Research Paper

Identification of Armadillo repeat-containing X-linked protein 4 (ARMCX4) as a new serum biomarker for pancreatic cancer using quantitative proteomic analysis with tandem mass tags

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Abstract Pancreatic ductal adenocarcinoma (PDAC) accounts for 85−90% of all pancreatic tumors. The median survival of PDAC cases is less than 6 months, and the recent 5-year survival rate is about 8%. One of the main reasons for the poor prognosis is the lack of early diagnostic markers for PDAC. To overcome this problem and improve the outcomes of patients with PDAC, there is an urgent need to identify highly sensitive and specific markers for early detection. CA19-9 and CEA are widely used serum markers for PDAC, but are not sufficiently accurate for use as early diagnostic markers. In this study, we used the tandem mass tag (TMT) method to generate comparative protein profiles of culture supernatant of PDAC cell lines, and then compared the serum levels of candidate proteins to evaluate their ability to discriminate between PDAC cases and healthy volunteers. TMT labelling and LC-MS/MS were used for comparative analyses of secreted proteins in culture media obtained from six human PDAC cell lines to identify serum biomarkers for PDAC. In validation studies, the discriminatory power of candidate proteins was evaluated. Among 33 candidates, ARMCX4 was identified as a protein with increased levels in all six cell lines and was selected for further analysis. Serum sialylated ARMCX4 levels were significantly higher in early stage of PDAC patients than in healthy volunteers and patients with chronic pancreatitis ($p<0.01$). These results suggest that ARMCX4 may be a useful biomarker for detection of PDAC.

Key words: ARMCX4, CA19-9, pancreatic ductal adenocarcinoma, serum, biomarker

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and is one of the leading causes of cancer death[1)]. Despite improvements in therapeutic strategies, the estimated 5-year survival rate of PDAC is only 8%, mainly because the disease is often diagnosed at a very late stage[2)]. Thus, there is a need to detect PDAC in patients in a presymptomatic state, but a recent data compilation showed that about 50% of cases were in Stage IV at the time of diagnosis. Early diagnosis is difficult due to the anatomical characteristics of the pancreas, which is a long and thin organ located dorsal to the stomach and large intestine. This prevents clear images of the entire pancreas being obtained on abdominal ultrasonography[3)].

The rate of patients with PDAC with subjective symptoms at Stage 0 or 1, for which a favorable prognosis can be expected, is only 25%, and the positive rates of common tumor markers such as CEA and CA19-9 are extremely low

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in clinical practice[4], resulting in fewer opportunities for referrals to a hospital. The Japanese database of PDAC indicates 5-year survival rates of patients with diagnosis at Stages 0 (non-invasive), IA and IB of 85.8%, 68.7%, and 59.7%, respectively[4], but the diagnostic rates at these respective stages are only 1.7%, 4.1%, and 6.3%[1]. Patients with a tumor of ≤10 mm account for only 0.8% of cases[3]. Therefore, detection and diagnosis of PDAC at an early stage and earlier use of multidisciplinary therapy including surgical resection and chemotherapy are required to improve outcomes.

Recent developments of proteomics technology have allowed various approaches to identification of biomarkers. Serum/plasma proteome analyses with use of mass spectrometry (MS) is a useful tool to analyze peptides and proteins in biological samples, and has identified many candidate markers[5–7]. We have detected apolipoprotein C-1, C4b-binding protein α-chain and prosaposin as tumor markers for PDAC, but these markers have yet to reach practical use. In contrast, type 1 sugar chain-related markers, such as CA19-9, DUPAN-2 and Span-1, are used in clinical practice. In the current study, a glycoprotein Sambucus sieboldiana (SSA) lectin was used for comparative analysis using the tandem mass tag (TMT) method to examine cell secretory proteins and find new candidate tumor markers. An enzyme-linked immunosorbent assay (ELISA) using SSA lectin was established to evaluate the clinical importance of the candidate markers.

Materials and Methods

PDAC cell lines

Human PDAC cell lines (BxPC-3, PANC-1, MIA PaCa-2, Capan-2, Capan-1, and AsPC-1) were purchased from ATCC.

Patient serum samples

Blood samples were obtained from 34 patients with PDAC, 11 patients with chronic pancreatitis (PT), and 36 age/gender-matched healthy volunteers (HVs) as controls. Samples were obtained from patients histologically diagnosed with PDAC and PT in the Department of General Surgery, Chiba University Hospital, and from HVs at Kashiwado Hospital.

Magnetic bead sample preparation using a Glycoprotein Isolation Kit

Paramagnetic non-porous particles coupled with the SSA lectin ligand (MB-SSA; Bruker Daltonics, Bremen, Germany) were used to process the culture supernatant and serum samples. Binding, washing and elution solutions were prepared according to the manufacturer's instructions. MB-SSA binding solution (10 μL) and PDAC cell line culture supernatant (5 μL) were transferred to a 0.2-mL thin-walled tube. A homogenous magnetic-particle solution (10 μL) was added, mixed, and incubated for 5 min. The tubes were placed in an 8×12-well magnetic-bead separator (MBS; Bruker Daltonics) for 30 s for magnetic fixation of the MB-SSA particles. The supernatant was aspirated and the tubes were removed from the MBS device. Washing solution (100 μL) was added and carefully mixed with the magnetic beads. The tube was placed back in the MBS device and moved back and forth sequentially between adjacent wells on each side of the magnetic bar of the device. After fixation of the magnetic beads for 30 s, the supernatant was aspirated. This washing procedure was repeated three times. After the final washing step, the bound molecules were eluted by incubation with 10 μL MB-SSA elution solution for 1 min, before the eluate was collected using the MBS device.

Tandem mass tag (TMT) labelling

Reduction and alkylation were performed as described previously[8]. A TMT sixplex Isobaric Label Reagent Set (Thermo Fisher Scientific, Rockford, IL, USA) was used according to the manufacturer's instructions. Tubes containing the different isobaric chemical tags (0.8 mg each) were added to 41 μL of anhydrous acetonitrile and dissolved for 5 min with occasional vortexing at room temperature. TMT solution (20 μL) was added to each tube and allowed to react at room temperature for 60 min. A volume of 4 μL of 5% hydroxylamine was added to each sample and the mixture was incubated for 15 min to quench the reaction[8]. Samples were finally pooled and lyophilized. Analyses were performed using samples of 20 μL of glycoprotein-isolated PDAC cell line culture supernatant (n=6).

Protein identification and quantification by LC-MS/MS analysis

Trypsin-digested peptides were injected into a trap column (C18, 5 μm, 0.3×5 mm) (IonPac; Thermo Fisher Scien-

tific, Rockford, IL, USA) and an analytical column (C18, 3 μm, 0.075×120 mm) (C18 packed emitter column; Nikkyo Technos, Tokyo, Japan) attached to an HPLC system (Ultimate 3000; Thermo Fisher Scientific). The flow rate of the mobile phase was 300 nL/min. The solvent composition of the mobile phase was programmed to change in 120-min cycles, with varying mixing ratios of solvent A (2% acetonitrile and 0.1% formic acid) to solvent B (90% acetonitrile and 0.1% formic acid), as described previously[9]. Purified peptides from HPLC were moved to a hybrid ion-trap Fourier transform mass spectrometer (LTQ-Orbitrap XL; Thermo Scientific) and analyzed using previously established parameters[9]. A database search engine (Proteome Discoverer; Thermo Scientific) was used to identify and quantify proteins from the mass, tandem mass and reporter ion spectra of peptides. Peptide mass data were matched by searching the UniProt database (https://www.uniprot.org/proteomes/?query=*) using the search parameters: peptide mass tolerance=2 ppm and fragment tolerance=0.6 Da, with the enzyme parameter set to trypsin, allowing up to one missed cleavage variable modification, methionine oxidation, and cysteine alkylation. The minimum criteria for protein identification were filtered with Xcorr vs. charge state and a false discovery rate (FDR)＜1%.

ELISA

Immobilization of antibodies on a polystyrene microtiter plate: Anti-ARMCX4 polyclonal antibody (Proteintech Group Inc.) dissolved in PBS buffer was dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific) at 0.5 mg/well and incubated for 1 day at 4˚C. The plate was washed three times with PBS containing 0.05% Tween-20 (PBST). The microtiter plate was coated with 20% NOF102 containing 10% sucrose for 1 day at 4˚C.

ELISA conditions: After washing the microtiter plate with PBST, 100-μL aliquots of 10-times diluted plasma samples were added in duplicate to wells. The plates were incubated at room temperature for 1 h and then washed three times. HRP-conjugated SSA lectin (Vector Laboratories, Burlingame, CA, USA) in PBST (100 μL) was added to each well and the plate was incubated at room temperature for 30 min. The plate was washed three times and then 100 μL of TMB solution (Roche Diagnostics) was added. After room-temperature incubation for 10 min, 100 μL of stop solution was added and the absorbance at 450 nm was measured.

Other procedures

CA19-9 was measured using a Lumipulse Presto CA19-9 kit (Fujirebio, Tokyo, Japan). CEA was measured using a Lumipulse Presto CEA kit (Fujirebio).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 19 (SPSS, Chicago, IL, USA). Numerical data are presented as the mean±standard deviation (SD). Differences in non-parametric data between two groups were analyzed by Mann-Whitney *U*-test, with P＜0.05 considered significant. Receiver operating characteristic (ROC) curves were constructed to assess the sensitivity, specificity, and areas under the ROC curves (AUCs).

Results

Identification of ARMCX4 by TMT labeling and LC-MS/MS analysis (Table 1)

Establishment of an ELISA for sialylated ARMCX4

Within-run and between-run reproducibility: The precision of the assay was determined using two sialylated ARMCX4 concentrations of 1.8 and 10.5 AU/mL. Within-assay CVs were determined with eight replicates of each sample. Between-assay CVs were determined based on assays performed on 5 different days (two replicates of each sample per day). The within-run CV was 7.0−9.1% and the between-run CV was 9.4−11.6%.

Interference: Interference was assessed in samples containing 10.5 AU/mL sialylated ARMCX4. Potential interference materials were added to plasma at various concentrations. There was no substantial interference from hemoglobin (up to 5000 mg/L), free bilirubin (up to 207 mg/L), ditaurobilirubin (up to 204 mg/L), chyle (up to 1400 formazine turbidity units, equal to 1176 mg/L triglyceride), ascorbic acid (up to 500 mg/L), and rheumatoid factor (up to 500 U/L).

Sialylated ARMCX4 levels are increased in sera of patients with PDAC

Serum sialylated ARMCX4 levels were measured in 34 PDAC cases, 11 PT cases and 36 HVs (Table 1). As shown in Fig. 1, sialylated ARMCX4 levels in PDAC patients (6.8 ±5.0 AU/mL) were significantly greater than those in HVs (2.4±2.6 AU/mL) and PT patients (2.3±3.3 AU/mL) (PDAC vs. HVs: P＜0.001, PDAC vs. PT: P＜0.01; Mann−

Table 1. Protein identities revealed from mass spectrometry analysis

No.	Accession	Protein name	MIA PaCa-2	PANC-1	BxPC-3	CaPan-1	CaPan-2	ASPC-1
1	P02765	Alpha-2-HS-glycoprotein	✓	✓		✓	✓	
2	B3KQK3	Calumenin	✓		✓			✓
3	P31944	Caspase-14		✓	✓	✓		✓
4	P01024	Complement C3	✓	✓		✓	✓	
5	P01034	Cystatin-C	✓	✓	✓			✓
6	P81605	Dermcidin	✓		✓	✓	✓	
7	P15924	Desmoplakin	✓	✓	✓			✓
8	B4DUV1	Fibulin-1		✓	✓	✓	✓	✓
9	Q96RW7	Hemicentin-1	✓			✓		
10	L0R4T3	Histone H2B	✓	✓	✓			✓
11	Q86YZ3	Hornerin	✓		✓	✓	✓	✓
12	Q5T985	Inter-alpha-trypsin inhibitor heavy chain H2	✓	✓		✓	✓	
13	Q5T749	Keratinocyte proline-rich protein	✓		✓	✓	✓	
14	Q13751	Laminin subunit beta-3	✓	✓	✓			✓
15	Q13753	Laminin subunit gamma-2		✓	✓	✓	✓	✓
16	P61626	Lysozyme C	✓		✓	✓		✓
17	P08582	Melanotransferrin	✓	✓		✓	✓	
18	Q5H9A7	Metalloproteinase inhibitor 1	✓	✓	✓			✓
19	P80188	Neutrophil gelatinase-associated lipocalin	✓		✓	✓	✓	✓
20	Q96N83	Podocalyxin		✓	✓	✓	✓	
21	O14944	Proepiregulin	✓	✓		✓	✓	✓
22	P12273	Prolactin-inducible protein	✓			✓	✓	
23	P07602	Prosaposin	✓	✓	✓			✓
24	P41222	Prostaglandin-H2 D-isomerase		✓		✓	✓	✓
25	B4DNK4	Pyruvate kinase	✓		✓	✓	✓	✓
26	O75326	Semaphorin-7A		✓	✓			✓
27	P10599	Thioredoxin	✓		✓	✓	✓	
28	B3GN61	Truncated E-cadherin	✓	✓				✓
29	D6RBJ7	Vitamin D-binding protein	✓		✓	✓	✓	✓
30	C9JEV0	Zinc-alpha-2-glycoprotein	✓	✓				
31	P81605	Dermcidin		✓		✓	✓	✓
32	P02788	Lactotransferrin			✓	✓		✓
33	Q5H9R4	Armadillo repeat-containing X-linked protein 4	✓	✓	✓	✓	✓	✓

Whitney *U*-test). Serum sialylated ARMCX4 levels were measured in 34 PDAC according to UICC Stage (Fig. 2). Serum sialylated ARMCX4 levels for stage I, II, III and IV were 7.0±4.6 AU/mL, 5.8±4.3 AU/mL, 4.8±3.6 AU/mL and 9.5±2.4 AU/mL, respectively.

Sialylated ARMCX4 is superior to CA19-9 for early detection of PDAC

Serum levels of sialylated ARMCX4, CA19-9, and CEA in 34 PDAC cases according to UICC Stage (Stage I: n=13, Stage II: n=10, Stage III: n=9, Stage IV: n=2) and those in 36 HVs and 11 PT cases are shown in Table 2. Cutoffs for the three markers were set at levels that gave 95.4% specificity for PDAC compared with HVs and PT: 7.6 AU/mL, 37.7 U/mL and 5.2 ng/mL for sialylated ARMCX4, CA19-9, and CEA, respectively. In the 34 PDAC cases, the respective sensitivities of the markers were 44.1%, 41.1% and 32.3%. The sialylated ARMCX4 level was above the cutoff in 10 (52.6%) of 19 PDAC cases in which the serum level of CA19-9 was below its cutoff value.

ROC curves were used to evaluate the sialylated ARMCX4 and CA19-9 levels required for distinguishing PDAC cases from HVs and PT cases. The AUCs for distinguishing between cancer (PDAC) and non-cancer (HVs and

PT) were 0.875 for sialylated ARMCX4 and 0.775 for CA19-9. These results suggest that serum sialylated ARMCX4 expression may be a better diagnostic PDAC biomarker than serum CA19-9 expression (Fig. 3).

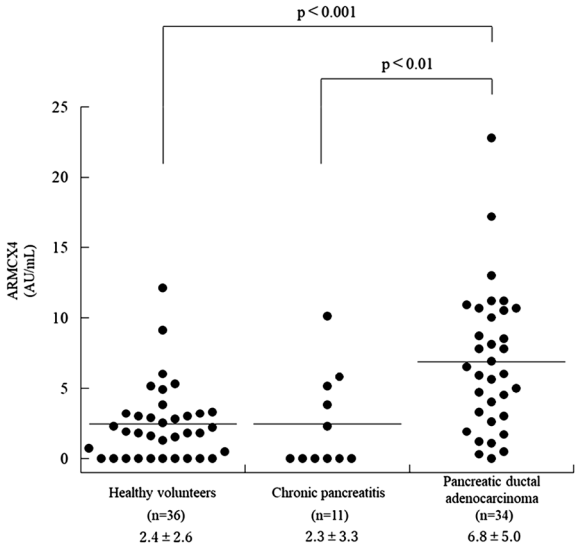

Fig. 1. Serum sialylated ARMCX4 levels in healthy volunteers, and patients with chronic pancreatitis and pancreatic ductal adenocarcinoma.

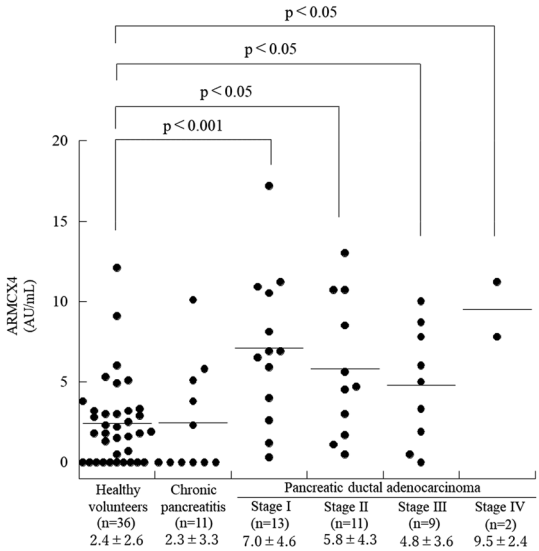

Fig. 2. Serum sialylated ARMCX4 levels in pancreatic ductal adenocarcinoma according to UICC Stage.

To evaluate whether sialylated ARMCX4 is useful for early detection of PDAC, serum ARMCX4, CA19-9 and CEA levels in patients with early stage PDAC (stages I and II) were compared with those in non-cancer cases (HVs and PT cases). In 24 early stage PDAC cases, the sensitivities of sialylated ARMCX4, CA19-9 and CEA at cutoff levels that gave 95.4% specificity were 45.8%, 29.2% and 20.8%, respectively. The AUCs of ROC curves for distinguishing between PDAC and non-cancer cases were 0.874 for sialylated ARMCX4 and 0.681 for CA19-9. Taken together, these results indicate that ARMCX4 is superior to CA19-9 as an early diagnostic biomarker for PDAC (Fig. 4).

Discussion

ARMCX4 was identified as a glycoprotein that was present in the culture supernatant of six examined cell lines of human PDAC. ARMCX4 is a member of the Armadillo repeat family of proteins and is involved in tumor development[10]. In this family, ARMCX1 has been shown to be

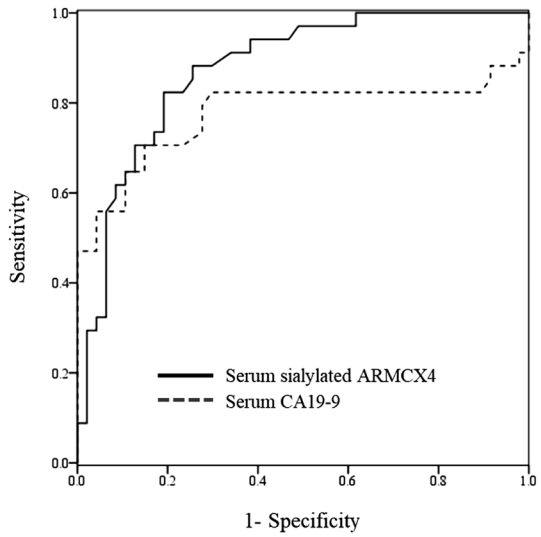

Fig. 3. ROC analyses of serum levels of sialylated ARMCX4 and CA19-9 between PDAC cases and non-cancer (HVs and PT) subjects.
The AUCs were 0.875 for sialylated ARMCX4 and 0.775 for CA19-9.

Table 2. Summary of the levels of sialylated ARMCX4, CA19-9 and CEA in sera of subjects

Biomarker	Non-cancer		Pancreatic cancer		
	HVs (n=36)	PT (n=11)	Stage I+II (n=24)	Stage III (n=9)	Stage IV (n=2)
Sialylated ARMCX4 (AU/mL)	2.4±2.6	2.3±3.3	8.0±4.9	4.8±3.6	9.5±2.4
CA19-9 (U/mL)	12.5±8.9	13.7±9.5	84.3±171.9	272.9±409.8	451.7±1368.5
CEA (ng/mL)	2.4±1.5	2.8±1.9	5.8±10.2	7.2±10.8	8.3±12.4

HVs=healthy volunteers; PT=pancreatitis. The levels are shown as mean±s.d.

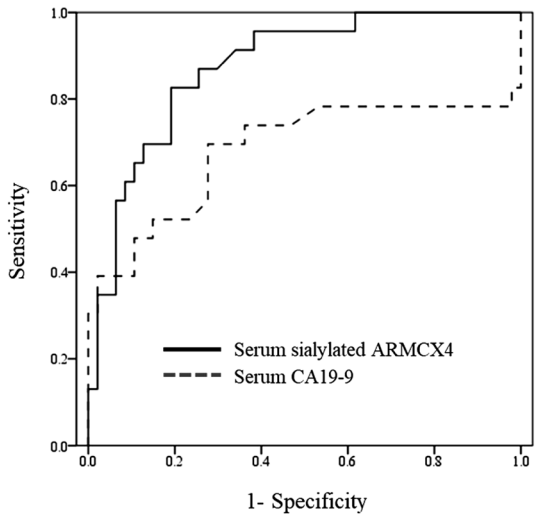

Fig. 4. ROC analyses for serum levels of sialylated ARMCX4 and CA19-9 between early stage (stages I and II) PDAC cases and non-cancer (HVs and PT) subjects.
The AUCs were 0.874 for sialylated ARMCX4 and 0.681 for CA19-9.

related to stomach cancer[11,12], prostate cancer[13], and urinary bladder cancer[14], and ARMCX2 is related to stomach cancer[12]; however, there is no report of a relationship of PDAC with ARMCX4.

Improvement of therapeutic outcomes of PDAC requires early detection and treatment with surgical resection. Screening tests in health checkups can be used for early detection of many cancers, but this is challenging for PDAC[15]. In blood tests, an increase of hepatic enzymes is found in many cases, and CA19-9 is also a specific marker of PDAC[16]. A nationwide study showed increases of CA19-9 and CEA in 69.0% and 18.0% of all registered cases of PDAC, respectively. This increase was higher in patients with advanced cancer, and there was almost no increase in early stage cases[16].

In recent years, new biomarkers have been found due to development of proteomics technique, but many of these biomarkers are still not used in clinical practice. Ideally in biomarker development, control and disease groups are compared using a semiquantitative method to select candidate markers in the discovery phase. Some candidate markers are then transferred to the validation phase that may include use of an ELISA.

This approach was used in this study to identify serum sialylated ARMCX4 as a new PDAC marker. Serum sialylated ARMCX4 levels were 2.4±2.6, 2.3±3.3, and 6.8 ±5.0AU/mL in HVs, patients with PT, and patients with PDAC, respectively, with a significant difference ($p<0.01$) between PDAC cases and HVs+PT cases, but not between PT cases and HVs. In addition, 46% of patients with stage I PDAC were positive for serum sialylated ARMCX4, whereas only 15% of these patients were positive for CA19-9. These results indicate that ARMCX4 is complementary to conventional markers and may be useful for detection of PDAC in clinical practice.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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