Discovery and analysis of pancreatic adenocarcinoma genes using cDNA microarrays

Gang Jin, Xian-Gui Hu, Kang Ying, Yan Tang, Rui Liu, Yi-Jie Zhang, Zai-Ping Jing, Yi Xie, Yu-Min Mao

AIM: To study the pathogenetic processes and the role of gene expression by microarray analyses in expediting our understanding of the molecular pathophysiology of pancreatic adenocarcinoma, and to identify the novel cancer-associated genes.

METHODS: Nine histologically defined pancreatic head adenocarcinoma specimens associated with clinical data were studied. Total RNA and mRNA were isolated and labeled by reverse transcription reaction with Cy5 and Cy3 for cDNA probe. The cDNA microarrays that represent a set of 4,096 human genes were hybridized with labeled cDNA probe and screened for molecular profiling analyses.

RESULTS: Using this methodology, 184 genes were screened out for differences in gene expression level after nine couples of hybridizations. Of the 184 genes, 87 were upregulated and 97 downregulated, including 11 novel human genes. In pancreatic adenocarcinoma tissue, several invasion and metastasis related genes showed their high expression levels, suggesting that poor prognosis of pancreatic adenocarcinoma might have a solid molecular biological basis.

CONCLUSION: The application of cDNA microarray technique for analysis of gene expression patterns is a powerful strategy to identify novel cancer-associated genes, and to rapidly explore their role in clinical pancreatic adenocarcinoma. Microarray profiles provide us new insights into the carcinogenesis and invasive process of pancreatic adenocarcinoma. Our results suggest that a highly organized and structured process of tumor invasion exists in the pancreas.

Abstract

INTRODUCTION

Pancreatic carcinoma is the 12th most common cause of cancer death in China, which is one of the most aggressive form of human tumors and is virtually incurable. Its incidence and mortality rates are almost identical, even after receiving surgical resection and adjuvant chemoradiotherapy, the overall 5-year survival rate is only 4.1%[1]. The etiology of pancreatic carcinoma is still unknown. There is clearly a need for novel and more effective diagnostic and therapeutic methods.

Oncogenesis and development of neoplasm is a complex multiphase process, which involves overexpression of oncogenes or inactivation of tumor suppressor genes, mutation or depletion of normal genes, pleiotropic effects and immunologic function. The genes are involved in vital processes of life, such as gene expression accommodation, immunology or cell differentiation, which are arranged in some gene clusters, in which all the members are being controlled in unity[2]. In the process of oncogenesis, there may exist some different clusters of tumor-related genes. Hence, it is important to find such unique gene clusters involved in carcinogenesis, invasion and metastasis processing.

Current focus of molecular profiling is the large-scale analysis of gene expression using new DNA array technology[3]. This powerful technology is being used to study many biological processes. The experimental or clinical goals range from insights to pathogenesis, cancer diagnosis and prediction of clinical outcome for identification of therapeutic targets. In this way, DNA array analysis is providing the first glimpse of a substantial improvement in our understanding of cancer biology and diagnosis. Identifying and sequencing a set of full-length cDNAs that represent all human genes would help in both gene discovery and functional analysis. It offers a
great opportunity to study the pathogenetic processes and molecular pathophysiology of pancreatic carcinoma.

In this study, we analyzed nine pancreatic adenocarcinomas using the cDNA microarray containing 4,096 human genes with the aim of understanding expression patterns and searching for carcinogenesis-related gene clusters and novel useful markers for the malignant potential of pancreatic carcinoma at the molecular level.

MATERIALS AND METHODS

Patients and tissue specimens

We analyzed samples of pancreatic head adenocarcinoma from nine patients (five males, four females, 51-71 years) who underwent pancreaticoduodenectomy at Shanghai Hospital, Shanghai, China, between November 1999 and May 2000. All samples were collected with informed consent and Ethics Committee approval. Samples were grossly dissected and snap-frozen in liquid nitrogen within 10 min of removal and stored at -80 °C. Initial diagnosis of each sample from the frozen section was later confirmed by detailed analysis of paraffin-embedded sections. Following the fourth Japanese edition of the Classification of Pancreatic Carcinoma (Japan Pancreas Society, 1993), the nine tumors were staged, including two stage I, two stage II, four stage III and one stage IVa. We isolated and purified total RNA from pooled, noncancerous, male adult human pancreas tissues and used as a reference “normal” sample for each microarray experiment.

RNA isolation

Tumor and normal tissue samples were ground into a fine powder in a 10-cm ceramic mortar (RNase-free) and total RNA was extracted according to the original single-step extraction procedure with slight modifications. Ground tissue was homogenized in Solution D containing 1% β-mercaptoethanol. After centrifugation, the supernatant was extracted with an equal volume of phenol:chloroform (5:1) and once with an equal volume of acidic phenol:chloroform (5:1), discarding the organic phase each time. The aqueous phase was then precipitated by adding an equal volume of isopropylalcohol at 4 °C, centrifuged for 2 h at 70% humidity, dried for 0.5 h at room temperature, and UV cross-linked at a dose of 65 mJ/cm. They were further treated with 2 g/L sodium dodecyl sulfate (SDS) for 10 min, distilled H2O for 10 min, and 2 g/L sodium borohydride (NaBH4) for 10 min at room temperature. The slides were dried again and made ready for use.

Probe preparation and hybridization

The fluorescent cDNA probes were prepared through reverse transcription of the isolated mRNAs and then purified. The RNA samples from healthy individuals were labeled with Cy3-dUTP and those from cancerous patients with Cy5-dUTP. The two color probes were then mixed, precipitated with ethanol and dissolved in 20 µL of hybridization solution. Microarrays were pre-hybridized with hybridization solution containing 0.5 mg/mL denatured salmon sperm DNA at 42 °C for 6 h. Fluorescent probe mixtures were denatured at 95 °C for 5 min, and the denatured probe mixtures were applied onto the pre-hybridized chip under a cover glass. Chips were hybridized at 42 °C for 15-17 h. The hybridized chips were then washed at 60 °C for 10 min each in solutions of 2× SSC and 2 g/L SDS, 0.1× SSC and 2 g/L SDS, and 0.1× SSC, and then dried at room temperature.

Data analysis

The chips were scanned with a ScanArray 3000 (GSI Lumonics, Billerica, MA, USA) at two wavelengths to detect emission from both Cy3 and Cy5. The acquired images were analyzed using ImaGene 3.0 software (BioDiscovery, Inc., Los Angeles, CA, USA). The intensities of each spot at the two wavelengths represent the quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each spot. Ratios of Cy3-Cy5 were computed for each location on each microarray. Overall intensities were normalized with a correction coefficient obtained using the ratios of 40 housekeeping genes (available at http://www.biodoor.com/).

We used the threshold value to define significant relative expression changes, which set at 2.0 for overexpression and at 0.5 for underexpression on the
basis of both the experimental variability in our data and the manufacturer’s established performance criteria. Data filtering with this algorithm identified the genes overexpressed by at least twofold and underexpressed by at least by 50% across, more than 66.7% (6/9) of all specimens. To minimize artifacts arising from low expression values, only the genes with raw intensity values for both Cy3 and Cy5 of >600 counts were chosen for differential analysis.

RESULTS

Sensitivity and reproducibility of the microarray system

The purity and concentration of isolated RNA were analyzed first by using UV spectrophotometer at absorbance wavelengths of 260 and 280 nm (A260 and A280). The average A260/A280 ratio was higher than 1.9. Furthermore, the integrity of the RNA sample was verified by electrophoresis on 10 g/L agarose gel stained with ethidium bromide. The quality of the RNA was assessed by the visualization of the 28S and 18S ribosomal RNA bands. The bands were distinct and sharp, without being diffused and smeared. The results indicated that mRNA preparation expressed continuous polyadenylated transcripts between 0.9 and 4.0 kb in length.

In order to access the “noise” in the differential expression assay, we employed self-comparison experiments. A sample of mRNA from a single fetal liver tissue was divided into two equal aliquots and labeled with Cy3-dUTP and Cy5-dUTP, respectively. The labeled samples were then mixed together and hybridized to the microarray. The results revealed that approximately 1% of the 4 096 cDNA clones showed more than 2.0-fold difference in signal intensity between the two channels. Furthermore, this “noise” in the data was shown upon analysis to occur at random array positions in each microarray experiment. Figure 1 shows the scatter plots of the within-slide normalization experiment. The Cy3/Cy5 log ratios from the different print tip groups were centered around zero, indicating that the types of systematic errors were minimized. The spots in the experiments are expressed differentially in cancer cells when compared with the normal cells. Scatter plot of the values of Cy3 and Cy5 fluorescent signals also revealed a pattern of tight distribution and clustered in an almost 45° diagonal line as expected.

In the 11 novel genes screened by our microarray experiments, an overexpressed clone in pancreatic adenocarcinomas was identified. The average Cy5/Cy3 ratio of the clone is 4.92. As we have reported recently, this clone is the full-length cDNA of the human gene S100P (GenBank accession number AF539739). The sequence is of 1 297 bp and encodes a protein identical to previously characterized human S100P, but it is much longer than the previously reported 439 bp. The cDNA is near full-length as confirmed by Northern blot analysis. We examined its distribution in tissues by using Northern blot and RT-PCR analysis, and found that it was abundantly expressed in many tissues including placenta, unlike the expression pattern of other S100 family genes.

DISCUSSION

DNA microarray technology has offered us a new insight into the secrets of life by monitoring the activities and
profiles of thousands clones simultaneously. The gene expression profiles can led us to mapping a cross-section of genetic activities and biological entity.

In this experiment, the genes identified as differentially expressed in microarrays revealed a wealth of information that pancreatic adenocarcinomas are complex tumors, as evidenced by the wide range of investigations. However, these findings not only provide novel insight into the biology of pancreatic carcinoma, but also serve to identify numerous new targets for development into serologic markers or therapeutic target. The differentially expressed genes in pancreatic adenocarcinomas included oncogenes and tumor suppression genes, cell-cycle-related genes, signal transduction factors, extracellular matrix and skeleton related genes, transcription factors, DNA damage and repair related genes and apoptosis-related genes (Tables 1 and 2).

The screened genes with good concordance in these pancreatic adenocarcinoma patients may have the potential to become candidates for tumor markers and the molecular target for gene therapy, whereas genes that show concordance in a patient subset may reflect different disease stages or physiological and genetic differences between the patients.

Griffin et al. reported that more than 70% of pancreatic adenocarcinomas possessed consistent chromosome abnormalities. The most frequent whole chromosomal gains were chromosomes 20 and 7, and

### Table 1: Representative list of highly expressed genes in pancreatic carcinoma

| Categories                        | Accessions     | Descriptions                     | Symbols     | Gene map locus | Average ratios |
|-----------------------------------|----------------|----------------------------------|-------------|----------------|---------------|
| Oncogenes                         | AF183421       | RAB22b, RAS oncogene family      | Rab22b      | 18p11.3        | 3.530         |
|                                   | NM_001175      | Rho GDP dissociation inhibitor   | Rho GDI     | 12p12.3        | 4.504         |
|                                   | NM_001788      | Cell division cycle 10           | CDC10       | 7p14.3-14.1    | 3.219         |
|                                   | NM_001798      | Cyclin-dependent kinase 2        | CDK2        | 12q13          | 2.853         |
|                                   | NM_002592      | Proliferating cell nuclear antigen | PCNA       | 20pter-p12     | 3.388         |
|                                   | NM_002835      | Protein tyrosine phosphatase, non-receptor type 12 | PTPN2 | 7q11.23         | 4.236         |
| Signal transduction               | NM_007039      | Protein tyrosine phosphatase, non-receptor type 1 | PTPN2 | 14q31.3         | 2.65          |
|                                   | NM_004721      | Mitogen-activated protein kinase kinase kinase 13 | MAPK13 | 3q25.29         | 2.361         |
|                                   | NM_000876      | Insulin-like growth factor 2 receptor | IGF2R | 6q26          | 2.192         |
|                                   | NM_000700      | Annexin I                        | ANXA1       | 9q12.21.2      | 4.092         |
| Extracellular matrix              | NM_002345      | Lumican                          | LUM         | 12q13.3-22.2   | 9.892         |
|                                   | NM_000889      | α 2 type I collagen              | COL1A2      | 7q22.1         | 11.638        |
|                                   | NM_000920      | α 1 type III collagen             | COL3A1      | 2q31           | 18.165        |
|                                   | M26576         | α 1 type IV collagen preproprotein | COL4A1 | 13q34         | 4.171         |
|                                   | NM_000393      | α 2 type V collagen preproprotein | COL5A2 | 2q14.3-32      | 3.677         |
|                                   | NM_011920      | Decorin                          | DCN         | 12q13.2        | 3.633         |
|                                   | NM_005862      | Secreted phosphoprotein 1        | SPP1        | 4q12-25        | 5.033         |
|                                   | NM_004385      | Chondroitin sulfate proteoglycan 2 | CSPG2  | 5q14.3         | 6.073         |
|                                   | NM_003380      | Vimentin                          | VIM        | 10q15          | 2.543         |
|                                   | NM_003158      | Caldesmon 1                       | CALD1       | 7q33           | 5.937         |
|                                   | NM_002926      | Fibronectin 1                     | FN1         | 4q34           | 18.298        |
|                                   | NM_003254      | Tissue inhibitor of metalloproteinase 1 | TIMP1  | Xp11.3-p11.23  | 13.791        |
| Cytoskeleton and motility         | NM_001613      | α 2 actin                         | ACTA2       | 10q23.3        | 2.79          |
|                                   | NM_006009      | Tubulin, α 3                      | TUBA3       | 12q12.14.3     | 2.647         |
|                                   | NM_005717      | Actin related protein complex subunit 5 | TPM1    | 15q22.1        | 2.113         |
| Cell surface antigen              | NM_002659      | Plasminogen activator, urokinase receptor | UPAR   | 19q13         | 6.073         |
|                                   | NM_001769      | CD9 antigen                       | CD9         | 12p13          | 2.614         |
| Enzymes                           | NM_003096      | Cathepsin K preproprotein         | CTSK        | 1q21           | 2.809         |
|                                   | NM_002654`     | Pyruvate kinase, muscle protein   | PKM2        | 15q22          | 3.187         |
| Cytokines                         | NM_005554      | Interferon 1 receptor 2           | IFNGR2      | 21q22.11       | 2.492         |
|                                   | NM_003641      | Interferon-induced transmembrane protein 1 | IFITM1 | 11 | 3.187 |
|                                   | NM_006438      | Interferon-induced transmembrane protein 2 | IFITM2 | 11p15.5 | 4.149 |
| Transcription factor              | NM_007315      | Signal transducer and activator of transcription 1 | STAT1 | 2q32.2-32.3 | 2.945 |
|                                   | BC007874       | Fructose biphosphatase 3          | FBP3        | 9q34.2         | 2.35          |
|                                   | NM_001530      | Hypoxia-inducible factor 1, α     | HIF1a        | 14q12.21       | 2.796         |
|                                   | NM_006940      | SRY-box 5 isoform A               | SOX5        | 12p12.1        | 2.671         |
|                                   | AF332129`      | Regulatory factor X, 4            | RFX4        | 12q            | 2.094         |
the chromosomal losses were much more frequent in chromosomes 18, 13, 12, 17, and 6. Structural abnormalities were frequently involved in chromosomes 1p, 6q, 7q, 17p, 1q, 3p, 11p, and 19q. From our microarrays, we found that the overexpressed genes in pancreatic adenocarcinomas are mainly located in chromosomes 1, 2q, 7q, 9q, 12, 14q, 15q, and 21q, and the downexpressed genes are mainly located in chromosomes 1p, 6q, 7q, 17p, 1q, 3p, 11p, and 19q. These results are similar to the previous reports. This phenomenon suggests the existence of acquired genomic alterations in pancreatic carcinomas.

Among the genes overexpressed in pancreatic adenocarcinomas, RAB22B and Rho GDP dissociation inhibitor (Rho GDI) are the members of Ras superfamily, whose Cy3/Cy3 ratios are 3.53 and 4.504, respectively. As it is well known that many pancreatic carcinoma cells show “addiction” to K-ras mutation, while normal cells appear resistant to suppression of K-ras-mediated signaling by antisense K-ras RNA expression adenosiviral vector[10]. So, overexpressed RAB22B may be the result of K-ras mutation in pancreatic adenocarcinoma. The Rho family proteins were found to reorganize cytoskeletons and regulate the cell migration via the activation of effector proteins. GTP-bound Rho is an active form, whereas the GDP-bound form is inactive. Rho GDI can block the conversion between the GTP- and GDP-bound forms. Expression of Rho family molecules has recently been reported in breast, lung, pancreas and colon carcinomas, and testicular germ cell tumors[12]. Moreover, three guanine nucleotide exchange factors (RCC1-like G

Table 2 Representative list of downregulated genes in pancreatic adenocarcinoma

| Categories            | Accessions | Descriptions                      | Symbols | Gene map locus | Average ratios |
|-----------------------|------------|-----------------------------------|---------|---------------|---------------|
| DNA injury and repair | NM_006763  | BTG family, member 2              | BTG2    | 1q22          | 0.141         |
|                       | NM_014877  | Helicase with zinc finger domain   | HELZ    | 17q24.2       | 0.337         |
|                       | NM_014140  | SWI/SNF-related matrix-associated |         |               |               |
| Tumor suppressor      | NM_000551  | Von Hippel-Lindau syndrome gene    | VHL     | 3p25          | 0.4           |
| Apoptosis             | NM_001229  | Caspase 9 isoform and preprotoxin | CASP9   | 1p36.3-36.1   | 0.29          |
| Cell-cycle dependent  | NM_002923  | Regulator of G-protein signaling 2 | RGS2    | 1q31          | 0.186         |
|                       | NM_005381  | Nucleolin                         | NCL     | 2q12          | 0.411         |
| Adhesive molecule     | NM_001078  | Vascular cell adhesion molecule 1  | VCAM    | 1p32          | 0.363         |
| Ribosomal protein     | BC001365   | Ribosomal protein L4               | RPL4    | 15q22         | 0.399         |
|                       | NM_005617  | Ribosomal protein S14              | RPS14   | 5q31-33       | 0.319         |
| Guanine nucleotide     | NM_001268  | RCC1-like G-exchanging factor      | CHC1L   | 13q14.3       | 0.363         |
| exchange factor       | NM_001960  | Eukaryotic translation elongation  | EEF1D   | 19p13.13      | 0.242         |
|                       | NM_001959  | Eukaryotic translation elongation  | EEF1B2  | 2q33-34       | 0.305         |
| Transcription factor  | NM_014900  | EBNA-2 co-activator (100 ku)       | p100    | 7q31.3        | 0.322         |
| Signal transduction   | NM_004301  | BRF1-associated factor BAF53a      |         |               |               |
|                       | NM_004236  | Thyroid receptor interacting protein 15 | SGN2 | 15q21.2      | 0.377         |
|                       | NM_002825  | Pleiotrophin                       | PTN     | 7q33-34       | 0.355         |
|                       | NM_002928  | Regulator of G-protein signaling 16 | RCS16   | 1q25-31       | 0.422         |
|                       | NM_005645  | TF-β-associated factor 13          | TAF2K   | 1p31          | 0.462         |
| Enzymes               | NM_002514  | IGFBP9                            | IGFBP9  | 8q24.1        | 0.376         |
|                       | NM_001979  | Epoxide hydrolase 2                | EPHX2   | 8q12-12       | 0.315         |
|                       | NM_001482  | Glycine amidinotransferase         | GATM    | 15q15.3       | 0.179         |
|                       | NM_000170  | Glycine dehydrogenase              | GLDC    | 9p22          | 0.428         |
|                       | NM_000362  | Aldo/hydrogenase 3 family member   | ALDH3A2 | 17p11.2      | 0.393         |
|                       | NM_005600  | Nitrilase 1                        | NIT1    | 1q21-22       | 0.358         |
|                       | NM_004990  | Methionine-RNA synthetase          | MARS    | 12p13.2       | 0.438         |
|                       | NM_001064  | Transketolase                      | TKT     | 3p14.3        | 0.317         |
|                       | NM_000221  | Ketohexokinase                     | KHK     | 2p23.3-2      | 0.309         |
exchanging factor, eukaryotic translation elongation factor 1-δ, eukaryotic translation elongation factor 1-δ, were downregulated in our microarrays, which implied that GDP-bound forms might be related to tumorogenesis of pancreatic adenocarcinoma. Furthermore, VHL gene, which has been confirmed as a tumor suppressor gene[13], was also downexpressed in pancreatic adenocarcinoma.

The result showed that pancreatic carcinoma cells are much more active than normal cells in many steps of multiple pathways of signal transduction. The stable state of normal somatic cells depends on the dynamic equilibrium of apoptosis and proliferation. Apoptosis-related genes were downexpressed in cancer. These findings revealed that the phenotypical similarities among different cancers are also reflected at the molecular level.

Gene expression profiling of pancreatic carcinoma has also provided new insights into the process of tumor invasion. In pancreatic carcinoma tissue, many invasion and metastasis related genes, such as ECM and cell skeleton related genes (type I collagen, type III collagen, type IV collagen, decorin, secreted phosphoprotein 1, vimentin, tissue inhibitor of matrix metalloproteinase 1, fibronectin 1,α2-actin, tubulin, tropomyosin 1, etc.), showed high expression level, reflecting the cellular components of the host stromal response seen in the presence of infiltrating carcinoma. Moreover, the urokinase-type plasminogen activator receptor (uPAR) was found highly expressed in pancreatic adenocarcinomas, which is a key molecule in the regulation of cell-surface plasminogen activation and, as such, plays an important role in many normal as well as pathologic processes[14]. Memarzadeh et al[15] concluded that uPAR is a useful prognostic marker for biologically aggressive forms of endometrial cancer. These phenomena suggest that poor prognosis of pancreatic carcinoma may have a solid molecular biological basis, and also indicate that a highly organized and structured process of tumor invasion exists in the pancreas.

The downregulated genes in the patients with pancreatic adenocarcinoma are also divided into distinct functional categories. Reduced expression was observed in genes encoding products that function in the apoptosis, immune system, cell regulation, DNA injury and repair processing and GTP/GDP signaling, which were in agreement with the previous reports[16,17].

In conclusion, the application of cDNA microarray technique for analysis of gene expression patterns is a powerful strategy to identify novel cancer-associated genes, and can rapidly explore their role in clinical pancreatic adenocarcinomas. Microarray profiles provide us new insights into the carcinogenesis and invasive process in pancreatic adenocarcinoma. Our results suggest that a highly organized and structured process of tumor invasion exists in the pancreas.

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