Overexpressed P-Cadherin/CDH3 Promotes Motility of Pancreatic Cancer Cells by Interacting with p120ctn and Activating Rho-Family GTPases

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Abstract

P-Cadherin/CDH3 belongs to the family of classic cadherins that are engaged in various cellular activities including motility, invasion, and signaling of tumor cells, in addition to cell adhesion. However, the biological roles of P-cadherin itself are not fully characterized. Based on information derived from a previous genome-wide cDNA microarray analysis of microdissected pancreatic ductal adenocarcinoma (PDAC), we focused on P-cadherin as one of the genes most strongly overexpressed in the great majority of PDACs. To investigate the consequences of overexpression of P-cadherin in terms of pancreatic carcinogenesis and tumor progression, we used a P-cadherin–deficient PDAC cell line, Panc-1, to construct a cell line (Panc1-CDH3) that stably overexpressed P-cadherin. Induction of P-cadherin in Panc1-CDH3 increased the motility of the cancer cells, but a blocking antibody against P-cadherin suppressed the motility in vitro. Overexpression of P-cadherin was strongly associated with cytoplasmic accumulation of one of the catenins, p120ctn, and cadherin switching in PDAC cells. Moreover, P-cadherin–dependent activation of cell motility was associated with activation of Rho GTPases, Rac1 and Cdc42, through accumulation of p120ctn in cytoplasm and cadherin switching. These findings suggest that overexpression of P-cadherin is likely to be related to the biological aggressiveness of PDACs; blocking of P-cadherin activity or its associated signaling could be a novel therapeutic approach for treatment of aggressive pancreatic cancers. (Cancer Res 2005; 65(8): 3092-9)

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the Western world and shows the worst mortality among malignancies, with a 5-year survival rate of only 4% (1–3). Approximately 30,700 patients are diagnosed with pancreatic cancer in the United States each year, and nearly 30,000 of them will die of the disease (3). Because most PDAC patients are diagnosed at an advanced stage, no effective therapy is available at present; surgical resection offers the only possibility for cure, but 80% to 90% of PDAC patients who undergo surgery relapse and die from this cancer (1, 2). Some approaches combining surgery with drugs such as 5-fluorouracil or gemcitabine, with or without radiation, can improve patients’ quality of life (1, 2), but those treatments have a limited effect on long-term survival due to the extremely aggressive and chemoresistant nature of PDACs. Hence, management of most PDAC patients is focused on palliative measures (1).

To overcome this dismal situation, development of novel therapies for PDACs, specifically through identification of molecular targets for new drugs, is an urgent issue. Earlier we generated precise expression profiles of PDACs using genome-wide cDNA microarrays consisting of ~27,000 genes, in combination with laser microdissection to obtain pure populations of cancer cells for testing (4). Among the genes being overexpressed in PDAC cells, we investigated P-cadherin (CDH3) as a novel target for molecular therapy.

P-Cadherin/CDH3 is a classic cadherin, a member of a molecular family of single-span transmembrane domain glycoproteins that function as important cell-cell adhesion molecules (5). The intracellular domain of classic cadherins interacts with various catenin proteins such as β- and γ-catenin, which bind to the same conserved site at the carboxyl termini of classic cadherins in a mutually exclusive way (6). In addition to their adhesive functions, classic cadherins modulate transduction of signals in the Wnt pathway and activate Rho-family GTPases by interacting with catenins (6). Therefore, alterations among cadherin molecules can affect not only the adhesive properties of a cell but also its signal transduction activity, which can affect many kinds of cell activities, organ development, and tumorigenesis (6). For instance, loss of epithelial (E)-cadherin function elicits active signals that support tumor-cell migration, invasion, and metastatic dissemination (7, 8), and also E-cadherin functions as a tumor suppressor of diffused-type gastric cancers although the detailed mechanism remains unclear (9). Nerve (N)-cadherin is up-regulated in some invasive cancers, with effects on cellular behaviors beyond adhesion specificity; for example, N-cadherin induces an invasive morphology and stimulates migration, invasion, and metastasis by incorporating fibroblast growth factor (FGF) receptor and by downstream signaling (10, 11). Regarding other classic cadherins, placental (P)-cadherin was first identified in mouse placenta (12); in humans its expression is not detectable in placenta but is present in a few organs such as mammary gland and prostate. Unlike the E- and N-cadherins, P-cadherin has not fully been investigated, so its roles in normal and tumor cells remain unclear.

In this study, we report overexpression of P-cadherin in the great majority of PDAC cells examined and show that this overexpression changes the subcellular localization p120 catenin (p120ctn) and increases the steady-state activity of Rho GTPases. Those changes correlated with increased motility of PDAC cells.

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Materials and Methods

Cell lines. PDAC cell line Panc-1 was purchased from the American Type Culture Collection (Rockville, MD). PK-45P, KLM-1, SUTT-2, and KP-1N were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). All cell lines were grown in RPMI 1640 (Sigma-Aldrich Corp., St. Louis, MO), which was supplemented with 10% fetal bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO2.

Antibodies. Monoclonal antibodies against P-cadherin, E-cadherin, N-cadherin, p120ctn, and Rac1 were obtained from BD Transduction Laboratories (Palo Alto, CA). Monoclonal antibodies against Cdc42 and myc-tag (9E10) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). P-Cadherin blocking antibody (NCC-CAD-299) and ACTB antibody (CP01-100UG) were purchased from Calbiochem (San Diego, CA).

Semiquantitative reverse transcription-PCR. Purification of cancer cells from primary pancreatic cancer tissues was described previously (4). Fresh surgical PDAC specimens were obtained from the PDAC patients who underwent surgical resection at the Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate rules for informed consent. RNAs from the purified populations of PDAC cells and from normal pancreatic ductal epithelial cells were subjected to two rounds of RNA amplification using T7-based in vitro transcription (Epicentre Technologies, Madison, WI) and synthesized to single-strand cDNA. From the human pancreatic cancer cell lines listed above, total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the recommended procedures of the manufacturer, treated with DNase I (Roche Diagnostic, Mannheim, Germany), and reverse transcribed to single-stranded cDNAs using oligo (dT) primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification and monitored their reactions using β-actin (ACTB) as a quantitative control. The primer sequences were 5′-CATCAGCAAGAACCTTTTCAACT-3′ and 5′-TCTCC-TTAGAAGAAGTGGGTTG-3′ for ACTB; 5′-CTGAGGGGGCCTAACAG-GAC-3′ and 5′-TACAGGATGCTTACACACTC-3′ for P-cadherin (CDH3); 5′-GCTTCGTAGCAGACATTGTC-3′ and 5′-AGTCTGAACCTGCGAAAAT-3′ for E-cadherin; and 5′-AAGTGGTTGGAAGAGACTGAGC-3′ and 5′-AGGATTATTGCAGAACGTCG-3′ for N-cadherin. All reactions involved initial denaturation at 94°C for 2 minutes followed by 21 cycles (for ACTB) or 28 cycles (for CDH3) at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Immunohistochemical staining. Conventional paraffin-embedded tissue sections from PDACs were obtained from surgical specimens that had been resected at the Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate rules for informed consent. Tissue sections from normal pancreas were purchased from Biochain Laboratories (Palo Alto, CA). Fresh surgical PDAC specimens were obtained from the PDAC patients who had been resected at the Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate rules for informed consent. Tissue sections from normal pancreas were purchased from Biochain (Hayward, CA). Tissue-microarray sections of pancreatic carcinomas (AccuMax Array) were purchased from Petagen Inc. (Seoul, South Korea), where 31 PDAC tissues and 2 endocrine tumor tissues were spotted in duplicate. The sections were deparaffinized and autoclaved for 15 minutes at 108°C in citrate buffer, (pH 6.0). Endogenous peroxidase activity was quenched by incubation for 30 minutes at 50°C in 3% hydrogen peroxide diluted in methanol. After incubation with fetal bovine serum for blocking, the sections were incubated with primary antibodies diluted in PBS containing 1% BSA for 1 hour at room temperature. Nonspecific binding was blocked by incubation with 5% nonfat dry milk in PBS for 30 minutes at room temperature. After washing with PBS, the cells were stained with FITC-conjugated secondary antibody (Santa Cruz) for 1 hour at room temperature. After washing with PBS, the sections were developed using the enhanced chemiluminescence kit (Amersham Biosciences).

Cell motility assay. Motility was assessed using cell-culture inserts with porous membranes (8.0 μm pore size; Becton Dickinson). After 12 hours of incubation, the number of migrated cells was estimated by counting three independent visual fields in a microscope with a ×100 objective. For the wound-healing migration assay, cells were grown to confluency in six-well plates for 2 days, and a scrape in the form of a cross was made through the confluent monolayers with a plastic pipette tip. Several wounded areas were marked for orientation and then photographed by phase-contrast microscopy. Another wound-healing migration assay was done in the presence of either P-cadherin–blocking antibody (100 μg/mL, NCC-CAD-299) or control mouse immunoglobulin G (IgG; 100 μg/mL, Sigma-Aldrich). At set times ranging from 12 to 24 hours in individual experiments, marked wounds were photographed again. The nonparametric Mann-Whitney U test (two-tailed) was used to study differences between Panc1-CDH3 and Panc1-Mock cells in the motility assays.

Small interfering RNA–expressing constructs. To down-regulate endogenous P-cadherin expression in PDAC cells, we used psi6BBSX0.1 vector for expression of short hairpin RNA against a target gene, as described previously (13). The U6 promoter was cloned upstream of the gene-specific sequence (19-nt sequence from the target transcript, separated from the reverse complement of the same sequence by a short spacer, TTCAGAGA), with five thyminides as a termination signal and a neo cassette for selection by geneticin (Sigma). The target sequences were 5′-GGAGAACGGCTGTTGTTG-3′ (si-CDH3) for P-cadherin and 5′-GA-AGCAGAGAAGTGGGGTG-3′ (si-EGFP) for a negative control. Cells from a human PDAC-derived line, KLM-1 and PK-45P, were plated onto glass coverslips within 10-cm dishes, and transfected with si-CDH3 or si-EGFP, using FuGEN6 according to the instructions of the manufacturer. Cells were selected by 400 μg/mL geneticin for 5 days. Some cells were transfected into a P-cadherin-null PDAC cell line, Panc-1, using FuGEN6 according to the recommended procedures of the manufacturer (Roche). A population of cells was selected with 800 μg/mL geneticin (Invitrogen). Clonal Panc-1 cells were subcloned by limiting dilution and transfected with pcDNA3.1(+)/myc-HisAneo vector for control purposes (Panc1-Mock). Expression of myc-tagged P-cadherin was assessed by Western blotting and immunofluorescence using anti–P-cadherin (BD Transduction) and anti-myc monoclonal antibodies. Some clones were established and designated Panc1-CDH3. The morphology of cells in each construct was analyzed by phase-contact microscopy.
harvested after 3 days for preliminary evaluation of a knockdown effect on P-cadherin by Western blotting with anti–P-cadherin antibody (BD Transduction Laboratories). After 5 days, the cells on glass coverslips were immunostained using anti-p120ctn antibody.

**Cell fractionation.** Cytoplasmic soluble fraction and insoluble fraction binding to plasma membrane were prepared essentially as described by Gout et al. (14). Panc1-CDH3 and Panc1-Mock cells were homogenized in CSK buffer [50 mmol/L NaCl, 10 mmol/L PIPES (pH 6.8), 3 mmol/L MgCl₂, 0.5% Triton X-100, 300 mmol/L sucrose] supplemented with Protease Inhibitor Cocktail Set III (Calbiochem) and 0.5 mmol/L sodium vanadate for 10 minutes at 4°C with gentle rocking. After centrifugation for 10 minutes at 4°C and 14,000 rpm, the supernatant constituted the Triton X-100–soluble fraction (S). The pellet was triturated in the same volume of SDS buffer [20 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 2.5 mmol/L EGTA, 1% SDS] and boiled at 100°C for 10 minutes. After centrifugation for 10 minutes at 4°C and 14,000 rpm, the supernatant constituted the Triton X-100–insoluble fraction from the pellet (P). Equal amounts of S and P fractions from each cell were loaded for SDS-PAGE and analyzed by Western blot using anti-p120ctn antibody (BD Transduction Laboratories).

**Affinity precipitation using the Cdc42/Rac1 interactive binding domain-glutathione-S-transferase.** A cDNA fragment encoding the Cdc42/Rac1 interactive binding domain (CRIB) of human p21-activated kinase-1 (amino acids 67-150) was amplified by PCR using primers containing appropriate restriction sites [5'-CGTGAATTCAGAGAGAGAAGAAGAGCC-3' and 5'-CTGAGATCTCTCTAGCTGACTTATCTGGAATTCTCAAGCTGACTTATCTGGAATTCTCAAGCTGACTTATCTGTAAAS3'- BamHI and EcoRI sites shown by underlines; ref. 15). The PCR-amplified product was cloned into pGEX-6P1 (Amersham Bioscience) and expressed in E. coli BL21 codon plus (Invitrogen) as a fusion protein with glutathione-S-transferase (GST). The fusion protein was purified with glutathione-Sepharose beads (Amersham Bioscience) in PBS containing 1% Triton X-100 and Protease Inhibitor Cocktail Set III. Concentrations of GST fusion protein on the beads were estimated by SDS-PAGE using a BSA standard, followed by Coomassie staining. GST-binding proteins conjugated with glutathione-Sepharose beads (CRIB-GST beads) were used for affinity precipitation in a GST pull-down assay for estimating the activity of Rac1/Cdc42 (16). After Panc1-CDH3 and Panc1-Mock cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 mmol/L MgCl₂, 0.5% Triton X-100, 5% glycerol, Protease Inhibitor Cocktail Set III], equal amounts of protein from each cell lysate were incubated with the CRIB-GST beads (8 μg of GST-fusion protein) in a volume of 100 μL at 4°C for 1 hour. The beads were washed five times with washing buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 mmol/L MgCl₂, 0.5% Triton X-100]; finally, the pellet of beads was suspended in 20 μL of Laemmli sample buffer (Bio-Rad). Bound Rac1/Cdc42 proteins were resolved on 12% polyacrylamide gels and detected by Western blotting using the respective monoclonal antibodies. The amount of each GTP-bound (active form) enzyme was normalized to the total amount of the GTPases present in whole unprecipitated cell lysates. Scanning and densitometric analyses were done with the Quantity One analysis system (Bio-Rad).

**Results.** Overexpression of P-cadherin in pancreatic ductal adenocarcinoma cells. Among dozens of genes that were up-regulated in PDAC cells during our genome-wide cDNA microarray analysis (4), we focused on P-cadherin (CDH3) for this study. Our microarray data on 20 microdissected PDAC cell populations had shown a high level of up-regulation of P-cadherin in all of the informative cases examined (4), and this time its overexpression was confirmed by reverse transcription-PCR (RT-PCR) in all of the 11 microdissected PDAC cell populations examined (Fig. 1A), which had been used for the previous microarray analysis. Immunohistochemical analysis at another series of PDAC tissues revealed strong signals of P-cadherin at the plasma membrane of cancer cells in all of 17 PDAC cases except one, whereas normal ductal and acinar

![Figure 1](https://example.com/image1.png)

**Figure 1.** Overexpression of P-cadherin in PDAC cells. A, RT-PCR confirmation of expression of P-cadherin and ACTB in microdissected PDAC cells (1–11) compared with normal pancreatic ductal epithelial cells (N) that were also microdissected. B, immunohistochemical study using anti–P-cadherin antibody. Intense staining was observed in most PDAC cells; left, two representative specimens where positive staining occurred mainly at the plasma membrane (original magnification, ×200). Acinar cells and normal ductal epithelium in normal pancreatic tissues showed very weak staining (right; original magnification, ×200).

In addition, tissue-microarray with other series of 31 PDAC tissues spotted showed that 26 of 31 PDACs expressed high levels of P-cadherin (data not shown).

**Down-regulation of endogenous N-cadherin by exogenous expression of P-cadherin.** To investigate the biological consequences of P-cadherin overexpression in PDAC cells, we transfected Panc-1 cells, in which P-cadherin expression was hardly detectable by RT-PCR and Western blotting, to establish cell lines that expressed wild-type P-cadherin stably and constitutively. As shown in Fig. 2A, a high level of P-cadherin in Panc1-CDH3 clones was confirmed by Western blotting with anti–P-cadherin antibody, but no expression was detected in Panc1-Mock cells. We also confirmed the localization of exogenously-induced P-cadherin at cell borders in Panc1-CDH3 cells (Fig. 2B).

Because several reports had indicated E-cadherin down-regulation concomitant with up-regulation of N-cadherin, and an association of those changes with tumor progression and metastatic disease (17–19), we examined whether induction of P-cadherin could affect endogenous expression of E-cadherin or N-cadherin (i.e., bring about cadherin switching) by Western blotting analysis. Analysis of several independent Panc1-derivative clones revealed drastic decreases in endogenous N-cadherin expression in Panc1-CDH3 cells compared with Panc1-Mock cells (Fig. 2A). Only slight changes were observed in E-cadherin expression levels among Panc1-CDH3 cells, but the precise effect of exogenous P-cadherin on E-cadherin expression was difficult to assess because parental Panc1 cells express very little E-cadherin
to start with. Immunocytochemical analysis also revealed decreased expression of N-cadherin at cell-cell borders in Panc1-CDH3 cells compared with Panc1-Mock cells (data not shown). Furthermore, whereas parental Panc-1 and Panc1-Mock clones exhibited spindle-shaped cells and fibroblastic morphology, Panc1-CDH3 cells typically displayed a cobblestone-like, epithelial morphology and adhered only loosely to each other (Fig. 2C). These results implied that introduction of P-cadherin into Panc-1 cells had altered their morphology and their ability to establish functional cell-cell contacts.

**Promotion of cell motility by overexpression of P-cadherin.**
We did a motility assay on uncoated membranes and an *in vitro* wound-healing migration assay. Figure 3A shows the average number of cells per field of view from three independent experiments. Panc1-CDH3 cells were significantly more motile than the control (Panc1-Mock) cells (*P* < 0.0001, Mann-Whitney *U* test). In the wound-healing migration assay, Panc1-CDH3 cells migrated more actively into the wound area than the control cells and 24 hours after formation of the wound they had filled in the wound area completely (Fig. 3B). To confirm that this rapid wound-healing effect was P-cadherin specific and independent of other cadherins, we did the same experiment using antibodies to P-cadherin to block its functional activity. As shown in Fig. 3C, the antibody against P-cadherin inhibited migration of Panc1-CDH3 cells in the wound-healing assay, but nonspecific IgG did not. On the other hand, anti-P-cadherin antibody did not affect the motility of Panc1-Mock cells, which showed no P-cadherin expression, indicating that this effect on cell motility is likely to be P-cadherin-specific. There was no significant difference between proliferation of Panc1-CDH3 and Panc1-Mock cells in counts of cell numbers or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, with or without blocking by anti-P-cadherin antibody, during this observed time (data not shown).

**Effect of P-cadherin overexpression on the subcellular localization of p120ctn.** p120ctn, one of the catenins that associate with the intracellular domain of cadherins, promotes both cell-cell adhesion and cell motility through regulation of Rho GTPases (20, 21). The most important aspect of p120ctn is the balance between its cadherin-bound form and the cytoplasmic pool; only cytoplasmic p120ctn can affect the activity of Rho GTPases (21, 22). The high affinity of cadherins for binding to p120ctn can sequester p120ctn at junctions, preventing the effect of p120ctn on the function of Rho GTPases (23). The increased motility of Panc1-CDH3 cells (Fig. 3A and B) led us to speculate that overexpression of P-cadherin might alter the levels of cytoplasmic p120ctn. We did immunocytochemical analyses to examine the subcellular distribution of p120ctn in Panc1-CDH3 and Panc1-Mock cells, and found that p120ctn was localized predominantly at the plasma membrane in parental Panc-1 and Panc1-Mock but accumulated extensively in the cytoplasm of Panc1-CDH3 cells (Fig. 3A). However, the total amounts of p120ctn protein in Panc1-CDH3 and Panc1-Mock cells, estimated by Western blotting, were the same (data not shown). As shown in Fig. 3B, the Triton X-100-based fractionation (14) of Panc1-CDH3 and Panc1-Mock cells revealed that apparently the insoluble fraction of p120ctn (P) was decreased in Panc1-CDH3 cells compared with Panc1-Mock cells, indicating that P-cadherin overexpression in Panc-1 cells decreased the amount of the insoluble and membrane-associated form of p120ctn. This is concordant with the immunocytochemistry results shown in Fig. 4A.

![Figure 2. Down-regulation of endogenous N-cadherin by overexpressed P-cadherin.](image)

**Figure 2.** Down-regulation of endogenous N-cadherin by overexpressed P-cadherin. A, Panc-1 cells were stably transfected with a CDNA encoding full-length P-cadherin (Panc1-CDH3). Mock-transfected Panc-1 cells served as controls (Panc1-Mock). Exogenous P-cadherin, endogenous expression of E-cadherin, and N-cadherin were detected in two sets of transfected clones on Western blots. Panc1-CDH3 showed a drastic decrease of endogenous N-cadherin expression compared with Panc1-Mock cells, implying “cadherin switching,” whereas E-cadherin expression in Panc1-CDH3 was not altered significantly. B, immunocytochemical staining of P-cadherin. Panc1-CDH3 cells were stained by anti-P-cadherin antibody at the borders. C, morphology of Panc1-CDH3 and Panc1-Mock cells analyzed by phase-contact microscopy. Panc1-CDH3 cells revealed typical cobblestone-like morphology whereas the Panc1-Mock cells remained spindle shaped.

We analyzed additional PDAC cell lines to clarify potential links between p120ctn distribution and endogenous expression levels of three classic cadherins (E-, N-, and P-cadherins). The expression of these classic cadherin in PDAC cell lines was assessed by Western blotting and RT-PCR. P-cadherin–expressing cell lines PK-45P, KLM-1, and KP-1N showed predominant accumulation of p120ctn in cytoplasm, whereas in a P-cadherin-null cell line,
Figure 3. Promotion of cell motility by P-cadherin overexpression. A, cell migration was estimated by means of transwell migration assays. The numbers of migrated cells were estimated 12 hours after seeding, then migrated cells were fixed, stained, and photographed. The experiment was repeated three times. Panc1-CDH3 cells were significantly more motile than the control Panc1-Mock cells ($P < 0.0001$, Mann-Whitney $U$ test). B, wound-filling ability of Panc1-CDH3 versus Panc1-Mock cells. Panc1-CDH3 cells frequently migrated into the wound area as lone cells after 12 hours and had filled in the wound after 24 hours, whereas the Panc1-Mock monolayer migrated very slowly into the wound. C, motility of Panc1-Mock (left) and Panc1-CDH3 cells (right), measured 12 and 24 hours after plating in the presence of function-blocking anti–P-cadherin antibody or control mouse IgG, in a wound-filling migration assay. Anti–P-cadherin antibody inhibited migration of Panc1-CDH3 cells whereas control mouse IgG did not. Anti–P-cadherin antibody did not affect the motility of Panc1-Mock cells that showed no P-cadherin expression.
Figure 4. Association of P-cadherin expression with cellular distribution of p120ctn. A, distribution of endogenous p120ctn in Panc1-CDH3 and Panc1-Mock cells, analyzed by immunostaining using anti-p120ctn antibody (green) and DAPI (blue). In Panc1-Mock cells, p120ctn was localized predominantly at the plasma membrane, but Panc1-CDH3 cells exhibited dissociation of p120ctn from plasma membrane and its accumulation in the cytoplasm. B, Western blot analysis of p120ctn after the Triton X-100 fractionation in Panc1-Mock cells and Panc1-CDH3 cells. The Triton X-100–soluble fraction (S) is expected to contain the cytoplasmic p120ctn and the insoluble fraction (P) of p120ctn is supported to be membrane-associated. The P and S fractions were standardized by triturating in the same volume of lysis buffer and loading the equal amount of the volume of S and P fractions from each cell to SDS-PAGE. The insoluble fraction of p120ctn (P) was decreased in Panc1-CDH3 compared with Panc1-Mock. C, four lines of PDAC cells stained using anti-p120ctn antibody (green) and DAPI (blue). In P-cadherin–expressing cells (KLM-1, PK-45P, and KP-1N), endogenous p120ctn was predominantly localized in the cytoplasm, but in SUIT-2 cells that express no P-cadherin, endogenous p120ctn was mainly localized at the plasma membrane as well as in Panc-1 cells. D, validation of knockdown effect on P-cadherin expression of KLM-1 and PK-45P by Western blotting after transfection of an siRNA-expression construct specific to P-cadherin (si-CDH3). The control construct (si-EGFP) showed no such effect. E, immunostaining using anti-p120ctn antibody (green) and DAPI (blue) after P-cadherin knockdown for 5 days in KLM-1 and PK-45P cells. The p120ctn protein was predominantly distributed in the cytoplasm in control cells (si-EGFP) as well as in nontreated KLM-1 and PK-45P, whereas it was localized mainly at the plasma membrane in KLM-1 cells and PK-45P where endogenous P-cadherin was knocked down (si-CDH3).
SUIT-2, p120ctn was predominantly localized in plasma membranes (Fig. 4C) as well as in parental Panc-1 cells. On the other hand, endogenous expression of either E-cadherin or N-cadherin did not correlate with p120ctn accumulation in cytoplasm at all.

Finally, we knocked down P-cadherin expression in KLM-1 and PK-45P cells, which have high levels of endogenous P-cadherin, by introducing a small interfering RNA (siRNA) expression construct specific to P-cadherin (si-CDH3). Five days after transfection of the siRNA-expressing plasmids under geneticin selection, we observed significant knockdown of P-cadherin expression in KLM-1 and PK-45P cells that resulted in predominant relocalization of p120ctn at the plasma membrane (Fig. 4D and E) of both cells. On the other hand, p120ctn was still localized mainly in the cytoplasm in the control cells (si-EGFP). These results strongly implied that P-cadherin expression could induce cytoplasmic accumulation of p120ctn in PDAC cells.

Promotion of cell motility by overexpressed P-cadherin through activation of Rac1 and Cdc42. Overexpression of p120ctn and its accumulation in cytoplasm seemed to increase cell motility through interaction with and activation of Rho GTPases such as Rac1 and Cdc42 (20–22). Rac1 and Cdc42 have crucial roles in cell motility; Rac1 regulates formation of lamellipodia and membrane ruffling, and Cdc42 regulates formation of filopodia (24, 25). To examine whether overexpression of P-cadherin in fact leads to activation of Rac1 and Cdc42, we did a GST pull-down assay using lysates of Panc1-CDH3 cells, and measured the amount of active (GTP-bound) Rac1 and Cdc42 showing high binding affinities to CRIB-GST beads (16, 26). When P-cadherin was overexpressed, the total amounts of Rac1 or Cdc42 (active and inactive forms) were slightly increased (Fig. 5A). In addition, their active fractions that were pulled down by GST beads (GTP-bound forms) were significantly increased in Panc1-CDH3 cells, compared with Panc1-Mock cells, on Western blots (Fig. 5A). Densitometric quantification of the Western blotting data in three independent experiments showed that the activated fraction of Rac1 was increased ~1.7-fold and that of Cdc42 ~1.6-fold in Panc1-CDH3 cells (Fig. 5B). The data indicated that P-cadherin–dependent activation of cell motility is likely to be associated with activation of Rac1 and Cdc42 through accumulation of cytoplasmic p120ctn in PDACs.

Discussion

We focused on the P-cadherin gene among dozens that were identified to be overexpressed in PDACs in an earlier genome-wide analysis (4). In this study, immunohistochemical analysis showed that P-cadherin was overexpressed in nearly 90% of another series of PDACs examined, suggesting that levels of this gene or its product could be applicable as a molecular marker for pancreatic cancer. A few previous studies of breast and endometrial cancers had suggested that P-cadherin expression might be an indicator of poor survival and aggressive behavior (27–29), and P-cadherin overexpression could be associated with poor survival and aggressive behavior of PDACs as well, but we do not have clinical data to verify that possibility. In the present study, to shed light on the role of P-cadherin in pancreatic carcinogenesis and PDAC progression, we established Panc-1 derivative cells that stably expressed wild-type P-cadherin (Panc1-CDH3) and showed that overexpression of exogenous P-cadherin promoted motility of the cancer cells.

Through the induction of P-cadherin in Panc-1 cells, we observed ‘cadherin switching’ (6, 17, 30), meaning changes in the expression of different cadherins. Cadherin switching plays a critical role during progression of some tumors [ref. 6; e.g., switching from E-cadherin to N-cadherin has been shown to enhance motility, invasiveness and metastatic potential of cancer cells (31)]. E-cadherin may suppress motility by increasing cell-cell adhesion (7, 8), whereas N-cadherin may increase motility and invasive behavior of tumor cells by incorporating FGF receptor and downstream signaling (11). In our study, we observed switching from N-cadherin to P-cadherin in Panc1-CDH3 cells and detected predominant expression of P-cadherin in several PDAC cell lines. Those findings suggested that switching from N-cadherin to P-cadherin is likely to be associated with pancreatic tumorigenesis or PDAC progression, especially in terms of increased motility of the cancer cells.

During our investigation of the molecular mechanisms involved in P-cadherin–mediated cell motility, we found that p120ctn, belonging to the cadherin/catenin family of cell adhesion proteins (32, 33), was playing an important role in the motility-promoting signal. Several recent reports have indicated that p120ctn can associate with all classic cadherin subtypes and is involved in the regulation of cell motility as well as cell adhesion (34–36). p120ctn is found in two forms, one bound to cadherins under the plasma

![Figure 5. Relationship between P-cadherin–induced motility and activation of Rac1 and Cdc42. A, Rho GTPase levels in lysates of Panc1-CDH3 and Panc1-Mock cells analyzed by Western blotting with ACTB (β-actin) serving as a quantitative control. Activities of the enzymes were determined by a pull-down assay using beads coated with the CRIB portion of p21-activated kinase-1 for specific binding of the GTP-bound forms of Rac1 and Cdc42. B, P-Cadherin overexpression led to significant augmentation of both activities in addition to increasing total Rac1 and Cdc42 in the lysates. B, quantitative results of three independent measurements of activity of the Rho GTPases in Panc1-CDH3 and Panc1-Mock cells. The amounts of active Rac1 and active Cdc42-GTP, estimated by densitometry, were normalized to the total amounts of the corresponding proteins in cell lysates (total Rac1 and total Cdc42 in A). Results are presented as ratios of activity of each Rho protein in Panc1-CDH3 cells to the activity of that protein in Panc1-Mock cells.](image-url)
membrane and the other in the cytoplasm (22, 37, 38). Different types of cadherins regulate cell movement by controlling the levels of p120ctn present in the cytoplasmic pool (22). Our immunocytochemical analyses of Panc1-CDH3 cells and other PDAC cell lines implied that cytoplasmic accumulation of p120ctn was significantly correlated with levels of P-cadherin expression but not with levels of E-cadherin or N-cadherin. We assume that alteration of p120ctn distribution may be caused by different affinities of p120ctn for each classic cadherin. Considering the switching from N-cadherin to P-cadherin in PDAC cell lines, P-cadherin should have a lower affinity for p120ctn than N-cadherin does.

In p120ctn-transfected fibroblasts that show increases of cytoplasmic p120ctn, activities of two Rho GTPases, Rac1 and Cdc42, are enhanced and migratory ability is augmented (20–22). Rho GTPases are considered to play essential roles in signaling events that regulate cadherin-dependent motility. Our estimation of the active fractions of Rac1 and Cdc42 in Panc1-CDH3 cells by pull-down assays revealed significant activation of both enzymes, indicating their involvement in the increased motility of Panc1-CDH3 cells.

Taken together, our findings indicate that overexpression of P-cadherin in pancreatic cancer cells increases cell motility by altering p120ctn trafficking and thereby enhancing activities of Rho family GTPases; those changes may be associated with the high degree of aggressiveness and potential for invasion and metastasis that are characteristic of PDACs. Inhibition of P-cadherin or P-cadherin–dependent p120ctn trafficking may represent a promising new approach to molecular therapy for aggressive PDACs.

References


Overexpressed P-Cadherin/CDH3 Promotes Motility of Pancreatic Cancer Cells by Interacting with p120ctn and Activating Rho-Family GTPases

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