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Verification of the effectiveness of fucosylated haptoglobin as a pancreatic cancer marker in clinical diagnosis

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ABSTRACT

Background: Fucosylated haptoglobin detected by *Pholiota squarrosa* lectin (PhoSL) that had specificity for fucose α1-6 was reported as an effective biomarker for several gastrointestinal diseases. The aim of this study was to verify Fucosylated haptoglobin detected by *Pholiota squarrosa* lectin (PhoSL-HP) as a pancreatic cancer (PC) marker using a new method of PhoSL-ELISA.
Methods: PhoSL-HP in sera from 98 PC patients and 158 non-PC samples including 32 intraductal papillary mucinous neoplasm (IPMN) patients, 21 chronic pancreatitis (CP) patients and 105 non-pancreatic disease controls (NPDC) were measured. We compared sensitivities, specificities and areas under the curves (AUC) of PhoSL-HP, CA19-9 and CEA as single markers. We also evaluated PhoSL-HP as combination marker by comparing AUC of CA19-9 combined with PhoSL-HP or CEA.
Results: The sensitivities of PhoSL-HP, CA19-9 and CEA for PC were 58%, 76% and 42%, respectively. Although the specificity of PhoSL-HP for NPDC was inferior to both of CA19-9 and CEA, that for pancreatic diseases was higher than both of CA19-9 and CEA. Combined CA19-9 with PhoSL-HP, the AUC was significantly higher at 0.880 than single use of CA19-9 at 0.825 in case of distinguishing PC from other pancreatic diseases. In contrast, the AUC of CA19-9 was not elevated significantly when combined with CEA.
Conclusion: PhoSL-HP would be a useful marker for PC and have sufficient complementarity for CA19-9.

Introduction

Pancreatic cancer (PC) is one of the most lethal forms of cancer, with an overall 5-year survival rate of approximately 5% [1]. Subjective symptoms of PC in early stages are so poor that the detection of PC is very difficult [2]. As the typical symptoms such as jaundice and lumbar backache are mainly recognized at the late stage of PC, it is very difficult to appropriately treat PC with surgery or chemotherapy [3]. Under such circumstances patients with suspected cancer are screened by tumor markers, ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI) and diagnosed as PC by either of endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNAB) or cytology/biopsy by endoscopic retrograde cholangiopancreatography (ERCP) [4,5]. The sensitivities for detecting PC and diagnostic accuracies of pathological analyses represented by EUS-FNAB and ERCP-related procedures are very reliable. It was reported that the accuracy of EUS-FNAB was more than 90% in pancreatic masses smaller than 10 mm and that the results of ERCP-related procedures showed a sensitivity of 100%, a specificity of 83.3%, and an accuracy of 95% in a fine surveillance system [4,6]. Therefore, if new tumor marker can

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detect patients suspected of PC more accurately than existing markers, diagnosis and treatment efficiency of PC are thought to be improved. Carbohydrate antigen 19–9 (CA19-9) is mostly used as the marker for detecting PC, however the true positive rate is insufficient [7]. And it is well-known that carcinoembryonic antigen (CEA) is commonly used with the combination of CA19-9 but false positives are often obtained in case with combination use [8]. Furthermore, both CA19-9 and CEA often elevate in not only patients with PC but also benign diseases of pancreas represented by chronic pancreatitis (CP) and intraductal papillary mucinous neoplasm (IPMN) [9–13] and it is difficult to discriminate PC from such benign diseases of pancreas.

Fucosylation, the addition of fucoses to N-linked or O-linked glycan of proteins can occur through multiple types of glycosidic linkages such as α1-3, α1-4 and α1-6. Some types of fucosylation have been involved in cancer and inflammation [14]. Accordingly, it is thought that fucosylations of proteins are considered as potential markers of cancer and inflammatory diseases.

It was reported that fucosylated haptoglobin was increased in the serum of patients with PC [15–17]. Fucosylated haptoglobin was evaluated as the biomarker for PC using *Aleuria aurantia* lectin (AAL) [18] that bound for α1-3, α1-4 and α1-6 fucose residues. As the results, AAL reactive haptoglobin (AAL-HP) was significantly increased in patients with PC [19,20] and tended to be increased in patients with CP [20]. *Pholiota squarrosa* lectin (PhoSL) [21] that had high specificity for α1-6 fucose (core fucose) was also evaluated for detecting fucosylated haptoglobin. It was reported that PhoSL reactive haptoglobin (PhoSL-HP) was more effective in CP diagnosis than in PC [22]. However, we improved the sensitivity of PhoSL-HP detection method significantly by selection of immobilized mouse monoclonal antibody and using high concentration urea as denaturing reagent of fucosylated haptoglobin, and then reevaluated PhoSL-HP as PC marker. Urea does not affect the specificity of PhoSL and enhances only the sensitivity, so the concentration of α1-6 fucose in the sample is reliably reflected in the measurement results. Consequently PhoSL-HP levels of PC measured by improved method were higher than those of CP, colorectal cancer, hepatocellular carcinoma and cholangiocarcinoma, and it was suggested that PhoSL-HP had sufficient specificity for PC [23].

In this study, we evaluated the usefulness of PhoSL-HP in clinical diagnosis as a marker that contributed to improve diagnostic accuracy of pancreatic cancer. We focused on the discrimination performance of PC and other pancreatic diseases such as chronic pancreatitis and IPMN. Accordingly, the ability of PhoSL-HP for discrimination of pancreatic cancer from other pancreatic diseases and the complementarity of PhoSL-HP for CA19-9 were evaluated.

Materials and methods

Ethics statement

This study on human subjects was approved by institutional review boards of Hokkaido University Hospital (Clinical Research approval number 013–0012, 016–0145), Yokosuka mutual aid hospital and Ishikawacho medical clinic. Written informed consent was obtained from each participant. All experiments were done in accordance with the ethical guideline of the 2008 Declaration of Helsinki.

Serum samples

Serum samples were collected at medical institutions involving a total of 151 patients which were patients with PC, IPMN and CP and 105 non-pancreatic disease controls (NPDC). The presences of pancreatic cancers were confirmed through cytodiagnosis by ERCP or tissue biopsy by EUS-FNA in all PC patients. The absences of cancer among the 32 IPMN patients and 21 CP patients were confirmed by any of diagnostic imaging methods such as ultrasonography, CT, MRI, EUS and ERCP. The patients having both of PC and CP were classified as PC patients. The patients with invasive carcinoma derived from IPMN were also classified as PC patients. The pancreatic cancers were staged according to the 6th edition of the General Rules for the Study of Pancreatic Cancer by the Japan Pancreas Society: stage I, invasive carcinoma with tumor diameter of <20 mm (T1) confined within the pancreas, along with the absence of regional lymph node metastasis (N0) and distant metastasis (M0); stage II, invasive carcinoma with tumor diameter of >20 mm confined within the pancreas (T2)/T1 with regional lymph node metastasis (N1); stage III, T2 with N1/invasive carcinoma with invasion to the bile duct/duodenum/adjacent connective tissue; stage IVa, invasive carcinoma with invasion to the superior mesenteric/common hepatic artery/celiac trunk/portal vein system/neural plexus; stage IVb, invasive carcinoma with distant metastasis. For patients with IPMN and CP, they were diagnosed in accordance with International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas and the revised Japanese clinical diagnostic criteria 2010 for chronic pancreatitis, respectively. NPDC were examinees with no cancer, no pancreatic disease and no other serious underlying diseases confirmed by tumor marker tests and imaging tests including any of ultrasonography, CT and MRI at Hokkaido University Hospital, Yokosuka mutual aid hospital and Ishikawacho medical clinic from 2013 to 2016. This study was comprised of NPDC (n = 105), and patients with PC (n = 98), IPMN (n = 32) and CP (n = 21).

All procedures including blood sampling and serum separation by centrifugation were carried out in accordance with standard operating procedures of each medical institution. And all serum samples were stored at less than −20 °C until use.

Measurement of serum PhoSL-HP

Fucosylated haptoglobin was purified from the culture medium of human hepatocellular carcinoma cells, HepG2 (RIKEN Bio Resource Center, Ibaraki, Japan) stimulated by recombinant human interleukin 6 (IL-6; Wako Pure Chemical Industries, Osaka, Japan). The medium was added to anti-haptoglobin antibodies (Medical & Biological Laboratories Co., Nagoya, Japan) immobilized column, and fucosylated haptoglobin was isolated. The concentration of purified fucosylated haptoglobin was measured according to a known haptoglobin standard (ERM-DA470; Sigma-Aldrich).

PhoSL-HP ELISA was performed to quantitatively determine the serum core-fucosylated haptoglobin levels. Purified fucosylated haptoglobin from HepG2 medium (0–100 mU/mL, 50 μL), or 1:500 diluted serum was placed into each well of microtiter plate that was coated with 12 μg/mL mouse monoclonal anti-haptoglobin antibody (J-Oil Mills, Tokyo, Japan) digested with PNGase-F. The plate was incubated for 1 h at room temperature and then washed three times with PBS-T. For the measurement of serum fucosylated haptoglobin, biotinylated PhoSL diluted with 5 M urea with 0.1% Blockace (Megmilk Snow Brand Co., Ltd., Tokyo, Japan) and 1% polyethylene glycol 200 in distilled water were added to each well, and the plate was then incubated for 30 min at 4 °C. After the plate was washed three times, streptavidin and TMB reactions were performed. Purified fucosylated haptoglobin (0–100 mU/mL, 50 μL) was used as a calibration standard for the measuring PhoSL-HP. The concentrations of serum PhoSL-HP were calculated using standard curves. In order to confirm that addition of urea does not inhibit antigen-antibody reaction, standard haptoglobin from HepG2 medium was detected by adding rabbit polyclonal anti-haptoglobin antibody instead of PhoSL together with 5 M urea.

Measurement of serum CA19-9 and CEA

CA19-9 and CEA of all serum samples from NPDC and the patients were measured using commercially available kits, E test TOSOH II CA19-9 and E test TOSOH II CEA (TOSOH, Tokyo, Japan).

Statistical analysis

The difference in age between disease groups was tested with ANOVA followed by Tukey's test. And the differences in gender and smoking ratio were tested by Ryan's method. The Steel-Dwass test was performed to identify statistically significant differences between groups. The correlations between the markers were estimated based on Spearman's correlation coefficient. The diagnostic performance of the scoring systems was assessed by analyzing receiver operating characteristic (ROC) curves. Delong's test [24] was performed to identify statistically significant differences of area under the curves (AUC) between CA19-9 as a single marker and the combination markers of CA19-9 with PhoSL-HP or CA19-9 with CEA. These tests were performed using R statistic software (https://www.r-project.org/).

Results

Patient characteristics

A total of 105 NPDC and 151 patients including 98 patients with PC, 32 patients with IPMN and 21 patients with CP were tested in this study. The 105 NPDC samples were examinees with no cancer and no severe pancreatic disease confirmed by work-up. Table 1 showed their characteristic data. The mean ages were matched among each group of the patients and NPDC. With regard to 98 PC patients, the mean age was 67.3 years (range: 44–80 years) and male/female ratio was 46/52. The stages were determined as I (n = 1), II (n = 2), III (n = 10), IVa (n = 34) and IVb (n = 51) based on the General Rules for the Study of Pancreatic Cancer by the Japan Pancreas Society as described above. The most of PC patients were in late-stage of the disease.

Confirmation of PhoSL-HP quantitative assay

It was confirmed whether adding 5 M urea, a protein denaturing agent, to the antigen-antibody reaction affects the quantitative assay of PhoSL-HP. As a result of sandwich ELISA using two anti-HP antibodies, it was confirmed that 5 M urea does not inhibit reaction between haptoglobin and mouse monoclonal anti-haptoglobin antibody immobilized on the plate. As the result of measuring 3 lots of standard PhoSL-HP, it was confirmed that the standard deviation of the calibration curve was sufficiently low (Fig. 1A). In addition, it was confirmed that there was almost no change as the result of measuring PhoSL-HP of a sample which was stored at −80 °C. for 1 year after blood collection and calculating the ratios of PhoSL-HP (Fig. 1B). Samples showing 110% or more or 90% or less do not affect the sensitivity and specificity because they are samples with extremely low PhoSL-HP.

Measurement of markers including PhoSL-HP

Serum PhoSL-HP levels were significantly higher in the patients with PC (median: 6.2 U/mL) than in NPDC (median: 3.5 U/mL) and in the patients with IPMN (median: 3.9 U/mL) and CP (median: 3.9 U/mL) as shown in Fig. 2A. The differences between NPDC and the patients with IPMN and CP were not significant. These results indicated that PhoSL-HP was useful in clinical diagnosis because the patients with PC were clearly discriminated from the patients with pancreatic diseases including IPMN and CP. Compared to NPDC and the patients with IPMN and CP, serum CA19-9 was also significantly higher in the patients with PC (Fig. 2B). Serum CEA levels were also higher in the patients with PC than in NPDC and the patients with IPMN, however, the difference between PC and CP was not significant (Fig. 2C).

ROC analysis and evaluation as a single marker

In ROC analysis, three groups were set up to verify the clinical usefulness of PhoSL-HP. Group 1 was composed of NPDC and the patients with PC, IPMN and CP. Group 2 was composed of the patients with PC, IPMN and CP, and group 3 was composed of NPDC and the patients with PC. ROC curves of PhoSL-HP, CA19-9 and CEA were shown in Fig. 3. In all groups, the cut off value of PhoSL-HP was determined as 5.7 U/mL using Youden Index. It was the same as our previous report (23). There were cases in which either PhoSL-HP, CA 19–9, or CEA was below the minimum detection sensitivity, but in order to fairly compare the three markers,

Table 1
Characteristics of the subjects in this study.

	Pancreatic cancer	Intraductal Papillary Mucinous Neoplasm	Chronic pancreatitis	Non-pancreatic disease controls
Number	98	32	21	105
Age (range, years)	67.3 (44–80)	67.5 (46–80)	57.0 (36–74)	64.7 (38–79)
P-Value v.s. NPDC[a]	0.1381	0.3589	0.0805	–
Gender				
Male	46	11	15	98
Female	52	21	6	7
P-Value v.s. NPDC**	<0.0001	<0.0001	0.0026	–
History of Smoking	41%	39%	71%	12%
P-Value v.s. NPDC**	<0.0001	<0.0001	<0.0001	–
History of Alcohol Use	54%	55%	89%	77%
P-Value v.s. NPDC**	0.0006	0.0208	>0.1000	–
Diabetes	36%	11%	42%	19%
P-Value v.s. NPDC**	>0.1000	>0.1000	>0.1000	–
Cancer Stage				
I	1	–	–	–
II	2	–	–	–
III	10	–	–	–
IVa	34	–	–	–
IVb	51	–	–	–

[a] Tukey' test, **Ryan's method.

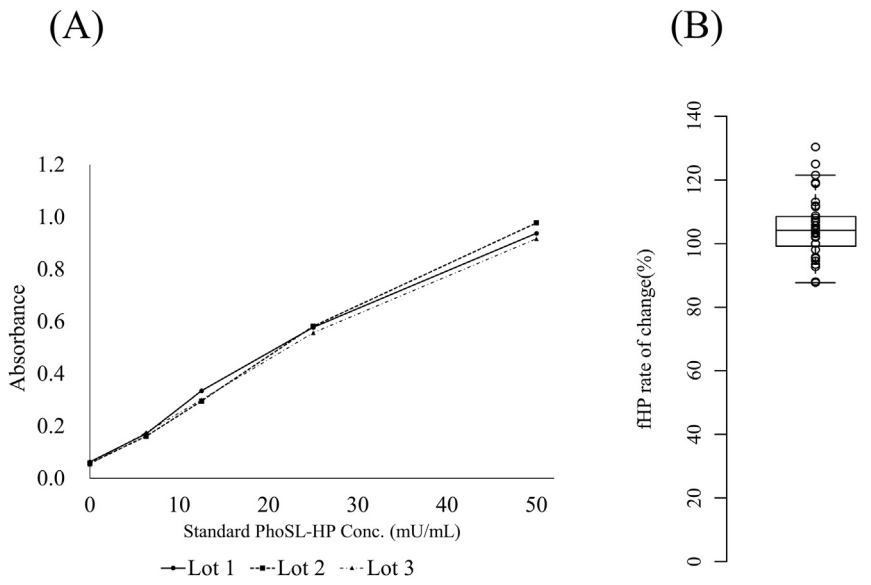

Fig. 1. Calibration curves of 3 lots of standard PhoSL-HP(A) and PhoSL-HP ratios of samples stored for 1 year at −80 °C (B).

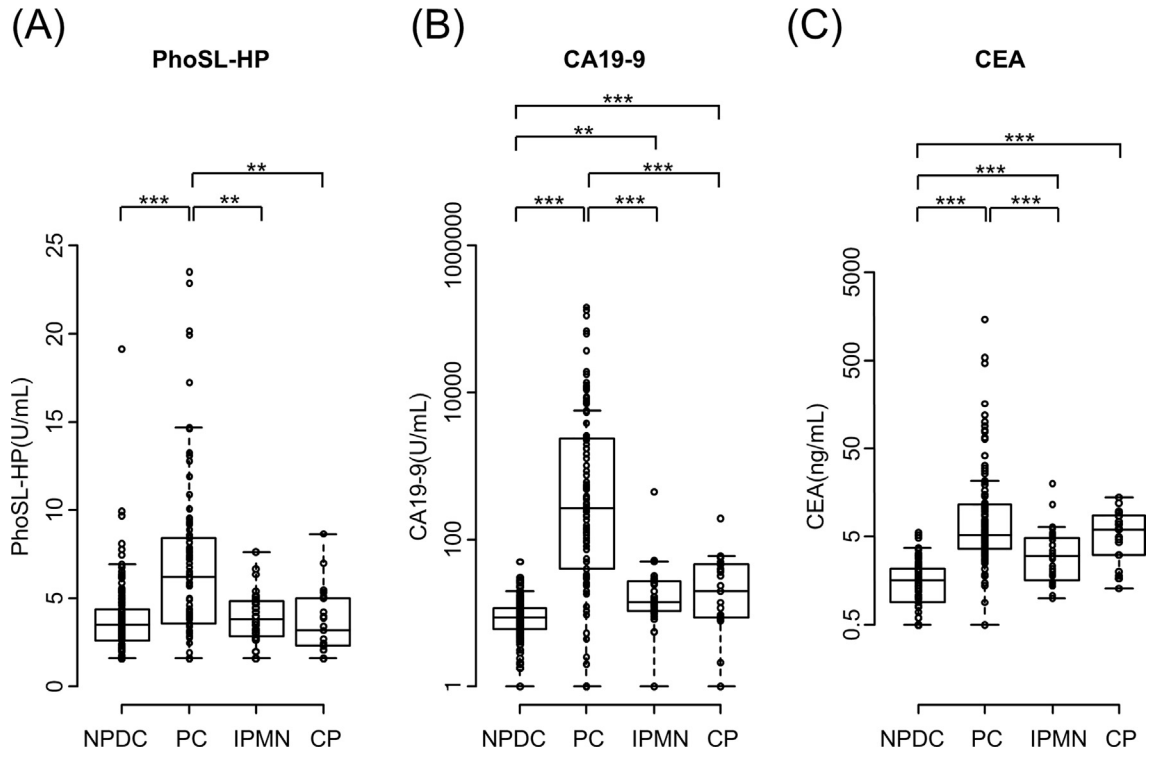

Fig. 2. Boxplot analysis of PhoSL-HP (A), CA19−9 (B) and CEA (C). The x-axis indicates the case classification, and the y-axis indicates the PhoSL-HP, CA19-9 and CEA level respectively. Significant differences among the four groups were determined using Steel-Dwass tests; $^{**}P < 0.01$, $^{***}P < 0.001$. The boxes indicate interquartile ranges for each group of specimens. The bar represents the median value.

analyses were performed for all cases used in this study. The AUCs of PhoSL-HP were almost constant in all groups, and they were 0.731 (in Group 1, 2 and 3). The AUCs of PhoSL-HP were inferior to those of CA19-9 (in Group 1, 2 and 3) and CEA (in Group 1 and 3), however the AUC of PhoSL-HP in Group 2 was superior to CEA. These results indicated that PhoSL-HP was more useful than CEA in clinical diagnosis because patients with PC were clearly discriminated from patients with IPMN and CP. With regard to Group 2, the discrimination ability of PC and CP, PC and IPMN was also evaluated in Fig. 3D–F. CA 19−9 and CEA tended to have higher discrimination abilities between PC and IPMN than PC and CP, but PhoSL-HP was opposite. In particular, in discrimination between PC and CP, the AUC of PhoSL-HP was higher than that of CEA.

Table 2 showed the medians and positive rates of PhoSL-HP, CA19-9 and CEA in each group. Positive rates of CA19-9 and CEA were calculated in accordance with their cut off values 37 U/mL and 6.5 ng/mL, respectively based on the values used at Hokkaido University Hospital. The sensitivity of PhoSL-HP for PC was 58% that

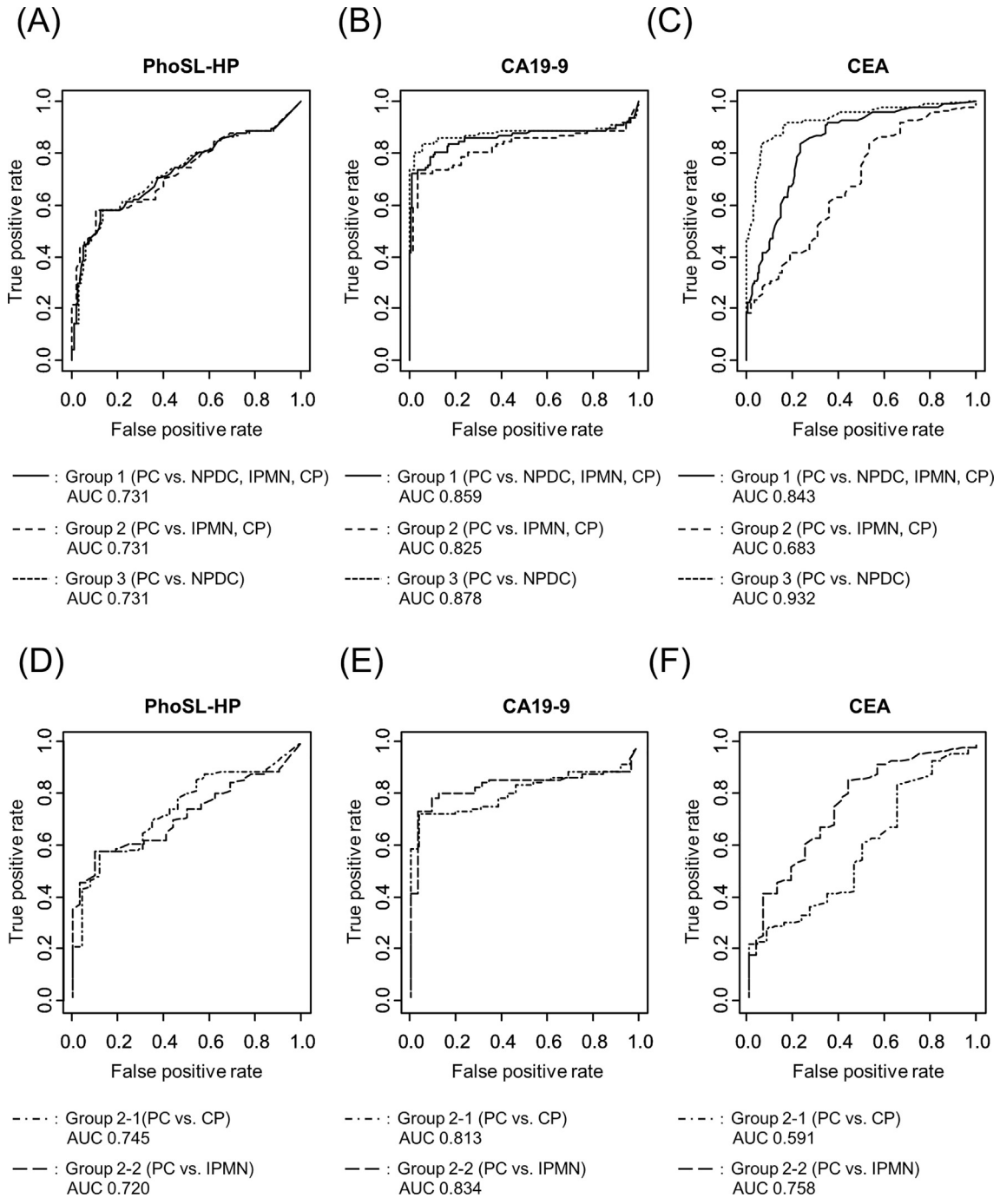

Fig. 3. ROC curves and AUC values of PhoSL-HP (A), CA19–9 (B) and CEA (C) for distinguishing patients with PC from NPDC and patient with IPMN and CP. Solid line represents curve for PC vs. NPDC, IPMN and CP (Group 1). Dashed line represents curve for PC vs. IPMN and CP (Group 2) and dotted line represents curve for PC vs. NPDC (Group 3). In Group 2, ROC curves and AUC values of PhoSL-HP (D), CA19-9 (E) and CEA (F) for distinguishing patients with PC from CP or IPMN. Dotdashed line represents curves for PC vs. CP and long dashed line represents PC vs. IPMN.

was inferior to CA19-9 (76%) but was superior to CEA (42%). Although 10% of patients with CP were falsely detected by PhoSL-HP, more patients were falsely detected by CA19-9 (33%) and CEA (43%) than PhoSL-HP. On the other hand false positive rate of PhoSL-HP for NPDC at 13% was higher than those of CA19-9 and CEA at 2% and 0%, respectively. As shown in Table 2 and Fig. 4, PhoSL-HP tended to be elevated in PC at early stages and not to be affected by stage progression. CA19-9 tended to increase as PC stage progressed, and CEA increased in only late stage of PC.

Correlation analysis and evaluation as combination marker

Spearman's rank correlation coefficients between PhoSL-HP, CA19-9 and CEA were calculated. The correlation coefficient between PhoSL-HP and CA19-9 was 0.281 and that of between PhoSL-HP and CEA was 0.279. Both of them were lower than the correlation coefficient between CA19-9 and CEA that was 0.502.

In order to verify the complementarities for CA19-9 of PhoSL-HP and CEA, combination analysis was performed. Table 3 showed the

Table 2
Medians and positive rates of the markers.

PhoSL-HP (U/mL)	No.	25% Interquartile	Median (range)	75% Interquartile	Positive No.	%
Non-pancreatic disease controls	105	2.6	3.5 (1.6–19.1)	4.6	14	13%
Pancreatic cancer	98	3.7	6.2 (1.6–23.5)	8.4	57	58%
Stage I	1	–	8.6	–	1	100%
Stage II	2	–	9.3 (8.4–10.1)	–	2	100%
Stage III	10	3.3	5.9 (1.6–22.9)	8.3	5	50%
Stage IVa	34	4.3	6.3 (2.5–23.5)	8.3	22	65%
Stage IVb	51	3.3	5.9 (1.6–19.9)	8.2	27	53%
IPMN	32	3	3.9 (1.6–7.6)	4.8	3	9%
Chronic pancreatitis	21	2.3	3.9 (1.6–8.7)	5	2	10%
CA19-9 (U/mL)	No.	25% Interquartile	Median (range)	75% Interquartile	Positive No.	%
Non-pancreatic disease controls	105	6	8.7 (1.0–50.1)	12	2	2%
Pancreatic cancer	98	42.7	270.0 (1.0–1.4×10^5)	2290.9	74	76%
Stage I	1	–	81.9	–	1	100%
Stage II	2	–	89.9 (25.6–316)	–	1	50%
Stage III	10	37.2	230.3 (1.0–1.9×10^4)	346.7	7	70%
Stage IVa	34	26.3	149.8 (1.0–1.2×10^4)	467.7	23	68%
Stage IVb	51	114.8	1273.0 (1.0–1.4×10^5)	8128.3	42	82%
IPMN	32	10.7	13.7 (1.0–448.7)	26.3	3	9%
Chronic pancreatitis	21	8.7	20.0 (1.0–194.5)	46.8	7	33%
CEA (ng/mL)	No.	25% Interquartile	Median (range)	75% Interquartile	Positive No.	%
Non-pancreatic disease controls	105	0.9	1.6 (0.5–5.6)	2.2	0	0%
Pancreatic cancer	98	3.6	5.3 (0.5–1.5×10^3)	11.5	41	42%
Stage I	1	–	5.1	–	0	0%
Stage II	2	–	4.8 (4.4–5.2)	–	0	0%
Stage III	10	3.1	5.2 (0.5–21.0)	6.8	3	30%
Stage IVa	34	3.3	5.2 (1.4–30.1)	7.2	12	35%
Stage IVb	51	3.7	6.7 (0.9–1.5×10^3)	28.8	26	51%
IPMN	32	1.8	3.0 (1.0–20.0)	4.8	2	6%
Chronic pancreatitis	21	3.1	6.1 (1.3–14.1)	8.7	9	43%

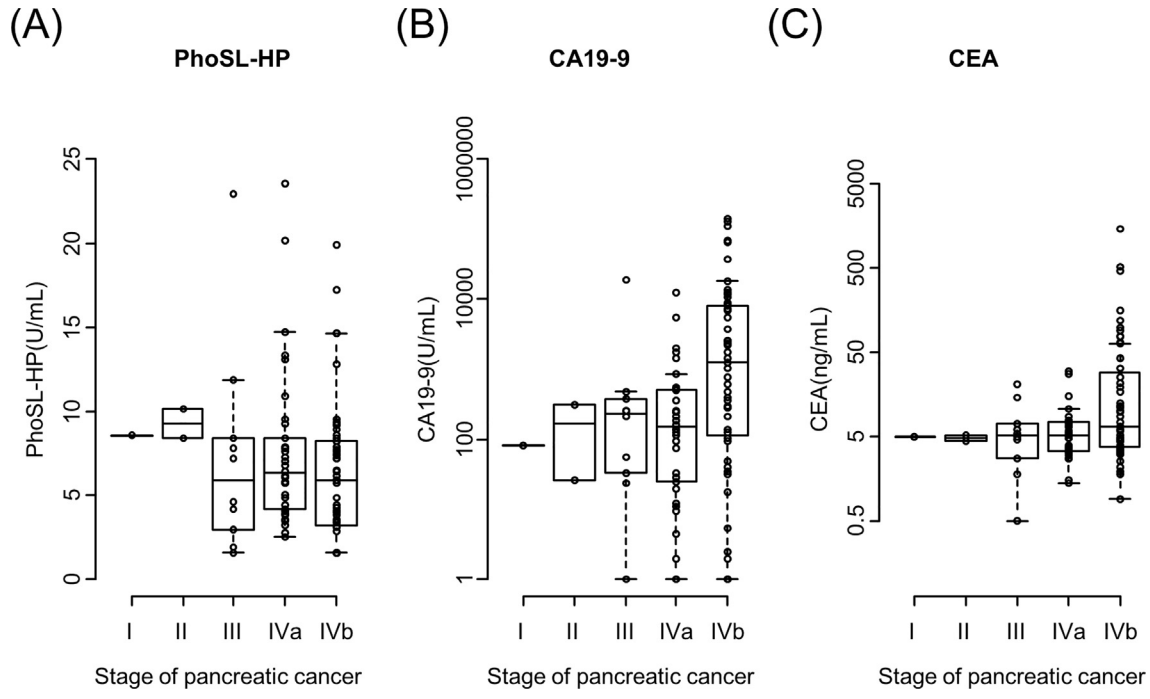

Fig. 4. Boxplot analysis for PC stage dependency of PhoSL-HP (A), CA19–9 (B) and CEA (C). Patients with PC in stage I (n = 1), stage II (n = 2), stage III (n = 10), stage IVa (n = 34) and stage IVb (n = 51) were investigated.

sensitivities and specificities in Group 1, 2 and 3 of each marker in single and combination use. The sensitivity of CA19-9 for detecting PC increased when combined with PhoSL-HP from 76% to 90%. And that also increased when combined with CEA from 76% to 86%. The specificities of CA19-9 were reduced when combined with PhoSL-HP from 92% to 81% (in Group 1), 81%–74% (in Group 2), 67%–

Table 3
Sensitivities and specificities of markers.

Marker (Cut off)	Sensitivity	Specificity				
		Group 1	Group 2	Group 2-1	Group 2-2	Group 3
		(NPDC, IPMN, CP)	(IPMN, CP)	(CP)	(IPMN)	(NPDC)
PhoSL-HP (5.7 U/mL)	58%	88%	91%	90%	91%	87%
CA19-9 (37 U/mL)	76%	92%	81%	67%	91%	98%
CEA (6.5 ng/mL)	42%	93%	79%	57%	94%	100%
CA19-9 (37 U/mL) + PhoSL-HP (5.7 U/mL)	90%	81%	74%	62%	81%	85%
CA19-9 (37 U/mL) + CEA (6.5 ng/mL)	86%	88%	68%	50%	88%	98%

62% (in Group 2–1), 91%–81% (in Group 2-2) and 98%–85% (in Group 3). Those were also reduced when combined with CEA from 92% to 88% (in Group 1) and from 81% to 68% (in Group 2), 67%–50% (in Group 2–1), 91%–88% (in Group 2-2) and not reduced in Group 3. In Group 2 not containing NPDC, the specificity of combination of CA19-9 and PhoSL-HP (74%) exceeded that of combination of CA 19–9 and CEA (68%), whereas the specificity of CA 19–9 and CEA was superior in Groups 1 (88%) and 3 (98%) than those of combination of CA19-9 and PhoSL-HP (81% and 85%).

Fig. 5 showed the ROC curves of combination of two markers. When combining CA19-9 with PhoSL-HP, the AUCs were significantly increased from 0.859 to 0.896 in Group 1 (Fig. 5A) and from 0.825 to 0.880 in Group 2 (Fig. 5B). However, the difference in Group 3 (PC vs. NPDC) was not significant (Fig. 5E). And when combining CA19-9 with CEA, the AUCs were also significantly increased from 0.859 to 0.919 in Group 1 (Fig. 5F) and from 0.878 to 0.970 in Group 3 (Fig. 5J). But in Group 2, the AUC did not increase significantly (Fig. 5G). It was suggested that the combination of CA19-9 and PhoSL-HP was more effective than that of CA19-9 and CEA in clinical diagnosis where patients with PC needed to be discriminated from the patients with pancreatic diseases including IPMN and CP.

Discussion

We verified the effectiveness of PhoSL-reactive core-fucosylated haptoglobin, namely, PhoSL-HP as a pancreatic cancer marker in clinical diagnosis. CA19-9 and CEA had high positive rates for pancreatic benign disease as previously reported [13], which was consistent with our study. The positive rates in CP of CA19-9 (33%) and CEA (43%) were higher than PhoSL-HP (10%), and this difference contributed to the higher specificity of PhoSL-HP in Group 2 (vs IPMN and CP). The finding is an important and clinically available feature of PhoSL-HP for differentiation of pancreatic diseases, which CA19-9 and CEA don't possess. In addition, although PhoSL-HP could show false positives of around 10% in any of NPDC, CP and IPMN, the median value did not change among NPDC, CP and IPMN, and there was no difference in the distribution of each group, so AUCs of Group 1–3 did not change. In particular, PhoSL-HP was elevated in early cancers when PhoSL-HP tended to show a low value in chronic pancreatitis. Although there are still many unclear points in the mechanism, as the result of correlation analysis with various biochemical test values, a negative correlation with HDL cholesterol level has been confirmed, and it could be related to lipid metabolism [25].

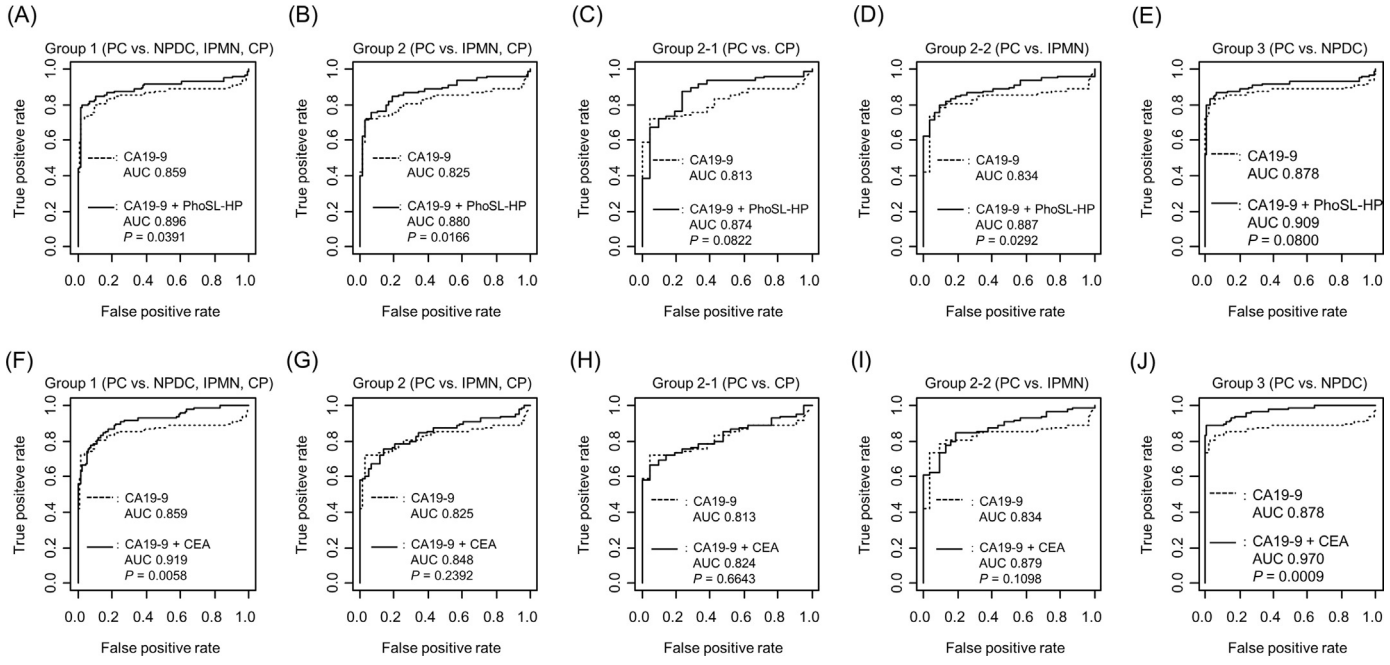

Fig. 5. ROC curves of CA19–9 and combination of CA19–9 and PhoSL-HP in Group 1 (A), Group 2 (B), Group 2–1 (C), Group 2–2 (D) and Group 3 (E) and curves of CA19–9 and combination of CA19–9 and CEA in Group 1 (F), Group 2 (G), Group2-1 (H), Group 2–2 (I) and Group 3 (J). Solid line represents curves for the combination of CA19-9 and PhoSL-HP or CA19-9 and CEA. Dotted line represents curve for CA19-9. Significant differences between AUCs of CA19-9 alone and combination of CA19-9 and PhoSL-HP or CA19-9 and CEA were determined using Delong's tests.

The correlation coefficient between PhoSL-HP and CA19-9 or CEA was lower than that between CA19-9 and CEA. This may be caused by the different detection mechanism of PhoSL-HP from CA19-9 or CEA, and showed higher complementarity of PhoSL-HP to CA19-9 than CEA to CA19-9. The AUC of PhoSL-HP was not so high as a single marker, however when combined CA19-9 and PhoSL-HP, the AUC was significantly increased than CA19-9 alone in Group 1 and 2. The low correlation coefficient between PhoSL-HP and CA19−9 may contribute the significant increase of AUC in combination use, and accordingly PhoSL-HP may well compensate the ability of CA19-9. The AUC of CA19-9 was significantly elevated when combined with CEA in Group 1 and 3 but not 2. There were 8 PC patients considered to be Lewis antigen negative because CA 19-9 levels were below the minimum detection. PhoSL-HP detected 5 out of the 8 PC specimens, while CEA detected 4 PC specimens. These results indicated that the complementarity of PhoSL-HP for CA19-9 in patients of PC, IPMN and CP was superior to that of CEA.

It is occasionally difficult to distinguish PC from other benign diseases of the pancreas including CP and IPMN [26,27]. One reason for this difficulty is caused by the higher false positive rates of CA19-9 and CEA for CP and IPMN as described above [9–13]. Thus, in this study we compared the serum PhoSL-HP levels of PC patients and non-PC samples including IPMN and CP patients with a view of using PhoSL-HP in clinical diagnosis. As the result, it was confirmed that PhoSL-HP showed highest values in patients with PC and did not increased in both NPDC and patients with other pancreatic diseases including IPMN and CP. PhoSL-HP showed low values even if inflammation occurred in the pancreas. These results indicated that PhoSL-HP was a useful and unique PC marker in clinical diagnosis where PC had to be discriminated from pancreatic diseases including IPMN and CP.

It is well known that detection of PC in an early stage is very difficult and most patients are detected in late stages [2,3]. As previously reported, PhoSL-HP was increased at the early stage of PC and maintained the higher level throughout the cancer stage [23], and the same tendency was also observed in this study. Although the numbers of patients with PC in stage I ($n = 1$), II ($n = 2$) and III ($n = 10$) were very small, and the most patients were in stage IVa ($n = 34$) and IVb ($n = 51$), PhoSL-HP could be the useful marker for the detection of PC in early stage (Fig. 4). PhoSL-HP in the early stage of PC showed higher serum level, although it seemed not to have dependency on PC stage. This result is the first finding which suggests that PhoSL-HP is useful for the early diagnosis of pancreatic cancer. In order to verify this possibility, further study should be conducted on patients with early stage PC.

Fucosylated haptoglobin detected by AAL that bound for α1-3, α1-4 and α1-6 fucosylated proteins was reported to be increased in PC, CP [19,20], hepatocellular carcinoma [28], non-alcoholic steatohepatitis [29] and colorectal cancer [30]. And AAL-HP was also reported to increase in prostate cancer with Gleason score dependency [31]. On the other hand, with regard to fucosylated haptoglobin detected by PhoSL which bound specifically to α1-6 fucosylated proteins, the increases in colorectal cancer [32] and CP [22] were reported. The increase of PhoSL-HP in CP was different from our present study. This would be caused by the difference of the antibody coated on plate and measurement sensitivity of previous and present studies. In our present study, we reselected antibodies for ELISA to specifically detect pancreatic cancer patients. Because there are various haptoglobin forms in human sera, it is thought that this discrepancy is due to the type of anti-haptoglobin antibody used in each assay. Furthermore we introduced denaturing reagent, urea, which improved the measurement sensitivity of PhoSL-HP. The detected absorbance value of standard PhoSL-HP improved more than 4 times [23]. As a result of improvement of the measurement method, it was confirmed that PhoSL-HP shows high value in PC, compared to pancreatic diseases such as CP and IPMN, and it was considered to be an effective marker for PC diagnosis. Further study will be needed to evaluate PhoSL-HP levels in colorectal cancer, hepatocellular carcinoma and prostate cancer with using our present improved method.

Recently, some candidate markers such as Apo-AII isoform [33–35], ICAM1 [36] and serum APN/CD13 [37] were reported to be effective in the detection of PC. Those markers showed higher AUCs, however those markers were increased in patients with not only PC but also CP. It can be suggested that PhoSL-HP is the most preferable marker for the purpose of discriminating PC from CP in clinical diagnosis.

In conclusion, we showed that PhoSL-HP is a useful marker for PC and have sufficient complementarity for CA19-9. Although further clinical research is required, PhoSL-HP would be contributed to the realization of more accurate PC diagnosis than current clinical practice.

Conflicts of interest

Naoya Sakamoto obtained research fund from TRSS limited company.

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