Anti-patched-1 Antibodies Suppress Hedgehog Signaling Pathway and Pancreatic Cancer Proliferation

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Abstract. *Background: The hedgehog (Hh) signaling pathway is aberrantly activated in many human carcinomas including pancreatic cancer and regulates tumor cell growth. Over-production of sonic hedgehog (Shh), a ligand of the Hh signaling pathway, increases the Hh signaling activity through transmitting the signal to patched-1 (Ptch1), the receptor of the Hh signaling pathway. Materials and Methods: α-Ptch1 antibodies were raised against an oligo-peptide, designed according to the Ptch1 amino-acid sequence. The specificity of α-Ptch1 was examined by immunoblotting and immuno-fluorescence, and biological effects were detected by RT-PCR and cell proliferation assay using two pancreatic cancer cell lines, Panc1 and SUIT-2. Results: α-Ptch1 recognized a 160 kDa protein as shown by immunoblotting and cell surface staining of pancreatic cancer cells. Incubation with α-Ptch1 suppressed Hh signaling activity and proliferation of pancreatic cancer cells. Conclusion: These results provide a new strategy for controling Hh dependent development of pancreatic cancer and other Hh related carcinomas.*

Pancreatic cancer is one of the most lethal human carcinomas with overall 5-year survival rate of <5%, partially because of the difficulty of diagnosis at an early stage (1). Despite the complete surgical removal of the tumor, most patients develop the disease again as metastases or local recurrence (2). Thus, it is a task of great urgency to establish a new medical strategy for the treatment of pancreatic cancer.

Abbreviations: Hh, hedgehog; Shh, sonic hedgehog; Ptch1, patched-1; Smo, smoothened; α-Ptch1, antibodies against patched1.

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Key Words: Pancreatic cancer, hedgehog signaling pathway, patched-1, sonic hedgehog, antibodies.

Recently, the hedgehog (Hh) signaling pathway has been reported to be aberrantly activated in pancreatic cancer in a ligand dependent manner (3-9). Without ligand stimuli, transmembrane protein patched-1 (Ptch1) suppresses another transmembrane protein smoothened (Smo), resulting in the silencing of the signal. In pancreatic cancer cells, aberrantly over produced sonic hedgehog (Shh), one of three Hh ligands, binds Ptch1 and inhibits the suppressive effect of Ptch1 on Smo, which activates Gli (glioma-associated oncogene) to transcript Hh target genes (6). The activated Hh signaling pathway promotes tumor cell growth, metastases and invasion. The Smo antagonist cyclopamine has been shown to inhibit the Hh signaling pathway, and suppress tumor cell growth *in vitro* (6) and in xenografts (4). Cyclopamine also increased the E-cadherin level and reduced the invasive ability of pancreatic cancer cells *in vitro*, and inhibited the metastatic spread of immortal pancreatic cells stably over-expressing Gli1 in a xenograft model (2). These findings indicate that the Hh signaling pathway is a useful theraputic target in human pancreatic cancer. However cyclopamine is an alkaloid and not precisely targeting the hedgehog pathway.

Antibodies which recognize an oligopeptide of Ptch1 on the cancer cell surface and have the ability to silence Shh stimuli to Ptch1 were raised in the present study. The aim was to find a new strategy for controling pancreatic cancer and other Hh dependent carcinomas by precisely controling the Hh activity to avoid the side effects. The antibodies might be also useful as a tool of marking and targeting cancer-cell surface *in vivo* for the diagnosis and the drug-delivery system of anti-cancer drugs.

Materials and Methods

Cell culture, reagents and antibodies. Human pancreatic cancer cell lines (Panc1 and SUIT-2), and human breast carcinoma cell lines (SK-BR-3, BT-474, MCF-7 and MDA-MB-231) were maintained at 37˚C under a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies)

and antibiotics (100 units/mL penicillin, and 100 μg/mL streptomycin Meijiseika, Tokyo, Japan). Anti-Ptch1 anti-serum (α-Ptch1) was prepared by Sigma (Deisenhofen, Germany) by the KLH (keyhole limpet hemacyamin)-MBS method (10), briefly, an oligo-peptide customized according to the amino-acid sequence of Ptch1 (KADYPNIQH) as an antigen was conjugated to the KLH by cystein and injected to rabbits. Rabbit serum was collected before (pre-serum) and after (α-Ptch1) immunization. Another anti-Ptch1 serum (G-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoblotting. Whole-cell extraction was performed with M-PER® Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and the whole-cell extract (100 μg) was separated by electrophoresis on SDS-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schnell BioScience, Dassel, Germany). Blots were then incubated with pre-serum (1:500), α-Ptch1 (1:500) or G-19 (1:500) as primary antibodies overnight at 4˚C. Then the blots were incubated with horseradish peroxidase-linked secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 h. Immunocomplexes were detected with an ECL® plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad).

Immunostaining of cell line. Panc1 cells ($2x10^4$/well) were seeded onto pre-underlaid Poly-L-lysine coated cover glass (Asahi Techno Glass Corporation, Chiba, Japan) in 24-well plates and were incubated overnight in 10% FBS-RPMI. Immunostaining was performed as previously described with some modification (11). In brief, cells were fixed in 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100 and incubated with primary antibodies (α-Ptch1 or pre-serum) followed by secondary antibodies. After mounting in Vectorshield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), samples were examined by Laser confocal microscopy (Nikon ECLIPS E600, Tokyo, Japan). Exposure time for recording was manually fixed to be the same for pre-serum and α-Ptch1. The antibodies and dilutions used were as follows: pre-serum (1/200) or α-Ptch1 (1/200) and AlexaFluor 488 goat anti-rabbit IgG (1:400; Molecular Probes, Eugene, OR, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed according to a method described previously (9). Briefly, total RNA was extracted from each cell line with the guanidinium thiocyanate-phenol-chloroform single-step method. For the reverse transcription reaction, pd(N)6 Random Hexamer (GE Healthcare UK Ltd, Buckinghamshire, England) were used for priming. *Gli1* sense (5'-TCT GCC CCC ATT GCC CAC TTG -3') and antisense (5'-TAC ATA GCC CCC AGC CCA CTT G-3') primers yielded a 480-bp product. *Ptch1* sense (5'-CGG CGT TCT CAA TGG GCT GGT TTT-3') and antisense (5'-GTG GGG CTG CTG TCT CGG GTT CG-3') primers yielded a 376-bp product. *β-Actin* sense (5'-CCA GGC ACC AGG GCG TGA TG-3') and antisense (5'-CGG CCA GCC AGG TCC AGA CG-3') primers gave rise to a 436-bp product. Amplification conditions comprised an initial denaturation for 2 min at 94˚C followed by 30 cycles of 94˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 1 min. Amplification of each gene was in the linear range. The RT-PCR products were separated on ethidium bromide-stained 2% agarose gels. Semi-quantitative analysis was done with Molecular Imager FX Pro (Bio-Rad).

Proliferation assay. Cells ($2x10^4$/well) seeded in 48-well plates in complete culture medium were incubated overnight. After a few brief washes with RPMI, the medium was changed to 2% FBS-RPMI containing α-Ptch1 or pre-serum. After incubation for 4 days, cells were harvested by trypsinization and viable cells were counted by flow cytometry [Coulter Counter (Beckman Coulter, Fullerton, CA, USA)].

Results

Immunoblotting analysis of α-Ptch1. An extract of Panc1, a pancreatic cancer cell line, was prepared as a source of Ptch1 protein, and subjected to immunoblotting. α-Ptch1 recognized a 160 kDa band of the Panc1 extract (Figure 1A) in contrast to the pre-serum control. Extracts of 4 breast cancer cell lines, SK-BR-3, BT-474, MCF-7 and MDA-MB-231, and Panc1, were subjected to immunoblotting with α-Ptch1 or G-19. α-Ptch1 visualized the same 160 kDa bands in all 5 extracts of the cancer cell lines, consistent with the band recognized by G-19 (Figure 1B). These results indicated that α-Ptch1 antibodies have affinity with Ptch1.

Cell surface protein recognition. The localization of the target protein recognized by α-Ptch1 was checked by immunofluorescence. α-Ptch1 exclusively stained the cell surfaces (Figure 2 middle) in the same pattern as phase contrast images (Figure 2 right). When the α-Ptch1 and phase contrast images were changed to green and red, respectively, overlying of both images showed a yellow color because of the colocalization of the two images (data not shown). In contrast pre-serum stained almost nothing (Figure 2 left). Since Ptch1 is located on the cell membrane, these images confirmed that α-Ptch1 specifically recognized Ptch1.

α-Ptch1 and activity of the Hh signal pathway in pancreatic cancer cells. The effect of α-Ptch1 on Hh signaling activity in pancreatic cancer cells was analyzed by RT-PCR. Because Gli1 and Ptch1 are not only the components of Hh signaling but also target genes of Gli1 trans-activation, the mRNA levels of Gli1 and Ptch1 as markers of the activity of Hh signaling pathway were examined. Panc1 cells were incubated with α-Ptch1, and subjected to RT-PCR. α-Ptch1 suppressed the expression levels of both Gli1 and Ptch1 in contrast to pre-serum, but did not suppress β-actin, an internal control (Figure 3).

Pancreatic cancer cell proliferation. Panc1 proliferation was suppressed by α-Ptch1 in comparison to pre-serum (Figure 4a). The suppressive effect of α-Ptch1 was further examined using a different pancreatic cancer cell line, SUIT-2. α-Ptch1 significantly suppressed proliferation of SUIT-2 in

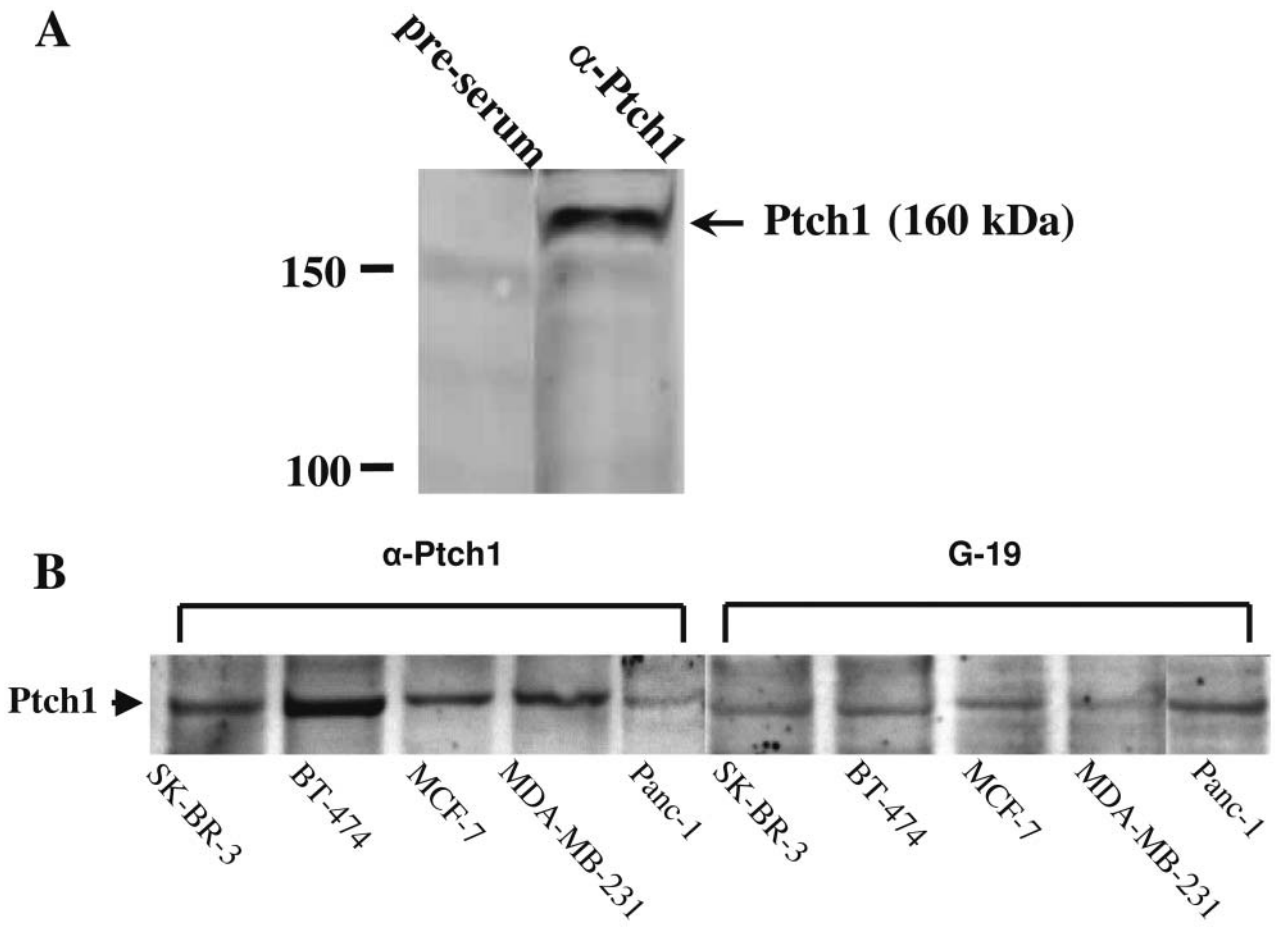

Figure 1. *α-Ptch1 recognized 160 kDa protein in immunoblotting analysis. (A) The extract of Panc1 was analyzed by immunoblotting with control pre-serum or α-Ptch1 as first antibodies. α-Ptch1 recognized a 160 kDa band in the Panc1 extract, but pre-serum did not. (B) Multiple extracts of cancer cell lines, as indicated, were analyzed by immunoblotting with α-Ptch1 or G-19. Both α-Ptch1 and G-19 recognized the same 160 kDa band in all extracts.*

comparison to pre-serum in a dose dependent manner (Figure 4B). Proliferation of a colon cancer cell line DLD1 was not attenuated by α-Ptch1 (data not shown), consistent with the fact that the Hh signaling pathway is not activated in most colon cancers (7, 12).

Discussion

Ectopic activation of the Hh signaling pathway has been demostrated in pancreatic cancer development (4) and Shh over-production is the major course of this activation (3). We have previously reported that inflammatory stimuli induced over-expression of Shh through NF-κB activation in pancreatic cancer (6). Suppression of the aberrantly activated Hh signaling pathway attenuated proliferation, invasion and metastases of pancreatic cancer (2). In the present study, antibodies raised against Ptch1 had a suppressive effect on Hh signaling activity and pancreatic cancer cell proliferation. Ptch1 is trans-membrane protein, located on the plasma membrane (13, 14). Shh binds Ptch1 and transmits signals to the sterol sensing domain (SSD) (15), which suppresses Ptch1 and inhibits Smo (16, 17). Because we aimed to suppress the activity of the Hh signaling pathway and proliferation of cancer cells, the target sequence of α-Ptch1 was located in one of the two extra-cellular arms, the putative docking site of the Hh ligand and Ptch1 to avoid stimuli from Shh to the SSD (13, 14, 18-20). As shown by RT-PCR, α-Ptch1 had the ability to suppress Hh signaling activity in pancreatic cancer cells.

We examined two pancreatic cancer cell lines for confirmation of α-Ptch1 effect on Hh activity and cancer proliferation. α-Ptch1 suppressed the proliferation of Panc1 less than that of SUIT-2, consistent with the fact that Panc1 is less dependent on the Hh signaling pathway than other pancreatic cancer cell lines (2).

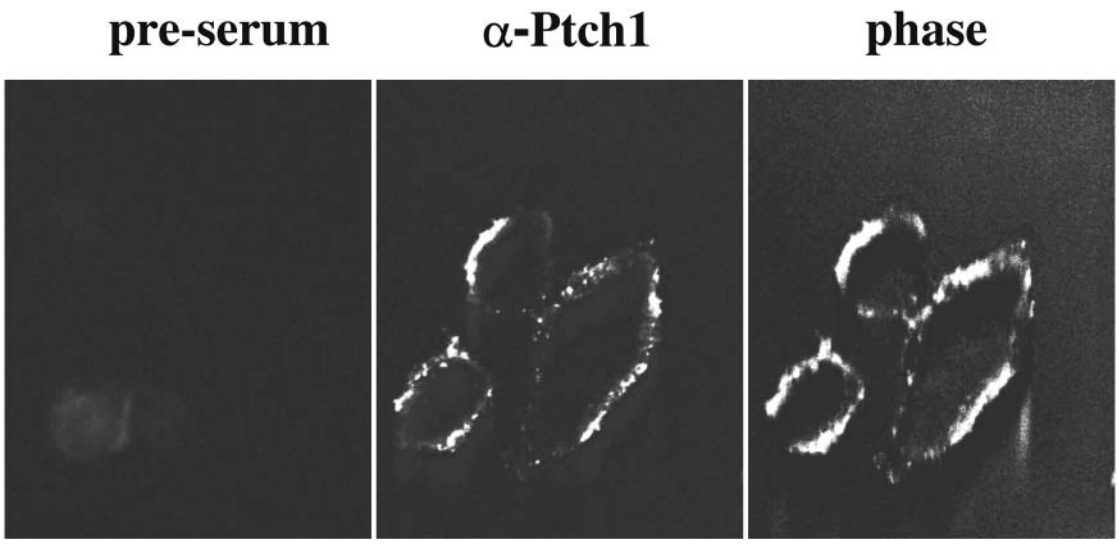

Figure 2. *α-Ptch1 recognized a cell surface protein. Panc1 cells were stained with control pre-serum (left) or α-Ptch1 (middle) as first antibodies and subjected to immunofluorescence using laser confocal microscopy. α-Ptch1 exclusively showed cell surface images, in a similar pattern to phase contrast images (right) in contrast to the pre-serum.*

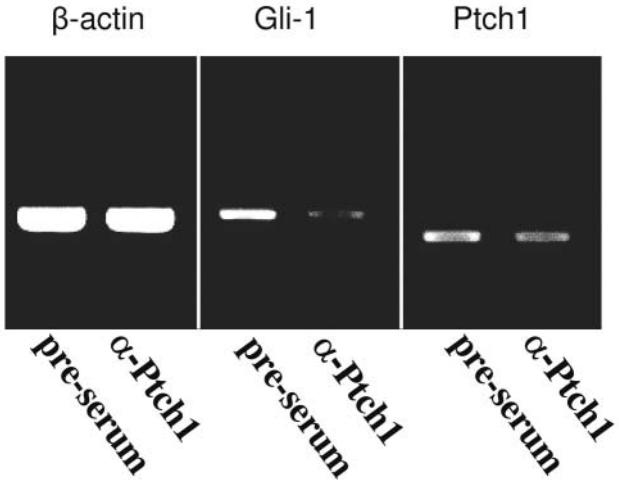

Figure 3. *α-Ptch1 antibodies suppressed the Hh signal pathway activity in pancreatic cancer cells. Panc1 cells were incubated with control pre-serum or α-Ptch1, and subjected to RT-PCR with primers for Gli1(480 bp) and Ptch1 (376 bp) with β-actin (436 bp) as internal control, as indicated. α-Ptch1 suppressed Gli1 and Ptch1 mRNA expression compared with pre-serum, while no significant differences were detected in the levels of β-actin mRNA expressions with both sera.*

The effects of α-Ptch1 on Hh signaling activity and cancer cell growth highlight the significance of the Hh pathway and Ptch1 as targets of pancreatic cancer treatment. Further research for the identification of small molecules inhibiting the function of target peptides may shed new light on pancreatic cancer treatment and other Hh related carcinomas.

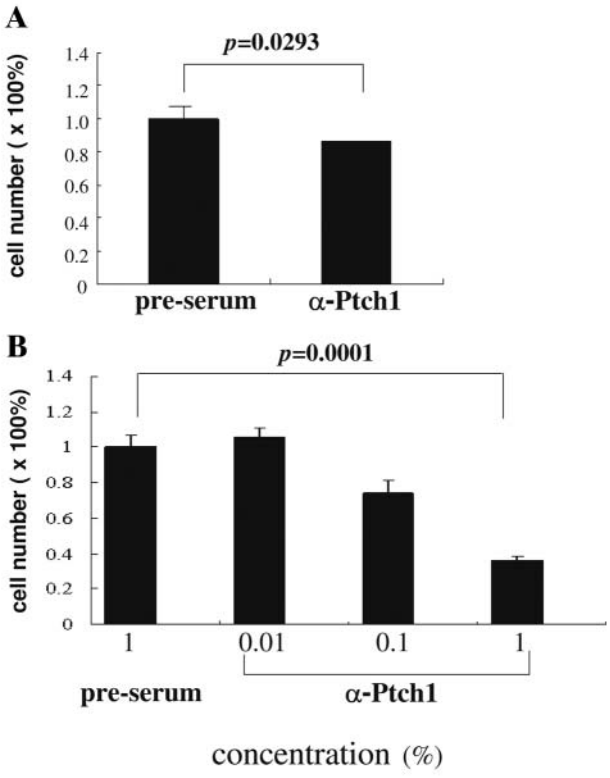

Figure 4. *α-Ptch1 suppressed pancreatic cancer cell proliferation. Panc1 (A) and SUIT-2 (B) were incubated with 1% (or the indicated concentrations) of α-Ptch1 or control pre-serum for 4 days and cell numbers were counted by flow-cytometry. Cell numbers relative to those of pre-serum are indicated. Proliferation of Panc1 was suppressed by α-Ptch1 in contrast to pre-serum (A). Proliferation of SUIT-2 was significantly suppressed by α-Ptch1 in contrast to pre-serum in a dose dependent manner (B).*

Acknowledgements

This study was supported by General Scientific Research Grants 18390350, 18659372 and 17591414 from the Ministry of Education, Culture, Sports, and Technology of Japan. We thank Kaori Nomiyama for skillful technical assistance.

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Received April 23, 2007
Revised August 1, 2007
Accepted August 14, 2007