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Development of a New Diagnostic Tool for Pancreatic Cancer: Simultaneous Measurement of Antibodies against Peptides Recognized by Cytotoxic T Lymphocytes

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Summary: One of the longstanding challenges in the treatment of pancreatic cancer, the fifth most common cancer worldwide, is to establish a simple and reliable diagnostic marker for the disease. This study examined whether or not the simultaneous measurement of plasma levels of IgG antibodies (IgGs) reactive to peptides recognized by cytotoxic T lymphocytes was useful for screening of pancreatic cancer. Sixty-three kinds of peptides were tested for their reactivity to plasma IgGs of pancreatic cancer patients with Luminex system followed by discriminatory analysis of the results using the Statistical Discovery Software. Under these circumstances, 83% of subjects with pancreatic cancer and 12% of healthy donors were diagnosed as having pancreatic cancer, respectively. These results suggest that the simultaneous measurement of IgGs reactive to these peptides could potentially be useful as a new diagnostic tool to screen for pancreatic cancer.

Key words pancreatic cancer, diagnosis, anti-peptide antibody, discriminatory analysis

INTRODUCTION

Development of new diagnostic tools has contributed to a remarkable improvement in the prognosis of many cancers, but few improvements have been achieved for the diagnosis of pancreatic cancer. Measurements of the serum levels of carcinoembryonic antigen (CEA) [1], sialylated Lewis blood group antigen CA19-9 [2], Du-PAN-2 [3], SPan-1 antigen [4], Mesothelin [5], prostate stem cell antigen [6] are not sufficient as reliable diagnostic markers for pancreatic cancer [7]. The failure to find an adequate marker may be in part due to insufficient understanding of the host-tumor interaction in pancreatic cancer. We reported a large number of cancer-associated antigens and the peptides that were recognized by cytotoxic T lymphocytes (CTLs) with the major histocompatibility antigen complex (MHC) class I antigen-restricted manner [8-11]. These antigens are highly expressed in pancreatic cancer cells. Further, we found that significant levels of IgGs specific to these peptides were detectable in the plasma of cancer patients, including pancreatic cancer patients [12,13]. In the present study, therefore, we examined whether or not measurement of plasma levels of IgG antibodies (IgGs) reactive to these peptides recognized by CTLs were useful for screening of pancreatic cancer.

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Abbreviations: CC, colon cancer; CEA, carcinoembryonic antigen; CTLs, cytotoxic T lymphocytes; DMSO, dimethyl sulfoxide; EDC, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; FIU, fluorescent intensity units; GC, gastric cancer; HDs, healthy donors; IgGs, IgG antibodies; MHC, major histocompatibility antigen complex; PC, pancreatic cancer; SD, standard deviation.

MATERIALS AND METHODS

Samples

The Institutional Ethical Committees of Kurume University School of Medicine, Kansai Medical School, and Yamaguchi University School of Medicine approved this study protocol. After informed written consent was obtained, the plasma samples were collected from 47 patients with stage 3 or 4 pancreatic cancer (n=6 and 42, respectively). Staging of pancreatic cancer was performed by Union International Contre le Cancer (UICC) classification of pancreatic cancer (6th edition, 2002). Plasma samples from patients with 15 patients with stage 1, 2, 3a, 3b, and 4 (n=1, 1, 2, 2, and 9, respectively) colon cancer, 32 patients with stage 2, 3a, 4 and recurrence (n=2, 2, 13, and 15 respectively) of gastric cancer, and 42 healthy donors (HDs) were also provided for the study. Staging of colon and gastric cancers were performed by UICC classification of colon cancer (5th edition, 1997) and Japanese classification of gastric carcinoma (he 13th edition), respectively.

Peptides

The list of the peptides used in this study is given in Table 1. All these peptides were derived from the cancer-associated antigens, and were recognized by the CTLs with MHC-class I-restricted manner as reported previously [9-13]. These peptides were purchased from Bio-Synthesis, Inc. (Lewisville, TX, USA). Each peptide was dissolved in dimethyl sulfoxide (DMSO), stored at −80°C, and diluted with saline just before use.

TABLE 1.
Peptides used in this study

Peptides with HLA-A24 binding motif		Peptides with HLA-A2 binding motif	
Name	Sequence	Name	Sequence
ART1-170:	EYCLKFTKL	CEA-233:	VLYGPDAPTI
ART4-13:	AFLRHAAL	CEA-379:	TLLSVTRNDV
CEA-390:	PYECGIQNEL	CEA-605:	YLSGANLNL
CEA-425:	TYYRPGVNL	CypB-129:	KLKHYGPGWV
CypB-91:	DFMIQGGDF	CypB-172:	VLEGMEVV
EGFR-124:	NYDANKTGL	EGFR-479:	KLFGTSGQKT
EGFR-54:	MFNNCEVVL	EGFR-1138:	YLNTVQPTCV
EGFR-800:	DYVREHKDNI	EIF-51:	RIIYDRKFL
EZH2-291:	KYDCCFLHPF	EZH2-120:	FMVEDETVL
EZH2-735:	KYVGIEREM	EZH2-569:	KQCPCYLAV
Her2-342:	CYGLGMEHL	Her2-444:	TLQGLGISWL
Her2-458:	LFRNPHQAL	Her2-484:	QLFRNPHQAL
Her2-553:	EYVNARHCL	HNR-140:	ALVEFEDVL
Lck-208:	HYTNASDGL	HNR-501:	NVLHFFNAPL
Lck-486:	TFDYLRSVL	Lck-246:	KLVERLGAA
Lck-488:	DYLRSVLEDF	Lck-422:	DVWSFGILL
MRP3-1293:	NYSVRYRPGL	MAP-294:	GLLFLHTRT
MRP3-503:	LYAWEPSFL	MAP-432:	DLLSHAFFA
PAP-213:	LYCESVHNF	PAP-112:	TLMSAMTNL
PSA-152:	CYASGWGSI	PSA-170:	KLQCVDLHV
PSA-248:	HYRKWIKDTI	PSA-178:	VISNDVCAQV
PSMA-624:	TYSVSFDSL	PSMA-4:	LLHETDSAV
PTHrP-102:	RYLTQETNKV	PSMA-441:	LLQERGVAYI
PTHrP-110:	KVETYKEQPL	PSMA-711:	ALFDIESKV
SART1-690:	EYRGFTQDF	PTHrP-42:	QLLHDKGKSI
SART2-161:	AYDFLYNYL	PTHrP-59:	FLHHLIAEI
SART2-899:	SYTRLFLIL	SART3-302:	LLQAEAPRL
SART2-93:	DYSARWNEI	SART3-309:	RLAEYQAYI
SART3-109:	VYDYNCHVDL	UBE-208:	ILPRKHHRI
SART3-315:	AYIDFEMKI	UBE-43:	RLQEWCSVI
		UBE-85:	LIADFLSGL
		WHS-103:	ASLDSDPWV
		WHS-141:	ILGELREKV

Preparation of xMAP beads

The xMAP carboxylate beads and Luminex system platform were obtained from Luminex Corp. (Austin, TX, USA) as reported previously [14]. The 96-well filter plates (MABVN12) and vacuum manifold apparatus (MAVM 09601) were from Millipore Corp. (Bedford, MA, USA). Biotinylated goat anti-human IgG (gamma chain-specific) (BA 3080) was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Streptavidin-PE (S-866) was purchased from Molecular Probes (Eugene, OR, USA). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, 22980) was obtained from PIERCE (Rockford, IL, USA). Peptides were coupled to xMAP beads according to the modified manufacturer's instructions as reported previously [14]. In brief, 100 μl of xMAP beads were washed with 0.1M MES buffer, pH 7.0, followed by mixing with 100 μl of peptide (1 mg/ml in 0.1M MES buffer, pH 7.0). The peptide-loaded beads were then incubated with EDC (1 mg/ml) at room temperature for 30 min in darkness, and then incubated twice more under the same conditions, and the beads were washed with 0.05% Tween 20-PBS. Finally, the beads were treated with 2-aminoethanol for 15 min at room temperature in darkness, then washed twice and re-suspended with 1 ml of 0.05% NaN_3 in Block-Ace.

Anti-peptide antibody measurement by flowmetry assay

Peptide-specific IgG levels in plasma were measured by flowmetry assay using the Luminex system as reported previously [14]. In brief, plasma was incubated with 170 μl of the peptides-coded beads for 2 hrs at room temperature in a 96-well filter plate on a plate shaker. After incubation, the plate was washed using a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (gamma chain-specific) for 1 hr at room temperature on a plate shaker. The plate was then washed, 100 μl of streptavidin-PE was added to the wells, and the plate was incubated for 40 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween 20-PBS into each well, and the plate was placed for 3 min on a plate shaker.

Statistics

The statistical significance of the data was determined using a two-tailed Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant. Discriminatory analysis with the Statistical Discovery Software (SAS Institute Inc., Cary, NC, USA) was employed for diagnosis of pancreatic cancer.

RESULTS

Antibodies against peptides

First, we used a serial dilution of samples to determine whether or not the levels of IgGs reactive to these peptides in the plasma of pancreatic cancer patients were dose-dependent. The level of antibody was given in fluorescent intensity units (FIU). The results showed that the levels of antibodies to a part of these peptides were detectable in the majority of samples with a dose-dependent manner according to the serial dilution of samples; representative positive cases of anti- SART3-109 and anti-EGFR-479 are shown in Fig. 1. In contrast, antibodies to some other peptides were under detectable levels in the samples (data not shown). Based on these results, 100-fold dilutions of samples were used for the assays in the following experiments.

Next, we investigated the levels of anti-peptide antibodies in the plasma of the following 4 groups: 47 pancreatic cancer patients, 15 colon cancer patients, 32 gastric cancer patients, and 42 healthy donors. Representative results of each group were given in Fig. 2. These results showed that the levels of IgGs against of these 63 peptides tested were different from each other both at the individual level and group level. When a Student-*t*-test was used for the statistical analysis, the levels of anti-SART3-109 were significantly ($P<0.05$ by a two-tailed Student's *t*-test) higher in the pancreatic patients than in the non-cancer subjects (Table 2). Furthermore, the levels of anti-EGFR-479 were significantly higher in the pancreatic patients than in both the non-cancer subjects and gastric cancer patients (Table 2). In addition, the levels of IgG reactive to as many as 26 of 63 peptides tested in the plasma of pancreatic cancer patients were significantly higher than those of gastric cancer patients.

Cumulative analysis

From a diagnostic point of view, the cut-off level of antibodies was set as the mean plus standard deviation (SD) of healthy donors, and if an antibody level was higher than the cut-off level, the patients was judged as being positive for pancreatic cancer. Under

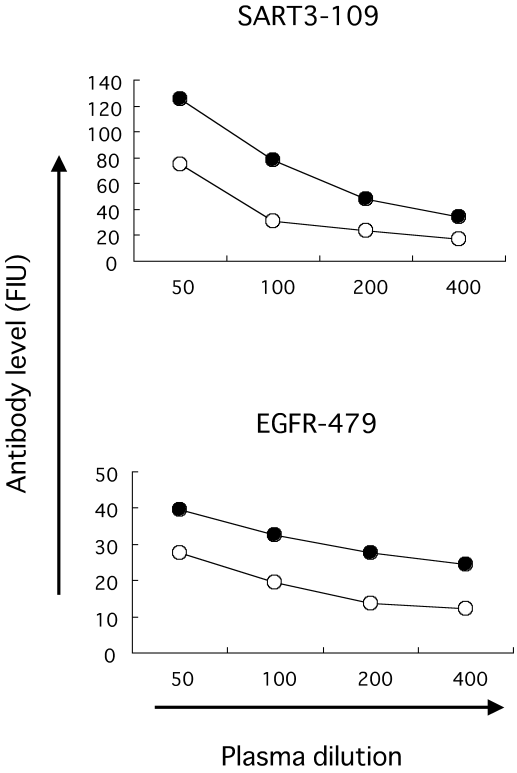

Fig. 1. Dose-dependency of IgGs reactive to certain peptides. The antibody levels in the plasma collected from cancer patients and healthy donors were measured by means of a multiplex beads suspension array using the Luminex system described in the Materials and Methods. A different dilution of samples was provided for the measurement. Values represent the fluorescence intensity units (FIU) of antigen-specific IgGs. Two cases, one with pancreatic cancer (●) and the other with healthy donor (○), are shown in the figure.

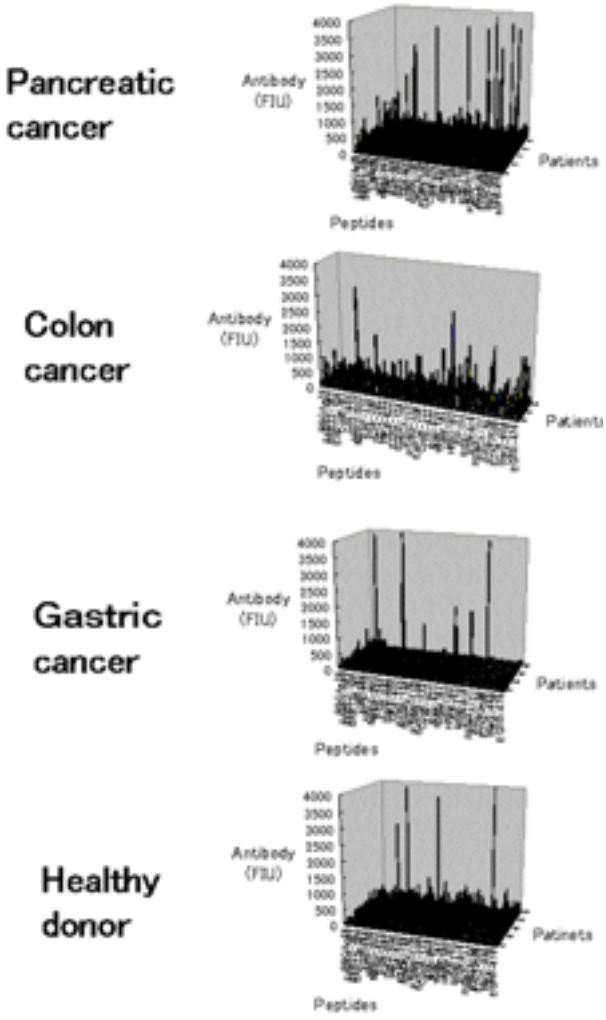

Fig. 2. Levels of anti-peptide antibodies in the four different groups. The levels of anti-peptide antibodies were measured using 100 times dilution of the plasma of the following 4 groups: 47 pancreatic cancer patients, 15 colon cancer patients, 32 gastric cancer patients, and 42 healthy donors. Representative results of each group were given in the figure.

these conditions, the positive cases were 20, 15, 9, 8, and 8 out of 47 pancreatic cancer patients when the antibody levels against SART3-109, EGFR-479, PAP-112, EGFR-54, and CEA-425 were used as biomarkers (Table 3). On the other hand, the false-positive cases were 9, 4, 4, 2, and 3 healthy donors, respectively.

We then conducted a cumulative analysis to determine the marker with the highest diagnostic value for pancreatic cancer. The results showed that 64% of subjects with pancreatic cancer patients and 31% of healthy donors were diagnosed as having pancreatic cancer, respectively, when a case showing significantly elevated levels of IgGs against at least one of the these 5 peptides was judged as positive for pancreatic cancer (Table 3). Under the same conditions, 80% of colon cancer patients and 47% of gastric cancer patients were diagnosed as having colon and gastric cancer, respectively.

Discriminatory analysis

We then conducted discriminatory analysis with the Statistical Discovery Software. Under these circumstances, 39 out of 47 (83%) of subjects with pancreatic cancer and 5 of 42 (12%) of healthy donors were diagnosed as having pancreatic cancer, respectively (Fig. 3). False negative cases were 8 cases (17%), and they were falsely diagnosed as gastric cancer (n=5), healthy donors (n=2), and colon cancer (n=1). On the other hand, 5 healthy donors were

TABLE 2.
Anti-peptide antibody in the plasma of pancreatic, colon, gastric cancer patients and those of the HD subjects

Peptides	Pancreatic Cancer mean (SD)	Colon Cancer mean (SD)	Gastric Cancer mean (SD)	Healthy Donor mean (SD)
SART3-109	361 (224)*	567 (778)	518 (1146)	168 (66)
EGFR-479	579 (1335)*, **	109 (113)	81 (64)	60 (55)
MRP3-503	12 (22)***	2 (35)	5 (11)	9 (17)
UBE-208	111 (217)**	21 (32)	22 (21)	29 (31)
PTHrP-42	34 (54)**	8 (7)	7 (8)	17 (19)
SART2-161	16 (24)**	4 (8)	3 (8)	11 (21)
MAP-432	29 (38)**	7 (4)	6 (9)	17 (21)
EGFR-54	82 (102)**	41 (44)	35 (21)	40 (26)
CEA-233	42 (69)**	11 (13)	9 (8)	13 (18)
CypB-91	59 (92)**	15 (17)	15 (13)	20 (17)
PSM-624	61 (98)**	10 (9)	13 (14)	16 (16)
EGFR-800	45 (73)**	12 (12)	10 (8)	15 (12)
CypB-129	112 (192)**	24 (19)	25 (23)	32 (31)
Lck-246	72 (116)**	26 (25)	22 (18)	21 (19)
WHS-103	77 (144)**	11 (10)	17 (24)	23 (24)
WHS-141	100 (191)**	14 (16)	16 (19)	22 (28)
EIF-51	176 (337)**	47 (24)	37 (38)	44 (51)
PTHrP-59	61 (114)**	8 (12)	11 (14)	19 (18)
CEA-605	169 (333)**	35 (39)	28 (32)	38 (48)
PTHrP-110	44 (65)**	10 (11)	11 (10)	16 (16)
SART2-93	80 (142)**	9 (7)	17 (25)	20 (24)
ART4-13	77 (161)**	9 (7)	11 (10)	18 (19)
Lck-488	84 (137)**	29 (18)	25 (24)	24 (35)
PSA-178	42 (62)**	10 (10)	9 (7)	13 (12)
PSMA-441	40 (65)**	10 (10)	9 (7)	12 (14)
PSMA-711	33 (54)**	6 (5)	8 (8)	14 (16)
SART3-315	45 (73)**	7 (6)	9 (10)	15 (17)
ART1-170	72 (136)**	15 (17)	17 (22)	15(17)
EZH2-291	21 (34)**	3 (7)	7 (15)	19 (24)
SART3-309	47 (98)	13 (18)	8 (7)	13 (12)
CEA-425	109 (190)	29 (30)	22 (24)	24 (30)
SART3-302	55 (90)	8 (8)	68 (271)	19 (17)
UBE-43	260 (640)	51 (56)	141 (412)	81 (154)
Her2-484	48 (83)	10 (6)	17 (20)	13 (14)
CEA-379	45 (83)	10 (11)	11 (11)	13 (15)
CypB-172	42 (77)	11 (10)	10 (10)	12 (12)
Lck-422	52 (58)	28 (22)	27 (29)	22 (15)
PAP-112	112 (300)	15 (20)	15 (17)	24 (21)
Lck-486	81 (155)	23 (29)	16 (15)	26 (33)
PAP-213	72 (126)	15 (12)	205 (1017)	54 (110)
Her2-458	253 (707)	15 (17)	18 (23)	24 (29)
Her2-553	95 (162)	29 (37)	25 (25)	29 (24)
CEA-390	60 (106)	13 (15)	13 (15)	17 (20)
EZH2-735	74 (145)	13 (13)	17 (20)	18 (19)
Her2-444	260 (725)	22 (27)	21 (22)	36 (54)
HNR-140	61 (107)	12 (15)	13 (15)	18 (19)
Lck-208	68 (141)	20 (23)	27 (34)	24 (25)
HNR-501	120 (273)	27 (38)	29 (42)	36 (50)
Her2-342	24 (57)	14 (35)	4 (8)	10 (12)
EGFR-124	73 (123)	14 (12)	31 (78)	72 (189)
PSMA-4	49 (119)	6 (9)	74 (370)	14 (17)
EZh2-569	42 (85)	12 (14)	9 (8)	13 (12)
PSA-152	85 (278)	14 (56)	16 (27)	24 (37)
SART2-899	0 (33)	0 (6)	0 (7)	0 (17)
PSA-248	105 (171)	21 (16)	138 (474)	324 (1059)
PTHrP-102	128 (253)	49 (47)	32 (29)	34 (45)
MRP3-1293	164 (291)	75 (94)	52 (51)	45 (47)
PSA-170	120 (247)	55 (100)	27 (31)	31 (35)
EGFR-1138	103 (181)	44 (51)	58 (59)	33 (29)
EZH2-120	74 (132)	64 (134)	30 (28)	24 (32)
MAP-294	335 (952)	81 (75)	75 (78)	42 (43)
UBE-85	10 (22)	6 (5)	7 (15)	11 (15)

Antibody levels of plasma in pancreatic cancer patients against each peptide were significantly ($P<0.05$) higher than those of *HD, **gastric cancer, and ***colon cancer patients by a Student's *t*-test

TABLE 3.
Cumulative analysis of antibody levels against 5-different peptides

Patients	Positive numbers (%) reactive to each peptide					Cumulative Positive numbers (%)
	SART3-109	EGFR-479	PAP-112	EGFR-54	CEA-425	
Pancreas Cancer	20 (42.6%)*	15 (31.9%)	9 (19.1%)	8 (17.0%)	8 (17.0%)	30 (63.8%)
Colon Cancer	11 (73.3%)	4 (26.7%)	3 (20.0%)	3 (20.0%)	2 (13.3%)	12 (80.0%)
Gastric Cancer	15 (46.9%)	1 (3.1%)	1 (3.1%)	0	1 (3.1%)	15 (46.9%)
HD	9 (21.4%)	4 (9.5%)	4 (9.5%)	2 (4.8%)	3 (7.1%)	13 (31.0%)

* The cut-off level of antibodies was set as the mean plus standard deviation (SD) of non-cancer subjects, and if an antibody level was higher than the cut-off level, the patients was judged as being positive for pancreatic cancer. Numbers of positive cases and positive % was shown in parenthesis.

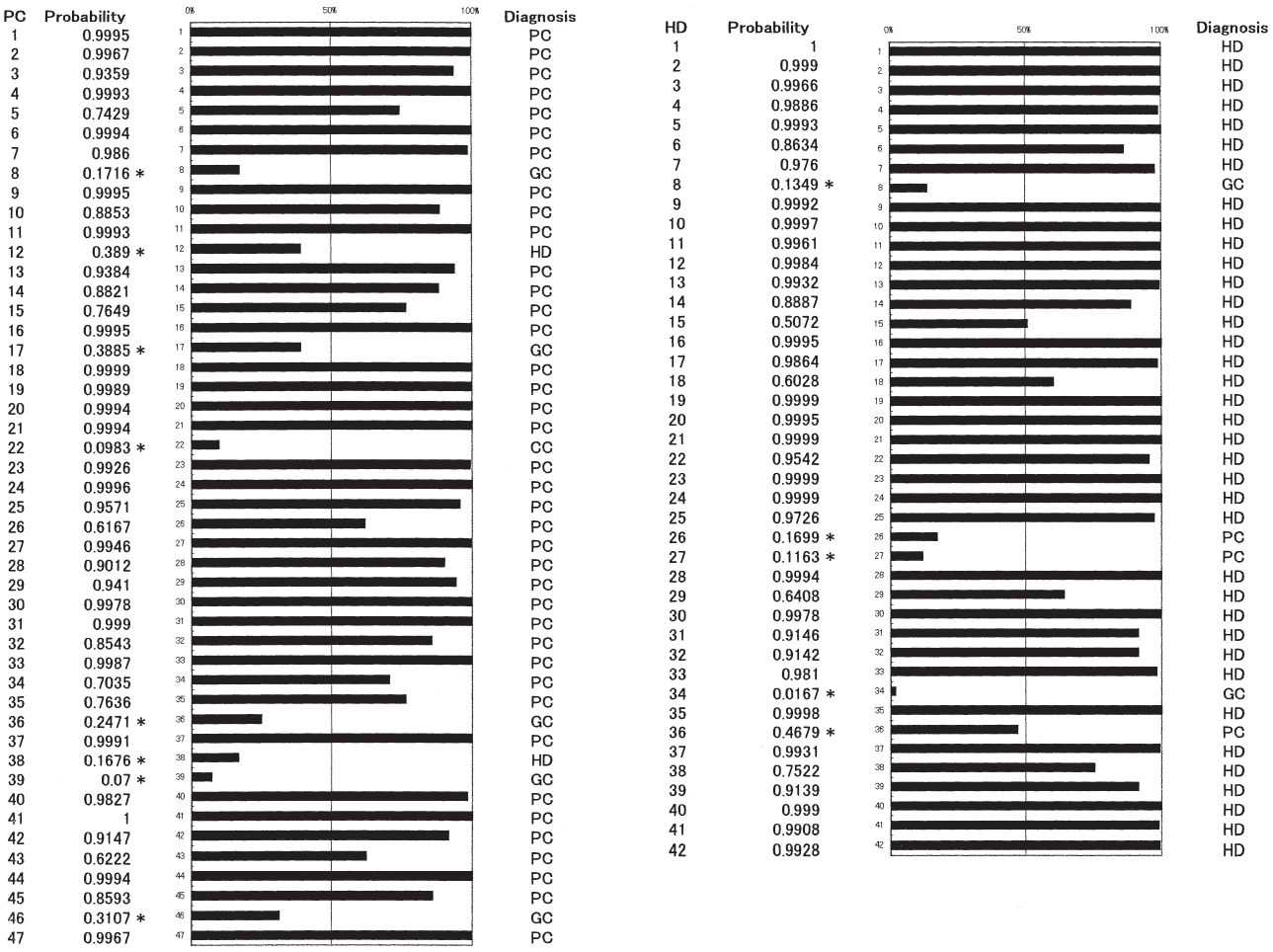

Fig. 3. Discriminatory analysis. Discriminatory analysis with the Statistical Discovery Software was carried out. Under these circumstances, 39 out of 47 (83%) of subjects with pancreatic cancer and 5 of 42 (12%) of non-cancer subjects were diagnosed as having pancreatic cancer, respectively. PC: pancreatic cancer, GC: gastric cancer, CC: colon cancer, HD: healthy donor.

TABLE 4.
*Summary of the result of the discriminatory analysis**

Patients	Diagnosis made by discriminating analysis			
	Pancreatic Cancer	Gastric Cancer	Colon Cancer	Healthy Donor
Pancreatic Cancer (n=47)	38	5	1	3
Gastric Cancer (n=32)	2	28	1	1
Colon Cancer (n=15)	1	2	11	1
Healthy Donor (n=42)	5	2	0	35

* The summary of the discriminatory analysis was shown. As a result, 38 of 47 patients (83%) with pancreatic cancer and 7 of 42 healthy donors (12%) were diagnosed as having pancreatic cancer, respectively. Under the same conditions, 73% of colon cancer patients or 88% of gastric cancer were diagnosed as having colon or gastric, respectively

falsely diagnosed as pancreatic cancer (n=3) or gastric cancer (n=2). The summary is given in Table 4. Under the same conditions, 11 of 15 (73%) of colon cancer patients or 27 of 32 (84%) of gastric cancer were diagnosed as having colon or gastric cancer, respectively.

DISCUSSION

We reported in this study that the levels of IgGs to SART3-109 and EGFR-479 peptides were elevated in the plasma of patients with relatively advanced stages of pancreatic cancer (stage 3 or 4) as compared to the levels in healthy donors. The higher levels of antibodies could be explained partly by the higher expression of SART3 and EGFR in pancreatic cancer cells, although the tumor samples were not available to measure the expression levels. Namely, the higher levels of SART3 and EGFR expression in pancreatic cancer cells could induce a much stronger immune responses, resulting higher levels of production of IgGs reactive to these peptides as compared to the regular levels of immune responses to these peptides in healthy donors.

Measurement of serum levels of CEA [1], sialylated Lewis blood group antigen CA19-9 [2], DuPAN-2 [3], or any other antigens available at the present time is not sufficient as a reliable diagnostic marker of pancreatic cancer [4-7]. However, their combined use significantly increases the rate of diagnosis of pancreatic cancer, though its level is still not satisfactory as reliable markers [7,15]. Like these markers, our results showing the elevation of IgG against certain peptides among the 63 peptides tested are not sufficient as reliable markers of pancreatic cancer even if cumulative analysis was employed.

We then conducted the discriminatory analysis, which is suggested to be superior to the other statistical analyses when a pattern of each group is different from each other, in addition to the difference at the level of an individual case as shown above [16]. Subsequently, our results showed that 83% of patients with pancreatic cancer and 12% with non-cancer subjects were diagnosed as having pancreatic cancer, respectively. These results encouraged us to further investigate the possibility to use this simultaneous measurement of IgGs as a new diagnostic tool for pancreatic cancer. Therefore, the results of this small scale study shall be confirmed by a large scale study. Furthermore, samples from pancreatic cancer patients in earlier stages should also be studied. Regardless of these limitations, the simultaneous measurement of IgGs reactive to these peptides could potentially be useful as a new diagnostic tool to screen for pancreatic cancer.

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