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Circulating cancer-associated extracellular vesicles as early detection and recurrence biomarkers for pancreatic cancer

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Abstract

Early detection of pancreatic ductal adenocarcinoma (PDAC) is essential for improving patient survival rates, and noninvasive biomarkers are urgently required to identify patients who are eligible for curative surgery. Here, we examined extracellular vesicles (EVs) from the serum of PDAC patients to determine their ability to detect early-stage disease. EV-associated proteins purified by ultracentrifugation and affinity columns underwent proteomic analysis to identify novel PDAC markers G protein-coupled receptor class C group 5 member C (GPRC5C) and epidermal growth factor receptor pathway substrate 8 (EPS8). To verify the potency of GPRC5C- or EPS8-positive EVs as PDAC biomarkers, we analyzed EVs from PDAC patient blood samples using ultracentrifugation in two different cohorts (a total of 54 PDAC patients, 32 healthy donors, and 22 pancreatitis patients) by immunoblotting. The combination of EV-associated GPRC5C and EPS8 had high accuracy, with area under the curve values of 0.922 and 0.946 for distinguishing early-stage PDAC patients from healthy controls in the two cohorts, respectively, and could detect PDAC patients who were negative for CA19-9. Moreover, we analyzed 30 samples taken at three time points from 10 PDAC patients who underwent surgery: before surgery, after surgery, and recurrence as an early-stage model. These proteins were detected in EVs derived from preoperative and recurrence samples. These results indicated that GPRC5C- or EPS8-positive EVs were biomarkers that have the potential to detect stage I early pancreatic cancer and small recurrent tumors detected by computed tomography.

KEYWORDS
early diagnosis, extracellular vesicles, liquid biopsy, pancreatic ductal adenocarcinoma, proteomic analysis

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is predicted to overtake colorectal cancer as the second leading cause of cancer deaths by 2025[1] and currently has the worst prognosis among the most common cancers, with an overall 5-year survival rate of less than 10%.[2] Tumor stage is the main prognostic determinant, and early-stage tumors are associated with longer survival than locally advanced or metastatic tumors.[3] The reason for this is that although surgical resection is the only treatment for pancreatic cancer, patients who have metastasis to blood vessels, lymph nodes, or other organs are not eligible for surgery. Unfortunately, however, because of the lack of typical early symptoms and highly aggressive biological characteristics, most pancreatic cancer cases are diagnosed at an advanced stage and are not eligible for curative surgery[4,5] leading to dismal clinical outcomes. For certain cancer types, screening tests using molecular biomarkers are helpful for identifying cancer in individuals who have no symptoms. Although CA19-9 is the most commonly used biomarker for the diagnosis and management of patients with pancreatic cancer, this biomarker does not possess the accuracy required for screening asymptomatic populations.[6,7] Therefore, CA19-9 is used in conjunction with imaging to direct diagnostic and treatment decisions in patients with suspected PDAC or other periampullary diseases, and not for screening purposes. Therefore, the development of screening or diagnostic biomarkers for early pancreatic cancer detection is a major goal for improving the poor prognosis of PDAC patients.

In the past decade, extracellular vesicles (EVs) have gained attention as novel tumor biomarkers to detect various cancer types and disease statuses.[8,9] EVs are lipid membranous vesicles that are actively released from almost all types of cells, including normal cells as well as abnormal cells such as cancer cells.[10] One of the characteristics of EVs is that they are molecularly complex entities that carry lipids, soluble and transmembrane proteins, various RNA species, including mRNA and miRNA, and DNA. Moreover, the total molecular composition of EVs varies depending on the type and functional state of the cell of origin and even the disease state. Therefore, disease-specific or related molecules of EVs can be a signature of cancer cells and used to identify disease-related biomarkers.[11,12] For this purpose, molecules that can be used as biomarkers should be identified. In particular, finding specific molecules from EVs present in body fluids is valuable because it can easily lead directly to biomarkers. However, it is not easy to obtain pure EV proteins from body fluids, especially blood.[13] Therefore, the critical point is to purify EVs that do not contain major proteins such as albumin, transferrin, and immunoglobulin G in the blood, especially for proteomic analysis.

In this study, we purified circulating EV proteins using improved methods for purification based on ultracentrifugation and affinity column approaches. In addition, we compared the proteomic profiles of purified serum EV proteins derived from non-PDAC donors and PDAC patients. From the results of the proteomic analysis, EV-associated GPRC5C and EPS8 were selected as candidate PDAC biomarkers. Furthermore, we evaluated the clinical utility of GPRC5C- or EPS8-positive serum EVs as novel PDAC biomarkers, focusing on their associations with tumor stage and serum CA19-9, and their changing levels after surgery and at the early stage of recurrence.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

All serum samples from healthy donors and pancreatitis patients were purchased from BizCom Japan: healthy donors for the proteomic analysis (n = 7), verification cohort (n = 18), and validation cohort (n = 14), and pancreatitis patients for the verification cohort (n = 14) and validation cohort (n = 8). Serum samples from PDAC patients for the proteomic analysis (n = 15) and verification cohort (n = 27) were purchased from BizCom Japan. Collection and usage of PDAC patient sera for the validation cohort (n = 27) were approved by Osaka University Institutional Review Board (approval no. 664). To evaluate monitoring markers for recurrence, we obtained serum samples taken at three time points from 10 enrolled patients: before surgery, after surgery, and at recurrence. This experiment involving human subjects was approved by the National Cancer Center Hospital East Institutional Review Board (approval no. 2007-060). Sera were aliquoted and stored at −80°C until use, and freeze-thawing was avoided as much as possible before use. Informed consent was obtained from all patients and healthy donors. Participant clinical information is provided in Tables S1, S2, S3, and S4.

2.2 | Purification of EV proteins for proteomic analysis

For proteomic analysis, 3 ml serum from patients of each stage (stages II, III, and IV) and from healthy donors were collected (3–8 donors per group), and EVs were enriched by ultracentrifugation. After washing, EVs were lysed by detergent (M-PER Mammalian Protein Extraction Reagent, Thermo Scientific) and the buffer was exchanged for PBS using spin columns (Amicon Ultra – 0.5 ml 3 K; Merck). EV proteins with contaminating serum proteins were purified by affinity columns (Agilent Human 14 Multiple Affinity

Removal System Spin Cartridges for the Depletion of High-Abundant Proteins from Human Proteomic Samples; Agilent). Before injection of the samples, EV proteins were cleaned up with a 0.22-μm spin filter, and then the cleaned samples were loaded onto the spin cartridge. The flow-through fractions were collected and concentrated using 2 kDa cut spin columns (Vivacon 500, 2000 MWCO; Sartorius). LC–MS/MS analysis was performed using these purified EV proteins (Figure 1A).

Additional materials and methods are available in Appendix S1.

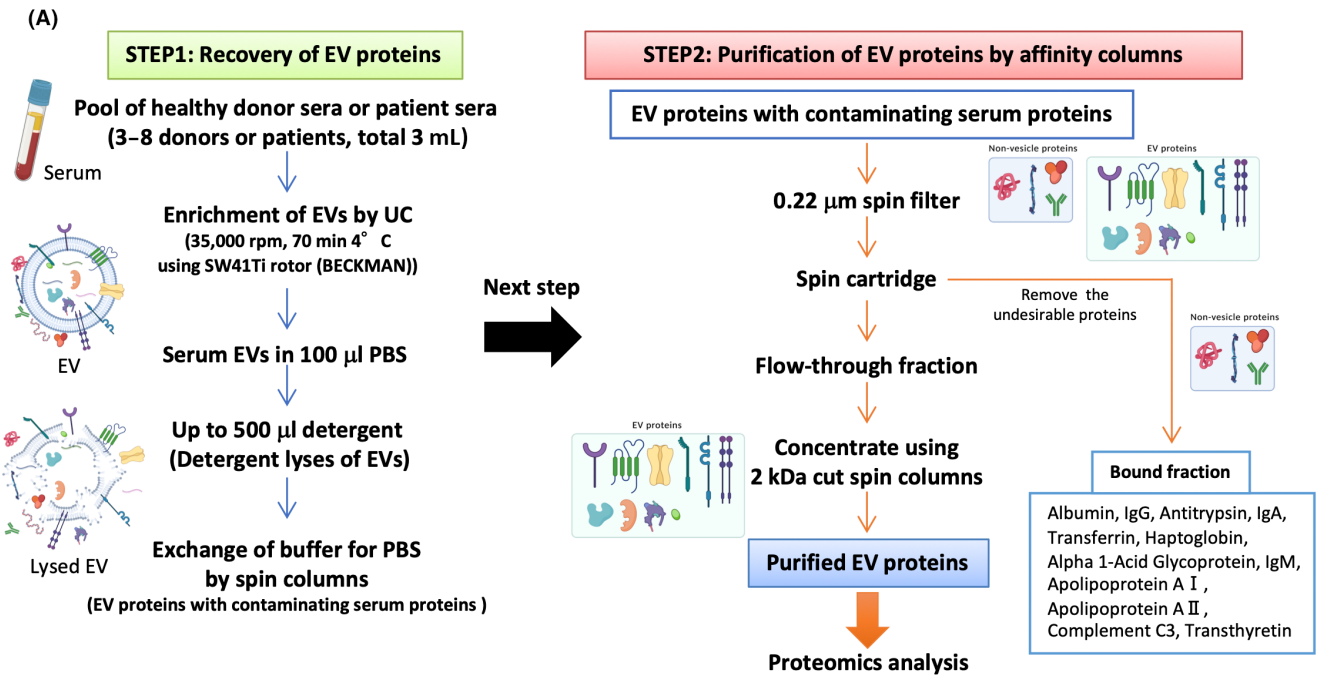
3 | RESULTS

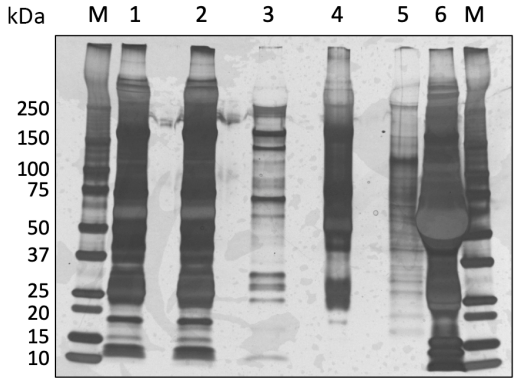
3.1 | Hunting for pancreatic cancer biomarkers from proteome profiles of serum EVs

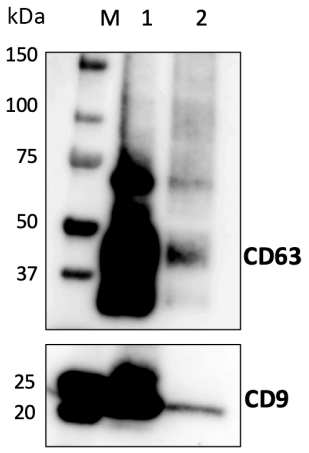
When hunting for biomarkers by proteomic analysis, high concentrations of unwanted serum proteins in ultracentrifuged samples are likely to impede the identification of EV-associated proteins with potential use as disease biomarkers. Therefore, to remove nonvesicular proteins and enrich EV-associated proteins, we first investigated the purification methods using ultracentrifugation and blood protein affinity columns. The experimental workflow is depicted in Figure 1A. After purification, we checked the efficiency of this method in removing potential contaminating proteins from EV protein fractions using silver staining (Figure 1B). As shown in Figure 1B, EV protein fractions obtained from this method (lane 3) exhibited decreased protein content of the concentrate compared with EVs derived from serum by simple ultracentrifugation (lane 1). This result indicated that the affinity column removed major serum proteins such as albumin and transferrin IgG and decreased the protein content. To confirm the recovery of EV-associated proteins, CD9 and CD63, known as EV markers, were detected by immunoblotting (Figure 1C). Although the amount of CD9 and CD63 detected was lower than that of the simple ultracentrifugation methods, the EV protein fraction purified by our current method also contained these markers, indicating that we were able to obtain EV-associated proteins. Therefore, purified serum EV proteins from non-PDAC donors (healthy donors) and PDAC patients (stages II, III, and IV) were subjected to proteomic analysis. As a result, we identified a total of 541–613 proteins in each group, and 418 proteins overlapped between the non-PDAC and PDAC groups (Figure 1D). To narrow down candidate proteins as PDAC biomarkers, we selected those detected exclusively in PDAC but not non-PDAC. Moreover, we listed 50 proteins as candidate proteins detected in the stage II group, which is the earliest stage among the three stages, and in at least one of the remaining two groups. To narrow down the list of 50 candidate proteins, we used ExoCarta (http://exocarta.org), a database that allows us to search for molecules contained in EVs (exosomes), and have selected those that are much more probably to be found in EV contents. Then we selected the candidate proteins from among them that belong to cellular components identified by gene ontology as "membrane" to narrow the focus to EV membrane proteins. Finally, we focused on five of these proteins and performed immunoblotting to confirm the detection of these proteins in the same samples. The results showed that epidermal growth factor (EGF) receptor pathway substrate 8 (EPS8) and G protein-coupled receptor class C group 5 member C (GPRC5C) were detected in the samples of stage II and stage IV groups, but not in the healthy donor group, as well as in the proteomic analysis (Figure 1E).

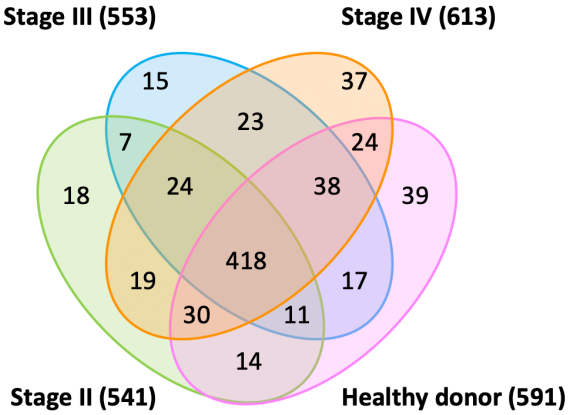
3.2 | Characteristics of GPRC5C and EPS8 in pancreatic cancer

GPRC5C and EPS8 were selected for further analysis. Before the verification and validation study using patient sera, we used public databases to characterize *GPRC5C* and *EPS8* and performed experiments in cell lines. We used the Oncomine database (https://www.oncomine.org/resource/login.html) to analyze the differential expression of *GPRC5C* or *EPS8* in pancreatic cancer patients and healthy controls (Figure 2A,B). It was found that *EPS8* expression was significantly elevated in pancreatic cancer tissue compared with the corresponding normal tissue, whereas *GPRC5C* expression was not. We further explored the relationship between gene expression in tumors and tumor malignancy, especially in the survival of patients with pancreatic cancer, using publicly available datasets (https://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq). Kaplan–Meier curve and log rank test analyses revealed that the high expression level of *EPS8* was significantly associated with poor overall survival and relapse-free survival of all the patients with PDAC (Figure 2E,F). However, *GPRC5C* differed from *EPS8* as well as the results of the expression analysis of tumors in Oncomine (Figure 2C,D). In other words, we found that decreased tumoral expression of *GPRC5C* was associated with poor prognosis. Taken together, these results suggested that *EPS8* is a possible oncogene and *GPRC5C* is a possible tumor suppressor gene. Next, we analyzed the relationship between the expression of EPS8 and GPRC5C in pancreatic cancer cells and EVs secreted from those cells by immunoblotting. As shown in Figure 2G, GPRC5C was highly expressed in noncancerous cells and cancer cells but not in SW1990 cells, whereas EPS8 was upregulated in hTERT-HPNE cells, which are noncancerous (Figure 2G). To confirm the amount of GPRC5C and EPS8 in EVs, we collected EVs from these six cell lines and performed immunoblotting. EV-associated GPRC5C and EPS8 were detected only in EVs derived from PANC1, Capan-1, and SW1990 cells (Figure 2H). These results indicated that the intracellular expression of GPRC5C and EPS8 did not correlate with their levels secreted by EVs, and the amount loaded to EVs was increased in specific cancer cells. Furthermore, EVs derived from these three cell lines were positive for both GPRC5C and EPS8. Therefore, because pancreatic cancer cells, but not all, loaded and secreted GPRC5C and EPS8 into EVs, and noncancerous cells did not load these proteins, GPRC5C and EPS8 in EVs could be biomarkers for pancreatic cancer.


(A)
STEP1: Recovery of EV proteins
Pool of healthy donor sera or patient sera
(3–8 donors or patients, total 3 mL)
Serum
Enrichment of EVs by UC
(35,000 rpm, 70 min 4° C
using SW41Ti rotor (BECKMAN))
EV
Serum EVs in 100 μl PBS
Up to 500 μl detergent
(Detergent lyses of EVs)
Lysed EV
Exchange of buffer for PBS
by spin columns
(EV proteins with contaminating serum proteins)
Next step
STEP2: Purification of EV proteins by affinity columns
EV proteins with contaminating serum proteins
0.22 μm spin filter
Non-vesicle proteins
EV proteins
Spin cartridge
Remove the
undesirable proteins
Non-vesicle proteins
Flow-through fraction
EV proteins
Concentrate using
2 kDa cut spin columns
Bound fraction
Albumin, IgG, Antitrypsin, IgA,
Transferrin, Haptoglobin,
Alpha 1-Acid Glycoprotein, IgM,
Apolipoprotein A Ⅰ,
Apolipoprotein A Ⅱ,
Complement C3, Transthyretin
Purified EV proteins
Proteomics analysis


(B)
kDa
M 1 2 3 4 5 6 M
250
150
100
75
50
37
25
20
15
10


(C)
kDa
M 1 2
150
100
75
50
37
CD63
25
20
CD9


(D)
Stage III (553)
Stage IV (613)
15
37
23
7
24
24
18
38
39
418
19
17
30
11
14
Stage II (541)
Healthy donor (591)

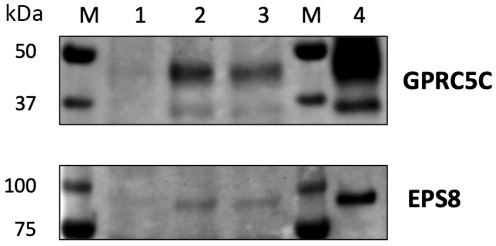

(E)
kDa
M 1 2 3 M 4
50
37
GPRC5C
100
75
EPS8

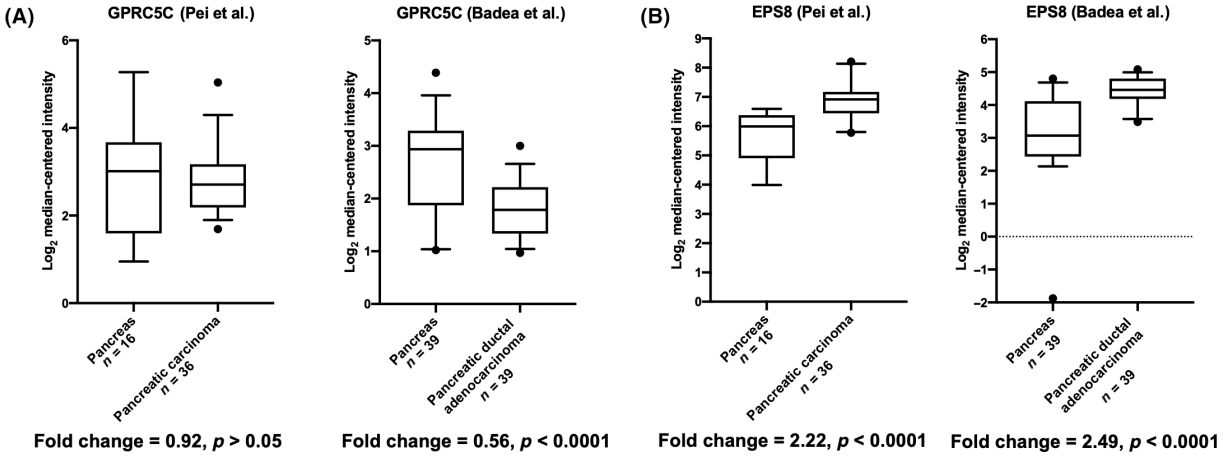
FIGURE 1 Discovery of a novel EV-associated PDAC biomarker in serum. (A) Workflow of the purification of EV proteins from serum. The detailed method is described in the Section 2. The figure was prepared using BioRender (www.biorender.com). (B) Silver staining of each purification step sample. Lane 1, EVs derived from serum by ultracentrifugation; lane 2, EV proteins with contaminating serum proteins; lane 3, purified EV proteins; lane 4, bound fraction proteins (major serum proteins); lane 5, 1 μg EVs derived from cell lines; lane 6, 1 μl serum; M, marker. Each lane was loaded with proteins extracted from 100 μl serum (excluding lanes 5 and 6). (C) Detection of EV marker proteins in EVs by simple isolation methods and purified EV protein fraction. EV proteins derived from healthy donor serum were immunoblotted with anti-human CD63 and CD9 antibodies. Lane 1, enriched EVs by simple ultracentrifugation; lane 2, purified EV proteins; M, marker. Each lane was loaded with EVs from 50 μl serum. (D) Venn diagram of serum EV proteins. Venn diagram showing the overlap of serum EV proteins identified in PDAC patients and healthy donors. Each value in the diagram shows the number of identified proteins by LC–MS/MS. (E) Validation of the results of proteomic analysis. Upregulation of GPRC5C and EPS8 in EVs from pancreatic cancer patient serum was confirmed by immunoblotting analysis using the same samples used in the proteomic analysis. Lane 1, healthy donors; lane 2, PDAC patients (stage II); lane 3, PDAC patients (stage IV); PANC1 EVs; M, marker. Each lane (excluding lane 4) was loaded with EVs from 100 μl serum.

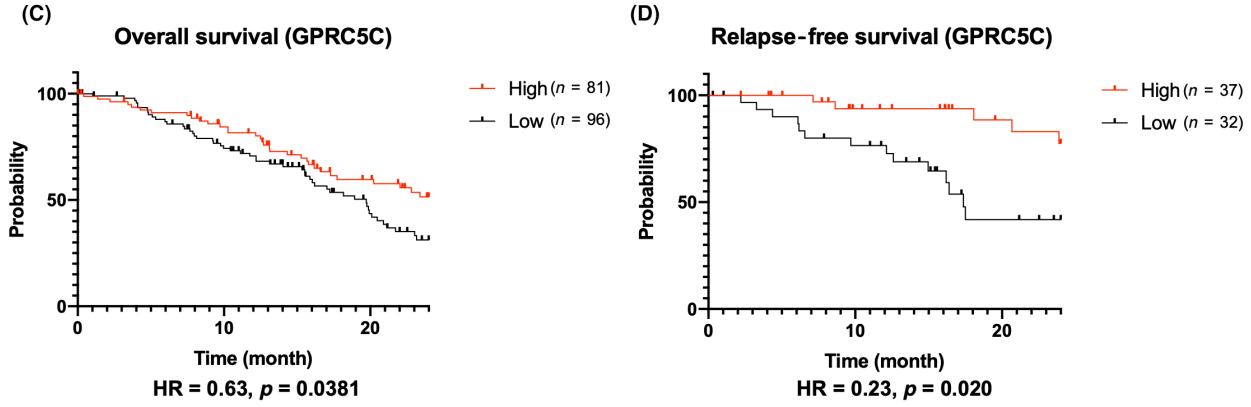
3.3 | Verification of GPRC5C- or EPS8-positive EVs as PDAC biomarkers using immunoblotting

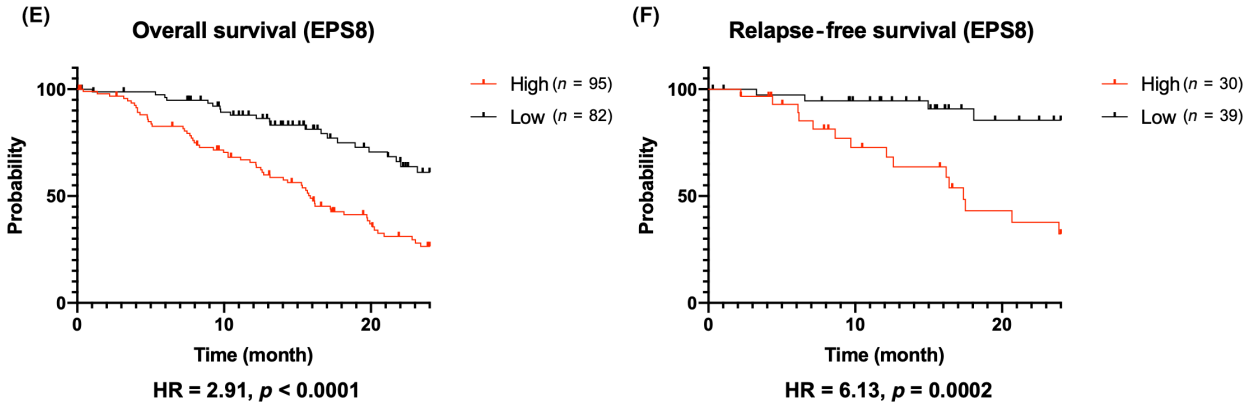
To verify the potency of GPRC5C- or EPS8-positive EVs as PDAC biomarkers, we first tested whether EV-associated GPRC5C and EPS8 could be detected by immunoblotting using serum from a small number of healthy controls, pancreatitis patients, and PDAC patients (n = 4; each group; Figure 3A,B). The EVs used for immunoblotting were collected using a different method from that used for the proteomic analysis because of the need to analyze a large number of samples. In other words, EVs were collected by a simple pellet-down method using ultracentrifugation. As shown in Figure 3A,B, GPRC5C and EPS8 were rarely detected in sera from healthy donors and slightly in sera from patients with pancreatitis. However, these proteins were often detected in the sera of PDAC patients. Furthermore, similar to the results of the proteomic analysis, GPRC5C and EPS8 could be detected in EVs derived from the sera of stage II patients (PC1 and 3). From this result, we found that GPRC5C and EPS8 could be quantified by immunoblotting even in EVs collected by a simple pellet-down method. Next, we performed immunoblotting of GPRC5C and EPS8 derived from serum EVs of patients with PDAC (n = 27), patients with pancreatitis (n = 14), and healthy controls (n = 18). Detailed data related to clinical sample characteristics are provided in Table S2. The relative amount of GPRC5C and EPS8 is shown in Figure 3C. GPRC5C- or EPS8-positive EVs were significantly more abundant in PDAC patient serum than in healthy controls. However, GPRC5C and EPS8 levels were not significantly different between PDAC patients and pancreatitis patients. Additionally, to assess the diagnostic performance of EV-associated GPRC5C and EPS8, we performed a receiver operating characteristic (ROC) curve analysis in PDAC (Figure 3D, left) and took the area under the ROC curve (AUC). The AUC for GPRC5C was 0.722, and the AUC for EPS8 was 0.786. Combined ROC curve analysis using GPRC5C and EPS8 revealed an AUC of 0.854 for distinguishing PDAC patients (n = 27) from healthy controls (n = 18). Furthermore, we further evaluated the diagnostic value of EV-associated GPRC5C and EPS8 levels for early-stage PDAC patients (stages I and IIA; Figure 3D, middle). The AUC of GPRC5C was 0.733 and the AUC for EPS8 was 0.811. Combined ROC curve analysis using GPRC5C and EPS8 revealed an AUC of 0.922 for distinguishing early-stage PDAC patients (n = 10) from healthy controls (n = 18). These results indicated that the diagnostic performances of EV-associated GPRC5C and EPS8 were better for early-stage disease rather than for all stages. In other words, EV-associated GPRC5C and EPS8 are more suitable for early-stage PDAC patient discrimination. Moreover, we also analyzed their performance in distinguishing between early-stage PDAC and chronic pancreatitis using the ROC curve. For the detection of early-stage pancreatic cancer compared with chronic pancreatitis, the AUC values for GPRC5C, EPS8, and the combination of GPRC5C and EPS8 were 0.740, 0.770, and 0.790, respectively (Figure 3D, right). These results indicated that GPRC5C and EPS8 may be moderate biomarkers, with AUC values of ~0.8, although their performance in distinguishing between pancreatic cancer and pancreatitis patients was lower than that between pancreatic cancer patients and healthy individuals.

3.4 | Comparison of GPRC5C- or EPS8-positive EVs with conventional tumor marker CA19-9

To further evaluate the performances of EV-associated GPRC5C and EPS8, we compared them with CA19-9, a conventional tumor marker. We were able to obtain CA19-9 values in 15 of the 27 samples shown in Figure 3C. The values of CA19-9, EPS8, and GPRC5C for these samples are summarized in Table 1, and we determined the optimal cutoff values for EV-related GPRC5C and EPS8 using the Youden index. At these cutoff values (GPRC5C, 1.210; EPS8, 1.197), the sensitivity of GPRC5C and EPS8 was 51.9% and 66.7%, and their specificity was 88.9% and 83.3%, respectively. Importantly, GPRC5C and EPS8 were able to detect seven of the 10 patients that were negative for CA19-9 (cutoff value: 37 U/ml). In addition, the values of GPRC5C and EPS8 were correlated, and similar to the cell lines (Figure 2H), GPRC5C and EPS8 tended to be double positive. However, no significant correlations were found between the serum levels of CA19-9 and EV-associated GPRC5C or EV-associated EPS8; the Pearson's correlation coefficient (R) was 0.134 between CA19-9 and GPRC5C, and 0.139 between CA19-9 and EPS8 (Figure S1). These results indicated that EV-associated GPRC5C and EPS8 could be useful novel biomarkers for distinguishing patients with PDAC from patients without the disease.


(A)
GPRC5C (Pei et al.)
GPRC5C (Badea et al.)
Log2 median-centered intensity
Pancreas n = 16
Pancreatic carcinoma n = 36
Pancreas n = 39
Pancreatic ductal adenocarcinoma n = 39
Fold change = 0.92, p > 0.05
Fold change = 0.56, p < 0.0001
(B)
EPS8 (Pei et al.)
EPS8 (Badea et al.)
Fold change = 2.22, p < 0.0001
Fold change = 2.49, p < 0.0001


(C)
Overall survival (GPRC5C)
Probability
Time (month)
High (n = 81)
Low (n = 96)
HR = 0.63, p = 0.0381
(D)
Relapse-free survival (GPRC5C)
High (n = 37)
Low (n = 32)
HR = 0.23, p = 0.020


(E)
Overall survival (EPS8)
High (n = 95)
Low (n = 82)
HR = 2.91, p < 0.0001
(F)
Relapse-free survival (EPS8)
High (n = 30)
Low (n = 39)
HR = 6.13, p = 0.0002

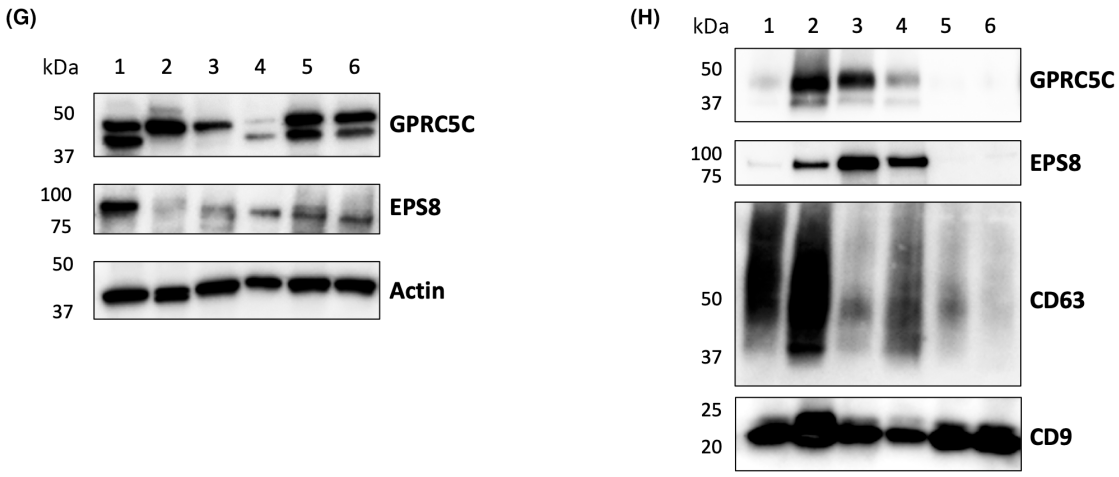

(G)
kDa
GPRC5C
EPS8
Actin
(H)
CD63
CD9

FIGURE 2 Expression analysis of GPRC5C and EPS8 in pancreatic tumor tissue and pancreatic cancer cell lines. Analysis of the mRNA levels of *GPRC5C* (A) and *EPS8* (B) in Oncomine database-derived pancreatic cancer patients. Box plots derived from gene expression data in the Oncomine database (two different studies; Pei et al.[33] and Badea et al.[34]) comparing the expression of *GPRC5C* and *EPS8* mRNA in normal tissue and pancreatic cancer. The black line in the box represents the median. Fold change and *p*-values are shown. Kaplan–Meier plots showing overall survival (C; GPRC5C, E; EPS8) and relapse-free survival (D; GPRC5C, F; EPS8) in pancreatic cancer. Survival probability and relapse-free survival are represented on the *y*-axis, and time is represented on the *x*-axis. Black curve corresponds to low *GPRC5C* or *EPS8* mRNA expression and red curves to high *GPRC5C* or *EPS8* mRNA expression. The number of PDAC patients is shown in the figure legend. HR, hazard ratio. The expression levels of GPRC5C and EPS8 in pancreatic cancer cell lines, pancreatic cell line (noncancer cell line), and EVs derived from these cell lines. (G) Whole-cell lysates and (H) EV-derived conditioned medium were analyzed by immunoblotting using anti-GPRC5C and anti-EPS8 antibodies. β-Actin was used as a control. CD63 and CD9 were used as EV protein markers. EV samples were loaded with equivalent total numbers of EV particles as determined by nanoparticle tracking analysis (NTA). Lane 1, hTERT-HPNE (noncancer cell); lane 2, PANC1; lane 3, Capan-1; lane 4, SW1990; lane 5, BxPc3; lane 6, MIAPaca2.

3.5 | Validation of GPRC5C- or EPS8-positive EVs in an independent cohort of PDAC and evaluation of monitoring markers for recurrence

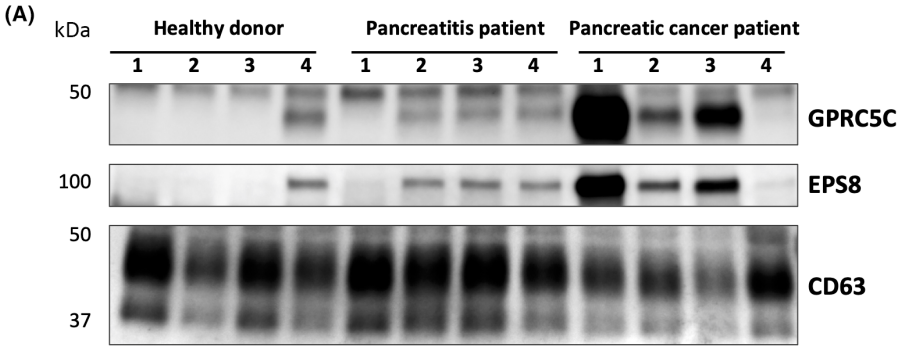
We extended our investigation using another cohort from a different hospital to validate the results obtained in the first verification cohort. The validation cohort included 27 PDAC patients, including 15 stage I patients, eight patients with chronic pancreatitis, and 14 healthy donors. We analyzed GPRC5C- or EPS8-positive EVs in the validation cohort using the same procedure as the verification cohort. As in the verification cohort, sera from patients with PDAC contained significantly more GPRC5C- or EPS8-positive EVs than those from healthy donors (Figure 4A). Importantly, there were also samples with high amounts of GPRC5C- or EPS8-positive EVs in the sera of patients with stage I early pancreatic cancer (indicated by pink dots in Figure 4A). As in the verification cohort, we performed a ROC curve analysis in the validation cohort. The AUC for GPRC5C was 0.852, and the AUC for EPS8 was 0.921. Combined ROC curve analysis using GPRC5C and EPS8 revealed an AUC of 0.952 for distinguishing PDAC patients ($n = 27$) from healthy controls ($n = 14$; Figure 4B, left). Regarding the performance of distinguishing between early stages (stage 0 or I) and healthy donors, the AUC of GPRC5C and EPS8 was 0.891 and 0.915, respectively. The combined ROC curve analysis using GPRC5C and EPS8 revealed an AUC of 0.946 for distinguishing early-stage PDAC patients ($n = 16$) from healthy controls ($n = 14$; Figure 4B, right). Moreover, we sought to check whether GPRC5C- or EPS8-positive EVs levels changed after surgery and at tumor recurrence using serum samples obtained from another hospital. We obtained serum samples taken from 10 patients at three time points: before surgery, after surgery, and at recurrence when the small pancreatic tumor was detected by regular checkup of computed tomography (CT) after surgery, and analyzed these sera using the same procedure. The amount of GPRC5C- or EPS8-positive EVs was decreased at the postoperative time point compared with that at the preoperative time point in more than half of the patients, and an elevated amount was then observed at the recurrence time point (Figures 4C and S2). This novel finding could be used as a biomarker to monitor the minimal amount of recurrence that can barely be detected by regular checkups of CT. Taken together, GPRC5C- or EPS8-positive EVs were shown to be biomarkers that have the potential to detect stage I early pancreatic cancer and small recurrent tumors detected by CT.

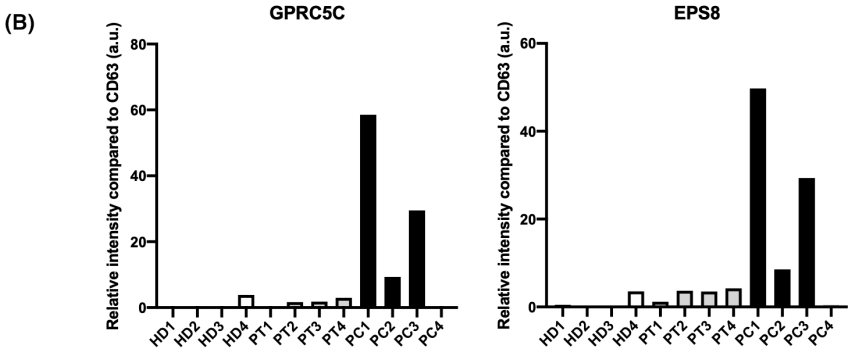
4 | DISCUSSION

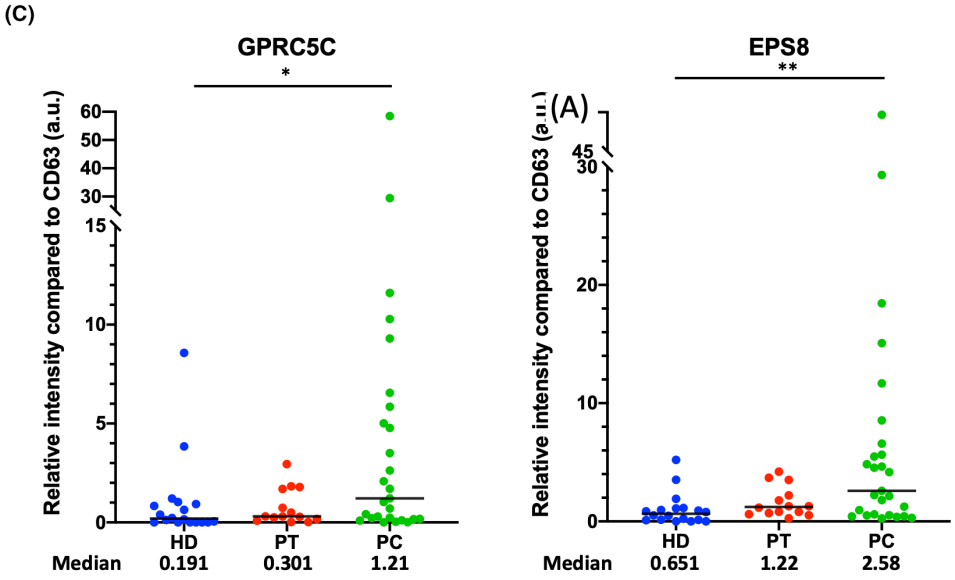
In this study, we identified novel pancreatic cancer biomarkers, EV-associated GPRC5C and EPS8, using our improved EV-associated protein purification method and proteomic analysis. GPRC5C and EPS8 had the ability to detect early-stage pancreatic cancer, which is difficult to identify with the conventional tumor marker, CA19-9.

GPRC5C is an orphan receptor that belongs to the GPRC5 family and is involved in renal acid–base homeostasis. There is only one report describing the function of GPRC5C in cancer, which states that knockdown of *GPRC5C* promotes the proliferation of breast cancer cells.[14] In fact, our analysis using the Oncomine database showed that the expression of *GPRC5C* tended to be decreased at the tumor site, and the results of the Kaplan–Meier plot suggested that it may be a tumor suppressor gene (Figure 2A,C,D). However, *GPRC5C* expression analysis using cell lines showed that GPRC5C expression was not high in noncancerous cells (Figure 2G). Nonetheless, GPRC5C levels in EVs were not correlated with the expression level of GPRC5C in cells, and GPRC5C could be detected in EVs derived from some, but not all, cancer cells (Figure 2H). Recently, it was reported that GPRC5C-positive EVs were secreted from the apical side of epithelial cells, and ALIX was required for GPRC5C-positive EV secretion but not the ceramide-dependent pathway.[15] Therefore, the discrepancy between the amount of GPRC5C in EVs and its expression level in cells may indicate that the secretory pathway of EVs is distinct in different cells. On the basis of the above findings, it is considered that GPRC5C-positive EVs were detected in the sera of PDAC patients because the secretory pathway for GPRC5C-positive EVs was activated in some pancreatic cancer cells. Nevertheless, the results of the Kaplan–Meier plot showed that high expression of GPRC5C in tumors is associated with a good prognosis, but further studies are needed to determine how high levels of EV-associated GPRC5C in the blood affect prognosis.

EPS8 was initially identified as a substrate for the EGF receptor, enhancing EGF-dependent mitogenic signals. Upregulation of EPS8 in pancreatic tumor tissue has been reported,[16] and our analysis using the


(A)
kDa
Healthy donor
Pancreatitis patient
Pancreatic cancer patient
1 2 3 4 1 2 3 4 1 2 3 4
50
GPRC5C
100
EPS8
50
37
CD63


(B)
GPRC5C
EPS8
Relative intensity compared to CD63 (a.u.)
HD1 HD2 HD3 HD4 PT1 PT2 PT3 PT4 PC1 PC2 PC3 PC4


(C)
GPRC5C
*
EPS8
**
Relative intensity compared to CD63 (a.u.)
HD PT PC
Median 0.191 0.301 1.21
Median 0.651 1.22 2.58

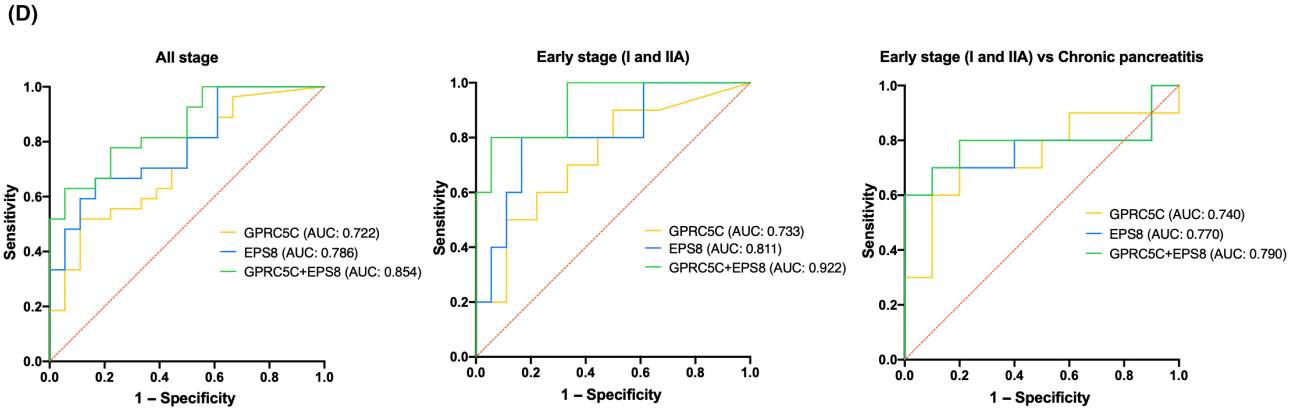

(D)
All stage
GPRC5C (AUC: 0.722)
EPS8 (AUC: 0.786)
GPRC5C+EPS8 (AUC: 0.854)
Early stage (I and IIA)
GPRC5C (AUC: 0.733)
EPS8 (AUC: 0.811)
GPRC5C+EPS8 (AUC: 0.922)
Early stage (I and IIA) vs Chronic pancreatitis
GPRC5C (AUC: 0.740)
EPS8 (AUC: 0.770)
GPRC5C+EPS8 (AUC: 0.790)
Sensitivity
1 – Specificity

FIGURE 3 Analysis of EV-associated GPRC5C and EPS8 in serum. (A) Detection of EV-associated GPRC5C and EPS8 in EVs from patient serum by immunoblotting. Serum EVs derived from healthy donors, pancreatitis patients, and pancreatic cancer patients were analyzed for GPRC5C and EPS8 by immunoblotting. CD63 was used as a loading control ($n = 4$ for each group). (B) Quantification of band intensity by immunoblotting of GPRC5C and EPS8. The band intensity of GPRC5C or EPS8 was calculated using ImageQuant and normalized to that of the band intensity of CD63. The relative level of GPRC5C or EPS8 was calculated by the average value of healthy donors. HD, healthy donor; PT, acute pancreatitis patient; PC, pancreatic cancer; PC1, stage IIA; PC2, stage III; PC3, stage IIA; PC4, stage IIB. (C) Dot plots of the semiquantitative immunoblotting analysis of EV-associated GPRC5C and EPS8. The relative amount of GPRC5C and EPS8 corresponding to CD63 was quantified by densitometry and normalized to the average value of healthy donors. Horizontal bars indicate the median values. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with healthy donor group. HD, healthy donors ($n = 18$); PT, pancreatitis patients ($n = 14$); PC, pancreatic cancer patients ($n = 27$). (D) ROC curves of GPRC5C, EPS8, and the combined markers for the diagnosis of pancreatic cancer. Left: To evaluate the diagnostic significance of GPRC5C and EPS8, ROC curves and AUC analyses was performed for GPRC5C and EPS8 and are presented to distinguish the healthy donor group ($n = 18$) from the pancreatic cancer patients ($n = 27$). Middle: Biomarker performance was assessed in clinically confirmed early-stage (I and IIA) pancreatic cancer samples ($n = 10$) compared with healthy donors ($n = 18$) or chronic pancreatitis ($n = 10$; right). The AUC value is represented in each box.

TABLE 1 List of the value of CA19-9, EPS8, and GPRC5C

	Age	Sex	Stage	CA19-9 (U/ml)	GPRC5C (a.u.)	EPS8 (a.u.)
Patient 1	54	F	IIA	0.6	0.18	0.43
Patient 2	58	F	IIB	1.1	6.55	4.83
Patient 3	67	M	IIB	2.8	4.78	11.68
Patient 4	64	M	IIA	5.3	1.70	4.18
Patient 5	51	M	III	11.0	9.30	8.54
Patient 6	44	M	III	13.5	0.16	2.14
Patient 7	56	F	III	13.5	5.01	4.54
Patient 8	37	M	IIB	19.0	0.09	0.60
Patient 9	62	M	IIB	21.7	0.03	0.51
Patient 10	59	F	IB	22.0	0.29	1.25
Patient 11	62	M	IIB	112	0.24	0.39
Patient 12	66	F	IIB	166	11.60	18.45
Patient 13	58	M	IIA	349	58.48	49.67
Patient 14	65	F	III	365	2.08	5.65
Patient 15	55	M	IIA	1490	3.51	4.64

Oncomine database also showed the upregulation of EPS8 in tumor tissue (Figure 2B). In addition, some reports indicated that a high expression level of EPS8 promotes cancer malignancy, such as cellular proliferation and migration in various cancers, including pancreatic cancer.[17,18] Moreover, it has been reported from studies in pancreatic cancer cell lines that EVs derived from metastatic pancreatic cancer cell lines contain high levels of EPS8.[19] However, because that study did not aim to develop biomarkers, EV-associated EPS8 in patient serum or plasma has not been analyzed, and its potential for utility as a pancreatic cancer biomarker was not evaluated. In this study, which differed from the above analysis of cell line-derived EVs, we identified EPS8 from an analysis using patient sera and demonstrated that EPS8 is a valuable biomarker for pancreatic cancer.

In this study, we analyzed the amount of GPRC5C and EPS8 proteins in EVs from five pancreatic cancer cell lines. Although we found that EVs from three cell lines contained these proteins, not all pancreatic cancer cells secrete GPRC5C- and EPS8-positive EVs (Figure 2H). Conversely, GPRC5C- or EPS8-positive EVs were not always detected in the sera of PDAC patients, even in the results from clinical samples (Figures 3A,C and 4A,C). These results indicated that some types of pancreatic cancer cells might secrete GPRC5C- and EPS8-positive EVs, while others do not. Therefore, given the diversity of cancers, it may still be challenging to detect cancer with 100% accuracy using only one or two markers. Moreover, as the tumor microenvironment constitutes multiple cell types, including cancer cells and noncancer cells such as immune cells, fibroblasts, and endothelial cells, it is possible that cells other than cancer cells secrete GPRC5C- or EPS8-positive EVs. Because analysis of postoperative serum samples shows a decrease in serum levels of GPRC5C- or EPS8-positive EVs, these EVs probably originate from tumor tissue; however, the present study could not determine which cells secrete these EVs.

Pearson correlation analysis showed no correlations between the level of EV-associated GPRC5C or EPS8 and CA19-9 values (Figure S1). In other words, as shown in Table 1, patients who were negative for CA19-9 could be detected by EV-associated GPRC5C or EPS8. However, some patients could not be detected by EV-associated GPRC5C or EPS8 and were positive for CA19-9, and therefore these can be used as independent markers and may be combined to increase the diagnostic performance. Moreover, regarding CA19-9, although it is the most widely used biomarker for PDAC, it has several limitations that should be considered when interpreting serum levels in the clinical setting. One of them is that CA19-9 is a sialylated Lewis blood group antigen[20]; therefore, CA19-9 cannot be used as a marker in the 5%–20% of people who do not produce a specific sialylated antigen.[21,22] CA19-9 will be falsely negative in this population, reducing its effectiveness as a diagnostic marker. Therefore, it is considered that EV-associated GPRC5C or EPS8 as a PDAC biomarker may be of particular value in terms of the detection of PDAC and monitoring recurrence in people who do not express the sialyl Lewis blood group antigen. Furthermore, CA19-9 is often increased in benign diseases, including chronic and acute pancreatitis and other benign pancreatobiliary diseases.[23,24] In addition to this problem, CA19-9 has been reported to be increased in multiple advanced gastrointestinal cancers, such as stomach, colorectal, and biliary cancer, as well

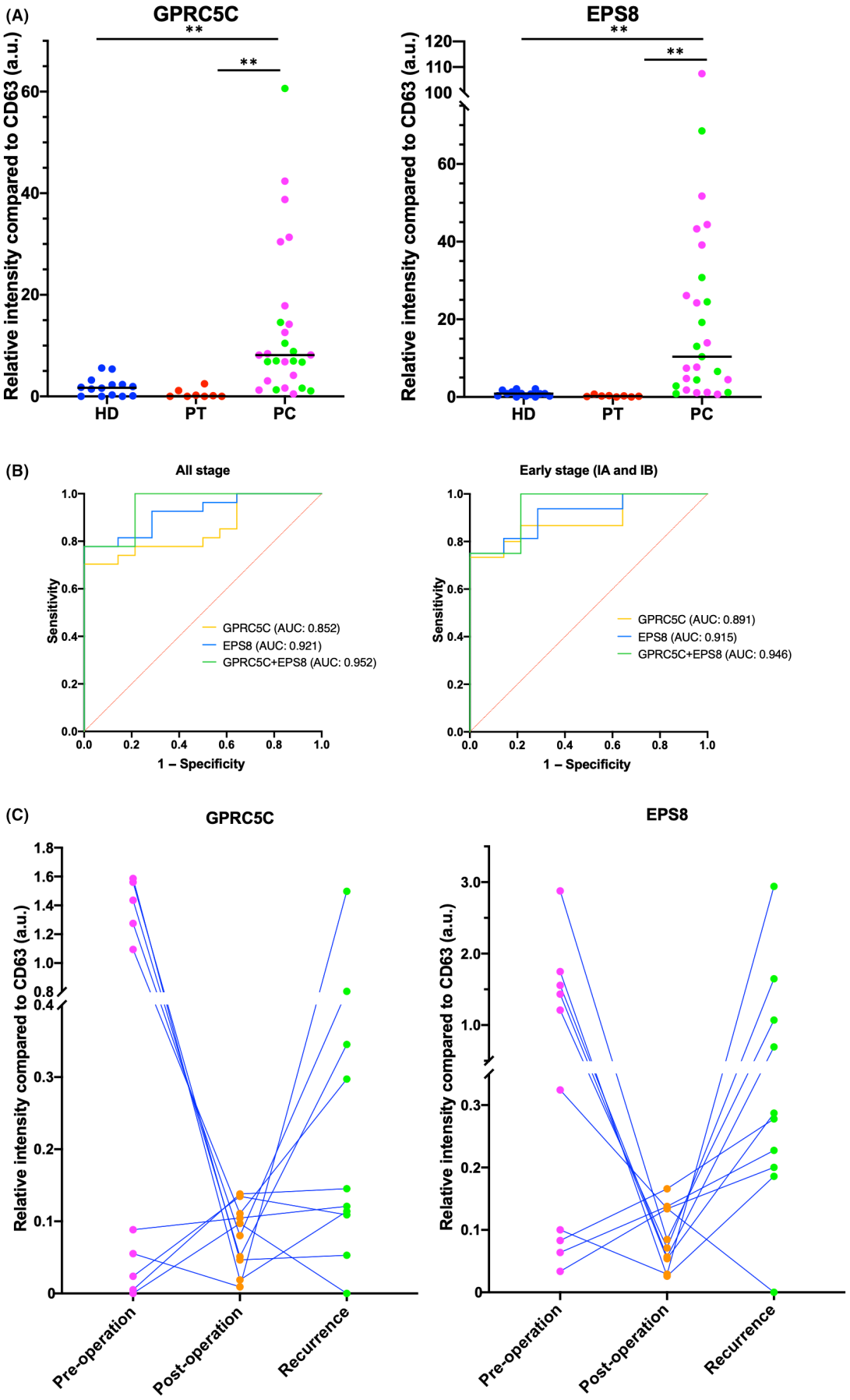

(A)
GPRC5C
EPS8
Relative intensity compared to CD63 (a.u.)
HD
PT
PC
**
(B)
All stage
Early stage (IA and IB)
Sensitivity
1 – Specificity
GPRC5C (AUC: 0.852)
EPS8 (AUC: 0.921)
GPRC5C+EPS8 (AUC: 0.952)
GPRC5C (AUC: 0.891)
EPS8 (AUC: 0.915)
GPRC5C+EPS8 (AUC: 0.946)
(C)
GPRC5C
EPS8
Relative intensity compared to CD63 (a.u.)
Pre-operation
Post-operation
Recurrence

FIGURE 4 Validation of the ability to detect small pancreatic tumors. (A) Dot plots of the semiquantitative immunoblotting analysis of EV-associated GPRC5C and EPS8 using a different cohort from the verification cohort. Horizontal bars indicate the median values. The pink dots indicate stages 0 and I patients. $^{**}p < 0.01$ compared with the healthy donor group or the pancreatitis group. HD, healthy donors ($n = 14$); PT, chronic pancreatitis patients ($n = 8$); PC, pancreatic cancer patients ($n = 27$). (B) ROC curves of GPRC5C, EPS8, and the combined markers for the diagnosis of pancreatic cancer. Left: To evaluate the diagnostic significance of GPRC5C and EPS8, ROC curves and AUC analyses were performed for GPRC5C and EPS8 and are presented to distinguish the healthy donor group ($n = 14$) from pancreatic cancer patients ($n = 27$). Right: Biomarker performance was assessed in clinically confirmed early-stage (0 and I) pancreatic cancer samples ($n = 16$) compared with healthy donors ($n = 14$). The value of AUC is represented in each box. (C) The transition values of EV-associated GPRC5C and EPS8. EV-associated GPRC5C and EPS8 from 10 paired serum samples at three time points (pre-surgery, post-surgery, and recurrence) were detected by immunoblotting. The relative amount of GPRC5C and EPS8 corresponding to CD63 were quantified by densitometry.

as pancreatic cancer.[25] In the present study, although some pancreatitis patients in the verification cohort had elevated amounts of serum EV-associated GPRC5C or EPS8, we showed that EV-associated GPRC5C or EPS8 could be capable of distinguishing between patients with chronic pancreatitis and those with pancreatic cancer, especially in the validation cohort (Figures 3A and 4A). Another important point to using these biomarkers, these biomarkers can detect early-stage PDAC patients. This point is different from conventional tumor markers such as CA19-9, whose value increases as the tumor size increases. As is true with most EV-based biomarkers, EV-based biomarkers are suitable for detecting early-stage cancers.[26,27] This is because cancer cells utilize EVs for their progression, including metastasis,[28,29] and may actively secrete more EVs associated with malignant transformation in the early stages to help and provide a favorable environment for their survival. Therefore, EVs derived from tumors may be more easily detected in blood at early stages. However, because of the limited sample size, further research should be conducted with larger sample sizes to confirm EV-associated GPRC5C or EPS8 effect more accurately. Especially concerning the evaluation of monitoring markers for recurrence, the number of samples analyzed is minimal. Therefore, we could not find the characteristics of patients who show a pattern in which the level of GPRC5C or EPS8 in serum EVs falls once after surgery and then rises again after recurrence. In other words, the transition pattern of these marker expressions does not differ depending on the site of recurrence and also seems to have no relationship with the recurrence time (Figure S2). Furthermore, regarding the specificity for other cancers, we have not analyzed the effectiveness of EV-associated GPRC5C or EPS8 as biomarkers for other tumors in this study, and further study is necessary to clarify their specificity.

EV-based biomarkers provide an additional and powerful diagnostic component in the field of liquid biopsies. In fact, it has been reported that many EV-related molecules can be potential pancreatic cancer biomarkers.[30–32] Although there is no doubt that these EV-related molecules have superior potential as biomarkers, their clinical application has not been realized, and several challenges are being considered. In other words, most EV-related biomarker studies were only undertaken in a laboratory setting with optimized EV isolation and detection methods that are not applicable to the hospital environment. Moreover, whether these results hold up in larger patient cohorts is important. The present study is no exception and has these problems. In particular, because we used immunoblotting to detect these EV markers, we needed to purify EVs by ultracentrifugation. Therefore, it is necessary to consider the lack of throughput and the quantitativeness and reproducibility of immunoblotting. It will be necessary to incorporate other methods, for example, the ExoScreen method that we developed previously,[12] into a simple and throughput EV detection system. The above challenges need to be addressed for their use in clinical practice; however, we believe that EV-associated GPRC5C and EPS8 will contribute to the early detection and monitoring of PDAC recurrence and help in the design of potential curative surgical options.

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DISCLOSURE

The authors declare the following competing interests: Yusuke Yoshioka and Takahiro Ochiya are inventors in a patent (PCT/JP2017/025115) on PDAC biomarkers. Hideshi Ishii, Tetsuya Nakatsura and Takahiro Ochiya are Associate Editors of Cancer Science. The remaining authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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