

# Serum fucosylated receptor expression-enhancing protein 5 as a biomarker for early stage pancreatic cancer

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**Abstract.** Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and is a leading cause of cancer mortality. 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. The aim of the present study was to search for protein biomarkers for PDAC in serum extracellular vesicles (EVs) using mass spectrometry, and to validate identified biomarkers using an enzyme-linked immunosorbent assay (ELISA) of sera from patients. Comprehensive and targeted proteomic analyses for biomarker discovery and verification were performed using EVs from serum of patients with PDAC, patients with chronic pancreatitis (PT) and healthy volunteers (HVs). For validation, the discriminatory power of candidate proteins was evaluated using an ELISA. Of the 3,043 proteins analyzed, 45 were identified as potential biomarkers, with receptor expression-enhancing protein 5 (REEP5) selected

for further analysis. The serum level of fucosylated REEP5 was significantly higher in PDAC cases compared with in PT and HVs ( $P < 0.001$ ). The areas under the curve (AUC) of the receiver operator characteristic were 0.928 for fucosylated REEP5 and 0.805 for carbohydrate antigen 19-9 (CA19-9) in PDAC vs. non-cancer controls. The AUCs were 0.962 for fucosylated REEP5 and 0.810 for CA19-9 in stages I and II of PDAC. These results indicate that fucosylated REEP5 is a novel serum EV biomarker for detection of early stage PDAC. Further analysis in a larger cohort is warranted to evaluate the clinical utility of fucosylated REEP5 as a biomarker for PDAC.

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and is a leading cause of cancer mortality (1). Despite improvements in therapy, the estimated 5-year survival rate of PDAC is still only 11%, mainly because the disease is often diagnosed at a very late stage (2); a previous study reported that ~50% of cases were in stage IV at the time of diagnosis. Thus, there is a need to detect PDAC in patients in a presymptomatic state. However, 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 subjective symptoms (such as weight loss and abdominal pain) in PDAC at stage 0 or I, for which a favorable prognosis can be expected, is only 25%, and the positive rates of common tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are extremely low in clinical practice (4), resulting in fewer opportunities for referrals to a hospital. The Japanese PDAC database indicates 5-year survival rates 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  $\leq 10$  mm account for only

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*Abbreviations:* AFP,  $\alpha$ -fetoprotein; AUC, area under the curve; DIA, data-independent acquisition; ELISA, enzyme-linked immunosorbent assay; EVs, extracellular vesicles; FDR, false discovery rate; HV, healthy volunteer; MS, mass spectrometry; PBST, PBS containing 0.05% Tween-20; PDAC, pancreatic ductal adenocarcinoma; PT, chronic pancreatitis; PTS, phase transfer surfactant; REEP5, receptor expression-enhancing protein 5; ROC, receiver operating characteristic; SD, standard deviation; sLex, sialyl Le<sub>x</sub>; SRM, selected reaction monitoring

*Key words:* biomarker, extracellular vesicles, pancreatic cancer, receptor expression-enhancing protein 5, serum

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.

Identification of biomarkers in extracellular vesicles (EVs), which are small membrane bound vesicles of endocytic origin released by various cell types, is of increasing interest. EVs exhibit pleiotropic functions, including immune response modulation, antigen presentation and intercellular communication (5,6). EVs are surrounded by a lipid bilayer membrane, making them readily accessible, highly stable and an ideal source for discovery of novel biomarkers (7). Thus, biomarkers from EVs have been used to clarify the pathogenesis, diagnosis and therapeutic strategies for several malignant diseases (such as lung, liver and colorectal cancer) (8-11). Also, the dynamic range of blood proteins is extremely wide. If a candidate protein is present only in small amounts, its detection in total blood protein may be challenging, even with use of selected reaction monitoring (SRM) (8). Therefore, EV proteins are promising candidates for biomarkers.

Previous advances in mass spectrometry (MS) have enabled detection and quantification of thousands of proteins in cells, tissues and biological fluids. As a powerful analytical tool, MS has been instrumental in elucidating the fundamental role of EVs in cancer, such as modulating the tumor micro-environment, mediating immune evasion and contributing to metastasis (12,13). MS-based proteomics is also useful for exploring EVs as a source of diagnostic, prognostic (severity of outcome) and predictive (treatment response) biomarkers. Comprehensive shotgun proteomics using data-independent acquisition (DIA) or data-dependent acquisition is commonly used for biomarker discovery. Biomarker candidates identified through comprehensive proteomics can be validated using targeted proteomics approaches, such as SRM or parallel reaction monitoring (8,14-16).

The aim of the present study was to identify and verify a serum EV-based protein biomarker for diagnosis of PDAC using DIA and SRM. To overcome the difficulty of consistent extraction of EVs from large sample sets, a clinically applicable enzyme-linked immunosorbent assay (ELISA) was developed for validation of potential targets in serum.

## Materials and methods

**Patient serum samples.** In the first cohort, blood samples were obtained from 20 patients (mean age, 64±5 years; 12 male and 8 female) with PDAC, 10 with chronic pancreatitis (PT) (mean age, 55±10 years; 6 males and 4 females) and 10 age- and sex-matched healthy volunteers (HVs) as controls (mean age, 60±4 years; 6 males and 4 females). To validate the results, serum levels of candidate proteins were measured in a second cohort of 20 patients with PDAC (mean age, 66±8 years; 12 males and 8 females), 10 with PT (mean age, 56±12 years; 6 males and 4 females) and 10 age- and sex-matched HVs (mean age, 62±6 years; 6 males and 4 females). In a third cohort, blood samples were obtained from 63 patients with PDAC (mean age, 67±9 years; 35 males and 27 females), 10 with PT (mean age, 58±10 years; 6 males and 4 females) and 30 age- and sex-matched HVs (mean age, 62±10 years; 17 males and 13 females). Blood samples were collected at the Department

of General Surgery at Chiba University Hospital (Chuo, Japan) between December 2019 and May 2022, from patients who were histologically diagnosed with PDAC or PT. The samples from HVs were collected at the Kashiwado Hospital (Chuo, Japan) between August and December 2015 (Table I). All blood samples were processed according to a standardized protocol (17), and serum aliquots were frozen until subsequent analysis. None of the patients had undergone therapy, such as radiation, chemotherapy or surgery, prior to serum sample collection. The ethics committee at each institution approved the protocols and written informed consent was obtained from all patients and HVs.

**Isolation of EVs.** EV isolation was performed using a MagCapture Exosome Isolation Kit PS (FUJIFILM Wako Pure Chemical Corporation) (18-20). In short, 200  $\mu$ l serum was centrifuged at 1,200 x g for 20 min at 4°C. The pre-cleared serum sample was filtered through a 0.45- $\mu$ m Spin-X centrifuge tube (Corning, Inc.) and incubated with magnetic beads. The beads were washed with PBS, the EVs eluted with 100  $\mu$ l elution buffer and 80  $\mu$ l eluate was used for proteomics analysis.

**Protein extraction from EVs and digestion.** EV samples were lysed to extract proteins using a phase transfer surfactant (PTS) protocol (21). Separated EV fractions were lysed with PTS buffer (12 mM sodium deoxycholate, 12 mM sodium lauroyl sarcosinate, 50 mM ammonium bicarbonate), and boiled for 5 min at 95°C. EV proteins were reduced with 10 mM tris (2-carboxyethyl) phosphine (FUJIFILM Wako Pure Chemical Corporation) for 30 min at 37°C and allowed to cool to room temperature and alkylated with 20 mM iodoacetamide (Nacalai Tesque, Inc.) for 30 min at 37°C in the dark. The mixture was incubated in a final concentration of 21 mM L-cysteine (Nacalai Tesque, Inc.) for 10 min at room temperature. Trypsin and Lys C digestion [1  $\mu$ l 1.0  $\mu$ g/ $\mu$ l trypsin solution (Roche Diagnostics) and 2  $\mu$ l 1 mAU/ $\mu$ l Lys C (FUJIFILM Wako Pure Chemical Corporation)] was performed overnight at 37°C. After surfactant removal, the digested peptides were desalted using StageTips (22).

**Liquid chromatography (LC)-MS/MS DIA and data analysis.** LC was performed using an UltiMate 3000 Nano LC system (Thermo Fisher Scientific, Inc.) with an HTC-PAL autosampler (CTC Analytics AG) and a trap column for sample injection (0.075x20 mm, Acclaim PepMap RSLC Nano-Trap Column; Thermo Fisher Scientific, Inc.). MS and MS/MS were performed using a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc.) with a nano LC-MS interface for peptide ionization using nano-electrospray ionization in positive ion mode (AMR Inc.). Peptides were separated using an analytical column (75  $\mu$ m x 30 cm) packed in-house with 1.9  $\mu$ m resin (ReproSil-Pur C18-AQ; Dr. Maisch HPLC GmbH) at a flow rate of 280 nl/min with a 105-min gradient from 5 to 30% solvent B [solvent A, 0.1% formic acid (FA) and 2% acetonitrile; solvent B, 0.1% FA and 90% acetonitrile]. MS data were acquired in DIA mode. Full mass spectra were obtained in the range of 398-802 m/z with the following parameters: Resolution 140,000, AGC target 3x10<sup>6</sup> ions, maximum injection time 200 ms and normalized

Table I. Clinical characteristics of participants.

| Characteristic        | First cohort  |              |                | Second cohort |              |                | Third cohort  |              |                |
|-----------------------|---------------|--------------|----------------|---------------|--------------|----------------|---------------|--------------|----------------|
|                       | HVs<br>(n=10) | PT<br>(n=10) | PDAC<br>(n=20) | HVs<br>(n=10) | PT<br>(n=10) | PDAC<br>(n=20) | HVs<br>(n=30) | PT<br>(n=10) | PDAC<br>(n=62) |
| Age, years, mean ± SD | 60±4          | 55±10        | 64±5           | 62±6          | 56±12        | 66±8           | 62±10         | 58±10        | 67±9           |
| Sex, n                |               |              |                |               |              |                |               |              |                |
| Male                  | 6             | 6            | 12             | 6             | 6            | 12             | 17            | 6            | 35             |
| Female                | 4             | 4            | 8              | 4             | 4            | 8              | 13            | 4            | 27             |
| UICC tumor stage, n   |               |              |                |               |              |                |               |              |                |
| I                     | -             | -            | 4              | -             | -            | 5              | -             | -            | 21             |
| II                    | -             | -            | 8              | -             | -            | 9              | -             | -            | 22             |
| III                   | -             | -            | 4              | -             | -            | 4              | -             | -            | 15             |
| IV                    | -             | -            | 4              | -             | -            | 2              | -             | -            | 4              |

UICC, Union for International Cancer Control; HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma.

collision energy of 27. MS/MS spectra were obtained with the following parameters: 8 m/z isolation window with resolution 70,000, AGC target  $3 \times 10^6$  ions, maximum injection time 'auto', overlapping window pattern and normalized collision energy of 27.

Raw MS data were analyzed using DIA-NN software (version 1.7.12; Demichev Lab, Department of Biochemistry, Charité Universitätsmedizin). Database searches were performed using the *Homo sapiens* UniProt database (<https://www.uniprot.org/>), a contaminant database (23) and a decoy database (created from forward database by DIA-NN) in which all protein sequences were in reverse order. The search parameters were: 7-30 peptide length, carbamidomethyl modification of cysteine residues (+57.021 Da), up to two cleavage sites missing, protein names from FASTA for implicit protein grouping, robust LC (high precision) for quantification strategy and retention time-dependent for cross-run normalization. The false discovery rate (FDR) for precursor ions was set to 1%.

*Quantitation of target peptides by SRM using SI-peptide as the internal standard.* SRM was performed for quantification of the target peptides, using an HTC-PAL autosampler, a Paradigm MS2 Nano-LC system (Michrom Bioresources, Inc.) and a triple quadrupole mass spectrometer (TSQ-Vantage; Thermo Fisher Scientific, Inc.) as previously described (8). The peptides used for SRM measurements were the two most intense peptides selected from those identified using LC-MS/MS (DIA). SRM transition lists (precursor m/z and product m/z pairs) for each peptide were created using Skyline software (version 20.2) (24) and optimized by monitoring the transitions of the synthetic isotope-labeled-peptide mixture (8,16,25). The transition list is shown in Table II. SI peptides with a C-terminal  $^{15}\text{N}$ - and  $^{13}\text{C}$ -labeled arginine or lysine (isotopic purity >99%) were obtained from JPT Peptide Technologies (crude purity). An SI peptide mixture was added to the digested EV proteins purified from each serum sample. The amount of SI peptide added was set to be close to the amount of endogenous peptides. Transition peaks obtained

using SRM measurements were manually inspected to select peaks with heights  $\geq 1,000$ , signal-to-noise ratios  $\geq 10$  or coefficient variation (CV) area ratios <35% between transitions for each peptide (8,16). Accuracy was ensured by removing transitions due to non-specific background interference or coelution. The ratio of the peak area of the endogenous peptide to that of the isotope-labeled peptide internal standard was used as the quantification value of the endogenous peptide.

*Western blotting analysis of serum.* Protein determination was performed using NANODROP LITE (Thermo Fisher Scientific, Inc.). For western blotting, serum samples were separated by electrophoresis on 10-20% gradient gels (DRC Co., Ltd.). REEP5 and albumin in total serum (5.0  $\mu\text{g}$  protein loaded per column) was measured in patients with PDAC and HVs. Proteins were subsequently transferred to an Immobilon-P transfer membrane (Merck KGaA) using a MINICA-MP (DRC Co., Ltd.). The membranes were blocked with 5% skimmed milk in PBS at 37°C for 1 h. After blocking, individual lanes containing the serum profile were cut into strips. These membrane strips were incubated individually at room temperature for 1 h with an anti-receptor expression-enhancing protein 5 (REEP5) polyclonal antibody (1,000-fold dilution; cat. no. 4929; Proteintech Group, Inc.). A peroxidase-conjugated anti-rabbit immunoglobulin (1,000-fold dilution; cat. no. P0260; Proteintech Group, Inc.) was used as the secondary antibody. These membrane strips were incubated individually at room temperature for 1 h. Antigen detection was performed using the Pierce ECL Plus Western blotting substrate (Thermo Fisher Scientific, Inc.).

*Lectin ELISA.* Serum fucosylated REEP5 was quantified using a lectin ELISA. An anti-REEP5 polyclonal antibody (Proteintech Group, Inc.) dissolved in PBS buffer was dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific, Inc.) at a concentration of 0.5  $\mu\text{g}$ /well and incubated at 4°C for 1 day. The plate was subsequently washed three times with PBS containing 0.05% Tween-20 (PBST). Next, the

Table II. Transition list of receptor expression-enhancing protein 5 for selected reaction monitoring analysis.

| Peptide sequence | Isotope label type | Precursor, m/z | Product, m/z | Precursor charge | Product charge | Fragment ion |
|------------------|--------------------|----------------|--------------|------------------|----------------|--------------|
| ETADAITK         | Light              | 424.721632     | 719.393395   | 2                | 1              | y7           |
| ETADAITK         | Light              | 424.721632     | 618.345717   | 2                | 1              | y6           |
| ETADAITK         | Light              | 424.721632     | 547.308603   | 2                | 1              | y5           |
| ETADAITK         | Light              | 424.721632     | 432.28166    | 2                | 1              | y4           |
| ETADAITK         | Light              | 424.721632     | 361.244546   | 2                | 1              | y3           |
| ETADAITK         | Heavy              | 428.728732     | 727.407594   | 2                | 1              | y7           |
| ETADAITK         | Heavy              | 428.728732     | 626.359916   | 2                | 1              | y6           |
| ETADAITK         | Heavy              | 428.728732     | 555.322802   | 2                | 1              | y5           |
| ETADAITK         | Heavy              | 428.728732     | 440.295859   | 2                | 1              | y4           |
| ETADAITK         | Heavy              | 428.728732     | 369.258745   | 2                | 1              | y3           |
| ATVNLLGEEK       | Light              | 537.295496     | 901.498923   | 2                | 1              | y8           |
| ATVNLLGEEK       | Light              | 537.295496     | 802.430509   | 2                | 1              | y7           |
| ATVNLLGEEK       | Light              | 537.295496     | 688.387582   | 2                | 1              | y6           |
| ATVNLLGEEK       | Light              | 537.295496     | 575.303518   | 2                | 1              | y5           |
| ATVNLLGEEK       | Light              | 537.295496     | 462.219454   | 2                | 1              | y4           |
| ATVNLLGEEK       | Heavy              | 541.302595     | 909.513122   | 2                | 1              | y8           |
| ATVNLLGEEK       | Heavy              | 541.302595     | 810.444708   | 2                | 1              | y7           |
| ATVNLLGEEK       | Heavy              | 541.302595     | 696.401781   | 2                | 1              | y6           |
| ATVNLLGEEK       | Heavy              | 541.302595     | 583.317717   | 2                | 1              | y5           |
| ATVNLLGEEK       | Heavy              | 541.302595     | 470.233653   | 2                | 1              | y4           |

microtiter plate was coated with 20% NOF102 (NOF Co., Ltd.) containing 10% sucrose and incubated at 4°C for 1 day. After washing with PBST, 100  $\mu$ l aliquots of 10-fold diluted plasma samples were added in duplicate to the wells. The plates were incubated at room temperature for 1 h and washed three times. HRP-conjugated LCA lectin (Vector Laboratories, Inc.) in PBST (100  $\mu$ l) was added to each well, followed by incubation at room temperature for 30 min. The plate was washed three times, and 100  $\mu$ l TMB solution (Roche Diagnostics) was added. After a 10 min incubation at room temperature, 100  $\mu$ l stop solution was added, and the absorbance at 450 nm was measured. Reproducibility and potential interference were assessed as previously reported (7,26).

*Other procedures.* CEA and CA19-9 levels were measured using Lumipulse Presto CEA and CA19-9 kits, respectively (Fujirebio). REEP5 was measured using a Human REEP5 ELISA Kit (cat. no. HUF105962; Assay Genie). DUPAN-2 (cat. no. 04770) and SPan-1 (cat. no. 04799) concentrations were determined using an enzyme immunoassay (EIA) from MBL International Co.

*Statistical analysis.* Each sample was measured in triplicate. Statistical analysis was performed using IBM SPSS Statistics 19 (IBM Corp.). Numerical data are presented as the mean  $\pm$  standard deviation (SD). Differences in non-parametric data between groups were analyzed using Kruskal-Wallis tests, with  $P < 0.05$  considered to indicate a statistically significant difference. The Steel-Dwass test was used for the post hoc analysis. Receiver operating characteristic (ROC) curves were

constructed to evaluate sensitivity, specificity and the area under curve (AUC).

## Results

*DIA proteomic analysis for discovery of PDAC biomarker candidates in serum EVs.* To identify PDAC biomarker candidate proteins in serum EVs, DIA proteomic analysis was performed using EV fractions prepared from sera using the phosphatidylserine affinity method (MagCapture). Serum EVs were collected from 20 patients with PDAC, 10 patients with PT and 10 age and sex-matched HVs (first cohort). Extracted proteins were digested using the PTS method. LC-MS/MS analysis and a subsequent database search identified 3,049 proteins (FDR <1%). In the DIA analysis, 14 proteins had quantified levels that were 2.0x higher in PDAC compared with in PT and HVs, with significant differences ( $P < 0.01$ ; Table III). Particular attention was paid to REEP5, which had the highest fold-change (Fig. 1; Table III).

*Verification of biomarker candidates using SRM.* An SRM verification assay of selected biomarker candidate proteins in the serum EVs was performed. A total of two target REEP5 peptides (ETADAITK and ATVNLLGEEK) with the highest intensity in DIA were selected. Individual EV fractions were prepared from the second cohort (HV, n=10; PT, n=10; PDAC, n=20) for SRM analyses. The quantitative value of each peptide was calculated as the ratio of the peak area of the endogenous target peptide to the isotope-labeled peptide internal standard. This value for each peptide was normalized using the

Table III. PDAC biomarker candidate proteins in extracellular vesicles identified using data-independent acquisition proteomic analysis.

| Number | Accession number | Protein name                            | P-value (HVs + PT vs. PDAC) | Fold-change |
|--------|------------------|---|-----------------------------|-------------|
| 1      | O14817           | Tetraspanin-4                           | P<0.01                      | 2.10        |
| 2      | P04264           | Keratin, type II cytoskeletal 1         | P<0.01                      | 2.54        |
| 3      | P07602           | Prosaposin                              | P<0.01                      | 2.07        |
| 4      | P10646           | Tissue factor pathway inhibitor         | P<0.01                      | 2.15        |
| 5      | P20073           | Annexin A7                              | P<0.01                      | 2.41        |
| 6      | P30626           | Sorcin                                  | P<0.01                      | 2.06        |
| 7      | P35527           | Keratin, type I cytoskeletal 9          | P<0.01                      | 2.31        |
| 8      | P35754           | Glutaredoxin-1                          | P<0.01                      | 2.29        |
| 9      | P50995           | Annexin A11                             | P<0.01                      | 2.27        |
| 10     | P69891           | Hemoglobin subunit gamma-1              | P<0.01                      | 2.05        |
| 11     | Q00765           | Receptor expression-enhancing protein 5 | P<0.01                      | 2.63        |
| 12     | Q5SY68           | Protein S100-A7-like 2                  | P<0.01                      | 2.21        |
| 13     | Q6E0U4           | Dermokine                               | P<0.01                      | 2.35        |
| 14     | Q86UF1           | Tetraspanin-33                          | P<0.01                      | 2.21        |

In the analysis, 14 proteins had quantified values 2.0x higher in PDAC compared with in HVs and PT, with significant differences (P<0.01). HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma.

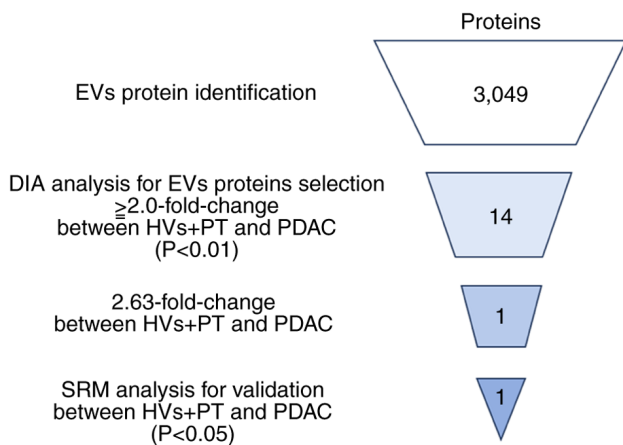


Figure 1. PDAC biomarker discovery in EVs. Overview of biomarker candidate selection, showing the selection method at each step and the number of remaining candidate proteins. EVs, extracellular vesicles; HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; DIA, data-independent acquisition; SRM, selected reaction monitoring.

average quantitative value of peptides from EV marker protein CD9 because the amount of EVs obtained from each serum sample was variable. Of the REEP5 peptides, ETADAITK was significantly higher in PDAC compared with PT and HVs (both P<0.05, Kruskal-Wallis test; Fig. 2A; Table IV). No significant difference in ATVNLLGEEK levels was observed in PDAC compared with HVs and PT (Kruskal-Wallis test; Fig. 2B; Table IV).

*REEP5 is detectable in total serum using western blotting.* REEP5 expression was subsequently assessed in serum. Western blotting of total serum from PDAC cases and HVs showed that REEP5 was detectable in whole serum (Fig. 3).

*Establishment of an ELISA for fucosylated REEP5.* The precision (within-run and between-run reproducibility) of the assay was determined using two fucosylated REEP5 concentrations of 0.05 and 0.32 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 8.1-9.8% and the between-run CV was 9.9-12.0%. Interference was assessed in samples containing 0.32 AU/ml fucosylated REEP5. Potential interference materials were added to plasma at various concentrations. There was no substantial interference from hemoglobin (up to 5,000 mg/l), free bilirubin (up to 207 mg/l), ditaur bilirubin (up to 204 mg/l), chyle (up to 1,400 formazine turbidity units, equal to 1,176 mg/l triglyceride), ascorbic acid (up to 500 mg/l) and rheumatoid factor (up to 500 U/l) (data not shown).

*Quantification of serum REEP5 using ELISA.* The serum REEP5 levels in the third cohort (HVs, n=30; PT, n=10; PDAC, n=63) were 0.16±0.04, 0.16±0.03 and 0.21±0.12 ng/ml in HVs, PT and PDAC, respectively (Fig. 4A), with no significant difference between HVs and PDAC cases (P=0.305).

*Serum fucosylated REEP5 is significantly increased in PDAC.* Fucosylated oligosaccharides are often used as cancer biomarkers (27-31). For example, CA19-9, a fucosylated oligosaccharide [sialyl Le<sub>a</sub> (NeuAc<sub>2</sub>-3Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-R)] is a biomarker for pancreatic cancer. Therefore, we hypothesized that fucosylated REEP5 might be a biomarker for early stage pancreatic cancer, and an ELISA was developed for fucosylated REEP5. Serum fucosylated REEP5 in the third cohort was significantly higher in PDAC (0.12±0.10 AU/ml) compared with in HVs (0.03±0.01 AU/ml) and PT (0.03±0.01 AU/ml) (PDAC vs. HVs and PT, both P<0.001; Kruskal-Wallis test; Fig. 4B).

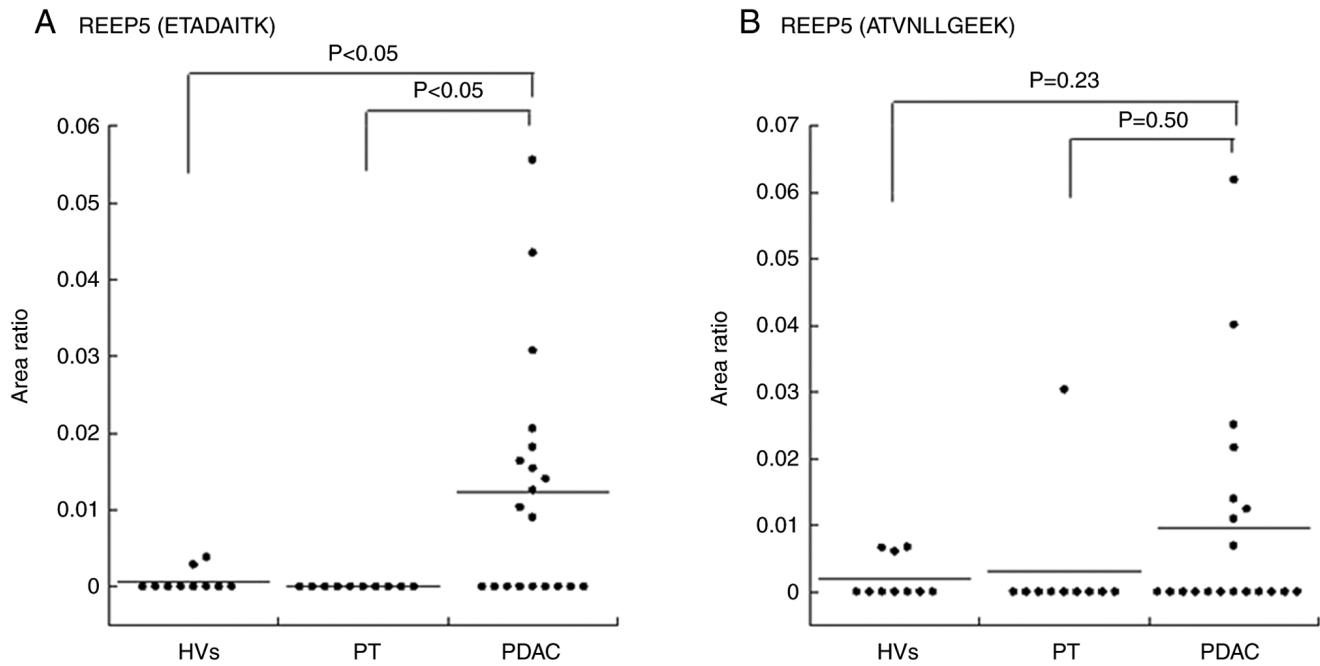


Figure 2. Verification of biomarker candidate proteins using targeted proteomics. The two REEP5 peptides with highest intensity (A) ETADAITK and (B) ATVNLLGEEK were selected. Relative quantitation of peptides of biomarker candidates was performed using selected reaction monitoring analysis. HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; REEP5, receptor expression-enhancing protein 5.

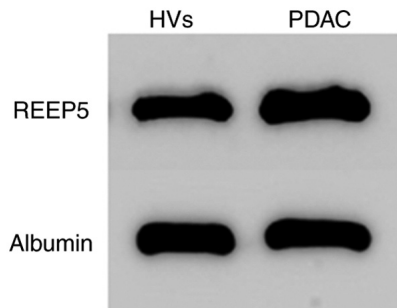


Figure 3. REEP5 protein is detected in total serum. Representative western blot of REEP5 and albumin in total serum (5.0  $\mu$ g protein loaded per column) of patients with PDAC and HVs. REEP5, receptor expression-enhancing protein 5; HVs, healthy volunteers; PDAC, pancreatic ductal adenocarcinoma.

*Serum fucosylated REEP5 is superior to CEA, CA19-9, DUPAN-2 and SPan-1 for early detection of PDAC.* Serum levels of fucosylated REEP5, CEA and CA19-9 in 62 PDAC cases in Union for International Cancer Control stages I (n=21), II (n=22), III (n=15) and IV (n=4) and in 30 HVs and 10 PT cases are shown in Table V and Fig. 5. Cutoffs for the three markers were set at levels that gave 95.0% specificity for PDAC compared with HVs and PT: 0.05 AU/ml for fucosylated REEP5, 5.5 ng/dl for CEA and 37.7 U/ml for CA19-9. In the 62 PDAC cases, the sensitivities of fucosylated REEP5, CEA and CA19-9 were 75.8, 17.7 and 62.9%, respectively (data not shown). The fucosylated REEP5 level was above the cutoff in 20 of 22 PDAC cases (90.0%) in which the serum level of CA19-9 was below its cutoff value (data not shown). The AUCs of ROC curves were 0.928 for fucosylated REEP5 and 0.805 for CA19-9 for distinguishing cancer (PDAC) from non-cancer (HV and PT) cases (Fig. 6A). The ratio of serum fucosylated REEP5 to total serum REEP5 in the third cohort

was significantly higher in PDAC ( $0.52 \pm 0.20$  AU/ml) compared with in HVs ( $0.17 \pm 0.05$  AU/ml) and PT ( $0.18 \pm 0.02$  AU/ml) (PDAC vs. HVs and PT,  $P < 0.001$ ; Kruskal-Wallis test) (data not shown). Serum fucosylated REEP5 and the ratio of serum fucosylated REEP5 to total serum REEP5 differed significantly among HVs, PT and PDAC, showing a consistent trend. Thus, serum fucosylated REEP5 may be a useful biomarker for detecting pancreatic cancer, and may be an improved diagnostic PDAC biomarker compared with serum CA19-9.

To evaluate whether fucosylated REEP5 is useful for early detection of PDAC, serum REEP5 and CA19-9 levels in early stage PDAC (stages I and II) cases were compared with those in non-cancer cases (HV and PT). In 43 early stage PDAC cases, the sensitivities of fucosylated REEP5, CEA and CA19-9 at cutoffs that gave 95.0% specificity were 83.7, 14.0 and 60.5%, respectively (Fig. 5). The sensitivities of DUPAN-2 and SPan-1 at their respective cutoffs (DUPAN-2, 150 U/ml; SPan-1, 30 U/ml) were 20.9 and 41.9%, respectively (Table VI). The AUCs of ROC curves for distinguishing early stage PDAC from non-cancer cases were 0.962 for fucosylated REEP5 and 0.810 for CA19-9. These results indicate that REEP5 is superior to CA19-9 as an early diagnostic biomarker for PDAC (Fig. 6B). In early-stage PDAC cases, the sensitivity of fucosylated REEP5 in combination with CEA and CA19-9 was 67.4 and 86.4%, respectively, and the specificity was 85.7 and 100.0%, respectively. The AUCs of ROC curves for distinguishing PDAC (stages I and II) from non-cancer cases for fucosylated REEP5 alone and in combination with CEA and CA19-9 were 0.962, 0.857 and 0.999, respectively (Fig. 6C).

## Discussion

REEP5 is a member of a protein family that comprises REEP1 through REEP6. This family serves crucial roles

Table IV. Verification of biomarker candidate proteins using targeted proteomics.

| Protein name | Sequence   | Area ratio  |             |             |
|--------------|------------|-------------|-------------|-------------|
|              |            | HVs         | PT          | PDAC        |
| REEP5        | ETADAITK   | 0.001±0.001 | 0.000±0.000 | 0.012±0.016 |
| REEP5        | ATVNLLGEEK | 0.002±0.003 | 0.003±0.010 | 0.010±0.017 |

Data are shown as mean ± SD. Relative quantitation of peptides of biomarker protein candidates was performed using selected reaction monitoring analysis. HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; REEP5, receptor expression-enhancing protein 5.

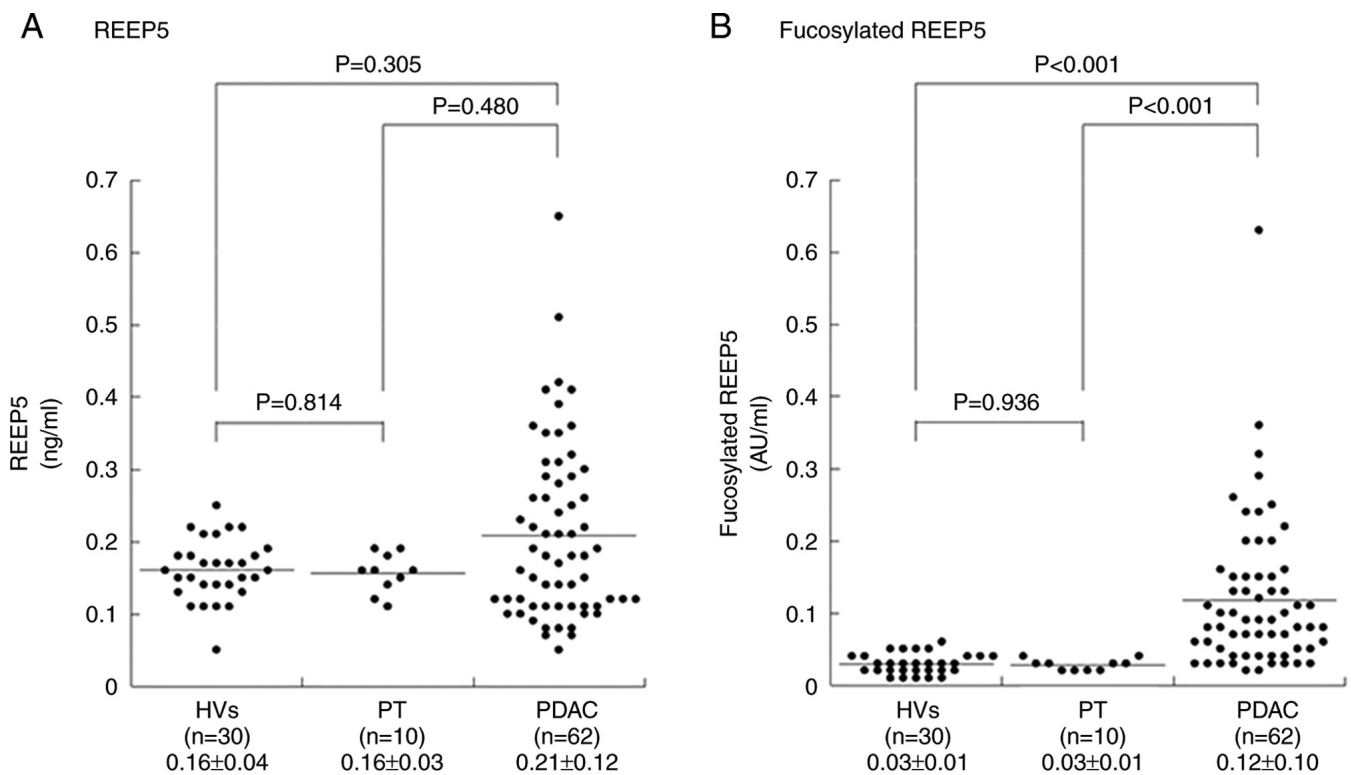


Figure 4. REEP5 and fucosylated REEP5 levels in HVs and in PT and PDAC. (A) Serum REEP5 levels in the third cohort were 0.16±0.04, 0.16±0.03 and 0.21±0.12 ng/ml in HVs, PT and PDAC, respectively. (B) Serum fucosylated REEP5 levels in the third cohort were 0.03±0.01, 0.03±0.01 and 0.12±0.10 AU/ml in HVs, PT and PDAC, respectively. HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; REEP5, receptor expression-enhancing protein 5.

in various cellular processes, including the shaping and remodeling of endoplasmic reticulum morphology (32-34), regulation of the microtubule cytoskeleton (35) and transport and expression of G protein-coupled receptors (36), among other key pathophysiological functions. REEP3 expression is markedly elevated in pancreatic cancer compared with normal tissues (37), and elevated REEP3 expression is associated with unfavorable overall and relapse-free survival, establishing it as a notable adverse prognostic marker in pancreatic cancer (37). REEP5 serves a key role in maintaining normal function of the cardiac sarcoplasmic reticulum (38,39). Chiamvimonvat and Song (40) showed that REEP5, as the only REEP family protein enriched in the heart, is a regulator of cardiac excitation-contraction coupling. REEP5 knockdown affects C-X-C motif chemokine

receptor 1 and downregulates the IL-8-mediated cellular response, which reduces growth and invasion of lung cancer cells (41), whereas REEP5 is downregulated in breast cancer tissues (42).

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 numerous cancers, but this is challenging for PDAC (43). In blood tests, hepatic enzymes are often increased and CA19-9 is a diagnostic marker of PDAC (43). A previous report showed greater elevation of CA19-9 in advanced stage cases and a smaller increase with 53% sensitivity in early stage I and II cases (44). Indeed, patients with early stage I and II of PDAC in the cohort of the present study showed 60.5% sensitivity for CA19-9.

Table V. Serum levels of fucosylated REEP5, CEA and CA19-9 in the third cohort.

| Marker                   | Non-cancer |           |             | PDAC, stage |               |                 |  |
|--------------------------|------------|-----------|-------------|-------------|---------------|-----------------|--|
|                          | HVs        | PT        | I           | II          | III           | IV              |  |
| Fucosylated REEP5, AU/ml | 0.03±0.01  | 0.03±0.01 | 0.11±0.06   | 0.14±0.13   | 0.12±0.08     | 0.07±0.07       |  |
| CEA, ng/ml               | 2.7±1.5    | 2.8±1.9   | 3.2±2.3     | 3.3±1.8     | 3.7±2.9       | 4.3±1.2         |  |
| CA19-9, U/ml             | 12.4±9.2   | 18.6±18.2 | 222.8±398.6 | 501.6±842.4 | 552.2±1,000.9 | 1,788.9±1,715.9 |  |

Data are shown as mean ± SD. HVs, healthy volunteers; PT, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; REEP5, receptor expression-enhancing protein 5.

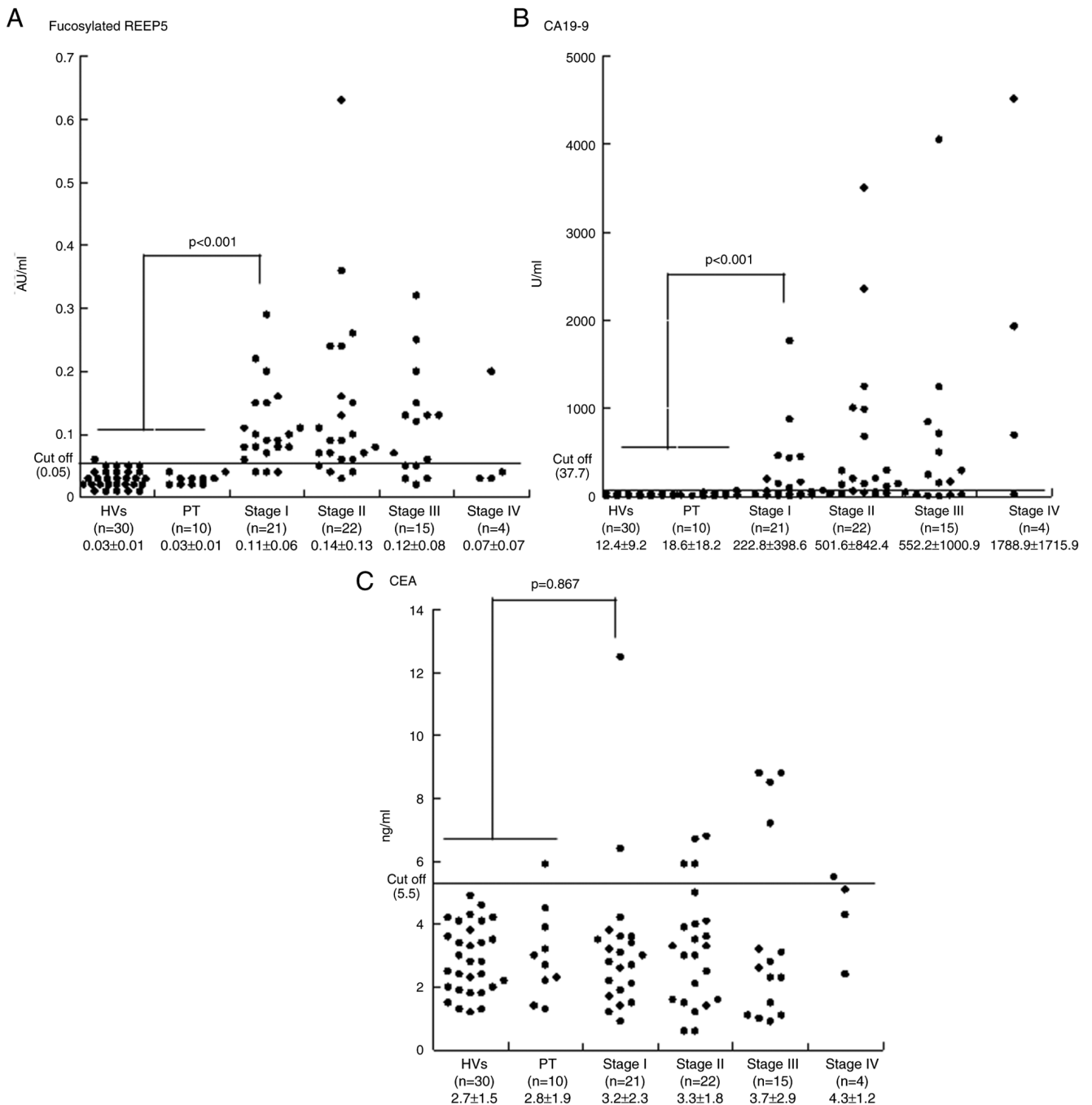


Figure 5. Fucosylated REEP5, CA19-9 and CEA levels in HVs and patients with chronic pancreatitis and PDAC (stage I-IV). (A) Serum fucosylated REEP5, (B) serum CA19-9 and (C) serum CEA levels in the third cohort. REEP5, receptor expression-enhancing protein 5; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; HVs, healthy volunteers; PDAC, pancreatic ductal adenocarcinoma.

Table VI. Serum levels of DUPAN-2 and SPan-1 in the third cohort.

| Marker        | Sensitivity (%) | PDAC, stage |             |             |               |
|---------------|-----------------|-------------|-------------|-------------|---------------|
|               |                 | I           | II          | III         | IV            |
| DUPAN-2, U/ml | 20.9            | 233.7±451.4 | 301.1±514.4 | 573.1±615.6 | 1,031.3±623.8 |
| SPan-1, U/ml  | 41.9            | 43.7±52.0   | 67.0±105.0  | 129.5±183.1 | 292.0±357.7   |

Data are shown as mean ± SD. PDAC, pancreatic ductal adenocarcinoma.

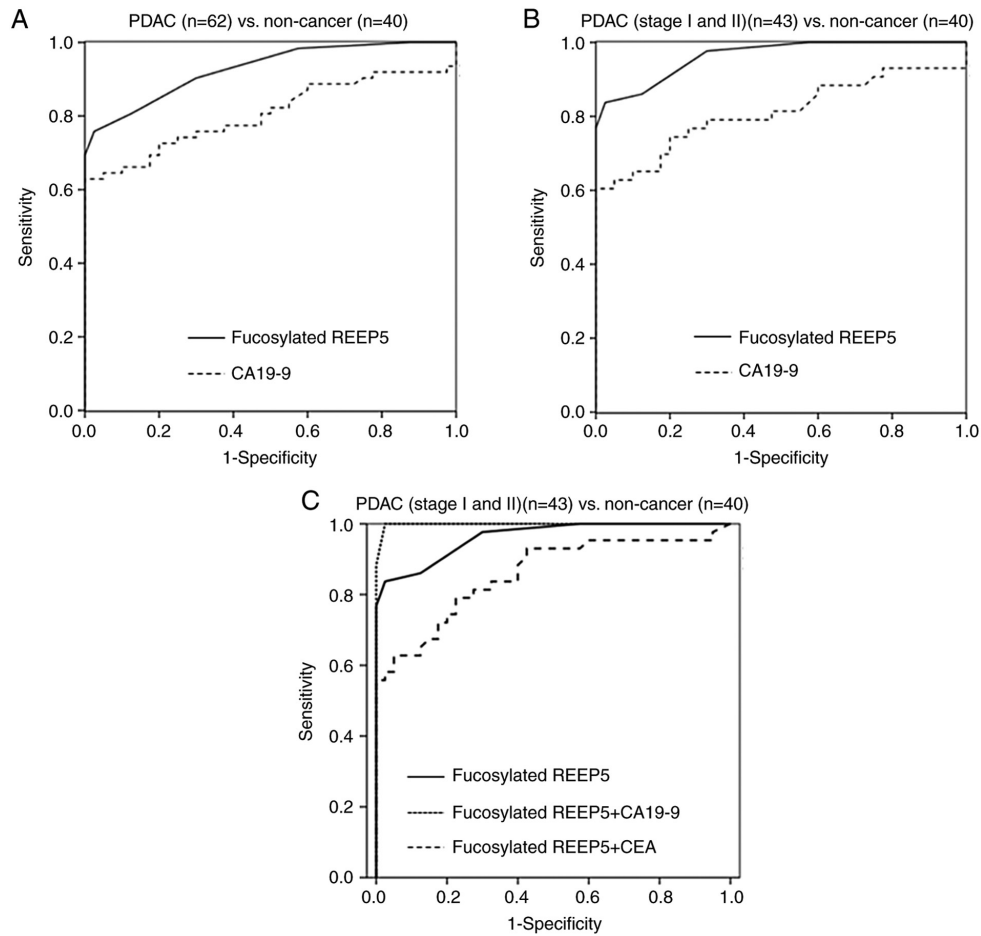


Figure 6. Fucosylated REEP5 is superior to CA19-9 for early detection of PDAC. (A) AUCs were 0.928 for fucosylated REEP5 and 0.805 for CA19-9 for distinguishing cancer (PDAC) from non-cancer (healthy volunteers and chronic pancreatitis) cases. (B) AUCs for distinguishing PDAC from non-cancer cases were 0.962 for fucosylated REEP5 and 0.810 for CA19-9. (C) AUCs for distinguishing PDAC (stage I and II) from non-cancer cases were 0.857 for fucosylated REEP5 combined with CEA, and 0.999 for fucosylated REEP5 combined with CA19-9. REEP5, receptor expression-enhancing protein 5; CA19-9, carbohydrate antigen 19-9; PDAC, pancreatic ductal adenocarcinoma; CEA, carcinoembryonic antigen; AUC, area under the curve.

In previous years, new biomarkers have been found through advances in proteomics techniques, but a number are still not used in clinical practice (17). Ideally in biomarker development, control and disease groups are compared using a semiquantitative method to search for candidate markers in the discovery phase. The identified candidates are subsequently transferred to a validation phase that may include an ELISA, which is the most common assay in current use. However, it is often difficult to reproduce MS-based quantitative results for the candidate markers using an ELISA, as seen in the present results. The reason why REEP5 was increased in EVs of PDAC

compared to HVs and PT, but not in serum, may be because EV proteins were measured using MS, which is more sensitive than ELISA. Thus, the use of MS may be more appropriate to quantify candidate biomarker proteins identified by proteomic analysis. Alternatively, the level of soluble REEP5 protein in serum may be the same among HVs, PT and PDAC, but increased in EVs in PDAC. In this case, the increased secretion of REEP5 in EVs may be due to the high expression of REEP5 protein in pancreatic cancer tissue.

Fucosylated oligosaccharides and proteins have been used as cancer biomarkers since they were demonstrated to be

increased in serum of patients with cancer several decades ago. Fucosylated  $\alpha$ -fetoprotein (AFP), which is referred to as the AFP-L3 fraction after the measurement method, has been used clinically as a biomarker for hepatocellular carcinoma since 1996 in Japan and 2005 in the United States (27,28). Similarly, a fucosylated oligosaccharide, sialyl Lea (sLex), has been used as a biomarker for pancreatic cancer (29). Binding of sLex or sLea on cancer cells to E-selectin on endothelial cells facilitates tumor metastasis (30), and expression of these proteins is associated with a poor prognosis of colon cancer (31).

Fucosylated complement component 4 binding protein  $\alpha$  (C4BPA) has previously been reported as an early diagnostic marker for pancreatic cancer (17). Serum C4BPA was elevated in PDAC compared with HVs and PT ( $P < 0.01$ ), with serum fucosylated C4BPA more significantly elevated in PDAC ( $P < 0.0001$ ). In early stage pancreatic cancer, the increase in fucosylation was more prominent compared with the increase in total C4BPA, possibly due to activation of fucosylation enzymes in PDAC (45). Similarly, REEP5 was low in most PDAC cases and did not differ markedly from those in HVs or PT. By contrast, fucosylated REEP5 levels were significantly higher in PDAC cases compared with in HVs and PT, although some samples did not show increased levels of fucosylated REEP5.

These findings provided a basis for investigation of fucosylated proteins and to develop an ELISA for REEP5. Using this approach, serum fucosylated REEP5 was identified as a new PDAC biomarker. Serum fucosylated REEP5 levels were  $0.03 \pm 0.01$ ,  $0.03 \pm 0.01$  and  $0.12 \pm 0.10$  AU/ml in HVs, PT and PDAC, respectively, and differed significantly between PDAC and HV and PT cases, but not between PT and HVs. Most stage I PDAC cases (83.7%) were positive for serum fucosylated REEP5, whereas only 60.5% of these cases were positive for CA19-9. These results indicate that serum fucosylated REEP5 is complementary to conventional markers and may be useful for detection of PDAC in clinical practice.

The present study has several limitations. First, the screen did not find previously identified tumor markers, most of which are glycoproteins, including CA 19-9. It might be because the methodology used did not target carbohydrate-containing proteins. Second, although EV proteins were focused on for biomarker discovery, the biomarker candidate protein REEP5 was assessed using whole serum. This is due to the difficulties to use whole serum for biomarker discovery by MS and to analyze EVs for verification. The dynamic range of blood proteins is extremely wide which is a disadvantage for measurement using MS. If a candidate protein is present only in small amounts, its detection in total blood protein may be challenging, even with use of SRM (8). On the other hand, consistent extraction of EVs from large sample sets for biomarker verification is laborious, therefore whole serum was used for verification. The use of whole serum instead of EVs may be the reason why the results of REEP5 could not be reproduced. Third, the present study is a retrospective study involving a small sample size. Further validation in independent, prospective, large-scale cohorts is necessary to establish fucosylated REEP5 as a more promising serodiagnostic biomarker for PDAC compared with CA19-9.

Finally, fucosylated REEP5 is associated with PDAC, but its potential biological functions and mechanisms in

the development and progression of PDAC remain unclear. Understanding the expression and localization of REEP5 in PDAC, as well as the impact of its fucosylation, is essential. Core fucosylation comprises the transfer of  $\alpha$ -1,6-fucose to the innermost GlcNAc residue of N-glycans by fucosyltransferase 8 (FUT8) (46). Core fucosylation mediated by FUT8 is one of the most important glycan modifications involved in cancer and inflammation (46). Watanabe *et al.* (47) reported that FUT8 expression increases gradually from normal pancreatic duct to adenoma and then carcinoma in an intraductal papillary mucinous neoplasm, a mucinous cystic tumor of the pancreas with malignant transformation potential. This suggests that increased FUT8 expression, and thus activation of the core fucosylation reaction, may serve a key role in the malignancy of pancreatic cancer (45). The functional analysis of REEP5 fucosylation is challenging due to the unidentified enzymes responsible for the modification and the unknown fucosylation sites on the protein.

In conclusion, the present study established a novel lectin ELISA for the measurement of serum fucosylated REEP5. Serum fucosylated REEP5 has notable diagnostic ability with potential applications for PDAC.

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#### Availability of data and materials

The data generated in the present study may be found in the ProteomeXchange Consortium via the jPOST repository under accession number PXD066048 or at the following URL: <https://repository.jpostdb.org/entry/JPST003936>. The rest of the data generated in the present study may be requested from the corresponding author.

#### Authors' contributions

KS was a major contributor in writing the manuscript. ST and TT contributed to manuscript drafting or critical revisions on the intellectual content. RY, MS, SM and JA carried out the proteomics experiments and the bioinformatics analysis. ST, HT and MO contributed to collecting the samples, the ethics application and interpretation of the data. KS and TT confirm the authenticity of all the raw data. KS, RY, MS, SM, JA, ST, HT, MO and TT contributed to the analysis and interpretation of the data. All authors read and approved the final version of the manuscript.

#### Ethics approval and consent to participate

The study was performed in accordance with the guidelines established by the Declaration of Helsinki and was approved by the Ethics Committee of Chiba University (approval nos. 2155 and M10831), Azabu University (approval nos. 2539

and 0129) and Kashiwado Memorial Foundation (approval no. 002).

### Patient consent for publication

Written informed consent was provided by patients for collection and publication of clinical data and information.

### Competing interests

The authors declare that they have no competing interests.

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