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# Isolation of tissue-type plasminogen activator, cathepsin H, and non-specific cross-reacting antigen from SK-PC-1 pancreas cancer cells using subtractive hybridization

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**Abstract** **We have used subtractive hybridization to isolate cDNAs overexpressed in SK-PC-1 pancreas cancer cells. Forty-five independent clones corresponding to 11 genes were identified. Their expression in cultured pancreas cancer cells, normal pancreas tissue, and normal exocrine pancreas cultures was examined by Northern blotting. cDNA clones can be grouped into two broad categories: (1) those corresponding to genes expressed at high levels both in tumor cell lines and in primary cultures of normal pancreas, but not in normal tissue (i.e. thymosin β4[3], cytokeratin 18, β-actin, pyruvate kinase and mitochondrial genes); and (2) those corresponding to genes expressed at high levels in pancreas cancer cultures but not in normal pancreas tissue or cultured cells (i.e. tissue-type plasminogen activator and cathepsin H). The overexpression of these proteases in pancreas cancers suggests that they play a role in the aggressive biological behavior of this tumor.**

*Key words:* Pancreas cancer; Tissue-type plasminogen activator; Thymosin β4; Non-specific cross-reacting antigen; Subtractive hybridization

## 1. Introduction

Pancreas cancer is a highly aggressive tumor that currently represents the fourth cause of cancer death in the United States. The majority of tumors arise in the exocrine component and are classified as 'ductal adenocarcinomas' on the basis of their microscopic appearance [1]. Despite the fact that a number of molecular alterations have been reported which are associated with this tumor, including mutations in K-*ras*, p53, and MTS-1 genes [2], there are few clues about the aggressive nature of its biological behavior. Epigenetic alterations also contribute to tumor progression. For example, overexpression of growth factor receptors and their ligands, such as c-*met*/hepatocyte growth factor, can lead to the activation of autocrine and paracrine loops enhancing tumor cell motility, protease secretion, and invasiveness in vitro and in experimental models [3]. The biological relevance of these alterations is suggested by their association with a worse prognosis [4].

To get further insight into the molecular basis of pancreas cancer, we have sought to identify cDNAs which are overexpressed in pancreas cancer cells and may have a role in tumor progression. Several facts hamper the use of pancreas cancer tissue for these studies: (1) very few patients undergo tumor resection and it is difficult to obtain sufficient tissue to prepare a cDNA library, (2) unlike other tumors, surgical specimens from pancreas cancer generally contain a substantial proportion of non-neoplastic epithelial cells (i.e. ductal proliferation with the histological appearance of chronic obstructive pancreatitis), inflammatory cells, and connective tissue. For example, in a recent study of pancreas cancer tissues, only 7/33 microdissected specimens contained 50% of tumor cells and could be subjected to allelotypic analysis [5]. Because of these difficulties, we have performed a subtraction between transcripts from SK-PC-1 pancreas cancer cells and normal pancreas tissue. SK-PC-1 cells were chosen because they were derived from a ductal adenocarcinoma and grow as a monolayer of polarized cells, develop transepithelial resistance, and express molecular markers characteristic of ductal cells such as cytokeratins and mucins [6]. In addition, they contain double minutes and may harbor the amplification of genes important for tumor progression [6].

We report here the isolation and identification of the cDNA clones obtained with this strategy as well as their expression in pancreas cancer cultures displaying a wide spectrum of phenotypes and in normal exocrine pancreas cultures recently developed in our laboratory [7].

## 2. Materials and methods

### 2.1. Cell culture

Tumor cell lines were maintained in Dulbecco's modified Eagle's medium (gibco-BRL, Gaithersburg, MA) supplemented with 10% fetal bovine serum (Gibco-BRL) at 37°C in 5% $CO_2$ atmosphere. Pancreas cancer cell lines IMIM-PC-1, IMIM-PC-2, SK-PC-1, and SK-PC-3 have been described recently [6]. Normal exocrine pancreas cultures displaying a ductal cell phenotype were established as described elsewhere [7].

### 2.2. RNA isolation

Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction procedure [8]. Fresh normal pancreas tissue was obtained from organ donors and processed as described by Vilá et al. in order to isolate the exocrine component [7]. Poly $(A)^+$ RNA was prepared with the polyATract mRNA Isolation System (Promega, Madison, WI) according to manufacturer's instructions. Poly $(A)^+$ RNA from fresh pancreatic tissue was prepared by affinity chromatography on oligo(dT)-cellulose columns.

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**R.P. and G.B. share first authorship having made equivalent contributions to this article.

*Abbreviations:* Tβ4, thymosin β4; tPA, tissue type plasminogen activator; NCA, non-specific cross-reacting antigen; MTS-1, multiple tumor suppressor 1 gene; HMG1, high mobility group 1; CEA, carcinoembryonic antigen; CK, cytokeratin.


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*2.3. Subtractive hybridization*

cDNAs were synthesized on oligo(dT)-Dynabead particles (Dynal, Oslo, Norway) from SK-PC-1 and normal pancreatic tissue poly $(A)^+$ RNA. Target cDNA (SK-PC-1) labelling and subtractive hybridization were carried out as described by Rodriguez and Chandler, with minor modifications [9]. Briefly, target cDNA was used to synthesize radioactive fragments by random priming [10] with Ready-Prime using [$\alpha$-$^{32}$P]dCTP (Amersham Iberica, Madrid, Spain); labelled fragments were recovered by elution and hybridized to an excess of normal pancreas tissue cDNA (driver) immobilized onto Dynabeads for two consecutive rounds. Non-hybridized fragments were recovered and used as a probe to screen a SK-PC-1 cDNA library.

*2.4. Construction and screening of the cDNA library*

Poly $(A)^+$ RNA from 2 day post-confluent cultures of SK-PC-1 cells was used to construct a library in LambdaZAP II according to the manufacturer's instructions (Stratagene, La Jolla, CA). The library was amplified before use. The subtracted probe was used to screen the SK-PC-1 cDNA library according to standard methods [10]. Positive clones were picked and subjected to three rounds of purification using the labelled subtracted probe. Isolated clones were excised in vivo and recovered as Bluescript plasmids. For dot hybridization, 10 ng of plasmid DNA from each isolated clone was spotted onto a nylon Hybond-N membrane (Amersham) and hybridized to labelled cDNA probes.

*2.5. Northern blot analysis*

Total RNA (15 μg) from cultured cells or tissues was electrophoresed on 1% agarose-formaldehyde gels, transferred to nylon filters, and hybridized to [$^{32}$P]dCTP labelled probes. Hybridizations were carried out overnight at 42°C, followed by washing at 60°C to final 0.2×SSC, and then autoradiography. The quality and quantity of the transferred RNA were assessed by ethidium bromide staining.

*2.6. DNA sequencing*

Sequencing of both strands of the isolated cDNA clones was performed by the dideoxynucleotide chain termination method either automatically, in an Applied Biosystem 373A DNA sequencer with Taq polymerase, or manually with Sequenase (United States Biochemical, Cleveland, OH) using universal primers (M13, T3, and T7) or internal specific primers. Sequences were compared against databases with the FASTDB and BLAST algorithms.

## 3. Results and discussion

We used two rounds of hybridization of the transcribed cDNA derived from SK-PC-1 cells with the cDNA derived from normal pancreatic tissue to obtain a probe enriched for genes overexpressed in the tumor cells. This probe was used to screen a SK-PC-1 cDNA library and led to the isolation of 45 clones, designated HPT for human pancreas tumor. Dot blotting analysis of several isolated clones (Fig. 1) confirmed that this method effectively enriched the probe for transcripts overexpressed in the tumor cells. All clones were purified and 300–400 nucleotide long sequences were obtained in order to determine identity to known genes. The results of such analysis, the homology to known genes and the expression pattern of each clone in cultured cells and tissue are summarized in Table 1.

The pattern of expression of the isolated clones was examined by Northern blotting with total RNA from SK-PC-1 and normal pancreas tissue: all isolated cDNAs hybridized preferentially with RNA from the tumor cells. To examine the relationship between the levels of expression, cell differentiation, and the transformed phenotype, we used four pancreatic tumor cell lines selected on the basis of their differentiation properties. SK-PC-1 and IMIM-PC-2 cells grow as a polarized monolayer, form domes, develop transepithelial resistance when cultured on permeable filters, and express markers typical of pancreatic ductal cells such as cytokeratin (CK) 7 and the MUC1 glycoprotein, indicating that they are well differentiated. In contrast, IMIM-PC-1 and SK-PC-3 cells are not polarized, pile up, and express low or undetectable levels of CK7 and MUC1, indicating that they are less differentiated [6]. In addition, we examined transcript levels in RNA samples from primary cultures of normal exocrine pancreas recently developed in our laboratory. These cells proliferate in vitro during 8–10 days and can be maintained for up to one month [7]. During the first days of culture, a phenotypic switch takes place which involves loss of acinar characteristics and the acquisition of a phenotype that is undistinguishable from that of normal pancreatic duct cells: growth as a polarized monolayer, expression of CK7, CK19, MUC1, and the cystic fibrosis transmembrane conductance regulator, and response to secretin [7].

Table 1. Identification and pattern of expression of cDNA clones obtained from screening the SK-PC-1 library with 'tumor enriched sequence' probes

| Homology | SK-PC-1 | Normal pancreas | | $n$[a] |
|---|---|---|---|---|
| | | Tissue | Culture | |
| tPA | +++[b] | – | – | 3 |
| NCA | ++ | – | – | 2 |
| Cathepsin H | +++ | ± | + | 1 |
| HMG1 | ++ | + | + | 1 |
| Pyruvate kinase | + | – | + | 2 |
| α-Tubulin | +++ | ± | ++ | 2 |
| Mitochondrial RNAs | +++ | + | ++ | 5 |
| β-Actin | ++ | ± | +++ | 3 |
| Ferritin H chain | +++ | ± | +++ | 3 |
| CK18 | +++ | + | +++ | 3 |
| Tβ4 | +++ | + | +++ | 20 |

[a]*n* indicates the number of independent clones isolated homologous to the same gene.

[b]The level of expression for each clone was visually estimated from the intensity of the autoradiographic signals obtained by dot blot or Northern hybridization.

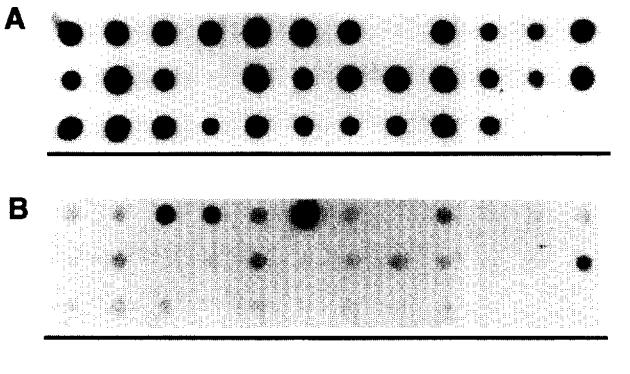


Fig. 1. Expression of SK-PC-1-derived purified clones analyzed by dot blotting. cDNA clones were spotted onto replica nitrocellulose filters and hybridized to labeled SK-PC-1 cDNA (A) and to labeled normal pancreas cDNA (B). The position of each clone is indicated in the bottom panel.

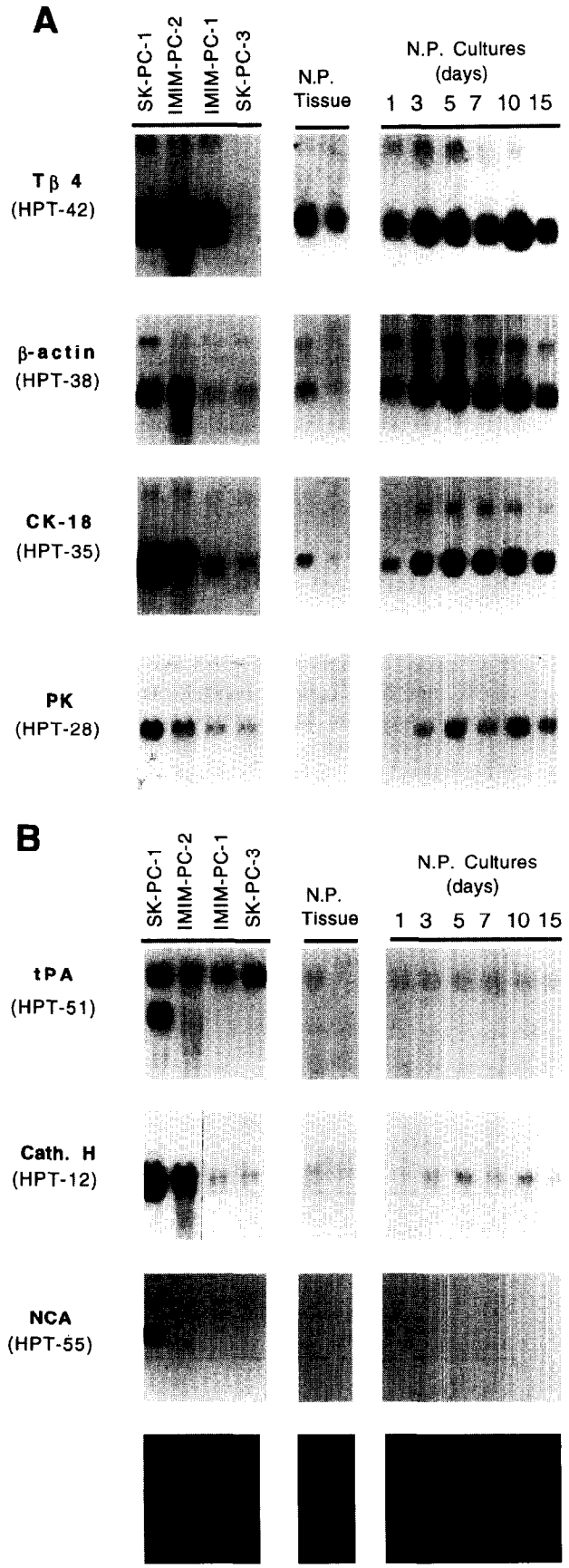


Fig. 2. Northern blotting analysis of the expression of selected clones in tumor cell lines and normal pancreas. Total RNA (15 μg) from the indicated tumor cell lines, two independent specimens of normal exocrine pancreas tissue (N.P.), and normal exocrine pancreas epithelium cultured for various time periods, was transferred to nylon filters and hybridized to: (A) Tβ4, β-actin, CK18, and pyruvate kinase (PK); (B) tPA, a 400 bp fragment from the 5′ region of the HPT-51 clone, cathepsin H, and NCA. A slower migrating band in the hybridization with the tPA probe results from cross-hybridization with 28S ribosomal RNA given by the high GC content of the probe used. Photographs of the 28S and 18S ribosomal RNA in ethidium bromide-stained gels are shown below the autoradiographs to normalize RNA deposits.The same filters were utilized to examine the expression of all clones shown in panels A and B. After each hybridization reaction, filters were stripped by incubation in boiling distilled water for two consecutive rounds, dried, and exposed for autoradiography for 48 h to evaluate for complete stripping before a new probe was added.
←

As summarized in Table 1, the clones isolated from the SK-PC-1 library can be classified into two broad categories according to the levels of expression of the corresponding transcripts: (1) those expressed at high levels both in differentiated pancreas tumor cells and in normal primary cultures, but not in normal tissue, and (2) those expressed at high levels in differentiated pancreatic tumor cells but not in normal tissue or normal primary cultures.

### *3.1. Clones corresponding to genes expressed at high levels in cultures of both pancreas cancer and normal pancreas epithelium*

This group includes genes whose products are involved in the regulation and formation of the cytoskeleton (Tβ4, β-actin, CK18, α-tubulin) and in cell metabolism (pyruvate kinase, mitochondrial genes, ferritin heavy chain, and HMG1). With few exceptions, the levels of expression of these transcripts are low or undetectable in normal pancreas tissue (see Table 1 and Fig. 2A).

#### *3.1.1. Genes encoding cytoskeleton-associated proteins.*

CK18 and β-actin transcripts are detected in all pancreas cancer cell lines, although higher levels are present in those with a more differentiated phenotype. Lower levels of expression are observed in normal pancreas tissue and in normal exocrine pancreas cultures. In the latter, CK18 and β-actin mRNAs are induced around days 3–5 of culture, as shown in Fig. 2. It is possible that their expression is activated as a consequence of cell proliferation in vitro; however, the high levels of expression detected at day 15 (Fig. 2A and data not shown), when normal epithelial cells no longer proliferate, do not support this hypothesis. CK18 protein has been detected in all pancreas cancer tissues and cell lines examined [6,11] although there has been no systematic study aimed at quantitating RNA or protein levels, or at carefully assessing its relationship to cell differentiation. Despite the fact that transcriptional activation of CK18 has been reported in cells transformed by mutated Ha-*ras* [12], our results do not support a relationship between CK18 levels and *ras* gene mutations in the four pancreas cancer cell lines examined since all of them harbor a mutated K-*ras* gene [13].

A remarkable finding was the very high proportion of the isolated cDNA clones (20/45) corresponding to Tβ4, possibly a reflection of the very high levels of expression detected, particularly in cultures of pancreas cancers and normal pancreas epithelium (Table 1 and Fig. 2A). In the exponential

growth phase, the levels of Tβ4 detected in pancreas cancer cells were much higher than in other lines studied, i.e. HT-29 colon cancer and SK-MEL-23 melanoma (data not shown). Furthermore, high levels were also detected in normal pancreas epithelium at days 10–15 of culture. Thus, although Tβ4 expression has been associated with cell proliferation in lymphoid cells [14], our data do not support a straightforward relationship between the increased expression of this transcript and cell proliferation. The precise significance of this overexpression in tumor cells is unknown. Beta-thymosins are the major actin-binding proteins in cells [15] and a role in differentiation and metastasis has been proposed in a variety of cellular systems. In astrocytes and microglia from rat cerebellum, Tβ4 and Tβ10 are selectively regulated during cell differentiation and their tissue expression pattern is maintained in cultured cells [16]. Tβ10 has also been isolated by subtractive cloning from metastatic melanoma cells and its participation in tumor metastasis through regulation of actin polymerization has been proposed in this tumor system [17].

*3.1.2. Genes whose products are involved in cell metabolism.* The expression of M1- and M2-type pyruvate kinase, ferritin heavy chain, and mitochondrial tRNAs is high both in pancreatic tumor cells and in normal cultures (Table 1 and Fig. 2A). Recent reports have implicated both pyruvate kinase and mitochondrial genes in cancer development. Increased expression and genomic insertion of mitochondrial genes have been described in association with cancer [18]. High expression of these genes has also been described in trophoblasts from normal placenta, a tissue defined as pseudomalignant, and it has been proposed that increased expression of these genes may be an early event in cancer development [19]. The L-pyruvate kinase gene is slightly active in the normal and neoplastic endocrine pancreas but it is not transcribed in the normal exocrine pancreas in vivo, despite the fact that its promoter remains in a permissive state for re-expression [20]. Our results show that both normal cultures and cancer cells express pyruvate kinase and mitochondrial genes at elevated levels when compared with the normal tissue (Table 1 and Fig. 2A), suggesting that such overexpression may not be directly related to the neoplastic state. Instead, it seems to associate better with the ductal phenotype of pancreas cancer and the acinar-to-ductal phenotypical switch accompanying the culture of normal pancreas epithelium.

### 3.2. Clones corresponding to genes expressed at high levels exclusively in tumor cells

This group comprises cDNAs encoding tissue-type plasminogen activator (tPA), cathepsin H, and non-specific cross-reacting antigen (NCA). Fig. 2B shows that tPA transcripts are present at high levels in SK-PC-1 cells, at lower levels in IMIM-PC-2 cells, and are undetectable in less differentiated pancreas cancer cells, in normal pancreas tissue and normal exocrine pancreas cultures. High levels of tPA, similar to those of SK-PC-1 cells, have been detected in MZPC-2 and MZPC-3 (data not shown), two cell lines which also display a differentiated ductal phenotype.The relevance of the findings reported above is strengthened by the fact that tPA overexpression at the RNA and protein levels occurs both in pancreas cancer cells and tissues (R.P., unpublished results). These results indicate that overexpression of tPA is associated with the neoplastic phenotype rather than with the differentiation program activated in cells derived from the exocrine pancreas. Secreted proteases have a prominent role in the degradation of extracellular matrix components during tissue remodelling and tumor invasion [21]. Overexpression of the plasminogen activator urokinase, together with its receptor, has been associated with invasive tumor cells in many types of cancers [22], and inhibition of receptor expression by antisense oligonucleotides results in a reduction of invasiveness [23,24]. In addition, uPA levels appear to be a prognostic factor in several types of cancer [22]. Fewer studies have analyzed the expression of tPA in tumors and its possible role in cancer progression, possibly because this protease is thought to participate in plasmin activation mainly in the vascular compartment [25]. In this regard, tPA expression has been reported in ovarian cancer cultures but not in normal ovary epithelium [26].

High levels of expression of cathepsin H gene are also detected in pancreas cancer cells showing a well differentiated phenotype, but not in less differentiated cancer cells nor in normal cultures (Fig. 2B). Little information is available regarding the expression of the thiol proteases in normal pancreas and pancreas cancer. Yamaguchi et al. have reported the secretion of cathepsin L by HPC-YP pancreas cancer cells and have suggested that a dimer of the mature form and the proenzyme is formed. However, no comparison with normal pancreas was made [27]. Tumor cells often secrete high levels of lysosomal enzymes such as cathepsins, indicating a defective intracellular processing. Upon secretion, these proteases may also contribute to the degradation of pericellular matrix and to tumor cell invasiveness [28]. In the context of these observations, our results indicate that overexpression of tPA and cathepsin H, and possibly other proteases that have been identified recently [29], is associated with cancer of the exocrine pancreas.

The expression pattern of NCA was very similar to that of tPA: high levels in well differentiated cell lines, but not in poorly differentiated cell lines, and undetectable levels in normal pancreas cultures (Fig. 2 and data not shown), indicating a relationship to the neoplastic phenotype rather than to cell differentiation. In a recent report, a mouse monoclonal antibody detecting NCA-related molecules showed strong reactivity with pancreas cancers and cDNA clones corresponding to the NCA gene have been isolated from a cDNA library derived from GER pancreas cancer cell line [30], supporting our own findings. NCA belongs to the carcinoembryonic antigen (CEA) family together with CEA, biliary glycoprotein, the CEA-gene family members, and pregnancy-specific glycoprotein. CEA and biliary glycoprotein mediate homophyllic interactions but the function of NCA and the other members of the CEA family is currently unknown. Antibodies have been extensively used to examine the expression of molecules belonging to the CEA family in normal and tumor tissues but the interpretation of these studies is hampered because of the immunological relatedness of CEA and NCA. Recent observations utilizing monoclonal antibodies that distinguish between the two proteins suggest that NCA is more widely overexpressed in human tumors than CEA [31,32].

## 4. Conclusions

Our strategy has led to the isolation of cDNAs corresponding to transcripts overexpressed in pancreas cancer. Those related to proteins involved in the structure and regulation

of the cytoskeleton and in cell metabolism appear to associate with the ductal differentiation observed in most tumors of the exocrine pancreas. Others, such as tPA, cathepsin H, and NCA, seem to be more directly related to the neoplastic phenotype. Because Southern blotting failed to reveal any evidence for amplification of the genes encoding tPA or NCA (data not shown), other mechanisms are likely to account for the high transcript levels detected and more work is necessary in this area.

The results of this study provide new clues to the study of cancer of the exocrine pancreas. Detection of the products of genes that are overexpressed may provide important information and be useful in monitoring the disease.

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