The Role of S100A6 in Pancreatic Cancer Development and Its Clinical Implication as a Diagnostic Marker and Therapeutic Target

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Abstract Recent microarray analyses showed that the S100 family contains members that are candidate diagnostic markers or therapeutic targets. In the present study, to evaluate the involvement of S100A6 in pancreatic cancer and its clinical usefulness for diagnosis, we examined S100A6 mRNA expression in pancreatic tissues and pancreatic juice from patients with different pancreatic diseases. To investigate the role of S100A6 in carcinogenesis of pancreatic cancer and the potential of S100A6 as a diagnostic marker for early detection of pancreatic cancer, we did immunohistochemistry and microdissection-based mRNA analysis of pancreatic normal ducts, pancreatic intraepithelial neoplasias, and invasive ductal carcinomas. We also used *in vitro* experiments and microarray analysis with RNA interference to evaluate the functional role of S100A6 and its potential as a therapeutic target for pancreatic cancer. S100A6 mRNA levels were significantly higher in carcinoma specimens than in nonneoplastic tissues. In pancreatic juice, there was a significant difference in S100A6 expression between patients with carcinoma and those with nonneoplastic disease. Receiver operating characteristic curves revealed that S100A6 might be a useful marker for diagnosis of pancreatic cancer. Immunohistochemistry and microdissection-based analysis showed differential expression of S100A6 among normal ducts, pancreatic intraepithelial neoplasias, and invasive ductal carcinomas. *In vitro* data showed that inhibition of S100A6 decreased proliferation and invasiveness of cancer cells, and these findings were supported by microarray data. Our present results suggest that quantitation of S100A6 mRNA is a promising tool for diagnosis of pancreatic cancer, and that S100A6 may be a promising therapeutic target for pancreatic cancer.

Pancreatic cancer is the fifth most common cause of tumor-related deaths in the industrialized world (1, 2). Fewer than 10% to 20% of patients are candidates for surgery at the time of presentation, and <20% of patients who undergo curative resection are alive after 5 years (3, 4). Despite recent progress, there is no modality for early detection of pancreatic cancer. With the exception of a recent report describing successful use of adjuvant chemotherapy in the ESPAC-1 trial (5), there has been no report of effective treatment of advanced pancreatic cancer, including local and metastatic disease. To improve the prognosis of patients with pancreatic cancer, we need effective screening strategies and effective treatments for the disease once it has been detected.

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Microarray analysis allows simultaneous monitoring of the expression of thousands of genes and is a powerful tool for identifying genes associated with pancreatic carcinoma. Microarray analyses recently showed expression of S100A2 and S100A6 to be up-regulated in pancreatic cancer (6–8). S100 family proteins are small Ca^{2+}, Zn^{2+}, and Cu^{2+} binding proteins of the EF-hand type and have been implicated in regulation of a variety of intracellular and extracellular processes, including cell proliferation, differentiation, cell-cell communication, cell structure, energy metabolism, motility, contraction, and intracellular signaling. In addition, protein chip analyses showed up-regulation of S100A6 in pancreatic cancer (9) and overexpression of S100A2 was observed in non–small cell lung cancer with metastasis (10). Taken together, the data suggest that S100A2 and S100A6 are promising diagnostic markers and/or therapeutic targets for pancreatic cancer.

In the present study, we quantitatively measured the expression of S100A2 and S100A6 mRNAs in pancreatic cancer cell lines, pancreatic tissues, and pancreatic juice by real-time PCR. S100A6 mRNA levels in tumor specimens from pancreas and pancreatic juice samples from patients with pancreatic cancer were increased significantly compared with levels in nonneoplastic tissues or pancreatic juice from patients with nonneoplastic diseases. The preoperative diagnostic accuracy of quantitative analysis of S100A2 and S100A6 mRNAs in pancreatic juice was compared by receiver operating characteristic (ROC) curve analysis (11). To investigate the role of

S100A6 in carcinogenesis and the usefulness of quantitative analysis of S100A6 mRNA for detection of early pancreatic cancer, immunohistochemical studies and microdissection-based quantitative analysis of mRNA were done in normal ducts, pancreatic intraepithelial neoplasias, and invasive ductal carcinomas (IDC). In addition, to evaluate the functional role of S100A6 and its possible therapeutic implications, we inhibited expression of S100A6 mRNA using RNA interference (RNAi) and investigated its effect on proliferation and invasiveness of pancreatic cancer cells *in vitro*. Finally, we screened oligonucleotide microarrays to identify transcripts influenced by S100A6 and found several cancer-related genes whose expression was up-regulated or down-regulated by inhibition of S100A6.

Materials and Methods

Cell lines, pancreatic tissues, and pancreatic juice. The following 14 pancreatic cancer cell lines were used: ASPC-1, BxPC-3, KP-1N, KP-2, KP-3, Panc-1, and Suit-2 (Dr. H. Iguchi, National Kyushu Cancer Center, Fukuoka, Japan); MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan); Capan-1, Capan-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, VA). Cells were maintained as described previously (12). Tissue samples were obtained at the time of surgery at Kyushu University Hospital, Fukuoka, Japan. Thirty-three tissue samples were obtained from cancerous lesions of the resected pancreas of patients with primary pancreatic ductal carcinoma, and 39 normal tissue samples were taken from the peripheral soft tissue away from the tumor or from normal pancreatic tissue resected due to bile duct cancer. The tissue samples were removed as soon as possible after resection, embedded in ornithine carbamyl transferase compound (Sakura, Tokyo, Japan), snap-frozen for analysis by microdissection, and stored at −80°C or fixed in formalin, embedded in paraffin for immunohistochemistry or H&E staining. All tissues adjacent to the specimens were examined histologically, and the diagnoses were confirmed. Pancreatic juice

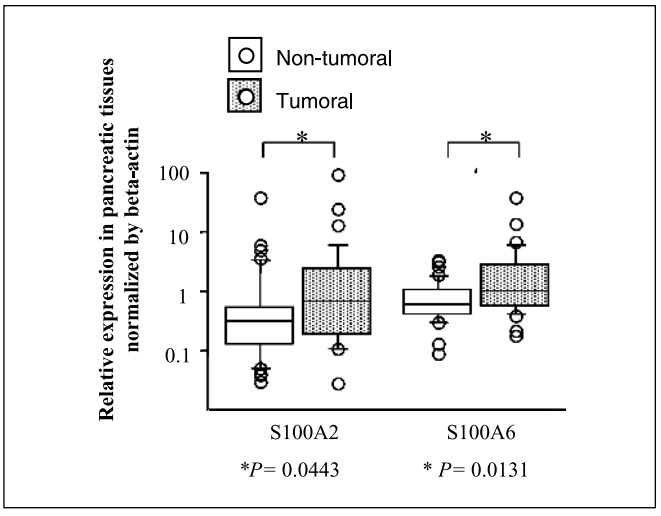

Fig. 1. Relative expression of S100A2 and S100A6 mRNA in tumoral and nontumoral tissues. We used 33 tumoral and 39 nontumoral pancreatic tissues for whole tissue analyses. Total RNA (10 ng) extracted from snap-frozen tissues was used for analysis of S100A2, S100A6, and β-actin mRNA expression with real-time PCR. When *β-actin* was used as the reference gene, there was a significant difference in the relative expression of S100A6 between tumoral and nontumoral tissues. Levels of S100A2 did not differ significantly between tumoral and nontumoral tissues.

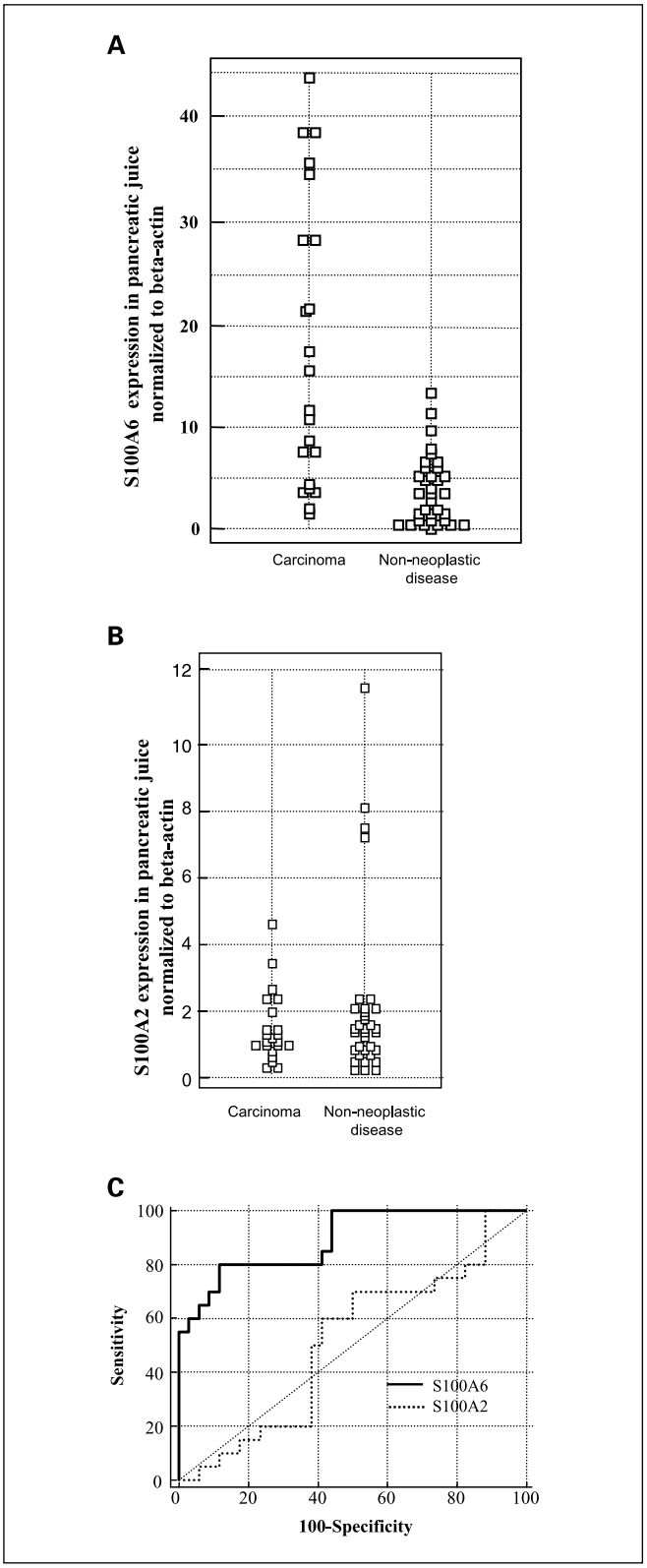

Fig. 2. Quantification of S100A6 and S100A2 in pancreatic juice. Relative expression of S100A6 (*A*) and S100A2 (*B*) in pancreatic juice was normalized to β-actin expression. There was a significant difference in S100A6 mRNA expression between carcinoma juice samples and nonneoplastic samples. *C,* sensitivity of each marker was determined at several specificity levels. ROC curve analyses showed that the discriminating ability of S100A6 was higher than that of S100A2.

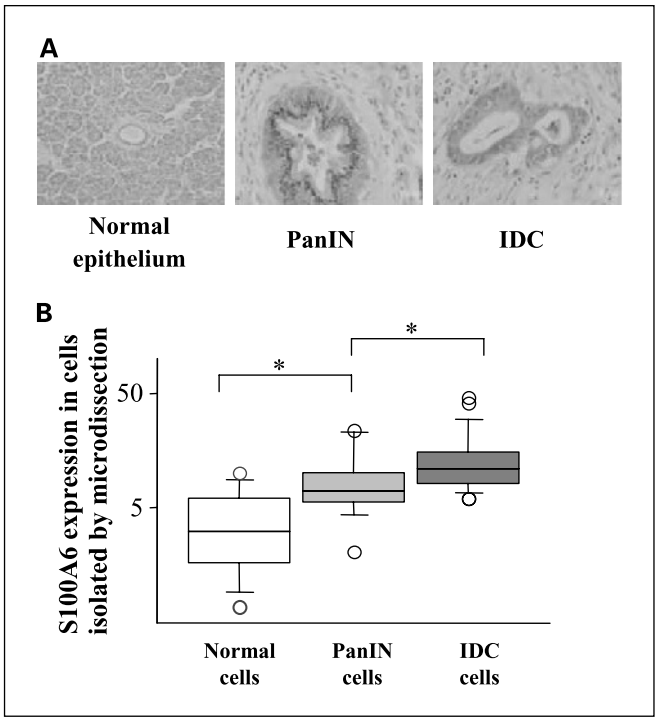

Fig. 3. Immunohistochemistry studies and microdissection-based quantitative analysis of S100A6. *A,* immunoreactivities for S100A6; representative images of normal ducts (*left*), high-grade pancreatic intraepithelial neoplasias (*middle*), and IDC (*right*). Original magnification, ×400. Differential expression of S100A6 protein was observed among IDCs, high-grade pancreatic intraepithelial neoplasias, and normal duct. *B,* we used a microdissection technique to isolate IDC cells, high-grade pancreatic intraepithelial neoplasia cells, and normal ductal cells from frozen sections, and total RNA extracted from these cells was subjected to quantitative analysis of S100A6 mRNA with real-time PCR. Differential expression of S100A6 mRNA was observed among IDCs, high-grade pancreatic intraepithelial neoplasias, and normal duct ($P = 0.028$, pancreatic intraepithelial neoplasia cells versus IDC cells; $P = 0.0034$, pancreatic intraepithelial neoplasia cells versus normal duct cells).

samples were collected from 56 patients who underwent endoscopic retrograde cholangiopancreatography for suspected malignancy of the pancreas at Kyushu University Hospital between January 1, 2002 and October 7, 2004 (13, 14). RNA was extracted from the cellular components of pancreatic juice samples.

The diagnosis of pancreatic ductal adenocarcinoma was confirmed by histologic examination of resected specimens or by the presence of liver metastasis. The diagnosis of pancreatitis or cholelithiasis was made based on either histologic examination of resected specimens or clinical observation with conventional diagnostic imaging for at least 6 months. Written informed consent was obtained from all patients, and the study was conducted according to the Helsinki Declaration.

Quantitative assessment of S100A2 and S100A6 mRNA levels by real-time PCR. Total RNA was extracted from cell pellets of pancreatic juice, cultured cells, and isolated cells by a microdissection technique with a PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA) or High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) with DNase I (Roche Diagnostics) treatment according to the manufacturer's instructions. We designed specific primers (S100A2 forward primer, AGCTTTGTGGGGGAGAAAGT and reverse primer, CAGTGATGAGTGCCAGGAAA; S100A6 forward primer, AAGCTGCAGGATGCTGAAAT and reverse primer, CCCTTGAGGGCTTCATTGTA; β-actin forward primer, AAATCTGGCACCACACCTTC and reverse primer, GGGGTGTTGAAGGTCTCAAA) and did BLAST searches to ensure the specificity of these primers. Quantitative reverse transcription-PCR was done with a QuantiTect SYBR Green Reverse Transcription-PCR kit (Qiagen, Tokyo, Japan) with a LightCycler Quick System 350S (Roche Diagnostics) as described previously (15). Each sample was run twice, and all samples showing >10% deviation of reverse transcription-PCR measurements were tested a third time. The level of expression of mRNA for each gene was calculated on a standard curve constructed from values of total RNA from the Capan-1 pancreatic cancer cell line. Expression of each gene was given as the ratio of expression of each target gene mRNA to that of β-actin mRNA.

Immunohistochemical studies. Thirty-three tumoral tissues containing IDC cells were obtained from patients who underwent surgery for pancreatic cancer. Forty-two nontumoral tissues containing normal ducts and 18 tissues containing pancreatic intraepithelial neoplasia lesions were obtained, some from these patients who underwent surgery for pancreatic cancer and some from other patients with pancreatic cancer or with other diseases. Sections of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and dehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS. Sections were incubated for 20 minutes with a protein-blocking solution consisting of PBS containing 1.5% normal goat serum (DAKO, Glostrup, Denmark) and then incubated with the appropriate dilution of mouse monoclonal S100A6 (Sigma, St. Louis, MO) antibody overnight at 4°C. Sections were then incubated with the appropriate dilution of biotinylated anti-mouse IgG (Vectastain Elite Avidin-Biotin Complex kit, Vector Labs, Burlingame, CA) for 30 minutes. Immunocomplexes were visualized with stable 3,3′-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan). The sections were rinsed with distilled water and counterstained with hematoxylin for 10 seconds.

Evaluation of degree of antibody reactivity. The degree of monoclonal anti-S100A6 reactivity with each tissue section was scored as the percentage of stained normal or neoplastic epithelial cells in the section (0 points for no cells stained, 1 point for <20%, 2 points for 20-75%, and 3 points for >75% of cells stained), and the intensity of immunoreactivity was graded on a scale of 0 to 3. The total score was the product of the scores for the intensity and extent of staining. Negative cases had a score of 0, weakly positive cases had a score of 1 to 3, moderately positive cases had a score of 4 to 6, and strongly positive cases had a final score of >6, as described previously (7). Pathologists who judged the intensity and extent of staining were blinded to the results of the other study.

Microdissection-based quantitative analysis of S100A6 mRNA. Frozen tissue samples were cut into 8-μm sections. One section was stained with H&E for histologic examination. IDC cells, pancreatic intraepithelial neoplasia cells, and normal pancreatic ductal epithelial cells were isolated selectively with a laser microdissection and pressure catapulting system (PALM Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocol. After microdissection, total RNA was extracted from the selected cells and subjected to real-time PCR for quantitative measurement of S100A6 as described previously (16).

Inhibition of S100A6 mRNA by small interfering RNA. Inhibition of S100A6 expression was achieved by RNAi. We designed two S100A6-targeting small interfering RNAs (siRNA: target sequence,

Table 1. S100A6 immunoreactivity in pancreatic tissue

Product	Normal (%)	Pancreatic intraepithelial neoplasias (%)	IDCs (%)
Negative	34 (81.0)	0 (0)	0 (0)
Weak	7 (16.7)	6 (33.3)	5 (15.2)
Moderate	1 (2.4)	12 (66.7)	11 (33.3)
Strong	0 (0)	0 (0)	17 (51.5)
Total	42	18	33

AAGCCCTCAAGGGCTGAAAAT for siRNA-K1 and AAGCTGCAGGATGCTGAAATT for siRNA-K2) and did BLAST searches to ensure the specificity of these siRNAs. To verify the specificity of the knockdown effect, we used control siRNA provided by Qiagen. Panc-1, CFPAC-1, and ASPC-1 cells were transfected with the indicated 100 pmol siRNA with Nucleofector (Amaxa Biosystems GmbH, Köln, Germany) according to the manufacturer's instructions. Cells were harvested at the indicated time after transfection. Total RNA was extracted from each sample, and the expression of target mRNA was evaluated by real-time PCR.

Cell proliferation assay. Cells were transfected with the indicated 100 pmol siRNA and seeded in 24-well plates at a density of 5×10^4 per well. The number of cells was investigated at the indicated time by measuring the fluorescence intensity of propidium iodide as described previously (17).

Invasion assay. Invasiveness of cancer cells was evaluated by counting the number of cells invading through a Matrigel-coated transwell as reported previously (12). Briefly, transwell inserts with 8-μm pores were coated with Matrigel (40 or 20 μg/well). Cancer cells were transfected with the indicated siRNA and seeded in the Matrigel-coated transwell inserts. After 48 or 72 hours of incubation, cells invading to the lower surface of the Matrigel-coated membrane were counted under a light microscope.

Oligonucleotide array hybridization and data analysis. Global gene expression profiling was done with oligonucleotide microarrays (Human Genome U133 Plus 2.0 Chips, Affymetrix, Santa Clara, CA). Briefly, total RNA (10 μg) was reverse transcribed with the One-Cycle cDNA Synthesis Kit (Affymetrix). The cDNA was *in vitro* transcribed with the GeneChip Expression 3′-amplification reagents for IVT Labeling (Affymetrix). The cRNA (20 μg) was fragmented and hybridized to an HG-U133 Plus 2.0 GeneChip (Affymetrix) with a standard procedure (45°C, 16 hours). Washing and staining were done in a Fluidics Station 450 (Affymetrix) with protocol EukGE-WS2v4 and scanning in an Affymetrix GeneChip 3000 scanner. Signal intensity for each transcript (background subtracted and adjusted for noise) and detection call (present, absent, or marginal) were determined with Microarray Suite Software 5.0 (Affymetrix).

Statistical analysis. Data for clinical samples were analyzed by Mann-Whitney *U* test, because normal distribution was not obtained. To evaluate the ability of measurement of S100A6 expression to differentiate carcinoma from nonneoplastic diseases, we constructed ROC curves by calculating the sensitivities and specificities of data for each marker at several predetermined cutoff points using the MedCalc statistical software package, version 7.6 (MedCalc, Mariakerke, Belgium; ref. 18). We used MedCalc software to do a statistical analysis of the difference in ROC curves, according to the method described by Hanley and McNeil (19). Differences in immunohistochemistry data were analyzed by Kruskal-Wallis test if comparisons involved three groups and by Mann-Whitney *U* test if comparisons involved two groups. For *in vitro* experiments, values are expressed as mean ± SD. One-way ANOVA was used to analyze differences among three groups, and Student's *t* test was used for differences between two groups. $P < 0.05$ was considered statistically significant for all tests.

Results

Quantitative analysis of S100A2 and S100A6 mRNA levels in cancerous and nonneoplastic pancreatic tissues. We measured S100A2 and S100A6 mRNA levels in 33 tumoral and 39 nontumoral pancreatic tissues (whole tissue). To quantify target gene expression, we used *β-actin* as a reference gene (Fig. 1A). Tumoral tissues showed significantly higher expression of S100A2 and S100A6 than that shown by nontumoral tissues (S100A2, $P = 0.0443$; S100A6, $P = 0.0131$).

We did the ROC curve analysis to compare the abilities of S100A2 and S100A6 to discriminate between tumoral and nontumoral tissues. The areas under the ROC curves of S100A2 and S100A6 for tumoral tissues and nontumoral tissues were 0.638 [95% confidence intervals (95% CI), 0.517-0.748] and 0.671 (95% CI, 0.550-0.777), respectively (data not shown). There was no significant difference between areas of S100A2 and S100A6 in tissues analysis.

Quantitative analysis of S100A2 and S100A6 mRNA levels in pancreatic juice. We measured S100A2 and S100A6 mRNA levels in pancreatic juice samples from 56 patients with different pancreatic diseases (pancreatic carcinoma, $n = 22$; nonneoplastic disease, $n = 34$: pancreatitis, $n = 28$ and cholelithiasis, $n = 6$). Expression of S100A6 mRNA was significantly higher in carcinoma samples than in nonneoplastic samples ($P < 0.0001$), whereas there was no significant difference in S100A2 mRNA expression ($P = 0.6749$; Fig. 2A and B).

The ROC curves for S100A2 and S100A6 mRNA expression are shown in Fig. 2C. The sensitivity of each marker was determined at several specificity levels. The area under the ROC curve was 0.893 for S100A6 (95% CI, 0.778-0.960) and 0.510 for S100A2 (95% CI, 0.371-0.649). A significant difference was observed between the areas (difference between areas, 0.382; 95% CI, 0.208-0.557; $P < 0.001$). These data suggest that the discriminating ability of S100A6 was higher than that of S100A2.

These data are not consistent with data obtained by analysis of pancreatic tissues. The use of bulky tissue consisting of various cell types including stromal cells and acinar cells for tissue analysis may have contributed to these conflicting results (20). The difference in S100A6 expression may be greater in analysis of pancreatic juice than in analysis of bulky tissues because most cells in pancreatic juice are epithelial cells. Conversely, the difference in S100A2 expression was less in pancreatic juices

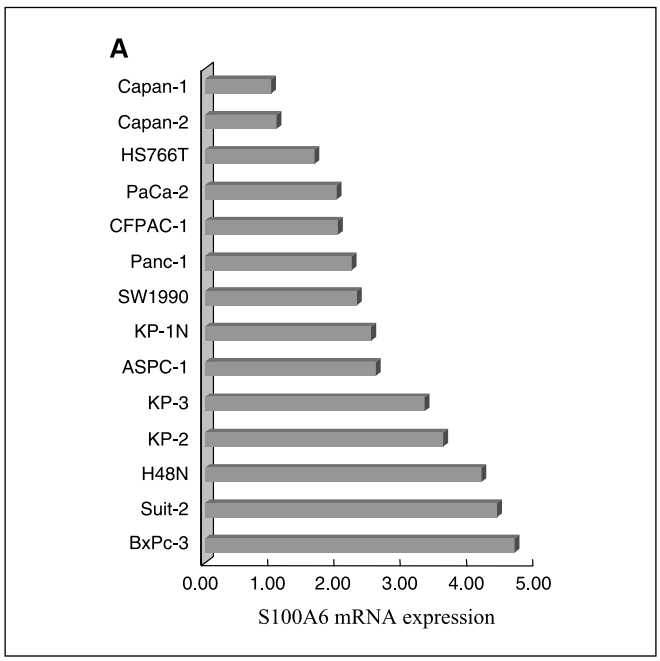

Fig. 4. *A,* S100A6 mRNA expression in 14 pancreatic cancer cell lines. expression of S100A6 mRNA was normalized to that of β-actin mRNA. Values are expressed relative to 1.00 for expression in Capan-1 cells. All 14 pancreatic cancer cell lines were positive for expression of S100A6 mRNA. The median value was 2.04. Panc-1, CFPAC-1, and ASPC-1 cells showed moderate expression of S100A6 mRNA.

than in bulky tissues. In the analysis of pancreatic juice, most pancreatic juice samples used as normal counterparts were derived from patients with chronic pancreatitis. These chronic pancreatitis-affected pancreatic juice samples include massive numbers of hyperplastic epithelial cells. These cells, in addition to the carcinoma cells, may express high levels of S100A2 and lead to false-positive results.

Differential expression of S100A6 in invasive ductal carcinomas, high-grade pancreatic intraepithelial neoplasias, and normal ducts. To confirm that quantification of S100A6 mRNA in pancreatic juice may be a valid method for detecting early pancreatic cancer, we compared expression of S100A6 mRNA and protein in high-grade pancreatic intraepithelial neoplasias with expression in normal ducts or IDCs. Representative immunohistochemistry data obtained with the anti-S100A6 antibody are shown in Fig. 3A. S100A6 was expressed diffusely at moderate or high levels in 84.8% of IDCs, whereas S100A6 immunoreactivity was negative in >80% of normal ducts (Table 1). In high-grade pancreatic intraepithelial neoplasias, S100A6 staining was weak or moderate, with granular staining localized in the supranuclear regions (Fig. 3A; Table 1).

We used a microdissection technique to isolate normal duct cells, pancreatic intraepithelial neoplasia cells, or IDC cells from frozen sections and quantified expression of S100A6 mRNA in these cells. S100A6 mRNA was differentially expressed in normal duct cells, pancreatic intraepithelial neoplasia cells, and IDC cells (Fig. 3B). There were significant differences in the levels of S100A6 mRNA among pancreatic intraepithelial neoplasia cells, IDC cells, and normal duct cells ($P = 0.028$, pancreatic intraepithelial neoplasia cells versus IDC cells; $P = 0.0034$, pancreatic intraepithelial neoplasia cells versus normal duct cells).

S100A6 mRNA expression in 14 pancreatic cancer cell lines. To confirm expression of S100A6 mRNA in pancreatic cancer cell lines and to select cell lines appropriate for *in vitro* RNAi experiments, S100A6 mRNA expression was measured in 14 pancreatic cancer cell lines. As shown in Fig. 4A, all 14 pancreatic cancer cell lines expressed S100A6 mRNA. The median value of expression was 2.4. For subsequent experiments, we selected Panc-1, ASPC-1, and CFPAC-1 cells because they expressed moderate levels of S100A6 and because conditions for siRNA transfection with Nucleofector had been established.

Effect of inhibition of S100A6 on cell proliferation and invasion. To investigate the potential of S100A6 as a therapeutic target, we used RNAi assays. The degree of inhibition of S100A6 mRNA expression induced by siRNA against S100A6 is shown in Fig. 4B. S100A6-targeting siRNAs K1 and K2 inhibited S100A6 mRNA levels in Panc-1 cells to 30% and to <10% of control cells, respectively, from 24 to 48 hours after transfection (Fig. 4B, *a*). In CFPAC-1 cells, similar results were observed 48 hours after transfection (Fig. 4B, *b*). Inhibition of S100A6 expression in Panc-1 cells suppressed cell proliferation significantly in an inhibition rate-dependent manner 4 days after transfection (Fig. 4C, *a*). In addition, in CFPAC-1 cells, inhibition of S100A6 mRNA suppressed cell proliferation 5 days after transfection (Fig. 4C, *b*).

We then investigated the effect of inhibition of S100A6 mRNA on the invasive potential of pancreatic cancer cells. A photograph of Panc-1 cells, which were invading through the Matrigel (40 μg)–coated membrane 48 hours after transfection of control siRNA or siRNA against S100A6, is shown in Fig. 5A. The number of invading cells was significantly reduced in an inhibition rate-dependent manner when the cells were transfected with siRNAs against S100A6. When siRNA-K2 against S100A6 was transfected, the number of invading cells was decreased to 31.8% that of control siRNA-transfected cells (Fig. 5B). We did the same analyses (Matrigel, 20 μg; incubation time, 72 hours) with CFPAC-1 cells and obtained similar results (Fig. 5C). The proliferation assay revealed that there was no significant difference in proliferation of Panc-1 cells 2 days after transfection and of CFPAC-1 cells 3 days after transfection of siRNA against

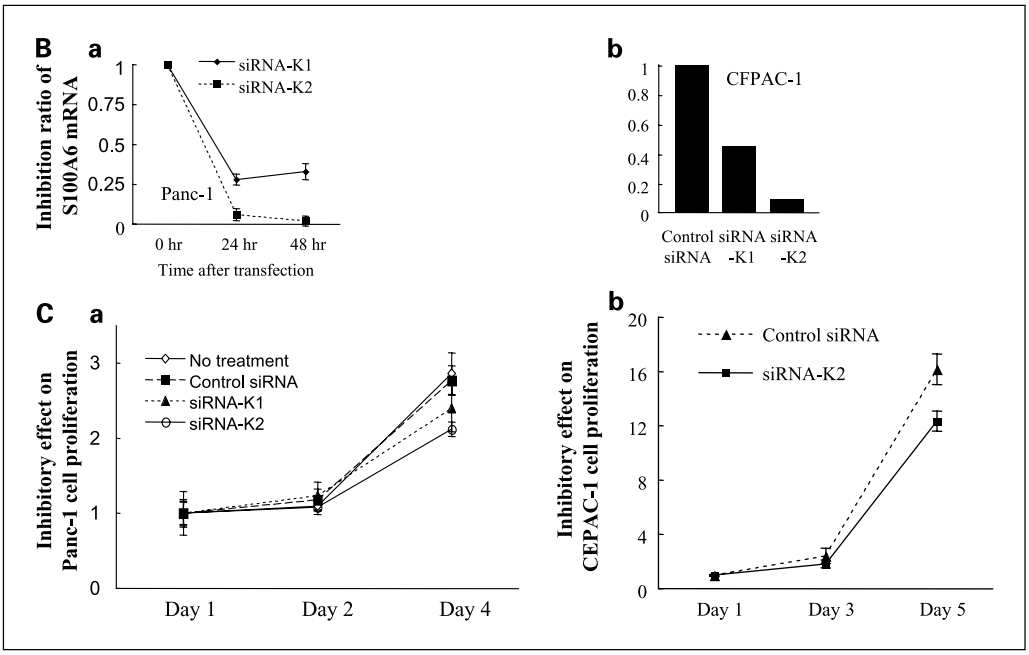

Fig. 4 *Continued. B,* RNAi-induced inhibition of S100A6 mRNA. Two siRNAs against S100A6 were designed (siRNA-K1 and siRNA-K2). siRNA (100 pmol) was transfected into cancer cells with Nucleofector reagent. S100A6 mRNA expression was evaluated in Panc-1 (*a*) and CFPAC-1 (*b*) cells at 24 hours (*a*) and/or 48 hours (*a* and *b*) after transfection. Both siRNAs showed efficient inhibition of transcript expression. Inhibition by siRNA-K2 was much stronger than that by siRNA-K1. *C,* effect of inhibition of S100A6 mRNA on cell proliferation of Panc-1 (*a*) and CFPAC-1 (*b*) cells. Cells were seeded after transfection of siRNA and evaluated by Propidium iodide assay on days 1, 2, and 4 (*a*) or on days 1, 3, and 5 (*b*) after transfection. Proliferation of Panc-1 and CFPAC-1 cells was inhibited. The degree of inhibition of cell proliferation was correlated with that of S100A6 mRNA in Panc-1 cells.

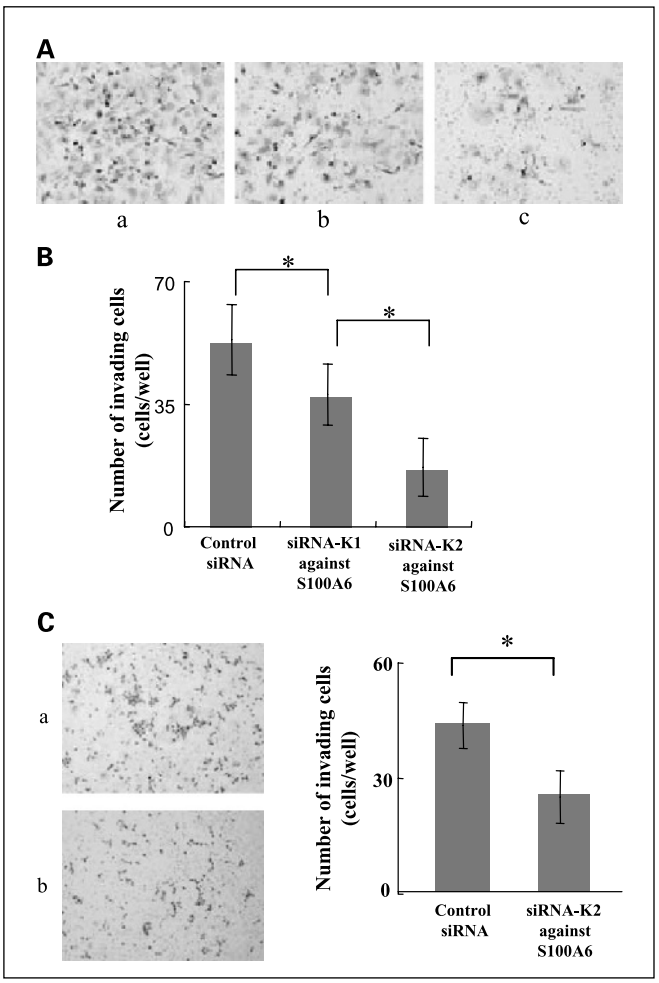

Fig. 5. Effect of inhibition of S100A6 mRNA on invasive potential of Panc-1 (*A* and *B*) and CFPAC-1 (*C*) cells. For invasion assay, cells transfected with siRNA were seeded in a cell migration chamber with the inner well coated with Matrigel (40 μg for Panc-1, 20 μg for CFPAC-1) and incubated for 48 hours (Panc-1) or 72 hours (CFPAC-1). The number of invading cells through the Matrigel-coated membrane was then counted. *A,* representative photomicrograph of Panc-1 cells treated with control siRNA (*a*), siRNA-K1 against S100A6 (*b*), and siRNA-K2 against S100A6 (*c*). Original magnification, ×100. *B,* there were significant differences in the number of invading cells between the groups ($P = 0.030$, control siRNA versus siRNA-K1 against S100A6; $P = 0.005$, siRNA-K1 versus siRNA-K2). *C,* representative photomicrograph of CFPAC-1 cells transfected with control siRNA (*a*) or siRNA-K2 against S100A6 (*b*). Original magnification, ×100. There was a significant difference between the two groups ($P = 0.0018$).

S100A6 (Fig. 4C). Therefore, it is unlikely that decreased invasiveness caused by transfection of siRNA against S100A6 merely reflects a decreased number of cells in the siRNA-treated cultures. The invasive behavior of cancer cells in the *in vitro* invasion assay is thought to be independent of proliferative activity (21, 22).

Global change in gene expression patterns induced by inhibition of S100A6. To elucidate the molecular mechanism underlying the function of S100A6, we examined global changes in gene expression in pancreatic cancer cells (Panc-1) 48 hours after transfection of siRNA against S100A6 or control siRNA. To identify specific genes that may be regulated by S100A6, we screened for transcripts with at least 2-fold differences in signal intensity (false discovery rate for 2-fold difference, 1.41%). We eliminated transcripts whose detection calls were absent to minimize detection of false up-regulation or down-regulation. Of the 20,244 transcripts analyzed, only 100 (0.49%) showed a ≥2-fold decrease in expression in S100A6-targeting siRNA-transfected cells compared with that of control siRNA-transfected cells. Only 130 (0.64%) transcripts showed a ≥2-fold increase in S100A6-targeting siRNA-transfected cells. Most of the genes identified as differentially expressed have not been reported previously in association with S100A6 expression. Fifteen known genes up-regulated or down-regulated by inhibition of S100A6 are listed in Tables 2 and 3. Notably, several genes down-regulated by inhibition of S100A6 are associated with the proliferative properties or apoptosis of cancer cells. In particular, a never in mitosis gene a (*NIMA*)–related kinase 2, serine/threonine kinase 6 (Aurora A), and centromere protein A are involved in cell proliferation, especially in mitotic regulation (23–25). Human ovarian *β-A inhibin*, which forms a homodimer, activin A, and cytokine *gro-β* were up-regulated by inhibition of S100A6, These genes are known to be negative regulators of cell proliferation (26–29). In addition, *activating transcription factor 3* and *protocadherin* β_2 are reported to be involved in cancer invasion (30–33). Therefore, up-regulation of these genes may lead to inhibition of motility or invasion of cancer cells. These findings suggest that proliferation or invasion/metastasis-promoting genes and growth or tumor suppressor genes may be direct or indirect targets of S100A6 activity.

Discussion

This is the first report of quantitative analyses of S100A6 mRNA levels in pancreatic tissues, microdissected pancreatic cancer cells, and pancreatic juice from patients with pancreatic cancer. S100A6 mRNA expression levels in tumoral tissues and carcinoma juice samples were significantly higher than those in nontumoral tissues and nonneoplastic juice, respectively. Microdissection-based quantitative analysis of mRNA revealed that S100A6 was differentially expressed in normal ducts, high-grade pancreatic intraepithelial neoplasias, and IDCs. These results were confirmed by immunohistochemistry, suggesting that stepwise up-regulation of S100A6 is related to carcinogenesis of pancreatic cancer and that accurate quantification of S100A6 could be used as a screening test in individuals with radiologic evidence of pancreatic cancer, or in high-risk populations. Most S100 proteins are released into the extracellular space. If S100A6 is also released and measurable in the serum, quantitative analysis with serum could also be a promising tool for screening of pancreatic cancer. However, thus far, there have been no reports on S100A6 expression in serum.

The sensitivity of cytology to detect cancerous cells in pancreatic juice varies from 30% to 80%, even in the hands of experienced cytologists (34, 35). Many investigators have reported qualitative analyses of alterations, such as K-ras or p53 mutations, in pancreatic juice (36–39). However, qualitative analyses of DNA mutations are problematic because of the frequent presence of K-ras or p53 mutations in pancreatic intraepithelial neoplasias or hyperplastic duct (40–42). It has been reported that quantitative analysis of Ras mutations is a useful strategy for diagnosis of pancreatic carcinoma (43). However, quantitation of DNA mutations merely reflects the component ratio of cells with mutations against cells without mutations, meaning that this is not a

Table 2. List of 15 down-regulated genes after transfection of siRNA against S100A6

Fold change	*P*	Gene bank ID	Gene name (symbol)	Putative cellular function
0.070	0.0288	NM_014624.2	S100 calcium-binding protein A6 (*S100A6*)	Proliferation, invasion
0.343	0.0229	D38553.1	HCAP-H	Mitotic regulation
0.362	0.0057	M29550.1	Calcineurin A1	Antiapoptotic effect
0.382	0.0147	AB077208.1	Thymidylate synthase	Metabolism (related to drug sensitivity)
0.382	0.0006	NM_003651.1	Cold shock domain protein A	Repressor of transcription
0.387	0.0086	AJ278112.1	DEP domain containing 1	Cell cycle control
0.391	0.0007	BG391171	Thymopoietin (*TMPO*)	Proliferation
0.392	0.0118	AV725328	Prion protein	Antiapoptotic effect
0.397	0.0230	NM_018492.1	T-cell originated protein kinase (*TOPK*)	Related to MAPK
0.399	0.0252	AU153848	GTPase-activating protein	Signal transduction
0.414	0.0309	BE045993	Opa-interacting protein 5	Not characterized
0.414	0.0267	Z25425.1	Never in mitosis gene a (*NIMA*) – related kinase 2 (NEK2)	Mitotic regulation
0.416	0.0002	NM_003158.1	Serine/threonine kinase 6 (*STK6*/*Aurora-A*/*STK15*)	Mitotic regulation
0.417	0.0345	BC002703.1	Centromere protein A	Related to mitotic behavior of chromosomes
0.419	0.0081	AF213040.1	Mutant cyclin-dependent kinase-associated protein phosphatase	Cell cycle regulator

Abbreviation: MAPK, mitogen-activated protein kinase.

suitable method for differentiating mutated malignant cells from mutated nonmalignant cells, such as atypical hyperplasia and pancreatic intraepithelial neoplasias. However, quantitation of target mRNA expression reflects the component ratio of cells expressing target mRNA to cells that do not express the target mRNA; it also reflects the difference in expression of the target mRNA between malignant cells and nonmalignant cells, even if both cell types express the target mRNA. This suggests that

Table 3. List of 15 up-regulated genes after transfection of siRNA against S100A6

Fold change	*P*	Gene bank ID	Gene name (symbol)	Putative cellular function
6.986	0.0085	AW003173 stanniocalcin 1	Cell metabolism, cellular calcium/phosphate homeostasis.	
4.643	0.0164	M13436.1	Human ovarian β-A inhibin	Negative regulator of cell proliferation, tumor suppressor activity
4.115	0.0312	NM_000450.1	Selectin E precursor cell	Adhesion molecules
3.947	0.0361	BC002827.1	Tropomyosin 4	Actin-binding proteins involved in the contractile system of the cytoskeleton of cells
3.471	0.0078	NM_005114.1	Heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase 1 precursor	The heparan sulfate biosynthetic enzyme family
3.127	0.0056	M57731.1	Cytokine gro-β	Antiproliferative effect
2.981	0.0009	NM_004591.1	Small inducible cytokine subfamily A (Cys-Cys), member 20 (*SCYA20*)	Antiproliferative or proliferative effect (cell type specific)
2.934	0.0247	AK023795.1	Metalloproteinase with thrombospondin type 1 motifs ADAMTS1 (*ADAMTS1*)	Antiangiogenic activity/inhibitor of endothelial cell proliferation
2.928	0.0248	AF043337.1	Interleukin 8 COOH-terminal variant (*IL8*)	Chemoattractant
2.904	0.0216	AF240698.1	Retinol dehydrogenase homologue isoform-1 (*RDH*)	Retinoid metabolism
2.833	0.0091	AF240697.1	Retinol dehydrogenase homologue isoform-2 (*RDH*)	Retinoid metabolism
2.595	0.0191	NM_001674.1	Activating transcription factor 3 long isoform	Proapoptotic, antiproliferative, and anti-invasive effect
2.585	0.0092	NM_018936.1	Protocadherin β_2	Cell-cell connections
2.579	0.0337	NM_005771.1	Retinol dehydrogenase homologue (*RDHL*)	Retinoid metabolism, colonocyte differentiation
2.564	0.0092	NM_006290.1	TNFα-induced protein 3 (*TNFAIP3*)	Inhibit NF-κB activation and TNF-mediated apoptosis

Abbreviations: NF-κB, nuclear factor-κB; TNF, tumor necrosis factor.

quantitative analysis of mRNA is a promising diagnostic tool for identifying pancreatic cancer in clinical specimens, provided that target mRNA expression levels differ sufficiently between malignant and nonmalignant cells.

Stulik et al. (44) and Komatsu et al. (45) observed differential expression of S100A6 in normal, preneoplastic, and neoplastic colonic mucosa by immunohistochemistry, consistent with results of our present immunohistochemistry and microdissection-based mRNA analyses of normal pancreatic ductal cells, high-grade pancreatic intraepithelial neoplasias, and IDCs. High-grade pancreatic intraepithelial neoplasias are regarded histologically as precancerous or noninvasive cancerous lesions (46). Therefore, differential expression of S100A6 during carcinogenesis suggests that quantitative analysis of S100A6 mRNA levels in pancreatic juice may be a promising modality not only to distinguish pancreatic cancer from nonneoplastic disease but also to detect early pancreatic cancer. In the present study, we used the whole cell pellet from pancreatic juice, which consisted of atypical cells and normal epithelial cells. There is still room to improve the sensitivity and specificity of this testing strategy. For example, to improve accuracy of the quantitative analysis, microdissection should be used to isolate atypical cells from pancreatic juice for testing. The study of cells microdissected from pancreatic juice is now in progress in our laboratory.

Although analysis of S100A6 expression has been reported, the role of S100A6 is not well understood. In the present study, RNAi experiments clearly showed that S100A6 is involved not only in cell proliferation but also in invasion of pancreatic cancer cells. Our oligonucleotide microarray data revealed that inhibition of S100A6 affects expression of some genes, such as *activin A* and *activating transcription factor 3*, that are involved in cell proliferation and invasion. Tonini et al. (47) and Breen and Tang (48) showed that S100A6 regulates cell cycle progression or proliferation, in keeping with our proliferation assay data. We show that inhibition of S100A6 expression suppresses invasive potential as well as proliferation. Komatsu et al. (45) reported that, in colorectal adenocarcinoma with or without metastasis, differential expression of S100A6 was observed by immunohistochemistry. The data suggest that S100A6 is clinically applicable for diagnosis and staging of pancreatic cancer and for determining prognosis. Interference with the multiple activities of S100A6 is an attractive strategy for therapeutic intervention.

In conclusion, results of our expression analyses suggest that S100A6 is up-regulated during the early phase of carcinogenesis of pancreatic cancer and that quantification of S100A6 mRNA may be useful for diagnosis and possibly for early detection of pancreatic cancer. Our data also indicate that S100A6 is associated with both progression and invasion of pancreatic cancer. Although these results need to be verified in an independent set of experiments, our findings suggest that S100A6 may be a promising diagnostic marker and therapeutic target for pancreatic cancer.

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