

Adenylate cyclase-associated protein 1 overexpressed in pancreatic cancers is involved in cancer cell motility

Ken Yamazaki¹, Masaaki Takamura², Yohei Masugi¹, Taisuke Mori¹, Wenlin Du¹, Taizo Hibi³, Nobuyoshi Hiraoka⁴, Tsutomu Ohta⁴, Misao Ohki⁴, Setsuo Hirohashi⁵ and Michiie Sakamoto¹

Pancreatic cancer has the worst prognosis among cancers due to the difficulty of early diagnosis and its aggressive behavior. To characterize the aggressiveness of pancreatic cancers on gene expression, pancreatic cancer xenografts transplanted into severe combined immunodeficient mice served as a panel for gene-expression profiling. As a result of profiling, the adenylate cyclase-associated protein 1 (CAP1) gene was shown to be overexpressed in all of the xenografts. The expression of CAP1 protein in all 73 cases of pancreatic cancer was recognized by immunohistochemical analyses. The ratio of CAP1-positive tumor cells in clinical specimens was correlated with the presence of lymph node metastasis and neural invasion, and also with the poor prognosis of patients. Immunocytochemical analyses in pancreatic cancer cells demonstrated that CAP1 colocalized to the leading edge of lamellipodia with actin. Knockdown of CAP1 by RNA interference resulted in the reduction of lamellipodium formation, motility, and invasion of pancreatic cancer cells. This is the first report demonstrating the overexpression of CAP1 in pancreatic cancers and suggesting the involvement of CAP1 in the aggressive behavior of pancreatic cancer cells.

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Pancreatic cancer is a leading cause of cancer death worldwide.^{1,2} Only about 20% of pancreatic cancer can be surgically resected with curative intent at the time of diagnosis.³ Mainly due to the difficulty in early detection and frequent metastatic dissemination, the 5-year survival rate for pancreatic cancer patients remains below 5%.^{3,4} Considering that vascular involvement, lymph node metastasis, and neural invasion have been proposed as prognostic factors,^{5–7} it seems that metastasis is responsible for the aggressive behavior of pancreatic cancer. Pancreatic cancer appears to acquire genetic aberrations with successive alteration of genes involved in the regulation of cell proliferation. The alterations include activating mutations of the KRAS gene^{8,9} that occur early in the stage of pancreatic carcinogenesis, and inactivations by the deletion and mutation of the CDKN2A,¹⁰ TP53,¹¹ and SMAD4 genes.¹² In addition to cell proliferation, cell motility is one of the factors associated with cancer metastasis; however, no alteration of such genes has been revealed in pancreatic cancer. Therefore, alteration of the

expression level of genes by epigenetic or transcriptional manner may be a cue to elucidate the mechanism of cancer metastasis.

Recent developments in the technology of human genome research enabled us to obtain genome-wide gene-expression profiles, and vast numbers of marker molecule candidates for pancreatic cancer have been proposed.^{13–18} In this study, xenografts of clinical specimens orthotopically transplanted into severe combined immunodeficient (SCID) mice¹⁹ were utilized as a panel for gene-expression profiling of pancreatic cancer. Among commonly overexpressed genes in pancreatic cancer xenografts, we further evaluated the adenylate cyclase-associated protein 1 (CAP1) gene, which encodes an actin monomer-binding protein (reviewed in reference²⁰). The reorganization of actin filament is essential for cell migration and is regulated by actin-binding proteins in some signaling pathways.^{21,22} Therefore, we examined the role of overexpression of CAP1 in cell motility and the pathology of pancreatic cancer.

¹Department of Pathology, School of Medicine, Keio University, Tokyo, Japan; ²Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; ³Department of Surgery, School of Medicine, Keio University, Tokyo, Japan; ⁴Cancer Genomics Division, National Cancer Center Research Institute, Tokyo, Japan and ⁵Pathology Division, National Cancer Center Research Institute, Tokyo, Japan

Correspondence: Professor M Sakamoto, MD, PhD, Department of Pathology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.

E-mail: msakamot@sc.itc.keio.ac.jp

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MATERIALS AND METHODS

Tissue Samples

Pancreatic cancer and non-neoplastic pancreas tissues were obtained from patients who underwent surgical resection at Keio University Hospital and the National Cancer Center Hospital, Japan. All experiments using human samples were approved by the ethics committees of the National Cancer Center and of Keio University, School of Medicine. Cancer tissue fragments from 12 patients were orthotopically implanted into SCID mice as described previously.¹⁹

Microarray Analyses

Total RNA of each xenograft was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and then RNeasy (QIAGEN, Valencia, CA, USA). Biotinylated cRNA was prepared from 5 µg of total RNA and then hybridized to GeneChip HG-U133A (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Each profile was normalized as the mean signal of all probe sets at 1000. Microarray data are accessible from our in-house database, Genome Medicine Database of Japan (GeMDBJ; <https://gemdbj.nibio.go.jp/dgdb/>).

Antibodies

We used two antisera against human CAP1 protein. Both a rat polyclonal antibody (a kind gift from Dr Kenji Moriyama, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan)²³ and a rabbit polyclonal antibody recognize the C-terminal domain of human CAP1. Each antibody raises similar results in Western blot and immunohistochemical analyses, and the specificity was further confirmed by antigen absorption tests as in a previous report²⁴ for the rabbit polyclonal antibody (data not shown).

Western Blot Analyses

Lysates of pancreatic cancers, non-neoplastic pancreas tissues, and pancreatic cancer cells were loaded onto 10% polyacrylamide-SDS gels, separated by electrophoresis, and blotted onto PVDF membranes by the semidry transfer method. Anti-CAP1 and anti-actin (Sigma, St Louis, MO, USA) antibodies were hybridized to the membranes overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were probed to the membranes and visualized by exposure to X-ray films with the ECL system (GE Healthcare UK Ltd., Buckinghamshire, UK).

Immunohistochemical Analyses

We reviewed the medical records of all consecutive patients who underwent resection with curative intent for pancreatic cancer from 1995 to 2004 at Keio University Hospital, Japan. For immunohistochemistry, 73 invasive ductal pancreatic adenocarcinomas were analyzed. Formalin-fixed and paraffin-embedded sections were incubated with anti-CAP1 antibody overnight at 4°C. The primary antibody was visualized using an ImmPRESS Anti-Rabbit Ig Kit (Vector Laboratories,

Burlingame, CA, USA) with diaminobenzidine. The sections were counterstained with hematoxylin and then mounted. Histologic diagnoses were made according to the Japanese Pancreas Society classification.²⁵

Statistical Analyses

The χ^2 -test was used when appropriate to determine the correlations between clinicopathological variables and CAP1 immunolabeling. Survival rates were calculated by the Kaplan–Meier method, and the log-rank test was applied. In the 73 consecutive patients, 4 patients who suffered in-hospital death and 15 patients without follow-up data were excluded for survival analysis. Taken together, follow-up data of 54 patients were utilized for survival analysis. All statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA).

Immunocytochemical Analyses

Human pancreatic cancer cell lines PANC-1, CFPAC-1, and Hs 766T were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells cultured on culture slides (BD Biosciences, San Diego, CA, USA) were fixed with 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and probed with anti-CAP1 antibody overnight at 4°C. The slides were rinsed with PBS, covered with FITC-labeled secondary antibody with rhodamine-phalloidin (Invitrogen), and visualized using an Axiovert 200 microscope, an AxioCam CCD camera, and the AxioVision software (Carl Zeiss MicroImaging Inc., Tokyo, Japan). Infiltrating lymphocytes served as a positive control for CAP1 staining.

RNA Interference

For knockdown of CAP1, two siRNA molecules (siCAP1A and siCAP1B) were synthesized by QIAGEN. Their target sequences were AAACCGAGTCCTCAAAGAGTA and AAACAGATGGCTGCCATGCTT, respectively. As a control, negative control siRNA (nonsilencing siRNA) was purchased from QIAGEN. CFPAC-1 and PANC-1 cells were dispersed into collagen I-coated six-well plates at 5×10^4 cells/well and cultured overnight under normal conditions. The following day, cells were transfected with siRNA in serum-free medium using Oligofectamine (Invitrogen) for 4 h at 37°C with 5% CO₂. After 4-h transfection, an equal volume of medium, supplemented with 20% fetal bovine serum (FBS), was added and the transfectants were cultured until further analyses. To observe lamellipodium formation, the transfectants (48 h after transfection) were serum starved for 24 h, and then stimulated with serum (by changing serum-free medium for medium supplemented with 10% FBS) for 30 min.

Migration Assay

CFPAC-1 and PANC-1 cells transfected for 48 h with siCAP1A, siCAP1B, or negative control siRNA were suspended in RPMI medium (Invitrogen) supplemented with

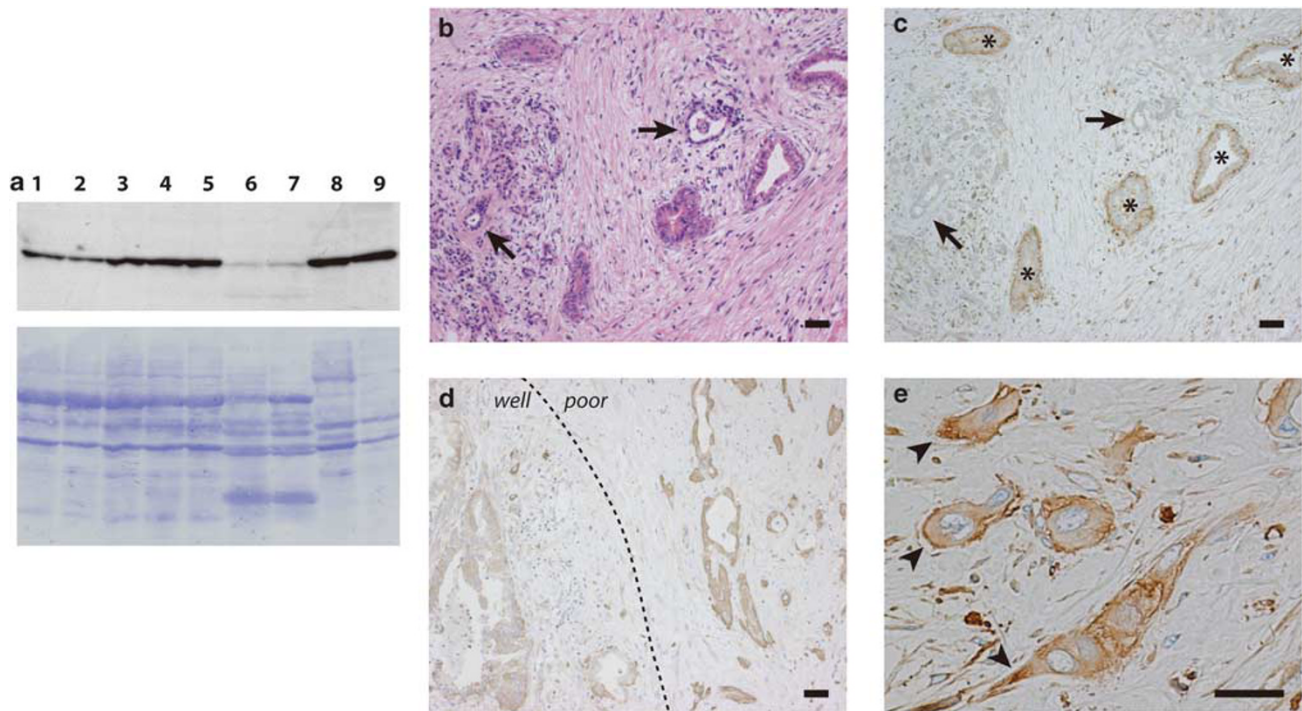


Figure 1 CAP1 overexpression in pancreatic cancers. (a) Expression level of CAP1 protein in pancreatic cancer tissues (lanes 1–5), non-neoplastic pancreas tissues (lanes 6 and 7), and pancreatic cancer cells, Hs 766T (lane 8) and PANC-1 (lane 9) was assessed by Western blot analysis (top). Equal protein load in samples was confirmed by Coomassie brilliant blue staining (bottom). (b) Pancreatic cancer tissue stained with hematoxylin and eosin containing non-neoplastic pancreatic ducts (arrows). (c) Most tumor cells (asterisks) were CAP1-positive whereas non-neoplastic duct cells (arrows) were negative. (d) In a lesion containing well- and poorly differentiated tumor cells, CAP1 was highly expressed in poorly differentiated cells. (e) CAP1 was detected strongly at the edge of tumor cells (arrowheads). Scale bars = 25 μ m.

0.5% FBS, and 5×10^4 (CFPAC-1) or 2×10^4 (PANC-1) cells treated with each siRNA were dispersed to each of three independent upper chambers of BD BioCoat Control Culture Inserts (six-well plates, pore size = 8 μ m; BD Biosciences). Culture medium containing 10% FBS was added to the bottom chamber. Following 24-h (CFPAC-1) or 20-h (PANC-1) culture at 37°C with 5% CO₂, the cells on the upper surface of the membrane in the chambers were removed by rubbing with cotton-tipped swabs. Cells migrating to the lower surface of the membrane were stained with Diff-Quik (Kokusai Shiyaku, Kobe, Japan). The number of migrated cells on three independent membranes was counted under microscope. Percent migration was represented as the mean number of migrated cells with each siRNA treatment divided by the mean number of migrated control cells.

RESULTS

Gene-Expression Profiling of Pancreatic Cancer Xenografts

We compared the gene-expression profiles of 12 pancreatic cancer xenografts with those of normal human pancreas tissues. As the samples were xenografts of human pancreatic cancer tissues grown in SCID mice, tumor cells derived from humans and stromal cells from mice coexisted in each sam-

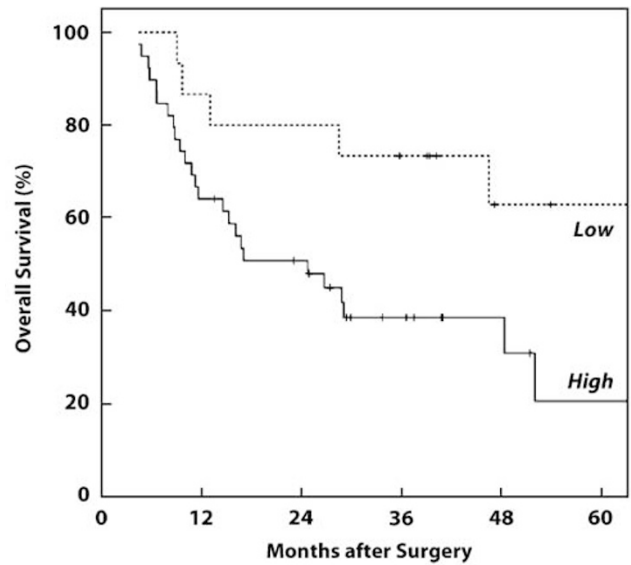
ple. As Affymetrix's GeneChip microarray can distinguish a one-base difference, the probes can identify cRNA derived from human tumor cells in the samples, although some probes detect cRNA derived from both species. We hybridized cRNA derived from each of the xenografts and normal human pancreas tissues to human gene-expression array HG-U133A. More than 10-fold upregulated and highly expressed (signals at ≥ 5000) genes in $\geq 10/12$ of the xenografts were extracted from about 22 000 probe sets. To improve the value as marker molecules, probe sets with a signal at > 1000 in normal human pancreas were then filtered out, and finally 57 genes were extracted (Supplementary Table 1). Half of them (26 genes indicated by asterisks in Supplementary Table 1) have been listed as overexpressed genes in pancreatic cancers by microarray analyses in published papers.^{13–18} This agreement suggested that xenografted specimens are able to serve as a panel for pancreatic cancer.

CAP1 Overexpression in Pancreatic Cancers

As the rapid turnover of actin filament is one of the important factors involved in cell migration and invasion, we focused on a gene encoding an actin-binding protein, CAP1, commonly overexpressed in xenografts. To confirm CAP1 protein expression in pancreatic cancers, Western blot analysis of CAP1 was performed using surgical specimens

Table 1 Characteristics of 73 pancreatic cancer patients on the basis of CAP1-immunolabeling

	CAP1-positive rate		P value (χ^2 -test)
	<75%	≥75%	
<i>Age (years old)</i>			
<65	11	19	0.213
≥65	10	33	
<i>Gender</i>			
Male	12	34	0.509
Female	9	18	
<i>Differentiation</i>			
Well	5	17	0.325
Moderately+poorly	16	35	
<i>Stroma</i>			
Intermediate	4	21	0.082
Scirrhus	17	31	
<i>Infiltrative type</i>			
β	8	19	0.901
γ	13	33	
<i>Lymphatic invasion</i>			
0-1	16	27	0.056
2-3	5	25	
<i>Vascular invasion</i>			
0-1	12	23	0.317
2-3	9	29	
<i>Neural invasion</i>			
0-1	13	14	0.005
2-3	8	38	
<i>Resection margin</i>			
Negative	18	31	0.032
Positive	3	21	
<i>Pathological tumor status (T)</i>			
1-3	13	26	0.356
4	8	26	
<i>Pathological node status (N)</i>			
0-1	19	32	0.015
2-3	2	20	
<i>Pathological metastasis status (M)</i>			
0	20	44	0.211
1	1	8	



Number at risk						
Month	0	12	24	36	48	60
CAP1 low	15	13	12	10	5	4
CAP1 high	39	25	18	9	5	2

Figure 2 Kaplan–Meier survival analysis. Overall survival (%) of patients with ≥75% (high) and <75% (low) CAP1-positive rate are indicated by solid and dotted lines, respectively.

(Figure 1a). Pancreatic cancer tissues (lanes 1–5) and cells (Hs 766T and PANC-1 in lanes 8 and 9, respectively) highly expressed CAP1 protein in comparison with non-neoplastic tissues of the pancreas (lanes 6 and 7). Immunohistochemical (IHC) analyses of CAP1 on pancreatic cancer specimens showed that CAP1 was highly expressed in tumor cells (asterisks in Figure 1c) as compared with pancreatic ducts (arrows in Figure 1c) and acini (73/73 cases). On the other hand, some inflammatory cells exhibited strong CAP1 positivity. In some lesions containing both well- and poorly differentiated tumor cells, poorly differentiated cells showed strong staining for CAP1 as compared with adjacent well-differentiated cells (Figure 1d). Furthermore, strong staining for CAP1 was shown, especially at the edge of the cells (arrowheads in Figure 1e).

CAP1 Overexpression Associates with Poor Prognosis

When CAP1 positivity was defined as stronger staining in tumor cells than in normal ductal cells, the mean rate of CAP1-positive tumor cells was 78.5% (standard deviation = 17.9) in 73 cases of pancreatic cancer. We classified the 73 cases into two groups, a higher level CAP1-expression group (≥75% CAP1-positive rate) and a lower level CAP1-expression group (<75%). As shown in Table 1, the CAP1-positive rate was significantly associated

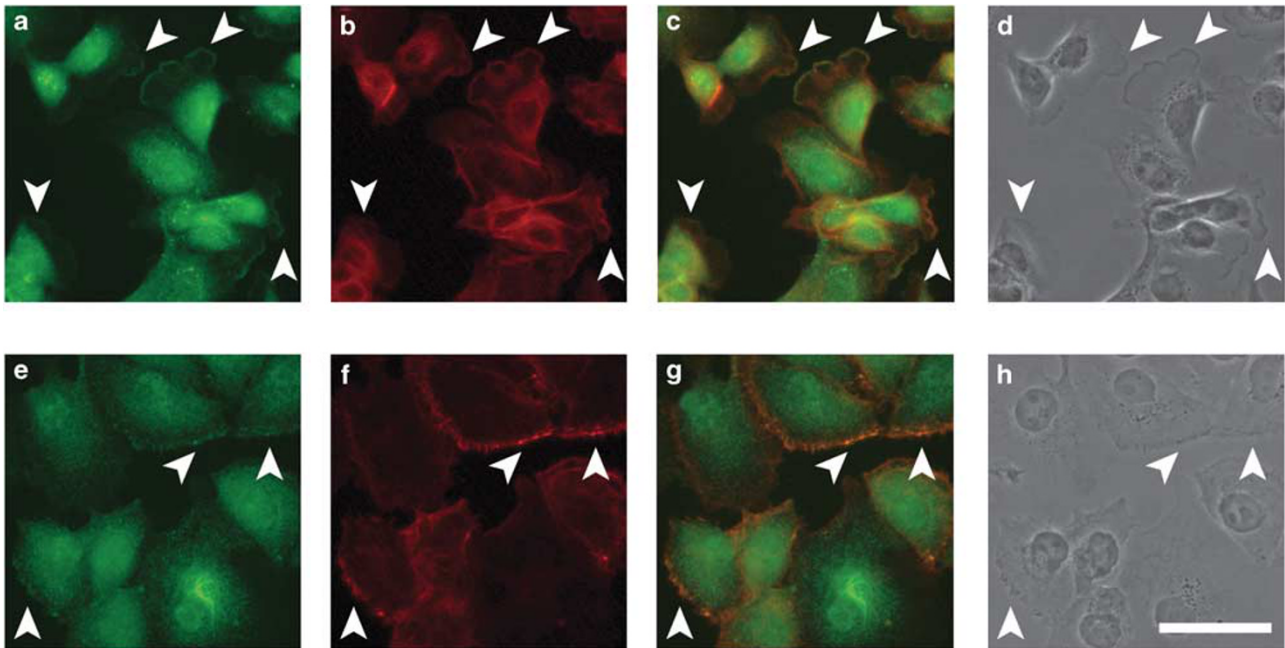


Figure 3 Subcellular localization of CAP1. CFPAC-1 (a–d) and PANC-1 (e–h) cells probed with anti-CAP1 antibody (a, e) and phalloidin-rhodamine (b, f). (c, g) Merged images. (d, h) Phase-contrast images. Arrowheads indicate the leading edge of the lamellipodium with CAP1–actin colocalization. Scale bar = 50 μ m.

with neural invasion ($P = 0.005$ by χ^2 -test), resection margin ($P = 0.032$), and lymph node metastasis ($P = 0.015$). Kaplan–Meier survival analysis revealed the poor outcome of patients in the high-level CAP1-expression group (Figure 2), and 5-year survival rates were 21% in the high level group and 63% in the low level group ($P = 0.021$, log-rank test). These results indicate that the expression level of CAP1 is associated with invasion to neurons and lymph nodes, which can be associated with shorter survival of patients with pancreatic cancer.

Defect in Lamellipodium Formation by CAP1 Knockdown

By immunocytochemical analyses (Figure 3), CAP1 was detected mainly in the perinuclear cytoplasm. In cells spreading lamellipodia, CAP1 and actin were colocalized to the leading edge of the lamellipodium (arrowheads). As lamellipodium formation is seen in migrating cells, CAP1 localization to the leading edge of the lamellipodium seems to be involved in cell migration.

To analyze the role of CAP1 in cell function, knockdown of CAP1 in pancreatic cancer cells was performed using two small interfering RNA (siRNA) molecules (siCAP1A and siCAP1B), because CAP1 was highly expressed in all eight pancreatic cancer-derived cells examined by microarray analyses (microarray data are accessible from our in-house database, GeMDBG). Figure 4a shows the results of Western blot in which CAP1 expression in CFPAC-1 and PANC-1 transfectants was reduced with either siRNA molecule. In the

two siRNA molecules, siCAP1A seems to be more effective in suppressing CAP1 protein than siCAP1B. Considering the localization of CAP1 to the leading edge of the lamellipodium, to examine the role of CAP1 in lamellipodium formation, knockdown cells were stimulated with serum. Culture medium was exchanged to RPMI 1640 medium without serum 2 days after siRNA transfection. After serum starvation for 24 h, the transfectants were stimulated with serum by changing the medium to normal culture medium supplemented with 10% FBS. By serum withdrawal, lamellipodium formation was suppressed in CFPAC-1 cells transfected with any siRNA (Figure 4b). Within 15 min after serum stimulation, control cells extended lamellipodia around the circumference of the cell clusters (arrowheads), whereas lamellipodium protrusion of CAP1-knockdown cells was still suppressed or was only modest (arrows). In PANC-1 cells, shrunken lamellipodia by serum withdrawal began to extend by serum addition, although the recovery of lamellipodium extension was delayed in CAP1-knockdown cells compared with control cells (Supplementary Figure 1). These results demonstrate that CAP1 knockdown affected lamellipodium formation.

Reduction of Cell Motility by CAP1 Knockdown

As protruding lamellipodia are seen in migrating cells, the role of CAP1 in cell motility was evaluated by migration assays using CAP1-knockdown cells (Figure 4c). In both cell lines (CFPAC-1 and PANC-1), the migrated cell number was decreased by CAP1 knockdown. Of note, the ratio of the

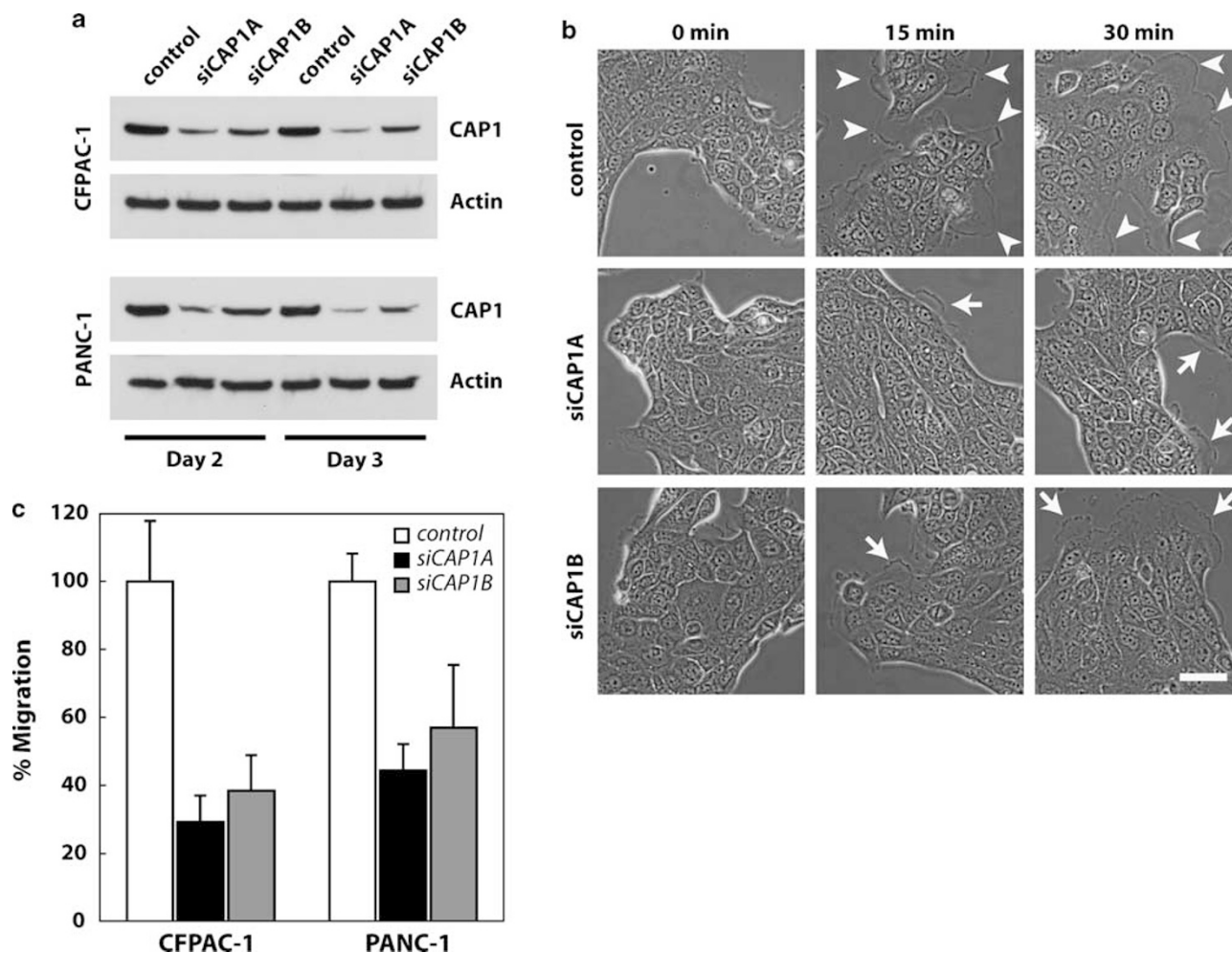


Figure 4 Effects of CAP1 knockdown. (a) Western blot analyses of CAP1 and actin using lysates of CFPAC-1 cells and of PANC-1 cells treated for 2 and 3 days with siCAP1A, siCAP1B, or negative control siRNA. (b) Microscopy of CFPAC-1 transfectants serum-starved (0 min) and serum-stimulated for 15 and 30 min. Scale bar = 50 μ m. Arrowheads and arrows indicate lamellipodia of control cells and CAP1-knockdown cells, respectively. (c) Migration assays showed reduced motility of CFPAC-1 and PANC-1 cells transfected with siCAP1A and siCAP1B as compared with control cells. Percent migration is represented as the mean number of migrated cells per field under a microscope against control cells. Bars reveal standard deviations.

migrated cell number was more decreased in siCAP1A transfectants in comparison with siCAP1B transfectants. This knockdown effect was consistent with that in suppressing CAP1 protein with the two siRNA molecules (Figure 4a). Considering that cell proliferation was not affected by RNAi (Supplementary Figure 2), the decreased number of migrated cells seems to be dependent on the reduced cell motility by CAP1 knockdown. Reduced cell motility by CAP1 knockdown was seen in another pancreatic cancer cell line, AsPC-1 (data not shown).

DISCUSSION

As a result of microarray analyses of pancreatic cancer xenografts, CAP1 was extracted as a candidate marker for pancreatic cancer. And IHC analyses showed its overexpression in 73/73 cases of pancreatic cancer examined. These results demonstrate that CAP1 overexpression was a

common feature in pancreatic cancers, and suggest that xenografting is likely to be a useful method for profiling cancers containing abundant stromal cells. IHC analyses also showed heterogeneous expression of CAP1, which was up-regulated in poorly differentiated tumor cells than in well-differentiated tumor cells. It is reported that reduced E-cadherin expression is detected frequently in poorly differentiated tumor cells than in well-differentiated tumor cells,^{26,27} and reduced E-cadherin expression is regarded as a predictor of poor outcome in pancreatic cancer. It is possible that a reciprocal transcriptional regulation of CAP1 and E-cadherin occurs in poorly differentiated tumor cells. Expression levels of CAP1 in pancreatic cancer specimens were significantly correlated with the presence of neural invasion and lymph node metastasis. Neural invasion and lymph node metastasis of pancreatic cancer are associated with poor outcome,⁵⁻⁷ and patients with high-level CAP1 expression had a poor outcome.

These results suggest that CAP1 overexpression promoted invasion and metastasis, which can be associated with the poor outcome of patients with pancreatic cancer.

Adenylate cyclase-associated proteins are multifunctional proteins with several structural domains involved in actin binding, adenylate cyclase association, SH3 binding, and oligomerization.²⁰ Yeast CAP is involved in adenylate cyclase activation in the RAS signaling pathway;²⁸ however, its function is not apparent in mammals. Further studies are needed to elucidate the molecular function of CAP1 in the RAS signaling. Both mammal and yeast CAPs interact with actin²⁹ and play a role in actin turnover.²³ In some reports about CAP1 mutations, the predicted functions of CAP1 have been identified from their phenotypes. Deletion of yeast CAP results in an abnormally large cell size, random budding pattern, and abnormal actin distribution.^{30,31} A CAP knockout mutant of *Dictyostelium* revealed aberrations in cell polarization, F-actin organization, and phototaxis.³² Loss of *Drosophila* CAP results in various developmental defects and problems in maintaining oocyte polarity.^{33,34} In addition to those genetic studies, CAP1 knockdown results in aberrant cell morphology and reduced migration in mouse cells.³⁵ These reports suggest that a failure of actin turnover by aberrations in CAP1 orthologs induces abnormalities in cell morphology, polarity, and motility. In our study, protruding of the lamellipodium was induced by serum stimulation and was inhibited by CAP1 knockdown, suggesting that CAP1 was involved in serum-induced lamellipodium formation. In addition, cell motility was reduced by CAP1 knockdown. As remodeling of the actin filament and formation of the actin meshwork occurred at the leading edge of the lamellipodium where CAP1 and actin colocalized, retardation of actin turnover by CAP1 knockdown seems to be a cause of the defect in lamellipodium formation and reduced cell motility. Considering that expression levels of CAP1 in clinical specimens were associated with neural invasion and lymph node metastasis, it seems that CAP1 overexpression was involved in enhanced cell motility and promoted invasion and metastasis of pancreatic cancer.

In conclusion, CAP1 was shown to be commonly overexpressed in pancreatic cancers, and to be associated with enhanced cell motility; therefore, CAP1 overexpression may be involved in the aggressive behavior of pancreatic cancers.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declared no conflict of interests.

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