Peer Review File

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Reviewer 1:

Comment 1: The title and the entire manuscript: Although the present title aims to review the role of extracellular vesicles in the precision medicine of pancreatic cancer, some sections of the manuscript do not sufficiently confine the scope of discussion to the field of precision medicine. For instance, Section 2 is dedicated to discussing the role of EVs in cancer progression, which does not directly relate to the tailored approach to individual patients based on risk profiles and predicted therapy response - the core definition of precision medicine. Furthermore, the manuscript includes some cases based on cell lines or animal models, and the evidence derived from these references seems not directly relate to the practice of precision medicine. The authors should emphasize more on literature cases based on clinical datasets.
Response to the reviewer: Thank you for your feedback. While we acknowledge that some sections of our manuscript may appear broader in scope, our intent was to provide the necessary background and clinical context for the application of precision medicine in pancreatic cancer. We would like to clarify that due to the limited availability of clinical datasets/ trials specific to EVs in PC, we have focused on providing a comprehensive overview of the existing research landscape, which includes preclinical studies, cell line models, and animal models.

Comment 2: On pages 14-16, Table 1: The table lacks diagnostic performance data of using EV cargo content for diagnostic purposes or treatment response monitoring. Including data such as sensitivity, specificity, and AUC would help readers better understand the potential of EVs in precision medicine of pancreatic cancer. Moreover, the function of EV cargo content in the table is not limited to early cancer detection, which contradicts the description on lines 167-168 in the manuscript.
Response to the reviewer: Thank you for your valuable feedback on our manuscript regarding the diagnostic potential of EV cargo content in pancreatic cancer. In response to your suggestion, we have included diagnostic performance data, such as sensitivity, specificity, and AUC, in Table 1 on pages 14-16. However, we would like to acknowledge the knowledge gap that currently exists in this field. Despite our efforts, we were unable to obtain sufficient data to provide these specific diagnostic performance metrics for all EV cargo contents in the context of PC.
Changes to the manuscript: The table number is modified to **Table 2**. It includes 2 additional columns (PC patient sample size, sensitivity and specificity). We have made changes to lines 167-168, 'Given the importance of EVs released by cancer cells in PC progression, the functional cargo carried by EVs shows potential as biomarkers for PC (Table 2).'

Comment 3: On pages 6-7, Section 3: The manuscript's discussion on using EV cargo content for diagnostic purposes or treatment response monitoring is insufficient. Many types of EV cargo content have shown potential diagnostic or monitoring value, including but not limited to miRNA, mRNA, circRNA, and protein. The authors could consider making this section a focal point of the article, by providing segmented discussion based on different types of EV cargo content to enhance clarity. The table could also be reorganized according to the types of EV cargo content.

Response to the reviewer: We appreciate your valuable suggestion for improving the discussion on EV cargo content for diagnostic purposes and treatment response. Regarding your recommendation for a segmented discussion based on different types of EV cargo content, we acknowledge the potential value of such an approach. We would like to clarify that the scope of this review article may not permit an exhaustive segmented discussion on this specific aspect. We have taken your valuable input into consideration and restructured the table 2. Addressing to the comment, we will make the following changes to the EV cargo content in the table.

Changes in the manuscript:

Table 2: The table has been modified according to the reviewer's suggestion. We have modified the table based on types of cargo (miRNA, mRNA, circRNA, proteins, DNA) experimental approach in analysis, sample size and relevance to PC. Further, we have modified the title of the table, 'Potential EV biomarkers in PC'. This table will help enhance the clarity and focus of the discussion on EV cargo content as diagnostic markers. By incorporating these changes, we aim to provide a more comprehensive and organized discussion of EV cargo content as potential diagnostic tools for pancreatic cancer. (Refer to Table 2 in the manuscript)

Comment 4: On pages 6-7, Section 3: The discussion of the potential and advantages of using EVs as biomarkers for pancreatic cancer is unclear. The authors should thoroughly explain the differences and advantages of using EVs derived from pancreatic cancer as biomarkers compared to other detection methods for precision medicine, such as cell-free DNA (cfDNA) and circulating tumour cells. For example, the reviewer would like to recommend referring to the paper, Zhao X, Ma Y, Dong X, et al. "Molecular characterization of circulating tumor cells in pancreatic ductal adenocarcinoma: potential diagnostic and prognostic significance in clinical practice." Hepatobiliary Surg Nutr. 2021;10(6):796-810, and Yoshioka Y, Shimomura M, Saito K, et al. "Circulating cancer-associated extracellular vesicles as early detection and recurrence biomarkers for pancreatic cancer." Cancer Sci. 2022 Oct;113(10):3498-3509, as the papers deal with the view of circulating tumor cells as well as EVs in pancreatic ductal adenocarcinoma.

Response to Reviewer: In response to your comment, we have provided a comprehensive explanation of the differences and advantages of using EVs derived from PC as biomarkers compared to cell-free DNA and ctDNA. Further, we have included an additional table and references suggested by the reviewer.

Changes to the manuscript:

Section added to line 159, page 5:' **EVs vs cancer biomarkers**

Scientific efforts are being made to detect early symptoms of the disease in high-risk individuals. This is particularly important in PC, where understanding the disease tumorigenesis, monitoring therapy, and predicting the progress is essential. High-risk individual compromise of family history, genetic mutation associated with PC, suffering from diabetes and chronic pancreatitis. Given the asymptomatic onset of PC, the paramount focus lies on early detection using reliable biomarkers. Minimally invasive biomarkers such as cell-free DNA and circulating tumour cells (CTCs) have yielded promising results [29]. These biomarkers, however, pose a challenge for large-scale applications due to their low concentration in blood plasma, difficult isolation techniques, and long-term storage and handling challenges. Moreover, EV cargo-derived RNA is considered to be more reliable than cell-free DNA as they are resistant to degradation [30]. In addition, EVs are comparatively highly concentrated in the blood and EV-derived cargo is unique to disease type [31, 32]. In order to fully harness the diagnostic and prognostic potential of EVs in PC, it is imperative that practical challenges need to be addressed. The variability in the isolation of EVs and contamination of albumin in plasma/serum samples can affect the downstream application of EVs. Utilisation of different isolation techniques may result in varying EV concentrations, which consequently impacts the purity and yield of retrieved EVs. Further, EVs composition can evolve according to the pathological and physiological conditions of PC. Future studies need to focus on EV expression based on tumour staging and detecting PC in early stages. Additionally, there is a need to increase the sample size based on high-risk population, chronic pancreatitis/ benign pancreatic conditions and existing genetic predisposition. Table 1 provides a comprehensive list of potential EVs, highlighting their advantages and disadvantages in comparison to other biomarkers.'

Table 1: Comparison of PC derived EVs vs other cancer biomarkers

Potential Biomarker	Sample Type	Diagnostic Value in PC patients	Advantages	Disadvantages	References
CA19.9	Serum	Sensitivity: 79-81% Specificity: 82-90%	Relatively easy collection Reliable marker for treatment response and monitoring	Poor screening marker Elevated expression in benign jaundice, pancreatitis, ovarian cancer or other malignancies.	[72]
CTCs	Serum/Plasma	Sensitivity: 75% Specificity: 96.4% AUC: 0.867 95% CI: 0.798-0.935	Correlated with poor prognosis	Low concentration in Serum/Plasma Lack of evidence in large scale clinical setting Variable in isolation techniques	[30, 73, 74]
cf-DNA	Plasma	Combination of 5mC and 5hmC prediction model Sensitivity: 93.8% Specificity: 95.5% AUC: 0.99	Correlated with poor prognosis	Utility is limited to identifying existing mutation in clinical setting Lacks evidence in large scale clinical setting	[75]
EVs	Plasma/ serum/ pancreatic juice	GPC1+ study Sensitivity: 95-100% Specificity: 95-100%	Correlated to early detection, prognostic marker and potential tumour staging marker	Variability in isolation techniques Lacks evidence in high quality production in clinical setting	[76-87]

Comment 5: On pages 7-8, Section 4: The manuscript's discussion of using EVs as therapeutic tools is insufficient. The emphasis should be on discussing the differences and advantages of using EVs instead of other carriers as drug delivery vehicles and detailing the mechanisms and potential applications of interrupting EV production and targeting molecular signalling as therapeutic tools. Instead of simply listing related literature cases, the authors should provide a comprehensive discussion in this regard.

Response to the Reviewer: Thank you for your constructive feedback. We have taken your comments into consideration and made significant improvements to the content. In response to your suggestion, we have enhanced the discussion in Section 4 (pages 7-8) by emphasizing the differences and advantages of using EVs as drug delivery vehicles compared to other carriers. Additionally, we have included a comprehensive table in the manuscript that highlights both clinical and preclinical studies related to the use of EVs in pancreatic cancer. This table provides readers with a clear overview of the various studies and their findings, further enhancing the understanding of the therapeutic potential of EVs in the context of pancreatic cancer.

Changes made to manuscript: Line added in 217, page 7: 'Rapid advancements in therapeutic tools have ushered in a new era of treatment strategies, where both biological and artificial vehicles are being harnessed for enhanced therapeutic outcomes (52-54). These remarkable tools share certain key physiological characteristics, such as their nanoscopic size and their capacity to efficiently deliver drugs to specific targets. Among these therapeutic tools, sEVs have emerged as particularly promising agents due to their unique capabilities (55). Notably, sEVs have demonstrated the remarkable ability to traverse the formidable barrier of the blood-brain barrier, allowing for precise drug delivery to targeted sites. Unlike the artificial nanoparticles, the sEVs will not be eliminated by phagocytic system. Small EVs constitute of

range of proteins that help in bypassing immunological response. This intrinsic ability to elude immune detection makes sEVs an appealing choice for therapeutic interventions. Further, sEVs are comparatively more efficient as drug carriers than liposomes. As sEVs exhibit a distinctive characteristic of circulating in the bloodstream for prolonged time in comparison to liposomes. Moreover, sEVs offer distinct advantage as drug carrier by accommodating significantly higher quantities of drugs compared to traditional liposomes.'

Line added in 222-223, page 8: The investigation of the application of EVs in therapies is still in its early phase (Table 3).

Line added in 264, page 10: 'EVs have emerged as a promising tool for improving PC patients' overall survival. By engineering EVs to carry pharmacological drug and specific surface modification may enable them to selectively recognise and bind to PC cells. The pharmaceutical drug may include chemotherapeutic agents, novel targeted therapies or siRNAs are designed to inhibit signalling pathways associated to PC progression. In conclusion, the PC derived EVs are small vesicles secreted by cells that can be engineered to deliver therapeutic agents directly to cancer cells. They can also traverse biological barriers and efficiently transport bioactive molecules that may interfere with the molecular mechanisms driving pancreatic cancer. Moreover, EVs can be analysed to provide diagnostic and prognostic value in pancreatic cancer. As research in EV-based therapies continues to evolve, it offers a beacon of hope for PC patients in need of more effective and tailored therapeutic options.'

Line 449, Page 17: **Table 3 Preclinical and Clinical Trials of PC derived EVs**

Disease Model	Trial	Pharmacological drug	Administration	References
Mouse	Preclinical	siPAK4	Intratumoral injection	[110]
Mouse	Preclinical	Gemcitabine	Intravenous injection	[111]
Mouse	Preclinical	siKRASG12D	Intraperitoneal injection	[112]
Mouse	Preclinical	siKRASG12D	Intraperitoneal injection	[62]
Mouse	Preclinical	siKRASG12D and miRNA-145-5p	Intratumoral injection	[113]
Human	Clinical (Phase 1)	siRNA KrasG12D	Intravenous injection	[114]

Reviewer 2:

The manuscript by Panda et al. is a very well written mini-review which provides a comprehensive picture of the role of extracellular vesicles in pancreatic cancer, by both biological and translational points of view. However, I think that some relevant papers have not been included among the reference, especially concerning miRNAs, which are among the most studied RNAs in EVs.

I recommend the authors to include some recent papers in their manuscript since would bring further enrichment to their paper:

Di Pace, A. L., Pelosi, A., Fiore, P. F., Tumino, N., Besi, F., Quatrini, L., Santopolo, S., Vacca, P., & Moretta, L. (2023). MicroRNA analysis of Natural Killer cell-derived exosomes: the microRNA let-7b-5p is enriched in exosomes and participates in their anti-

tumor effects against pancreatic cancer cells. Oncoimmunology, 12(1), 2221081. https://doi.org/10.1080/2162402X.2023.2221081

Vannini, I., Rossi, T., Melloni, M., Valgiusti, M., Urbini, M., Passardi, A., Bartolini, G., Gallio, C., Azzali, I., Bandini, S., Ancarani, V., Montanaro, L., Frassineti, G. L., Fabbri, F., & Rapposelli, I. G. (2023). Analysis of EVs from patients with advanced pancreatic cancer identifies antigens and miRNAs with predictive value. Molecular therapy. Methods & clinical development, 29, 473–482. https://doi.org/10.1016/j.omtm.2023.05.009

Roy, J. W., Wajnberg, G., Ouellette, A., Boucher, J. E., Lacroix, J., Chacko, S., Ghosh, A., Ouellette, R. J., & Lewis, S. M. (2023). Small RNA sequencing analysis of peptide-affinity isolated plasma extracellular vesicles distinguishes pancreatic cancer patients from non-affected individuals. Scientific reports, 13(1), 9251. https://doi.org/10.1038/s41598-023-36370-3

Response to the reviewer: We appreciate your recommendations for additional references, and we have included the suggested papers in our revised manuscript to further enrich the discussion regarding miRNAs and other relevant aspects of EVs in pancreatic cancer.

Changes to the manuscript:

Citation added to Table 2,' Di Pace, A. L., Pelosi, A., Fiore, P. F., Tumino, N., Besi, F., Quatrini, L., Santopolo, S., Vacca, P., & Moretta, L. (2023). MicroRNA analysis of Natural Killer cell-derived exosomes: the microRNA let-7b-5p is enriched in exosomes and participates in their anti-tumor effects against pancreatic cancer cells. Oncoimmunology, 12(1), 2221081. https://doi.org/10.1080/2162402X.2023.2221081'

Citation added in line 180, page 6: 'Vannini, I., Rossi, T., Melloni, M., Valgiusti, M., Urbini, M., Passardi, A., Bartolini, G., Gallio, C., Azzali, I., Bandini, S., Ancarani, V., Montanaro, L., Frassineti, G. L., Fabbri, F., & Rapposelli, I. G. (2023). Analysis of EVs from patients with advanced pancreatic cancer identifies antigens and miRNAs with predictive value. Molecular therapy. Methods & clinical development, 29, 473–482. https://doi.org/10.1016/j.omtm.2023.05.009' and 'Roy, J. W., Wajnberg, G., Ouellette, A., Boucher, J. E., Lacroix, J., Chacko, S., Ghosh, A., Ouellette, R. J., & Lewis, S. M. (2023). Small RNA sequencing analysis of peptide-affinity isolated plasma extracellular vesicles distinguishes pancreatic cancer patients from non-affected individuals. Scientific reports, 13(1), 9251. https://doi.org/10.1038/s41598-023-36370-3'