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Identification of Disease-specific Genes in Chronic Pancreatitis Using DNA Array Technology

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Objective
To use DNA arrays to analyze the differential gene expression patterns in the normal pancreas and in pancreatic diseases.

Summary Background Data
Genome-wide gene expression analysis will provide new insights into gene function and cause of disease.

Methods
RNA was extracted from eight normal pancreatic specimens, eight specimens with chronic pancreatitis (CP), and eight pancreatic cancer (PCa) tissues. Poly A(+) RNA was purified, reverse-transcribed, and converted into cRNA using biotinylated nucleotides. The HuGeneFL DNA array containing 5,600 full-length human genes was used for analysis.

Results
First, normal pancreatic tissues were analyzed in comparison with a panel of other normal tissues (colon, liver, prostate, lung, lymph node). This analysis revealed 11 signature genes that were selectively expressed in the pancreas (e.g., pancreatic elastase-IIA). Comparison of the expression of 5,600 genes between the normal pancreas, CP, and PCa specimens showed that the expression of 34 genes was decreased in CP tissues compared with normal pancreatic tissues, and that the expression of all of these genes was simultaneously decreased in PCa. In addition, the expression of 157 genes was increased in CP tissues compared with the normal pancreas. Of those, 152 genes were simultaneously increased in PCa. Thus, only 5 of 5,600 genes were significantly overexpressed in CP compared with both normal pancreas and PCa.

Conclusions
The majority of alterations observed in CP are present in PCa, and the number of genes whose expression is selectively deregulated in CP is surprisingly small. These results may provide new insight into the pathobiology of CP and help identify certain molecular alterations that might serve as targets for new diagnostic tools and disease-specific therapy.

Chronic pancreatitis (CP) is a long-lasting inflammatory disease of the pancreas characterized by irreversible and progressive destruction of the whole organ, resulting in severe exocrine and endocrine insufficiency.[1,2] Heavy alcohol consumption is the main etiologic factor in Western industrialized countries.[2–4] However, less common causes, including nutritional factors, gene mutations (hereditary CP), metabolic disturbances, congenital anomalies of the ductal system (pancreas divisum), and acquired pancreatic duct obstructions, have also been shown to be etiologic factors in CP.[2,3,5,6] Despite our better understanding and characterization of the underlying etiology in recent years, in approximately 15% of the patients the etiology of CP is unknown (idiopathic CP).[4,7]

The morphologic changes of CP are well described by histopathologic analysis. They include acinar cell degeneration, dilatation of the duct system with or without intraductal protein plugs and stones, necrosis, and replacement

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of the lost parenchyma by dense fibrous tissue. The destruction of the pancreatic parenchyma is accompanied by infiltrating leukocytes, followed by tissue remodeling, with ductal cell proliferation and ductular hyperplasia.

Although the etiologic factors and the morphologic changes in CP have been more clearly identified in the past decade, the pathogenetic mechanisms of CP (with the exception of hereditary CP) remain enigmatic, and none of the recently proposed pathophysiologic concepts[3,5,8] can conclusively explain the morphologic, functional, and clinical picture of the disease. Our knowledge about the mechanisms and the time course of tissue destruction and remodeling in CP is still limited by the fact that tissue specimens for detailed analysis are mainly available from patients with advanced disease, in whom surgical treatment is normally performed.

In recent years, modern molecular biology techniques have provided important clues to clarify the morphologic changes and pathophysiologic aspects of CP. Thus, several genetic and epigenetic changes have been observed in CP, including *K-RAS* mutations[9] and altered expression patterns of several mitogenic growth factors and their receptors.[10] In addition, in the past few years there has been growing evidence of the involvement of the immune system and its signaling components (e.g., chemokines) in the pathophysiology of CP.[8,11–13] Further, genetic studies have expanded our knowledge of another important topic, the relationship between CP and pancreatic adenocarcinoma.[14–16] A deeper understanding of the biologic and molecular mechanisms underlying CP could help in the development of new diagnostic and therapeutic strategies in this disorder. However, understanding and evaluating biologic systems with approximately 30,000 to 40,000 biologically active genes,[17,18] such as a human cell, requires new technologies; the traditional gene-by-gene or protein-by-protein approach is not sufficient to meet the magnitude of the task. DNA arrays represent a first step forward, providing a systematic way to survey concomitant RNA expression of a large number of genes. Therefore, for the first time in the pancreas, we analyzed alterations in gene expression patterns in chronic pancreatitis compared with both the normal pancreas and pancreatic cancer (PCa), using the powerful DNA array technology to assess simultaneously the expression of 5,600 human genes.

METHODS

Patients and Tissue Sampling

Eight CP tissue samples were obtained from two male and six female patients (median age 46 years; range 38–51 years). All individuals had alcohol-induced, histologically confirmed CP. Eight PCa tissue samples were obtained from three male and five female patients (median age 63 years; range 43–80 years). According to the Union Internationale Contre le Cancer (UICC) classification, there were two stage 2, five stage 3, and one stage 4 duct cell adenocarcinomas. All individuals had histologically confirmed pancreatic ductal adenocarcinoma. In addition, normal human tissue samples were obtained through an organ donor program from eight previously healthy individuals (five male donors, three female donors; median age 50 years; range 37–69 years).

Freshly removed tissue samples were snap-frozen in liquid nitrogen immediately on surgical removal and maintained at −80°C until use. Histology was confirmed by frozen section in all cases. The studies were approved by the Human Subjects Committee at the University of Bern, Switzerland.

DNA Arrays and Designs

The HuGeneFL DNA array used in this study was fabricated by Affymetrix Inc. (Santa Clara, CA). It contained about 7,000 sequences representing 5,600 full-length human genes. The sequences were selected from three database exemplars from Unigene supplemented with additional genes from GenBank and the Institute for Genomic Research (Rockville, MD).

Transcript Profiling Using DNA Arrays

Total RNA was extracted by the guanidine isothiocyanate method from snap-frozen human tissues. Poly $(A)^+$ RNA was isolated using Oligo(dT)-cellulose kits from Amersham Pharmacia Biotech (Piscataway, NJ). Two and a half micrograms of poly $(A)^+$ RNA was converted into double-stranded cDNA by reverse transcription (GIBCO BRL Life Technologies, Grand Island, NY) using the T7-T24 primer (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG (dT_{24})). The double-strand cDNA product was cleaned up by phenol/chloroform/isoamyl extraction using phase lock gels (Eppendorf, Westbury, NY). Double-stranded cDNA was converted into cRNA using the in vitro transcription MEGAscript T7 kit from Ambion (Austin, TX) and biotinylated nucleotides as described.[19] The in vitro transcription product was purified using RNeasy mini columns from Qiagen (Valencia, CA) and fragmented as described.[19] Hybridization of fragmented in vitro transcription product to oligonucleotide arrays was performed as suggested by the manufacturer (Affymetrix). All 24 pancreatic tissue samples were subjected to RNA extraction and transcript profiling.

Computational Analysis of Data

Primary analysis of array data was performed using the Affymetrix GeneChip software. Differential expression was defined as a change of at least threefold versus respective controls.

Probe Synthesis

For further analysis of the CP-specific genes, probes were generated for subsequent Northern blot analysis. The COMP cDNA probe consisted of a 209-base pair fragment of human COMP cDNA (GenBank accession number L32137). The CRISP-3 cDNA probe consisted of a 171-base pair fragment of human CRISP-3 cDNA (GenBank accession number X95240). Probes were subcloned into the pGEMT-easy vector (Promega Biotechnology, Madison, WI). A 190-base pair fragment of the mouse 7S cytoplasmic cDNA that cross-hybridizes with human 7S RNA was used to verify equivalent RNA loading in the Northern blot experiments.[20,21] For Northern blot analysis, cDNA probes were labeled with α-^{32}P-dCTP using a random primer labeling system (Roche Diagnostics, Rotkreuz, Switzerland).

Northern Blot Analysis

To verify the results obtained by DNA array expression analysis, Northern blot analysis was carried out. To this end, total RNA was extracted by the guanidine isothiocyanate method, size fractionated on 1.2% agarose/1.8 mol/L formaldehyde gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalency.[20,21] The RNA was capillary transferred onto nylon membranes and cross-linked by ultraviolet irradiation. Prehybridization, hybridization, and washing were performed under conditions appropriate for cDNA probes, as previously reported in detail.[20,21] Membranes were then exposed at −80°C to Kodak BioMax x-ray films using intensifying screens for 1 to 2 days. All membranes were rehybridized with the 7S cDNA probe to access equivalent RNA loading, as previously reported.[20,21] Densitometric analysis of autoradiographs was performed using the Image Pro plus program (Media Cybernetics, Silver Spring, MD). Relative mRNA expression levels were calculated for each sample and normalized to the corresponding 7S signal.

Statistical Analysis

Expression data from DNA array and Northern blot analysis are calculated as mean ± SEM unless indicated otherwise. For statistical analysis, the Student *t* test was used. Significance was defined as $P < .05$.

RESULTS

We first analyzed normal human pancreatic tissues in comparison with a panel of other normal tissues (colon, liver, prostate, lung, lymph node). This analysis revealed 11 signature genes that were selectively expressed in the pancreas but not in the other normal human tissues. These signature genes were triglyceride lipase, microsomal dipeptidase, pancreatic elastase IIA, pancreatic secretory trypsin inhibitor, pancreatic lipase-related protein 2, MAP kinase-activated protein kinase, protein disulfide isomerase, pancreatic zymogen granule membrane protein GP-2, Na,K-ATPase gamma subunit, carboxyl ester lipase, and procarboxypeptidase A1 (Table 1). This analysis confirmed the feasibility of our approach by identifying 11 pancreas-specific genes out of a panel of 5,600 genes.

Table 1. IDENTIFICATION OF PANCREATIC SIGNATURE GENES

Gene	GenBank Accession Number
Triglyceride lipase	J05125
Microsomal dipeptidase	J05257
Pancreatic elastase IIA	M16652
Pancreatic secretory trypsin inhibitor	M20530
Pancreatic lipase-related protein 2	M93284
MAP kinase-activated protein kinase 2	U12779
Protein disulfide isomerase (PDIp)	U19948
Pancreatic zymogen granule membrane protein	U36221
Na,K-ATPase gamma subunit	U50743
Carboxyl ester lipase	X54457
Procarboxypeptidase A1	X67318

Comparison of the expression of 5,600 genes between the normal pancreas (n = 8) and CP (n = 8) as well as PCa tissues (n = 8) showed that the expression of 157 genes (footnote 1) was increased in CP compared with the normal pancreas (Fig. 1, 2). Of those, 152 genes were simultaneously increased in PCa tissues. In addition, the expression of 34 genes (footnote 2) was decreased in CP compared with normal pancreatic tissues (see Fig. 2), and the expression of all of these genes was simultaneously decreased in PCa tissues. Thus, there was altered expression of 3.4% (191/5,600) of the analyzed genes in CP versus the normal pancreas.

Next, we carried out further analysis of the genes whose expression was increased or decreased in CP. To this end, genes were grouped according to their function and their involvement in certain pathways. As summarized in Table 2, genes overexpressed in CP belonged to a number of different groups. The first group included cell surface receptors, growth factors, cytokines, and transcription factors. Exemplary genes whose expression was increased in CP are shown in Table 2. Another major group of genes overexpressed in CP included genes encoding for proteins involved in cellular adhesion, extracellular matrix formation, and integral membrane composition. The third large group of genes included those coding for proteins involved in immunity and defense mechanisms such as antigen processing, presentation, and recognition. Further, there were a number of other genes overexpressed in CP, including those involved in protein metabolism, modification, and maintenance, DNA/RNA metabolism, and carbohydrate metabolism.

Table 3 summarizes genes whose expression was decreased in CP compared with the normal pancreas. The

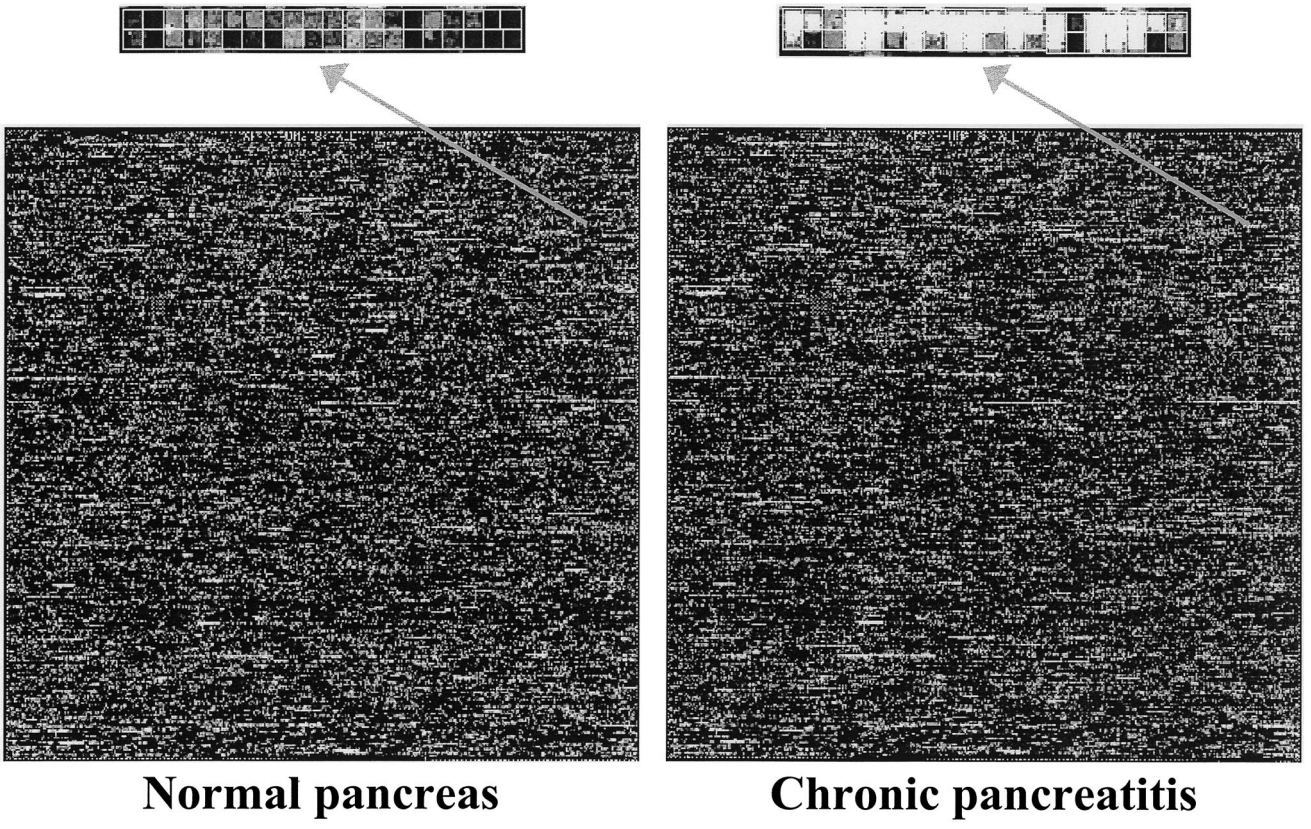

Figure 1. Example of a DNA array probed with RNA isolated from a normal pancreatic specimen (left panel) and a chronic pancreatitis specimen (right panel). Magnifications of corresponding areas are shown on the top. The depicted gene is SPARC/osteonectin, which exhibited an average 51-fold increase in chronic pancreatitis compared with normal tissues when all eight samples were compared.

largest groups included genes involved in growth, development, differentiation, and proliferation; genes encoding for proteins of the extracellular matrix; integral membrane proteins; and structural proteins. Again, there were several other genes whose expression was decreased in CP, including those involved in protein metabolism, DNA/RNA metabolism, and carbohydrate metabolism, as well as several enzymes.

Interestingly, 5 of 5,600 genes were significantly overexpressed in CP compared with both normal pancreas and PCa (Table 4).

Mucin-6

Mucins belong to a family of large glycoproteins that are thought to play a major role in protecting the gastrointestinal tract from acid, proteases, pathogenic microorganisms, and mechanical trauma. Expression of mucin-6 has been shown in the developing pancreas.[22,23] In addition, mucin-6 protein has been detected in material obstructing small pancreatic ducts in patients with cystic fibrosis.[24] In the present study, we found 10.9 ± 5.1-fold increased expression levels of mucin-6 in CP compared with the normal pancreas and a 3.3 ± 1.5-fold increase when compared with PCa.

Germline Oligomeric Matrix Protein

Germline oligomeric matrix protein (COMP) is expressed at high levels in the matrix of chondrocytes. Mutations of the COMP gene have been observed in patients with pseudoachondroplasia and one form of multiple epiphyseal dysplasia.[25–30] In the present study we found a 124.9 ± 69.0-fold increase of COMP mRNA expression levels in CP tissues compared with the normal pancreas and a 2.9 ± 1.6-fold increase compared with PCa. These results were confirmed by Northern blot analysis, which showed a 63 ± 25.8-fold increase of COMP mRNA expression levels in CP tissues compared with the normal pancreas, and a 4.4 ± 1.8-fold increase compared with PCa tissue specimens (Fig. 3). The COMP mRNA transcript size of approximately 2.8 kb was in agreement with previous reports.[31]

TPSB1

Tryptase is a serine protease that is selectively concentrated in the secretory granules of mast cells and is secreted during the coupled activation–degranulation response of these cells. DNA arrays revealed increased expression of

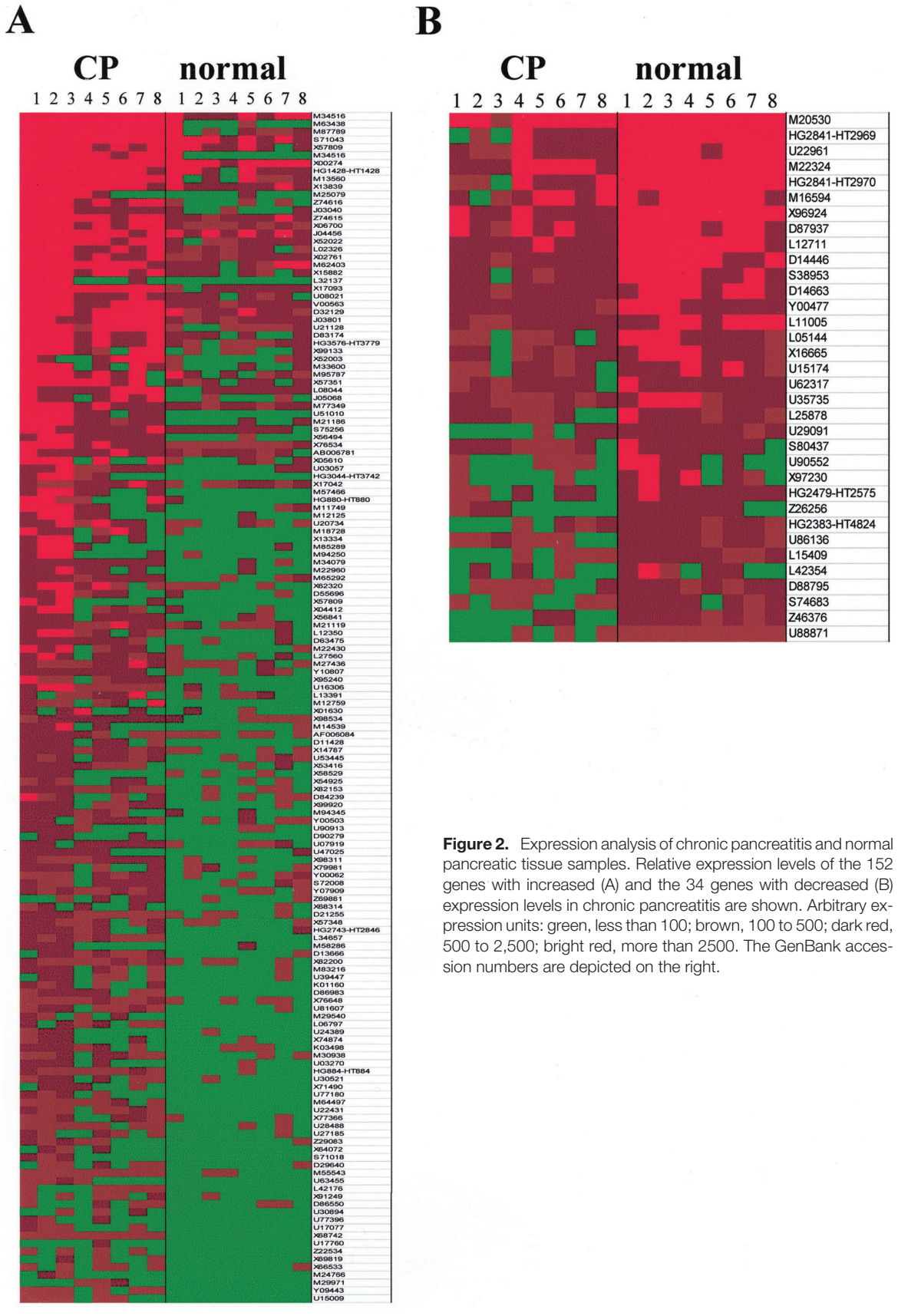

Figure 2. Expression analysis of chronic pancreatitis and normal pancreatic tissue samples. Relative expression levels of the 152 genes with increased (A) and the 34 genes with decreased (B) expression levels in chronic pancreatitis are shown. Arbitrary expression units: green, less than 100; brown, 100 to 500; dark red, 500 to 2,500; bright red, more than 2500. The GenBank accession numbers are depicted on the right.

Table 2. GENES EXPRESSED AT INCREASED LEVELS IN CHRONIC PANCREATITIS SUMMARIZED IN GROUPS ACCORDING TO THEIR FUNCTION

Function/Pathway	No. of Genes	Subgroup	Exemplary Gene	GenBank Accession Number
Signal transduction and regulation	25	Cell surface receptors	tumor necrosis factor receptor	M58286
		Growth factors	epithelin 1	X62320
		Cytokines	macrophage inflammatory protein 3 beta	U77180
		Transcription factors	helix-loop-helix basic phosphoprotein (G0S8)	L13391
Growth and development, differentiation and proliferation	13		insulin-like growth factor binding protein 5	L27560
Enzyme: oxidoreductases	6		lysyl oxidase-like protein gene	U24389
Enzyme: transferases	10		6-O-methylguanine-DNA methyltransferase	M29971
Enzyme: hydrolases	12		synovial phospholipase A-2	M22430
Enzyme: miscellaneous	4		cyclophilin C	S71018
Transporters and pumps	6		vacuolar proton ATPase, subunit D	X71490
Protein modification and maintenance	7	Proteases, chaperones	type I interstitial collagenase	X54925
Protein metabolism	8		collagen binding protein 2	D83174
Carbon and carbohydrate metabolism	8		M1-type and M2-type pyruvate kinase	X56494
Nucleic acid synthesis and modification	3		5′ nucleotidase	X55740
DNA/RNA metabolism	8		DNA-binding protein Ku (p70/p80)	M30938
Cellular adhesion proteins	30	Integrins, MHC	integrin, alpha subunit	X68742
Extracellular matrix proteins	38	Collagens, fibronectins	collagen alpha 1(V) chain	D90279
Integral membrane proteins	37		PMP-22 glycoprotein	D11428
Localized and structural proteins, cytoskeleton, microfilaments	14	Actins	vascular smooth muscle alpha-actin	X13839
Localized and structural proteins, organelle, nucleus	18		nucleolar protein p40	U86602
Kinesis, actin-based motility	5		fibroblast muscle-type tropomyosin	M12125
Drug and xenobiotic metabolism	2		nicotinamide N-methyltransferase	U08021
Environmental responses, wound healing	9		thrombospondin 2	L12350
Immunity and defense	38	Antigen processing and presentation	HLA class-I (HLA-A26) heavy chain	D32129
		Antigen recognition	IgG Fc binding protein	D84239

The total number of genes is larger than 157 because some genes participate in more than one pathway group.

tryptase III in CP compared with both the normal pancreas (11.1 ± 4.1-fold) and PCa (8.3 ± 3.3-fold).

Rearranged Immunoglobulin Lambda Light Chain

Six subgroups of lambda L chains are recognized; they are presumably determined by separate but closely linked loci. The light chains are encoded by three gene segments, V(L), J(L), and C(L), which are separated in the genomes of cells undifferentiated with regard to antibody gene expression. In the present study we found 26.2 ± 10.8-fold increased expression levels of the rearranged immunoglobulin lambda light chain in CP compared with the normal pancreas and a 5.4 ± 2.2-fold increase compared with PCa.

Cysteine-Rich Secretory Protein-3

Cysteine-rich secretory protein-3 (CRISP-3) belongs to a family of secreted glycoproteins. Its expression has been shown in the salivary gland, pancreas, and prostate and in less abundance in the epididymis, ovary, thymus, and colon.[32] Using DNA arrays, we showed increased expression of CRISP-3 in CP compared with both the normal pancreas (21 ± 6.5-fold) and PCa (4.1 ± 1.3-fold). These results were confirmed by Northern blot analysis of CRISP-3 expression in CP, PCa, and normal pancreatic tissues. This analysis revealed a 37 ± 12.5-fold increase of CRISP-3 mRNA levels in CP tissues compared with the normal pancreas and a 5.4 ± 1.8-fold increase compared with PCa tissue specimens (see Fig. 3). The transcript size of CRISP-3 mRNA (approximately 2.2 kb) was consistent with the known length of the full-length CRISP-3 cDNA (2,124 base pairs).[33]

Table 3. GENES EXPRESSED AT DECREASED LEVELS IN CHRONIC PANCREATITIS SUMMARIZED IN GROUPS ACCORDING TO THEIR FUNCTION

Function/Pathway	No. of Genes	Exemplary Gene	GenBank Accession Number
Signal transduction and regulation	1	HOX2H	X16665
Growth and development, differentiation and proliferation	5	von Hippel-Lindau disease tumor suppressor	L15409
Enzyme: oxidoreductases	3	aldehyde oxidase (hAOX)	L11005
Enzyme: transferases	4	transketolase (tk)	L12711
Enzyme: hydrolases	3	aminopeptidase N/CD13	M22324
Enzyme: miscellaneous	2	phosphoenolpyruvate carboxykinase (PCK1)	L05144
Transporters and pumps	3	L-type calcium channel	Z26256
Protein modification and maintenance	3	bone marrow serine protease gene (medullasin)	Y00477
Protein metabolism	3	pancreatic secretory trypsin inhibitor (PSTI)	M20530
Carbon and carbohydrate metabolism	4	hexokinase II	Z46376
DNA/RNA metabolism	2	telomerase-associated protein TP-1	U86136
Adhesion and molecular recognition	1	butyrophilin (BTF5)	U90552
Extracellular matrix proteins	5	HFREP-1	D14446
Integral membrane proteins	5	RACH1	U35735
Structural proteins	7	mitochondrial citrate transport protein	X96924
Kinesis, actin-based motility	2	HsPex7p (HsPEX7)	U88871
Drug and xenobiotic metabolism	2	p33/HEH epoxide hydrolase (EPHX)	L25878

Note that the total number of genes is larger than 34 since some genes participate in more than one pathway group.

DISCUSSION

The pathogenesis of CP is still not completely understood. Long-term alcohol intake is known to be the most prominent etiologic factor in the Western world, but its mechanisms of action have not been clearly elucidated. However, recent advances in cell and molecular biology have revealed complex interactions between inflammatory cells and pancreatic parenchymal cells, which can explain various pathophysiologic characteristics of CP, such as site of action and modalities of activation of inflammatory cells, mechanisms of tissue destruction and remodeling, and pain generation.[8] Further, enhanced expression of a variety of growth factors and growth factor receptors is present in most CP samples.[10] These factors are most likely produced by the remaining pancreatic acinar and ductal cells, and thus might directly influence the morphologic changes that occur in CP. These factors also have the potential to recruit and stimulate inflammatory cells, which in turn can induce the expression of growth factors in pancreatic acinar and ductal cells. Current research is designed to shed light on the complicated network of factors and cells that influence each other in the pathogenesis of this disease. In addition, recent

Table 4. GENES SIGNIFICANTLY OVEREXPRESSED IN CHRONIC PANCREATITIS COMPARED WITH BOTH THE NORMAL PANCREAS AND PANCREATIC CANCER

		fold increase									
		CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	all CP	all CP
		versus									
Gene	GenBank Accession Number	normal	normal	normal	normal	normal	normal	normal	normal	normal	cancer
MUC6	L07517	26.8	25.4	0.8	1.4	0.3	0.3	0.3	32.6	10.9	3.3
COMP	L32137	145.5	320.9	516.8	1.0	1.0	1.0	11.6	1.0	124.9	2.9
TPSB1	M33493	19.8	13.9	33.4	2.5	2.0	13.5	0.1	3.5	11.1	8.3
Rearranged Ig-lambda light chain	X57809	52.1	38.0	81.7	1.0	1.0	1.0	1.0	33.9	26.2	5.4
CRISP-3	X95240	51.7	8.7	3.1	1.0	8.0	31.7	36.5	27.1	21.0	4.1

Data are calculated as mean.
CP, chronic pancreatitis.

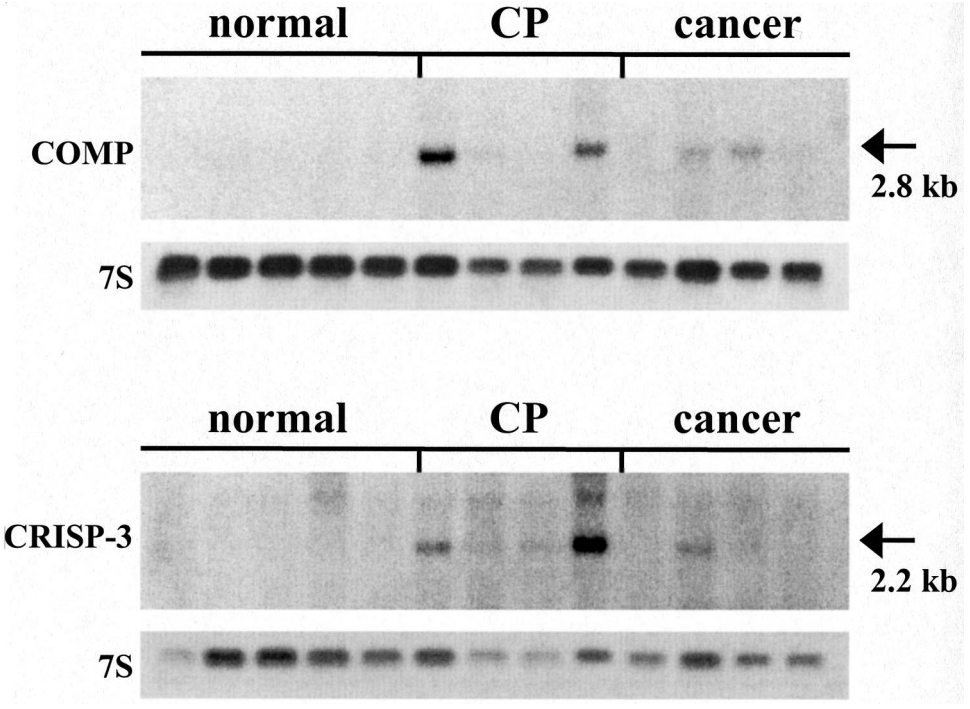

Figure 3. Northern blot analysis of germline oligomeric matrix protein (COMP) (A) and cysteine-rich secretory protein-3 (CRISP-3) (B) mRNA in pancreatic tissue samples obtained from normal controls (normal) and chronic pancreatitis and pancreatic cancer tissues. Total RNA (20 μg) was subjected to Northern blot analysis and probed with the ^{32}P-labeled COMP and CRISP-3 cDNA, respectively. The blot was subsequently rehybridized with a 7S cDNA probe to verify equivalent RNA loading. The approximate sizes of the COMP and CRISP-3 mRNA transcripts are indicated on the right.

genetic studies have led to the identification of various genes that can cause or at least lower the threshold for the development of CP.

DNA array expression analysis is a powerful new technology that allows the simultaneous analysis of the expression of multiple genes. Today, the main application of DNA arrays is expression analysis, such as comparative expression profiling of different tissues (e.g., diseased vs. normal tissue). Currently, the vast number of available DNA arrays are designed for expression analysis. Comparative expression profiling has been performed in several different settings. One of the main points of interest is the analysis of genes involved in carcinogenesis. Up to now, several different human tumors, such as ovarian cancer,[34] lung cancer,[35,36] breast cancer,[37–39] colon cancer,[40] prostate cancer,[41–43] rhabdomyosarcoma,[44] melanoma,[45] and head and neck cancers,[46,47] have been investigated by DNA array analysis to identify cancer-specific genes, genes involved in metastasis formation,[48] and genes that regulate the response toward chemotherapy.[49] The present study is the first, to our knowledge, that investigates differential expression using DNA arrays in a chronic inflammatory disorder: CP. Our results reveal that the expression of a vast number of human genes is deregulated in CP (approximately 3.4% of the analyzed 5,600 genes). However, the present analysis used RNA extracted from whole tissue specimens, including several different cell types (e.g., acinar cells, ductal cells, islet cells, inflammatory cells). In the future, it would be interesting to obtain microdissected tissue sections to compare expression profiles of different cellular subtypes in CP (e.g., normal ducts, proliferating ducts, hyperplastic ducts). However, at present, current protocols for fluorescent labeling of RNA/cDNA demand large quantities of RNA, which impedes the use of microdissected RNA on DNA arrays. In addition, in the case of the pancreas, the isolation of high-quality RNA from microdissected tissue compartments is extremely demanding because of the high RNAse content of the pancreas, and has up to now not been reported. However, future innovations will likely overcome these problems.

Further, CP (as well as PCa) is a complex disease, and therefore each patient may have a specific makeup of alterations. In the present study we investigated eight tissue samples in each group. Obviously, a larger panel of tissues would be helpful to delineate characteristic changes and correlate those with clinic pathologic parameters. In addition, the real sensitivity of the DNA array approach is not known, nor is it known to what extent several important changes that occur in CP are represented by this approach (e.g., expression of certain genes by acinar cells, islet cells, and inflammatory cells). Nonetheless, our results point to the complex epigenetic alterations in CP and help to identify those genes whose further analysis in this disease is promising. Another important conclusion of the present analysis is that almost all changes in the expression of genes (186/191 genes) observed in CP are also present in PCa. Chronic pancreatitis is considered a risk factor for the development of pancreatic adenocarcinoma, mainly on the basis of epidemiologic evidence,[50,51] but the underlying biologic mechanisms are still poorly understood. So far, neither morphologic[52] nor molecular genetic studies have convincingly shown a progression from CP to PCa. However, overexpression of a variety of growth factors and their receptors that are present in CP[10] has also been reported at higher

levels for PCa,[53] suggesting that molecular alterations in CP might act as precancerous factors under certain circumstances. Our results of concomitant changes observed in CP and PCa might be in agreement with the assumption that CP is a risk factor for PCa. Alternatively, these observations could be explained, at least in part, by the presence of CP-like changes in almost all PCa samples, which might result in similar expression patterns in these two disorders. Thus, the correlation between CP and PCa may simply reflect a pathologic pancreas rather than linked pathologic pathways.

Our results also indicate that the number of key genes whose expression is deregulated in CP compared with both the normal pancreas and PCa is surprisingly small. We could identify five genes whose expression was significantly increased in CP. Mucin-6, a large glycoprotein, has been identified in the developing pancreas and in material in small pancreatic ducts in patients with cystic fibrosis and concomitant CP.[24] The role of this gene in the pathophysiology of alcoholic CP is currently completely unknown, and further studies are needed to address this topic. Germline oligomeric matrix protein has been implicated in the pathogenesis of two hereditary disorders of the skeletal system (pseudoachondroplasia and one form of multiple epiphyseal dysplasia).[25–30] Preliminary studies have shown COMP expression in degenerating acinar cells in CP and in CP-like lesions next to PCa but not in the cancer cells themselves (unpublished observation). It will be interesting to evaluate the expression and function of COMP in CP. Tryptase is a protease that is selectively concentrated in the secretory granules of mast cells, and these cells are increased in chronic inflammatory processes with fibrous tissue deposition. Recently, we have shown that the total number of mast cells is significantly higher in CP than in the normal pancreas and PCa, that they are localized both in fibrous tissue and in the residual acinar parenchyma, and that the number of mast cells correlates positively with the histopathologic grade (extent of fibrosis and intensity of inflammation) of CP.[54] Rearranged immunoglobulin lambda light chain is probably involved in specific immunologic changes observed in CP; its exact function and source, however, remain to be determined. The last upregulated gene in CP is CRISP-3. This gene encodes for a glycoprotein whose presence has been previously detected in the normal pancreas.[32] However, that this protein might be important in the pathogenesis of CP was previously not known.

In conclusion, there are complex changes in the expression profile in CP compared with the normal pancreas. Most of the changes observed in CP are also present in PCa, but a small number of key genes are selectively upregulated in CP. These results may provide new insights into the pathobiologic changes of CP and might also help to identify certain molecular alterations that could serve as potential targets for new therapies in CP.

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Footnote 1

GenBank accession numbers of 152 genes expressed at increased levels in CP:

AB006781, AF006084, D11428, D13666, D21255, D29640, D32129, D55696,D63475, D83174, D84239, D86550, D86983, D90279, HG1428-HT1428, HG2743-HT2846, HG3044-HT3742, HG3576-HT3779, HG880-HT880, HG884-HT884, J03040, J03801, J04456, J05068, K01160, K03498, L02326, L06797, L08044, L12350, L13391, L27560, L32137, L34657, L42176, M11749, M12125, M12759, M13560, M14539, M18728, M21119, M21186, M22430, M22960, M24766, M25079, M27436, M29540, M29971, M30938, M33600, M34079, M34516, M34516, M55543, M57466, M58286, M62403, M63438, M64497, M65292, M77349, M83216, M85289, M87789, M94250,, M94345, M95787, S71018, S71043, S72008, S75256, U03057, U03270, U07919, U08021, U15009, U16306, U17077, U17760, U20734, U21128, U22431, U24389, U27185, U28488, U30521, U30894, U39447, U47025, U51010, U53445, U63455, U77180, U77396, U81607, U90913, V00563, X00274, X01630, X02761, X04412, X05610, X06700, X13334, X13839, X14787, X15882, X17042, X17093, X52003, X52022, X53416, X54925, X56494, X56841, X57348, X57351 X57809, X57809 X58529, X62320, X64072,, X66533, X68314 X68742, X69819, X71490, X74874, X76534, X76648, X77366, X79981, X82153, X82200, X91249, X95240, X98311, X98534, X99133, X99920, Y00062, Y00503, Y07909, Y09443, Y10807, Z22534, Z29083, Z69881, Z74615, Z74616

Footnote 2

GenBank accession numbers of 34 genes expressed at decreased levels in CP:

D14446, D14663, D87937, D88795, HG2383-HT4824, HG2479-HT2575, HG2841-HT2969, HG2841-HT2970, L05144, L11005, L12711, L15409, L25878, L42354, M16594, M20530, M22324, S38953, S74683, S80437, U15174, U22961, U29091, U35735, U62317, U86136, U88871, U90552, X16665, X96924, X97230, Y00477, Z26256, Z46376

Discussion

PROF. J. NEOPTOLEMOS: I feel very honored to be asked to discuss this paper from one of the few groups that have helped to provide a fundamental understanding to the pathogenesis of the chronic pancreatitis (CP).

Dr. Helmut Friess and his colleagues from the University Department of Surgery at Bern, Switzerland, and Roche Genomic and Information Sciences, Hoffmann-La Roche Inc., Nutley, New Jersey, have analyzed eight tissues with CP and compared these to eight tissues with pancreatic ductal carcinoma and a similar number of normal pancreata obtained from transplant donors.

Messenger RNA from these tissues was amplified and differential expression determined on a DNA microarray with sequences from 5,600 selected genes. They identified 11 signature genes for the pancreas, which were not present in human colon, liver, prostate, lung, or lymphatic tissue. This of itself is a useful contribution.

Dr. Friess and colleagues also identified reduced gene expression of 34 genes in CP, but interestingly all 34 genes were also decreased in pancreatic cancer. They do not say if other genes were uniquely reduced in pancreatic cancer.

Similarly, they identified increased expression in 157 genes in CP, and again curiously 152 of these same genes were also decreased in pancreatic cancer. Only five genes were uniquely increased in CP. These were mucin-6 (contributes to cell-surface glycoproteins), germline oligometric matrix protein or COMP (associated with chondrocytes), tryptase-3 (secretory granules of mast cells), rearranged immunoglobulin light chain, and cysteine-rich secretory protein-3 or CRISP-3 (a secreted glycoprotein). They confirmed differential expression by Northern blots in two of these (COMP and CRISP-3).

Of course, it is not clear whether these five genes are particularly interesting and whether their detailed investigation in future studies would throw any new light into the pathogenesis of CP. Out of the remaining 187 genes with increased/decreased expression, only 42 are mentioned, and these are either predictable or not especially interesting at this stage. It would be much more helpful to mention all the genes with differential expression and to indicate the levels of differential expression both with respect to pancreatic ductal adenocarcinoma as well as normal pancreata.

The development of gene microarray technology is likely to have a major impact on differential diagnosis of pancreatic tumors, prognosis, and targeted gene therapies. Undoubtedly it will provide important roles too in determining the pathogenesis of CP. This paper represents the first demonstration of this approach in the field of CP.

Nevertheless, there are serious limitations to the methodology. Only patients with advanced CP were included (i.e., only patients requiring resection for symptomatic CP). Whole tissue specimens were used, so we do not know the variable contribution of acinar, ductular, and "inflammatory" vascular, matrix, and other stromal cells. The prospects for camouflage of valuable data are immense. Thus, ultimately the most useful data will derive from the use of microdissected tissue.

The pancreas is notoriously difficult from which to extract high-quality RNA. Thus, there is likely to be biased differential preservation of quality mRNA. Moreover, the amplification steps for tissue mRNA are also likely to show differential biases.

The actual sensitivity of the DNA microarrays is relatively poor, perhaps so much so that important changes are likely to be missed. We know, for example, that pancreas cancer will have reduced expression of $p16^{INK4a}$, while p53 levels are likely to be increased, but there is no mention of either of these.

The data as reported in this paper tells us little that is new in the understanding of the pathogenesis of CP. Perhaps these data are hidden in the long list of inferred genes differentially expressed but not named individually.

The expression of important differentially expressed genes will need to be confirmed by Northern blots or real-time RT-PCR, protein levels by Western analysis, and sites of expression by immunohistochemistry. Moreover, their biologic relevance will need to be assessed in experimental systems.

There seems little doubt that this kind of approach will greatly facilitate the scientific investigation of CP. Undoubtedly other technologies such as proteomics, which can identify novel, differentially expressed proteins, will also contribute significantly.

I conclude by congratulating the authors on a landmark paper.

Dr. H. Friess (closing): Dear Professor Neoptolomos, thank you for your comments and your questions. There are some questions which I would like to specifically address.

The first of your questions deals with the aim of our study. In this study we never aimed to elucidate the complete pathophysiology of chronic pancreatitis. However, our DNA-chip array analysis allows us to detect genes which might be critical in the pathogenesis of chronic pancreatitis and which are worthy of being studied further in this context. This also influences the further studies which we are doing now. We have identified five specifically upregulated genes out of these 5,600 genes, and we are going on to do more DNA array analysis on more human genes identified in the meantime. The industry is able to fabricate larger arrays due to technical developments. Therefore, we will start up soon with an array which contains more than 12,000 genes, and we hope to identify some more interesting genes which are altered in chronic pancreatitis in comparison to the normal pancreas and pancreatic cancer.

As we have shown in our paper and also in the presentation, we of course are going on to analyze in more detail those genes which are upregulated. We have already presented some data for COMP and CRISP-3. For further analysis, we are applying Northern blot analysis to reconfirm the expression data obtained by the DNA analysis.

You made some critical comments, saying that expression analysis is difficult to evaluate by DNA-chip analysis. We agree with you; however, due to the time limitations on our presentation, we could not show a comparison of the results of the chip analysis and the Northern blot analysis. However, the results are very similar. And of course we are going to perform in situ hybridization to identify the place where these mRNAs are synthesized. For example, CRISP-3 is synthesized in acinar cells, and we believe that with some additional work we can come up with some very interesting results for the pathogenesis of chronic pancreatitis.

Tissue contamination is always a problem when this new technology is applied to RNA isolated from whole tissue homogenates, which we have used in our study. However, I have to admit that microdissection in the pancreas is very difficult to perform successfully. It takes some time to process the tissue taken out of storage at −80°C, and this time is very critical for RNAse activation, which limits the isolation of high-quality pancreatic RNA. Therefore, microdissection in the pancreas is very demanding, and we have to improve our technique to use microdissection in conjunction with DNA-chip arrays.

The quality of RNA which we have used in our study was excellent because the tissues were frozen in the operating room in liquid nitrogen immediately after removal. The quality of total RNA was checked on ethidium bromide gels, and mRNA which was subsequently isolated also showed no signs of degradation.