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Evolving utility of exosomes in pancreatic cancer management

Anoop TM *et al*. Role of exosomes in pancreatic cancer

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Abstract

Despite the development of newer oncological treatment, the survival of patients with pancreatic cancer (PC) remains poor. Recent studies have identified exosomes as essential mediators of intercellular communications and play a vital role in tumor initiation, metastasis and chemoresistance. Thus, the utility of liquid biopsies using exosomes in PC management can be used for early detection, diagnosis, monitoring as well as drug delivery vehicles for cancer therapy. This review summarizes the function, and clinical applications of exosomes in cancers as minimally invasive liquid biomarker in diagnostic, prognostic and therapeutic roles.

Key Words: Pancreatic cancer; Exosomes; Biomarker; Liquid biopsy; Clinical applications; Circulating biomarkers

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Core Tip: The determination and identification of biomarkers using liquid biopsy can enable the early detection, monitoring, therapeutic interventions, risk of relapse, therapeutic targets and identification of resistance mechanisms in pancreatic cancer (PC). There has been a recent interest in use of exosomes as biomarker in PC management. Exosomes loaded with multiple diagnostic molecules can be isolated from different body fluids and can be used for making the exosome markers-based liquid

biopsy more attractive for initial tumor detection, monitoring, and prognostic assessment of PC.

INTRODUCTION

Pancreatic cancer (PC) is associated with poor survival outcome with a 5-year survival of 5%-10%, and a short median survival of 6-8 mo after cancer diagnosis[1]. Most individuals diagnosed with advanced disease are symptomatic whereas early stages of the cancer are generally asymptomatic and often undiagnosed. Hence diagnosis is often made after dissemination. Surgery is the only curative treatment[2]. Regrettably, a large number of patients present with unresectable or metastatic disease at the time of diagnosis. Early detection of PC is essential for treatment with curative intent, typically by surgical resection in combination with neoadjuvant or adjuvant chemotherapy and chemo radiation. The majority of patients can have local recurrence or systematic metastasis even after resection. Screening methods for PC often relies on carbohydrate antigen 19-9 (CA 19-9). CA 19-9 demonstrates relatively low sensitivity and specificity in diagnosing PC[3]. Hence a diagnostic test with high sensitivity and specificity and capable of distinguishing PC at early stages from benign diseases is highly recommended. Table 1 shows common circulating biomarkers for PC[4-19]. Comparison of usefulness of various Liquid biopsies used in PC is shown in Table 2[20-30].

BIOGENESIS OF EXOSOMES

There is an emerging role of molecular profiling of liquid biopsies for cancer diagnosis and prognostication. Extracellular vesicles (EVs) derived from various body fluids and serum. There are four subclasses of EV based on different sizes like Exosomes (30-150 nm), Oncosomes (100-1000 nm), Ectoderms (100-1000 nm) and Apoptotic bodies (200-2000 nm). Exosome or Exosomes derived proteins, *etc.* are believed to serve as reliable molecular biomarkers. The circulating vesicles in the blood that originate from tumor cells contains immense proteomic and genetic information to monitor cancer progression, metastasis, and drug efficacy[31-33]. Exosomes were originally introduced

during the culture of sheep reticulocytes *in vitro* by Johnstone *et al*[34]. Exosomes are EVs that are endosomal in origin with a diameter of 40-160 nm (average, 100 nm). The formation of cancer cell derived exosomes is depicted in Figure 1. Initially, exosomes are formed by inward invagination of plasma membrane to form an early endosome. These endosomes form nano-sized vesicles resulting in formation of multi vesicular body (MVB) that contain intraluminal vesicles which contain cytoplasmic components including various nucleic acids and soluble proteins[35]. These intra luminal vesicles are released to the extracellular environment by fusing the MVBs with the plasma membrane. Then with the help of exocytosis, exosomes are released in to circulation.

Exosomes contains many molecules like heat shock proteins, RNAs, DNAs, GTPase, CD63, CD81, CD9, CD82, cholesterol, sphingomyelin, and ceramides. Exosomes facilitate both the transport of essential substances like nucleic acids and proteins into various recipient cells and the communications between cells. The main sources of exosomes are plasma, serum, urine, bile, saliva and breast milk. The secreted exosomes have various cellular functions in cell-to-cell interactions and might be pivotal in the occurrence and development of tumour progression and metastasis[36]. Exosomes have definite role in inflammation, coagulation, and embryo implantation in pregnancy. However, cancer cells are capable of secreting 10 times than normal cells. Hence tumour derived exosomes can provide vast information on cancer. Furthermore, exosomes are potential surrogates of the original cells, hence they are useful for understanding cell biology.

Oncosomes are tumor derived cells and they contain different oncogenic molecules that can modify the cells to encourage cancer growth. Tumor cell-secreted exosomes are responsible for paracrine signalling during tumor progression, tumor-stromal interactions, proliferative pathway activation, and immunosuppression[37]. Tumor derived exosomes enters the cells by a variety of methods, depending on the cells that secrete them and the target cells. Metastatic breast cancer derived exosomes use transcytosis to cross the brain endothelial cells, while the “CDC42-dependent endocytic pathway” was utilized to enter astrocytes during brain metastasis[38].

EXOSOMES IN INITIATION OF PC AND METASTASIS

There are increasing evidence that exosomes are involved in the pathogenesis of development of pancreatic inflammation as well as related cancer initiation. Repeated episodes of pancreatitis are a strong risk factor which can eventually increase the risk of PC. The pathogenesis and evolution of many pancreases precancerous conditions, including diabetes mellitus and pancreatitis, have been linked to crucial involvement of exosomes[39]. Exosomes can participate in promoting the transformation of various precancerous like intraductal papillary malignant neoplasm to PDAC. Exosomes are a key factor in initiating angiogenesis, cell migration, and epithelial-mesenchymal transition[40]. Cancer-associated fibroblasts, tumor-associated macrophages and pancreatic stellate cells can promote exosomes, that could promote growth, proliferation, drug resistance, EMT, migration, invasion and metastasis of PC[41]. Interestingly, exosomes initiated from PC cells contains tumor suppressor components which can inhibit the cancer cell proliferation and this could open the pandora box of potential therapeutic value of exosomes[42]. Exosomes promote cancer cell proliferation and initiate metastasis by delivering carcinogenic proteins, cytokines, adhesion molecules and miRNA. Thus, initiate proliferation of tumour by activation of different pathways like phosphoinositide 3-kinase/AKT serine/threonine kinase 1 (Akt) and mitogen-activated protein kinase pathways[43]. The features like weight loss and new-onset diabetes are characteristics of the paraneoplastic effect of PC which mostly precede the diagnosis of the PC. The biological reason of PC-associated diabetes is due to exosomal adrenomedullin, endoplasmic reticulum stress which may result in β-cell dysfunction and diabetes[44]. There is emerging evidence that suggest role of exosome-mediated immunosuppression in PC. The exosomes have clear role in communications between tumor and immune cells and supposed to have a dynamic role in tumor immunity regulation. Gemcitabine chemotherapy is considered a standard treatment for PC either in combination or monotherapy, based on evidence from many studies which shown a better survival rate and more clinical benefits with

median overall survival (OS) of 5 mo to 7 mo[45]. Most of patients with PC ultimately present with rapid disease progression even following chemotherapy with gemcitabine. Tumor derived exosomes can induce the progression of chemotherapy resistance in cancer cells. Chemotherapy agents could also be secreted from the extracellular matrix by exosomes is another reason for chemotherapy resistance. When exposed to gemcitabine, exosomal CAFs which are inherently insensitive to gemcitabine may also leads to chemotherapy resistance. CAF exosome secretion inhibition could decrease proliferation and drug resistance[46].

ISOLATION OF EXOSOMES

There are various methods to isolate and characterize exosomes based on their physical and chemical properties. Most popular methods are ultracentrifugation, size exclusion chromatography, magnetic activated cell sorting, membrane filtration and various commercial kits[47]. Western blotting and flow cytometry can be used to analyze and detect exosome markers. Transmission electron microscopy and nanoparticle tracking analysis are other methods to detect exosome.

Liquid biopsy to analyze exosome biomarkers could guide the diagnosis and prognosis of PC. Therefore, the identification of reliable predictive biomarkers for diagnosis and prognosis is an unmet need in PC management. The most common methods for isolation for exosome are summarized in Table 3[48-56] and quantifying methods for exosome are presented in Table 4[48-64]. Methods like Western blotting and enzyme linked immunosorbent assays needs large amounts of sample and extensive technical steps for detection. The comparison of various isolation methods used for exosomes are given in Table 5[65-76].

EXOSOMES AS DIAGNOSTIC BIOMARKER IN PC

At present scenario, early diagnosis of PC is very difficult and most are diagnosed at late stage. Mostly CT imaging are used for diagnosis and treatment. CA19-9 which is used in clinical practice has a low specificity and poor ability to distinguish benign

pancreatic diseases from PC[77]. Thus, the search for novel early diagnostic markers is a concern for PC diagnosis and treatment. Exosomes are excessively produced in excess by malignant tumours. They also carry information about the tumour genetics and microenvironment, which determines its behaviour and its prognosis[78]. Circulating biomarkers are non-invasive and inexpensive for monitoring disease[79]. The circulating molecular tumor markers are circulating tumor cells, cell-free DNA, cell-free RNA, circulating tumor proteins, and exosomes. When compared to circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), exosomes are considered as the best diagnostic biomarkers in PC with a sensitivity of 93% and a specificity of 92%[80]. The ctDNA group had similar specificity 0.92 (95%CI 0.88-0.95) but lowest sensitivity. Thus, ctDNA was useful for the diagnosis of PC rather than screening. Whereas CTCs exhibits medium sensitivity and lowest specificity compared to others. The sensitivity, specificity and AUC of ctDNA were 0.6400, 0.9200, and 0.9478 respectively. Glypican 1 (GPC1) is expressed in the serum of patients with PC but not in benign pancreatic disease. Melo *et al*[19] described that Glypican-1 identifies cancer exosomes and could detects early PC. It was described that GPC1+ circulating exosomes could be used as prognostic biomarker in pre- and post-surgical patients. The GPC1+ circulating exosomes could distinguish PC precursor lesions from healthy individuals and benign pancreatic disease. The circulating GPC1+ exosomes levels were higher in PC precursor lesions than the levels in the healthy donor group and benign pancreatic diseases. There is supporting evidence that there is a potential use in early detection of pancreas cancer. Melo *et al*[19] study shows that circulating GPC1+ exosomes exhibit a sensitivity of 100% and specificity of 100%; with a positive predictive value of 100% and a negative predictive value of 100% compared to CA 19-9 which was inferior in distinguishing between pancreas cancer and healthy controls.

The functional role of MicroRNAs has a greater opportunity in developing both prognostic and diagnostic markers. MiRNA -based biomarkers can help in the early diagnosis of disease. A recent study evaluated the expression patterns of miR-21, miR-155, miR-17-5p, and miR-196a in circulating exosomes as biomarkers for PC. The

expression profile of miR-17-5p and miR-21 were increased in PC patients, The increased expression of miR-17-5p was seen in unresectable pancreatic patients[81]. miR-155 and miR-21 are over-expressed in PDAC, and can distinguish PC from benign lesions[82]. Upregulated miR-221-3p and miR-212 is associated in PDAC and they are responsible for cancer proliferation in PDAC cells. miR-128 expression is decreased in PC while non-cancerous tissue has a normal level of miR-128. Gemcitabine resistance is associated with downregulation of miRNA200b, miRNA-200c, let-7b, let-7c, let-7d, and let-7e. miR-155 and miR-1246 also have been related to gemcitabine resistance. There are miRNA that function as tumor suppressors in pancreatic ductal cancer like miR-99b, miR-100, miR-99a, miR-34a, miR-148a, miR-200a, miR-200b, and miR-200c. MicroRNAs expression profiles showed that miR-143, miR-29c, miR-148b, miR-150, and miR-96, were present in PDAC and chronic pancreatitis whereas miR-196b, miR-203, miR-196a, miR-210, miR-222, miR-210, miR216, miR-375, and miR-217, were expressed only in pancreatic carcinoma[83]. miR-190, miR-196a, miR-222, miR-15b, miR200b, miR-95, and miR-221 are elevated in pancreatic adenocarcinoma[84]. Nakamura *et al*[85], developed an exosome-based signature for non-invasive and early detection of PDAC. Previous research studies showed that serum Ephrin type-A receptor 2 in exosomes (Exo-EphA2) was expressed highly in PC cells. A study by Wei *et al*[86] the evaluated role of serum Exo-EphA2 as a potential diagnostic biomarker in PC. Serum Exo-EphA2 were higher in PC than in non-cancer pancreatic disease. Exo-EphA2 in combination with CA 19-9 was more useful to discriminate early stage of PC from non-cancer pancreatic disease. Alkaline phosphatase placental-like 2 presents in PC EVs has a potential application in liquid biopsy-based diagnostic tests. Shin *et al*[87] developed ALPPL2 direct and sandwich aptamer-linked immobilized sorbent assay for EVs, which could sensitively and specifically detect membrane protein,17 could be a potential biomarker for early diagnosis of PDAC. Recently, there are reports of exosomal migration inhibitory factor (MIF) may be an attractive sensitive biomarker for PC. MIF is an immunostimulatory cytokine associated with tumorigenesis. Costa-Silva *et al*[88] reported that the pancreatic exosomes are capable of inducing premetastatic niche formation in liver. They

demonstrated that the exosome education-induced liver metastasis was abolished by silencing of exosomal MIF. The combined use of exosomal glypican-1 and MIF is a promising tool to identify very early stages of PC. The potential of MIF as a target for the treatment of PDAC should be explored in future.

EXOSOMES AS PROGNOSTIC BIOMARKER IN PC

The level of circulating Exo-EphA2 was higher in PC patients when compared to that of healthy controls, suggesting it could be a diagnostic and prognostic marker for PC. In a study by Wei *et al*[86] found that high expression of Exo-EphA2 in PC was associated with shorter OS. Exosomal KRAS mutations seems better than CA 19-9 Levels for the prognostic surveillance in PDAC[17]. A study by Tsuchida *et al*[89] revealed that KRAS mutation detected at baseline with a mutation frequency above 5% indicated poor clinical outcome following monitoring in the treatment course of patients with metastatic PDAC. Costa-Silva *et al*[88] found that MIF was markedly higher in exosomes from stage I PDAC patients who later developed liver metastasis. Thus, it is suggested that higher exosomal MIF may be a prognostic marker for the development of PDAC liver metastasis. Potential role of exosomal biomarkers for prognosis evaluation in PDAC was evaluated in a systematic review and meta-analysis, involving eleven studies comprising 634 patients and seen that detection of positive exosomal biomarkers increased risk of mortality and progression across disease stages. Positive exosomal biomarkers preoperatively had higher risk of mortality in resectable stages than positive exosomal biomarkers in unresectable stages[90].

The better understanding of the prognostic role of miRNAs in PC can be done by profiling miRNAs at different stages of cancer. In a study by Takahasi *et al*[91], authors suggest that plasma exosomal miR-451a may be useful to predict recurrence and prognosis in PDAC patients. The miR-451a had a significant association with tumor, stage and showed the highest upregulation in the stage II patients who showed recurrence after surgery. In a retrospective clinical study by Namkung *et al*[92] comprising 200 pancreatic ductal adenocarcinoma tissue samples, miRNA-574-5p,

miRNA-1244, miRNA-145, miRNA-328, miRNA-26b, and miRNA4321 showed association with OS and disease-free survival. Poor survival outcomes have been seen in PDAC with lower expression of *miR-183* and *miR-34a* as well as high expression of *miR-1290*, *miR-155*, *miR-203*, *miR-222*, and *miR-10b*[93]. Similarly, Microarray-based expression profiling of miRNAs derived from exosomes study revealed that miR-451a was the highest upregulated miRNA in stage II patients who developed recurrence after surgery. It was seen that survival rates of the high exosomal miR-451a patients were significantly worse than those of the low miR-451a patients[94].

TREATMENT MONITORING IN PC

Currently, one of the most common biomarkers used for so long to monitor the therapeutic responses in PC is CA 19-9. Exosomes have a significant role in monitoring response to therapy and disease progression. Melo *et al*[19] clearly demonstrated that all 190 patients with PDAC serum had higher GPC1+ exosomes than healthy individuals and was an independent prognostic marker for disease specific survival. In view of the fact that CA19-9 is not a reliable marker that correlates with clinical evolution of PC, a combination of CA19-9 together with exosome derived GPC1 could be explored for treatment monitoring and disease progression. Besides early diagnosis and prognosis, clinical utility of exosome proteins is evolving for personalized and posttreatment disease monitoring[95]. Circulating exosomal PD-L1 is an attractive option in disease monitoring. Recently, Chen *et al*[96] study explains the rationale for the application of exosome PD-L1 as a predictor for anti-PD-1 therapy.

EXOSOMES AS DRUG CARRIERS, THERAPEUTIC TARGETS AND TREATMENT

Currently, innovators are exploring the utility of exosomes for biomedical applications. Many advanced drug delivery systems that used to deliver various anticancer and antiviral agents explore the use of polymeric nanoparticles and liposomes to encapsulate drug and thus utilize for drug delivery.

Exosomes can be used as therapeutic drugs carriers because of favourable bioavailability, biocompatibility, ability to penetrate biological membranes and immunogenicity[97]. Exosomes can be used as transporters, therapeutic targets and therapeutic drugs.

Due to the favorable bioavailability and biocompatibility with the characteristics of exosomes, there appears a greater future of exosomes used either as parental exosomes or artificially modified exosomes for drug delivery vehicle. To avoid systemic toxicity, drugs can be encapsulated in exosomes and transferred to target cells[98]. Exosomes possess better biocompatibility as drug carriers. It is generally considered that injected exosomes shed from endogenous cells are tolerated with minimal immune reaction. The cargos can be delivered into the tumor microenvironment with the utility of exosomes[99]. Kamerkar *et al*[100] studied modified exosomes for cancer prevention and treatment and revealed that exosomes had a longer retention time in the circulation. Engineered exosomes specialized for malignant KRAS G12D were more successful in targeting oncogenic KRAS. Recent evidence suggests that safety and efficacy of exosomes in treating PC is not far. Exosome-based therapies for cancers have been developed due to the easy permeability of the exosome membrane, low toxic side effects and low immunogenicity. Paclitaxel -loaded exosomes have shown a great potential for delivery of chemotherapy and treatment of drug-resistant cancers[101]. Because of their rapid clearance from blood circulation after systemic administration, targeted delivery of exosomes is highly restricted. The rapid clearance after injection limits their applications for effective and durable therapeutic action. However, recent studies on modification of exosomes for targeted delivery *via* direct modification and genetic engineering to circumvent this limitation is promising. The use of MSCs-derived exosomes loaded with KRAS G12D siRNA to treat metastatic pancreas cancer (NCT03608631) is promising[102]. Mittal *et al*[103] also showed the efficacy of administration of micelles of gemcitabine and the tumor suppressor *miRNA-205* for the treatment of pancreas. Masamune *et al*[104] found that hypoxic environment in PC can release several angiogenic factors that may induce proliferation and angiogenesis.

Understanding of these interactions under hypoxia is critical for angiogenic regulation in PDAC, which will also help to develop new anti-angiogenesis therapeutic strategies[105].

CONCLUSION

Even though there are several limitations in implementing exosome analysis clinically, it is a promising diagnostic and therapeutic tool for PC. The role of exosomes in cancer treatment continues to evolve.

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Figure Legends

Figure 1 Diagrammatic representation of formation of cancer cell derived exosomes.
MVB: Multi vesicular body.

Table 1 Circulating biomarkers in pancreatic cancer

Biomarker	Type	Role in pancreatic cancer
CA19-9	Protein	Widely used biomarkers to aid in the diagnosis[4] Poor screening tool in asymptomatic patients Elevated in many benign gastrointestinal conditions as well as other malignancies, including pancreatitis, cirrhosis, cholangitis, and colorectal cancer[5] 5%-10% of the Caucasian population possesses a Lewis a-/b- genotype and thus does not express CA19-9
CEA	Glycoprotein	Elevated across several cancers[6] Non specific Inferior sensitivity of CEA compared to ca19-9[7]
CA125	Glycoprotein	Associated with ovarian cancer, CRC and cholangiocarcinoma[8] Speriority to CA19-9 in predicting resectability of PC, along with correlating with metastasis-associated disease burden
Anti-MUC1 antibody	Antibody	Anti-MUC1 antibody assays showed a sensitivity and specificity of 77% and 95%, respectively, in discriminating pancreatic cancer from pancreatitis[9]
CTCs	Tumour cells	CTCs had moderate diagnostic value in PC[10] Sveral studies have demonstrated isolation of CTCs regardless of stage among localized, locally advanced, or metastatic patients

		Conflicting evidence on CTC positivity is correlated with survivability In ombination with CA19-9, it was reported to have a superior sensitivity and specificity of 97.8% and 83.3% respectively, compared to when used in isolation[11] The presence of CTCs in 54/72 patients with confirmed PDAC (sensitivity = 75.0%, specificity = 96.4%, AUROC = 0.867, 95%CI = 0.798-0.935, and $P < 0.001$)[12] A cut-off of ≥ 3 CTCs in 4 mL blood could differentiate between local/regional and metastatic disease (AUROC = 0.885; 95%CI = 0.800-0.969; and $P < 0.001$)
cfDNA	DNA	Plasma ctDNA quantification of hot-spot mutations in KRAS and GNAS are useful in predicting tumor burden in patients diagnosed with PC[13] Digital PCR provided accurate tumor-derived mutant KRAS detection in plasma in resectable PC and improved post-resection recurrence prediction compared to CA19-9[14] Detection of plasma cfDNA mutations and copy number alterations may be helpful in pancreatic cancer prognosis and diagnosis Its sensitivity and specificity in identification of clinically relevant KRAS mutations was 87% and 99% respectively[15]
Cell-free RNA	RNA	Higher expression of lncRNA MALAT1 has been shown to correlate with poorer PDAC survival[16]

		Several microRNAs have also been associated with PDAC (*i.e.*, miR-21 and miR-155), and correlate with tumor stage or prognosis[17]
EVs	Exosomes	KRAS G12D mutations were identified in 7.4% of control patients, 67% of localized PDAC, 80% of locally advanced PDAC, and 85% of metastatic PDAC patients[18] GPC1 EVs could be detected in both pancreatic precursor lesions and pancreatic cancer, and could distinguish between any evidence of malignancy and healthy patients with an AUC of 1 (100% sensitivity, 100% specificity)[19] miRNA isolated from EVs revealed a cocktail of miRNAs (miR-1246, 4644, 3976, 4306) upregulated in 83% of pancreatic cancer derived EV Glypican-1 exosomes are a potential biomarker for PC

CA: Carbohydrate antigen; PDAC: Pancreatic ductal adenocarcinoma; PC: Pancreatic cancer; CEA: Carcinoembryonic antigen; CTCs: Circulating tumour cells; cfDNA: Cell-free DNA; EVs: Extracellular vesicles; AUROC: Area under the curve.

Table 2 Comparison of usefulness of various liquid biopsies in pancreatic cancer

Item	CTC[20-22]	Ct DNA[21,23,24]	Exosomes[20,25-28]	CA 19-9[20,21,28-30]
Origin	Viable tumor cells	cfDNA, viable tumor cells, CTCs	DNA, proteins, lipids, RNAs metabolites, and tumor cells	Ductal cells in the pancreas, biliary system, and epithelial cells in the stomach, colon, uterus, and salivary glands
Samples used	Plasma	Frozen plasma, urine and other biofluids	Frozen plasma, urine and other biofluids	Plasma
Methods	CellSearch, MACS, Dynabeads, microfluidic, SE-iFISH, CD45/CEP8/DAPI staining-FISH, anti-EpCAM Portal-vein blood	Real-time quantitative PCR, digital PCR, droplet digital PCR, next-generation sequencing; commercial liquid biopsy platforms: GuardantTM (breast, colon, and lung cancers and multi-cancer detection) FoundationOne® (multi-cancer detection);	Ultracentrifugation, ExoChip, precipitation, size-based isolation immunoaffinity-based isolation microfluidics-based isolation	Radio immuno assay

		signateraTM (colorectal cancer), Galleri (multi-cancer detection), CancerSEEK (multi-cancer detection), TempusTM (multi-cancer detection), Caris (bioinformatics testing of both circulating DNA and RNA)		
Mutation analysis	Yes	Yes	Yes	No
Drug delivery vehicle	No	No	Yes	No
Sensitivity	76.0%	65.0%	50.0%-85.0%	78.2%
Specificity	68.0%	75.0%	90.0%	82.8%
Usage in clinics	Diagnosis of PDAC, prognosis/prediction of PDAC	Diagnosis of PDAC; monitoring Treatment efficacy; monitoring of disease progression	Diagnosis and prognosis of PDAC; prognosis/prediction of PDAC	Combining ct DNA with CA 19-9 levels could improve diagnostic sensitivity to 98%, and specificity to

					97%; monitoring treatment efficacy; monitoring of disease progression

PDAC: Pancreatic ductal adenocarcinoma; CTC: Circulating tumour cells; CA: Carbohydrate antigen; ctDNA: Circulating tumor DNA.

Table 3 Different isolation methods for exosome

Method	Sample volume	Time	Ref.
Ultracentrifugation	Low	Approximately 5 h	[48,49]
Density-gradient	Low	Approximately 5 h	[50]
Nanopillar	30 μL	Approximately 10 min	[51]
Acoustic-based	0.4-0.7 μL/min	< 30 min	[52]
Inertial lift force-based	70 μL/min	> 4 h	[53]
Surface-modified	4-16 μL/min	< 1 h	[53-55]
Nanoshearing	Not mentioned	< 3 h	[56]

Table 4 Different quantifying methods for exosome

Method	Size range	Specificity	Time	Ref.
Nanoparticle tracking analysis	10 nm-2 μm	Immunoaffinity	< 1 h	[48]
Dynamic light scattering	10 nm-8 μm	Size	< 1 h	[57]
Electron microscopy	10 nm	Size	< 1 h	[58,59]
Nanopore	> 10 nm	Size	< 1 h	[60,61]
Magnetic resonance	Wide range	Immunoaffinity	< 10 min	62
Electrochemical and plasmonic	Depends on binding	Immunoaffinity	< 10 min	[63,64]

Table 5 Comparison of various isolation methods for exosomes

Conventional isolation of exosomes				
Methods	**Advantages**	**Disadvantages**	**Clinical use**	**Ref.**
Ultracentrifugation	Widely used; high purity; protein and RNA components are not affected	Highly labour intensive; time-consuming; yields are typically low extensive training of personnel needed; expensive; inappropriate for the extraction of exosomes from a small amount of serum samples	Functional study of exosomes	[65,66]
Ultrafiltration	High yield; simple; less time-consuming; do not require the use of special equipment	Low purity, clogging of pores	Study of sample concentration; used in combination with other methods	[67]
Precipitation	Widely used; economical	Co-isolation of non-EV particles	For studies with very low purity requirements that do not require omics studies	[68]
Size exclusion chromatography, OR, and gel filtration	Fast, reliable, and inexpensive; maintain the	Nanoscale contaminants like lipoproteins; extensive laboratory	Suitable for exosome research in those requiring	[69]

	biological activity and integrity of exosomes; high purity	equipment requirements	high purity, omics, and large volume samples	
Immunoaffinity capture	Convenient; not affected by exosome size; no need for expensive instruments	Expensive; low capacity; low yields	Suitable for the Separation of specific exosome subgroups	[70]
Emerging isolation methods				
Stirred ultrafltration	Do not rely on equipment; less time consuming; reduces the destruction of exosomes during the process	Moderate purity of isolated exosomes; loss of exosomes during the process	Isolating exosomes from culture supernatant of bone marrow mesenchymal stem cells	[71]
ExoTIC (exosome total isolation chip)	Simple, easy-to-use, modular, and facilitates high-yield and high-purity EV isolation from biofluids	Special equipment requirements; lack of tests on clinical samples	Efficiently isolate EVs from small sample volumes; EV-based clinical testing from fingerprick quantities (10-100 µL) of blood	[72,73]

3D ZnO Nanoarrays	Multifunction; high sensitivity; downstream analysis is possible; enhance the capture of exosomes at a high flow rate	Relatively expensive	Widely used in biosensing and analysis aspects, powerful tools for effective purification and molecular analysis of exosome	[74,75]
Nano plasmon-enhanced scattering	Rapid, high-throughput, sensitive, and specifc method for the detection of exosomes from trace samples depending on the amount of scatter area, based on calculation of the proportion of the area that contains scattered light	High reagent cost; complex statistical tools; low capacity	Uses antibodies against the cellular markers CD81, CD63, and CD9, which are enriched on most exosome membranes	[76]

SEC: Size exclusion chromatography; EV: Extracellular vesicle.

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