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Identification of Genes With Specific Expression in Pancreatic Cancer by cDNA Representational Difference Analysis

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cDNA representational difference analysis (cDNA-RDA) is a polymerase-chain-reaction-coupled subtractive and kinetic enrichment procedure for the isolation of differentially expressed genes. In this study, the technique was used to isolate novel genes specifically expressed in pancreatic cancer. cDNA-RDA was done on cDNA reverse transcribed from a poly(A)$^+$ mRNA pool made from 10 cancer tissues (tester) by using as a driver a cDNA from a poly(A)$^+$ mRNA pool made from a combination of 10 tissues of chronic pancreatitis and 10 healthy pancreatic tissues. The use of chronic pancreatitis in addition to healthy pancreas mRNA in the driver preparation eliminated the influence of stromal tissue components present as contamination in the cancer-specific preparations. Such cDNA-RDA led to the isolation of 16 distinct, cancer-specific gene fragments. These were confirmed to be overexpressed in pancreatic cancer tissues by Northern blot analysis. Sequence analysis revealed homologies to five genes previously implicated in the carcinogenesis of the pancreas or other tissues. Eleven fragments had no significant homology to any known gene and thus represent novel candidate disease genes. The experiments demonstrate that cDNA-RDA is a reproducible and highly efficient method for the identification of novel genes with cancer-specific expression. *Genes Chromsom. Cancer 19:97–103, 1997.*

INTRODUCTION

Pancreatic cancer is the fifth most common cause of cancer-related deaths in industrialized countries, with a dismal prognosis, an increasing incidence, and no or only ineffective means of treatment (Myers et al., 1989; Murr et al., 1994). The development of new treatment modalities and diagnostic and preventive approaches requires an understanding of the molecular mechanisms of the complex multistep process of tumorigenesis in the pancreas. Despite its importance as a health problem with a major socioeconomic impact, the mechanisms of carcinogenesis in pancreas are poorly understood. Thus, it is of major importance to identify novel genes involved in pancreatic carcinogenesis. In this context, the analysis of differences between tumor and healthy tissue at the level of mRNA offers a very good prospect for the isolation of candidate disease genes.

Two methods based on polymerase chain reaction (PCR) are available to identify changes of gene expression between cancer and normal tissues. Differential-display PCR (DD-PCR; Liang and Pardee, 1992) relies on random-primed amplification of total RNA of two different populations, aiming at the identification and isolation of differential bands in sequencing gels. Representational difference analysis (RDA) is a combination of subtraction and kinetic enrichment coupled to subsequent amplification, originally developed for genomic DNA to isolate differences between complex genomes (Lisitsyn et al., 1993, 1995). Whereas DD-PCR amplifies fragments from all represented mRNA species, RDA has the advantage of eliminating fragments present in both populations, leaving only the differences. Recently, RDA protocols were adapted to the examination of differential gene expression between two mRNA populations (Hubank and Schatz, 1994). This technique offers the means to reduce rapidly the number of candidate genes in a highly specific manner. The aim of the present study was to use RDA on cDNA populations to isolate novel differentially expressed genes of potential importance to biomedical analyses of pancreatic carcinomas.

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MATERIALS AND METHODS

Pancreatic Tissues

Human pancreatic tissue from patients with adenocarcinoma of the pancreas and chronic pancreatitis and from organ donors was provided by the Department of Visceral and Transplantation Surgery of the University of Bern (Switzerland) with written consent from each patient and after approval by the local ethics committee. The histological diagnosis was confirmed for each individual tissue block by standard light microscopic evaluation of sections stained with hematoxylin and eosin.

Oligonucleotides for RDA

The sequences of the primer and adapter oligonucleotides were: R-Bam-24, d(AGCACTCTCCAGCCTCTCACCGAG); R-Bam-12, d(GATCTGCCGTGA); J-Bgl-12, d(GATGTGTTCATG); J-Bgl-24, d(ACCGACGTCGACTATCCATGAACA); N-Bgl-12, d(GATCTTCCCTCG); and N-Bgl-24, d(AGGCAACTGTGCTATCCGAGGGAA).

RNA Extraction

RNA from shock-frozen human pancreatic tissues was extracted by using a standard guanidinium thiocyanate extraction, followed by centrifugation in a cesium chloride gradient (Pharmacia, LKB, Freiburg, Germany). Poly(A)$^+$-RNA was isolated by using oligo-d(T) coupled to magnetic beads (DYNABEADS™, Dynal®, Germany) according to the manufacturer's instructions. Quality was checked by standard techniques mostly based on gel electrophoresis and hybridizations to the subsequently blotted RNA size separation. Preparations used for the experiments had to be of the same size distribution and purity.

cDNA-RDA

Preparation of representations

cDNA was synthesized by using reverse transcriptase (Superscript, Life Technologies, USA) and oligo-d(T) primers. Two micrograms of the double-stranded cDNA populations were digested with *Dpn*II, phenol extracted and ethanol precipitated, followed by a blunt-end ligation with an adapter molecule (R-Bam-12/24). To allow also the detection of messages present at a higher level in the tester than in the driver (rather than present in the tester only and absent in the driver), very low copy sequences were removed by melt depletion. The ligations were denatured (6 min, 98°C) and reannealed for 2.5 h at 67°C after adding NaCl to 1 M final concentration; under such conditions, very low copy sequences remain single stranded and will not be amplified subsequently. The DNAs were diluted and PCR amplified by using the R-Bam-24 oligonucleotide as primer (20 cycles of 1 min each at 95°C and 3 min at 72°C). PCR products were phenol extracted, ethanol precipitated, and resuspended at 0.5 µg/µl to form the representations. Both tester and driver representations were digested with *Dpn*II to remove the R adapters and then phenol extracted and ethanol precipitated. The tester representations were gel purified and ligated to the J-Bgl-12/24 adapter.

Selective amplification

For the first selection cycle, tester and driver DNA were mixed in a 1:100 ratio. After denaturation (6 min, 98°C), the salt concentration was adapted to 1 M with NaCl and the DNA was allowed to anneal (48 h, 67°C). The DNA was diluted and PCR amplified by using the J-Bgl-24 primer (10 cycles of 1 min at 95° C and 3 min at 70° C). Several identical PCRs were combined, phenol extracted, isopropanol precipitated, resuspended in water, and digested with mung bean nuclease (35 min, 30°C). The digest was heated (5 min, 95°C), chilled on ice, and PCR amplified by using the J-Bgl-24 primer (18 cycles of 1 min at 95°C and 3 min at 70°C). The PCR product was phenol extracted, isopropanol precipitated, and resuspended at 0.5 µg/µl to form the first difference product (DP1). For the generation of difference product 2 (DP2), the J-adapter was replaced by the N-Bgl-12/24 adapter. This DNA was mixed with driver in a 1:800 ratio. Hybridization and PCR amplification (using the N-Bgl-24 primer) were done as described for DP1, except that the extension was done at 72°C. To generate the tester for the last selection cycle, the N adapter was substituted by the J adapter. Such DP2-DNA was mixed with driver in a 1:400,000 ratio. The product of the PCR amplification with the J-Bgl-24 primer formed the final difference product (DP3).

Sequence Analysis

DP3-DNA was electrophoresed in a 2.5% agarose TAE gel. Distinct bands were excised, purified, and cloned into the Bluescript KS plasmid vector (Stratagene, USA). Plasmid DNA was prepared by using the Qiagen plasmid mini kit (Qiagen, Hilden, Germany). Sequencing was done on a 373A Automated Sequencer (Applied Biosystems, Foster City, USA) using T3 and T7 primers (Eurogentec, Seraing, Belgium) and the Prism™ DyeDe-

oxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City) according to the manufacturer's recommendations. BLASTX and BLASTN (Altschul et al., 1990) analyses were used to screen for DNA and protein homologies.

Northern Blot Analysis

Per lane, 30 µg of total RNA from pancreatic cancer, pancreatic control, or chronic pancreatitis tissues were size fractionated on 1.0% agarose, 6% formaldehyde denaturing gels and transferred to Hybond N^+ membranes (Amersham, UK). All Northern blots were hybridized with ^{32}P-labeled probe of 18s rRNA to confirm equal loading of total RNA in the individual lanes. RDA fragments were labeled by random hexamer priming using $\alpha[^{32}P]$dCTP as a radioactive nucleotide. Hybridization was done in 6×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.5), 5×Denhard's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.1 µg/µl yeast t-RNA at 42°C. Stringent washes were done in 0.2×SSC, 0.1% SDS at 65°C. The product was exposed to X-ray films at −70°C for 5–14 days.

RESULTS AND DISCUSSION

For three tissues—pancreatic cancer, chronic pancreatitis, and healthy pancreas—10 tissue samples each were taken from different individuals. Equal fractions of total RNA were pooled to obtain three RNA populations representing the respective tissue type devoid of interindividual differences as much as possible, thus concentrating the study more on common differences among the three tissue types. After mRNA isolation and cDNA synthesis, the DNA was digested with *Dpn*II (Hubank and Schatz, 1994) because RDA relies on the generation of simplified versions of reduced complexity (representations) of the initial cDNA populations. Subsequent to the addition of adapter molecules, the fragments were PCR amplified. The following representations were prepared: *Tester* (10 pancreatic cancer tissues), *Driver 1* (10 healthy pancreatic tissues), and *Driver 2* (10 healthy pancreatic tissues plus 10 chronic pancreatitis tissues).

For each RDA, the *Tester* was mixed with one of the driver representations; by an iterative process of subtractive and kinetic enrichment and subsequent selective PCR amplification (Hubank and Schatz, 1994), DNA fragments that were present in the tester and absent in the driver were selected. The selectivity of the procedure is influenced by the ratio of tester to driver, with values as high as 1:8,000,000 having been reported for genomic RDA (Lisitsyn et al., 1995). However, sequences overexpressed in cancer tissues may not all be completely absent from normal tissues and could be eliminated by using such very stringent conditions. By using an adapted version of the RDA protocol, the detection of differences in the magnitude of expression rather than absolute differences was possible. Prior to RDA, the representations were subjected to melt depletion, the rationale being to suppress further patient-specific differences within each representation. This procedure leaves very rare sequences single stranded and thus nonamplifiable. The tester: driver ratios were checked during the experiments and adjusted where necessary. After three cycles of RDA with ratios of 1:100, 1:800, and 1:400,000, distinct bands could be seen in agarose gels of the differing products with decreasing amounts of background signal (Fig. 1). These bands were excised, cloned, sequenced, and hybridized onto Northern blots to verify the expression patterns (Fig. 2).

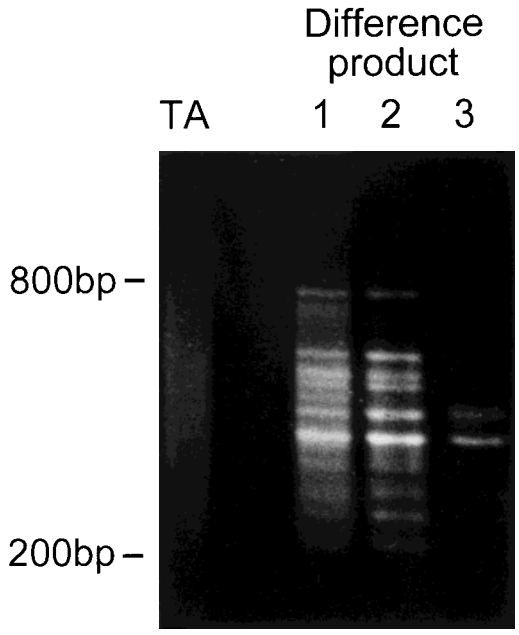

Figure 1. Agarose gel electrophoresis of the original *Tester* amplicon (**TA**) and the difference products, obtained after the first (**1**), second (**2**), and third (**3**) cycles of RDA with *Driver 2*.

Initially, the *Tester* was analyzed with a driver made from 10 normal pancreatic tissues (*Driver 1*), which led to the isolation of 24 unique gene fragments (Table 1). Most fragments were expressed in both cancer tissue and chronic pancreatitis tissue samples. Chronic pancreatitis is an inflammatory disease of the pancreas characterized by a strong stromal reaction (fibrosis and interstitial inflammation) similar to that observed in pancre-

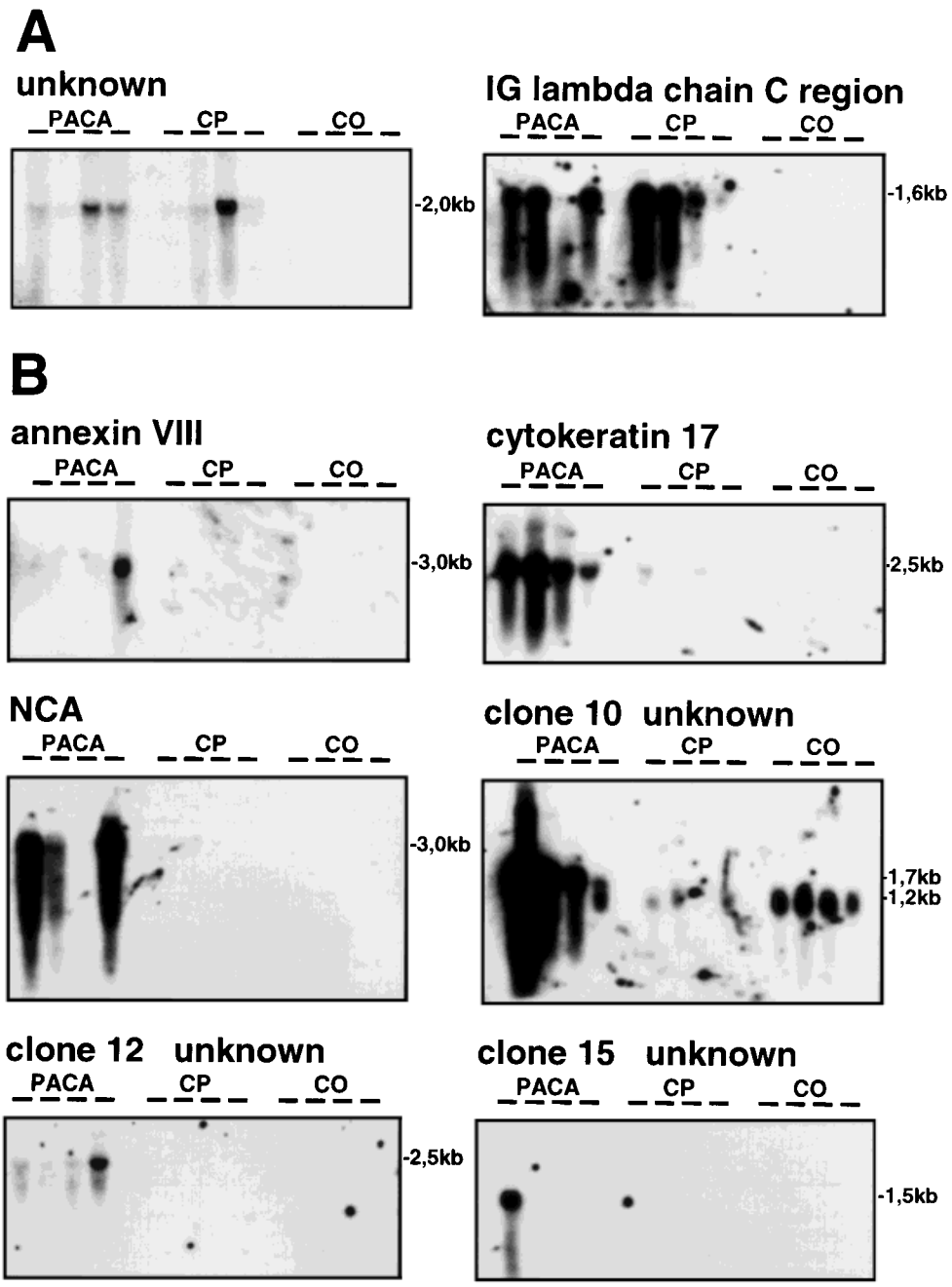

Figure 2. Northern blots of individual RNA preparations (PACA, pancreatic cancer; CP, chronic pancreatitis; CO, normal pancreatic tissue) were hybridized with cloned RDA products. **A:** Expression pattern of two DP3 fragments (clone 13/24, unknown; clone 7/24, Ig-lambda chain C region; see Table 1) obtained from RDA with *Driver 1*, made from healthy pancreas. The method identified gene fragments that were absent in the control tissues. However, they were also expressed in chronic pancreatitis tissue. To suppress this effect, cDNA of pooled mRNA from 10 chronic pancreatitis tissue samples was added to *Driver 1* to obtain *Driver 2*. This method led to the identification of 16 gene fragments with cancer-tissue-specific expression as verified by Northern blot analysis. **B:** Six examples are shown. Clone 10 identified a transcript that exhibited differential splicing in cancer tissue (1.7 kb instead of 1.2 kb).

atic cancer tissues. Sequence analysis revealed that the majority of these fragments were most likely derived from stromal and inflammatory cells (see Table 1 and Fig. 2A) rather than being specific to cancer cells. Due to the strong desmoplastic reaction present in most of the pancreatic cancer tissues (for a review, see Klöppel, 1993), these fragments represent a common problem that has so far hampered attempts to isolate differentially expressed sequences specific for pancreatic cancer.

TABLE 1. Sequences of RDA Fragments Expressed in Pancreatic Cancer and Chronic Pancreatitis Tissues

Clone	Accession	Sequence homology
1/24	U52841	2 collagen VI exon 1
2/24	U52870	EST F12018
3/24	U53081	EST T04974
4/24	U53082	EST R14209
5/24	U53083	FK-506 binding protein
6/24	U53084	human germline oligomeric matrix protein
7/24	U53085	Ig lambda chain C region
8/24	U53086	Ig 4b
9/24	U53087	pro-1(I) collagen
10/24	U53088	fetal elastin 3′ untranslated region
11/24	U53089	pro-1 type 3 collagen
12/24	U53090	EST T81388
13/24	U53091	none
14/24	U53092	pro-2 (V) collagen
15/24	U53093	EST R64072
16/24	U53094	EST T04974
17/24	U53095	Platelet derived growth factor-Receptor
18/24	U53096	type VI collagen 3
19/24	U53097	non-specific cross-reacting antigen
20/24	U53098	nicein B2
21/24	U53099	metalloproteinase inhibitor 1 precursor mRNA
22/24	U53100	glycerolaldehyde 3-phosphate dehydrogenase
23/24	U53101	collagenase type IV
24/24	U53102	EST H01775

Fragments isolated with *Driver* 1 are listed. Only homologies with a sequence similarity of >80% over at least 50 bp were included.

To suppress the influence of stromal tissue components, pooled mRNA from 10 chronic pancreatitis tissue samples was mixed with the pooled mRNA from the 10 healthy control pancreatic tissues of *Driver 1*, eventually to form *Driver 2*. The use of *Driver 2* led to the identification of 16 distinct gene fragments, of which 13 were overexpressed in pancreatic cancer tissues only (see Fig. 2B and Table 2). On Northern blots (Fig. 2B), nine of the 16 gene fragments were expressed at low levels and required prolonged exposure times (10–14 days at −70°C by using intensifying screens). Clones 4 and 8 did not detect a transcript in any of the pancreatic tissues (cancer, chronic pancreatitis, or control) on a Northern blot, but the transcript could be amplified from the cancer tissue RNA by RT-PCR. These gene fragments were obviously expressed below the level of detection of standard Northern blot analysis with total RNA. Not unexpectedly, cDNA-RDA was thus confirmed to be more sensitive than Northern blot analysis. Both clones did hybridize weakly to Southern blots of the initial *Tester* representation but not to the *Driver* representations (data not shown), indicating the good degree of representation in the amplicons of the original RNA. By hybridization to Northern blots, clone 10 was found to be a special product of the cDNA-RDA selection insofar as the transcript is differentially spliced, with the cancer-specific splice variant being about 500 bp longer than the transcript in normal cells (Fig. 2B).

Sequence analyses of the cloned fragments revealed homologies to five genes that have already been associated with carcinogenesis in the pancreas or in other tissues (Table 2). Two of the isolated fragments were homologous to different portions of the fibronectin (*FN1*) gene. Northern blot hybridizations of both fragments showed that they were highly overexpressed in pancreatic cancer and that they were also expressed at a much lower level in chronic pancreatitis tissue samples (Table 2). Fibronectins are adhesive glycoproteins that have variable primary structures due to cell type-specific splicing of the precursor mRNA. They have a role in various biological phenomena, such as cell adhesion, mobility, and differentiation (Yamada et al., 1985). Fibronectin molecules produced by tumor cells have been reported to contain different portions of splice variants from normal cells (Castellani et al., 1986; Borsi et al., 1987; Gutman and Kornblihtt, 1987; Carnemolla et al., 1989; Borsi et al., 1992). Numerous studies have evaluated the potential of measuring plasma levels of fibronectin as tumor marker (Ylätupa et al., 1995, and references therein). Although results have been contradictory so far, cellular fibronectin was reported to be elevated in the serum of a high proportion of patients with gastrointestinal tract tumors.

Clone 7 was homologous to annexin VIII. Annexin VIII is a calcium-dependent phospholipid-binding protein with increased expression in acute promyelocytic leukemia and has been identified as a blood anticoagulant (Sarkar et al., 1994, and references therein). Recent studies have demonstrated that annexin VIII is predominantly localized at the plasma membrane, and a role in signal transduction in acute promyelocytic leukemia cells has been suggested (Sarkar et al., 1994). Increased expression of other members of the annexin family has been shown in the hamster model of nitrosobis-induced pancreatic cancer (Vishwanatha et al., 1993).

The gene of insulin growth factor-like binding protein 1 (*IGFBP1*) was isolated by cDNA-RDA as another tester-specific sequence. It was one of the RDA gene fragments that could not directly detect a transcript in any of the pancreatic cancer tissues used in standard Northern blot analysis due to a lack of sensitivity. Insulin growth factor-like binding

TABLE 2. Sequences and Expression Patterns of Pancreatic Cancer-specific RDA Fragments

Clone	Accession no.	Sequence homology	% ID[a]	Expression[b] PACA	CP	CO	Transcript
1	U54592	—	—	+	−	−	2.0 kb
2	U54593	human fibronectin (alternatively spliced without ED-A)	98% 362 bp	+++	+	−	>8.0 kb
3	U54594	human fibronectin (Exon 1–3)	99% 330 bp	+++	+	(+)	>8.0 kb
4	U54595	human small insulin-like growth-factor binding protein 1	99% 279 bp	−	−	−	—
5	U54596	human EST H43243	99% 266 bp	++	−	−	5.0 kb
6	U54597	—	—	+	−	−	4.0 kb
7	U54598	human annexin VIII	98% 356 bp	+	−	−	3.0 kb
8	U54599	—	—	+	−	−	4.0 kb
9	U54600	—	—	−	−	−	—
10	U54601	—	—	+++	−	−	1.7 kb
				−	+	++	1.2 kb
11	U54602	human cytokeratin 17 (Exon 6–8)	98% 269 bp	+++	(+)	−	2.5 kb
12	U54603	—	—	++	−	−	2.5 kb
13	U54604	—	—	+	−	−	6.0 kb
14	U54605	human EST N57606	98% 247 bp	+	−	−	2.0 kb
15	U54606	—	—	++	−	−	1.5 kb
16	U54607	human non-specific cross-reacting antigen (NCA)	99% 243 bp	+++	(+)	−	3.0 kb

The gene fragments were isolated with *Driver* 2.

[a]ID = level of sequence homology.

[b]Expression patterns observed by Northern blot analysis were scored as follows: − = not detectable; (+) = detectable as weak signal only after extended exposure (at least 10 days); + = weak signal; ++ = moderate signal; +++ = strong signal. PACA = pancreatic cancer; CP = chronic pancreatitis; CO = control pancreatic tissue. The transcript sizes were determined on Northern blots.

proteins are postulated to carry out several functions, including control of insulin growth factor interactions with cell-surface receptors and modulation of biological actions of the insulin growth factors (Clemmons et al., 1993). Finding this gene seems somehow surprising, because it was postulated as a potential tumor suppressor. However, similarly to TP53, a well-known tumor suppressor, overexpression in pancreatic carcinomas has been described for 50–60% of the cases analyzed, which is intriguingly close to a detected gene mutation rate of 45–80% (Barton et al., 1991; Casey et al., 1993).

Clone 11 showed homologies to cytokeratin 17 and was highly overexpressed in pancreatic cancer tissues. Several members of the cytokeratin family including cytokeratin 17 may be expressed in pancreatic cancer cells (Osborn and Weber, 1983) and may be molecular markers for cell differentiation and neoplastic transformation in the exocrine pancreas (Vilá et al., 1994). Clone 16 was found to be homologous to the sequence of NCA (nonspecific cross-reacting antigen), which is a member of the CEA family of tumor markers (Tawagari et al., 1988).

Of the remaining gene fragments, two (clones 5 and 14 in Table 2) showed homologies to expressed sequence tags with unknown function generated in the context of the genome project. In the same way as the eight gene fragments with no homologies to sequences in the database, they represent novel genes. Because all known genes isolated in this approach have already been associated with malignant cells, these new sequences may be derived from new disease genes or disease-associated genes. Full-length clones for most of these sequences have been isolated and are being used for further characterization in ongoing experiments.

cDNA-RDA was a powerful and highly efficient method reproducibly to identify novel cancer-related genes of potential biomedical importance. The addition of chronic pancreatitis to the driver ensured the isolation of cancer-specific, differentially expressed gene fragments by suppressing products of stromal origin. Thus, cDNA-RDA is the only available method that eliminates the influence of stromal tissue components without the necessity to enrich for cancer cells by elaborate processes (e.g., tissue microdissection or nude mouse xenografts). In addition, even genes that were differentially expressed at a low expression level could be identified by cDNA-RDA due to the intrinsically high sensitivity and selectivity of the technique, which is difficult to impossible to achieve

with other methods such as differential hybridizations (e.g., Gress et al., 1992). cDNA-RDA studies the entire set of transcribed genes in one experiment, whereas only much simpler subsets are analyzed simultaneously by the differential-display technique (Liang and Pardee, 1992). By including a melt depletion step and by adapting the tester:driver ratios, cDNA-RDA allowed not only the detection of absolute differences in gene expression but also variations in the scale of expression, which are the most frequent events leading to cancer-specific differential gene expression. The number of genes with altered expression in tumors should be more than the 16 that have actually been identified. In the DP2 and especially the DP1 amplicons (Fig. 1), more but nevertheless distinct bands could be seen, indicating that this low number resulted from the high ratio of driver:tester applied for the isolation of DP3. An influence of variable amplification rates between fragments, eventually leading to the extinction of poorly amplified DNA, cannot be ruled out entirely, although the selectivity of the RDA process should more than compensate for such an effect. Besides the ability to detect differences in expression levels, cDNA-RDA also seems well suited to detect cancer-specific splice variants. In summary, this study has shown the feasibility of using cDNA-RDA in approaches aiming at the isolation of cancer-specific expressed genes and has led to the identification of a number of genes clearly associated with pancreatic cancer, thus representing novel candidate disease genes.

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