SPANDIDOS PUBLICATIONS

Increased levels of IgG antibodies against peptides of the prostate stem cell antigen in the plasma of pancreatic cancer patients

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Abstract. 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 plasma levels of IgG antibodies (IgGs) reactive to peptides derived from the prostate stem cell antigen (PSCA), which is highly expressed in pancreatic cancer cells, were elevated in patients with pancreatic cancer. Fifty-seven kinds of peptides encoded by PSCA were tested for their reactivity to plasma IgGs of pancreatic cancer patients. The results showed that the levels of IgGs specific to each of the 10 different peptides in the plasma of pancreatic cancer patients were significantly higher than those of non-cancer subjects. Eighty percent of subjects with and 18% of subjects without pancreatic cancer were diagnosed as having pancreatic cancer, respectively, when those cases showing significantly elevated levels of IgGs against at least one of the three peptides of PSCA at positions 2-11, 85-95, and 109-118 were judged as positive for pancreatic cancer. These results indicate that the measurement of IgGs reactive to these PSCA-derived peptides can provide novel information on the host-tumor interaction in pancreatic cancer, and could potentially be used as a new diagnostic tool to screen for pancreatic cancer.

Introduction

The 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 the carcinoembryonic antigen (CEA) (1), the sialylated Lewis blood group antigen CA19-9 (2), Du-PAN-2 (3,4), the SPan-1 antigen (5), or Mesothelin (6,7) are not sufficient as reliable diagnostic markers for pancreatic cancer (8). The failure to find an adequate marker could be in part due to our insufficient understanding of the host-tumor interaction in pancreatic cancer. Certain studies have reported several new tumor antigens that are highly expressed in pancreatic cancer cells. One of them is the prostate stem cell antigen (PSCA). PSCA is a glycosylphosphatidyl-inositol-linked cell surface antigen that is expressed in normal prostate cells, and is up-regulated in pancreatic cancer (9,10). In addition, we have reported a dozen pancreatic cancer-associated antigens that were recognized by the cellular immune system as a complex formed of a peptide and the major histocompatibility antigen complex (MHC) class I antigen (11). We have also reported that significant levels of IgGs specific to certain cancer-associated antigens were detectable in the plasma of pancreatic cancer patients prior to the vaccination (12-14). In the present study, therefore, we examined whether or not the plasma levels of IgG antibodies reactive to PSCA-derived peptides were elevated in pancreatic cancer patients, and found that IgGs reactive to 10 of the 57 kinds of PSCA-derived peptides tested were elevated in these patients.

Materials and methods

Samples. The Institutional Ethics Committees of Kurume University School of Medicine, Yamaguchi University School of Medicine, and Kansai Medical School approved this study protocol. After written informed consent was obtained, the plasma samples were collected. Plasma samples were collected from 40 patients with stage 3 or 4 pancreatic cancer, and 60 non-cancer subjects (16 patients with urolithiasis, 16 with IgA nephropathy, and 28 healthy donors). Plasma samples from patients with colon cancer (n=16), gastric cancer (n=16), and prostate cancer (n=40) were also provided for the study.

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Key words: pancreatic cancer, prostate stem cell antigen, antibody, diagnosis, peptide

Table I. Peptides encoded by PSCA used in this study.

Peptide	Sequence
PSCA: HLA-A2 and -A24	
PSCA 5-13	LLALLMAGL
PSCA 7-15	ALLMAGLAL
PSCA 43-51	QLGEQCWTA
PSCA 109-117	LLPALGLPAL
PSCA 76-84	DYYVGKKNI
PSCA 4-12	VLLALLMAG
PSCA 106-114	ILALLPALG
PSCA 21-29	LLCYSCKAQV
PSCA 70-79	QVDDSQDYYV
PSCA 108-117	ALLPALGLPA
PSCA 27-36	KAQVSNEDCL
PSCA: HLA-A3 supertype	
PSCA 74-82	SQDYYVGKK
PSCA 3-11	AVLLALLMA
PSCA 105-113	AILALLPAL
PSCA 108-116	ALLPALGLL
PSCA 46-54	EQCWTARIR
PSCA 44-52	LGEQCWTAR
PSCA 73-81	DSQDYYVGK
PSCA 82-91	KNITCCDTDL
PSCA 43-52	QLGEQCWTAR
PSCA 55-64	AVGLLTVISK
PSCA 52-61	RIRAVGLLTV
PSCA 42-51	TQLGEQCWTA
PSCA 18-27	GTALLCYSCK
PSCA 73-82	DSQDYYVGKK
PSCA 76-85	DYYVGKKNIT
PSCA: Overlap peptides	
PSCA 1-10	MKAVLLALLM
PSCA 5-14	LLALLMAGLO
PSCA 10-19	MAGLALQPGT
PSCA 11-20	AGLALQPGTA
PSCA 16-25	QPGTALLCYS
PSCA 21-30	LLCYSCKAQV
PSCA 26-35	CKAQVSNEDC
PSCA 31-40	SNEDCLQVEN
PSCA 36-45	LQVENCTQLG
PSCA 41-50	CTQLGEQCWT
PSCA 46-55	EQCWTARIRA
PSCA 51-60	ARIRAVGLLT
PSCA 56-65	VGLLTVISKG
PSCA 61-70	VISKGCSLNC
PSCA 66-75	CSLNCVDDSQ
PSCA 71-80	VDDSQDYYVG
PSCA 81-90	KKNITCCDTD
PSCA 86-95	CCDTDLCNAS
PSCA 91-100	LCNASGAHAL
PSCA 96-105	GAHALQPAAA
PSCA 101-110	QPAAAILALL
PSCA 106-115	ILALLPALGL
PSCA 111-120	PALGLLLWGP
PSCA 116-123	LLWGPGQL

Table I. Continued.

Peptide	Sequence
PSCA: Overlap peptides	
PSCA 2-11	KAVLLALLMA
PSCA 3-12	AVLLALLMAG
PSCA 4-13	VLLALLMAGL
PSCA 6-15	LALLMAGLAL
PSCA 7-16	ALLMAGLALQ
PSCA 8-17	LLMAGLALQP
PSCA 9-18	LMAGLALQPG

PSCA, prostate stem cell antigen. The numbers following PSCA represent the numeric order of the amino acids of the PSCA molecules.

Peptides. The list of PSCA-derived peptides used in this study is given in Table I. i) We prepared 26 kinds of peptides with HLA-A2, -A3 or -A24 binding motifs based on our previous observation that IgG antibodies specific to peptides with HLA-A2, -A3 or -A24 binding motifs were frequently found in the plasma of cancer patients, including patients with pancreatic cancer (12-16). ii) We prepared 24 kinds of deca-peptides covering the entire sequence of PSCA. Preliminary experiments indicated that the peptides containing the N-terminus portions of PSCA were reactive to the patients' plasma (data not shown). iii) We additionally prepared 7 kinds of peptides covering positions 1 to 20 of the N-terminal sequence. 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.

Preparation of xMAP beads. The xMAP carboxylate beads and Luminex® system platform were obtained from Luminex Corporation (Austin, TX, USA). The 96-well filter plates (MABVN12) and vacuum manifold apparatus (MAVM 09601) were from Millipore Corporation (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). The peptides were coupled with the xMAP beads according to the modified manufacturer's instructions as reported previously (13). In brief, 100 μl of the xMAP beads were washed with 0.1M MES buffer, pH 7.0, followed by mixing with 100 μl 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, then incubated twice more under the same conditions, and the beads were washed with 0.05% Tween-20 PBS (PBST). 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 0.05% NaN_3 in Block Ace.

Anti-peptide antibody measurement by flow cytometry assay. Peptide-specific IgG levels in the plasma were measured by

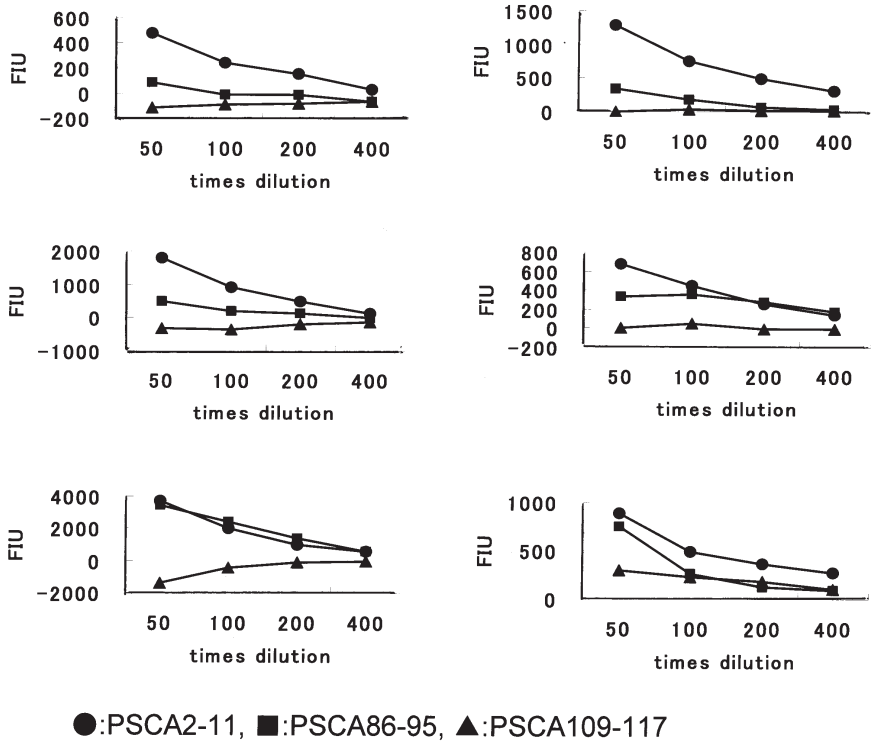

Figure 1. Dose-dependency of IgG antibodies (IgGs) reactive to prostate stem cell antigen (PSCA) peptides. The antibody levels in the plasma collected from 6 different pancreatic cancer patients were measured by means of a multiplex beads suspension array using the Luminex® system described in 'Materials and methods'. Values represent the fluorescence intensity units of the antigen-specific Igs.

flow cytometry assay using the Luminex® system as reported previously (13). In brief, the plasma was incubated with 170 μl of the peptide-coded beads for 2 h 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 biotinylated goat anti-human IgG (gamma chain-specific) for 1 h at room temperature on a plate shaker. The plate was then washed, 100 μl streptavidin-PE were 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 PBST into each well, and the plate was then placed for 3 min on a plate shaker.

Adsorption test of antibodies against peptides. For the preparation of the peptide immobilized ELISA plate for the antibody absorption test, the peptides were diluted in 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (Pierce), as reported previously (13). ELISA plates were coated overnight at 4˚C with the target peptides (20 μg/well). The wells were rinsed three times with 0.05% PBST. The plates were blocked overnight at 4˚C with Block Ace. To test the specificity of the anti-peptide IgG, 100 μl/well of plasma samples (1:1000 dilution with 0.05% PBST) were absorbed with the immobilized peptide (20 μg/well) in wells kept for 2 h at room temperature. The absorption was repeated three times, and then the level of the peptide-specific IgG in the resultant supernatant was measured.

Statistics. The statistical significance of the data was determined using the two-tailed Student's t-test. A P-value of <0.05 was considered statistically significant.

Results

Antibodies against PSCA peptides. First, we used a serial dilution of the samples to determine whether or not the levels of IgGs reactive to the PSCA peptides in the plasma of pancreatic cancer patients were dose-dependent. The level of the antibody was given in fluorescent intensity units (FIU). The results showed that the levels of antibodies to the PSCA-derived peptides at positions 2-11 (PSCA 2-11) and 86-95 (PSCA 86-95) in pancreatic cancer patients were gradually decreasing according to the serial dilution of the samples; representative cases are shown in Fig. 1. Although the level was relatively low, the antibody titers in non-cancer subjects also decreased in a dilution-dependent manner (data not shown). Based on these results, 100-fold dilutions of the samples were used for the assays in the following experiments.

Next, we investigated the levels of anti-peptide antibodies in the plasma of pancreatic cancer patients [n=40, (31 males and 9 females; mean age, 65 years)] and those of the age-matched healthy donors [n=29, (16 males and 13 females; mean age, 64 years)]. The results showed that the levels of IgGs against 15 of the 57 peptides tested 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 II).

We then provided the plasma from the different types of cancer, and investigated the levels of anti-peptide antibodies reactive to these 15 peptides in patients with pancreatic (n=40), prostate (n=38), colon (n=20), and gastric cancer (n=20). Plasma samples from subjects without cancer [n=60, (16 patients with urolithiasis, 16 with IgA nephropathy, and 28 healthy donors)] were used as the control. The levels of antibodies reactive to each of the 10 of the 15 peptides shown

Table II. Antibodies against PSCA-encoded peptides in pancreatic cancer patients and the age-mached non-cancerous controls.

	Pancreatic cancer patients		Non-cancerous control		
Peptides	Mean	SD	Mean	SD	P
PSCA 2-11	1516	964.5	813	438.5	0.0001
PSCA 3-11	412	220.8	160	139.4	0.0000
PSCA 3-12	424	239.5	192	146.1	0.0000
PSCA 8-17	504	367.2	226	144.4	0.0001
PSCA 18-27	402	362.7	223	192.9	0.0105
PSCA 27-36	428	518.3	215	251.5	0.0281
PSCA 43-51	533	796.2	240	349.0	0.0435
PSCA 44-52	999	1672.3	404	520.1	0.0398
PSCA 51-60	536	562.5	277	291.9	0.0155
PSCA 55-64	565	577.7	279	282.0	0.0086
PSCA 56-65	589	677.1	312	337.9	0.0297
PSCA 86-95	4323	3481.1	2115	2349.9	0.0025
PSCA 105-113	262	283.7	147	187.8	0.0472
PSCA 108-117	415	485.1	225	273.9	0.0431
PSCA 109-117	175	457.8	45	458.0	0.0417

PSCA, prostate stem cell antigen; SD, standard deviation. The IgG levels of antibodies against each of the peptides were significantly higher in the plasma of pancreatic cancer patients (n=40) than in those of the age-matched controls, including the healthy donors (n=29).

in Table II were significantly higher in the pancreatic than in the non-cancer subjects (Table III). Furthermore, the levels of antibodies to each of the 8, 4, or 2 of these 10 peptides in the plasma of colon, gastric, or prostate cancer patients were significantly higher than those of non-cancer subjects, respectively. In addition, the antibody levels to PSCA 86-95 in pancreatic or colon cancer patients were also significantly higher than those of prostate cancer patients, which in turn were higher than those of non-cancer subjects.

Lack of correlation between the level of CA19-9 and antibody levels against 10 peptides. The correlation between the level of CA19-9, one of the standard biomarkers for the diagnosis of pancreatic cancer, and the levels of antibodies against each of the 10 peptides shown in Table III was investigated in 20 pancreatic cancer patients whose CA19-9 values were available for the study. However, the serum levels of CA19-9 did not correlate with the levels of antibodies reactive to any of the 10 peptides in the plasma of these 20 pancreatic cancer patients (data not shown).

Cumulative analysis with antibodies against 3 different peptides. From a diagnostic point of view, the cut-off level of antibodies was set as the mean plus standard deviation (SD) of the non-cancer subjects, and if an antibody level was higher than the cut-off level, the patients were judged as being positive for pancreatic cancer. Under these conditions, the positive cases were 22, 4, 4, 9, 8, 7, 8, 21, 11, and 12 out of 40 pancreatic cancer patients when the antibody levels against PSCA 2-11, 3-11, 3-12, 18-27, 27-37, 44-52, 51-60, 86-95, 108-117, and 109-118 were used as biomarkers. On the contrary, the false-positive cases were 6, 3, 3, 4, 4, 4, 6, 4, and 3 out of 60 non-cancer subjects. We then conducted a cumulative analysis to determine the marker with the highest diagnostic value for pancreatic cancer. The results showed that 80% of subjects with and 18% of subjects without pancreatic cancer were diagnosed as having pancreatic cancer, respectively, when those cases showing significantly elevated levels of IgGs against at least one of the three peptides of PSCA at positions 2-11, 85-95, and 109-118 were judged as positive for pancreatic cancer. Under the same conditions, 80% of the colon cancer patients, 90% of gastric cancer, and 45% of the prostate cancer patients were diagnosed as having colon, gastric, and prostate cancer, respectively (Table IV).

Table III. Antibodies against PSCA-encoded peptide fragments in cancer and non-cancer subjects.

	Pancreas				Colon			Gastric				Prostate			Non-cancer	
Peptides	Mean	SD	vs NC	vs Prost	Mean	SD	vs NC	Mean	SD	vs NC	vs Prost	Mean	SD	vs NC	Mean	SD
PSCA 2-11	1516	964.5	0.0000		1515	778.0	0.0000	1786	659.2	0.0000	0.0182	1190	1238.8	0.0056	565	712.4
PSCA 3-11	412	220.8	0.0166		463	294.2	0.0142	438	225.8	0.0143		845	2756.8		220	542.5
PSCA 3-12	424	239.5	0.0130		474	380.9	0.0312	460	268.5	0.0131		676	2048.4		237	488.1
PSCA 18-27	402	362.7	0.0441		652	814.2	0.0430	366	411.3			966	3325.5		247	380.7
PSCA 27-36	428	518.3	0.0340		560	738.0		289	312.7			783	2757.7		210	448.6
PSCA 44-52	999	1672.3	0.0354		1545	2267.7	0.0365	509	625.4			1224	3419.8		389	746.9
PSCA 51-60	536	562.5	0.0064		765	926.3	0.0219	387	403.3			990	3180.8		233	468.3
PSCA 86-95	4323	3481.1	0.0000	0.0014	3113	1666.5	0.0000	3841	2212.5	0.0000	0.0081	2150	2202.7	0.0127	1075	1818.4
PSCA 108-117	934	1358.6	0.0052		1549	2206.3	0.0190	648	837.1			1072	3007.4		277	485.6
PSCA 109-118	124	463.6	0.0182		376	1067.9		-80	363.3			247	1718.5		-130	387.7

PSCA, prostate stem cell antigen; SD, standard deviation; Prost, prostate. Antibodies against each of the PSCA-encoded peptide fragments in colon (n=20), gastric (n=20), pancreatic (n=40), and prostate (n=40) cancers, and the same from non-cancer subjects (n=60), including those with urolithiasis (n=16) and IgA nephropathy (n=16) and healthy donors (n=28), were tested.

SPANDIDOS PUBLICATIONS Cumulative analysis of antibodies against different epitopes of PSCA molecules.

Patients	Number of patients	Positive cases[a] PSCA 2-11 (%)	PSCA 86-95 (%)	PSCA 109-118 (%)	Positive (%)
Pancreas	n=40	22 (55)	21 (52.5)	12 (30)	32 (80)[b]
Colon	n=20	14 (70)	11 (55)	5 (25)	16 (80)
Gastric	n=20	15 (75)	14 (70)	2 (10)	18 (90)
Prostate	n=40	14 (35)	8 (20)	6 (15)	19 (45)
Non-cancer	n=60	6 (10)	6 (10)	3 (11.1)	11 (18.3)[b]

PSCA, prostate stem cell antigen. [a]The cut-off level of antibodies was set as the mean plus standard deviation (SD) of the non-cancer subjects, and if an antibody level was higher than the cut-off level, the patients were judged as being positive for pancreatic cancer. [b]Eighty percent of subjects with and 18% of subjects without pancreatic cancer were diagnosed as having pancreatic cancer, respectively, when those cases showing significantly elevated levels of IgGs against at least one of the three peptides of PSCA at positions 2-11, 85-95, and 109-118 were judged as positive for pancreatic cancer.

Specificity of anti-peptide activities. The specificity of anti-peptide activities was investigated by absorption tests. Plasma samples from pancreatic patients were incubated with each of the 3 peptides shown in Table IV. The reactivity was largely reduced by absorption with the corresponding peptide, but not with the irrelevant peptides tested in the case of PSCA 2-11 and 86-95 (data not shown). In the cases of PSCA 2-11 and 109-118, however, the reactivity was not reduced by absorption with the corresponding peptide nor with the irrelevant peptides tested.

Discussion

We reported in this study that the levels of IgGs to 10 of the 57 peptides of PSCA were elevated in the plasma of patients with relatively advanced stages of pancreatic cancer (stage 3 or 4) relative to the levels in the non-cancerous controls. The higher levels of antibodies could be explained partly by the higher expression of PSCA in pancreatic cancer cells (9,10), although the tumor samples were not available to measure the expression levels of PSCA. Namely, the higher levels of PSCA expression in pancreatic cancer cells could induce a much stronger immune response to PSCA, resulting in higher levels of production of IgGs reactive to PSCA-derived peptides as compared to the regular levels of immune responses to these peptides in non-cancer subjects. Among these 10 peptides, 4 peptides (PSCA 2-11, 3-11, 3-12, and 8-17) shared the common 4 amino-acid sequence (LLMA), suggesting that this sequence could be one of the dominant epitopes.

The specificity of the anti-PSCA 86-95 activity was confirmed, whereas the broad reactivity of the anti-PSCA 2-11 or 109-118 activity was shown by means of an absorption test. This broad reactivity could be due to the shared B cell epitope between the PSCA 109-118 peptide and unidentified immunodominant epitopes, although the details are unclear at the present time.

One particular study revealed than PSCA-positive circulating tumor cells were found in the blood of patients with gastrointestinal cancers (17), which could be responsible for the higher levels of IgGs reactive to the PSCA-derived peptides in colon and gastric cancer patients. Namely, the plasma of colon and gastric cancer patients showed higher levels of IgGs against 8 and 4 peptides among the 10 peptides to which the plasma of pancreatic cancer patients also showed the higher levels as compared to those of the non-cancer subjects. In contrast, IgGs to only 2 of these 10 peptides were found to be elevated in prostate cancer patients, regardless of the fact that PSCA was expressed in the normal prostate with a much higher expression in prostate cancer cells (18). This discrepancy needs to be confirmed by a study of larger scale.

The measurement of the serum levels of the carcinoembryonic antigen (CEA) (1), the sialylated Lewis blood group antigen CA19-9 (2), Du-PAN-2 (3,4), and the SPan-1 antigen (5), or Mesothelin (6,7) are not sufficient as reliable diagnostic markers for pancreatic cancer when used individually (8). However, their combined use significantly increases the rate of diagnosis for pancreatic cancer (8,19).

In this study, we found no correlation between the levels of CA19-9 and those of anti-PSCA-derived peptides. Therefore, the combined measurement of CA19-9 and antibody levels to the PSCA peptides shown in this study could increase the diagnostic rate for pancreatic cancer. This issue, however, needs to be confirmed by a study of larger scale. In addition, the correlation of markers other than CA19-9 and the antibody levels to these 3 PSCA peptides should also be studied.

Our results showed that 80% of subjects with and 18% of subjects without pancreatic cancer were diagnosed as having pancreatic cancer, respectively, when those cases showing significantly elevated levels of IgGs against at least one of the three peptides of PSCA at positions 2-11, 85-95, and 109-118 were judged as positive for pancreatic cancer. Some of the false-negative cases could have depressed the immune responses to PSCA. Alternatively, the tumor samples of the false-negative cases expressed no or low levels of PSCA, although the tumor samples from the patients were not measured in this study. False-positive cases can show stronger immune responses either to self-antigens in a non-specific manner or to non-self antigens that share the epitope homology with the three PSCA peptides.

A large scale study is needed to confirm the elevation of IgGs to PSCA-derived peptides in the plasma of pancreatic cancer patients. Furthermore, samples from pancreatic cancer

patients in earlier stages should also be studied. Regardless of these limitations, the measurement of IgGs reactive to these PSCA-derived peptides could provide novel information on the host-tumor interaction in pancreatic cancer, and could potentially be used as a new diagnostic tool to screen for pancreatic cancer.

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