined their expression in several pancreatic tissue samples by immunohistochemistry using specific antibodies against DJ-1 and HSP27. Negative controls without the primary antibody or without any antibody had no signal. Both healthy pancreatic cells and malignant tumor cells demonstrated cytoplasmic signals for DJ-1 and HSP27 in all tissue samples examined, but in every case with higher signal intensities in tumor cells (Fig. 2). Quantitative differences between the expression of these interesting proteins in healthy pancreatic cells and malignant tumor cells were as clear as in ProteinChip array results. Furthermore, we carried out IHC assays to a number of additional proteins, including PEBP, cystatin B, and cyclophilin A (see Fig. 2 in the online Data Supplement).

To further confirm that the localized DJ-1 and HSP27 are identical to the peaks found by ProteinChip analysis, areas of similar size from tumorous and healthy tissue that were previously analyzed in IHC were obtained by tissue laser microdissection. In protein lysates from the pancreatic tumor fraction, we detected signals identical in mass to the interesting peaks obtained with the initial SELDI-MS analysis on proper arrays (Q10 and CM10). In the protein lysate from IHC-negative areas, these peaks were absent (Fig. 3). We also examined by Western blot the expression levels of DJ-1 and HSP27 in lysates derived from an independent set of pancreatic tissue specimens (see Fig. 3 in the online Data Supplement). We detected strong signals corresponding to DJ-1 and HSP27 in the majority of analyzed probes.

ANALYSES OF HSP27 IN SERUM
In addition to the tissue samples, we quantified HSP27 in a sample set of sera derived from pancreatic cancer patients and controls ($n = 72$) by use of an independent ELISA analysis. The concentration of HSP27 in serum from tumor patients was found to be significantly higher.
than in serum from controls (P < 0.001). The median for controls was 0.76 μg/L, and for patients with pancreatic carcinoma, 2.93 μg/L (Fig. 4). We constructed ROC curves for HSP27 serum concentration, resulting in a sensitivity of 100% at a specificity of 84% and a cutoff of 1.33 μg/L. The area under the ROC curve was calculated as 0.985 (Fig. 4).

Discussion
Despite enormous efforts, relevant markers useful for screening have been established in only a few tumor types (13, 14), and no studies have found markers for early detection of pancreatic cancer (15–19). 2-DE, especially in combination with microdissection, seems an appropriate tool (19), but proteins in the peptide interval, as well as those of high hydrophobicity or of extreme isoelectric points, are difficult to separate and hence are typically neglected, resulting in a loss of potentially interesting proteins. Additionally, the sensitivity is low compared with MS.

In this study, we used protein-profiling SELDI MS and 2-DE to find biomarkers that might enable earlier tumor detection. Only a small number of protein-profiling studies in pancreas tumor have so far been performed using SELDI technology (20–22). Our study improves on this approach by using samples of pure microdissected cells derived from central pancreatic tumor areas, tumor margin, and adjacent healthy tissue. We detected a small number of signals that discriminated well between the 3 sample groups. For identification of these signals, we separated histologically checked tissue specimens from pancreatic tumors and healthy pancreatic tissue using 2-DE followed by analysis of peptide mass fingerprints using SELDI MS. We excised 95 protein spots from those that were obviously different in 2-DE gels and, using the methodology described in Melle et al. (23), identified 29 proteins unequivocally. Two proteins identified in this
manner, DJ-1 and HSP27, matched well to different signals found in prior protein-profiling assays. Both proteins were up-regulated in pancreatic cancer and discriminated well between central tumor and tumor margin vs healthy tissue and between central tumor and healthy tissue. We confirmed the identities of both proteins in immunodepletion assays and further characterized them by immunological techniques. In an independent set of pancreatic tumor tissue specimens that had not been assessed earlier by ProteinChip technology, we also detected strong signals corresponding to DJ-1 and HSP27 by Western blot analysis. We also analyzed the identified HSP27 specifically in serum by a corresponding ELISA, with strong signals corresponding to DJ-1 and HSP27 by Western blot analysis. We also confirmed the identities of both proteins in immuno-depletion assays and further characterized them by immunological techniques. In an independent set of pancreatic tumor tissue specimens that had not been assessed earlier by ProteinChip technology, we also detected strong signals corresponding to DJ-1 and HSP27 by Western blot analysis. We also analyzed the identified HSP27 specifically in serum by a corresponding ELISA, with exactly the same results for HSP27 as found in prior protein profiling.

Our evidence suggests that the concentration of DJ-1 is increased in pancreatic carcinomas and that this increase distinguishes pancreatic tumor tissue from adjacent healthy tissue. DJ-1 is a conserved protein, coded by the gene PARK7 (Parkinson disease 7), that is reported to be involved in diverse cellular processes including cellular transformation, control of protein–RNA interaction, oxidative stress response, and control of male infertility. The PARK7 gene is associated with an autosomal recessive, early onset Parkinson disease (24). Recent reports show that PARK7 is overexpressed in a number of cancer types, including breast, lung, and prostate. It partly obtains its transforming activity by an RNA helicase named Abstrakt (25, 26). In primary breast cancer samples, DJ-1 negatively regulates the PTEN tumor suppressor and thus produces overexpressed hyperphosphorylation of PKB/Akt and increased cell survival (27). In a proteomic analysis of gastric cancer, DJ-1 was detectable only in metastatic tumor tissue vs nonmetastatic tumor tissue and healthy gastric tissue (28). Based on this fact, it seems likely that DJ-1 contributes to the metastatic potential of a tumor.

HSP27 is a powerful molecular chaperone whose main function is to prevent the aggregation of nascent and stress-accumulated misfolded proteins. It is able to interact directly with various components of the tightly regulated programmed cell death machinery, upstream and downstream of the mitochondrial events, and seems to play a role in the proteasome-mediated degradation of selected proteins. HSP27 is associated with poor prognosis in gastric, liver, and prostate carcinoma and osteosarcomas (29, 30). Data concerning the prognosis potential of HSP27 in the above cancer types are conflicting because a recent study showed that in gastric cancer, HSP27 was not detectable in metastatic tumors and could be found only in samples derived from nonmetastatic tumors (28). To date, only a few studies are available that report an association of differential expression of HSP27 and pancreatic carcinoma, and the results of these investigations are partly conflicting (31, 32).

Whereas our study identified DJ-1 and HSP27 as potential new biomarkers for the early detection of pancreatic cancer, further studies with larger sample sizes using cancerous and healthy tissue acquired by noninvasive sampling methods are required.

In conclusion, we show that a proteomic procedure composing tissue microdissection, protein profiling by ProteinChip technology, separation and identification of interesting proteins by 2-DE, peptide mass fingerprinting, and SELDI MS as well as confirmation of these proteins using immunological techniques is able to identify and characterize differentially expressed proteins that could serum markers for pancreatic carcinoma. The clinical relevance of these findings will require further study.

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