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ORIGINAL ARTICLE – PANCREATIC TUMORS

# Pancreatic Juice Exosomal MicroRNAs as Biomarkers for Detection of Pancreatic Ductal Adenocarcinoma

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## ABSTRACT

**Background.** Pancreatic ductal adenocarcinoma (PDAC) is a lethal neoplasm because of difficulties in early detection. Several studies have recently suggested that exosomes may have potential as novel biomarkers. This study aimed to isolate exosomes from pancreatic juice and to investigate whether exosomal microRNAs (ex-miRs) could be used as biomarkers for PDAC.

**Methods.** Pancreatic juice was collected from patients with PDAC and chronic pancreatitis (CP) by endoscopic retrograde pancreatography. Exosomes were extracted by ultracentrifugation. The presence of exosomes was confirmed by electron microscopy and Western blotting using anti-CD63, -CD81, and -TSG101 antibodies. Relative levels of ex-miR-21 and ex-miR-155 were quantified and compared between PDAC and CP patients.

**Results.** A total of 35 pancreatic juice samples (27 PDAC and 8 CP) were collected. Relative levels of both ex-miR-21 and ex-miR-155 were significantly higher in PDAC patients compared with CP patients ($p < 0.001$ and $p = 0.008$, respectively). By contrast, no significant difference was apparent in relative levels of miR-21 and miR-155 in whole pancreatic juice from PDAC patients compared with CP patients ($p = 0.08$ and $p = 0.61$, respectively). Ex-miR-21 and ex-miR-155 levels discriminated PDAC patients from CP patients with area under the curve values of 0.90 and 0.89, respectively. The accuracies of ex-miR-21 levels, ex-miR-155 levels, and pancreatic juice cytology were 83%, 89%, and 74%, respectively. When combining the results of ex-miR profiling with pancreatic juice cytology, the accuracy was improved to 91%.

**Conclusions.** We successfully extracted exosomes from pancreatic juice. Ex-miRs, including ex-miR-21 and ex-miR-155, in pancreatic juice may be developed as biomarkers for PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is a lethal neoplasm with a 5-year survival rate of < 10%.[1] Surgery is the only curative treatment, but unfortunately most patients are diagnosed with locally advanced or metastatic PDAC. Therefore, identification of biomarkers for early and definitive PDAC diagnosis is crucial. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is currently used to diagnose PDAC. Although EUS-FNA is a safe and well-established technique, a risk of gastric wall implantation or dissemination of tumor cells via the puncture tract is associated with the procedure.[2,3] In such cases, endoscopic retrograde pancreatography (ERP) with subsequent cytological assessment of pancreatic juice can also be used to diagnose PDAC; however, the sensitivity of

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pancreatic juice cytology (PJC) has been reported as 20–60%.[4–6] Therefore, identification of unique PDAC-associated molecules in pancreatic juice for the diagnosis of PDAC is an important goal.

MicroRNAs (miRs) are 18–23 nucleotide non-coding RNAs that modulate the translation of specific mRNAs and play important roles in tumorigenesis.[7] MiRs are dysregulated in various tumor types and are expected to become novel biomarkers of various neoplasms.[8–12] Recently, miRs have also been identified in the exosomes, which are small membrane vesicles, 50–100 nm in diameter, that are secreted from various cell types and identified in several body fluids. Exosomes can transfer nucleic acids such as miRs between cells.[13] Exosomal miRs (ex-miRs) are protected from endogenous RNase activity and are expected to be stable, even in body fluids containing high levels of RNases. In PDAC patients, several studies have reported alterations in the levels of ex-miRs in blood (electronic supplementary Table S1),[14–20] but ex-miRs in pancreatic juice have not yet been evaluated as diagnostic markers.

Because PDAC contacts pancreatic juice directly in the pancreatic duct, we hypothesized that the ex-miR profile of pancreatic juice derived from PDAC patients might be a more accurate diagnostic tool than ex-miR levels in blood. This study had two major aims: (1) to purify exosomes from pancreatic juice samples; and (2) to investigate ex-miR levels as potential biomarkers for PDAC diagnosis. We focused specifically on miR-21 and miR-155 levels based on our previous work.[21]

## METHODS

### *Patients*

This study was approved by the Ethics Committee of Kyushu University (no. 30-120) and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration. Thirty-five subjects underwent ERP and subsequent collection of pancreatic juice samples at Kyushu University Hospital, Japan, from 2011 to 2014, and the clinicopathological characteristics of these individuals were evaluated. Twenty-seven patients had PDAC and eight patients had chronic pancreatitis (CP). A diagnosis of PDAC was made by pathology or cytology, while diagnosis of CP was made by pathology or clinical criteria based on imaging results at the time of initial diagnosis and during a follow-up period of at least 12 months.

### *Pancreatic Juice Samples*

Pancreatic juice samples were obtained via preoperative ERP, as previously reported,[21] and stored at − 80 °C until analyses. Briefly, a 5-Fr wedge pressure balloon catheter was inserted into the main pancreatic duct during ERP. Pancreatic juice was collected through the catheter for 10 min after intravenous administration of 1 μg of secretin (ChiRhoClin, Inc., Burtonsville, MD, USA) dissolved in 5 mL of 0.9% saline.

### *Exosome Isolation from Pancreatic Juice*

Exosomes were extracted by ultracentrifugation according to previously reported protocols for serum, plasma,[22] and bile.[23] Five-hundred microliters of pancreatic juice was centrifuged at $300\times g$ for 10 min at 4 °C, and then the supernatant was centrifuged at $16{,}500\times g$ for 20 min at 4 °C to remove cellular debris. Next, the supernatant was filtered through a 0.2-μm filter and the filtrate was ultracentrifuged at $140{,}000\times g$ for 70 min at 4 °C. The efficient relative centrifugal force was determined with size distribution and concentration by nanoparticle tracking analysis (NTA). The exosomal fraction was collected from the pellets and resuspended in 40 μL of phosphate-buffered saline.

### *Visualization and Verification of Exosomes*

Transmission electron microscopy (TEM) was used to visualize vesicles extracted from pancreatic juice. The vesicles were analyzed for nanoparticle size distribution and concentration by NTA using a NanoSight LM10-HS instrument (Nanosight, Amesbury, UK). To confirm the presence of exosomes, expression of protein markers commonly found in exosome preparations was assessed by Western blotting using anti-CD63, anti-CD81 (Life Technologies Corporation, Carlsbad, CA, USA; diluted 1:250), anti-TSG101 (Abcam, Cambridge, UK; diluted 1:500), and anti-CANX (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan; diluted 1:10,000) antibodies.[24]

### *MicroRNA (MiR) Extraction*

MiR was extracted from either (1) exosomes purified from 500 μL of pancreatic juice, or (2) 500 μL of whole pancreatic juice using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final RNA volumes were normalized to 30 μL; thus, 1 μL of each sample was representative of approximately 17 μL of pancreatic juice. RNAs were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an

Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) (electronic supplementary Fig. S1).

### *Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction*

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed with a CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) using the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. For measurement of miR-21, miR-155, and miR-16 levels, we performed two-step qRT-PCR with primers specific for miR-21 (5′-UAGCUUAUCAGACUGAUGUUGA-3′), miR-155 (5′-UUAAUGCUAAUCGUGAUAGGGGU-3′), and miR-16 (5′-UAGCAGCACGUAAAUAUUGGCG-3′) [designed by Applied Biosystems) following the manufacturer's protocol. PCR amplifications were performed in triplicate for each sample. We used the ΔCT method to calculate relative miR expression, with miR-16 serving as the internal control according to previous reports.[9,11,25]

### *Evaluation of Pancreatic Juice Exosomal miR (Ex-miR) Stability and Diagnostic Value*

We aimed to compare the stability of purified ex-miRs and free miRs in bulk pancreatic juice; the latter were obtained from whole pancreatic juice, which contains other molecules in addition to ex-miRs. To determine the effects of storing exosome samples or pancreatic juice samples at room temperature or 37 °C, we examined the expression level of miR-21 in each sample over time.

To evaluate the diagnostic value of ex-miRs as biomarkers of PDAC, the expression levels of miR-21 and miR-155 in exosomes (ex-miRs) and free miRs in whole pancreatic juice (free-miRs) were analyzed using qRT-PCR. We also compared ex-miRs with traditional biomarkers such as serum carbohydrate antigen (CA) 19-9 levels and PJC. For cytology, atypical cells were defined as positive results.

### *Statistical Analyses*

Statistical analyses were performed using JMP 12.2.0 (SAS Institute, Inc., Cary, NC, USA). Comparisons of continuous and categorical variables were made using the Wilcoxon signed-rank test. Receiver operating characteristic (ROC) curves were generated, and area under the curve (AUC), sensitivity, and specificity values were calculated to evaluate the diagnostic value of candidate miRs. The cut-off points were determined using the Youden index. AUCs for miRs and CA19-9 were compared using Z tests. Differences between groups were considered significant when the two-tailed $p$ value was $< 0.05$.

## RESULTS

### *Patient Characteristics*

Electronic supplementary Table S2 shows the clinicopathological characteristics of the study population. Although the median serum CA19-9 level was higher in PDAC patients than in CP patients, this difference did not reach statistical significance ($p = 0.10$). Only PJC was a significant indicator of PDAC ($p = 0.0002$). A summary of patient characteristics is provided in Table 1. There were no adverse events related to the endoscopic procedures, including post-ERP pancreatitis.

### *Characterization of Exosomes Extracted from Pancreatic Juice*

The presence of 50–100 nm rounded vesicles was observed by TEM, consistent with the features of exosomes (Fig. 1a).[13] The subsequent NTA showed abundant small vesicles (Fig. 1b) with sizes normally distributed around a mode of 79 nm and an overall concentration of $1.06 \times 10^{11}$ particles/mL (Fig. 1c). The median concentration of vesicles in pancreatic juice samples collected randomly from five PDAC patients was 1.45 (0.75–1.86) $\times 10^{11}$ particles/mL. Western blotting showed that CD63, CD81, and TSG101 were present in the vesicles obtained from pancreatic juice, while CANX was not (Fig. 1d).

### *Stability of Ex-miRs in Pancreatic Juice*

After storing whole pancreatic juice at room temperature for up to 48 h, the levels of free-miR-21 and ex-miR-21 were stable (electronic supplementary Fig. S2a). On the other hand, when stored at 37 °C, ex-miR-21 levels were stable, while free-miR-21 decreased over time (electronic supplementary Fig. S2b). This finding demonstrated that ex-miRs in pancreatic juice were stable even in the pancreatic duct, where the temperature is usually 37 °C.

### *Expression of Ex-miR-21 and Ex-miR-155 in Pancreatic Juice*

Pancreatic juice samples from all 35 patients were available for analyses of ex-miRs; however, those from two PDAC patients (PDAC-12 and PDAC-27 in electronic supplementary Table S2) had insufficient volumes for analyses of free-miRs. The median Ct value of miR-16 was

**TABLE 1** Characteristics of patients with pancreatic ductal adenocarcinoma and chronic pancreatitis

| | PDAC ($N$ = 27) | CP ($N$ = 8) | $p$-Value |
|---|---|---|---|
| Age, years [median (range)] | 71 (47–79) | 59.5 (38–79) | 0.15 |
| Sex (male/female) | 17/10 | 6/2 | 0.52 |
| Location (head/body/tail) | 7/10/10 | – | |
| Stage[a] (0/IA/IB/IIA/IIB/III/IV) | 1/0/1/3/13/2/7 | – | |
| T factor (cis/1/2/3/4) | 1/1/3/19/3 | – | |
| CA19-9, U/mL (median (range)) | 107.3 (0.6–1562) | 16.65 (4.3–110.3) | 0.10 |
| PJC (positive/negative) | 18/9 | 0/8 | 0.0002* |

*PDAC* pancreatic ductal adenocarcinoma, *CP* chronic pancreatitis, *CA19-9* carbohydrate antigen 19-9, *PJC* pancreatic juice cytology, *UICC* Union for International Cancer Control

*$p < 0.05$

[a]UICC classification

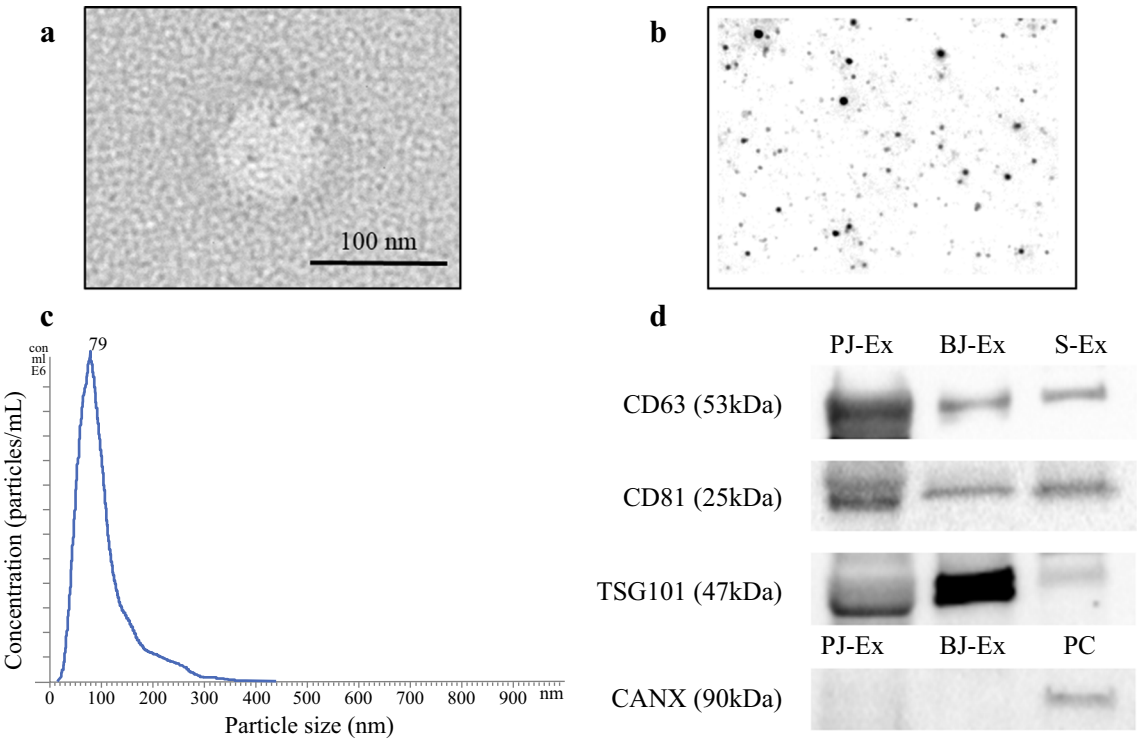


**FIG. 1** **a** Small vesicles in pancreatic juice imaged using transmission electron microscopy with the negative stain method. **b** Small vesicles visualized by nanoparticle tracking analysis. **c** The mode of the particle size distribution and the concentration of small vesicles isolated from pancreatic juice were 79 nm and $1.06 \times 10^{11}$ particles/mL, respectively. **d** Exosomes isolated from pancreatic juice, bile, and serum were analyzed by Western blotting using anti-CD63, -CD81, -TSG101, and -CANX antibodies. CD63, CD81, and TGS101 were present in the exosomes, while CANX was absent. The positive control for CANX was the cell pellet lysate derived from bile

26.6 (18.1–31.2), which was stable and sufficient for internal control. Relative levels of ex-miR-21 and ex-miR-155 were significantly higher in PDAC patients compared with CP patients (Figs. 2a, c). By contrast, there were no significant differences observed in the relative levels of free-miR-21 and free-miR-155 in PDAC and CP patients (Figs. 2b, d). Compared with tissue samples, the relative levels of ex-miRs were equivalent or higher (electronic supplementary Fig. S3). There was a significant difference between the AUC values for ex-miR-21 and free-miR-21 ($p$ = 0.007) (Fig. 3a). Similarly, there was a significant difference between the AUC values for ex-miR-155 and free-miR-155 ($p$ = 0.0004) (Fig. 3b).

### *Comparison of the Diagnostic Values of Ex-miR Quantitation, Serum CA19-9 Levels and Pancreatic Juice Cytology*

The AUC values for ex-miR-21 and ex-miR-155 levels were significantly higher than that for serum CA19-9 levels

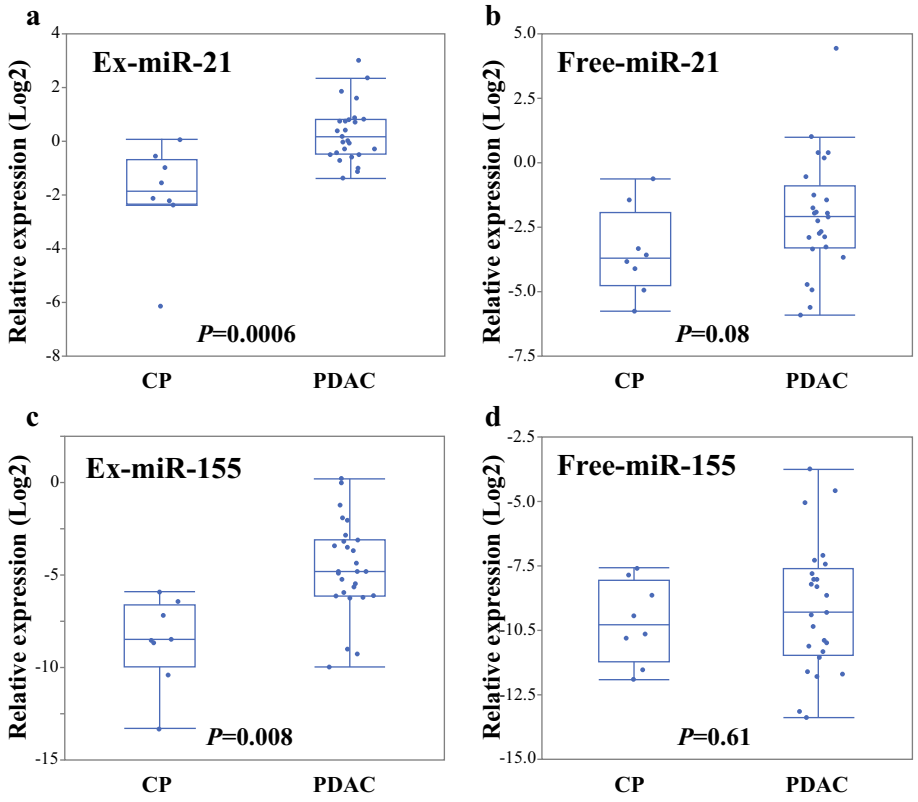


**FIG. 2** Expression of miR-21 and miR-155 in patients with PDAC and CP. **a** Relative expression levels of miR-21 in exosomes purified from pancreatic juice (ex-miR-21) were significantly higher in PDAC patients compared with CP patients ($p = 0.0006$). **b** There was no significant difference in the relative expression of miR-21 in whole pancreatic juice (free-miR-21) obtained from PDAC and CP patients ($p = 0.08$). **c** Relative levels of miR-155 in exosomes purified from pancreatic juice (ex-miR-155) were significantly higher in PDAC patients compared with CP patients ($p = 0.008$). **d** There was no significant difference in the relative expression of miR-155 in whole pancreatic juice (free-miR-155) obtained from PDAC and CP patients ($p = 0.61$). *CP* chronic pancreatitis, *PDAC* pancreatic ductal adenocarcinoma, *Ex-miR* exosomal microRNA

($p = 0.04$ and $p = 0.04$, respectively) (Figs. 3c, d). The cut-off values for ex-miR-21 and ex-miR-155 levels were determined and were used to stratify patients into two groups (positive or negative). The results for each subject are shown in electronic supplementary Table S2. Both ex-miR-21 and ex-miR-155 levels were elevated in pancreatic juice of patients with carcinoma in situ (stage 0). Table 2 shows a comparison of the diagnostic value of ex-miR-21, ex-miR-155, and PJC. The accuracies of ex-miR-21 (83%) and ex-miR-155 (89%) were superior to that of PJC (74%). When combining the results of ex-miR-21 with ex-miR-155, the sensitivity, specificity, and accuracy were 96%, 75%, and 91%, respectively. Many PDAC patients with false-negative PJC findings could be diagnosed via positive results from ex-miR levels. Taking positive results of either ex-miR-21/ex-miR-155 levels or PJC as the diagnostic criterion for PDAC, the sensitivity of the combined test was 93%/93% and the specificity was 88%/88%.

## DISCUSSION

This study is the first report describing ex-miRs in pancreatic juice, as well as their diagnostic value as stable biomarkers for PDAC. We successfully isolated exosomes from pancreatic juice with high purity and concentration. Zheng et al.[26] have reported the isolation of exosomes from pancreatic juice using ultracentrifugation. In their study, the ultracentrifugation step (100,000×*g*, 70 min) was performed according to the protocol for plasma.[22] In the present study, we conclusively applied ultracentrifugation (140,000×*g*, 70 min). Because of the higher viscosity compared with blood, higher centrifugation speeds should be necessary for exosome purification from pancreatic juice. Additionally, pancreatic juice contains many impurities. Therefore, filtration may also be needed, as per the described protocol for bile.[23,27] The concentration of exosomes isolated using our protocol was approximately 200 times higher than that previously reported,[26] and thus our method appears to be superior for isolating exosomes from pancreatic juice.

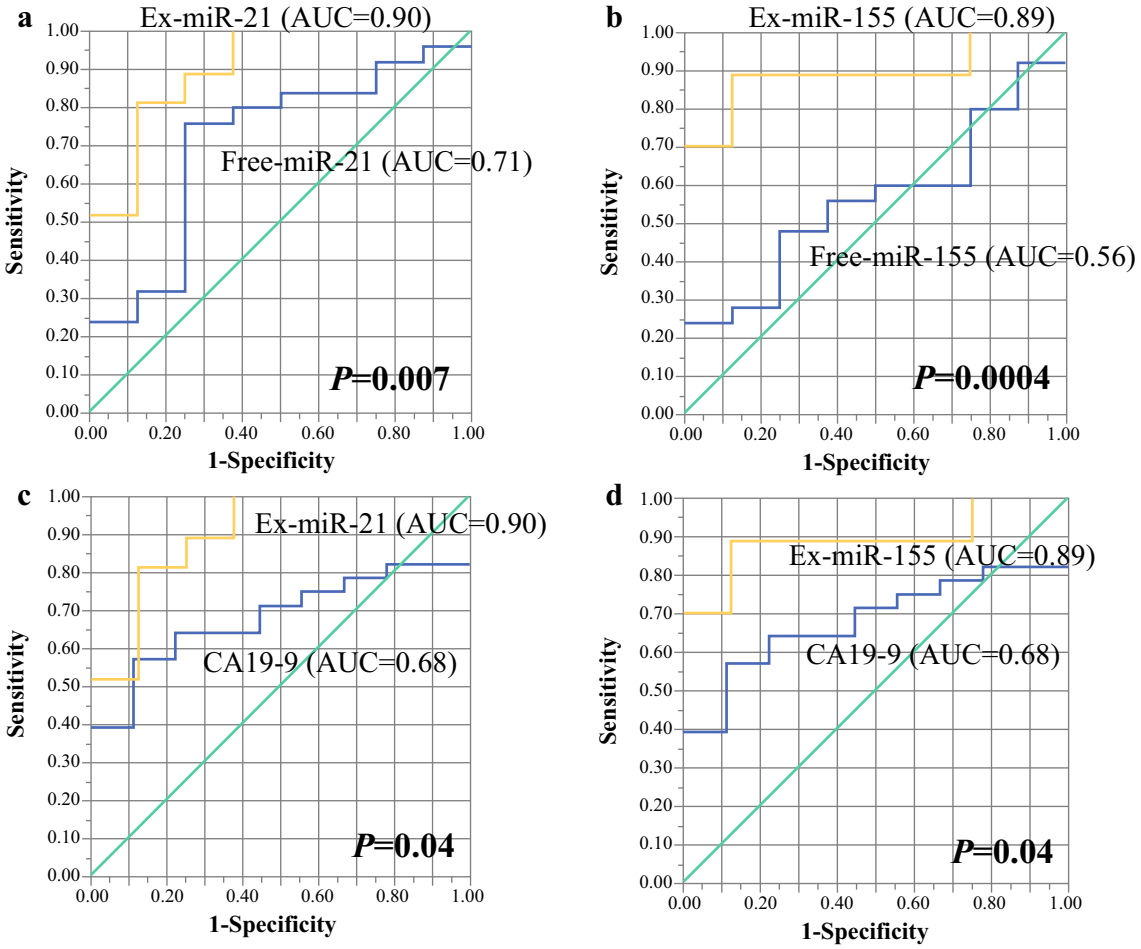


**FIG. 3** **a**, **b** ROC curves of miRNA expression levels. **a** ROC curves of expression levels of miR-21. There was a significant difference in the diagnostic value of miR-21 levels in exosomes (ex-miR-21) compared with levels in whole pancreatic juice (free-miR-21), with AUC values of 0.90 and 0.71, respectively ($p = 0.007$). **b** ROC curves of expression levels of miR-155. There was a significant difference in the diagnostic value of miR-155 levels in exosomes (ex-miR-155) compared with levels in whole pancreatic juice (free-miR-155), with AUC values of 0.89 and 0.56, respectively ($p = 0.0004$). **c**, **d** ROC curves of miR expression level and serum CA19-9 level. (**c**) Ex-miR-21 level versus serum CA19-9 level. There was a significant difference in the diagnostic value of ex-miR-21 level and CA19-9 level, with AUC values of 0.90 and 0.68, respectively ($p = 0.04$). **d** Ex-miR-155 level versus serum CA19-9 level. There was a significant difference in the diagnostic value of ex-miR-155 level and CA19-9 level, with AUC values of 0.89 and 0.68, respectively ($p = 0.04$). *ROC* receiver operating characteristic, *Ex-miR* exosomal microRNA, *AUC* area under the curve, *CA19-9* cancer antigen 19-9

**TABLE 2** Diagnostic value of exosomal miR-21 level, exosomal miR-155 level, and pancreatic juice cytology for pancreatic ductal adenocarcinoma

| | TP | FN | FP | TN | Sensitivity (%) | Specificity (%) | Accuracy (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|---|---|---|
| Ex-miR-21 | 22 | 5 | 1 | 7 | 81 | 88 | 83 | 96 | 58 |
| Ex-miR-155 | 24 | 3 | 1 | 7 | 89 | 88 | 89 | 96 | 70 |
| Ex-miR-21/155 | 26 | 1 | 2 | 6 | 96 | 75 | 91 | 93 | 86 |
| PJC | 18 | 9 | 0 | 8 | 67 | 100 | 74 | 100 | 47 |
| Ex-miR-21/PJC | 25 | 2 | 1 | 7 | 93 | 88 | 91 | 96 | 78 |
| Ex-miR-155/PJC | 25 | 2 | 1 | 7 | 93 | 88 | 91 | 96 | 78 |

*TP* true positive, *FN* false negative, *FP* false positive, *TN* true negative, *PPV* positive predictive value, *NPV* negative predictive value, *Ex-miR* exosomal microRNA, *PJC* pancreatic juice cytology, *PJ-Ex* pancreatic juice exosomes, *BJ-Ex* bile exosomes, *S-Ex* serum exosomes, *PC* positive control

Our previous study analyzing the expression levels of miRs extracted from cell pellets derived from pancreatic juice, demonstrated that relative levels of miR-21 and miR-155 distinguished PDAC patients from CP patients.[21] In that study, relative levels of miRs in pancreatic juice from patients with cytological positive results were not significantly different from levels in patients with negative results.[21] This finding suggests that elevated miR levels in the pancreatic juice of PDAC patients might not be derived from atypical cells, but instead from acellular miRs, including ex-miRs. In the present study, we demonstrated that expression levels of ex-miR-21 and ex-miR-155 were significantly higher in PDAC patients compared with CP patients. In contrast, no significant differences were observed in the relative levels of free-miR-21 and free-miR-155 between these two groups. Since whole pancreatic juice, which contains other miRs in addition to ex-miRs, was unstable (likely because miRs not enclosed in exosomes were easily degraded), we concluded that analyses of ex-miRs rather than free-miRs were preferable. Furthermore, because of their instability, the results of analyses based on free-miRs were inconsistent; free-miRs could not distinguish PDAC patients from CP patients in this study, while a significant difference was observed in our previous study.[21] Taken together, ex-miRs may be more useful and stable as biomarkers for PDAC detection than free-miRs, in any body fluids, including pancreatic juice.

MiR-21 is upregulated in various cancers and targets tumor-suppressive mRNAs.[28] Goto et al.[20] demonstrated that elevated serum ex-miR-21 expression was diagnostic of PDAC. By contrast, miR-155 has been reported to target transcripts related to antiapoptosis and tumorigenicity.[29] Mikamori et al.[17] investigated that an increase in cellular miR-155 expression led to exosome release by the cell and subsequent exosome-mediated delivery of chemoresistance-related substances, including miR-155, to other cancer cells. Que et al.[14] analyzed the diagnostic value of serum ex-miR-155. Compared with levels of these ex-miRs in blood,[14,20] the measurement of ex-miR-21 and ex-miR-155 levels in pancreatic juice could represent a more feasible strategy for PDAC diagnosis. We surmise that exosomes in pancreatic juice reflect tumor cells directly, while those in blood reflect the various cell types in the body because exosome content and quantity reflect the pathophysiological state of the cells from which they are emitted.[27]

We compared the diagnostic values of ex-miRs in pancreatic juice with those of serum CA19-9 levels and PJC. Of note, both ex-miR-21 and ex-miR-155 levels were diagnostic of early-stage PDAC when CA19-9 levels were still within normal limits. When combining positive findings for ex-miR-21 or ex-miR-155 levels with those of PJC, the sensitivity of the combined test was improved to 93%, while the specificity remained at 88%. PJC is usually assessed using cell pellets, while exosomes are extracted from the supernatant (which is usually discarded during PJC). Pancreatic juice is a precious material, and analysis of all of its components using different methods may lead to more accurate diagnoses.

A limitation of our study was that the sample size was not sufficient to precisely estimate the diagnostic values of ex-miRs. Although we tried to evaluate pancreatic juice from patients with intraductal papillary mucinous neoplasm (IPMN), exosomes were unable to be reliably extracted from IPMN fluids because of their mucin content, which confers very high viscosity. Another limitation was that the ERP procedure for obtaining pancreatic juice was invasive and resulted in the possible occurrence of post-ERP pancreatitis. Less-invasive biomarkers have recently been reported using duodenum fluid,[30–32] which can be obtained during upper gastrointestinal screening tests using gastrointestinal endoscopy. Our next step will be analysis of the duodenum fluid.

## CONCLUSIONS

This study showed that ex-miR-21 and ex-miR-155 levels in pancreatic juice effectively discriminated patients with PDAC from those with CP. Furthermore, the combination of ex-miRs with PJC was more sensitive than the single tests. These findings indicate that ex-miRs, including ex-miR-21 and ex-miR-155, represent novel biomarkers of PDAC and that their quantitation in pancreatic juice could be used as a complementary test for further confirmation of diagnoses.

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