

PCDI, a Novel Gene Containing PDZ and LIM Domains, Is Overexpressed in Several Human Cancers

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ABSTRACT

In an effort to discover novel genes differentially expressed in human pancreatic cancer, we have identified a gene named *PCDI* (pancreatic cancer derived) that is up-regulated in pancreatic dysplasia and cancer relative to normal pancreatic ductal epithelium. We cloned the full length (4572 bp) of this gene, which encodes a novel protein of 1064 amino acids containing a PDZ domain and a LIM domain. An alternatively spliced form with a deletion of 30 bp in the coding region was also found. *In situ* hybridization results showed that *PCDI* is highly expressed in a significant percentage of colon, breast, liver, lung, pancreas, stomach, and prostate tumor tissues but is expressed in very few normal tissues. Northern blot hybridization confirmed the overexpression of *PCDI* in colon and breast tumor tissues and also showed strong expression of *PCDI* in the heart as well as in HeLa cells. Real-time quantitative reverse transcription-PCR verified the overexpression of *PCDI* in primary colon tumors or in liver metastases relative to normal colon tissues in five of eight patients. The *PCDI* gene maps to human chromosome 13q21.33. Because of its high levels of expression in neoplastic tissues and the presence of both PDZ and LIM domains, we suggest that *PCDI* may play an important role in cytoskeletal reorganization during carcinogenesis.

INTRODUCTION

Differential display PCR has been used extensively to search for genes differentially expressed under altered conditions in a broad range of fields (1, 2). Genes discovered to be differentially expressed under pathological conditions and further validated by expression analyses in human tissues may serve as candidates for potential therapeutic drug targets or diagnostic markers (3–5). Those coding for proteins that contain established functional domains offer the most options for further study (6, 7) because functional domains in novel proteins can provide valuable information about the biological function of the proteins in cells or organisms.

The PDZ domain is a well-established multifunctional protein-protein interaction motif that exists in a variety of dissimilar proteins interacting mainly with the cytoskeleton (8–10). A typical PDZ domain consists of 80–120 amino acids and can bind to the consensus sequence Ser/Thr-X-Val/Leu/Ile at the COOH termini of other proteins. PDZ domains are also reported to interact with internal consensus sites, other PDZ domains, and other protein motifs. For example, the PDZ domain of neuronal nitric oxide synthase binds to the second PDZ domain of PSD-95 (11); the COOH terminus of Fas binds to the third PDZ domain of PTP-BAS, and this interaction participates in Fas-mediated apoptosis of T cells (12). InaD is a multi-PDZ domain protein that functions as a scaffold for assembly of signaling molecules of the *Drosophila* vision system (13). The LIM domain is another important protein-protein interaction domain that is composed of two specialized zinc fingers joined by a 2-amino acid spacer (14–16). LIM proteins form a diverse group including transcription

factors and cytoskeletal proteins. The LIM domain interacts with other LIM domains and with many different protein domains including the PDZ domain. LIM domains are thought to mediate specific contacts between members of functional complexes and to regulate the activity of some of the constituent proteins.

Recently, a family of proteins containing both the PDZ and LIM domains has been discovered. The members include LIM-kinase 1, LIM-kinase 2, ALP, RIL, enigma, enigma homology protein, Cypher 1, and CLP36 (17–22). These proteins all seem to participate in cytoskeleton organization. LIM-kinase 1 plays a critical role in Rac signaling. It phosphorylates and inactivates the actin binding/depolymerizing factor cofilin, inducing actin cytoskeletal reorganization (23, 24). The PDZ domain of LIM-kinase 1 controls its nuclear export, and the LIM domains negatively regulate its kinase activity (25, 26). The PDZ domain of ALP binds to the spectrin-like motifs of α -actinin-2, and ALP is up-regulated during muscle differentiation (18, 27). Cypher 1 binds to protein kinase C through its LIM domains and associates with α -actinin-2 via its PDZ domain (21). The LIM domains of enigma bind to protein kinases, whereas its PDZ domain binds to the actin-binding protein β -tropomyosin (20).

In this study, we report cloning of a novel gene encoding a new member of the family of proteins containing both PDZ and LIM domains. This gene, which we name *PCDI*, was discovered through differential display and was found to be highly overexpressed in ductal epithelium from patients with pancreatic dysplasia and cancer relative to normal pancreatic ductal epithelium. Various analyses, including ISH,² Northern blot hybridization, and real-time quantitative RT-PCR in the Lightcycler (Roche Diagnostics, Indianapolis, IN) have shown that *PCDI* is overexpressed in a variety of human tumors. We also report that *PCDI* is localized to human chromosome 13q21.33.

MATERIALS AND METHODS

Differential Display. Differential display analysis was performed using the Hieroglyph mRNA profile kit (Genomix, Foster City, CA). RNA was extracted from primary cultures of ductal epithelial cells obtained from patients with normal pancreas, nonspecific pancreatitis, pancreatic dysplasia, and pancreatic carcinoma. Briefly, 2 μ g of total RNA prepared by the guanidinium method were reverse-transcribed with anchored oligo(dT)₁₈ primer in a 10- μ l reaction volume. Two μ l of each reaction were subjected to PCR using 200 primer pairs to profile gene expression. [α -³²P]dCTP (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) was included in the PCR reaction. The PCR products were then separated on 6% sequencing gels using a GenomixLR sequencer. The dried gels were subjected to autoradiography on Kodak BioMax films (33 \times 61 cm). Differentially expressed bands were then excised, reamplified, subcloned, and sequenced.

ISH. ISH was performed to detect *PCDI* mRNA expression in tissues with gene-specific oligonucleotides as probes using the Super Sensitive ISH Detection System kit from Biogenex Laboratories, Inc. (San Ramon, CA). All procedures were carried out as instructed in the protocol provided by the manufacturer.

² The abbreviations used are: ISH, *in situ* hybridization; RT-PCR, reverse transcription-PCR.

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Northern Blot Hybridization. Rapid-Hyb buffer (Amersham Life Science, Little Chalfont, United Kingdom) with 5 mg/ml denatured single-stranded sperm DNA was prewarmed to 65°C, and human colon tumor total RNA blots (Invitrogen, Carlsbad, CA) were prehybridized in the buffer with shaking at 65°C for 30 min. Gene-specific DNA probes (50 ng/reaction) labeled with [α -³²P]dCTP [3000 Ci/mmol (Amersham Pharmacia Biotech, Inc.); Prime-It RmT Kit (Stratagene, La Jolla, CA)] and purified with Probe-Quant G-50 Micro Columns (Amersham Pharmacia Biotech, Inc.) were added and hybridized to the blots with shaking at 65°C overnight. The blots were washed in 2× SSC and 0.1% (w/v) SDS at room temperature for 20 min, washed twice in 1× SSC and 0.1% (w/v) SDS at 65°C for 15 min, and then exposed to Hyperfilms (Amersham Life Science).

Real-time Quantitative RT-PCR in the Lightcycler. One μ g of human placenta total RNA (Clontech, Palo Alto, CA) was reverse-transcribed with oligo(dT)₁₈ primer at 42°C for 1 h and then heated at 94°C for 5 min in a total reaction volume of 20 μ l (1st-Strand cDNA Synthesis Kit; Clontech). The reaction mixture was used as the 1× template standard for PCR in the Lightcycler. Serial dilutions from the 1× template standard were then prepared (10^{-1} ×, 10^{-2} ×, 10^{-3} ×, 10^{-4} ×, and 10^{-5} × template standards). Patient colon tissue was obtained at surgery and stored frozen in liquid nitrogen. The patient tissue samples were homogenized in Trizol reagent. Chloroform was then added to isolate RNA, followed by RNA precipitation with isopropanol. The RNA precipitates were washed with 75% ethanol, dried in air, and then dissolved in RNase-free distilled water. The total RNA samples were treated with RNase-free DNase I (2 units/ μ l; Ambion, Austin, TX), cleaned up using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA), and then reverse-transcribed with oligo(dT)₁₈ primer (1st-Strand cDNA Synthesis Kit; Clontech). PCR was performed in the Lightcycler using the following gene-specific primers: (a) β -actin, forward primer 5'-CGGAAATCGTGCATGACATTAAG-3' and reverse primer 5'-TGATCTCCTTCTGCATCCTGTCGG-3'; and (b) PCD1, forward primer 5'-TTCGTAGCATCAGTTGAAGCAGG-3' and reverse primer 5'-GGTGAACCAGCCTTCCATAGC-3'.

The 20- μ l PCR reaction mixture in each Lightcycler capillary contained 2 μ l of 10× PCR buffer II, 3 mM MgCl₂ (Perkin-Elmer, Foster City, CA), 140 μ M deoxynucleotide triphosphate, 1:50,000 SYBR Green I, 0.25 mg/ml BSA, 1 unit of Taq polymerase (Boehringer Mannheim, Indianapolis, IN), 0.175 μ M each primer, and 2 μ l of reverse transcription reaction mixture. The PCR amplification began with a 20-s denaturation at 95°C, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 1 s, and extension at 72°C for 30 s. At the end of the final cycle, PCR products were annealed at 60°C for 5 s and then heated slowly to 95°C at 0.2°C/s to measure the melting curves of specific PCR products. All experiments were performed in duplicate. Data analysis was performed using Lightcycler Software (Roche Diagnosis) with quantification and melting curve options.

Chromosome Mapping. A fragment of PCD1 cDNA was used as a probe to screen a P1 genomic DNA library, and a single clone was obtained (Genome Systems, St. Louis, MO). This P1 clone was then used to determine chromosomal localization of PCD1 by fluorescence ISH.

RESULTS

Cloning of a Novel Gene Overexpressed in Pancreatic Cancer through Differential Display. Using a differential display PCR assay, we identified a transcript that was differentially expressed in primary cultures of ductal epithelial cells from normal pancreas and pancreas from patients diagnosed with nonspecific pancreatitis, pancreatic dysplasia, and pancreatic cancer (Fig. 1). This transcript was highly expressed in pancreatic dysplasia and cancer samples but was not detected in normal and pancreatitis samples. We screened a human colon cell line cDNA library (HT29) to isolate a full-length cDNA and identified a 4572-bp cDNA. The open reading frame predicts a novel protein of 1064 amino acids, and an alternatively spliced form with a deletion of 30 bp in the coding region was also obtained (Fig. 2). The deduced amino acid sequence contains a PDZ domain in the middle and a highly conserved LIM domain at the COOH terminus (Fig. 3). We named this gene *PCD1*.

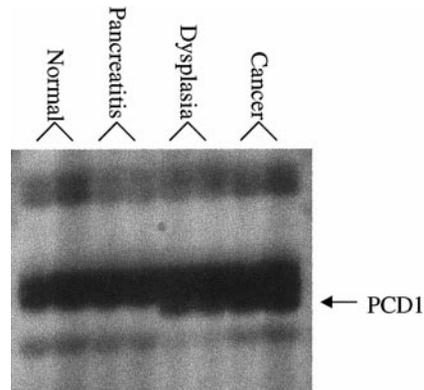


Fig. 1. Differential display of PCD1 transcript levels in primary cultures of ductal epithelial cells from normal human pancreas and pancreas from patients diagnosed with pancreatitis, pancreatic dysplasia, and pancreatic cancer. The PCD1 band is present at high levels in the dysplasia and cancer samples and is virtually absent from the normal and pancreatitis samples.

PCD1 Overexpression in Tumor Tissues Detected by ISH. ISH allows the detection of mRNA within a cell or tissue. We have performed ISH using *PCD1* gene-specific oligonucleotides to detect PCD1 mRNA expression in a variety of tumor tissues as well as normal tissues. Table 1 summarizes the mRNA expression of PCD1 in seven different tissues that we examined, and Fig. 4 shows the percentages of tissues that are positive for PCD1. For all seven tumor tissues, PCD1 is highly expressed in a significant portion of the samples tested; in contrast, few or no normal samples of each tissue showed positive PCD1 expression. Therefore, PCD1 expression is elevated in a significant portion of tumor tissue samples from colon, breast, liver, lung, pancreas, stomach, and prostate cancer patients. Representative examples of ISH results for each normal and tumor tissue, respectively, are shown in Fig. 5.

Northern Hybridization. To further confirm PCD1 overexpression in cancer, a PCD1 cDNA fragment was labeled and hybridized to Invitrogen human colon and breast tumor tissue total RNA blots (Fig. 6). Each blot has four pairs of tumor and normal total RNA samples from four different cancer patients. The samples were collected from whole tissues. The Northern analysis revealed that PCD1 was overexpressed in tumor tissue compared with normal tissue in one of the four colon cancer patients (patient 3) and in three of four breast cancer patients (patients 2–4). A shorter spliced form was also detected in breast tissue in addition to the 4.6-kb one. In addition, Northern hybridization was performed on a human multiple tissue mRNA blot and a human cancer cell line mRNA blot to analyze PCD1 expression in various tissues and cancer cell lines (Fig. 7). PCD1 is highly expressed in heart tissue as well as in HeLa cells, and the shorter form detected in breast tissue exists in heart tissue. It is noteworthy that PCD1 is detected as a spliced form of ~6 kb in skeletal muscle; this longer form is also present in lung and liver tissues.

Real-time Quantitative RT-PCR in the Lightcycler. Recently, real-time quantitative PCR has emerged as an excellent technology for detection of nucleic acids (28–31). We decided to use the Lightcycler instrument to perform real-time quantitative RT-PCR to investigate expression levels of PCD1 message in tumor tissues from eight colon cancer patients. The PCR reaction was monitored with SYBR Green I dye. The quantification assay is based on determination of the cycle crossing point, which represents the cycle when the PCR product begins to double with each cycle, *i.e.*, when the log-linear phase begins. A template dilution test was performed and demonstrated that the gene-specific primers for β -actin and PCD1 are capable of accurate, sensitive, and specific detection of expression levels for β -actin and PCD1, respectively (data not shown). For each colon cancer

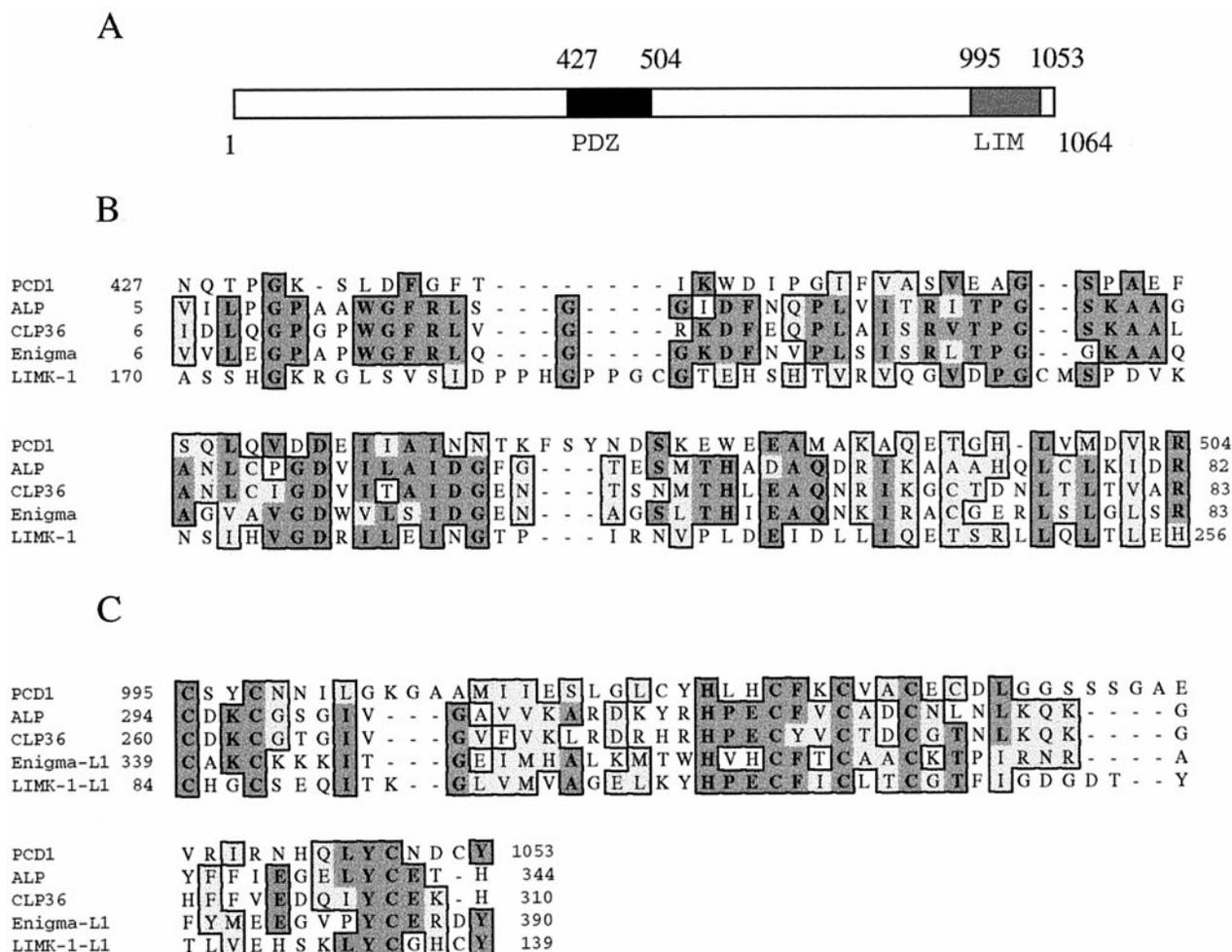


Fig. 3. A, schematic structure of the predicted PCD1 protein sequence. B, alignment of the PDZ domain of PCD1 with PDZ domains of other PDZ and LIM domain-containing proteins. C, alignment of the LIM domain of PCD1 with the LIM domains of other PDZ and LIM domain-containing proteins. The PCD1 LIM domain matches well with the LIM consensus motif $CX_2CX_{16-2}[^3H]X_2CX_2CX_{16-21}CX_{2-3}(C/H/D)$.

Table 1 Summary of PCD1 gene expression in tumor and normal tissues detected by ISH^a

Tissue	Tumor			Normal		
	+	-	Total	+	-	Total
Colon	65	73	138	4	44	48
Breast	76	103	179	3	26	29
Liver	51	0	51	1	7	8
Lung	20	0	20	0	8	8
Pancreas	7	0	7	0	4	4
Stomach	2	1	3	0	8	8
Prostate	4	0	4	2	12	14

^a +, strong expression; -, weak expression or no detectable expression.

tissue as compared with normal colon tissue in five of eight patients. This is consistent with the previous results from ISH and Northern blot hybridization (Figs. 4-6; Table 1). It is noteworthy that in one patient, PCD1 expression levels in primary tumor colon and metastatic liver tissue are dramatically decreased, not increased, relative to normal colon tissue.

Chromosomal Localization. PCD1 cDNA was used as a probe to screen a P1 genomic DNA library. A single clone was obtained and confirmed by sequencing with a PCD1 primer. This genomic clone was then used as a probe for chromosomal localization of PCD1 by fluorescence ISH. PCD1 maps to human chromosome 13q21.33 (data not shown).

DISCUSSION

We have cloned a novel gene named *PCD1*, which encodes a new member of the PDZ and LIM domain-containing protein family. The PDZ domain, named for the three proteins in which it was first recognized (PSD-95, Discs-Large septate junction protein, and ZO-1 tight junction protein), is a multifaceted protein-binding domain. PDZ domains have been found in more than 100 different proteins across eukaryotic species (9). For instance, PDZ domains are found in signaling molecules, such as Dlg tumor suppressor protein, neuronal nitric oxide synthase, and several protein tyrosine phosphatases (8).

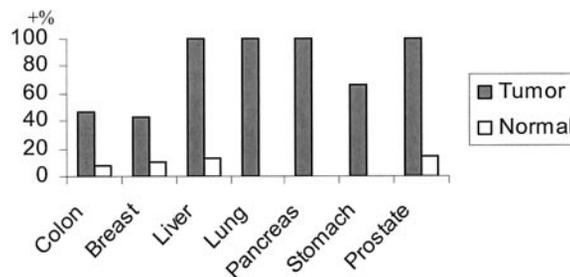


Fig. 4. The percentages of tumor and normal tissue samples expressing PCD1 by ISH analysis. Total numbers of patient samples tested for each cancer type are indicated, along with the absolute numbers of normal and tumor samples scoring positive for PCD1 mRNA expression.

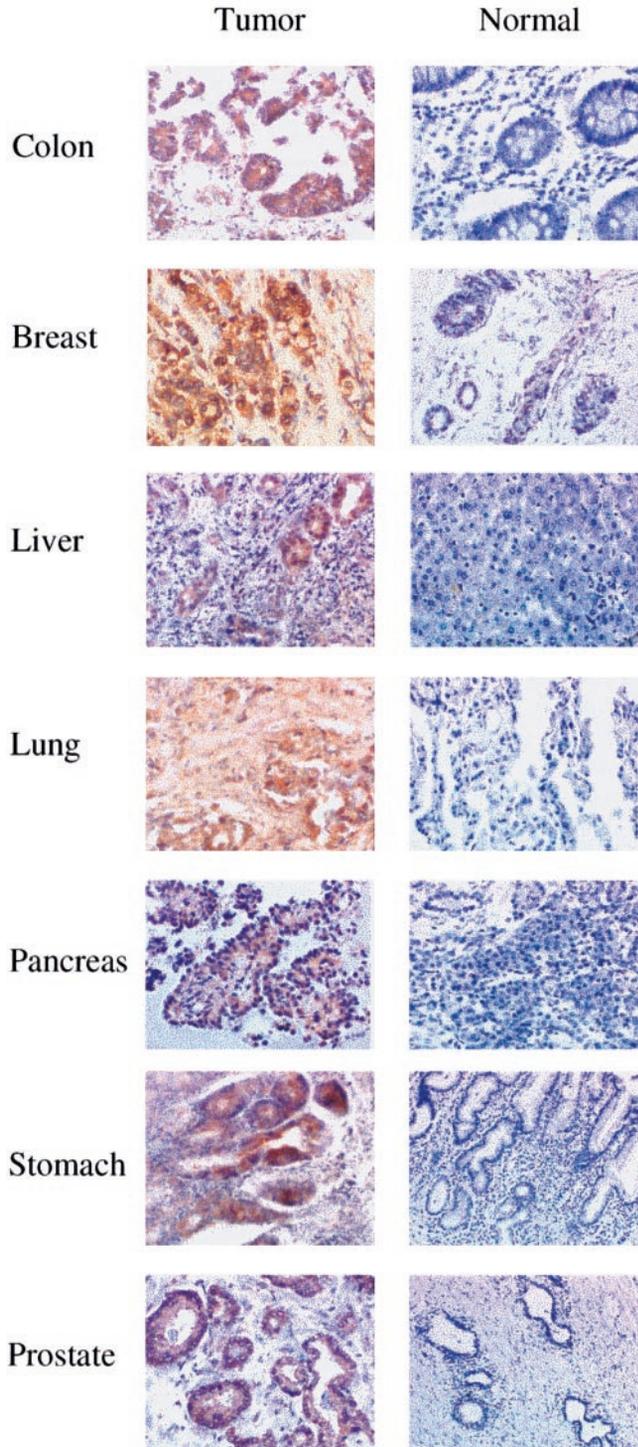


Fig. 5. Representative ISH results for each tumor and normal tissue examined using *PCD1* gene-specific oligonucleotides as probes.

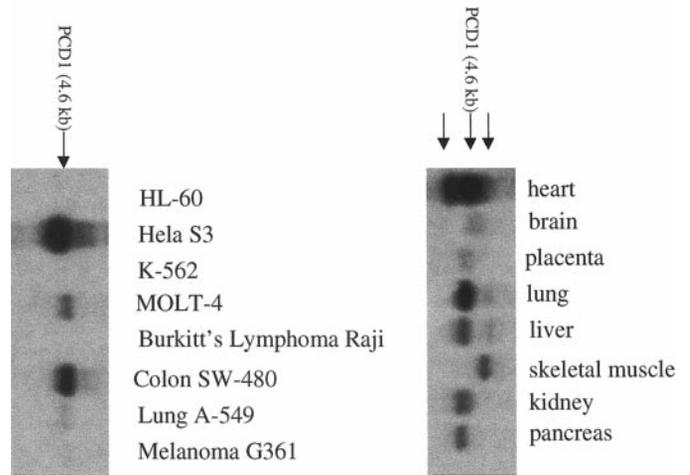
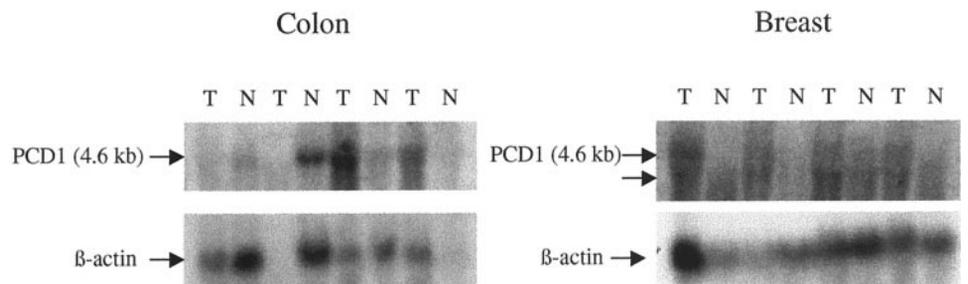


Fig. 7. Northern hybridization analysis of human multiple tissue and multiple cancer cell line blots (Clontech) to detect *PCD1* expression.

The majority of proteins that contain PDZ domains seem to be associated with the cytoskeleton at the cell cortex and act as scaffolds for diverse signaling complexes. However, at least one PDZ domain-containing protein, interleukin 16, is a secreted protein, although its PDZ domain is altered and does not exhibit the usual peptide-binding properties of PDZ domains (32). There are multiple distinct mechanisms for PDZ domain binding: PDZ domains can bind to specific recognition sequences at the COOH termini of target proteins; dimerize with other PDZ motifs; and interact with internal consensus sites, spectrin-like repeats, LIM domains, and unspecified sites (11, 13, 18, 19, 33–35). The LIM domain, named for the first three homeodomain proteins in which it was recognized (*lin-11*, *isl-1*, and *mec-3*), is a special cysteine-rich metal-binding structure that consists of two distinct zinc-binding subdomains (14, 15). LIM domains also mediate protein-protein interactions through the formation of dimers with identical or different LIM domains or by binding to other protein motifs. LIM domains have been found in diverse proteins that may also contain homeodomains, kinase domains, cytoskeletal components, or other structures. Proteins containing LIM domains are key players in a number of fundamental pathways controlling development. For example, knockout mice lacking the *lhx-1* gene fail to form anterior head structures (36); LMO2 regulates erythroid precursor cell differentiation (37). Recently, a group of proteins containing both the PDZ and LIM domains has emerged, including LIM-kinase 1, LIM-kinase 2, ALP, RIL, enigma, enigma homology protein, Cypher 1, and CLP36. These diverse proteins all appear to be involved in cellular cytoskeleton organization. For instance, LIM-kinase 1 regulates actin cytoskeletal reorganization by phosphorylating and inactivating cofilin in the Rac-mediated stimulus signaling pathway (23, 24). ALP and Cypher 1 interact with α -actinin-2 through their PDZ domains (18, 21).

PCD1 was identified through differential display PCR analysis

Fig. 6. Northern hybridization analysis of human colon and breast tumor tissue total RNA blots (Invitrogen) to detect *PCD1* expression. Total RNA samples from paired tumor and normal tissues of each patient are loaded adjacent to each other from left to right. *T*, tumor; *N*, normal. Sample pairs are numbered from left to right.



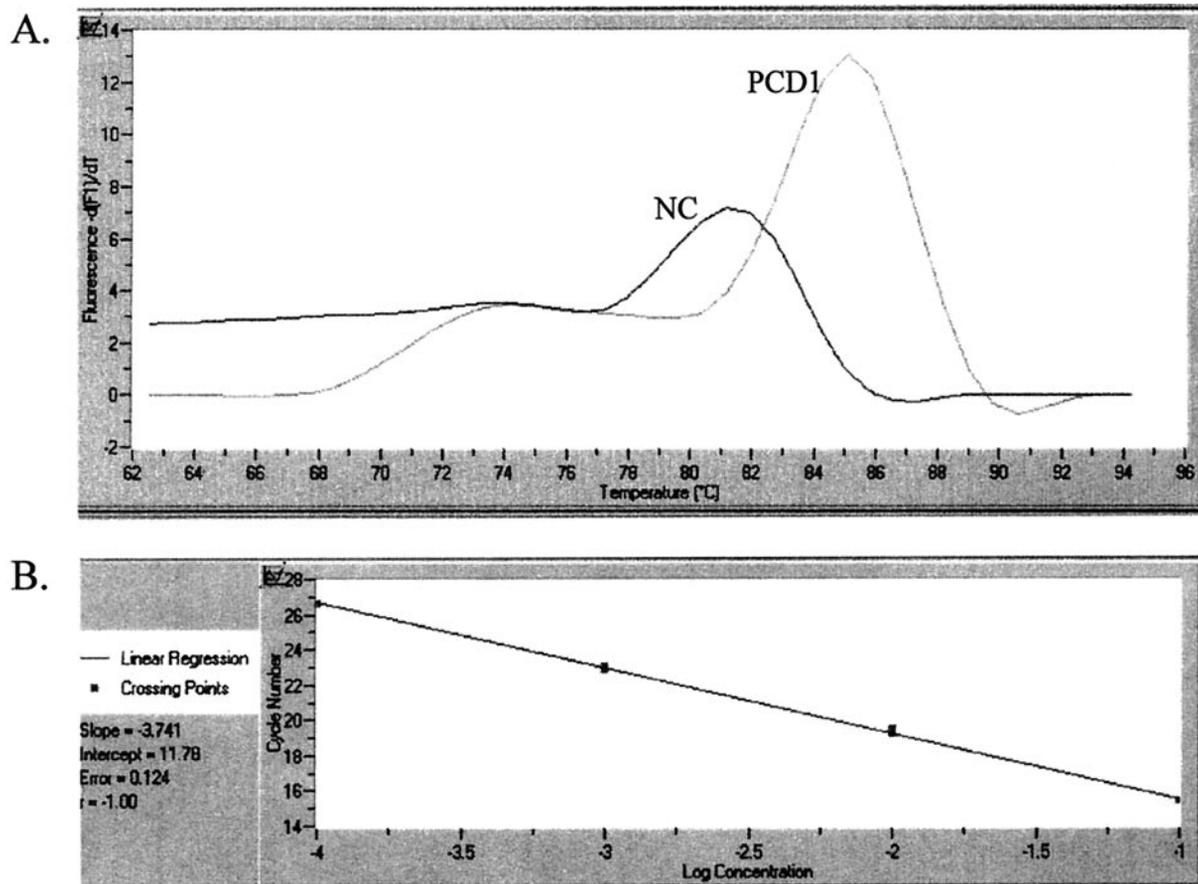


Fig. 8. PCD1 expression analysis by real-time quantitative RT-PCR in the Lightcycler. *A*, melting curve analysis of the specificity of the PCR amplification product of PCD1 in the Lightcycler. *NC*, nontemplate control. *B*, a standard curve established from detection of β -actin levels in the Lightcycler standard samples; 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} are regarded as the relative concentrations for the Lightcycler standard samples: $10^{-1}\times$, $10^{-2}\times$, $10^{-3}\times$, and $10^{-4}\times$, respectively. *C*, PCD1 gene expression levels in tissues from eight colon cancer patients. *N*, normal colon tissue; *PT*, primary tumor colon tissue; *MET*, metastatic liver tissue. The expression data (on the *Y* axis) have been adjusted by β -actin expression level and are thus relative values. All PCR reactions were performed in duplicate.

because it was found to be overexpressed in pancreatic dysplasia and cancer compared with normal and pancreatitis samples. Using ISH of tissues and Northern hybridization of human tumor tissue total RNA blots, we have examined PCD1 expression in a variety of cancers. The results indicate that PCD1 is overexpressed in all seven different cancers examined in a significant percentage of samples and suggest that the PCD1 gene product may participate in a common event shared by many cancer types. Also, real-time quantitative PCR in the Lightcycler was performed for total RNA samples prepared from patient tissues. Five of eight patients showed significant increases in PCD1 expression (>2 -fold) in primary tumor colon tissue or metastatic liver tissue relative to normal colon tissue, although one patient showed the opposite results (PCD1 expression levels in this unique patient may have been affected by physiological causes other than colon cancer). Thus, PCD1 may be a good target for cancer drug development or a useful cancer diagnostic marker once its biological function becomes more clear. Another PDZ and LIM domain-containing protein, RIL, was reported to be differentially regulated in cancer: it was down-regulated in H-ras-transformed cells (38). Some similarities in tissue distribution are notable between PCD1 and other PDZ and LIM domain-containing proteins. PCD1 shows a very high expression in the heart, among all the tissues examined. Intriguingly, human CLP36 was reported to be highly expressed in heart and skeletal muscle, and Cypher1 is expressed exclusively in cardiac and striated muscle (21, 22). PCD1 was detected as a spliced form of ~ 6 kb in skeletal muscle, whereas ALP was detected as a M_r 40,000 protein in skeletal muscle

instead of the normal M_r 36,000 protein (27). ALP is up-regulated during muscle differentiation.

Although the function of the PCD1 gene product is still unclear, we suggest that like its family members, it may also play a role in cytoskeletal organization. It may function as an adaptor or regulator through its PDZ and LIM domains, participating in functional complexes or signaling pathways in cytoskeletal reconstruction in cancer. It may also be involved in muscle differentiation like ALP. PCD1 could play an important role as a target in anticancer drug development or in the search for cancer diagnostic markers. The study of the function of PCD1 is under way, and elucidation of its biological function and its role in carcinogenesis will lead to a further understanding of the importance of PCD1 in cancer therapy or diagnosis.

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