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REVIEW ARTICLE

Clinical application of pancreatic juice-derived small extracellular vesicles of pancreatic ductal adenocarcinoma

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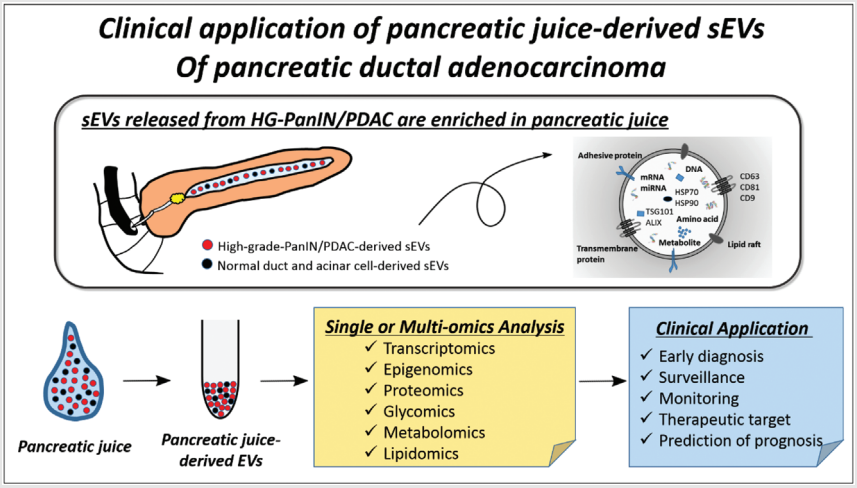
Graphical Abstract



Graphical Headlights

- Small extracellular vesicles (sEVs) released from pancreatic ductal carcinoma (PDAC) are enriched in pancreatic juice (PJ).
- PJ-derived sEVs are promising for the early diagnosis of PDAC.
- PJ-derived sEVs could be promising therapeutic targets.

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Abstract

Background: Recent imaging modalities have helped inthe early detection of pancreatic ductal adenocarcinoma (PDAC), resulting inimproved survival rates for patients with early-stage PDAC. However, preoperative pathological diagnosis of early-stage PDAC remains a challenge, particularly for small PDAC that is difficult to diagnose through standardendoscopic ultrasound-guided fine-needle biopsy. In this context, pancreaticjuice cytology has been re-evaluated as an important tool for the preoperativediagnosis of early-stage PDAC.

Main: Pancreatic juice (PJ) comes in directcontact with PDAC lesions in the pancreatic duct and thus may contain a fewHG-PanIN/PDAC cells and specific molecules. Additionally, the PJ may containconcentrated small extracellular vesicles (sEVs) that are released from cancerlesions. sEVs are double-layered lipid-bound particles that contain cargoassociated with the cell-of-origin, including proteins, microRNA, and RNA. sEVsreleased from cancer lesions found in body fluids, such as blood, urine, andsaliva, have already been studied as potential sources of diagnostic biomarkersfor cancer. PJ-derived sEVs could serve as a "liquid biopsy" for theearly diagnosis of PDAC. However, little is known about the existence,physiological status, and function of PJ-derived sEVs and their potentialutility as biomarkers for diagnostic, surveillance, and monitoring purposes oras therapeutic targets.

Conclusion: PJ-derived sEVs represent a promisingavenue for the early diagnosis of PDAC. The utility of these particles as biomarkersfor diagnostic, surveillance, and monitoring purposes, or as therapeutictargets, warrants further research. Understanding the existence, physiologicalstatus, and function of PJ-derived sEVs is crucial to unlocking their potentialas a valuable tool for overcoming PDAC.

KEYWORDS
biomarker, early diagnosis, high-grade PanIN, IPMN, liquid biopsy, pancreatic ductal carcinoma, pancreatic juice, small extracellular vesicle, treatment

1 | INTRODUCTION

While the 5-year overall survival of patients with pancreatic ductal adenocarcinoma (PDAC) has increased from <5% to 11% over the past three decades,[1] PDAC remains a devastating disease that is associated with a poor prognosis. Recently, an analysis of the Japan Pancreatic Cancer Registry by the Japan Pancreas Society revealed that the 5-year overall survival rate of patients with early-stage PDAC of <10 mm in diameter (Tis, T1a and T1b; T staging according to the 8th edition of the Union for International Cancer Control TNM classification), confined within the pancreas and without metastasis, is >80%.[2,3] Therefore, the detection of small PDAC, including its precursor lesions—high-grade pancreatic intraepithelial neoplasm (HG-PanIN)—followed by curative resection can be an attractive approach for improvement of the poor prognosis. However, despite tireless exploration by researchers,[4–10] there is no highly accurate biomarker that can be applied in the diagnosis and screening of small PDAC using non-invasively obtained samples, such as blood, urine and saliva. Furthermore, despite progress in imaging technology, the detection of small PDAC is extremely difficult, and moreover, HG-PanIN lesions do not necessarily form solid pancreatic mass.

Meanwhile, pancreatic juice cytology (PJC) during endoscopic retrograde pancreatography (ERP) has recently been reevaluated as an important diagnostic modality, especially for such small malignant pancreatic lesions.[3,11,12] In addition, PJ is a promising biomarker source for the early detection of small PDAC, despite its low cellularity and the associated risk of post-ERP pancreatitis.[13] From the viewpoint of the developmental process of PDAC, there is no doubt that PJ contains enriched cancer-specific molecules; the precursor lesion, PanIN arises in the lining of the ducts in the pancreas and then progresses into HG-PanIN and PDAC,[14,15] while PJ secreted from the exocrine glands is invariably directly exposed to these malignant lesions. On the other hand, small extracellular vesicles (sEVs) derived from human body fluids, such as plasma, serum and urine, have gathered a great deal of attention as a hopeful biomarker source for the diagnosis of cancer.[16] sEVS are initially formed by endocytosis in early endosomes to form multivesicular bodies (MVBs) and released into extracellular environment by fusion of MVBs with the plasma membrane of the parent cell [17] The concept of the sEVs-based diagnosis is mainly based on evidence that sEVs with a lipid bilayer membrane protect their unique biomolecular cargo, consisting of proteins, nucleic acids and lipids, from digestive enzymes and RNase, and cancer-derived sEVs have informative molecules, which have been shown to mostly be involved in cancer progression via intercellular communication.[18,19] Regarding the diagnosis of PDAC, PJ-derived sEVs would contain abundant sEVs released directly into pancreatic duct from PDAC. They would be sheltered from activated pancreatic enzymes that may degrade promising biomarkers, and thus might be an innovative and powerful diagnostic source, even for small PDAC, including HG-PanIN. However, little is known about the diagnostic and therapeutic potential of PJ-derived sEVs for PDAC.

We reviewed the latest studies on human PJ-derived sEVs and will discuss the challenges and perspective of PJ-derived sEVs-based early diagnostics, screening and therapeutics for PDAC.

2 | CHARACTERISTICS OF PANCREATIC JUICE-DERIVED sEVs AND THEIR POTENTIAL FOR CLINICAL APPLICATION

2.1 | Origin of sEVs in PJ

Firstly, sEVs in human PJ would be exclusively derived from the exocrine pancreas, which is responsible for the secretion of digestive enzymes, ions and water into the duodenum of the gastrointestinal tract.[20] The exocrine pancreas mainly consists of acinar cells and ductal epithelial cells, and both cells have a secretory mechanism: the former secretes various digestive zymogens, such as trypsinogen, chymotrypsinogen and proelastase, and the latter secretes bicarbonate-rich fluid to dilute and optimize the pH of the protein concentrate secreted by acinar cells.[21] To date, there have been no studies to investigate the types of cells that dominantly release sEVs into PJ, how to distinguish acinar cells-derived EVs from ductal cell-derived EVs, or how to distinguish tumour cell-derived sEVs from non-tumour cell-derived sEVs. Furthermore, the functional role of sEVs in PJ remains to be elucidated. However, prior accumulated evidence indicates that tumour cells would actively produce, release and utilize sEVs to promote tumour growth,[22,23] and biological fluids in close proximity to tumour cells likely serve as enriched sources of potential biomarkers.[24] Thus, PDAC-derived sEVs will be abundantly contained in PJ samples obtained from PDAC patients, and even from patients with the precursor stage, HG-PanIN.

2.2 | Collection of PJ for the isolation of sEVs

To our knowledge, there have been five reports on human PJ-derived sEVs, and these have mainly been related to the identification of biomarkers of PDAC (Table 1).[25–29]

TABLE 1 A list of pancreatic juice-derived extracellular vesicles as source of biomarkers in pancreatic cancer.

	Pancreatic juice collection	Sample volume	Isolation method	Detection method	Biomarker	Subjects (diagnosis, number of pts.)	Early-stage PDAC (UICC, number of pts.)	Reference (reference number)
1	During surgery	1 ml	dUC	LC-MS/MS	CEACAM 1 CEACAM 5 Tenascin C	PDAC 13 IPMN 8 -IPMC 4 and low grade 4 CP 3 retention cyst 1 duodenal adenoma 1	StageIA, 2 StageIA, 2	Zheng J, et al. 2018 [25]
2	During surgery	250 µl for dUC, diluted 4 times in PBS	dUC anti-CD63 magnetic beads	LC-MS/MS	MUC1, MUC4, MUC5AC MUC6, MUC16, CFTR, MDR1	PDAC 7 CP 7 AC 7	N/A	Osteikoetxea X, et al. 2018 [26]
3	ERP under secretin stimulation	500 µl	dUC	RT-qPCR	miR-21 miR-155	PDAC 27 CP 8	Stage 0, 1 StageIB, 1	Nakamura S, et al. 2019 [27]
4	Duodenal fluid under secretin stimulation	200 µl	PBP	RT-qPCR	miR-21 miR-25 miR-16	PDAC 53 IPMC 1 non-malignant 118	Resectable 10	Nesteruk K, et al. 2022 [28]
5	ERP ENPD tube	1 ml	dUC PBP SEC	–	–	IPMN 18 PDAC 7 CP 3 Pancreatitis 2 AIP 1 Pancreatic cyst 1	N/A	Tsutsumi K, et al. 2022 [29]

Abbreviations: AP, ampullary carcinoma; AIP, Autoimmune pancreatitis; CFTR, cystic fibrosis transmembrane conductance regulator; CP, chronic pancreatitis; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; dUC, differential ultracentrifugation; ERP, endoscopic retrograde pancreatography; ENPD, endoscopic naso-pancreatic drainage; IPMN, intraductal papillary mucinous neoplasm; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDR1, multiple drug resistance 1; N/A, not applicable; PBS, phosphate Buffered Saline; PBP, polymer-based precipitation; PDAC, pancreatic ductal adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; SEC, size-exclusion chromatography.

Among the subjects from whom PJ samples were collected, PDAC patients were most frequent, followed by patients with intraductal papillary mucinous neoplasm (IPMN), a precursor to pancreatic cancer. Patients with chronic pancreatitis (CP) were usually adopted as a control, and this setting seems appropriate because CP is one of the risk factors for the development of PDAC, and CP patients sometimes have a chance to undergo therapeutic ERCP with pancreatic stenting for pancreatic duct stenosis or pancreatic stones. In these reports, PJ-derived sEVs were isolated in all cases, but the co-existence of mucin in a PJ sample was sometimes an obstacle to the isolation of sEVs due to its viscosity, especially in IPMN patients.

Pure PJ samples can usually be collected during ERP; however, contamination with contrast agent, which is a little viscous and hyperosmotic, should be avoided. Endoscopic placement of a naso-pancreatic drainage (ENPD) tube is often useful for increasing the total amount of PJ that is collected, even in cases in which an insufficient amount of PJ can be collected during ERP and/or when a PJ sample is highly viscous, as we described in a previous study.[29] Alternatively, the collection of duodenal fluid close to the ampullary orifice during diagnostic endoscopic ultrasonography (EUS) after intravenous secretin stimulation was also reported.[28] This method is ideal for screening of PDAC detection because there is no risk of post-ERP pancreatitis; however, the contamination of duodenal contents is unavoidable. Thus, rigorous interpretation of the obtained data using these PJ samples might be needed, dependent on the method used to collect the sample. After the collection, the PJ sample is centrifuged at 2,000 ×*g* for 10 min for the removal of cells, and it is necessary to store the sample below −80°C until a further molecular biological analysis.

2.3 | Isolation and characterization of PJ-derived sEVs

For the isolation of PJ-derived sEVs, the applied volume of PJ samples was 200 µl to 1 ml, and a standard differential ultracentrifugation (dUC) protocol was used.[30] To identify a more suitable method allowing the isolation of 'pure' PJ-derived sEVs from a small volume of PJ samples, we compared the three common isolation methods, including dUC, polymer-based precipitation, and size-exclusion chromatography (SEC). [31] According to our analysis using transmission electron microscopy (TEM) and Western blotting of sEV markers, SEC could be an optimal isolation method due to its higher collection rate and the purity of PJ-derived sEVs. [29] SEC is a simple and reproducible sEVs collection method and readily recover morphologically intact functionally competent EVs from small volumes. [31] On the other hand, dUC has disadvantages such as low collection and high time consumption, and is unsuitable for viscous samples, [27] and the precipitation can yield higher sEVs amounts but achieves lower purity, because of co-precipitated proteins,and is time-consuming. [32] Further validation is needed with large numbers of PJ samples. Moreover, the development of high-throughput sEV isolation methods is strongly desired for clinical application.

Based on the Minimal Information for Studies of Extracellular Vesicles 2018 guidelines of the International Society for Extracellular Vesicles,[33] the characterization of isolated PJ-derived sEVs by tools such as TEM and Western blotting is crucial before a downstream assay. In those studies, TEM images revealed that the mode diameter of PJ-derived sEVs ranged from 40 to 138 nm depending on the findings of each report. As in plasma-derived sEVs, it was confirmed that positive protein markers of sEVs, such as CD63 and CD81 (these are non-tissue-specific tetraspanin) and TSG101 (a cytosolic protein recovered in EVs) were expressed, and that negative markers, such as calnexin and GM130 (present in endoplasmic reticulum and Golgi apparatus) were absent in PJ-derived sEVs.

2.4 | Impact of alkaline condition and pancreatic enzyme activity on sEVs existed in PJ

Human PJ chiefly consists of alkaline fluid with bicarbonate. Nakamura et al. reported that the expression of microRNA (miRNA)-21 in PJ-derived sEVs was stable in such alkaline conditions at 37°C for 48 h in vitro, suggesting miRNAs in sEVs would be stable in the pancreatic duct.[27] We investigated the stability of PDAC cell lines, PANC-1-derived sEVs under alkaline conditions and under protease activation, as well in vitro, and found that the morphology—as observed by TEM—of PANC-1-derived sEVs was unchanged under alkaline conditions for 12 h at 37°C, while the sEVs collapsed at 12 h after exposure to pancreatin (an activated pancreatic enzyme) at 37°C.[29] In addition, according to an in vitro evaluation using PJ samples with protease activation, the expression of TSG101 (one of the markers present within sEVs) obviously decreased in comparison to the control (no exposure) after more than 6 h of exposure to room temperature and after 3 h of exposure, thus suggesting that protease activity would certainly affect the degradation of sEVs in PJ. Considering the time required for degradation, collected PJ samples that have just flowed out into the ENPD tube would be suitable as PJ samples. Interestingly, protease activity was positive in 46% of PJ samples collected during ERP, and in over 80% of PJ samples collected using

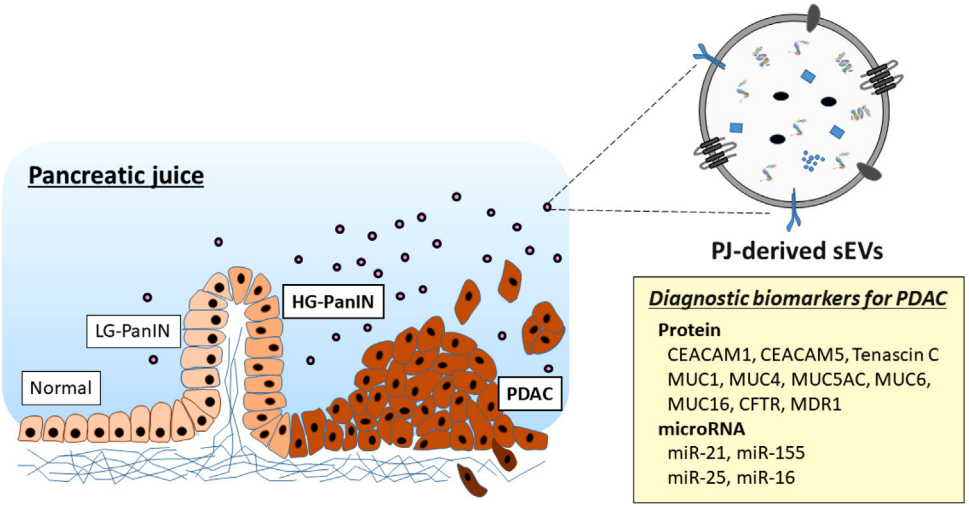

FIGURE 1 Potential biomarkers for high-grade PanIN and pancreatic ductal adenocarcinoma in pancreatic juice-derived small extracellular vesicles.

an ENPD tube. Therefore, it would be preferable to immediately store collected PJ samples in a freezer, and the addition of protease inhibitor may be useful for preventing degradation.[34]

2.5 | PJ-derived sEVs as a potential diagnostic and screening tool for PDAC

All encapsulating cargos, including protein, nucleic acids and lipids, into various body fluids-derived sEVs can be targets for cancer-specific biomarkers, and their potential application in the noninvasive diagnosis of cancer, screening and monitoring of therapeutic responses is strongly expected.[34–47] Regarding the cargo of PJ-derived sEVs, miRNAs and proteins have been studied to identify candidates diagnostic biomarkers for PDAC Figure 1. [25–29]

Nakamura et al. identified miR-21 and miR-155 in PJ-derived sEVs as potential diagnostic biomarkers of PDAC.[27] The authors investigated 35 patients (PDAC, $n = 27$; CP, $n = 8$) and found that the high expression of either miR-21 or miR-155 in PJ-derived sEVs resulted in an accurate diagnosis of PDAC in 83% and 89% of cases, respectively, while there was no significant difference in the expression of either miRNA in whole PJ samples between PDAC and CP patients. In addition, the accuracy improved from 74% to 91%, when the results of these two miRNA biomarkers in PJ-derived sEVs were combined with the results of PJC alone. Noesteruk et al. studied 54 malignant cases (PDAC, $n = 53$; IPMN, $n = 1$) and 118 non-malignant controls, such as high-risk individuals under surveillance due to a hereditary predisposition for PDAC or a family history of PDAC and also for neoplastic pancreatic cysts, using PJ samples that were prospectively collected from the duodenum.[28] They found that a combined panel of miR-21, miR-25 and miR-16 in PJ-derived sEVs, miR-210 in serum-derived sEVs and serum CA19-9 was a little better for distinguishing PDAC patients from controls in comparison to CA19-9 alone (Area Unver the Curve 0.91 and 0.85, respectively); however, this improvement was not sufficient for it to be applied in clinical practice. In particular, the miRNA expression patterns of each of five examined miRNAs were not correlated between PJ-derived sEVs and serum-derived sEVs. This result allows us to speculate that a comprehensive investigation of miRNAs in PJ-derived sEVs may be able to identify novel biomarkers, which are not detected in blood, that are strongly related to the development and progression of cancer. However, there have been no comprehensive miRNA analyses for the identification of unique biomarkers of PDAC, and there were too few patients with early-stage PDAC (PDAC of <10 mm in diameter and HG-PanIN, confined within the pancreas, without metastasis) in these two articles (there was only one such patient). Furthermore, due to tumour heterogeneity, the optimal combination of miRNAs in PJ-derived sEVs for the early detection of PDAC should be prospectively elucidated using a greater number of samples from high-risk patients.

Proteomic analyses of PJ-derived sEVs have been performed to identify biomarkers for PDAC.[25,26] Osteikoetxea et al. compared the proteomic profiles of four PDAC patients with those of four CP patients and identified candidate markers for PDAC, including cystic fibrosis transmembrane conductance regulation (the mutation of which was associated with an increased risk of developing PDAC),[48] MUC1, MUC4, MUC5AC, MUC6, MUC16 and MDR1 (multidrug resistance protein 1) proteins.[26] Zheng et al. studied 26 patients (PDAC, $n = 13$; IPMN, $n = 8$; other benign pancreatic diseases, $n = 5$) and

identified carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1, CEACAM 5 and tenascin C as candidate biomarkers for distinguishing PDAC from the other conditions.[25] Interestingly, validation by immunohistochemistry revealed that CEACAM 1/5 was strongly expressed on the membrane of tumour epithelial cells, while tenascin C, which has been implicated in cell growth and migration of PDAC,[49,50] was expressed strongly and more diffusely on the stroma. Cancer-associated fibroblasts within the stroma contribute to cancer progression via sEVs,[51] and stroma-derived EVs may also be released in the PJ; however, it is unknown whether this finding is observed in non-invasive PDAC, including HG-PanIN. Furthermore, transcriptomics such as circular RNA (circRNA) and long non-cording RNA (lncRNA),[40–43] epigenomics, glycomics,[44] metabolomics[45] and lipidomics,[46,47] which have been used to investigate sEVs-derived from various biofluids for potential clinical application, can also be used to identify biomarkers in PJ-derived EVs.

2.6 | PJ-derived sEVs as a potential therapeutic target for PDAC

Tumor-derived EVs have roles in cancer progression, including cell proliferation and metastasis in various cancers.

Hoshino et al. showed that tumour-derived sEVs taken up by organ-specific cells prepared the pre-metastatic niche. They revealed the distinct integrin expression patterns of sEVs, in which integrins $\alpha 6\beta 4$ and $\alpha 6\beta 1$ were associated with lung metastasis, while integrin $\alpha v\beta 5$ was linked to liver metastasis.[52] Thus, the analysis of sEV integrin in PJ could contribute to predicting pre-metastatic organs and prevent metastasis to a specific organ. In addition, remnant pancreatic cancer is rarely seen in clinical practice. Miyasaka et al. reported that remnant pancreatic cancer developed more frequently after the resection of cancer in the pancreatic body-tail.[53] Makohon–Moore et al. revealed that precancerous neoplastic cells, such as PanINs, can move through the pancreatic ductal system and progress into PDAC with the accumulation of genetic and epigenetic changes.[54] Furthermore, initial PDAC-derived sEVs, which flow through the pancreatic duct from the body-tail to the head, could initiate the development of remnant PDAC through cell-to-cell communication. If so, the identification of a specific target related to the uptake of sEVs into pancreatic ductal cells could prevent remnant pancreatic cancer. Thus, PJ-derived EVs may be a novel target for preventing metastasis of PDAC and metachronous PDAC. To identify therapeutic targets, further experiments should be conducted to investigate the functional roles of PJ-derived sEVs.

3 | CHALLENGES AND FUTURE PERSPECTIVE

In this review, we focused on the characteristics of human PJ-derived sEVs and their potential for clinical application, especially as an innovative diagnostic tool for the early diagnosis of PDAC, and an emerging therapeutic target for inhibiting cancer progression and metastasis and preventing recurrence. With the integration of PJ-derived sEVs and advanced omics technologies, it might be possible to achieve a precise diagnosis of early-stage PDAC and epoch-making treatment for metastatic and/or recurrent PDAC.

Despite the expectations raised in prior investigations, there are still some issues to be solved in relation to PJ-derived sEV-based biomarkers and therapeutic target discovery, to progress from the investigation stage to clinical application.[55–58] First, the development of a simple, reproducible and high-throughput method for the isolation of PJ-derived sEVs is essential for widespread clinical use. Second, a reliable normalization method is needed for the analysis of sEVs, for both miRNA biomarkers and protein biomarkers, although the alignment of sample volumes, compensation using synthetic nonhuman miRNAs, cel-miR-39, or the expression of multiple miRNAs, and certain sEVs markers (e.g., CD9 and CD63) have been commonly used. Certainly, these two matters are common, but they still remain unsolved tasks for examining body fluid-derived sEVs. Third, PJ is sometimes viscous in comparison to other body fluid, as mentioned above. The development of a novel technology for the isolation of sEVs in viscous PJ samples is highly desirable. This development would amount to a great step forward in the establishment of a method for distinguishing malignant IPMN from benign IPMN, is also a major problem in clinical practice. Fourth, large-scale prospective longitudinal cohort studies are needed to identify reliable biomarkers for diagnostics, screening and surveillance. In addition, we should separately address the definitive diagnosis of early-stage PDAC, including HG-PanIN, and screening tests for PDAC. While the collection of pure PJ using ERP via the papilla of Vater is ideal for precise inspections of PDAC, non-invasive sampling (e.g., duodenal fluid) is more suitable for screening. As control subjects, symptomatic CP patients and IPMN patients with high-risk stigmata who need to undergo diagnostic or therapeutic ERCP are suitable for PJ collection via the papilla, while duodenal fluid collection close to the papilla is appropriate for many asymptomatic patients who have risk factors for the development of PDAC, such as family history, genetic disorders, diabetes mellitus, CP or IPMN[59,60] and who undergo screening esophagogastroduodenoscopy and EUS.

Recently, some retrospective cohort studies suggested distinctive imaging features that can be used to suspect small PDAC including HG-PanIN, including: focal pancreatic parenchyma atrophy, stenosis of the pancreatic duct and main pancreatic duct dilation.[12,61] Especially for patients without a pancreatic mass, repeated PJC using ENPD is a feasible method that is important for the diagnosis of early-stage PDAC including HG-PanIN.[3,11,12] The combination of advanced imaging and PJ-based examination including cytology and PJ-derived sEVs-based biomarkers has great potential for application in the precise diagnosis of early-stage PDAC and could contribute to the improvement of the prognosis of PDAC. Furthermore, the exploration of HG-PanIN/PDAC-released sEVs in PJ will open a new field in tumourigenesis and the development of PDAC, and may bring new light on lethal PDAC.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest associated with this manuscript.

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