Analysis of gene expression profile of pancreatic carcinoma using cDNA microarray

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Abstract

AIM: To identify new diagnostic markers and drug targets, the gene expression profiles of pancreatic cancer were compared with that of adjacent normal tissues utilizing cDNA microarray analysis.

METHODS: cDNA probes were prepared by labeling mRNA from samples of six pancreatic carcinoma tissues with Cy5-dUTP and mRNA from adjacent normal tissues with Cy3-dUTP respectively through reverse transcription. The mixed probes of each sample were then hybridized with 12 800 cDNA arrays (12 648 unique human cDNA sequences), and the fluorescent signals were scanned by ScanArray 3 000 scanner (General Scanning, Inc.). The values of Cy5-dUTP and Cy3-dUTP on each spot were analyzed and calculated by ImaGene 3.0 software (BioDiscovery, Inc.). Differentially expressed genes were screened according to the criterion that the absolute value of natural logarithm of the ratio of Cy5-dUTP to Cy3-dUTP was greater-than-0.69.

RESULTS: Among 6 samples investigated, 301 genes, which accounted for 2.38 % of genes on the microarray slides, exhibited differentially expression at least in 5. There were 166 over-expressed genes including 136 having been registered in Genebank, and 135 under-expressed genes including 79 in Genebank in cancerous tissues.

CONCLUSION: Microarray analysis may provide invaluable information on disease pathology, progression, resistance to treatment, and response to cellular microenvironments of pancreatic carcinoma and ultimately may lead to improving early diagnosis and discovering innovative therapeutic approaches for cancer.

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INTRODUCTION

The morbidity of pancreatic carcinoma has taken an upward trend all over the world. In occidental countries, the morbidity of pancreatic carcinoma has increased by 3 to 7 times in nearly thirty years, and pancreatic carcinoma has become one of the ten commonest malignant tumors. In China, the morbidity was 1.16/100 000 in Shanghai in 1963, and reached on 3.80/100 000 in 1974. Then, it took the 14th place of the morbidity of the malignant tumors, and jumped to fifth in 1984. The statistical results showed that it was 5.1/100 000, which was four times higher than that of twenty years before. In some medical centers, curative resections were given to minority of patients in early stage who were highly selected, and their five-year survival of these patients might even rise to 15 to 25 percent. But generally speaking, treatment of pancreatic cancer is still a serious challenge to us. The key problem to improve the current situation of treatment is to seek novel diagnostic markers, effective adjunctive therapy and mechanism of genesis and evolution of pancreatic cancer. Hence, more and more attention has been paid to the research on molecular pathology and related genes of pancreatic cancer.

Over the past decade, many studies involving pancreatic cancer have searched for cancer-causing gene. As a result, several cancer-related genes have been identified. DPC4, p53, and p16 are the three most frequently inactivated tumor suppressor genes. Other tumor suppressor genes that are altered in pancreatic cancer include BRCA2, ALK-5, MMK-4, and STK11. Mutations of K-ras oncogene are commonly seen in pancreatic cancer, with it’s incidence as high as 90 %. Some other cancer-related genes, such as Her-2/neu, COX-2, VEGF have also been reported to be overexpressed in pancreatic cancer. Development and progression of pancreatic cancer is a very complicated process, so it is reasonable to predict that many other genes, as yet undiscovered, might be potential tumor markers or drug targets.

Microarray is the technique that a large number of cDNAs are arranged orderly on the carrier, such as glass chip or else in high density. Data are obtained by examining the signals of fluorescence, analyzed and compared by computer software. A large number of genes can be examined simultaneously, accurately, and effectively in one experiment. In this study, we have used a high-density cDNA microarray technique to assess the gene expression profile of pancreatic carcinoma versus adjacent normal tissue. Several genes, we identified, may be involved in pancreatic tumorigenesis as well as it’s potential clinical biomarkers that may be used to improve early diagnosis, and to constitute potential novel therapeutic targets.

MATERIALS AND METHODS

Materials
cDNA microarray slides used in this study were fabricated in United Gene Technique, Ltd. Briefly, each slide has 12 800 spots, containing 112 genes as negative control, such as ripe U2 RNA gene (8 spots), HCV coat protein gene (8 spots), spotting solution (96 spots); and 40 housekeeping genes as positive control. Each slide has 12 648 unique human cDNA sequences. Six samples of pancreatic carcinoma were obtained from patients undergoing pancreaticoduodenectomy in department of general surgery, Changhai Hospital, the Second Military Medical University. All cases were proved pathologically as carcinoma of pancreatic head. Normal tissues as control were taken from tissue adjacent to the cutting margin of the carcinoma and proved pathologically to be free from tumor invasion. Samples were snap-frozen in liquid nitrogen within 15 to 20 minutes after resection and then stored at -80 °C.
Cy3-dUTP and Cy5-dUTP were purchased from Amersham Phamacia Biotech, Inc. and Oligotex mRNA Midi Kit from Quagen, Inc. ScanArray 3 000 scanner was manufactured by General Scanning, Inc. ImaGene3.0 software came from BioDiscovery, Inc.

Methods
Probe preparation Total RNA isolated from pancreas tissues and normal adjacent tissues by using modified single step extraction. Briefly, frozen tissues were crushed down and homogenized in solution D and 1 % mercaptoethanol. The supernatant was then extracted by phenol: chloroform (1:1) two times, and phenol: chloroform (5:1) and NaAc (PH=4.5) once. Afterward, the supernatant was precipitated in equal volume of isopropanol at -20 °C for 1.5 hours and was precipitated in LiCl for purification. Both kinds of mRNAs were purified using Oligotex mRNA Midi Kit. The fluorescent cDNA probes were prepared by reverse transcription and then purified, according to the protocol of Schena et al. 1995, The mRNA from normal tissue was labeled with Cy3-dUTP, and that from cancerous tissue with Cy5-dUTP. Then the two probes were mixed with equivalence, precipitated by ethanol, and resolved in 20 µl hybridization buffer (5×SSC + 0.4 % SDS, 50 % Formamide, 5×denhardt’s solution).

Hybridization Microarray slides were pre-hybridized in hybridization buffer, which contained 0.5 mg/ml denatured clupeina DNA, at 42 °C for 6 hours. After denatured at 95 °C for 5 minutes, the probes mixture were added on the pre-hybridized slides and sealed with cover glass. After hybridizing in HybChamber at 60 °C for 15 to 17 hours, the slides were subsequently washed in solutions of 2×SSC + 0.2 % SDS, 0.1×SSC+0.2 % SDS and 0.1×SSC respectively for 10 minutes, then dried at room temperature.

Scanning and analysis The slides were scanned by ScanArray 3 000 laser scanner at two wavelengths to obtain fluorescence intensities for both dyes (Cy3 and Cy5). The original value of each spot was normalized by the values of 40 housekeeping genes selected on the slides. The fluorescence intensities of Cy3 and Cy5 were analyzed and the ratios of Cy5 to Cy3 were calculated by ImaGene 3.0 software. The intensities of two fluorescent signals represented the quantities of two tagged probes. The ration of Cy5 to Cy3 of certain spot on the slides demonstrated that mRNA abundance of this gene expressed in cancerous tissues versus normal.

RESULTS
Verification of micrearray technique system There were 12 800 spots on each slide, including 6 170 known genes and 6 478 unknown ones. In order to monitor entire process of microarray technique system, negative and positive control genes were set on the slides. They were ripe U2 RNA gene (8 spots), HCV coat protein gene (8 spots), spotting solution (96 spots), which served as negative control; and 40 housekeeping genes as positive control. In present study, the individual result of six hybridization showed that all of positive control signals are distinct, and all of negative control signals were very low. These prove the reliability of the data.

Table 1 Over-expressed genes in pancreatic carcinoma

| Accession number | Gene name                              | Gene function                          | Ratio value |
|------------------|----------------------------------------|----------------------------------------|-------------|
| U06863           | Alpha 1 (III) collagen                 | Extracellular metrix                   | 33.89       |
| X91148           | Microsomal triglyceride transfer protein| Microsomal triglyceride transfer       | 21.89       |
| AF017986         | Secreted apoptosis related protein 1 (SARP1) | Repress apoptosis                      | 20.32       |
| Y00755           | Extracellular matrix protein BM-40     | Extracellular matrix                   | 20.23       |
| Z74616           | Alpha2 (I) collagen                    | Extracellular matrix                   | 12.52       |
| D32039           | Proteoglycan PG-M(V3)                  | Extracellular matrix                   | 11.90       |
| J03607           | 40-kDa keratin intermediate filament precursor | Extracellular matrix                   | 11.62       |
| AF144103         | Novel chemokine family member with altered expression in human head and neck squamous cell carcinoma | Cytokine                              | 9.93        |
| AF141201         | Ubiquitin fusion-degradation 1 protein (UFD1) | Protease                              | 8.29        |
| X02761           | Fibronectin (FN)                       | Extracellular matrix                   | 8.01        |
| U06863           | Fetuin-related protein precursor       | Nucleoprotein                          | 6.92        |
| M14219           | Chondroitin/ dermatan sulfate-proteoglycan (PG40) core protein | Extracellular matrix                   | 6.65        |
| M17783           | Glia-derived nexin (GDN)               | Extracellular matrix                   | 6.65        |
| U59877           | Low-Mr GTP-binding protein (RAB31)     | Protease inhibitor                     | 6.49        |
| AF000989         | Thymosin beta 4 Y isoform (TB4Y)       | Thymosin isofrom                       | 5.71        |
| L20688           | GDP-dissociation inhibitor protein (Ly-GDI) | Signal transduction                   | 5.62        |
| X57351           | 1-BD gene from interferon-inducible gene | Signal transduction                   | 5.51        |
| U18728           | Lumican                                | Extracellular matrix                   | 5.15        |
| L02326           | Lambda-like gene                       | Extracellular matrix                   | 4.77        |
| M14144           | Vimentin                               | Extracellular matrix                   | 4.64        |
| J05633           | Integrin beta-5 subunit                | Cytoadhesion molecule                  | 4.42        |
| AF070523         | JWA protein                            | -                                      | 4.32        |
| X3741            | Immunoglobulin-related 14.1 protein    | Immunity correlation                   | 4.28        |
| S83308           | Sry-related HMGB box gene (SOX5)       | Transcription factor                   | 4.16        |
| U05875           | Interferon gamma receptor accessory factor-1 (AF-1) | Immunity correlation                   | 3.92        |
| M17733           | Thymosin beta-4                        | Thymosin isofrom                       | 3.89        |
| M20259           | Thymosin beta-10                       | Thymosin isofrom                       | 3.75        |
| M36501           | Alpha-2-macroglobulin                  | Protease inhibitor                     | 3.72        |
| AF026977         | Microsomal glutathione S-transferase 3 (MGST3) | Oxidation-reduction                   | 3.62        |
| M14630           | Prothymosin-α                          | Nucleoprotein                          | 3.45        |
such as signal transduction, transcription and translation, these genes are involved in various cytobiological functions, such as signal transduction, transcription and translation, cell cycle, apoptosis, chromosome instability, tumor suppressor genes, enzyme, and "others". Moreover, some genes exhibit differently expression in pancreatic cancer in our study, as well as in other cancers. They are fibronectin[1], caltractin[6], glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[8], lipocortin II, uPAR[10] in hepatic cancer; FN[9], glutathione peroxidase[10], phospholipase A2[10], thymosin beta-10[11], uPAR[12], uracil DNA glycosylase[10] in colorectal cancer; N-cadherin[13], uPAR[14], alpha-2-macroglobulin[15], caltractin[6], syntenin[16] in gastric cancer; phospholipase A2[10]; in cholangiocarcinoma; fibronectin[10], glutathione peroxidase[16,21], prothymosin alpha[12,24], thymosin beta-10[21], uPAR[26], caltractin[6], GAPDH[27], proteoglycan PG-M(V3), syntenin[16], lumican[28] in mammary cancer and glutathione peroxidase[29], SPARC/osteonectin[29], thymosin beta-10[30], uPAR[31] in thyroid carcinoma. These data indicated that we have obtained the same results by using cDNA microarray as by other methods. Meanwhile, these confirmed the feasibility, accuracy and effectiveness of microarray as a method to investigate the expression profiles of pancreatic cancer. On the other hand, we might get the conclusion that genesis and progression of pancreatic carcinoma has not been covered in any study up to the present. The standard of determination for differentially expressed genes was that, the absolute value of natural logarithm of the ratio of Cy5 to Cy3 was greater than 0.69, that was to say change of gene expression was above 2 times, and the signal value of either Cy3 or Cy5 needed to be greater than 600.

### Judgement of differentially expressed genes
Hybridization of each couple of probes repeated two times. Among 6 samples investigated, 301 genes, which accounted for 2.38 % of genes on the microarray slides, exhibited differentially expression at least in 5. There were 166 over-expressed genes including 136 having been registered in Genebank, and 135 under-expressed genes including 79 in Genebank. Some of these genes, which took superior places of differentially expression, were listed in Table 1 and Table 2.

### Genes expressed differentially
Among 6 samples investigated, 301 genes, which accounted for 2.38 % of genes on the microarray slides, exhibited differentially expression at least in 5. There were 166 over-expressed genes including 136 having been registered in Genebank, and 135 under-expressed genes including 79 in Genebank. Some of these genes, which took superior places of differentially expression, were listed in Table 1 and Table 2.

### DISCUSSION
There are altogether 215 previously reported genes differently expressed in cancer tissues in our research, including urokinase plasminogen activator surface receptor (uPAR)[11], glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lumican[20], phospholipase A2[10], vascular cell adhesion molecule 1[4], which have been reported to play certain role in evolution of pancreatic carcinoma. However, the relationship between the majority of these genes and development of pancreatic carcinoma has not been covered in any study up to the present. These genes are involved in various cytobiological functions, such as signal transduction, transcription and translation, cytoskeleton, cell adhesion, extracellular matrix and matrix degradation, cell cycle and apoptosis, chromosome instability, tumor suppressor genes, enzyme, and "others". Moreover, some genes exhibit differently expression in pancreatic cancer in our study, as well as in other cancers. They are fibronectin[1], caltractin[6], glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[8], lipocortin II, uPAR[10] in hepatic cancer; FN[9], glutathione peroxidase[10], phospholipase A2[10], thymosin beta-10[11], uPAR[12], uracil DNA glycosylase[10] in colorectal cancer; N-cadherin[13], uPAR[14], alpha-2-macroglobulin[15], caltractin[6], syntenin[16] in gastric cancer; phospholipase A2[10]; in cholangiocarcinoma; fibronectin[10], glutathione peroxidase[16,21], prothymosin alpha[12,24], thymosin beta-10[21], uPAR[26], caltractin[6], GAPDH[27], proteoglycan PG-M(V3), syntenin[16], lumican[28] in mammary cancer and glutathione peroxidase[29], SPARC/osteonectin[29], thymosin beta-10[30], uPAR[31] in thyroid carcinoma. These data indicated that we have obtained the same results by using cDNA microarray as by other methods. Meanwhile, these confirmed the feasibility, accuracy and effectiveness of microarray as a method to investigate the expression profiles of pancreatic cancer. On the other hand, we might get the conclusion that genesis and progression of various neoplasms have some common mechanisms. Further study on these common mechanisms might lead us go into depth the knowledge of molecular biology of cancer, and find the key to the improvement of diagnosis and treatment of cancer.

Pancreatic carcinoma is one of the most malignant tumors, and is characterized by aggressive growth behavior and high incidence rate of recurrence. During proliferation of a primary tumor or the establishment of metastatic foci, there is...
continuous remodeling of the extracellular matrix including various degrees of biosynthesis, reformation and degradation. Among over-expressed genes, several genes, such as alpha1 (III) collagen, extracellular matrix protein BM-40, alpha2 (I) collagen, proteoglycan PG-M (V3), fibronectin, chondroitin/dermatan sulfate proteoglycan (PG40) core protein, lumican, vimentin, chondroitin sulfate proteoglycan versican V0 splice-variant, and versican V2 splice-variant core protein, component analysis related to extracellular matrix (ECM), take the superior places. Versican belongs to the family of large aggregating proteoglycans (PGs). In mammals, versican appears as four possible spliced variants, V_0, V_1, V_2, and V_3. It has been described that the versican-rich extracellular matrices exert an anti-adhesive effect on the cells, thus facilitating tumor cell migration and invasion. Besides decreasing cell adhesion, versican is also able to increase the cell proliferation. The study on melanoma has shown that this PG could serve as a good marker for primary malignant as well as metastatic lesion. Lumican is the member of the small leucine-rich proteoglycan (SLRP) family, whose members are known as keratocan, mimecan, decorin, biglycan, fibromodulin, epiphycan, osteoadherin, and lumican. SLRP proteins can modulate cellular behaviour, including cell migration and proliferation during embryonic development, tissue repair, and tumor growth. In breast cancer tissue, lumican mRNA is reported to be overexpressed. Furthermore, its high expression level was associated with high tumor grade, low estrogen receptor levels, and young age of patients. It is also found that lumican is not synthesized by the exocrine components of the normal pancreas, but that these cells ectopically synthesize and secrete the lumican in cancer tissues, which may play a role in pancreatic cancer cell growth. Extracellular matrix protein BM-40, an anti-adhesive protein, is proposed to modulate cell migration and vascular morphogenesis either by directly interacting with ECM proteins or by initiating a receptor-mediated signaling event. It may directly affect cell motility by inducing intracellular changes of cytoplasmic components; or indirectly promote cell migration by modulating the expression of proteolytic enzymes that degrade the ECM, such as collagenase, stromelysin and MMP-9. It may promote infiltration of tumor cells, serve as a cellular marker of invasion, and correlate to angiogenesis. Fibronectin connects with the cancer cell via its receptors, including integrins α_5β_1 and αvβ_3. The abilities in adhesion to fibronectin and migration increased markedly, after hepatocellular carcinoma cells were transfected with H-ras oncogene. In addition, fibronectin is the primary protein involved in the displacement of MMP-2 produced by adjacent normal cells to cancer tissues. MMP-2 can associate with the cell surface through its COOH-terminal and hemopexin-like domain via a number of mechanisms, including binding to cell-associated collagen I and IV. In our present study, we also find that collagen I and IV overexpress in cancerous tissues. This may enhance the fibronectin-induced displacement of MMP-2, and facilitates invasion of cancerous cells. Vimentin is also a component of ECM. Enhanced expression of the vimentin is associated with high degree of motility, poor differentiation and metastasis of prostate carcinoma. It has been shown that vimentin was immunohistochemically positive in basaloid squamous carcinoma of esophagus. Moreover, uPAR and cathepsin O2 are also found to be over-expressed in our study. All of these changes endow pancreatic cancer with the trait of aggressive growth, and may be potential markers of invasion.

On the other hand, recurrence to chemotherapy after resection remains a major obstacle to the cure of pancreatic cancer. It is well known that cancerous cells are surrounded by an extensive stroma of ECM at both primary and metastatic sites, which contains, among other proteins, fibronectin, laminin and collagen IV. Adhesion of pancreatic cancer cells to these proteins confers resistance to apoptosis induced by standard chemotherapeutic agents. The study on small-cell lung cancer showed that β1-integrin-mediated cell adhesion to ECM proteins results in tyrosine phosphorylation, which, weaken persistent chemotherapy-induced DNA damage, prevents caspase activation and apoptosis. Alpha-tubulin and uracil DNA glycosylase were also found to be contributed to resistance to chemotherapy. These genes over-express in pancreatic cancer in our study, and may play the same role. It is indicated that the regulation routes of these genes may be potential targets for treatment of pancreatic carcinoma.

There are still some genes that should be mentioned. Prothymosin alpha (PTA) is a nuclear protein that is present throughout the cell cycle. It has been shown that it binds histones in vitro and has been proposed to affect the chromatin state. PTA is expressed in various human tumor tissues of different origins, supporting the idea that PTA expression is required for tumor growth. Recent study has shown that in breast cancer, whose tumor with low or moderate PTA level demonstrated a statistically significant decreased rate of tumor recurrence and a statistically significant increased overall survival in comparison with those whose tumor had high PTA levels. It is proposed that PTA could be used as a predictor of the potential malignancy of breast tumors that might help to identify patients at high risk of fatality. Fas-binding protein and secreted apoptosis related protein-1 (SARP-1) are proposed to repress apoptosis. SARP-1 may inhibit phosphorylation of liberated β-catenin and degradation by ubiquitin-protease system via Wnt signaling way. Subsequently, β-catenin accumulates in cytoplasm, and cell apoptosis is suppressed. SARP-1 thus promotes excessive proliferation and transformation. Fas-binding protein can arrest Fas-induced apoptosis. Thymosin beta-4 and Thymosin beta-10 are also up-regulated. These genes may serve as markers for measuring proliferation of pancreatic cancer cells.

There are two tumor suppressors, B-cell translocation gene 2 (BTG2) and programmed cell death 4 (PDCD4), which are down-regulated in cancer tissues. PDCD4 gene is homologous to the mouse gene (MA-3/Pdcd4/A7-1), which is associated with apoptosis and is shown to suppress tumor promoter-induced neoplastic transformation. The ORF of human PDCD4 encodes a protein of 458 aa with a predicted molecular size of 50.6 kDa. It has been demonstrated that PDCD4 protein inhibits neoplastic transformation and must be down-regulated for progression to occur. BTG2, which is induced by p53, displays an antiproliferative activity in different cell types, such as fibroblasts and PC12 cells. It is well known that the control of the cell cycle plays an essential role in cell growth and in the activation of important cellular processes, such as differentiation and apoptosis. p53 and p35 are two molecules identified as key regulators of the cell cycle. Some suggestions came from a recent report, which showed that BTG2 interacted with a protein-arginine N-methyltransferase (Prmt1) by positively modulating its activity. Prmt1, in turn, has been found to bind the interferon receptors and to be required for interferon-mediated growth inhibition. A further investigation on BTG2 demonstrated that this gene inhibited G1/S progression in an Rb-dependent manner and that this effect was correlated with its ability to inhibit cyclin D expression. Furthermore, the impairment in the ability of BTG2 to lower cyclin D levels, seen in consequence of mutations of the BTG2 molecule, correlated with the extent of impairment in growth arrest.
the origin recognition complex (ORC) associates with specific DNA sequences near origins of replication to recruit other replication initiation factors during the G1 phase of the cell cycle. The other replication initiation factors, Cdc6p, Cdt1p, and the Mcm2-7p complex, associate with the origin sequence in an ORC-dependent reaction to form a pre-replicative complex (pre-RC). At the G1-S transition, the activation of cyclin-dependent kinases leads to the recruitment of elongation factors, CDC45, DNA polymerases, and RPA to the pre-RCs at origins. The action of these replication elongation proteins leads to the initiation of DNA synthesis, the hallmark of S phase. Geminin interacts with Cdt1p and prevents the recruitment of the Mcm2-7p complex to origins during S, G2, and early M phases of the cell cycle and thereby inhibits replication initiation, leading to the expectation that the protein acts as an inhibitor of cell proliferation42. The research on these under-expressed genes may render invaluable information to find novel targets for the treatment of pancreatic cancer.

In summary, though some genes have been missed in present study, the genes we have identified may be important to the tumorigenesis of pancreatic cancer, and potential to serve as tumor markers or drug targets.

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