PCA-1/ALKBH3 Contributes to Pancreatic Cancer by Supporting Apoptotic Resistance and Angiogenesis

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Abstract

The PCA-1/ALKBH3 gene implicated in DNA repair is expressed in several human malignancies but its precise contributions to cancer remain mainly unknown. In this study, we have determined its functions and clinical importance in pancreatic cancer. PCA-1/ALKBH3 functions in proliferation, apoptosis and angiogenesis were evaluated in human pancreatic cancer cells in vitro and in vivo. Further, PCA-1/ALKBH3 expression in 116 patients with pancreatic cancer was evaluated by immunohistochemistry. siRNA-mediated silencing of PCA-1/ALKBH3 expression induced apoptosis and suppressed cell proliferation. Conversely, overexpression of PCA-1/ALKBH3 increased anchorage-independent growth and invasiveness. In addition, PCA-1/ALKBH3 silencing downregulated VEGF expression and inhibited angiogenesis in vivo. Furthermore, immunohistochemical analysis showed that PCA-1/ALKBH3 expression was abundant in pancreatic cancer tissues, where it correlated with advanced tumor status, pathological stage and VEGF intensity. Importantly, patients with low positivity of PCA-1/ALKBH3 expression had improved postoperative prognosis compared with those with high positivity. Our results establish PCA-1/ALKBH3 as important gene in pancreatic cancer with potential utility as a therapeutic target in this fatal disease. Cancer Res; 72(18); 1–11. ©2012 AACR.

Introduction

Pancreatic cancer is one of the most deadly of all types of cancer in humans. The 5-year survival rate is one of the lowest, at 5% (1). Although systemic treatment including gemcitabine has recently been used for advanced pancreatic cancer, the effect of current chemotherapy is only modest (2, 3). Furthermore, at present, surgical resection offers the only chance for long-term survival for pancreatic cancer (4, 5). However, the disease is so aggressive that only about 20% of patients are indication for surgery at the time of diagnosis (6). Therefore, identification of novel targets and development of new therapeutic approaches are required against pancreatic cancer to improve patient prognosis.

Failure of apoptosis is one of the key features of tumor development (7). It is known that pancreatic cancer is highly resistant to apoptosis induced by various stimuli including chemotherapeutic agents (8, 9). Therefore, to reveal the resistance mechanism of the pancreatic cancer cells to apoptosis may lead to develop novel and effective strategies for the treatment of pancreatic cancer. Recently it has been reported that prostate cancer antigen-1 (PCA-1) was highly expressed in a few human cancers (10, 11) and was associated with apoptotic resistance of prostate cancer (12). To our knowledge, no studies have addressed the role of PCA-1 in pancreatic cancer.

PCA-1 has been found to be identical to ALKBH3, one member of human AlkB homologs (11). AlkB, an Escherichia coli protein, catalyzes the oxidative demethylation of 1-methyladenine and 3-methylcytosine in DNA and RNA (13, 14). At least 9 putative human AlkB homologs (ALKBH1-8, FTO) have been identified (15–19). Among human AlkB homologs, PCA-1/ALKBH3 has been reported to be critically involved in tumor development (20). Besides these physiological roles, PCA-1/ALKBH3 has been recently reported to be highly expressed in human actual cancer including prostate cancer (10) and non–small cell lung cancer (11). The following study has shown that silencing of PCA-1/ALKBH3 gene on prostate cancer cell by siRNA transfection induced apoptosis and suppression of cancer-cell invasion (24). Conversely, other previous studies have shown that overexpression of PCA-1/ALKBH3 makes COS-7 cells resistant to cell death due to an S,N-alkylation agent, methylmethylene sulfonate, acting primarily at the N7-position of guanine and the N3-position of adenine (13, 25). Taken together, it may be possible that PCA-1/ALKBH3 is critically involved in survival and invasion on cancer cells in certain human malignancies.

In this study, we hypothesized that PCA-1/ALKBH3 may have some roles in apoptosis resistance mechanism in
pancreatic cancer and therefore can be a potential therapeutic target. Using RNA interference method in vitro and in vivo, we investigated the biological roles of PCA-1/ALKBH3 in pancreatic cancer. Furthermore, we also tried to clarify its clinical importance in human pancreatic cancer.

Materials and Methods

Reagents, animal, and cell lines
Anti-human Ki67 monoclonal antibodies (MIB-1) were purchased from Zymed Laboratories. Anti-VEGF and anti-CD31 (PECAM-1) antibodies were purchased from Santa Cruz Biotechnology, Inc. Female C.B-17/scid-seid Jcl (SCID) mice (6-week-old) were obtained from CLEA Japan, Inc. All mice were maintained under specific pathogen-free conditions in the animal facility at Nara Medical University. All experiments were conducted under a protocol approved by our institutional review board. The human pancreatic cancer cell lines, PANC-1 and MIAPaCa-2 were obtained from RIKEN BioResource Center and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS.

Preparation of antiseras
Anti-PCA-1/ALKBH3 antiseras were prepared as described previously against a synthetic PCA-1/ALKBH3 peptide (amino acids 64 to 76) as the antigen (10). After a 0.5 mg aliquot of peptides was emulsified and injected into mice, blood was collected at 2-week intervals. The relative activity of antiseras against the synthetic peptide was tested by ELISA.

Extraction of total RNAs and real-time reverse transcriptase PCR analysis
Total RNA was isolated using RNAspin Mini (GE Healthcare UK Ltd.) and the first-strand cDNA was synthesized from 1 μg RNA using a ReverTra Ace qPCR RT Kit (TOYOBO) according to the instructions of the manufacturer. For real-time reverse transcriptase PCR analysis, cDNA was amplified in TaqMan Fast Universal PCR Master Mix (2 ×; Applied Biosystems) with gene-specific primers and probe on the StepOnePlus Real-Time PCR System (Applied Biosystems), according to the manufacturer’s instructions. Thermal cycling conditions were 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Real-time PCR experiments for each gene were carried out on 3 separate occasions. All primer/ probe sets were purchased from Applied Biosystems. The expression level of the housekeeping gene, β2-microglobulin was measured as an internal reference with a standard curve to determine the integrity of template RNA for all of the specimens. The ratio of mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β2-microglobulin).

Preparation of cell lysates and Western blot analysis
We resolved the cell lysates in SDS-polyacrylamide gels and transferred them onto polyvinylidene difluoride membranes (Millipore, Ltd.), which were then blocked in 5% skim milk at room temperature for 1 hour. The membranes were incubated with the indicated primary antibody for 1 hour, and then incubated with horseradish peroxidase–conjugated antimouse or antirabbit IgG (Amersham Pharmacia Biotech). We detected peroxidase activity on X-ray films using an enhanced chemiluminescence detection system (12).

siRNA transfection of PCA-1/ALKBH3
For our transfection analyses, PANC-1 and MIAPaCa-2 cells were seeded in 6-well plates and transfected either with control RNA (Santa Cruz Biotechnology) or with 100 nmol/L of siRNA of PCA-1/ALKBH3. Transfections were carried out using the Lipofectamine system (Invitrogen) in accordance with the manufacturer’s protocol when cells were achieved about 30% confluent. The PCA-1/ALKBH3 siRNA duplexes, generated with 3’-dTdT overhangs and prepared by QIAGEN, were chosen against the DNA target sequences as follows: (PCA-1/ ALKBH3 (1) target sequence: 5’-CAGAGAGGATAACT-TATCA-3’, PCA-1/ALKBH3 (2) target sequence: 5’-ATCGC-TATCATCTTTAGGCAA-3’).

Cell viability assay
Cells were cultured in medium containing FBS for 60 hours. After incubation, MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] reagent (Promega) was added and optical absorbance at 490 nm was measured using a microplate reader as previously described (26).

Apoptosis detection assay
After transfection with siRNA, cells were collected and stained by propidium iodide (PI) and FITC conjugated Annexin V according to the manufacturer’s protocol (TACS Annexin V-FITC kit, R&D system). Then, cells undergoing apoptosis were quantified by measurement of those bound Annexin V but negative for PI. All experiments were carried out at least 3 times in duplicate.

Generation of cells with stable PCA-1/ALKBH3 expression
PCA-1/ALKBH3 cDNA was ligated into pEBMulti-Neo vectors (Wako Pure Chemicals) at an EcoRV/BamHI site. ALKBH3 expression vectors or empty vectors were transfected into MIAPaCa-2 cells plated at 5 × 10³ cells/well using Lipofectamine 2000 (Life Technologies). The culture media were changed to media containing 900 μg/mL genetricin to select for cells with stable PCA-1/ALKBH3 expression, 24 hours after transfection.

Anchorage-dependent cell growth assay
An anchorage-dependent cell growth assay was carried out using WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, (Dojindo) according to the manufacturer’s instructions. Briefly, MIAPaCa-2 cells (200 cells/well) were seeded in a 96-well microplate up to a volume of 100 μL/well. After 1, 3, and 5 days, 10 μL of WST-1/1-methoxy-5-methylphenazinium methyl sulfone solution was added to each well. Absorbance was measured at 2 hours after the addition using a microplate reader with a test wavelength of 450 nm and a reference wavelength of 630 nm.
Anchorage-independent cell growth assay

MIAPaCa-2 cells were suspended in DMEM–10% FCS with 0.3% agar (Difco) and plated at 7,500 cells/well in 6-well plates that were precoated with 0.4% base agar in the same medium. After 2 weeks of culturing, cells were fixed and stained with 0.05% crystal violet in 50% methanol, and the number of colonies formed was counted. Images were then taken under a microscope. Groups or clusters of cells of diameter approximately 0.1 mm or larger were considered colonies.

In vitro invasion assay

Invasion assays were carried out using BD Biocoat Matrigel Invasion Chambers (24-well plates with an 8.0-μm pore size; BD Biosciences) according to the manufacturer’s instructions. In brief, 5 × 10⁴ cells in serum-free DMEM were plated in the upper chamber. DMEM supplemented with 20% FCS was placed in the lower chamber as a chemoattractant. After 24 hours of culturing, the invasive cells were fixed and stained using Diff-Quick stain solution (Sysmex). The number of invasive cells was counted under a microscope.

Microarray analysis

For microarray analysis, aRNA was amplified on the CodeLinkTM Bioarray (Applied Microarrays, Inc.), according to the manufacturer’s instructions. Microarray scanning was done using GenePixH000B (Molecular Devices). The data were analyzed using CodeLink TM Expression Analysis v5.0 software (Applied Microarray, Inc.).

Animal experimental protocol

In one in vivo model, cells (4 × 10⁶ PANC-1) were subcutaneously inoculated on one side of the ventral surface in the lower flank region of SCID mice. Treatment was started 10 days after tumor implantation. We locally injected either control RNA or 10 μmol/L of the PCA-1/ALKBH3 siRNA with AteloGene Local Use (total 0.1 mL; Koken Co.) 2 times a week for 2 weeks, according to manufactures protocol. At 4 weeks after tumor implantation, mice were sacrificed and tumors were taken for further analysis. In another in vivo model, cells (1 × 10⁶ PANC-1) were orthotopically implanted in the pancreas of SCID mice. One week after tumor implantation, we locally injected only once

![Figure 1](https://example.com/figure1.png)

Figure 1. Inhibition of PCA-1/ALKBH3 expression by PCA-1/ALKBH3 siRNA decreased cell proliferation and induced apoptosis in vitro. A, PANC-1 and MIAPaCa-2 cells were transfected with PCA-1/ALKBH3 siRNA or control RNA. The relative expression of PCA-1/ALKBH3 was determined by quantitative real-time PCR. The expression of mRNA level of PCA-1/ALKBH3 was strongly reduced in PANC-1 and MIAPaCa-2 cells when transfected with PCA-1/ALKBH3 siRNA for up to 72 hours. PANC-1: * n = 6 of each group; MIAPaCa-2: n = 3 of each group. B, cells were treated as above. Total protein lysates were extracted and subjected to immunoblotting analysis using antibodies against PCA-1/ALKBH3 and actin. The protein expression of PCA-1/ALKBH3 was also effectively suppressed in both cell lines. Lane 1, parental line; lane 2, control RNA; lane 3, PCA-1/ALKBH3 siRNA(1); lane 4, PCA-1/ALKBH3 siRNA(2). C, cells were treated as above. After 60 hours incubation, cell proliferation was determined by MTS assay. Cell proliferation was significantly suppressed by PCA-1/ALKBH3 gene silencing in these cells (n = 4 of each group). D, percentages of apoptotic cells were shown. Quantitation of apoptotic cells by flow cytometric analysis of PCA-1/ALKBH3 siRNA-treated PANC-1 and MIAPaCa-2 cells labeled with Annexin V and PI. Annexin V-positive and PI-negative cells were considered to be apoptotic cells. Data are presented as means ± SD (n = 6). **, P < 0.0001; *, P < 0.001, significant difference when the value of siRNA treatment was compared with that of the control.
either control RNA or 20 μmol/L of the PCA-1/ALKBH3 siRNA with AteloGene Local Use (total 0.1 mL; Koken Co. Tokyo Japan). At 2 weeks after tumor inoculation, the mice were killed and tumors were removed for further analysis. The tumor volume was calculated according to the formula $V = \frac{A \times B^2}{2}$ (mm$^3$), where $A$ is the largest diameter (mm) and $B$ is the smallest diameter (mm).

**In situ detection of apoptosis in tumor tissue sections**

Formalin-fixed and paraffin-embedded 5-μm-thick sections of all tumor samples were used to identify apoptotic cells by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using tumor TACS In Situ Apoptosis Detection Kit (R&D Systems, Inc.). The apoptotic index was calculated as the number of apoptotic cells in 10 high power fields ($\times 400$).

**Patients**

We examined 116 patients with pancreatic cancer who underwent surgery at Department of Surgery, Nara Medical University, between 1996 and 2008. The median age of the patients was 65.5 years, with a range of 33 to 82 years. Tissues were obtained from the resected specimens and then fixed in 10% phosphate-buffered formalin and embedded in paraffin. Tumors were classified according to the TNM staging system (27). Follow-up was until death or October 2011. Written informed consent was obtained from patients to use their surgical specimens and clinicopathological data for research purposes.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, deparaffinized, and rehydrated in a graded series of ethanol. Antigen retrieval was done by heating tissue sections using a Target Retrieval Solution, pH 9.0 (DAKO). To block endogenous peroxidase, sections were immersed in 0.3% solution of hydrogen peroxide in absolute methanol for 10 minutes at room temperature and washed in fresh PBS for 3 times, each of 5 minutes duration. Purified mouse anti-human PCA-1/ALKBH3 antibody diluted 1:200 with Antibody Diluent (DAKO) was added and incubated overnight at 4°C. Sections were washed in PBS for 3 times, each of 5 minutes duration, and then we use EnVision +, Mouse/HRP or Rabbit/HRP (DAKO) according to the instructions of the manufacturer. Sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and coverslipped. Authorized pathologists who had no knowledge of the patients’ clinical status and outcome evaluated immunohistochemistry for PCA-1/ALKBH3. Percentage of cells positive for PCA-1/ALKBH3 were expressed per >1,000 cells examined. Each sample was classified into 2 groups according to median of positivity. To count microvessels in murine pancreatic...
cancer tissue, 5 randomly selected areas were counted at ×200 magnification, and the summation was calculated.

Statistical analysis

Results were expressed as mean values ± standard deviation (SD) or as medians with ranges, and the Student t test, the chi-square test, Fisher’s exact test, or the Mann–Whitney U test were used for evaluating statistical significance. Kaplan–Meier survival calculations and the corresponding log-rank tests were carried out to determine differences in survival rates. A P value < 0.05 was considered statistically significant.

Results

Silencing of PCA-1/ALKBH3 inhibits tumor cell proliferation and induces apoptosis in human pancreatic cancer cell lines

To assess the roles of PCA-1/ALKBH3 in human pancreatic cancer, we first examined the effects of PCA-1/ALKBH3 down-regulation in vitro. Human pancreatic cancer cell lines, PANC-1 and MIAPaCa-2 were selected, because these cell lines express PCA-1/ALKBH3 mRNA and protein. Both mRNA and protein expressions of PCA-1/ALKBH3 were substantially reduced in PANC-1 and MIAPaCa-2 cells, when transfected with PCA-1/ALKBH3 siRNA for up to 72 hours (Fig. 1A and B). Cell proliferation for up to 60 hours was significantly suppressed by PCA-1/ALKBH3 gene silencing in these cells (Fig. 1C). Apoptosis detection assay showed a significant increase in the percentage of Annexin V-positive and PI-negative cells with downregulation of PCA-1/ALKBH3 (Control RNA, vs. PCA-1/ALKBH3 siRNA, PANC-1; 3.5 ± 0.3% vs. 29.0 ± 3.0%, P < 0.001, MIAPaCa-2; 1.1 ± 0.5% vs. 20.3 ± 2.4%, P < 0.001; Fig. 1D). Data suggested that PCA-1/ALKBH3 silencing induced apoptosis and restrained cell proliferation in PANC-1 and MIAPaCa-2 cells.

Overexpression of PCA-1/ALKBH3 promotes anchorage-independent growth and invasiveness of pancreatic cancer cell

To further investigate the role of PCA-1/ALKBH3 in pancreatic cancer cell, we then examined the effect of its overexpression in vitro. MIAPaCa-2 cells were episomally
transfected with ALKBH3 expression vectors or empty vectors and then selected using geneticin. Cell lysates were immunoblotted with anti-PCA-1/ALKBH3 and anti-β-actin antibodies. Higher expression of PCA-1/ALKBH3 was detected in cells transfected with pEBMulti-Neo-ALKBH3 than in those transfected with empty vectors (Fig. 2A). Next we investigated anchorage-dependent cell growth of MIAPaCa-2 cells overexpressing PCA-1/ALKBH3. Cell growth was assessed using the WST-1 assay on days 1, 3, and 5 after plating under standard culture conditions. Anchorage-dependent cell growth did not differ between cells overexpressing PCA-1/ALKBH3 and the control cells (Fig. 2B). In contrast, in the case of anchorage-independent cell growth, a higher colony number was observed for the cells overexpressing PCA-1/ALKBH3 than for the control cells, suggesting that ALKBH3 is an important molecule that regulates the anchorage-independent growth of pancreatic cancer cells (Fig. 2C). Furthermore, overexpression of PCA-1/ALKBH3 promoted the invasiveness of MIAPaCa-2 cells (Fig. 2D). These results suggested that PCA-1/ALKBH3 plays a significant oncogenic role in pancreatic cancer cell.

Effect of silencing PCA-1/ALKBH3 in pancreatic cancer in vivo

To evaluate the role of PCA-1/ALKBH3 in pancreatic cancer cell under physiological conditions, we then examined the effect of PCA-1/ALKBH3 siRNA in pancreatic cancer in vivo. PANC-1 cells were subcutaneously inoculated on SCID mice and treated with PCA-1/ALKBH3 siRNA in the presence of atelocollagen. By immunohistochemistry, PCA-1/ALKBH3 expression was successfully downregulated by in vivo PCA-1/ALKBH3 siRNA transfection (Fig. 3A). This was confirmed by Western blot analysis on pancreatic cancer tissues at 4 weeks after tumor implantation. Knockdown efficacy was of high degree (Fig. 3B). PCA-1/ALKBH3 downregulation induced substantial antitumor effect in vivo and significantly inhibited tumor growth (Fig. 3C). And the weights of PCA-1/ALKBH3 siRNA treated tumors at sacrifice were significantly reduced compared with controls (Fig. 3D).

To confirm the above in vivo data, we next used an orthotopic in vivo model. PANC-1 cells were orthotopically implanted on SCID mice and treated with PCA-1/ALKBH3

Figure 4. Effect of targeting PCA-1/ALKBH3 on pancreatic cancer in vivo. A, the representative micrographs show immunohistochemical staining for cell proliferation (Ki67) and apoptosis (TUNEL) in tumor tissues obtained from subcutaneous models. A significant decrease in Ki67 staining was evident in tumor tissue samples obtained from PCA-1/ALKBH3 siRNA-treated mice when compared with mice given control RNA. Conversely, a significant increase in TUNEL expression, representing apoptosis, was evident in tumors obtained from mice given PCA-1/ALKBH3 siRNA compared with controls. B, the number of TUNEL-positive cells. Apoptosis was significantly induced in PCA-1/ALKBH3 siRNA treated tumors compared with controls (control RNA, n = 5; PCA-1/ALKBH3 siRNA, n = 5). C, the representative micrographs show immunohistochemical staining for Ki67 and TUNEL-positive cells (arrows) in tumor tissues obtained from orthotopic model. A significant decrease in Ki67 staining was evident in tumor tissue samples obtained from PCA-1/ALKBH3 siRNA-treated mice when compared with mice given control RNA. Conversely, a significant increase in TUNEL expression, representing apoptosis, was evident in tumors obtained from mice given PCA-1/ALKBH3 siRNA compared with controls. D, apoptotic cells determined by TUNEL staining were significantly higher in PCA-1/ALKBH3 knockdown compare to control (control RNA, n = 5; PCA-1/ALKBH3 siRNA, n = 8).
siRNA in the presence of atelocollagen. As shown in Fig. 3E, PCA-1/ALKBH3 expression was also successfully downregulated by in vivo PCA-1/ALKBH3 siRNA transfection in this model. PCA-1/ALKBH3 downregulation induced substantial antitumor effect, thereby resulting in significant inhibition of tumor growth (Fig. 3F). Furthermore, the weights of PCA-1/ALKBH3 siRNA treated tumors at sacrifice were significantly reduced compared with controls (Fig. 3G).

Proliferation activity of subcutaneous PANC-1 tumors examined by Ki67 staining was significantly suppressed by PCA-1/ALKBH3 knockdown in vivo (Fig. 4A). Then, tumor cells undergoing apoptosis in the subcutaneous model were quantified by measurement of nuclear staining for TUNEL. As a result, apoptosis was frequently induced in cells with PCA-1/ALKBH3 silencing (Fig. 4A and B). In the orthotopic model, Ki67 staining was also significantly suppressed by PCA-1/ALKBH3 silencing (Fig. 4C). Apoptotic cells were significantly higher in PCA-1/ALKBH3 knockdown compared to control (Fig. 4D). Taken together, PCA-1/ALKBH3 silencing reduced cell proliferation and induced apoptosis in human pancreatic cancer cells in vitro and in vivo.

PCA-1/ALKBH3 silencing reduces VEGF expression in human pancreatic cancer

Next we explored the other underlying mechanism in effects of PCA-1/ALKBH3 silencing in tumors. To this end, we used microarray analysis to compare the gene expression profiles of PANC-1 cells transfected with PCA-1/ALKBH3 siRNA versus control RNA. We identified 106 genes that were 3 times up- or downregulated in PCA-1/ALKBH3 siRNA vs. control RNA (Supplementary Table S1). In these genes, we focused on VEGF that is a major stimulator of angiogenesis, because angiogenesis is a key mechanism in many human cancers. By qPCR, the mRNA expression of VEGF was significantly downregulated in PANC-1 and MIAPaCa-2 cells by PCA-1/ALKBH3 siRNA transfection in vitro (Fig. 5A). And PCA-1/ALKBH3 silencing also reduced protein expression of VEGF in vitro (Fig. 5B). In addition, VEGF expression was significantly decreased in PCA-1/ALKBH3 silenced tumors compared with controls in both subcutaneous and orthotopic models as shown by immunohistochemistry (Fig. 5C). Western blotting further confirmed that VEGF expression was strongly decreased in vivo subcutaneous models by this injection of PCA-1/ALKBH3 siRNA versus control RNA (Fig. 5D). PCA-1/ALKBH3 downregulation significantly decreased the numbers of micro vessels in tumors (Fig. 5E and F). Data

Figure 5. Effect of angiogenesis targeting PCA-1/ALKBH3 on pancreatic cancer. A, PANC-1 and MIAPaCa-2 cells were transfected with PCA-1/ALKBH3 siRNA. The relative expression of VEGF was determined by quantitative real-time PCR (PANC-1: PCA-1/ALKBH3 siRNA, n = 6; control RNA, n = 6; MIAPaCa-2: PCA-1/ALKBH3 siRNA, n = 3; control RNA, n = 3). **, P < 0.001; *** P < 0.0001. B, cells were treated as above. Total protein lysates were extracted and subjected to immunoblotting analysis using antibodies against VEGF and actin. Lane 1, parental line; lane 2, control RNA; lane 3, PCA-1/ALKBH3 siRNA(1); lane 4, PCA-1/ALKBH3 siRNA(2). C, the representative immunohistochemical staining for VEGF in tumor tissues obtained from mice. A significant decrease in VEGF was evident in tumor tissue samples obtained from PCA-1/ALKBH3 siRNA-treated mice when compared with mice given control RNA both in subcutaneous and orthotopic models. D, Western blotting indicated that VEGF expression was strongly decreased in vivo by local injection of PCA-1/ALKBH3 siRNA vs. control RNA. E, immunohistochemical staining for CD31 in tumor tissues obtained from mice. F, analysis of microvessel counts in tumors at 28-day after tumor cells injection between control RNA and PCA-1/ALKBH3 siRNA mice. The numbers of CD31 positive vessels within tumors treated with PCA-1/ALKBH3 siRNA were significantly lower than control mice (control RNA, n = 5; PCA-1/ALKBH3 siRNA, n = 5; P < 0.01).
suggested that PCA-1/ALKBH3 silencing not only reduced cell proliferation and induced apoptosis, but also decreased angiogenesis in human pancreatic tumor.

Clinical significance of PCA-1/ALKBH3 expression in human pancreatic cancer

We evaluated the PCA-1/ALKBH3 expression in 116 actual human pancreatic cancer tissues by immunohistochemistry. Positive staining was seen in all examined specimens of pancreatic cancer. On the other hand, PCA-1/ALKBH3 expression was not observed in noncancer tissues of the pancreas. Median positivity of PCA-1/ALKBH3 was 62.5%. Then all specimens were classified into 2 groups according to the median of positivity of PCA-1/ALKBH3 expression (Fig. 6A). We evaluated the correlation of the PCA-1/ALKBH3 expression with various clinicopathological findings. The 2 groups did not differ significantly with respect to age or histopathological grading. We found that tumors with a high positivity of PCA-1/ALKBH3 expression significantly correlated with advanced T status (T1, T2, T3 vs. T4, \( P = 0.0079 \)) and pathological stage (\( P = 0.0144 \); Table 1). These data suggested that PCA-1/ALKBH3 expression might be functionally important in tumor progression in pancreatic cancer. Then, we compared postoperative prognosis between 2 groups. Importantly, tumors with low positivity of PCA-1/ALKBH3 expression had significantly better postoperative prognosis compared with tumors with high positivity (Fig. 6B). Finally, we evaluated correlation between PCA-1/ALKBH3 and VEGF expression in actual human pancreatic cancer. We scored the intensity of immunostaining of VEGF as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining (Fig. 6C), and then classified score 0 and 1 as low intensity, and score 2 and 3 as high intensity. We found that tumors with a high positivity of PCA-1/ALKBH3 expression significantly correlated with high intensity of VEGF expression in human cancer (\( P \leq 0.0001 \); Table 1). These data indicated that PCA-1/ALKBH3 might play important roles in actual human pancreatic cancers and also be a potential therapeutic target for human pancreatic cancer.

Discussion

Humans are continuously exposed to agents that methylate DNA and RNA, such as tobacco-specific nitrosamines (28) and cellular S-adenosylmethionine (29). These agents may initiate abnormal methylation on genes. When DNA methylation is dysregulated, the harmful methylation contributes to diseases such as cancer (30–33). Therefore, the enzymatic functions of repairing these abnormalities are critical for maintaining DNA and RNA integrity. Recently, several demethylating enzymes have been studied very well. In these proteins, it has been reported that AlkB and its human homologs play an important role in demethylation of DNA (13, 14, 19, 20, 22, 23, 34). Among human AlkB homologs, ALKBH3 is known as a unique member of the AlkB superfamily that has a unique biochemical function in removing methylation from the messenger RNA (mRNA).

Figure 6. Immunohistochemical staining of human pancreatic cancer tissue for PCA-1/ALKBH3 and survival rate after surgery. A, representative case of high positivity (Hi) and low positivity (Low) of PCA-1/ALKBH3. Original magnification, \( \times 100 \). B, overall survival of 116 patients with pancreatic cancer in relation to tumor PCA-1/ALKBH3 status. Tumors with high positivity of PCA-1/ALKBH3 expression had significantly poorer prognosis compared with tumors with low positivity (\( P = 0.0255 \)). C, immunohistochemical analysis of VEGF in human pancreatic cancer tissues of no (score 0), mild (score 1), moderate (score 2), and strong (score 3) intensities.
to demethylate RNA besides repairing methylated DNA (13, 35–37). Several previous studies suggest that ALKBH3 may play physiological key roles in humans.

There are only limited studies that have recently described roles of AlkB family in cancer biology. For instance it has been reported that PCA-1/ALKBH3 was specifically expressed and contributed to cell survival and invasion in human prostate cancer (10, 24) and in human non–small cell lung cancer (11). In gastrointestinal tumor, it has been reported that in rectal cancer tumor tissues overexpressed PCA-1/ALKBH3 compared with normal rectal tissues, although the function and the role of PCA-1/ALKBH3 have not been addressed (38). It was also reported that PCA-1/ALKBH3 was one of the candidate gene associated with the risk of papillary thyroid cancer (39). Nevertheless, the roles of PCA-1/ALKBH3 in carcinogenesis were largely unknown. In other ALKBHs, ALKBH8 contributes to progression of human bladder cancer (12). The other homolog, ALKBH2, downregulation of it increases cisplatin sensitivity in human lung cancer cell line (40). By sharp contrast, the antitumor activity of ALKBH2 has been reported in gastric cancer. In that study, overexpression of ALKBH2 significantly inhibited the proliferation of cancer cells, and induced G1 arrest of the cell cycle, whereas ALKBH2 knockdown promoted cell growth and cell cycle progression of cancer cells (41). Thus, at present the roles of AlkB family in cancers were diverse, complex and controversial. In addition, there is no report that examined the roles of AlkB family in pancreatic cancer. Therefore, in this study, we were intrigued with PCA-1/ALKBH3 in pancreatic cancer and investigated precise roles of PCA-1/ALKBH3 in pancreatic cancer.

We found several important observations. First, we examined the biological mechanism of PCA-1/ALKBH3 on pancreatic cancer by using siRNA method in vitro. Cell proliferation was significantly suppressed by PCA-1/ALKBH3 gene silencing in both PANC-1 and MIAPaCa-2 cells. Furthermore, PCA-1/ ALKBH3 siRNA transfection induced apoptosis to these pancreatic cancer cell lines. These might be consistent with previous reports on prostate cancer (10, 24). On the other hand, we showed that overexpression of PCA-1/ALKBH3 in MIAPaCa-2 cells increased anchorage-independent growth and invasive potential. These data showed that PCA-1/ALKBH3 had oncogenic significance in pancreatic cancer cells. Second, there is no study to have examined the mechanism of PCA-1/ALKBH3 knockdown in physiological in vivo model. Then we investigated the in vivo roles of PCA-1/ALKBH3 downregulation in pancreatic cancer. We used subcutaneous and orthotopic xenograft tumor models used PANC-1 cells. PCA-1/ALKBH3 knockdown reduced the size of tumors in these models. As underlying mechanisms, we revealed that proliferation activity examined by Ki67 was significantly suppressed, and apoptosis evaluated by TUNEL was significantly induced in cancer tissues in both models.

Next, for a deeper understanding of functions of PCA-1/ ALKBH3 in pancreatic cancer, we focused on angiogenesis. Angiogenesis play a key role in tumor growth and metastasis. Since about 40 years ago, it has become to be known that the growth of any tumor depends on angiogenesis. Without angiogenesis, the tumor cannot grow beyond a few millimeters in diameter (42). VEGF is the most prevalent and dominant proangiogenic growth factor in the tumor microenvironment (43–45). Although most pancreatic cancers are hypovascular or avascular, the human pancreatic cancer cells reportedly overexpress pro-angiogenic molecules such as VEGF (46). We (47) and others (48) have shown that the intratumoral microvessel density is an independent prognostic factor in pancreatic cancer patients. Furthermore, in animal models, the antiangiogenic therapies have been effective in inhibiting pancreatic tumor development. Therefore, angiogenesis also plays a pivotal role in the growth of pancreatic tumors. Recently, antiangiogenic agents include the anti-VEGF antibody bevacizumab, has shown promise in the treatment of patients with several cancers (49, 50). Therefore, we examined involvement of PCA-1/ALKBH3 in VEGF. PCA-1/ALKBH3 knockdown also reduced both mRNA and protein expression of VEGF in PANC-1 and MIAPaCa-2 cells in vitro. These suggested that PCA-1/ALKBH3 correlated with not only apoptosis but also angiogenesis, and might be upstream of VEGF in cancer cells. The expression of VEGF was also significantly decreased by down-regulation of PCA-1/ALKBH3 in vivo. And then the number of

Table 1. Comparison of clinicopathological characteristics according to tumor PCA-1/ ALKBH3 expression

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<thead>
<tr>
<th>Characteristics</th>
<th>PCA-1 low (n = 59)</th>
<th>PCA-1 high (n = 57)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66 (42–81)</td>
<td>66 (33–80)</td>
<td>0.6480</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (55.9%)</td>
<td>34 (59.7%)</td>
<td>0.6854</td>
</tr>
<tr>
<td>Female</td>
<td>26 (44.1%)</td>
<td>23 (40.4%)</td>
<td></td>
</tr>
<tr>
<td>Histopathological grading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>17 (28.8%)</td>
<td>17 (29.8%)</td>
<td>0.6156</td>
</tr>
<tr>
<td>G2</td>
<td>34 (57.6%)</td>
<td>31 (54.4%)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>5 (8.5%)</td>
<td>8 (14.0%)</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>3 (5.1%)</td>
<td>1 (1.8%)</td>
<td></td>
</tr>
<tr>
<td>pT category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1/pT2/pT3</td>
<td>53 (89.8%)</td>
<td>40 (70.2%)</td>
<td>0.0079</td>
</tr>
<tr>
<td>pT4</td>
<td>6 (10.2%)</td>
<td>17 (29.8%)</td>
<td></td>
</tr>
<tr>
<td>pN category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>28 (47.5%)</td>
<td>19 (40.4%)</td>
<td>0.1214</td>
</tr>
<tr>
<td>pN1</td>
<td>31 (52.5%)</td>
<td>38 (59.6%)</td>
<td></td>
</tr>
<tr>
<td>pM category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM0</td>
<td>57 (96.6%)</td>
<td>50 (87.7%)</td>
<td>0.0735</td>
</tr>
<tr>
<td>pM1</td>
<td>2 (3.4%)</td>
<td>7 (12.3%)</td>
<td></td>
</tr>
<tr>
<td>UICC stage</td>
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<tr>
<td>IA/IB</td>
<td>14 (23.7%)</td>
<td>7 (12.3%)</td>
<td>0.0144</td>
</tr>
<tr>
<td>IIA/IIB</td>
<td>38 (64.4%)</td>
<td>29 (50.9%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>5 (8.5%)</td>
<td>14 (24.6%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2 (3.4%)</td>
<td>7 (12.3%)</td>
<td></td>
</tr>
<tr>
<td>VEGF intensity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>22 (37.3%)</td>
<td>42 (73.7%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Low</td>
<td>37 (62.7%)</td>
<td>15 (26.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: UICC, International Union Against Cancer.
microvessels in tumors treated with PCA-1/ALKBH3 siRNA was significantly lower compared to that in tumors treated with control RNA. Although PCA-1/ALKBH3 knockdown led to apoptosis in 29% of Panc-1 cells in vitro, it led to about 50% to 70% of tumor reduction compared with controls in vivo subcutaneous and orthotopic models. Taken together, data suggested that apoptosis induction and anti-angiogenesis induced by PCA-1/ALKBH3 knockdown might synergistically exert antitumor effect in vivo.

In our study, PCA-1/ALKBH3 knockdown induced not only apoptosis but also reduction of VEGF in 2 human pancreatic cancer cell lines. However, the relations of PCA-1/ALKBH3 and angiogenesis are still unknown. Therefore, further fundamental studies are clearly required. Collectively PCA-1/ALKBH3 might be a potential target against pancreatic cancer, because blockade of PCA-1/ALKBH3 induced both apoptosis and anti-angiogenesis.

Finally, we examined clinical importance of PCA-1/ALKBH3 in actual human pancreatic cancer, and found that PCA-1/ALKBH3 expression was abundant in most human pancreatic cancer tissues and was not seen in noncancer tissues. The expression of PCA-1/ALKBH3 may be helpful for the early diagnosis of pancreatic cancer. The tumor PCA-1/ALKBH3 expression in human pancreatic cancer significantly correlated with advanced tumor status and pathological stage, whereas it did not relate to lymph node and distant metastasis. These clinical data suggested that tumor PCA-1/ALKBH3 might be an important factor of proliferation and invasion rather than that of metastasis in pancreatic cancer. Therefore, further study to evaluate the serum level of PCA-1/ALKBH3 in pancreatic cancer patients may be warranted. In addition, patients with high positivity of PCA-1/ALKBH3 expression have significantly poor prognosis in comparison with low positivity of PCA-1/ALKBH3 expression. Furthermore, we showed correlation between positivity of PCA-1/ALKBH3 and intensity of VEGF expression in human cancer. These data further emphasized that PCA-1/ALKBH3 may be a promising therapeutic target against human pancreatic cancer.

In conclusion, we have shown for the first time that PCA-1/ALKBH3 participates in apoptotic resistance of cancer cells, proliferation of tumor and tumor angiogenesis in pancreatic cancer. An inverse correlation has been also observed between PCA-1/ALKBH3 expression and prognosis in pancreatic cancer patients. This study may provide the rationale of developing a novel cancer therapy targeting PCA-1/ALKBH3 for this fatal malignant disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: I. Yamato, M. Sho, K. Shimada, N. Konishi, K. Tsujikawa
Development of methodology: I. Yamato, M. Sho, K. Shimada, Y. Ueda, N. Konishi, K. Tsujikawa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Yamato, M. Sho, K. Shimada, K. Hotta, Y. Ueda, S. Yasuda, N. Konishi, K. Tsujikawa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Yamato, M. Sho, K. Hotta, K. Tsujikawa
Writing, review, and/or revision of the manuscript: I. Yamato, M. Sho, N. Konishi, K. Tsujikawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Sho, S. Yasuda, N. Shigi, K. Tsujikawa
Study supervision: M. Sho, K. Shimada, N. Konishi, K. Tsujikawa, Y. Nakajima

Grant Supports
This study was supported by the following grants: Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (M. Sho) and Kansai Biomedical Cluster project promoted by the Knowledge Cluster Initiative (stage-II) of the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (K. Tsujikawa).

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Received January 30, 2012; revised July 3, 2012; accepted July 17, 2012; published OnlineFirst July 23, 2012.

References


PCA-1/ALKBH3 Contributes to Pancreatic Cancer by Supporting Apoptotic Resistance and Angiogenesis

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Cancer Res  Published OnlineFirst July 23, 2012.

Updated version  Access the most recent version of this article at:
        doi:10.1158/0008-5472.CAN-12-0328

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