**S100A11, A Putative Tumor Suppressor Gene, Is Overexpressed in Pancreatic Carcinogenesis**

Kenoki Ohuchida,1 Kazuhiro Mizumoto,1 Seiji Ohhashi,1 Hiroshi Yamaguchi,2 Hiroyuki Konomi,1 Eishi Nagai,1 Koji Yamaguchi,1 Masazumi Tsuneyoshi,2 and Masao Tanaka1

**Abstract**

**Purpose:** Recent microarray analyses revealed that expression of *S100A11* is up-regulated in pancreatic cancer. The aim of the present study was to evaluate the association of *S100A11* with pancreatic carcinogenesis.

**Experimental Design:** We measured *S100A11* mRNA expression in various clinical samples related to pancreatic cancer and its precursor lesions, intraductal papillary mucinous neoplasm (IPMN) and pancreatic intraepithelial neoplasia, by quantitative reverse transcription-PCR.

**Results:** Levels of *S100A11* were significantly higher in pancreatic cancer (*n* = 22) and IPMN (*n* = 18) bulk tissues than in nonneoplastic bulk tissues (*n* = 22; *P* < 0.0001 for both). Levels of *S100A11* did not differ between pancreatic cancer and IPMN bulk tissues. In microdissection analyses, however, IPMN cells (*n* = 21) expressed significantly higher levels of *S100A11* than did cancer cells (*n* = 23; *P* = 0.003). The median level of *S100A11* expression was higher in pancreatic intraepithelial neoplasia cells (*n* = 6) than in cancer cells. In pancreatic juice analyses, cancer-related (*n* = 24; *P* = 0.004) and IPMN-related (*n* = 18; *P* = 0.001) juice expressed significantly higher levels of *S100A11* than did chronic pancreatitis–related juice (*n* = 23).

**Conclusions:** The present data suggest that expression of *S100A11*, a putative tumor suppressor gene, is increased in the early stage of pancreatic carcinogenesis and decreased during subsequent progression to cancer. Analysis of the *S100A11* level in pancreatic juice may be an effective tool for screening of patients with high-risk lesions that could progress to pancreatic cancer or detecting early-stage pancreatic cancer.

Pancreatic cancer is the fourth and fifth most common cause of tumor-related death in the United States and Japan, respectively (1, 2). Only a minority of cases in the early stage of the disease are amenable to curative surgical resection. Despite recent progress, there is no effective modality for early detection of pancreatic cancer. Thus, to improve the prognosis of patients with pancreatic cancer, a novel biomarker for early detection of the disease is needed.

S100 family proteins are small Ca2+-binding proteins of the EF-hand type, and they have been implicated in the regulation of a variety of intracellular and extracellular processes, including cell proliferation, differentiation, and intracellular signaling (3). *S100A11* was originally discovered as a homologue of rabbit calgizzarin in a cloning study of colorectal cancer cell lines (4). *S100A11* is expressed in skeletal muscle (5), and a mRNA study showed expression in placenta, heart, lung, and kidney (6). It was also reported that gastric and prostate cancers express high levels of *S100A11* (7, 8). Microarray analysis, a powerful tool for identifying genes associated with cancer, recently revealed that expression of *S100A11* is up-regulated in pancreatic cancer (9). The data suggest that *S100A11* may be a novel diagnostic marker for pancreatic cancer.

In the present study, to clarify the association of *S100A11* expression with pancreatic cancer, we examined expression of *S100A11* mRNA in 15 pancreatic cancer cell lines, in bulk pancreatic tissues, in cells isolated by microdissection, and in pancreatic juice by quantitative real-time reverse transcription-PCR (RT-PCR). In addition, to investigate the contribution of *S100A11* to pancreatic carcinogenesis, we examined the expression of *S100A11* mRNA in intraductal papillary mucinous neoplasm (IPMN) and pancreatic intraepithelial neoplasia (PanIN), both of which are thought to be precursor lesions of pancreatic cancer (10–12), and we compared levels of *S100A11* in these precursor lesions with those in pancreatic cancer.

**Materials and Methods**

Cell lines, pancreatic tissues, and pancreatic juice. Fifteen pancreatic cancer cell lines, ASPC-1, BxPC-3, KP-1N, KP-2, KP-3, Panc-1, Suit-2...
manufacturers' instructions. We designed specific primers (forward) and 5'-GGGGTGTTGAAGGTCTCAAA-3' (reverse) and 5'-AAATCTGGCACCACACCTTC-3' (forward) and 5'-GGGGTGTTGAAGGTCTCAAA-3' (reverse); and 18S rRNA, 5'-GATATGCATCGTCGTGCTG-3' (forward) and 5'-GATATGCATCGTCGTGCTG-3' (reverse) and did BLAST searches to ensure the specificity of each primer. Quantitative real-time RT-PCR was done with a Quantitect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) with a LightCycler Quick System 350S (Roche Diagnostics) according to the manufacturers' instructions. We designed specific primers [S100A11, 5'-GTCCTGATTGCCTGCTTGC-3' (forward) and 5'-ACCAGGCTCTGCTGCTTGC-3' (reverse); and 5'-AAATCTGGCACCACACCTTC-3' (forward) and 5'-GGGGTGTTGAAGGTCTCAAA-3' (reverse); and 18S rRNA, 5'-GATATGCATCGTCGTGCTG-3' (forward) and 5'-GATATGCATCGTCGTGCTG-3' (reverse) and did BLAST searches to ensure the specificity of each primer. Quantitative real-time RT-PCR was done with a Quantitect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) with a LightCycler Quick System 350S (Roche Diagnostics) as described previously (18). Each sample was run twice, and any sample showing >10% deviation of the RT-PCR values was 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 S100A11 mRNA was expressed as the ratio normalized to that of β-actin mRNA or 18S rRNA.

Microdissection-based quantitative analysis of S100A11 mRNA. Frozen tissue samples were cut into 8-µm-thick sections. One section was treated with H&E stain for histologic examination. IDC cells from 23 lesions, IPMN cells from 21 lesions, including 16 IPMNs with mild or moderate atypia and 5 borderline IPMNs with severe atypia, PanIN cells from 6 sections, including 4 PanIN-1B and 2 PanIN-2 lesions, and normal pancreatic ductal epithelial cells from 17 lesions were isolated selectively using a laser microdissection and pressure catapulting system (P.A.L.M. Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols. More than 100 cells could be obtained from 1 IDC or IPMN section, whereas 3 to 10 sections were needed to isolate normal ductal cells due to the lower number of normal ductal cells per section. Thus, similar numbers of cells were isolated from samples of IDC, IPMN, and normal ductal lesions. After microdissection, total RNA was extracted from the selected cells and subjected to real-time RT-PCR for quantitative measurement of S100A11 as described previously (19).

Results

S100A11 mRNA expression in 15 pancreatic cancer cell lines, HPDE, and 4 primary cultures of pancreatic fibroblasts. We investigated the levels of S100A11 mRNA expression in 15 pancreatic cancer cell lines, HPDE-E6E7 clone 6, which is immortalized by transduction with the E6/E7 genes of human papillomavirus 16, and primary cultured pancreatic fibroblasts. As shown in Table 1, 11 of the 15 pancreatic cancer cell lines and HPDE-E6E7 expressed higher levels of S100A11 than did the 4 primary cultures of normal pancreatic fibroblasts. Notably, HPDE-E6E7 (S100A11 expression value, 5.31) showed a relatively high level of S100A11 expression compared with that of pancreatic cancer cell lines (median S100A11 expression value, 2.41).

Quantitative analysis of S100A11 expression in bulk pancreatic tissues. We measured S100A11 mRNA levels in pancreatic cancer, IPMN, and nonneoplastic bulk tissues. To quantify S100A11 gene expression, we used β-actin as a reference gene (Fig. 1). Pancreatic cancer and IPMN bulk tissues contained significantly higher levels of S100A11 than nonneoplastic bulk tissues contained [pancreatic cancer versus nonneoplastic tissues, difference between the median levels, 1.57; 95% confidence interval (95% CI), 1.04-2.31; P < 0.0001; IPMN versus nonneoplastic tissues, difference between the median levels, 1.19; 95% CI, 0.73-1.66; P < 0.0001]. However, S100A11 expression did not differ between IPMN and pancreatic cancer bulk tissues.

Quantitative analysis of S100A11 expression in IDC, IPMN, and normal ductal cells. To accurately quantify expression of S100A11 in specific cells, we isolated IDC cells, nonmalignant IPMN cells, and normal ductal epithelial cells from frozen sections by microdissection. As shown in Fig. 2A, S100A11 expression was much higher in IDC and IPMN cells than in normal ductal cells (IDC cells versus normal ductal epithelial cells, difference between the median levels, 1.70; 95% CI, 0.72-2.53; P < 0.0001; IPMN cells versus normal ductal epithelial cells, difference between the median levels, 3.49; 95% CI, 2.25-4.74; P < 0.0001), which was consistent with the results of our bulk tissue analyses. However, IPMN cells expressed significantly higher levels of S100A11 than IDC cells expressed, which was not consistent with the results of our bulk tissue analyses. In bulk tissue analyses, IPMN and IDC tissues often contained more ductal cells than nonneoplastic tissues contained. Therefore, the S100A11 expression profile in the bulk tissue analyses may have reflected an epiphenomenon rather than a biological phenomenon. Taken together, these
data suggest that microdissection analyses are needed to investigate the biological differences between IDC, IPMN, and normal ductal cells.

Several previous publications suggested that expression of $\beta$-actin is altered in cancer (20). Therefore, to confirm that $\beta$-actin is a valid reference gene for normalization of expression, we normalized $S100A11$ expression to that of $\beta$-actin. Expression was calculated from a standard curve constructed with total RNA from the Capan-1 human pancreatic cancer cell line.

The present microdissection study included 16 samples of low-grade IPMA cells with mild or moderate atypia and 5 samples of borderline IPMA cells with severe atypia. As shown in Fig. 2B, the median value of $S100A11$ expression in borderline IPMA cells (median, 2.862) was lower than that in low-grade IPMA cells (median, 4.674), although the difference was not statistically significant ($P = 0.05$) after Bonferroni correction possibly due to the limited number of samples. However, borderline IPMA cells expressed significantly higher levels of $S100A11$ than did normal ductal cells (median, 0.587; difference between the median levels, 1.99; 95% CI, 0.11-4.51; $P = 0.01$) or low-grade IPMA cells (difference between the median levels, 3.79; 95% CI, 2.91-5.51; $P < 0.0001$).

Quantitative analysis of $S100A11$ expression in PanIN cells and comparison with IDC and normal ductal cells. In the present microdissection study, six samples of PanIN lesions were included. As shown in Fig. 2C, PanIN cells expressed significantly higher levels of $S100A11$ than did normal ductal cells (difference between the median levels, 2.80; 95% CI, 0.64-4.34; $P = 0.001$). In addition, the median value of $S100A11$ expression in PanIN cells (median, 3.586) was higher than that in IDC cells (median, 2.433), although the difference was not statistically significant ($P = 0.15$) possibly due to the limited number of samples. Notably, three of four PanIN-1B lesions expressed higher levels of $S100A11$ than the median value of all PanIN cells, and none of the two PanIN-2 lesions expressed levels of $S100A11$ higher than the median value.

Quantitative analysis of $S100A11$ expression in pancreatic juice. We measured $S100A11$ expression in pancreatic juice samples from 65 patients with various pancreatic diseases (pancreatic cancer, $n = 24$; IPMN, $n = 18$; chronic pancreatitis, $n = 23$). The levels of $S100A11$ expression were significantly higher in cancer (median, 2.06) and IPMN (median, 1.81) juice samples than the levels in pancreatitis (median, 0.87) juice samples (pancreatic cancer versus chronic pancreatitis, difference between the median levels, 0.99; 95% CI, 0.10-2.26; $P = 0.0043$; IPMN versus chronic pancreatitis, difference between the median levels, 1.02; 95% CI, 0.43-1.69; $P = 0.0008$; Fig. 3). $S100A11$ expression did not differ significantly between pancreatic cancer and IPMN juice samples.

Discussion

This is the first report of quantitative real-time RT-PCR-based analyses of $S100A11$ expression in pancreatic tissues, microdissected pancreatic ductal cells, and pancreatic juice samples from patients with pancreatic cancer. According to bulk tissue

![Fig. 1. Expression of $S100A11$ in bulk pancreatic tissues (pancreatic cancer, $n = 22$; IPMN, $n = 18$; nonneoplastic pancreas, $n = 22$). Total RNA (10 ng) extracted from snap-frozen tissues was used for analysis of $S100A11$ expression by real-time RT-PCR. $\beta$-Actin was used as a reference gene. Pancreatic cancer (median, 2.40) and IPMN bulk tissues (median, 2.04) expressed significantly higher levels of $S100A11$ than did nonneoplastic bulk pancreatic tissues (median, 0.97); pancreatic cancer versus nonneoplastic pancreas, $P < 0.0001$; IPMN versus nonneoplastic pancreas, $P < 0.0001$). $S100A11$ expression did not differ significantly between pancreatic cancer and IPMN bulk tissues.](www.aacjrournals.org)

Table 1. $S100A11$ expression in pancreatic cancer cells and pancreatic fibroblasts

<table>
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<th>Pancreatic cancer cells and fibroblasts*</th>
<th>$S100A11$ expression$^a$</th>
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$^a$Primary cultured pancreatic fibroblasts were derived from resected pancreatic tumors.

$^b$Relative $S100A11$ mRNA expression was normalized to that of $\beta$-actin. Expression was calculated from a standard curve constructed with total RNA from the Capan-1 human pancreatic cancer cell line.

$^c$Primary cultured pancreatic fibroblasts were derived from resected pancreas.
analyses, \textit{S100A11} expression was significantly higher in pancreatic cancer and IPMN than in nonneoplastic samples. To evaluate the association of \textit{S100A11} expression with pancreatic carcinogenesis, we investigated \textit{S100A11} levels in IPMN and PanIN, both of which are precursor lesions of pancreatic cancer. The microdissection-based analyses revealed that IPMN and PanIN expressed significantly higher levels of \textit{S100A11} than did normal ductal cells. In addition, IPMN cells expressed significantly higher levels of \textit{S100A11} than did IDC cells isolated by microdissection from the invasive component of pancreatic cancer. PanIN cells also seemed to express higher levels of \textit{S100A11} than did IDC cells, although the difference was not statistically significant possibly due to the limited number of samples. Furthermore, levels of \textit{S100A11} expression in low-grade IPMA or PanIN-1B seemed to be higher than those in borderline IPMA or PanIN-2, respectively. Memon et al. (21) reported that superficial bladder tumors expressed significantly higher levels of \textit{S100A11} than did invasive tumors. The data suggest that \textit{S100A11} might be overexpressed at the early stage of pancreatic carcinogenesis, such as in IPMN and PanIN, and then decrease during progression to a more malignant phenotype.

We found no significant differences in \textit{S100A11} expression between IPMN and IDC cells in bulk tissue and pancreatic juice analyses. Bulk pancreatic cancer tissues and pancreatic juice derived from patients with pancreatic cancer may contain noninvasive cancer cells and/or premalignant cells, which may express high levels of \textit{S100A11}, as well as invasive pancreatic cancer cells. Pancreatic juice samples may contain many low-grade malignant cells derived from lesions exposed directly to the lumen of the pancreatic duct rather than high-grade malignant cells seemingly trapped within the desmoplasic of the invasive component. Therefore, it may be difficult to discriminate pancreatic cancer from IPMN by analysis of \textit{S100A11} expression in pancreatic juice. However, our present data show that both pancreatic cancer and IPMN juice samples express significantly higher levels of \textit{S100A11} than chronic pancreatitis juice samples express, suggesting that measurement of \textit{S100A11} in pancreatic juice may be an effective technique for detecting early pancreatic cancer or high-risk lesions that might progress to pancreatic cancer.

HPDE was reported to have a near-normal genotype and phenotype (22, 23). In the present study, we used HPDE6-E6E7 clone 6 cells. In general, HPDE cells expressing the E6 and E7

Fig. 2. We used microdissection to isolate specific cells from frozen sections, and total RNA extracted from these cells was subjected to \textit{STO0A1I} measurement with quantitative real-time RT-PCR. \textit{\beta}-Actin was used as a reference gene. A. Analysis of \textit{STO0A1I} expression in IDC cells (\(n = 23\)), IPMN cells (\(n = 21\)), and normal ductal cells (\(n = 17\)). IPMN cells (median, 4.65) expressed highest levels of \textit{STO0A1I} (IPMN versus IDC, \(P = 0.0001\); IPMN versus normal duct, \(P < 0.0001\)). IDC cells (median, 2.43) also expressed significantly higher levels of \textit{STO0A1I} than did normal ductal cells (median, 0.59; IDC versus normal duct, \(P = 0.0001\)). B. Comparison between \textit{STO0A1I} expression in low-grade IPMA (\(n = 16\)) with mild or moderate atypia, borderline IPMA (\(n = 5\)) with severe atypia, and normal ductal cells (\(n = 17\)). The median value of \textit{STO0A1I} expression in low-grade IPMA (median, 4.67) was higher than that in borderline IPMA (median, 2.86), although the difference was not statistically significant after Bonferroni correction (\(P = 0.05\)). C. Comparison between \textit{STO0A1I} expression in PanIN cells (\(n = 6\)), IDC cells (\(n = 23\)), and normal ductal cells (\(n = 17\)). The median value of \textit{STO0A1I} expression in PanIN cells (median, 3.59) was significantly higher than that in IDC cells (median, 2.43), although the difference was not statistically significant (\(P = 0.15\)).
S100A11 Expression in Pancreatic Carcinogenesis

Fig. 3. Quantification of S100A11 mRNA in pancreatic juice. We measured S100A11 mRNA levels in pancreatic juice samples from a total of 65 patients with different pancreatic diseases (pancreatic carcinoma, n = 24; chronic pancreatitis, n = 23; IPMN, n = 18). Expression of S100A11 in pancreatic juice was normalized to that of β-actin. Expression of S100A11 in pancreatic cancer (median, 2.06) and IPMN (median, 1.81) tissues was significantly higher than that in chronic pancreatitis (median, 0.87) juice samples (pancreatic cancer versus chronic pancreatitis, P = 0.004; IPMN versus chronic pancreatitis, P = 0.0008), whereas there was no significant difference in S100A11 mRNA expression between pancreatic cancer and IPMN juice samples (pancreatic cancer versus IPMN, P = 0.092).

S100A11 Expression in Pancreatic Carcinogenesis

genes of human papillomavirus 16 undergo crisis between passages 9 and 12. Maintenance of these senescent cells by frequent medium replacement eventually led to the emergence of immortal cell lines, including HPDE6-E6E7 clones. We found that HPDE6-E6E7 clone 6 cells express remarkably high levels of S100A11 compared with levels in normal fibroblasts and pancreatic cancer cells. These results seemed to be inconsistent with our data from the microdissection-based analyses that normal ductal cells express lower levels of S100A11 than IPMN cells express. However, Ouyang et al. (23) reported that HPDE-E6E7 cells express high levels of several oncoproteins and tumor suppressor genes, including c-myc, Met/HGFR, and p16INK4, compared with expression in primary cultures of normal HPDE6 cells (22, 23). HPDE-E6E7 clones were reported to lack functional p53 and Rb pathways and showed positive telomerase activity (23). These data suggest that HPDE6-E6E7 clone 6 is not an appropriate control cell for normal ductal (nonneoplastic) cells; however, for comparison with cancer cells, these immortalized cells may serve as a control for premalignant duct cells with several oncogenic changes, which are not sufficient for full malignant transformation. Therefore, the results of analyses of HPDE cells suggest that expression of S100A11 is up-regulated in cells with some oncogenic changes.

Sakaguchi et al. (24) reported that S100A11 increases transcription of p21, a negative regulator of cell growth. In addition, expression of several known tumor suppressor genes, including p53 and p16INK4, is reported to be increased in premalignant lesions (25–27), similar to the present findings that S100A11 expression is elevated in noninvasive neoplasms, such as IPMA and PanIN, but decreased in invasive cancer, such as IDC. The data suggest that S100A11 may be a tumor suppressor gene. In conclusion, the present data suggest that overexpression of S100A11, a putative tumor suppressor gene, may be an early tumorigenic event and expression of S100A11 may be decreased during subsequent progression to a more malignant phenotype. Although the clinical application of S100A11 analysis may be limited for discrimination of pancreatic cancer from benign neoplasms, such as IPMN, S100A11 analysis in pancreatic juice may allow early detection of pancreatic cancer and effective screening of patients with high-risk lesions that may progress to pancreatic cancer, such as patients who have a family history of pancreatic cancer or who have chronic pancreatitis.

References

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