Genomic analysis of invasion-metastasis-related factors in pancreatic cancer cells

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Abstract. Pancreatic cancer is known to be an extremely lethal neoplasm, one of the reasons being that pancreatic cancer itself has an extremely high potential of invasion-metastasis. In our previous study, two pancreatic cancer cell lines with a different potential for invasion-metastasis, PC-1 with a low potential and PC-1.0 with a high potential of invasion-metastasis after intrapancreatic transplantation, were established in a Syrian golden hamster. To determine the invasion-metastasis-related factors, a cDNA microarray that represented a set of 27,000 genes was hybridized with a labeled cDNA probe and screened for molecular profiling analysis. Furthermore, Gene Ontology and Pathway differential expression of candidate genes was further validated using RT-PCR. One hundred and forty-one differentially expressed genes (>3.0-fold change) were identified in the present study, including 46 up-regulated genes (e.g., nup107, tjp-2 and MMP-13) and 95 down-regulated genes (e.g., Spc21, plau and CD44) in the PC-1.0 cells. Our present results suggest that a highly organized and structured process of tumor invasion-metastasis exists in the pancreas. Analysis of gene expression profiles by cDNA microarray provides useful information for clarifying the mechanism underlying this invasion and metastasis. Furthermore, the identification of invasion-metastasis-specific genes may allow us to develop new therapeutic and diagnostic targets for the invasion-metastasis of pancreatic cancer.

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Introduction

One of the most lethal features of pancreatic cancer is its apparent capacity for early invasion and metastasis to the liver and other organs. Apart from surgery, there is no effective therapy and even resected patients usually die within one year postoperatively. Reasons for the poor prognosis include the occurrence of local recurrences and/or distant metastasis after surgery. However, to date, the cellular and molecular mechanisms of the invasion-metastasis of pancreatic cancer remain unclear. Detection of the factors related to the differences in potential for invasion and metastasis of cancer cells could provide useful information for the development of new therapeutic methods to prevent the invasion and metastasis of pancreatic cancer.

To investigate the mechanisms of invasion-metastasis of pancreatic cancer, two hamster pancreatic cancer cell lines with a different potential for invasion and metastasis, PC-1 with a low potential and PC-1.0 with a high potential after intrapancreatic transplantation, were established from a pancreatic ductal carcinoma induced by N-nitrosobis (2-oxopropyl) amine (BOP) in a Syrian golden hamster in our previous investigation (1,2).

cDNA microarray is a new emerging technique in the post genomic era. Large-scale analysis of gene expression with cDNA microarray allows us to evaluate the gene expression profiles of hundreds to tens of thousands of genes in a single experiment (3). Therefore, the cDNA microarray is a promising tool to provide new insight into the mechanisms of cancer invasion and metastasis.

In the present study, we analyzed alteration in the invasion-metastasis-related gene expression patterns of 27,000 genes in highly invasive and metastatic pancreatic cancer cells (PC-1.0) in comparison to weakly invasive and metastatic pancreatic cancer cells (PC-1) utilizing powerful cDNA microarray technology.

Materials and methods

Cell lines and cell culture. Two hamster pancreatic cancer cell lines, weakly invasive and metastatic cells (PC-1) and highly invasive and metastatic cells (PC-1.0) were used. The

PC-1 cell line was established from pancreatic ductal/ductular adenocarcinomas induced by BOP in a Syrian golden hamster (1). The PC-1.0 cell line was established from a subcutaneous tumor produced after inoculation of PC-1 cells (2). *In vitro*, PC-1 cells grow mainly as island-like cell colonies, whereas PC-1.0 cells exhibit the growth pattern of single cells. *In vivo*, local expansion of PC-1 cells and local invasion of PC-1.0 cells are observed (1,2).

The PC-1 and PC-1.0 cells were incubated in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 U/ml penicillin G and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Preparation of total RNA. Total RNA of the PC-1.0 and PC-1 cells was extracted using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). After TRIzol puri-fication, RNA was further purified with RNeasy mini spin column kit (Qiagen, Valencia, CA, USA). The concentration and qualify of the RNA were assessed via spectrophotometry and agarose gel electrophoresis.

cDNA microarray and statistical analysis of data. Preparation of fluorescent dye-labeled DNA and hybridizations was performed according to the protocol of the reagent/kit manufacturers and previously reported methods (4). Briefly, RNA was reverse-transcribed into cDNA with Oligo(dT)15 (Promega) as primer and Superscript II choice for cDNA synthesis (Invitrogen) and subsequently labeled in red (Cy5) or in green (Cy3) (Amersham Pharmacia Biotech). Cy5- and Cy3-labeled cDNA was purified with a PCR purification kit (Qiagen). DNA was mixed with 30 μl hybridization solu-tion prior to loading onto a rat gene microarray (Capitalbio Inc., Beijing, P.R. China) which included 27,000 transcripts (Oligo library, Rat Genome version 3.0.5; Qiagen). Arrays were hybridized at 42°C overnight. The experiments were performed twice with reverse dye-labeled cDNA.

The microarray plates were scanned by LuxScan 10KA dual pathways laser scanner (Capitalbio), and images wereana-lyzed through GenePix Pro 4.0 image analysis software (Axon Instruments Co.). Genes were considered to be differentially expressed, integrated ratio of two experiments, at a change in increase (>3.00) or decrease (<0.33) in the ratio of expression levels between PC-1.0 and PC-1 cells.

Statistical analysis was carried out with the t-test, and the expression of a given gene was considered changed when the difference between means was significant (P<0.01).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from PC-1.0 and PC-1 cells, and an aliquot of 1 μg of total RNA from each sample was reverse-transcribed to cDNA using the SuperScript II kit (Life Technologies, Inc.) as previously described (5). The primers used for PCR amplification in this study are listed in Table I. Amplification was run for 30 cycles at 95°C for 5 min, 95°C for 40 sec, 55°C for 30 sec, 72°C for 1 min and finally extended at 72°C for 7 min.

Gene Ontology and Pathway analysis of differentially expressed genes. Using the Gene Ontology tool from http://

Table I. Primers used for the RT-PCR of PC-1.0 and PC-1 cells.

Gene name	Primer sequence	Product size (bp)
Actin	F: GTGGGGCGCCCCAGGCACCA R: CTCCTTAAGTCACGCACGATTCC	664
nup107	F: GACAGAAGAGGCACAACGAC R: ACCAGACTGTCCACCATCAC	309
tjp2	F: GCAGAGCGAACGAAGAGTATGG R: TGACGGGATGTTGATGAGGGT	245
MMP-13	F: CAGTCTTTCTTCGGCTTAG R: CAGGGTCCTTGGAGTGGTC	496
Spc21	F: GTGGTGCTGAGTGGCAGTAT R: CCAGTTCTGGCCTTCTTTGT	246
plau	F: AGAATTCACCACCATCGAGA R: ATCAGCTTCACAACAGTCAT	474
CD44	F: AAGGTGGAGCAAACACAACC R: AACTGCAATGCAAACTGCAAG	115

www.pantherdb.org, the differentially expressed genes were automatically assembled to categories of Biological process, Molecular function and Cellular component. Biologically related networks were automatically assembled from identified genes on microarrays by the BioRag (http://www.biorag.org), which enables the analysis of pathways among interested genes according to Kegg (http://www.genome.ad.jp/kegg) or GenMAPP (http://www.genmapp.org). The Fisher's exact test was performed to detect the significantly regulated gene and pathway, A P-value <0.01 was considered significantly overrepresented.

Results

Differentially expressed genes identified by cDNA microarray in the highly (PC-1.0) and weakly (PC-1) invasive and meta-static pancreatic cancer cells. To clarify the differentially expressed genes between highly (PC-1.0) and weakly (PC-1) invasive and metastatic cells, the expression level for each gene in the two pancreatic cancer cell lines was compared. Of the 27,000 genes analyzed through microarray experiments, a total of 141 genes revealed differential expression using a fold ratio >3 as the criteria for cut-off. Of the 141 genes, the expression of 46 genes (32.6%) was markedly increased in the highly invasive and metastatic cells (PC-1.0) as compared with the weakly invasive and metastatic cells (PC-1) (Table II). On the other hand, the expression of 95 genes (67.4%) was signifi-cantly decreased in the highly invasive and metastatic cells (PC-1.0) as compared with the weakly invasive and metastatic cells (PC-1) (Table III). The ratio represented the expression value in PC-1.0 cells compared with the expression level in PC-1 cells.

Validation of selected genes with RT-PCR. To verify the reli-ability of the microarray data, we selected three up-regulated

Table II. Genes up-regulated in highly invasive and metastatic cells (PC-1.0) compared with weakly invasive and metastatic cells (PC-1).

Gene name	Gene ID	Gene symbol	Description	Ratio
Mlp	ENSRNOG00000009113	NM_030862	MARCKS-like protein	71.9931
Aldr1	ENSRNOG00000009513	ALDR_RAT	Aldehyde reductase 1	33.6872
MMP-13	ENSRNOG00000008478	MM13_RAT	Matrix metallopeptidase 13	30.0071
MMP-12	ENSRNOG00000008993	MM03_RAT	Matrix metallopeptidase 12	26.2124
Col5a2	ENSRNOG00000003736	O70598	Collagen, type V, $\alpha 2$	20.8255
Tnni2	ENSRNOG00000020276	TRIF_RAT	Troponin 1, type 2	20.5716
Tjp2	ENSRNOG00000015030	P70625	Tjp2 protein	20.5690
MMP-3	ENSRNOG00000008993	MM03_RAT	Matrix metallopeptidase 3	20.5668
Snrpn	ENSRNOG00000022595	NM_130738	Small nuclear ribonucleoprotein N	17.1193
Syt8	ENSRNOG00000020245	NM_053325	Synaptotagmin 8	15.8243
S100a5	ENSRNOG00000011748	S105_MOUSE	S100 calcium binding protein A5	11.6390
Ndrg2	ENSRNOG00000010389	NM_133583	N-myc downstream regulated gene 2	11.4480
MMP-10	ENSRNOG00000008993	MM03_RAT	Matrix metallopeptidase 10	10.7218
Tf	ENSRNOG00000009434	TRFE_RAT	Transferrin	8.7425
Anxa6	ENSRNOG00000010668	ANX6_RAT	Annexin A6	6.5978
Nup107	ENSRNOG00000006541	N107_RAT	Nucleoporin 107	6.5895
Spnb3	ENSRNOG00000019564	SPCP_RAT	β-spectrin 3	6.3763
Cdk4	ENSRNOG00000025602	CDK4_RAT	Cyclin-dependent kinase 4	6.2099
Fap	ENSRNOG00000005679	NM_138850	Fibroblast activation protein	5.7157
Eno3	ENSRNOG00000004078	ENOB_RAT	Enolase 3,β	5.6429

The complete data of the cDNA microarray analysis is available upon request.

Table III. Genes down-regulated in highly invasive and metastatic cells (PC-1.0) compared with weakly invasive and metastatic cells (PC-1).

Gene name	Gene ID	Gene symbol	Description	Ratio
App	ENSRNOG00000001546	A4_RAT	Amyloid β (A4) precursor protein	0.1286
Col9a1	ENSRNOG00000012920	CA19_RAT	Procollagen, type IX, α 1	0.1269
CD44	ENSRNOG00000013562	CD44_RAT	CD44 antigen	0.1205
Serpinh1	ENSRNOG00000016831	HS47_RAT	Serine proteinase inhibitor 1, clade H	0.1190
Tcf4	ENSRNOG00000012405	ITF2_RAT	Transcription factor 4	0.1039
Chn2	ENSRNOG00000009411	CHIO_RAT	Chimerin (chimaerin) 2	0.0913
Plau	ENSRNOG00000010516	UROK_RAT	Plasminogen activator, urokinase	0.0895
Sphk1	ENSRNOG00000010626	NM_133386	Sphingosine kinase 1	0.0882
Apom	ENSRNOG00000000850	APOM_RAT	Apolipoprotein M	0.0850
Psmb8	ENSRNOG00000000456	PSB8_RAT	Proteosome subunit, β type 8	0.0844
Ldhb	ENSRNOG00000013000	LDHB_RAT	Lactate dehydrogenase B	0.0737
Spc21	ENSRNOG00000017036	SPC3_RAT	Microsomal signal peptidase 21 kDa subunit	0.0712
Klf4	ENSRNOG00000016299	NM_053713	Kruppel-like factor 4	0.0669
Cntn4	ENSRNOG00000005652	NM_053746	Contactin 4	0.0508
Ephx1	ENSRNOG00000003515	HYEP_RAT	Epoxide hydrolase 1	0.0493
Serpinb2	ENSRNOG00000002460	PAI2_RAT	Plasminogen activator inhibitor 2	0.0465
Pmp22	ENSRNOG00000003338	PM22_RAT	Peripheral myelin protein 22	0.0337
Pde1c	ENSRNOG00000012337	CN1C_RAT	Phosphodiesterase 1C	0.0322
Ngfrap1	ENSRNOG00000012646	NM_053401	Nerve growth factor receptor associated protein 1	0.0218
Hspb1	ENSRNOG00000023546	HS27_RAT	Heat shock 27 kDa protein 1	0.0177

The complete data of the cDNA microarray analysis is available upon request.

Table IV. Gene Ontology analysis – Molecular function.

GO Term	Total	P-value	Gene	Input symbol
GO:0004852 uroporphyrinogen-III synthase activity	1	0.0043	Uros	Rn30016380
GO:0000900 translation repressor activity	1	0.0043	Purb	Rn30006362
GO:0005131 growth hormone receptor binding	1	0.0086	Socs2	R002975_01
GO:0030161 calpain inhibitor activity	1	0.0086	Cast	R001975_01
GO:0046980 tapasin binding	1	0.0086	Tap2	Rn30000347
GO:0004308 exo-α-sialidase activity	1	0.0129	Neu1	R003273_01
GO:0005518 collagen binding	1	0.0172	Serpinh1	R003232_01
GO:0008538 proteasome activator activity	1	0.0172	Psme1	Rn30017518
GO:0008243 plasminogen activator activity	1	0.0214	Plau	Rn30009672
GO:0019838 growth factor binding	1	0.1738	axl	Rn30019093

Table V. Gene Ontology analysis – Biological process.

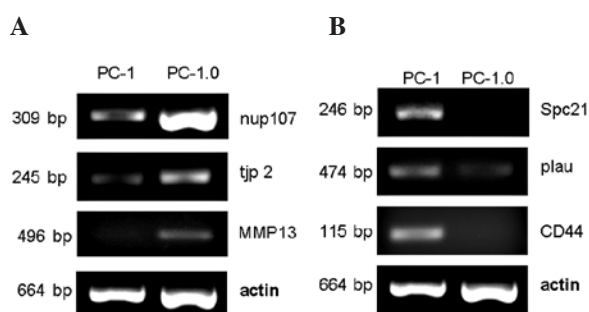| GO Term	Total	P-value	Gene	Input symbol
GO:0015914 phospholipid transport	1	1.10E-4	Plscr1	Rn30007316
GO:0006983 ER overload response	1	1.83E-4	Ddit3	Rn30006089
GO:0006955 immune response	4	0.0068	Tap2	Rn30000347
			Ada	R004405_01
			Psme1	Rn30017518
			Plscr1	Rn30007316
GO:0007034 vacuolar transport	1	0.0086	Vps26a	Rn30000282
GO:0031100 organ regeneration	1	0.0172	axl	Rn30019093
GO:0007520 myoblast fusion	1	0.0172	Cast	R001975_01
GO:0009968 negative regulation of signal transduction	2	0.0183	Socs2	R002975_01
			Rgs10	Rn30018565
GO:0050892 intestinal absorption	1	0.0257	Vdr	Rn30007787
GO:0001558 regulation of cell growth	1	0.0331	Igfbp6	Rn30010107
GO:0035023 regulation of Rho protein signal transduction	1	0.0382	Net1	Rn30016337

Figure 1. Validation of cDNA microarray data by RT-PCR. (A) The expression levels of up-regulated genes (nup107, tjp2 and MMP13) in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. (B) The expression levels of down-regulated genes (Spc21, plau and CD44) in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells.

genes (nup107, tjp2 and MMP13) and three down-regulated genes (Spc21, plau and CD44) to measure their expression levels by RT-PCR. The results were very similar to the cDNA microarray data on these genes and supported the reliability of our expression data (Fig. 1).

Gene Ontology and Pathway analysis of differentially expressed genes. Gene Ontology (GO) and Pathway analysis was applied in order to generate groups of genes that belong to similar biological processes correlated with invasion and metastasis of pancreatic cancer cells.

The differentially expressed genes between highly (PC-1.0) and weakly (PC-1) invasive and metastatic cells were summarized in Molecular function, Biological process and Cellular component, respectively. These are the three types of categories of GO analysis. The ten most correlated (the lowest P-value) GO categories of Molecular function, Biological Process and Cellular Component are presented in Tables IV, V and VI, respectively.

In addition, Pathway analysis of differentially expressed genes was also applied using the public database (Kegg and GenMAPP). The ten most correlated pathways obtained from

Table VI. Gene Ontology analysis – Cellular component.

Go Term	Total	P-value	Gene	Input symbol
GO:0005923 tight junction	1	0.0156	Tjp2	Rn30013789
GO:0005788 endoplasmic reticulum lumen	1	0.0340	Tap2	Rn30000347
GO:0030904 retromer complex	1	0.0043	Vps26a	Rn30000282
GO:0008537 proteasome activator complex	1	0.0172	Psme1	Rn30017518
GO:0005662 DNA replication factor A complex	1	0.0040	Purb	Rn30006362
GO:0042589 zymogen granule membrane	1	0.0214	Scamp1	R004473_01
GO:0005793 ER-Golgi intermediate compartment	1	0.0506	Serpinh1	R003232_01
GO:0005905 coated pit	1	0.0949	Vldlr	Rn30025704
GO:0019717 synaptosome	1	0.0382	Vamp3	Rn30017017
GO:0016020 membrane	1	0.6022	axl	Rn30019093

Table VII. Pathway analysis – Kegg.

Pathway name	Total	P-value
Pentose and glucuronate interconversions	4	0.0000
Antigen processing and presentation	8	2.7E-5
Starch and sucrose metabolism	3	3.15E-4
Porphyrin and chlorophyll metabolism	2	3.65E-4
Sphingolipid metabolism	5	3.65E-4
Fructose and mannose metabolism	5	9.51E-4
Phenylalanine, tyrosine and tryptophan biosynthesis	3	0.0011
Type I diabetes mellitus	4	0.0028
Metabolism of xenobiotics P450 by cytochrome	3	0.0028
SNARE interactions in vesicular transport	4	0.0035

Table VIII. Pathway analysis – GenMAPP.

Pathway name	Total	P-value
GTP binding	23	2.0E-6
Guanyl nucleotide binding	23	3.0E-6
Cytosol	15	4.0E-6
Endoplasmic reticulum	23	7.0E-6
Binding	21	8.0E-6
Cytoplasm	21	7.30E-5
Electron transport	20	2.11E-4
Magnesium ion binding	11	2.74E-4
Protein folding	14	3.52E-4
RNA binding	20	3.69E-4
Metabolism	21	3.73E-4

the Kegg and GenMAPP are listed in Tables VII and VIII, respectively.

The complete data of the GO and Pathway analysis is available upon request.

Discussion

To date, there have been some reports regarding the molecular mechanisms involved in the development of pancreatic cancer, including some reports utilizing cDNA microarray (6,7). However, thus far, most of these cDNA microarray studies have focused on the differences between pancreatic cancer tissue and normal tissue (8); few studies have investigated the mechanism of invasion and metastasis in pancreatic cancer cells using highly and weakly invasive and metastatic pancreatic cancer cell lines. Yet, these tissue samples have considerable disadvantages. They are highly complex and are usually composed of several different cell types and extracellular matrices; for example, non-neoplastic pancreatic tissue includes ductal and acinar cells, various neuroendocrine cells and mesenchymal cells. Thus, one has to be aware that using samples of tissue homogenates does not simply mean a comparison of neoplastic vs. non-neoplastic epithelial cells, but a complex mixture of genes of diverse origin, some of them deriving from epithelial cells. In contrast, one advantage of using cancer cell lines is that pure tumor cells are tested without any contamination from surrounding stromal elements.

In particular, the highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cell lines, which are established from the experimental pancreatic cancer model in our previous study (1,2), show an obviously different potential for invasion and metastasis (9,10). Therefore, this cell line model is suitable for the investigation of invasion-metastasis-related specific factors in pancreatic cancer.

In the present study, using cDNA microarray analysis, we found that a total of 141 genes were differentially expressed between the PC-1.0 and PC-1 cells, including 46 up-regulated genes and 95 down-regulated genes. We selected several differentially expressed genes (nup107, tjp-2, MMP-13, Spc21, plau and CD44) for validation by RT-PCR. The results of RT-PCR were in accordance with those of the cDNA microarray analysis. In addition, several of the identified genes (i.e., MMP-13, plau and CD44) have been previously reported to be correlated with invasion and metastasis (11-13), and the other differentially expressed genes (i.e., nup107, tjp-2 and Spc21) have not been reported to be associated with the invasion-metastasis of pancreatic cancer.

Of the identified genes not previously reported to be associated with the invasion-metastasis of pancreatic cancer, Nup107 is a critical component of the nucleoporin 107-160 subcomplex, which is the key building block of the nuclear-pore complex (NPC). From yeast to humans, the function of NPC is the regulation of nuclear import and export (14). The Nup107-160 complex thus additionally offers an attractive point for regulation of nuclear pore complex assembly (15). Although nup107 has been identified from the comparison of gene expression in highly and weakly invasive and metastatic pancreatic cancer cells in the present study, the molecular mechanism of involvement of nup107 in the invasion-metastasis of pancreatic cancer needs to be further tested and assessed.

Several studies have demonstrated that tight junction proteins (TJPs) associate with each other and directly and/or indirectly to actin filaments (16) and also recruit factors involved in signal transduction and the regulation of proliferation and differentiation (17). The zonula occludens (ZO) protein is one of the tight junction proteins and belongs to the membrane associated guanylate kinase-like (MAGUK) protein family. It includes three members, TJP1/ZO-1, TJP2/ZO-2 and TJP3/ZO-3 (18). mRNA levels of ZO-2 were found to be elevated in tumor tissues compared with controls using quantitative PCR. Moreover, ZO-2 exhibits a 23-amino acid truncation at the N-terminus, which may play a role in limiting tumor development in pancreatic cells. In another investigation, ZO-2 was found to be associated with the progression of breast cancer (19).

Moreover, Spc21 was identified as a down-regulated gene in this study, suggesting that dysregulation of this gene is likely to be associated with the invasion and metastasis of pancreatic cancer cells. Fish and ISH analysis for this gene demonstrated a significant correlation between genetic deletion and corresponding mRNA down-regulation, raising the possibility that the Spc21 gene may play a putative role as a tumor suppressor (20). However, little is known about the biological role of this gene, although it belongs to the peptidase S26B family and functions as part of the signal peptidase complex (20).

In conclusion, our results suggest that a highly organized and structured process of invasion and metastasis exists in the pancreas. Analysis of gene expression profiles by cDNA microarray can provide useful information for clarifying the mechanism underlying the invasion and metastasis of pancreatic cancer cells. Furthermore, the identification of invasion-metastasis-specific genes may allow us to develop new therapeutic and diagnostic targets for the invasion-metastasis of pancreatic cancer.

Acknowledgements

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