Clinical features of cystatin A expression in patients with pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is the most lethal malignancy known, with an extremely poor prognosis and a 5-year survival rate of <5% worldwide.1–3 Surgical resection is the only treatment available that can achieve a complete cure; however, only 15–20% of patients are diagnosed in the early (i.e., operable) stages.2,3 Chemotherapy is an alternative for unresectable PDAC; however, any survival benefit is limited. Therefore, it is extremely important to establish novel diagnostic biomarkers for PDAC, indicating curative surgical treatment.4

Peripheral blood (PB) contains a variety of immune-mediating cells, such as neutrophils, lymphocytes, and monocytes, which are involved in the host immune defense system and respond to various diseases, such as viral infection, metabolic disease, and cancers.5–8 Peripheral blood cells alter their gene expression profile in response to various diseases9–11 including PDAC.11 We previously investigated the gene expression features of patients with digestive cancers12 and found that these cancers affect the expression of the cysteine protease inhibitor cystatin A (CSTA) in PB cells.

In this study, we report that CSTA expression was upregulated in the CD4+ cells of patients with PDAC. We also observed that the serum CSTA level was higher in patients with PDAC compared with healthy controls. An immunohistochemical staining analysis of surgically resected PDAC tissues showed that CSTA and the lysosomal cysteine protease cathepsin B, which is inhibited by CSTA, were expressed in tumor tissues and tumor-infiltrating immune cells. Thus, CSTA and cathepsin B play an important role in the local cancer tissues and PB of patients with PDAC.

Materials and Methods

Patients. Nine patients with PDAC (male:female, 8:1; age, 70.8 ± 8.9 years) and seven healthy volunteers (male:female, 4:3; age, 61.0 ± 3.7 years) were subject to gene expression analysis of CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD15+ neutrophils, and CD19+ B cells in PB (Table 1). The groups were not different with respect to other clinical parameters. Another, larger cohort of 41 patients with PDAC (male: female, 30:11; age, 73.3 ± 11.3 years) and 20 healthy volunteers (male:female, 6:14; age, 61.1 ± 9.6 years) were also enrolled. In addition, serum CSTA concentrations in 36 patients with PDAC (male:female, 27:9; age, 71.2 ± 9.8 years) and 37 healthy volunteers (male:female, 18:19; age, 62.4 ± 7.7 years) were measured (Table 2). The groups were not significantly different with respect to other clinical parameters. In terms of the clinical background of PDAC patients for the gene expression analysis (Table 1) and serum CSTA concentration analysis, the frequency of patients with distant metastasis or stage IV disease was 77.8% and 63.8%, respectively.
Isolation of subpopulations of PB cells and flow cytometry. Peripheral blood cells were isolated from fresh heparinized venous blood using ACK lysing buffer in accordance with the manufacturer’s protocol (Lonza, Basel, Switzerland). The subpopulations of PB cells were isolated using a magnetic cell sorting system (Miltenyi, Cologne, Germany), and a magnet column (Miltenyi) according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was confirmed to be >95% by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Quantitative RT-PCR analysis. Total RNA was isolated from cells using a microRNA isolation kit (Stratagene, La Jolla, CA, USA) and was reverse-transcribed using 1 μg oligo(dT) primer and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Primer pairs and probes for CSTA, Tbet, Foxp3, γ-interferon (IFN-γ), transforming growth factor-β (TGF-β), and β-actin (Applied Biosystems, Foster City, CA, USA) were used for the mRNA expression analysis with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The relative gene expression levels were calculated with the 2 -ΔΔC T method using β-actin as the control gene. (11)

Serum CSTA concentrations. Serum CSTA concentrations were measured with a human ELISA kit (Biorad, San Francisco, CA, USA) according to the manufacturer’s protocol. The detection range was 0.313–20 ng/mL.

Immunohistochemical analysis of surgically resected pancreatic cancer tissues. For immunohistochemical staining, 4-μm tissue block sections were incubated overnight with rabbit anti-human CSTA mAb (clone EPR6941, dilution 1:500; Abcam, Cambridge, UK), mouse anti-human monoclonal cathepsin B antibody (clone CA10, dilution 1:200; Abcam), mouse anti-human monoclonal T-bet antibody (clone EPR9301, dilution 1:200; Abcam), mouse anti-human monoclonal Foxp3 antibody (clone 2B8, dilution 1:200; Abcam), mouse anti-human monoclonal IFN-γ antibody (clone IFNG/466, dilution 1:200; Abcam), mouse anti-human monoclonal tumor necrosis factor-α (TNF-α) antibody (clone 2B8B3, dilution 1:200; Abcam), mouse anti-human monoclonal TGF-β antibody (clone TB21, dilution 1:200; Abcam), mouse anti-human monoclonal interleukin (IL)-6 antibody (clone 10C12, dilution 1:50; Leica Biosystems, Newcastle, UK) or rabbit anti-human polyclonal IL-1β antibody (clone H-153, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) after heat-induced antigen retrieval. Following incubation with the antibody, the samples were incubated at room temperature for 1 h with anti-mouse immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Simple Staining Kit; Nichirei, Tokyo, Japan). After the benzidine reaction, the sections were lightly counterstained with hematoxylin.

Serum cytokine and chemokine concentrations in PDAC patients. Serum concentrations of cytokines/chemokines were measured using a Multiplex bead Immunoassay kit and Human Cytokine 27-Plex Panel (Invitrogen) including IFN-γ, IL-6, IL-1β, and TNF-α, according to the manufacturer’s protocol. Serum was reserved from six PDAC patients with positive CSTA expression and nine PDAC patients with negative CSTA expression.

Statistical analysis. Data are expressed as mean ± SE. The Mann–Whitney U-test was used to detect differences between the two groups. A P-value <0.05 was considered significant. Pearson correlation coefficients and multiple regression analysis were used to analyze correlations.

Results

Cystatin A expression and concentration were elevated in CD4 + T cells and sera of patients with PDAC. We isolated CD4 + T cell, CD8 + T cell, CD14 + monocyte, CD14 + neutrophil, and CD19 + B cell fractions from whole PB cells, and examined...
Cystatin A (CSTA) is a novel biomarker of pancreatic cancer

Cystatin A expression in the CD4+ T cells of nine patients with PDAC was significantly higher than in those of healthy volunteers, but no differences were observed for CD8+ T cells or CD19+ B cells, nor CD14+ monocytes or CD15+ neutrophils, both of which were originally abundant with CSTA expression (Fig. 1a). We also assessed CSTA expression in peripheral CD4+ T cells of PDAC patients was related to the specific subset of CD4+ T cells, such as antitumor helper T cells (Th1) or regulatory T cells (T-reg), which are characterized by inhibition of antitumor immunity, we measured the mRNA expression of molecules including transcriptional factors (T-bet for Th1, and Foxp3 for T-reg) and cytokines (IFN-γ for Th1, and TGF-β for T-reg) in CD4+ T cells of PDAC patients by quantitative RT-PCR, followed by correlation analysis. We did not observe any significant correlation with mRNA expression of CSTA and genes related to either antitumor helper Th1 or T-reg subsets, or these cytokines (Fig. S1). Thus, CD4+ T cells expressing CSTA in PB was not categorized into any specific conventional CD4+ subset.

Next, we measured serum CSTA concentrations in 36 patients with PDAC and 37 healthy volunteers. Serum CSTA concentration was significantly increased in patients with PDAC (Fig. 1c). When we defined the cut-off value as 0.313 ng/mL, which is the minimal detection limit of the ELISA kit, detection sensitivity was 16/36 (44.4%), and specificity was 35/37 (94.6%). Moreover, the positive predictive value was 16/18 (88.9%) and the negative predictive value was 35/37 (63.6%).

We also measured the serum cytokine concentration of 15 PDAC patients: an increment of CSTA expression in six patients (73%) were not associated with tumor cells expressing CSTA, and the positive predictive value was 35/37 (94.6%). Moreover, the positive predictive value was 16/18 (88.9%) and the negative predictive value was 35/37 (63.6%).

Comparison of serum CSTA concentration and clinical parameters in patients with PDAC. We further assessed the associations between serum CSTA concentration and several clinical parameters. Gender (Fig. 2a) and the presence of distant metastasis were not associated with serum CSTA concentration (Fig. 2b). However, serum CSTA positivity was significantly correlated with advanced clinical stage (III–IV; Fig. 2c). We also assessed serum concentrations of the tumor markers CEA and CA19-9 in patients with PDAC according to CEA and CA19-9 levels (<5 μL, ≥10, <30 μL, ≥30 ng/mL; n = 24/45/3) and CA19-9 level (<37 μL, ≥37, <200 μL, ≥200, <400 μL, ≥400 U/mL; n = 14/6/1/1/5). No association was detected between CEA level (Fig. 2d) and serum CSTA positivity. However, CA19-9 level ≥400 U/mL was correlated with CSTA positivity (Fig. 2e), as well as clinical stage (data not shown).

Expression of CSTA and cathepsin B in tumor tissues and tumor-infiltrating immune cells. To further investigate the features of CSTA-related pathophysiology in patients with PDAC, the expression of CSTA and cathepsin B, which is a lysosomal cysteine protease, was evaluated in the tumor tissues and tumor-infiltrating immune cells of 20 surgically resected PDAC tissues by immunohistochemical staining (Table 3). Cystatin A expression either in tumor cells or tumor-infiltrating immune cells was found in 16 of 20 patients (80%) (Table 3). Tumor-infiltrating immune cells expressing CSTA, mostly neutrophils, were found in 15 of 20 patients; among them, 11 patients (73%) were not associated with tumor cells expressing CSTA (Table 3). In tumor cells CSTA expression was found in 5 of 20 patients (25%) (Fig. 3a,b, Table 3), and cathepsin B in

![Fig. 1. Cysteine protease inhibitor cystatin A (CSTA) expression in peripheral blood cells and serum CSTA concentrations in patients with pancreatic ductal adenocarcinoma (PDAC). (a) CSTA expression was upregulated in the CD4+ T cells of nine patients with PDAC, but no differences in expression were detected in CD8+ T cells, CD4+ monocytes, CD15+ neutrophils, or CD19+ B cells, compared with seven healthy volunteers. (b) CSTA expression was upregulated in the CD4+ T cells of 41 patients with PDAC compared with 20 healthy volunteers. (c) Serum CSTA concentrations in 36 patients with PDAC were higher compared with those of 37 healthy volunteers. Detection sensitivity was 16/36 (44.4%), and specificity was 35/37 (94.6%). Moreover, the positive predictive value was 16/18 (88.9%) and the negative predictive value was 35/37 (63.6%).]
15 patients (75%) (Fig. 3c,d, Table 3). As for tumor-infiltrating immune cells, CSTA expression was not or weakly founded in mostly neutrophils (Fig. 4a,b, Table 3), while cathepsin B, were found in all 20 PDAC patients (100%) in mostly macrophages (Fig. 4c,d, Table 3). Thus, CSTA expression in PDAC tissues was found predominantly in tumor-infiltrating immune cells, and cathepsin B expression was found both in tumor cells and tumor-infiltrating immune cells.

We also undertook immunohistochemistry of PDAC tissues using a multicolor assay for transcriptional factors T-bet (Fig. S3A) and Foxp3 (Fig. S3B), and cytokines IFN-γ (Fig. S3C), TGF-β (Fig. S3D), TNF-α (Fig. S3E), IL-6 (Fig. S3F), and IL-1β (Fig. S3G), together with CSTA. We found a substantial number of tumor-infiltrating immune cells expressing IFN-γ and TGF-β (Fig. S3C,D); however, we did not find tumor-infiltrating immune cells expressing these cytokines concomitantly with CSTA, except that a very few cells expressing CSTA and IFN-γ were detected. We did not find any tumor-infiltrating immune cells expressing TNF-α (Fig. S3E), and rarely found cells expressing IL-6 (Fig. S3F) and IL-1β (Fig. S3G). Taken together, CSTA-expressing tumor-infiltrating immune cells were involved in PDAC tissues, which were independent from cells expressing cytokines including profibrogenic TGF-β, not TNF-α, suggestive of the fibrotic condition of PDAC tissues. (13)

Discussion

In this study, we identified upregulated expression of CSTA in CD4+ T cells of PB, as well as elevated serum concentrations of CSTA, in patients with PDAC. The increase in serum concentrations of CSTA was correlated with clinical stage. Cystatin A expression was observed mainly in tumor-infiltrating immune cells of surgically resected PDAC tissues, particularly neutrophils, and cathepsin B was observed in tumor cells as well as tumor-infiltrating immune cells, particularly macrophages.

Cystatin A is a member of the cystatin superfamily of cytoplasmic cysteine protease inhibitors. Cathepsin B belongs to the human cysteine protease cathepsin family, which has 11 members,(14) and is generally expressed in epithelial cells, immune-mediating cells, and lymphoid tissue. (15) It is a key acid hydrolase within the lysosome and represents one of the principal effectors of protein catabolism and autophagy. (16–18) Cystatin A inhibits the enzymatic activity of cathepsin B.
Table 3. Cystatin A and cathepsin B expression in 20 resected specimens of pancreatic ductal adenocarcinoma tumor tissue and infiltrating inflammatory cells

| Case no. | Age, years | Gender | Degree of inflammation | Stage | T category | N category | CRP, mg/dL | CEA, ng/mL | CA19-9, U/mL | Cathepsin B Tumor | Cathepsin B Ductal epithelium | Cathepsin B TIIC | Cystatin A Tumor | Cystatin A Ductal epithelium | Cystatin A TIIC |
|----------|------------|--------|------------------------|-------|------------|-----------|------------|------------|-------------|----------------|-------------------|----------------|----------------|-------------------|----------------|----------------|
| 1        | 71         | F      | Mild                   | II B  | 3          | 1         | 0.2        | 2.2        | 157         | +            | −                 | >100             | −               | −                 | −                 | 0              |
| 2        | 57         | M      | Moderate               | II B  | 3          | 1         | 0.1        | 4.3        | 293         | +            | −                 | >100             | −               | −                 | −                 | 0              |
| 3        | 61         | F      | Severe                 | II B  | 1          | 1         | 0.0        | 2.5        | 17          | +            | +                 | >100             | −               | −                 | >100              | 0              |
| 4        | 54         | F      | Mild                   | II B  | 3          | 1         | 0.1        | <2.0       | 54          | +            | −                 | 15               | +               | −                 | <10               | 0              |
| 5        | 70         | F      | Mild                   | II B  | 3          | 1         | 0.0        | <2.0       | 90          | +            | −                 | 62               | −               | −                 | >100              | 0              |
| 6        | 66         | M      | Moderate               | II B  | 3          | 1         | 0.1        | 2.8        | 56          | −            | −                 | >100             | +               | −                 | >100              | 0              |
| 7        | 60         | F      | Moderate               | II B  | 3          | 1         | 0.5        | 30.1       | 649         | +            | −                 | 35               | −               | −                 | 45                | 0              |
| 8        | 78         | M      | Moderate               | II A  | 3          | 0         | 0.5        | 4.5        | 23          | +            | −                 | <10              | +               | −                 | <10               | 0              |
| 9        | 77         | M      | Moderate               | II A  | 3          | 0         | 0.5        | <2.0       | 187         | +            | −                 | 39               | −               | −                 | <10               | 0              |
| 10       | 57         | M      | Moderate               | II B  | 3          | 1         | 0.1        | <2.0       | 402         | −            | −                 | 25               | −               | −                 | 14                | 0              |
| 11       | 65         | M      | Moderate               | II B  | 3          | 1         | 0.9        | 4.2        | 292         | −            | −                 | 31               | +               | −                 | <10               | 0              |
| 12       | 68         | F      | Severe                 | II B  | 3          | 1         | 2.7        | <2.0       | 50          | +            | −                 | >100             | −               | −                 | 45                | 0              |
| 13       | 62         | M      | Mild                   | II A  | 3          | 0         | 0.0        | <2.0       | 57          | +            | +                 | 55               | −               | −                 | 38                | 0              |
| 14       | 65         | M      | Moderate               | II A  | 3          | 0         | 0.0        | 3.4        | 184         | +            | −                 | >100             | −               | −                 | <10               | 0              |
| 15       | 59         | F      | Mild                   | II A  | 3          | 0         | 0.1        | <2.0       | 18          | −            | −                 | 33               | −               | −                 | 13                | 0              |
| 16       | 66         | M      | Moderate               | II A  | 3          | 0         | 0.1        | 3.2        | 9           | +            | −                 | <10              | −               | −                 | 0                 | 0              |
| 17       | 70         | M      | Moderate               | II B  | 3          | 1         | 0.1        | 1.5        | 85          | +            | −                 | 22               | +               | −                 | 0                 | 0              |
| 18       | 57         | F      | Moderate               | II A  | 3          | 0         | 0.1        | 4.9        | 7           | +            | −                 | 43               | −               | −                 | 0                 | 0              |
| 19       | 63         | M      | Mild                   | II B  | 2          | 0         | 0.9        | 2.5        | <1          | +            | +                 | 35               | −               | −                 | 46                | 0              |
| 20       | 64         | F      | Moderate               | II A  | 3          | 0         | 0.0        | 2.7        | 33          | −            | −                 | >100             | −               | −                 | <10               | 0              |

CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; CRP, C-reactive protein; F, female; M, male; TIIC, Tumor infiltrating immune cells.
The role of cathepsin B expression in tumor cells is controversial, and has been reported to be both progressive and regressive.\(^{19-22}\) Cathepsin B expression in patients with PDAC is related to prognosis and recurrence,\(^{23-25}\) although the mechanism remains to be elucidated. Cystatin A expression has been observed in tumor tissues, such as breast,\(^ {26}\) head and neck,\(^ {27}\) and lung\(^ {28}\) cancers, as well as hepatocellular carcinoma.\(^ {29}\) In the current study, cathepsin B was expressed in tumor cells, as well as tumor-infiltrating immune cells, especially macrophages, in PDAC tissues. In contrast, CSTA expression was observed mainly in tumor-infiltrating immune cells rather than tumor cells, which represents a different pathological feature to those of other cancer types.\(^ {26}\) In this context, CSTA could affect cathepsin during antigen-presenting processes involving macrophages and dendritic cells, resulting in an altered immune response of the host to local tumor cells.\(^ {32-33}\) The local pathological conditions mediated by cathepsin and CSTA in tumor cells and tumor-infiltrating immune cells affect tumor progression. The responses of cathepsin and the CSTA complex affect tumor progression and metastasis in laryngeal\(^ {34}\) and breast cancers.\(^ {26}\) Furthermore, tumor cells overexpressing CSTA showed a reduced capacity for lung or bone metastasis in a human esophageal squamous cell carcinoma xenograft model and a syngeneic mouse model of mammary gland tumorigenesis, respectively, suggesting that CSTA could act as a tumor metastasis suppressor in this complex.\(^ {35,36}\) Previously, there were some reports that the expression ratio of cathepsin B and cystatin families, such as cystatin B or cystatin C, in sera were significantly correlated with presence of cancer, prognosis, or lymph node metastasis in patients.
with colorectal cancer,\(^{(37)}\) esophageal cancer,\(^{(38)}\) and cholangiocarcinoma.\(^{(39)}\) Considering these, the ultimate consequences of the presence of CSTA-expressing cells and cathepsin-expressing cells in PDAC tissues, elevated CSTA concentration in sera, and increased expression of CSTA in peripheral CD4\(^+\) T cells of PDAC patients should be further investigated.\(^{(40)}\)

Intriguingly, we also observed that CSTA expression in the PB CD4\(^+\) T cells of patients with PDAC was higher than in those of healthy volunteers. In addition, serum CSTA concentrations in patients with PDAC were higher than those of healthy volunteers. Considering the involvement of cathepsin B and CSTA in tumor cells and tumor-infiltrating immune cells, it is possible that PB reflects CSTA activity in the local tumor microenvironment, as we previously reported the PB biological signature features of gene expression under inflammatory conditions in local cancer tissues.\(^{(9)}\) Thus, upregulated expression of CSTA in CD4\(^+\) T cells, and increased serum CSTA concentration in PB, is presumably a reflection of the local PDAC tumor environment involving cathepsin B and CSTA. The characteristics of peripheral CD4\(^+\) T cells expressing CSTA in PB should be further investigated, as they were not indicative of the conventional antitumor immune Th1 cells, nor T-reg, which is inhibitory to antitumor immunity. In addition, most tumor-infiltrating immune cells expressing CSTA were not relevant to CD4\(^+\) T cells. Further investigations are needed to disclose details of the roles of CSTA for cancer immunity.

In conclusion, the current study indicates that CSTA expression is involved in the PDAC inflammatory condition in local tumor tissues, including with respect to the environment associated with cathepsin B expression, as well as PB. Further investigations are needed to elucidate the significance of the CSTA-cathepsin axis in the pathophysiology of PDAC, to aid development of novel diagnostic and treatment approaches.

**Disclosure Statement**

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Gene expression of CSTA, transcriptional factors, and cytokines in peripheral CD4+ T cells of patients with pancreatic ductal adenocarcinoma.

**Fig. S2.** Serum concentrations of cytokines and cystatin A (CSTA) in patients with pancreatic ductal adenocarcinoma (PDAC).

**Fig. S3.** Immunohistochemical analysis of with pancreatic ductal adenocarcinoma tissues for transcriptional factors and cytokines related to CD4+ T subsets T-bet (A), Foxp3 (B), γ-interferon (IFN-γ) (C), and transforming growth factor-β (TGF-β) (D), as well as cytokines tumor necrosis factor-α (TNF-α) (E), interleukin (IL)-6 (F), and IL-1β (G), whose expression were correlated with those of CSTA in sera.