Original Article

Clinical Implications and Diagnostic Usefulness of Correlation Between Soluble Major Histocompatibility Complex Class I Chain-Related Molecule A and Protumorigenic Cytokines in Pancreatic Ductal Adenocarcinoma

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BACKGROUND: Tumor-derived soluble factors serve as mediators between tumors and surrounding microenvironment to promote tumor growth and metastasis under a complex network. The objective of this study was to evaluate the relationships between soluble major histocompatibility complex class I chain-related molecule A (sMICA) and 4 categories of cytokines (tumor-related proinflammatory, anti-inflammatory, chemotactic/proangiogenic, and growth-stimulatory) in the development and progression of pancreatic ductal adenocarcinoma (PDAC). **METHODS:** Serum levels of sMICA and 4-categorized cytokines were measured by enzyme-linked immunosorbent assay and chemiluminescent immunoassay, respectively, in 134 individuals (normal, n = 55; chronic pancreatitis, n = 25; PDAC, n = 54). Clinical implications of sMICA and tumor-related cytokines, their correlations, and diagnostic usefulness in PDAC were evaluated. **RESULTS:** Serum sMICA, which was associated with the development and progression of PDAC, correlated with interferon-γ negatively ($P = 0.024$), whereas it correlated positively with the anti-inflammatory cytokines interleukin-10 (IL-10) and IL-1 receptor antagonist, and the bifunctional cytokine tumor necrosis factor α, with respect to PDAC development ($P < .05$). sMICA also correlated positively with the chemotactic/proangiogenic cytokines vascular endothelial growth factor, soluble CD40 ligand, and IL-8, and the tumor growth-stimulatory cytokines epidermal growth factor and transforming growth factor α, with respect to PDAC development and/or progression. Logistic regression analysis validated the diagnostic usefulness of combination use of sMICA and its related cytokines to predict the presence of PDAC and distant metastasis in PDAC, superior to carbohydrate antigen 19-9. **CONCLUSIONS:** sMICA may be involved in tumor-associated angiogenesis and tumor growth either directly or indirectly by affecting corresponding cytokines as well as causing impairment of natural killer cell cytotoxicity in the development and progression of PDAC. A combination of sMICA and its related cytokines exhibited remarkable diagnostic potential in PDAC. ***Cancer*** **2013;119:233-44.** © *2012 American Cancer Society.*

KEYWORDS: cytokines, diagnostic accuracy, pancreatic ductal adenocarcinoma, relationship, soluble major histocompatibility complex class I chain-related molecule A.

Tumors in immunocompetent hosts are forced to develop and grow despite constant pressure from the immune system, consisting of cytotoxic immune cells and multiple soluble mediators such as cytokines and chemokines.[1] However, tumor cells can reprogram normal stroma to immunosuppressive tumorigenic stroma, which allows them to escape antitumor immunosurveillance, and ultimately leads to the development or evolution of the malignant process by the aberrant production of soluble mediators, which induce an imbalance between proinflammatory and anti-inflammatory cytokines.[2,3] Tumor-derived cytokines also serve as paracrine or autocrine growth factors to promote tumor growth and tumor-associated angiogenesis, which are critical steps for tumor expansion and acquisition of metastatic potential.[4]

Natural killer (NK) cells are the primary critical effectors in anti-tumor immunity.[5] They are mainly activated by natural killer group 2 member D (NKG2D) receptors.[6] In humans, major histocompatibility complex class I-related molecule A (MICA) and B (MICB) are major ligands of NKG2D, which are widely up-regulated on tumor surfaces in response to malignant transformation, thereby leading to tumor cell lysis and cytokine secretion induced by NK or T cells.[6] However, MICA/MICB proteins can be shed from the tumor cell surface into the extracellular milieu as a cytokine-like soluble form, impairing the cytotoxic response against tumors by reducing MICA/MICB expression on the tumor cell surface and down-regulating NKG2D on NK and T cells via internalization and degradation.[7] These immune escape strategies may influence tumor development and progression.[8] However, other mechanisms may exist in the sMICA/sMICB-associated tumorigenicity and invasiveness of cancer beyond these immune escape strategies.

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Pancreatic ductal adenocarcinoma (PDAC) is characterized as being surrounded by a dense desmoplastic microenvironment that produces multiple immunosuppressive cytokines, which can provide several immunological advantages that allow cancer cells to escape host antitumor immunity.[3,9-11] PDAC also produces multiple protumorigenic growth factors such as proangiogenic and growth-stimulatory cytokines.[12] Thus, cytokines may play crucial roles in the malignant transformation and evolution of PDAC, allowing it to become an intractable and aggressive disease.

In PDAC, MICA proteins also can be released from the cell surface into the extracellular tumor microenvironment; the resulting elevation of serum sMICA can affect the development and evolution of PDAC[13,14] through direct impairment of NK cell function via a decrease in MICA expression on the PDAC surface and down-regulation of NKG2D on NK cells.[7] However, circulating sMICA may interact with other soluble mediators, such as immune-related or protumorigenic cytokines produced by PDAC, which can affect the development and progression of PDAC. In general, soluble mediators in the tumor microenvironment are connected with one another.[15] In addition, the NKG2D-MICA system is interrelated with several cytokines and chemokines for the activation of NK cells and the proper expression of MICA on the cell surface.[16] Thus, exploration of the relationship between sMICA and dysregulated cytokines produced by PDAC along the carcinogenic process of this cancer can help to understand the detailed mechanism of sMICA-associated tumorigenicity and the evolution of PDAC, and can provide valuable information for effective diagnostic and therapeutic strategies for PDAC with respect to cancer immunology.

In this study, we evaluated the correlations between sMICA and 4 categories of cytokines (proinflammatory, anti-inflammatory, chemotactic/proangiogenic, and tumor growth-stimulatory cytokines) along the PDAC carcinogenic process, and clarified their clinical implications and diagnostic usefulness in PDAC using serum samples obtained from normal, chronic pancreatitis (CP), and PDAC subjects.

MATERIALS AND METHODS

Study Population

The study groups were composed of 55 healthy volunteers, 25 patients with CP, and 54 patients with PDAC who visited the Yonsei University Health System, Seoul, Korea, to allow for a statistical power greater than 0.8 with a statistical significance (α) of 0.05 by F-test (1-way ANOVA). All participants underwent medical history assessment, physical exam, chemical tests, and abdominal sonography. To confirm CP or PDAC, further radiologic tests such as computed tomography (CT) and histopathological tests were performed. Recruitment to each group was consecutively performed on the basis of these test results.

For the healthy group, sex- and age-matched healthy individuals who received a regular checkup and were revealed to have a normal pancreas without any risk factors of PDAC and abnormal lab findings were enrolled. For the CP and PDAC groups, patients with typical radiological and/or histopathological findings of CP or PDAC were enrolled, respectively. Other pancreatic malignancies except ductal adenocarcinoma were excluded. Patients who suffered from other acute or chronic illness, fever within 1 week, or other cancers were excluded. Individuals who received any medications or treatments for corresponding diseases were also excluded.

Tumor-node-metastasis (TNM) classification of PDAC was analyzed according to the American Joint Committee on Cancer (AJCC) *Cancer Staging Manual*, 6th edition. A pathologist at the Yonsei University Health System reviewed all histopathological information. Blood samples for index tests (serum levels of sMICA or cytokines) were prospectively collected at the time of recruitment based on the reference standard (diagnosis) and before beginning the corresponding treatments. Collected blood samples were stored at -80°C as the serum fraction until analysis. The Institutional Review Board of the Yonsei University Health System approved this research, and all participants provided written informed consent.

Measurement of Serum sMICA Levels Using Enzyme-Linked Immunosorbent Assay

Serum sMICA was detected using a sandwich ELISA as described.[13,14] In brief, monoclonal antibodies (mAbs) AMO1 and BAMO3 were used at 5.0 and 1.0 μg/mL, respectively, with recombinant sMICA*04 as the standard. Plates were coated with the capture mAb and then blocked by the addition of 15% BSA, washed, and incubated with normal or patient serum overnight at 4°C. Anti-mouse IgG2a-HRP (1:8,000 dilution; Southern Biotechnologies, Birmingham, Alabama) was used as the secondary antibody and the reaction was developed using the TMB peroxidase substrate system (KPL, Gaithersburg, Maryland). Absorbance was measured at 450 nm, and results were calculated using a calibration curve prepared from standards.

Measurement of Serum 4-Categorized Cytokines Levels Using a Chemiluminescent Immunoassay

Twenty tested cytokines were classified into 4 categories as follows: proinflammatory cytokines with antitumor activity comprising interleukin-1α (IL-1α), IL-1β, IL-2, IL-6, IL-12p70, IL-15, tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), and IFN-γ; anti-inflammatory cytokines with protumorigenic activity comprising IL-4, IL-10, IL-13, and IL-1 receptor antagonist (IL-1RA); chemotactic/proangiogenic cytokines comprising monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), interferon-inducible protein (IP)-10 (also known as CXCL10), vascular endothelial growth factor (VEGF), soluble CD40 ligand (sCD40L), and IL-8 (also known as CXCL8); and growth-stimulatory cytokines comprising epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). These classifications are not absolute, because many of these cytokines are pleiotropic and have overlapping activities.

Cytokines were measured using a commercially available MILLIPLEX MAP Human Cytokine/Chemokine Kit (Millipore, Billerica, Massachusetts). This kit allowed for the simultaneous quantification of all 20 tested cytokines. Briefly, the filter plate was prewetted with 200 μL assay buffer for 10 minutes at room temperature (RT), followed by vacuum removal of the assay buffer. Standard or control (25 μL) was added into the appropriate well, and 25 μL of assay buffer was added to the sample wells but not to the background well. Next, 25 μL of the appropriate matrix solution was added to the background, standard, and control wells, followed by the addition of 25 μL of sample into the appropriate wells. After mixing, 25 μL of beads were added and the plate was incubated overnight at 4°C with shaking. After incubation, the fluid was removed and the plate was washed twice. Detection antibodies (25 μL) were added, and the plate was incubated for 1 hour at RT with shaking. Streptavidin-phycoerythrin (25 μL) was added to each well containing 25 μL of detection antibodies and was incubated for 1 hour at RT with shaking. The fluid was then removed, the plate was washed, and 150 μL of sheath fluid was added. After resuspension for 5 minutes, the median fluorescent intensity (MFI) data were read on a Luminex 100 IS and analyzed using the logistic curve-fitting method to determine cytokine concentrations.

Measurement of Serum CA19-9 Levels

Serum CA 19-9 levels were measured using a Vitros-3600 automatic analyzer (Ortho Clinical Diagnostic, New York, New York) for comparison of cytokine diagnostic accuracy.

Statistical Analysis

All assays were performed in duplicate in a blinded fashion on the same day (1 month after the end date of recruitment, November 30, 2011) by a PhD who is an expert in this field. Each value is expressed as the mean of 2 determinations. All of the serum samples from the subjects recruited in this study were included in the final analysis. The Kruskal-Wallis test was used to compare serum levels of each tested value among more than 3 groups. Mann-Whitney *U* test was used to compare serum levels of each tested value between 2 groups. PDAC group was subdivided into 2 groups according to the presence or absence of distant metastasis: PDAC without metastasis (PDAC/M0) and PDAC with metastasis (PDAC/M1). Spearman's correlation analysis was performed to assess the correlations between soluble mediators and non-continuous variables, and Pearson's correlation analysis was performed to assess the correlations among soluble mediators. Receiver operator characteristic (ROC) curves were generated to calculate the area under the curve (AUC) to determine the cutoff point for the best sensitivity/specificity in order to predict the presence of PDAC or its distant metastasis. To confirm the diagnostic potential of the combined use of sMICA and specific cytokines in PDAC, we performed logistic regression analysis. P values $<.05$ were considered statistically significant. Statistical analyses were performed with SPSS version 13.0 software (SPSS, Chicago, Illinois).

We validated the final conclusions using independent test samples (validation dataset) to estimate the reproducibility of the results. Minimal sample size of each group for validation (noncancer vs PDAC/M0 vs PDAC/M1) was calculated to be 13, under the setting that there were 3 treatment groups, standard deviation within each group was 0.75, statistical significance was 0.05, and the statistical power was greater than 0.8 (by ANOVA test).

RESULTS

Serum Levels of sMICA and the 4-Categorized Cytokines According to Disease Groups

Serum levels of sMICA and cytokines from the 4 different functional categories were compared among disease groups (Table 1). Serum sMICA was significantly higher in PDAC groups compared with the normal group (7.08 ± 6.8 pg/ml, Kruskal-Wallis, $P < .05$) and CP group (44.10 ± 56.4 pg/ml, $P < .05$), and it was higher in the PDAC/M1 group (238.49 ± 19.1 pg/mL)

Table 1. Serum Levels of sMICA and 4-Categorized Cytokines According to Disease Groups

Soluble Factors	Normal (n = 55)	CP (n = 25)	PDAC/M0 (n = 31)	PDAC/M1 (n = 23)	Kruskal-Wallis[a] (*P*)
NK cell-related					
sMICA (pg/mL)	7.08 ± 6.8	44.10 ± 56.4	133.00 ± 67.1	238.49 ± 19.1	<.001
Proinflammatory					
IL-1α (ng/mL)	3.39 ± 1.9	0.10 ± 0.1	4.53 ± 24.1	0.10 ± 0.1	.094
IL-1β (ng/mL)	16.80 ± 107.2	0.72 ± 3.1	0.05 ± 0.2	3.18 ± 13.8	.459
IL-2 (ng/mL)	6.69 ± 20.8	1.17 ± 1.9	0.03 ± 0.1	0.02 ± 0.1	<.001
IL-12 (ng/mL)	23.51 ± 135.5	0.91 ± 2.7	3.49 ± 13.3	1.14 ± 4.0	.001
IL-15 (ng/mL)	3.08 ± 13.2	0.70 ± 1.3	3.32 ± 2.2	4.07 ± 4.1	.430
IFN-α (ng/mL)	11.77 ± 18.3	10.67 ± 16.7	23.92 ± 32.1	19.75 ± 25.4	.359
IFN-γ (ng/mL)	14.21 ± 22.4	3.35 ± 6.3	2.37 ± 4.2	2.28 ± 4.5	<.001
Anti-inflammatory					
IL-4 (ng/mL)	0.10 ± 0.7	0.01 ± 0.1	0.01 ± 0.1	0.01 ± 0.1	.594
IL-10 (ng/mL)	4.81 ± 17.0	2.32 ± 5.4	13.05 ± 32.8	21.15 ± 57.0	<.001
IL-13 (ng/mL)	1.32 ± 9.3	0.01 ± 0.1	0.01 ± 0.1	0.01 ± 0.5	.576
IL-1RA (ng/mL)	5.67 ± 23.0	8.20 ± 31.7	40.44±128.2	95.95 ± 350.7	.001
Bifunctional					
IL-6 (ng/mL)	4.42 ± 12.5	1.83 ± 21.7	18.55 ± 31.3	74.53 ± 292.3	<.001
TNF-α (ng/mL)	7.39 ± 8.8	5.52 ± 3.8	13.14 ± 7.4	16.45 ± 20.3	<.001
Chemotactic/angiogenic					
MCP-1 (ng/mL)	505.54 ± 184.1	489.75 ± 123.9	620.24 ± 476.3	1006.95 ± 213.2	.124
IP10 (ng/mL)	710.14 ± 178.2	542.45 ± 696.4	819.14 ± 723.5	772.42 ± 582.5	<.001
VEGF (ng/mL)	186.67 ± 198.7	142.11 ± 206.5	244.82 ± 261.2	316.64 ± 359.1	.206
sCD40L (ng/mL)	11554.15 ± 483.9	18648.82 ± 102.7	24590.95 ± 622.4	28291.95 ± 420.7	<.001
IL-8 (ng/mL)	55.85 ± 96.4	22.45 ± 36.3	39.44 ± 65.0	1117.08 ± 199.9	<.001
Growth-stimulatory					
EGF (ng/mL)	120.62 ± 113.5	251.80 ± 211.2	340.00 ± 325.7	462.87 ± 356.3	<.001
TGF-α (ng/mL)	8.36 ± 9.5	6.78 ± 7.5	9.88 ± 11.2	14.25 ± 11.4	.002

Abbreviations: CP, chronic pancreatitis; EGF, epidermal growth factor; IFN-α, interferon-α; IL-1α, interleukin 1α; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein-1; NK cells, natural killer cells; PDAC, pancreatic ductal adenocarcinoma; PDAC/M0, PDAC without distant metastasis; PDAC/M1, PDAC with distant metastasis; sCD40L, soluble CD40 ligand; sMICA, soluble MICA; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.
Serum levels of sMICA and cytokines were expressed as mean ± standard deviation.
[a] Statistical analysis was performed using a Kruskal-Wallis test. $P < .05$ (2-tailed) was considered to be statistically significant.

compared with the PDAC/M0 group (133.00 ± 67.1 pg/mL, $P < .05$).

We also found that serum levels of several proinflammatory cytokines including IL-2, IL-12p70, and IFN-γ were significantly reduced in the CP and PDAC groups compared with corresponding normal individuals (Kruskal-Wallis, all $P < .05$; Table 1), and these 3 cytokines were lower in cancer groups compared with noncancer groups (Mann-Whitney *U*, all $P < .05$). Between the PDAC/M0 and PDAC/M1 groups, there was no significant difference in these 3 cytokines (all $P > .05$; Table 1).

On the other hand, serum levels of several anti-inflammatory cytokines including IL-10 and IL-1RA were significantly elevated in PDAC groups compared to CP and normal groups (Kruskal-Wallis, all $P < .05$; Table 1). These cytokines were slightly higher in the PDAC/M1 group compared to the PDAC/M0 group, although the difference was not significant ($P > .05$).

Serum levels of IL-6 and TNF-α, which have bifunctional immune activities, were significantly elevated in PDAC groups compared to the CP and normal groups (Kruskal-Wallis, all $P < .05$). These 2 cytokines demonstrated a tendency to be slightly higher in the PDAC/M1 group compared to the PDAC/M0 group.

Serum levels of IP-10, sCD40L, and IL-8, which are generally considered as chemotactic and/or proangiogenic cytokines, were significantly different among disease groups (Kruskal-Wallis, all $P < .05$; Table 1). IP-10 and sCD40L were significantly higher in cancer groups compared with noncancer groups (Mann-Whitney *U*, all $P < .05$), whereas they were not significantly different between PDAC/M0 and PDAC/M1 groups ($P > .05$). IL-8 was significantly higher in the PDAC/M1 group compared to the PDAC/

Table 2. Spearman's Correlations Between Soluble Immune Mediators, Including sMICA and 4-Categorized Cytokines, and the Development or Progression Parameters of Pancreatic Ductal Adenocarcinoma

Soluble Factors	PDAC Development γ_s (*P*)	Size γ_s (*P*)	T Stage γ_s (*P*)	Node Metastasis γ_s (*P*)	Distant Metastasis γ_s (*P*)
NK cell-related					
sMICA	**0.732 (<.001)**	0.051 (.724)	0.095 (.510)	0.021 (.887)	**0.341 (.015)**
Proinflammatory					
IL-1α	0.082 (.330)	−0.038 (.764)	0.047 (.711)	−0.212 (.090)[a]	0.257 (.058)[a]
IL-1β	−0.098 (.248)	0.044 (.727)	0.003 (.980)	−0.119 (.346)	0.044 (.729)
IL-2	**−0.878 (<.001)**	−0.005 (.971)	0.055 (.671)	−0.198 (.122)	−0.046 (.720)
IL-12	**−0.280 (.001)**	−0.060 (.633)	0.032 (.803)	0.035 (.779)	−0.116 (.357)
IL-15	0.088 (.296)	0.024 (.852)	−0.133 (.290)	0.040 (.754)	0.042 (.737)
IFN-α	0.136 (.107)	−0.085 (.501)	−0.032 (.803)	0.162 (.198)	−0.085 (.501)
IFN-γ	**−0.325 (<.001)**	−0.249 (.051)[a]	−0.021 (.869)	−0.158 (.219)	−0.092 (.479)
Anti-inflammatory					
IL-4	−0.077 (.360)	0.002 (.998)	0.003 (.980)	0.011 (.901)	0.031 (.833)
IL-10	**0.710 (<.001)**	0.037 (.768)	−0.167 (.185)	0.209 (.094)[a]	0.085 (.503)
IL-13	0.009 (.913)	0.082 (.515)	0.109 (.387)	0.077 (.540)	0.144 (.253)
IL-1RA	**0.345 (<.001)**	0.041 (.747)	−0.181 (.149)	0.065 (.610)	0.076 (.548)
Bifunctional					
IL-6	**0.508 (<.001)**	0.052 (.681)	−0.167 (.184)	0.058 (.647)	0.020 (.875)
TNF-α	**0.645 (<.001)**	−0.082 (.516)	−0.213 (.100)	0.148 (.241)	0.058 (.646)
Chemotactic/angiogenic					
MCP-1	0.155 (.065)[a]	−0.068 (.592)	−0.091 (.473)	0.209 (.095)[a]	0.160 (.204)
IP10	**0.455 (<.001)**	−0.175 (.163)	−0.188 (.134)	−0.070 (.582)	−0.081 (.521)
VEGF	**0.223 (.010)**	0.244 (.072)[a]	0.031 (.820)	0.203 (.138)	**0.252 (.044)**
sCD40L	**0.519 (<.001)**	0.140 (.267)	0.111 (.377)	0.031 (.805)	0.144 (.252)
IL-8	0.131 (.126)	**0.281 (.028)**	0.070 (.586)	0.071 (.589)	**0.469 (.001)**
Growth-stimulatory					
EGF	**0.446 (<.001)**	0.154 (.221)	0.080 (.527)	0.216 (.084)[a]	**0.261 (0.036)**
TGF-α	**0.234 (.005)**	0.221 (.076)[a]	0.029 (.817)	0.149 (.238)	**0.278 (0.025)**

Abbreviations: EGF, epidermal growth factor; γ_s, Spearman's correlation coefficient; IFN-α, interferon-α; IL-1α, interleukin 1α; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein-1; NK cells, natural killer cells; PDAC, pancreatic ductal adenocarcinoma; sCD40L, soluble CD40 ligand; sMICA, soluble MICA; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

Statistical analysis was performed by Spearman's correlation test.

$P < .05$ (2-tailed) was considered to be statistically significant.

Bold *P* values are statistically significant.

[a] This value shows a tendency to be correlated with indicated paramenters although it is not statistically significant.

M0 group, although it was not significantly different between the cancer and noncancer groups (Table 1). Serum levels of MCP-1 and VEGF demonstrated a tendency to be elevated in cancer groups compared with noncancer groups.

Serum levels of EGF and TGF-α, the major growth factors of PDAC,[12] were also significantly elevated along the PDAC development and progression process (Kruskal-Wallis, all $P < .05$; Table 1). They were significantly higher in the PDAC/M1 group compared to the PDAC/M0 group (all $P < .05$).

Soluble Mediators Correlated With the Development and Progression of PDAC

Next, we evaluated the correlations between tested soluble mediators and the development of PDAC. Among the tested soluble mediators, IL-2, IL-12p70, and IFN-γ (proinflammatory cytokines) showed strong negative correlations with the development of PDAC (Spearman's correlation, all $P < .05$; Table 2). On the other hand, IL-10 and IL-1RA (anti-inflammatory cytokines) as well as IL-6 and TNF-α (bifunctional proinflammatory cytokines) correlated positively with the development of PDAC (Spearman's correlation, all $P < .05$; Table 2). sMICA also correlated positively with the development of PDAC ($P < .05$). In addition, IP-10, VEGF, and sCD40L (chemotactic/proangiogenic cytokines) as well as EGF and TGF-α (growth-stimulatory cytokines) showed a strong positive correlation with the development of PDAC (Spearman's correlation, all $P < .05$).

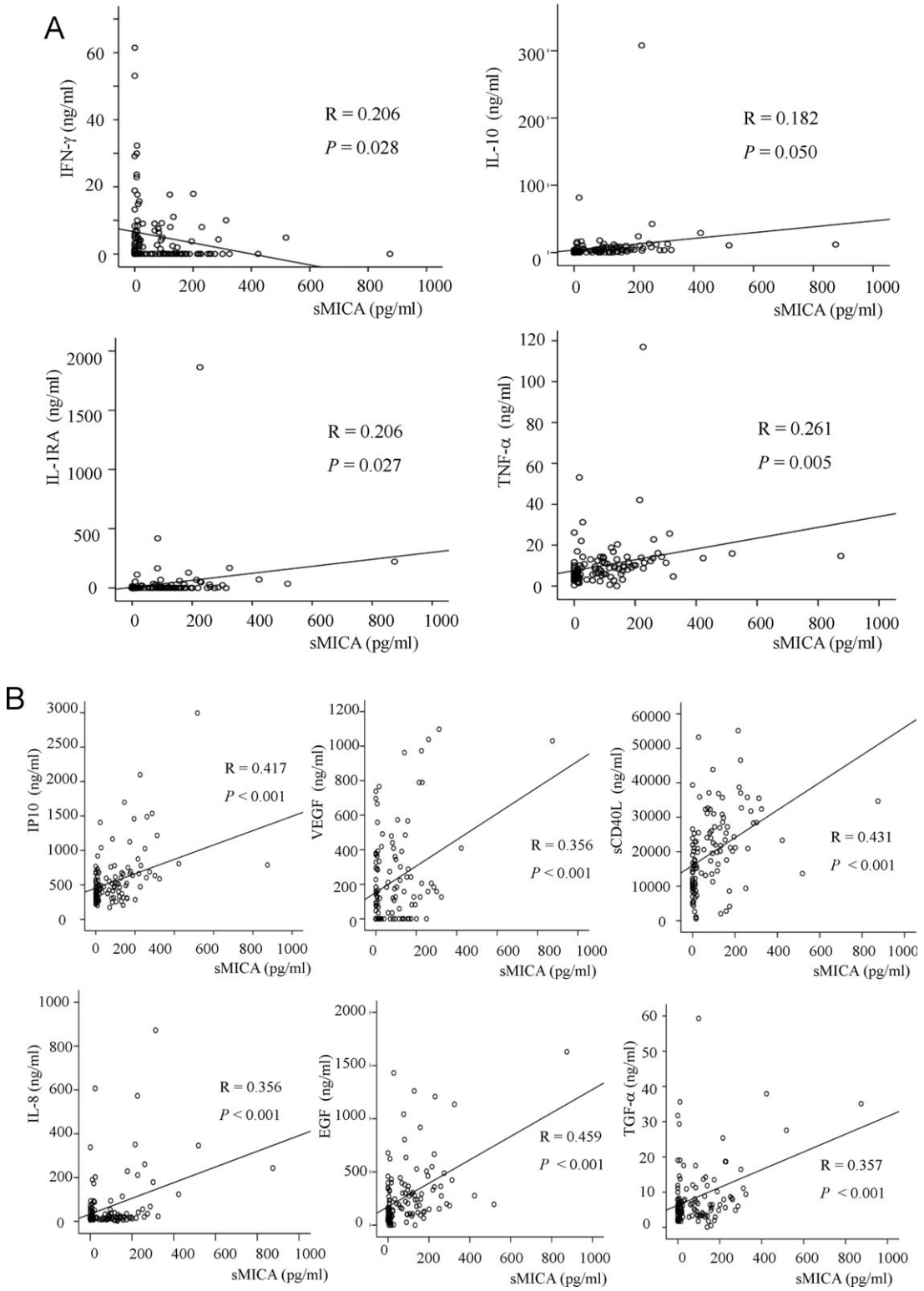


Figure 1. Linear regression graphs between soluble major histocompatibility complex class I chain-related molecule A (sMICA) and cytokines (epidermal growth factor [EGF], interleukin-8 [IL-8], interleukin-10 [IL-10], interleukin-1 receptor antagonist [IL-1RA], inducible protein-10 [IP10], soluble CD40 ligand [sCD40L], transforming growth factor-α [TGF-α], tumor necrosis factor-α [TNF-α], vascular endothelial growth factor [VEGF]) related to the development or progression of pancreatic ductal adenocarcinoma (PDAC) when serum samples were analyzed from all subjects—(A) immune-related, (B) proangiogenic or growth-stimulatory cytokines—and (C) from overt PDAC patients.

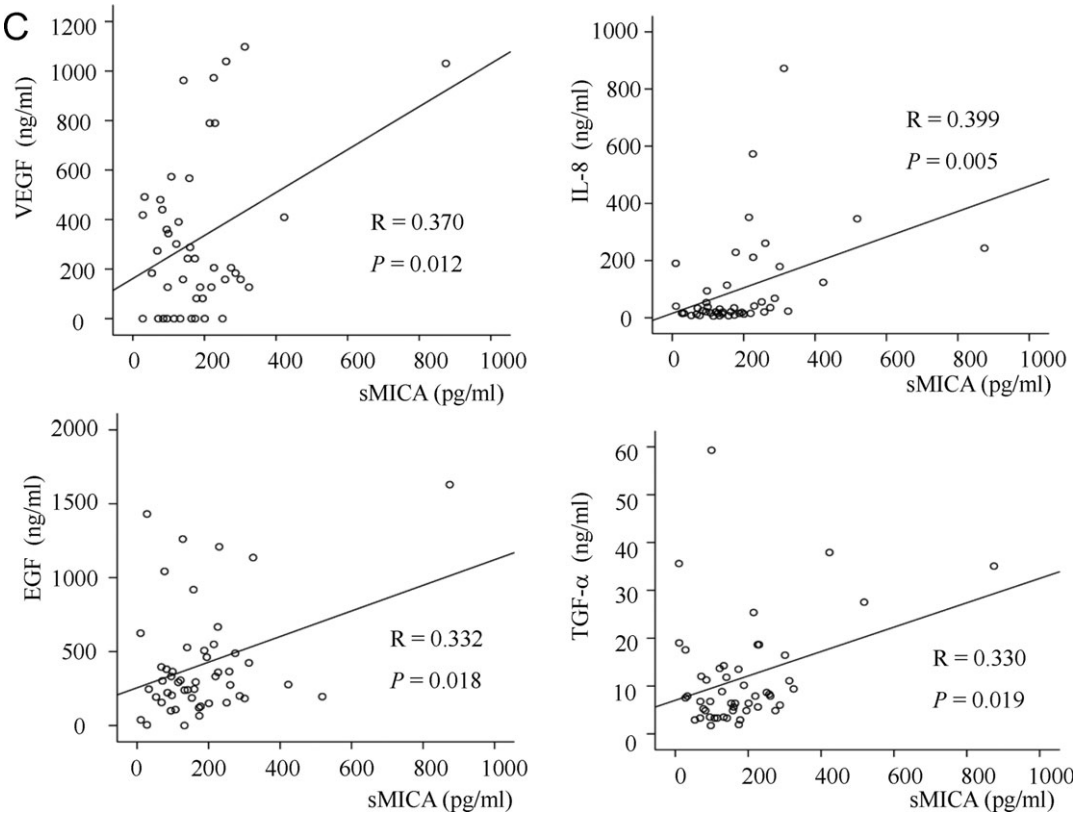


Figure 1. (Continued)

We also evaluated the correlations between serum levels of soluble mediators and pathological parameters of PDAC including size and TNM stage, reflecting the progression of PDAC. Most immune-associated cytokines including proinflammatory, anti-inflammatory, and bifunctional cytokines were not found to be involved in the progression of PDAC in this study; although IL-1α and IFN-γ demonstrated a tendency toward correlating with distant metastasis and tumor size, respectively (Table 2). On the other hand, VEGF and IL-8 (proangiogenic cytokines), EGF and TGF-α (growth-stimulatory cytokines), and sMICA showed meaningful correlations with the progression parameters of PDAC (Spearman's correlation, all $P < .05$; Table 2).

Correlations Between Serum sMICA and Cytokines in the Development and Progression of PDAC

We evaluated the correlations between sMICA and specific cytokines that were meaningful in the development of PDAC using serum samples from all subjects. The linear regression graphs showed that IFN-γ (proinflammatory) correlated with sMICA negatively, whereas IL-10 and IL-1RA (anti-inflammatory) as well as TNF-α (bifunctional) correlated with sMICA positively (Fig. 1A). IP-10, VEGF, sCD40L, and IL-8 (chemotactic/angiogenic cytokines) as well as EGF and TGF-α (growth-stimulatory cytokines) also correlated with sMICA positively (Fig. 1B). These results reflect that sMICA may have a relationship with these PDAC development-associated cytokines.

Next, we investigated cytokines related to sMICA in aspect of PDAC progression using serum samples from overt PDAC patients. No immune-associated cytokines correlated with sMICA with respect to the progression of PDAC. On the other hand, VEGF, IL-8, EGF, and TGF-α, which are proangiogenic or growth-stimulatory cytokines, were closely related with sMICA with respect to the progression of PDAC (Fig. 1C).

Taken together, these findings indicate that, among the 20 tested cytokines, sMICA correlated with 10 cytokines (IFN-γ, IL-10, IL-1RA, TNF-α, IP-10, VEGF, sCD40L, IL-8, EGF, and TGF-α) when all subjects were analyzed, which may reflect the development of PDAC, whereas sMICA was correlated with 4 cytokines (VEGF, IL-8, EGF,

Table 3. Diagnostic Accuracy of Combination Use of Serum sMICA and Specific Cytokines Related With Pancreatic Ductal Adenocarcinoma (PDAC) Development to Predict the Presence of PDAC

Variables	Sensitivity	Specificity
CA19-9	77.8%	76.9%
Single-marker panel		
sMICA	83.0%	80.0%
IL-10	87.0%	80.0%
TNF-α	83.1%	81.5%
sCD40L	75.3%	80.0%
IP-10	61.0%	81.5%
Two-marker panel		
sMICA, IL-10	84.8%	80.0%
sMICA, TNF-α	86.4%	80.0%
sMICA, sCD40L	92.4%	80.0%
sMICA, IP-10	87.9%	80.0%
Three-marker panel		
sMICA, IL-10, TNF-α	84.8%	80.0%
sMICA, IL-10, sCD40L	92.4%	80.0%
sMICA, IL-10, IP-10	87.9%	80.0%
sMICA, TNF-α, sCD40L	93.9%	80.0%
sMICA, TNF-α, IP-10	92.4%	80.0%
sMICA, sCD40L, IP-10	93.9%	80.0%
Four-marker panel		
sMICA, IL-10, TNF-α, sCD40L	90.9%	80.0%
sMICA, IL-10, TNF-α, IP-10	92.4%	80.0%
sMICA, TNF-α, sCD40L, IP-10	95.5%	80.0%
Five-marker panel		
sMICA, IL-2, IL-10, TNF-α, sCD40L	90.9%	80.0%

Abbreviations: CA19-9, carbohydrate antigen 19-9; EGF, epidermal growth factor; γ_s, Spearman's correlation coefficient; IFN-α, interferon-α; IL-1α, interleukin 1α; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein-1; PDAC, pancreatic ductal adenocarcinoma; sCD40L, soluble CD40 ligand; sMICA, soluble MICA; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.
For comparison among various-sized subpanels, the cut-point ensured a target specificity of around 80%.

Table 4. Diagnostic Accuracy of Combination Use of Serum sMICA and Specific Cytokines Related With PDAC Progression to Predict the Presence of Distant Metastasis in PDAC

Variables	Sensitivity	Specificity
CA19-9	70.3%	64.3%
Single-marker panel		
sMICA	78.6%	63.6%
VEGF	45.9%	66.7%
IL-8	70.3%	66.7%
EGF	54.1%	64.3%
TGF-α	59.5%	67.9%
Two-marker panel		
sMICA, VEGF	78.6%	70.6%
sMICA, IL-8	75.0%	68.4%
sMICA, EGF	67.0%	68.2%
sMICA, TGF-α	64.3%	68.2%
Three-marker panel		
sMICA, VEGF, IL-8	89.3%	85.7%
sMICA, VEGF, EGF	67.9%	82.4%
sMICA, VEGF, TGF-α	67.9%	82.4%
sMICA, IL-8, EGF	75.0%	84.2%
sMICA, IL-8, TGF-α	57.1%	84.2%
sMICA, EGF, TGF-α	53.6%	77.3%
Four-marker panel		
sMICA, VEGF, IL-8, EGF	82.1%	85.7%
sMICA, VEGF, IL-8, TGF-α	89.3%	85.7%
sMICA, IL-8, EGF, TGF-α	71.4%	84.2%
Five-marker panel		
sMICA, VEGF, IL-8, EGF, TGF-α	89.3%	85.7%

Abbreviations: CA19-9, carbohydrate antigen 19-9; EGF, epidermal growth factor; γ_s, Spearman's correlation coefficient; IFN-α, interferon-α; IL-1α, interleukin 1α; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein-1; PDAC, pancreatic ductal adenocarcinoma; sCD40L, soluble CD40 ligand; sMICA, soluble MICA; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

and TGF-α) when only PDAC patients were analyzed, which may reflect the progression of PDAC (Fig. 1).

Diagnostic Validity of Combined Use of Serum sMICA and Cytokines to Predict the Presence of PDAC and Its Distant Metastasis

Finally, we evaluated the diagnostic usefulness of serum sMICA and cytokines combination for PDAC, compared with CA19-9 (Tables 3, 4, and 5; Fig. 2). At first, we evaluated the diagnostic potential of serum sMICA and cytokines, which were meaningful in PDAC development, to predict the presence of PDAC as a single or multiple-marker panel using logistic regression analysis. ROC curves were generated for 5 selected soluble factors (sMICA, IL-10, TNF-α, sCD40L, and IP-10), which were related with PDAC development and whose AUC value was above that of CA19-9, for the prediction of PDAC (Fig. 2A). Logistic regression analysis demonstrated the 5 selected soluble factors showed superior or similar sensitivity/specificity for predicting the presence of PDAC as a single marker, compared to CA19-9. As multiple-marker panels, almost all combinations exhibited superior predictive utility for the presence of PDAC compared to CA19-9 (Table 3). In particular, the combination of sMICA, TNF-α, sCD40L, and IP-10 demonstrated the best predictability for the presence of PDAC (95.5% sensitivity at 80% specificity; Table 3).

We also evaluated the diagnostic potential of serum sMICA and cytokines, which were associated with distant metastasis in relation to sMICA, for predicting distant metastasis in PDAC. ROC curves and logistic regression results demonstrated these soluble mediators were not superior in diagnostic potential for predicting distant

Table 5. Diagnostic Accuracy of Combination Use of Serum sMICA and Specific Cytokines to Differentiate Chronic Pancreatitis and Pancreatic Ductal Adenocarcinoma

Variables	Sensitivity	Specificity
CA19-9	71.7%	76.2%
Single-marker panel		
sMICA	71.7%	78.3%
IL-10	96.1%	78.3%
TNF-α	88.2%	78.3%
IP-10	78.4%	78.3%
Two-marker panel		
sMICA, IL-10	97.9%	78.3%
sMICA, TNF-α	95.8%	78.3%
sMICA, IP-10	83.3%	78.3%
Three-marker panel		
sMICA, IL-10, TNF-α	97.9%	82.6%
sMICA, IL-10, IP-10	97.9%	78.3%
sMICA, TNF-α, IP-10	95.8%	78.3%
Four-marker panel		
sMICA, IL-10, TNF-α, IP-10	97.9%	87.0%

Abbreviations: CA19-9, carbohydrate antigen 19-9; EGF, epidermal growth factor; γ_s, Spearman's correlation coefficient; IFN-α, interferon-α; IL-1α, interleukin 1α; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein-1; PDAC, pancreatic ductal adenocarcinoma; sCD40L, soluble CD40 ligand; sMICA, soluble MICA; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.
For comparison among various-sized subpanels, the cut-point ensured a target specificity of around 80%.

metastasis in PDAC compared to CA19-9 (70.3% sensitivity at 64.3% specificity) as a single marker (Table 4; Fig. 2B). However, they showed superior predictive abilities as multiple-marker panels compared to CA19-9 in almost all cases (Table 4). Especially, the combination of sMICA, VEGF, and IL-8 exhibited up to 90% sensitivity/specificity for predicting distant metastasis in PDAC (89.3% sensitivity at 85.7% specificity; Table 4).

Because the differentiation between CP and PDAC is sometimes difficult in clinics, we evaluated whether combination use of sMICA and cytokines were useful for differentiating CP and PDAC. Logistic regression was performed using the 4 selected soluble factors (sMICA, IL-10, TNF-α, and IP-10), whose AUC value for differentiating CP and PDAC was above that of CA19-9 (Fig. 2C), and demonstrated superior sensitivity/specificity for differentiating between CP and PDAC compared to those of CA19-9 in almost all combinations (Table 5).

Validation of Diagnostic Usefulness of Combination Use of Serum sMICA and Cytokines Using Independent Validation Dataset

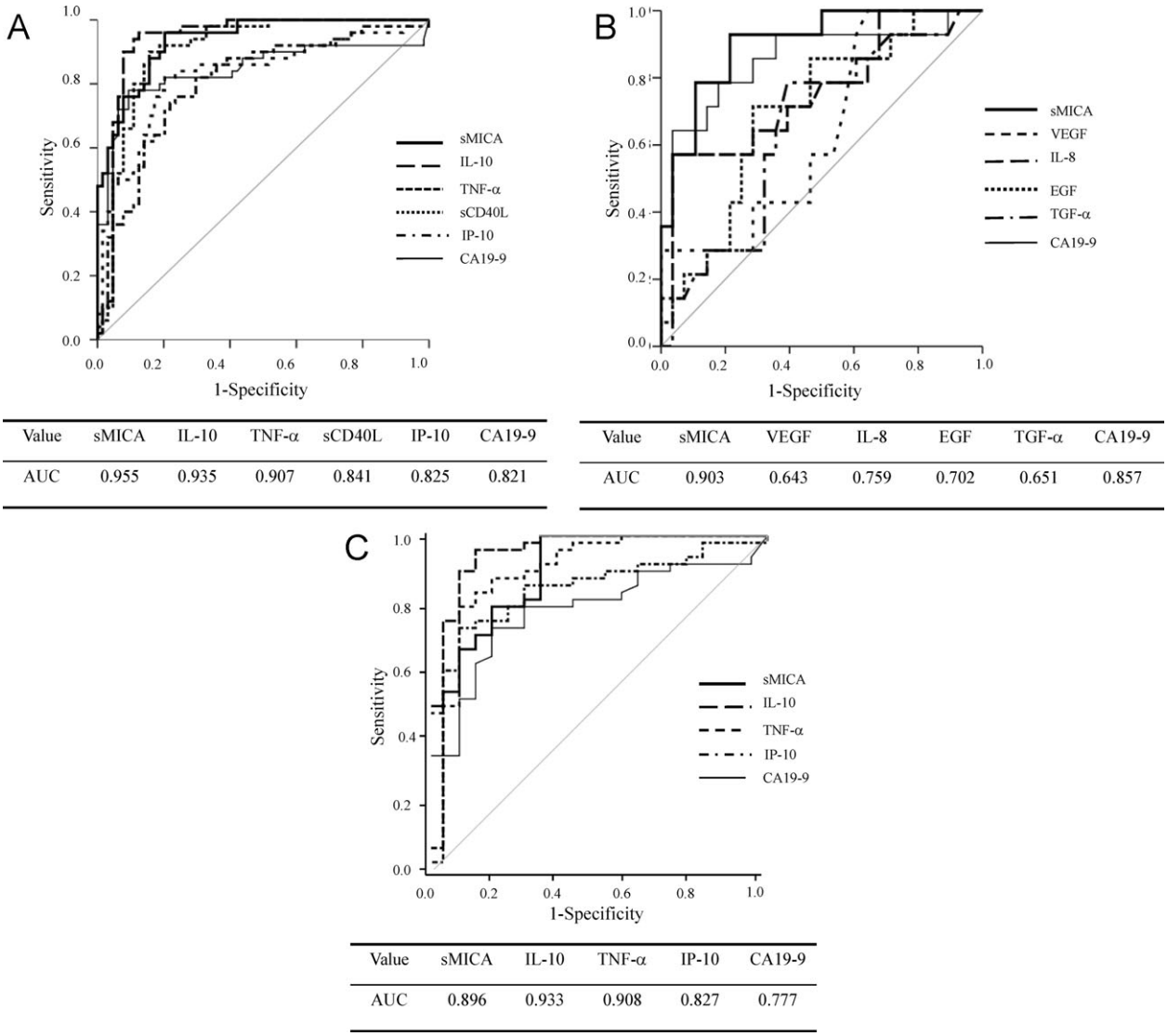
Overall expression patterns of sMICA and cytokines according to disease groups, and their correlations were similar to original results in validation dataset (data not shown). The diagnostic potential for the presence and distant metastasis of PDAC were also superior to CA19-9 in almost all cases similar to original results (Tables 6 and 7).

DISCUSSION

Here, we demonstrated that aberrantly expressed cytokines directed toward the development or progression of PDAC by impairing the anti-tumor immunity and promoting angiogenesis and tumor growth (Table 2). We also showed that sMICA, thought to affect the development and progression of PDAC through down-regulation of NK cell antitumor function, correlated with proangiogenic and growth-stimulatory cytokines as well as immunosuppressive cytokines (Fig. 1). These results imply sMICA is involved in tumor-associated angiogenesis and tumor growth stimulation either directly or indirectly by affecting corresponding cytokines as well as by causing impairment of NK cell cytotoxicity in the development and progression of PDAC.[7]

In our data, immune-associated cytokines were shown to be mainly involved in PDAC development rather than PDAC metastasis (Table 2), although we could not explain the reason for this in the present study. In regards to impairment of antitumor immunity, sMICA correlated with IFN-γ negatively (Fig. 1A). Because IFN-γ is predominantly produced by activated NK cells,[17] sMICA-induced down-regulation of NK cell function can induce reduction of IFN-γ, which may aggravate the impairment of antitumor immunity. Our results also suggest the probability that serum sMICA may cause an immunosuppressive state by increasing anti-inflammatory cytokines (IL-10 and IL-1RA), although how sMICA increases these cytokines cannot be explained in this study. It is well known that PDAC spontaneously secretes immunosuppressive substances such as IL-10.[3,10,11] sMICA may be correlated with these immunosuppressive substances under a certain mechanism, and finally induce a positive effect on PDAC development. TNF-α, the pluripotent proinflammatory cytokine, was positively associated with PDAC development in connection with sMICA in this study (Table 2; Fig. 1A) even though it is considered to have antitumor properties. TNF-α may act as a growth factor, as previous studies have suggested,[18] and sMICA may affect PDAC growth directly in connection with this cytokine.

IP-10 can act as a tumor growth factor,[19] although IP-10 is generally known as an anti-tumor cytokine. Thus, this cytokine may be associated with PDAC development, which is inconsistent with previous reports.



Value	sMICA	IL-10	TNF-α	sCD40L	IP-10	CA19-9
AUC	0.955	0.935	0.907	0.841	0.825	0.821

Value	sMICA	VEGF	IL-8	EGF	TGF-α	CA19-9
AUC	0.903	0.643	0.759	0.702	0.651	0.857

Value	sMICA	IL-10	TNF-α	IP-10	CA19-9
AUC	0.896	0.933	0.908	0.827	0.777

Figure 2. Receiver operator characteristic curves generated with sMICA and cytokines to predict the presence of (A) pancreatic ductal adenocarcinoma or (B) its distant metastasis, and to (C) differentiate chronic pancreatitis from pancreatic ductal adenocarcinoma. AUC indicates area under the curve; CA19-9, carbohydrate antigen 19-9; EGF, epidermal growth factor; IL-8, interleukin-8; IL-10, interleukin-10; IL-1RA, interleukin-1 receptor antagonist; IP10, inducible protein-10; sCD40L, soluble CD40 ligand; sMICA, soluble major histocompatibility complex class I chain-related molecule A; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

Because angiogenesis is a critical step for the initiation and progression of PDAC,[20] proangiogenic cytokines were expected to correlate with the development and/or progression of PDAC. As expected, these cytokines, including VEGF, sCD40L, and IL-8, correlated with the development and/or progression of PDAC (Table 2). sCD40L, which is known to promote angiogenesis through VEGF induction,[21] was expected to be associated with both the development and progression of PDAC. Actually, sCD40L was correlated with the development of PDAC (Fig. 1B), similar to a previous study on another cancer.[22] Unexpectedly, however, we did not observe a correlation with distant metastasis. IL-8, known to stimulate tumor growth directly or enhance angiogenesis in other cancers,[23] was expected to be associated with both the development and progression of PDAC. However, it did not correlate with the development of PDAC. Rather, it correlated with only the progression of PDAC in this study (Table 2). IL-8 was closely related with sMICA in both serum samples from all subjects and overt PDAC patients (Fig. 1B,C). VEGF correlated with both the development and progression of PDAC (Table 2), and it exhibited a

Table 6. Diagnostic Accuracy of Combination Use of Serum sMICA and Pancreatic Ductal Adenocarcinoma (PDAC)-Development–Related Cytokines to Prediction the Presence of PDAC in the Validation Dataset

Variables	Sensitivity	Specificity
CA19-9	73.1%	78.6%
Single-marker panel		
sMICA	88.5%	78.6%
IL-10	91.7%	84.6%
TNF-α	84.0%	78.6%
sCD40L	76.9%	78.6%
IP-10	80.8%	78.6%
Two-marker panel		
sMICA, IL-10	91.7%	84.6%
sMICA, TNF-α	88.0%	78.6%
sMICA, sCD40L	88.5%	78.6%
sMICA, IP-10	80.8%	78.6%
Three-marker panel		
sMICA, IL-10, TNF-α	95.7%	84.6%
sMICA, IL-10, sCD40L	91.7%	84.6%
sMICA, IL-10, IP-10	91.7%	84.6%
sMICA, TNF-α, sCD40L	96.0%	85.7%
sMICA, TNF-α, IP-10	84.0%	85.7%
sMICA, sCD40L, IP-10	88.5%	85.7%
Four-marker panel		
sMICA, IL-10, TNF-α, sCD40L	95.7%	84.6%
sMICA, IL-10, TNF-α, IP-10	95.7%	84.6%
sMICA, TNF-α, sCD40L, IP-10	96.0%	85.7%
Five-marker panel		
sMICA, IL-2, IL-10, TNF-α, sCD40L	95.7%	92.3%

Table 7. Diagnostic Accuracy of Combination Use of Serum sMICA and Specific Cytokines related With Pancreatic Ductal Adenocarcinoma (PDAC) Progression to Predict the Presence of Distant Metastasis of Pancreatic Ductal Adenocarcinoma in Validation Dataset

Variables	Sensitivity	Specificity
CA19-9	66.7%	71.4%
Single-marker panel		
sMICA	75.0%	71.4%
VEGF	66.7%	71.4%
IL-8	66.7%	71.4%
EGF	58.3%	71.4%
TGF-α	50.0%	71.4%
Two-marker panel		
sMICA, VEGF	75.0%	71.4%
sMICA, IL-8	72.7%	71.4%
sMICA, EGF	75.0%	71.4%
sMICA, TGF-α	75.0%	71.4%
Three-marker panel		
sMICA, VEGF, IL-8	81.8%	71.4%
sMICA, VEGF, EGF	75.0%	71.4%
sMICA, VEGF, TGF-α	75.0%	71.4%
sMICA, IL-8, EGF	81.8%	71.4%
sMICA, IL-8, TGF-α	81.8%	71.4%
sMICA, EGF, TGF-α	75.0%	71.4%
Four-marker panel		
sMICA, VEGF, IL-8, EGF	81.8%	71.4%
sMICA, VEGF, IL-8, TGF-α	81.8%	78.6%
sMICA, IL-8, EGF, TGF-α	81.8%	78.6%
Five-marker panel		
sMICA, VEGF, IL-8, EGF, TGF-α	81.8%	78.6%

Abbreviations: CA19-9, carbohydrate antigen 19-9; EGF, epidermal growth factor; γ_s, Spearman's correlation coefficient; IFN-α, interferon-α; IL-1α, interleukin 1α; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein-1; PDAC, pancreatic ductal adenocarcinoma; sCD40L, soluble CD40 ligand; sMICA, soluble MICA; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

strong correlation with sMICA in the development and progression of PDAC (Fig. 1B,C). These results imply that sMICA may be involved in tumor angiogenesis, thereby affecting the development and progression of PDAC.

EGF and TGF-α, which have mitogenic and minimal angiogenic activities in cancer and are important growth factors of PDAC,[12,24] were shown to play important roles in both the initiation and progression of PDAC (Table 2). We also observed that serum sMICA was closely correlated with these growth factors in terms of both the development and progression of PDAC (Fig. 1B,C). These results may imply sMICA can be involved in the direct stimulation of tumor growth by affecting growth factors of PDAC such as EGF and TGF-α in part in the development and progression of PDAC.

Because sMICA and cytokines are easily measurable in serum and they show a distinct expression pattern toward tumorigenesis and/or progression of PDAC, we assumed they can be available as useful biomarkers for predicting the presence or distant metastasis of PDAC. Previously, we verified serum sMICA as a desirable diagnostic biomarker for PDAC compared to CA19-9, a conventional tumor marker for PDAC.[14] Here, we also validated the diagnostic potential of the combination use of sMICA and specific cytokines to predict the existence of PDAC and its distant metastasis (Tables 3 and 4; Fig. 2A,B). Interestingly, we observed that combination use of sMICA and specific cytokines exhibited superior diagnostic potential for the presence and distant metastasis of PDAC compared to CA19-9 in almost all cases (Tables 3 and 4). It also exhibited superior sensitivity/specificity for differentiating between CP and PDAC compared to those of CA19-9 (Table 5).

In conclusion, we demonstrated that PDAC produces many soluble factors for suppressing anti-tumor immunity and promoting tumor initiation and metastasis.

sMICA, inducing the impairment of NK cell antitumor immunosurveillance, may be involved in angiogenesis and/or tumor growth either directly or indirectly through close relationships with corresponding cytokines that are engaged in these functions as well as down-regulation of antitumor immunity in PDAC. Finally, we confirmed the diagnostic usefulness of combination use of sMICA and specific cytokines for PDAC using an independent validation dataset (Tables 6 and 7). However, relative small sample size is still a limitation of this study. Thus, further large-scaled prospective study is needed. Nevertheless, we expect our results will provide valuable information to the field of cancer immunology for which to develop new soluble biomarkers or therapeutic approaches in PDAC.

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CONFLICT OF INTEREST DISCLOSURE

The authors made no disclosure.

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