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ORIGINAL ARTICLE

S100A4 mRNA is a Diagnostic and Prognostic Marker in Pancreatic Carcinoma

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Abstract
Objective The aim of this study is to evaluate the clinical significance of *S100A4* mRNA expression in pancreatic cancer.
Materials and Methods We obtained invasive ductal carcinoma (IDC) cells from ten lesions, intraductal papillary mucinous neoplasm (IPMN) cells from 20 lesions, and normal ductal cells from 20 normal pancreatic tissues by laser microdissection of frozen tissues. *S100A4* expression was examined in the microdissected cells and in formalin-fixed paraffin-embedded (FFPE) samples of 87 pancreatic cancers by quantitative reverse transcription-polymerase chain reaction.
Results IDC cells expressed higher levels of *S100A4* than IPMN cells ($P=0.002$) and normal ductal cells ($P<0.001$), although the difference between IPMN cells and normal ductal cells was not statistically significant ($P=0.070$). Analysis of FFPE samples revealed that high *S100A4* expression was significantly associated with a shorter overall survival ($P=0.023$). In immunohistochemical analysis, the extent of *S100A4* mRNA expression was significantly correlated with the expression of S100A4 protein ($P=0.028$).
Conclusion S100A4 could be a marker for malignancy in pancreatic tumors and for poor prognosis in patients with pancreatic cancer.

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Keywords S100A4 · Pancreatic carcinoma · Prognostic marker · Diagnostic marker · Formalin-fixed paraffin-embedded (FFPE)

Introduction

Pancreatic cancer is one of the most lethal tumors and is the fourth leading cause of tumor-related deaths in the industrialized world.[1,2] Only 10–20% of patients with pancreatic cancer have a chance of curative resection because most patients are at advanced stages of the disease at the time of diagnosis.[3,4] Therefore, early diagnosis of pancreatic cancer is critical to improve survival. On the other hand, many asymptomatic pre-invasive pancreatic neoplasms with cystic lesions have been found as a result of recent advances of diagnostic tools and screening strategy. This poses a dilemma for clinicians because it is often difficult to distinguish between pancreatic cancers and nonhazardous tumors. Intraductal papillary mucinous neoplasm (IPMN), which is recog-

nized as a precursor of pancreatic ductal adenocarcinoma, is representative of such neoplasms. Prognosis is favorable for patients with IPMN without invasion but poor for those with invasion, which accounts for a rate of death of about 30% of patients with IPMNs.[5] To determine the nature of pancreatic lesions preoperatively, novel modalities are needed. A promising approach is to measure molecular markers that could classify patients into different risk categories and aid clinicians in choosing suitable treatments for individual patients. To date, p53, transforming growth factor-β, basic fibroblast growth factor,[6] Bcl-2,[7–9] matrix-metalloproteinases,[10] β-catenin/E-cadherin,[11] vascular endothelial derived growth factor,[12,13] platelet-derived endothelial growth factor,[14,15] and human equilibrative nucleoside 1[16] have been suggested as biomarkers to predict the prognosis of pancreatic cancer patients. However, there are conflicting findings with regard to their validity as prognostic markers,[6] and none of the markers described above are used in clinical practice.

S100A4 is a member of the S100 family of calcium-binding proteins, which is characterized by two distinct EF-hand structural motifs.[17,18] *S100A4* is known to be overexpressed in many solid tumors, including breast carcinoma,[19] gastric carcinoma,[20] and colorectal adenocarcinoma,[21] while S100A4 has historically been referred to as fibroblast-specific protein 1 (FSP1), as a marker of fibroblasts.[22] There are also alternative names for S100A4 including mts1, pEL-98, 18A2, p9Ka, CAPL, and calvasculin. *S100A4* promotes cell motility and invasion in cancer[21,23–25] and induces remodeling of the extracellular matrix,[26–29] suggesting that *S100A4* is a mediator of tumor metastasis.[30] *S100A4* has also been reported to be a prognostic marker in a number of human cancers, including esophageal-squamous cancers,[31] non-small-cell lung cancers,[32] gastric cancers,[20] and bladder cancers.[33] In pancreatic cancers, it was reported that *S100A4* overexpression is associated with poor differentiation[34] and poor prognosis.[35,36] Recently, Mahon et al.[37] showed that S100A4 contributed to chemoresistance and the inhibition of apoptosis in pancreatic cancer.

The aim of this study was to evaluate the clinical significance of *S100A4* mRNA expression in pancreatic cancers as a diagnostic and prognostic marker. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we evaluated *S100A4* mRNA expression in invasive ductal carcinoma (IDC) cells, nonmalignant IPMN cells, and normal ductal cells of pancreatic tissues obtained by laser microdissection. Moreover, we investigated the association between *S100A4* expression and the prognosis of patients with pancreatic cancers using formalin-fixed paraffin-embedded (FFPE) samples.

Materials and Methods

Patients and Pancreatic Tissues Tissue samples were obtained from primary pancreatic tumors at the time of surgery at Kyushu University Hospital (Fukuoka, Japan) between 1992 and 2007. Normal pancreatic tissues were taken from peripheral tissues away from the tumor or from nonneoplastic pancreas resected due to bile duct disease. The tissue samples were removed as quickly as possible after resection, and a part of each sample was embedded in ornithine carbamyl transferase compound (Sakura, Tokyo, Japan), snap-frozen for analysis by microdissection, and stored at −80°C. The remainder was fixed in formalin and embedded in paraffin for pathological diagnosis. Tissues adjacent to the specimens were evaluated histologically according to the criteria of the World Health Organization.[38] Two pathologists were in agreement with regard to the pathological features of all cases, and the diagnoses were confirmed. In IPMNs, main-duct IPMNs or branch-duct IPMNs which were larger than 3 cm in diameter were removed on suspicion of being high-risk lesions. We only used IPMNs diagnosed with nonmalignant cystic tumors, which were confirmed to be intraductal papillary mucinous adenocarcinoma or intraductal papillary mucinous borderline tumor, not intraductal papillary mucinous carcinoma (IPMC), by pathological examination. Overall survival analysis was conducted for 87 patients who underwent pancreatic resection for pancreas cancer (85 ductal adenocarcinomas and two adenosquamous cell carcinomas). The patients comprised 53 men and 34 women with a median age of 65 years (range, 36–86 years). Survival was measured from the time of pancreatic resection, with death as the endpoint. Prognosis was examined in October 2008. The median observation time for overall survival was 16.3 months, ranging from 1 to 108 months. Sixty-four patients died during the follow-up, and the other patients were alive and censored. This study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

RNA Isolation from Microdissected Samples and FFPE Samples Frozen tissue samples were cut into 8-μm-thick sections. One section was stained with hematoxylin and eosin (H&E) for histological examination, and the diagnosis of target cells was confirmed by the expert pathologist. Target cells (IDC cells from ten lesions; IPMN cells from 20 lesions, excluding IPMCs; and normal ductal epithelial cells from 20 tissues with the histological appearance of normal pancreas) were isolated selectively with a laser-microdissection and pressure catapulting system (P.A.L.M.

Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocol.[39] We microdissected 500–1,000 target cells to perform reliable and reproducible measurements of mRNA levels. We obtained 20 μl of RNA per lesion with the concentration of 10–50 ng/μl. The 28S/18S rRNA ratios ranged from 0.5 to 2.5. Total RNA was extracted from microdissected cells by a microdissection technique using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and treated with DNase I (Roche Diagnostics) according to the manufacturer's instructions. The total RNA derived from FFPE samples was isolated using the RNeasy FFPE kit (Qiagen, Tokyo, Japan), as previously described.[40] We used FFPE samples from 87 IDC patients with available prognostic data. After a review of representative H&E-stained slides, four to seven sections of 5-μm thickness were obtained from FFPE blocks of pancreatic cancers for macrodissection. Adjacent normal tissues, including normal acinar tissues and adipose tissues, were removed macroscopically using a scalpel. Only the cancerous parts of the sections were used for the isolation of mRNA. The extracted RNA was quantified by reading the absorbance of 260 nm and 280nm (A260/280) with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction Quantitative RT-PCR was performed with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) for 40 cycles of 15 s at 94°C and 30 s at 55°C with a QuantiTect SYBR Green Reverse Transcription-PCR kit (Qiagen) according to the manufacturer's instructions.

We designed specific primers for *S100A4* (forward, 5′-atcgccatgatgtgtaacga-3′; reverse, 5′-cccaaccacatcagaggagt-3′) and *β-actin* (forward, 5′-aaatctggcaccacaccttc-3′; reverse, 5′-ggggtgttgaaggtctcaaa-3′) using primer 3 and performed BLAST searches to confirm primer specificity. The PCR product sizes of these primers are small (*S100A4*, 85 base pairs (bp); *β-actin*, 139 bp, respectively), which allowed accurate and sensitive qRT-PCR despite the fragmented RNA extracted from FFPE tissue specimens.[41,42] The *S100A4* and *β-actin* expression levels were calculated for all cases using a standard curve constructed with total RNA from SUIT-2, a pancreatic cancer cell line. One microliter of RNA was used in qRT-PCR despite the concentration of RNA. *S100A4* mRNA expression levels were normalized using *β-actin* as an internal control and expressed as the ratio of expression of *S100A4* mRNA to that of *β-actin* mRNA. All samples were run in triplicate. The accuracy and integrity of the PCR products were confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.).

Immunohistochemical Procedures and Evaluation Sections (4 μm thick) were cut from paraffin-embedded tissues, deparaffinized in xylene, and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was achieved by autoclaving the sections in citrate buffer at pH 6.0. A Histofine SAB-PO(R) kit (Nichirei, Tokyo, Japan) was used for immunohistochemical labeling. Each section was exposed to 10% non-immunized goat serum for 10 min to block nonspecific antibody binding, followed by incubation with a rabbit polyclonal anti-S100A4 antibody (NeoMarkers, Fremont, CA, USA; 1:100 dilution) at 4°C overnight. The sections were then sequentially incubated with a biotinylated anti-rabbit immunoglobulin solution for 20 min followed by peroxidase-labeled streptavidin for 20 min. The reaction products were visualized using 3,3′-diaminobenzidine as a chromogen, followed by nuclear counterstaining with hematoxylin. Cells were considered positively immunostained when nuclei and cytoplasm were stained. The distribution of stained S100A4 was evaluated as the percentage of stained cells, which was scored as 0, <5%; 1, 5–25%; 2, 26–50%; and 3, >51%, and as staining

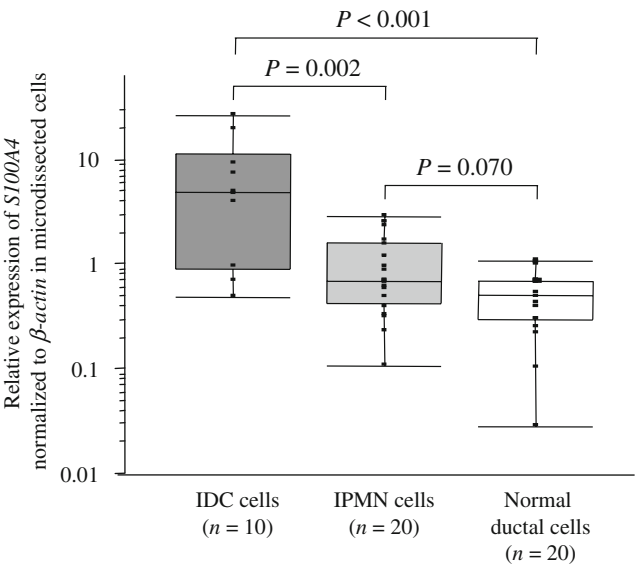

Figure 1 qRT-PCR analysis of *S100A4* mRNA expression in IDC, nonmalignant IPMNs, and normal ductal epithelial cells. IDC cells expressed higher levels of *S100A4* compared with IPMNs (P=0.002) and normal ductal cells (P<0.001). IPMNs tended to express higher levels of *S100A4* compared with normal ductal cells, although the difference did not reach statistical significance (P=0.070). The expression of *S100A4* was normalized to that of *β-actin.* The scale is logarithmic.

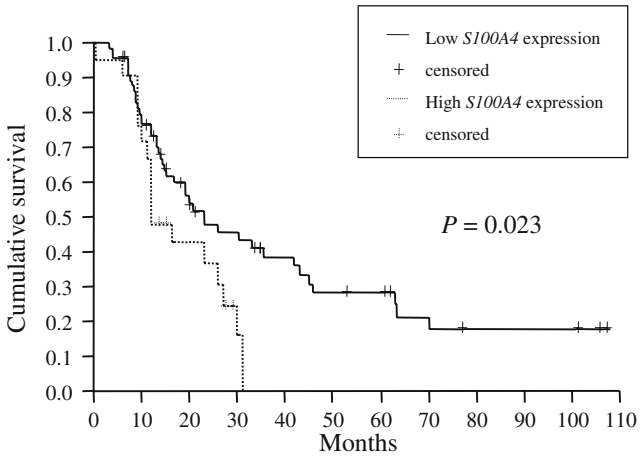

Figure 2 Overall survival after resection of pancreatic cancers with high *S100A4* expression versus low *S100A4* expression. High *S100A4* expression was significantly associated with shorter survival (P= 0.023). The S100A4 expression levels were normalized by *β-actin*.

intensity, which was scored as 0, no staining; 1, weak; 2, moderate; and 3, strong. When the multiplication product of the two scores was greater than 2, S100A4 was considered positively stained. In the immunohistochemical staining, we performed additional staining without primary antibodies in parallel to confirm that no staining was seen. All slides were evaluated independently by two investigators (NI and KN) without any knowledge of the background of each case.

Statistical Analysis Data were analyzed using the Kruskal–Wallis test if comparisons involved three groups and the Mann–Whitney U test if comparisons involved two groups. *S100A4* expression was split into high- and low-level groups using recursive descent partition analysis, as described by Hoffmann et al.[43] Survival curves were constructed with the Kaplan–Meier product-limit method and compared by log-rank test. The statistical significance was defined as a P value <0.05. All statistical analyses were performed with JMP 7.01 software (SAS Institute, Cary, NC, USA).

Results

Quantitative Analysis of S100A4 mRNA Expression in IDC, Nonmalignant IPMN, and Normal Ductal Epithelial Cells We measured the *S100A4* mRNA expression levels in IDC cells, nonmalignant IPMN cells, and normal ductal epithelial cells by qRT-PCR after laser-microdissection from frozen sections to determine whether *S100A4* is differentially expressed between pancreatic cancer cells and cells from nonmalignant tumors or normal ductal cells. *S100A4* mRNA expression was significantly higher in IDC cells than in IPMN (P=0.002) and normal ductal cells (P< 0.001), as shown in Fig. 1. IPMNs tended to express higher levels of *S100A4* compared with normal ductal cells,

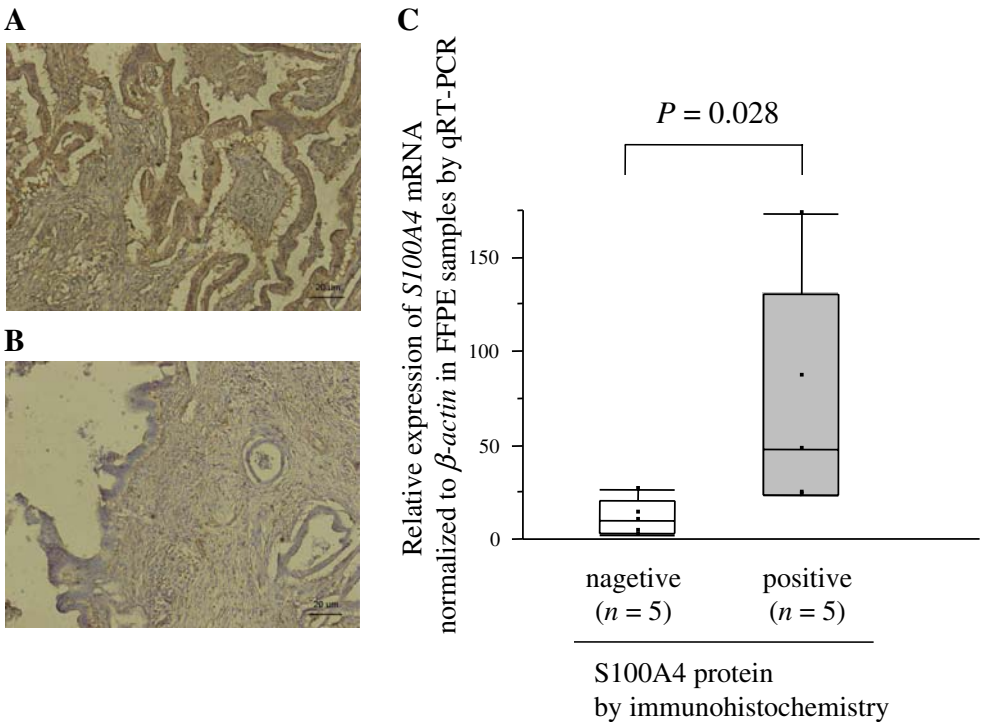

Figure 3 Positive (**a**) and negative (**b**) expression of S100A4 in pancreatic cancers (original magnification: ×100). **c** The extent of *S100A4* mRNA expression was correlated with the expression of S100A4 protein.

although the difference did not reach statistical significance (P=0.070).

S100A4 mRNA Expression Was Correlated with Prognosis of Patients with Pancreatic Cancers To investigate the correlation between *S100A4* expression and prognosis in patients with pancreatic ductal carcinomas, we isolated total RNA from FFPE samples from 87 patients with pancreatic cancers and measured the levels of *S100A4* expression. After normalizing *S100A4* mRNA expression to *β-actin* expression, we obtained two groups with high versus low *S100A4* expression (cutoff value, 20.5). The high- and low-expression *S100A4* groups comprised 21 and 66 cases, respectively. High *S100A4* expression was significantly associated with a shorter overall survival (P=0.023, Fig. 2). The median survival time of the patients with high and low S100A4 expression was 12 and 23 months, respectively.

S100A4 mRNA Expression Was Correlated with the Expression of S100A4 Protein S100A4 was immunoreactive in cytoplasm and nuclei of cancer cells (Fig. 3a, b). Cancer cells were highly stained with S100A4 compared with that in fibroblast in the stroma, even though S100A4 has been called "fibroblast-specific protein 1". The level of *S100A4* mRNA expression was significantly correlated with the expression of S100A4 protein, as shown in Fig. 3 (P=0.028).

Discussion

We measured the *S100A4* mRNA expression levels in IDC cells, nonmalignant IPMN cells, and normal ductal cells with qRT-PCR and found that IDC cells expressed the highest levels of *S100A4* among the cell types analyzed in the present study. To our knowledge, this is the first study to evaluate the correlation of *S100A4* expression in pancreatic cancers and IPMN. We have previously reported that IDC cells expressed higher levels of *S100A2*, another S100 family member, than premalignant cells and that IPMN cells with high-grade atypia expressed higher levels of *S100A2* than IPMN with low-grade atypia and normal ductal cells.[40] In the present study, a trend for a stepwise increase in *S100A4* mRNA expression from normal ductal cells to IDC cells was shown, suggesting that *S100A4* may also be involved in pancreatic carcinogenesis, similar to *S100A2*.

We quantitatively measured *S100A4* mRNA expression by qRT-PCR using FFPE samples of surgically resected pancreatic cancers. We found that high *S100A4* expression was significantly associated with a shorter overall survival, suggesting that *S100A4* mRNA could be a prognostic marker in pancreatic cancers. This finding supports a report of an immunohistochemical analysis of 62 surgical cases with pancreatic cancers, in which overexpression of S100A4 was significantly correlated with tumor size, tumor–node–metastases stage, and poor prognosis.[35] These consistent results also indicate that quantitative analysis of *S100A4* mRNA by qRT-PCR could be a reliable modality to contribute to the prediction of the prognosis of patients with pancreatic cancer. In fact, *S100A4* mRNA expression was correlated with the expression of S100A4 protein. The measurement of *S100A4* mRNA expression by qRT-PCR offers a high level of objectivity and quantitative performance compared with immunohistochemical examination. Additionally, the evaluation of *S100A4* mRNA expression of the tumor could be also performed from tiny tissue samples, resulting in a clinically informative technique.

Cytological specimens obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) and by endoscopic retrograde cholangiopancreatography (ERCP) have played an important role in the diagnosis of pancreatic cancer. However, cytological interpretation of clinical specimens obtained by these techniques is often difficult because samples are scant and bloody.[44–47] Therefore, molecular markers are needed to aid the diagnosis in indeterminate cytological samples.[48] The present study revealed that *S100A4* mRNA expression level was significantly higher in cancer cells than in nonmalignant IPMN cells or normal ductal cells. The merit of the analysis used in the present study is that we can sensitively and accurately measure the mRNA expression levels using gene-specific primers that generate short PCR products, even for tiny tissue samples or fragmented RNA obtained by EUS-FNA or ERCP. The measurement of *S100A4* mRNA for clinical samples could give clinicians important information, including tumor nature and the patient's prognosis, because *S100A4* expression was correlated with prognosis, although further studies are required to confirm this clinical application.

In summary, *S100A4* mRNA was expressed at higher levels in pancreatic cancer cells than in cells derived from nonmalignant tumors or nonneoplastic epithelium. The level of *S100A4* expression was significantly correlated with the prognosis of patients with pancreatic cancer. Thus, *S100A4* could be a marker of malignancy in pancreatic tumors and for poor prognosis in patients with pancreatic cancer.

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