Review

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Liquid Biopsy for Early Pancreatic Cancer Detection: Why Has It Not Yet Worked?

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Review

Liquid Biopsy for Early Pancreatic Cancer Detection: Why Has It Not Yet Worked?

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Simple Summary

Liquid biopsy is anticipated as a non-invasive complement to histopathology, yet its utility for early detection of pancreatic ductal adenocarcinoma (PDA) remains limited. The primary obstacles are biological—specifically minimal tumor shedding—rather than purely technical, hindering clinical implementation. We critically examine these barriers and the lack of standardization. Furthermore, we outline potential solutions and initiatives to overcome these challenges, incorporating our own clinical attempts and experimental data to bridge the gap toward meaningful clinical application.

Abstract

Despite extensive technological advances and an ever-growing body of literature, liquid biopsy has yet to achieve reliable early detection of pancreatic ductal adenocarcinoma (PDA). Numerous studies have investigated circulating tumor-derived components, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), extracellular vesicles (EVs), and circulating tumor cells (CTCs), primarily using peripheral blood samples; however, their clinical utility for early-stage disease remains limited. The fundamental obstacles are biological rather than purely technical: early PDA and its precursor lesions, such as pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasms (IPMN), are characterized by minimal tumor burden, low levels of nucleic acid shedding, and substantial background signals from non-neoplastic tissues. Increasing analytical complexity through multilayered liquid biopsy approaches, including analyses from pancreas-associated fluid, has not consistently translated into improved diagnostic performance and, in some cases, has amplified issues related to specificity, reproducibility, and interpretability. Moreover, molecular alterations detected in body fluids may reflect clonal expansion without inevitable malignant progression, raising concerns regarding overdiagnosis and clinical decision-making. Pre-analytical variability, lack of standardization, and limited access to tumor-adjacent fluids further hinder clinical implementation. Liquid biopsy should therefore be regarded as a complementary modality rather than a substitute for histopathological diagnosis, with its precise clinical role in early detection still ill-defined. In this review, we critically examine why liquid biopsy has not yet succeeded in early PDA detection, highlighting the key biological, technical, and clinical barriers that must be addressed to move the field beyond exploratory research toward meaningful clinical application.

Keywords: liquid biopsy; cell-free DNA (cfDNA); cell-free RNA (cfRNA); extracellular vesicles (EVs); non-coding RNA (ncRNA); pancreatic cancer (PDA)

1. Introduction

Recently, extensive research has been conducted on liquid biopsy as minimally invasive testing strategy using various body fluid samples, including blood, urine, and saliva, with the aim of enabling early cancer diagnosis. A wide range of tumor-derived targets in body fluids have been investigated for liquid biopsy, such as cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTCs), extracellular vesicles (EVs), and proteins[1–3] (Figure 1). In the field of pancreatobiliary diseases, gastrointestinal biofluids, duodenal fluid (DF) and pancreatic juice (PJ) collected during endoscopic procedure, can be analyzed in addition to blood or urine[4].

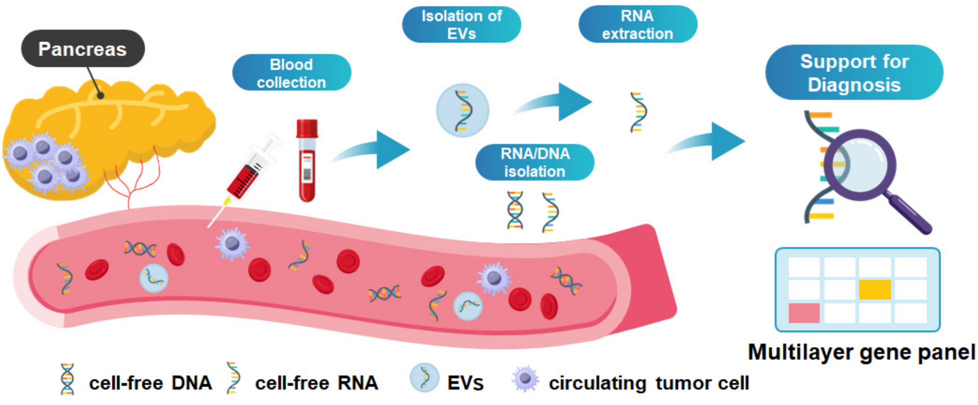

Figure 1. Overview of Liquid Biopsy for generating multilayer gene panel.

Despite substantial research efforts and a growing number of published studies, only a limited number of liquid biopsy approaches have progressed toward clinical implementation, and biomarkers that clearly outperform the conventional marker CA19-9 has not yet been established[5]. The underlying reasons are multifactorial and include challenges in achieving sufficient diagnostic accuracy and reproducibility, limited sensitivity for detecting early-stage disease, and pronounced tumor heterogeneity. To address these issues, diverse strategies are currently being explored across multiple disciplines, including advances in analytical technologies, assay design, and specimen selection [6–8].

In this review, we focus on the fundamental challenges in biomarker development for the early diagnosis of pancreatic cancer (PDA). We also summarize the current landscape of recent liquid biopsy research and ongoing efforts to overcome these limitations, with a particular emphasis on cfDNA, cfRNA, and EV-based approaches, including our investigative attempts.

2. Liquid Biopsy Using ctDNA: Current Limitations and Strategies to Address Them

The development of pancreatic cancer precursor lesions, such as pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasms (IPMN), is frequently associated with activating mutations in driver genes including *KRAS* and *GNAS[9]*. Subsequent inactivation of tumor suppressor genes, including *SMAD4*, *TP53*, and *CDKN2A*, promote progression of these precancerous lesions to invasive pancreatic ductal adenocarcinoma (PDA)[9]. Accordingly, DNA mutation analysis using cfDNA has been regarded as one of the most central targets in liquid biopsy-based approaches for PDA diagnosis[10].

Beyond somatic mutations, numerous PDA-specific DNA methylation markers have been identified, and their combined application has been proposed as a promising strategy for early diagnosis[11–14]. In addition, recent studies have demonstrated that cfDNA fragmentation patterns, including fragment sizes and end motifs, differs between patients with PDA and healthy individuals. Machine learning models integrating these fragmentomic features have been suggested to contribute

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to early diagnosis and, potentially, inference of the cell of origin of tumor[15]. Nevertheless, despite the clinical implemented of plasma cfDNA analysis for cancer genome profiling (CGP) tests in Japan, its application remains limited to therapeutic decision-making rather than primary cancer diagnosis[16].

Plasma cfDNA-based liquid biopsy offers several advantages, including minimally invasiveness, ease of sample collection enabling longitudinal monitoring, short turnaround time (TAT), and the ability to capture systemic tumor heterogeneity rather than information from a single lesion. However, cfDNA concentrations in peripheral blood are inherently low, highly fragmented, and susceptible to chemical degradation. Moreover, the proportion of tumor-derived ctDNA within total cfDNA is strongly influenced by tumor size, stage, and the proximity to circulating body fluids, resulting in limited mutation detection rate in early-stages of PDA[17].

Pre-analytical factors further complicate cfDNA analysis. Delays between sample collection and processing, as well as variations in storage conditions, can substantially affect the analytical results. For example, leukocyte lysis during blood storage releases genomic DNA, increasing background DNA levels and reducing variant allele frequency (VAF), thereby impairing the detection of low-frequency mutations[18]. Standardized protocols for plasma separation, storage, processing are therefore essential. Additionally, mutations arising from clonal hematopoiesis of indeterminate potential (CHIP), which increases with aging, represent a major source of false-positives findings. Because hematopoietic cells constitute the primary source of cfDNA is hematopoietic cells, CHIP-associated mutations , including those in *DNMT3A* and *TET2*, as well as PDA-relevant genes such as *TP53* and *BRCA1/2* may be misidentified as tumor-derived alterations[19,20], thereby reducing diagnostic specificity.

Genetic analysis of cfDNA can be broadly categorized into PCR amplification-based detection methods and sequencing-based genomic profiling approaches that enable broader genomic coverage. PCR-based techniques include real-time PCR, digital PCR (dPCR), and the extremely sensitive BEAMing method, which is particularly suited for detecting predefined hotspot mutations. These approaches offer high analytical sensitivity (approximately 0.01% for dPCR and BEAMing), relatively low cost, and simplified workflows; however, they are inherently limited in the number of target regions that can be interrogated simultaneously.

In contrast, sequencing-based cfDNA analysis incorporating molecular barcoding and error-suppression strategies allow for the detection of low-frequency variants at allele frequencies of approximately 0.1–1%, which is substantially lower than those detectable by standard target sequencing approaches without error correction. Compared with PCR-based assays, sequencing-based genomic profiling enables the simultaneous identification of a broader range of genomic alterations, including point mutations and copy number variations across multiple genes, thereby providing a more comprehensive overview of tumor-associated genomic changes[1,21].

To overcome these limitations, various strategies are currently being explored. These include the use of pre-amplification combined with dPCR to enhance sensitivity for low-abundance mutations, as well as multiplexed assay design developed by the authors to maximize information yield from limited sample volumes[22]. In parallel, specialized blood collection tubes capable of stabilizing nucleic acids (e.g., Streck, Becton Dickinson, and Roche) have been introduced, with expanding applications for both cfDNA and cfRNA preservation.

3. Liquid Biopsy Using EV and RNA: Complementary Approaches and Remaining Challenges

As discussed above, cfDNA-based detection of KRAS mutations has intrinsic limitations, particularly in early-stage disease. To complement its diagnostic performance, EVs and non-coding RNAs (ncRNAs) have emerged as alternative and potentially informative targets in liquid biopsy research.

ncRNAs are broadly classified based on a transcript length, small ncRNAs (<200 nucleotides) including microRNAs (miRNAs), and with long non-coding RNAs (lncRNAs) exceeding of 200 nucleotides[23,24]. Among these, miRNAs, typically 21–25 nucleotides in length, have been most extensively investigated as circulating biomarkers[25,26], whereas studies on lncRNAs remain relatively limited[24,27,28]. Several reports have demonstrated the diagnostic potential of miRNAs detected as cfRNA in body fluids. For example, Nakamura et al. validated a 13-miRNA signature for PDA detection, archiving an area under the curve (AUC) of 0.93[29]. Another study employing machine learning of urinary miRNA combinations reported high diagnostic accuracy for PDA, including early-stage disease [30].

EVs are membrane-bound vesicles secreted by virtually all cell types and are present in diverse body fluids, including blood, urine, breast milk, and saliva. EVs contain a variety of bioactive molecules, such as proteins and nucleic acids, reflecting the molecular characteristics of their cells of origin[31,32]. Recent studies have demonstrated the diagnostic potential of EV-associated markers in pancreatic diseases. For instance, elevated expression of MUC5AC in plasma EVs was shown to distinguish high-grade IPMNs from low-grade IPMNs with high sensitivity and specificity[33].

However, EV-based biomarker development faces substantial technical challenges, particularly regarding isolation methods, purity, and reproducibility. Commonly used EV isolation techniques, such as precipitation and ultracentrifugation, are prone to co-isolation of non-EV contaminants and inter-method variability[31]. To address these issues, we have developed a proprietary EV isolation platform, EViSTEP®, based on immunoprecipitation using antibody-coupled magnetic particles targeting the tetraspanin, combined with a pretreatment reagent that enhances EV–antibody interactions while reducing contaminating proteins[34]. Using this platform, we identified a previously unrecognized lncRNA, HEVEPA, which was significantly upregulated in serum EVs from PDA patients compared to healthy controls and IPMN patients, achieving an AUC of 0.86. Notably, HEVEPA elevation was observed specifically within EVs rather than as cfRNA, underscoring the value of EV-targeted biomarker discovery[34].

Despite these advances, standardization remains a major challenges in cfRNA and EV analyses, particularly with respect to endogenous reference controls. While several stable miRNAs (e.g., miR-149-3p, miR-2861, and miR-4463) have been proposed as cfRNA[35], no consensus has been established for EV-based normalization strategies. In our preliminary analysis, ACTB and B2M demonstrated relatively stable expression within EVs, and further validation studies are ongoing.

4. Recent Application and Studies as Liquid Biopsy

In recent years, several biomarkers have been introduced into clinical practice under health insurance coverage in Japan. Among these, the APOA2 isoform index (APO2-iTQ) has been implemented as an adjunctive marker for PDA diagnosis. Multiple studies have demonstrated significantly reduced APOA2-ATQ/AT levels in patients with PDA, with reported AUC values comparable to or exceeding those of CA19-9 in distinguishing across disease stages[36–39].

Another promising marker is EphA2-NF, a circulating N-terminal fragment of the receptor tyrosine kinase EphA2, which is frequently overexpressed in PDA cells. EphA2-NF is independent of CA19-9 and have been associated with poor prognosis among patients receiving gemcitabine plus nab-paclitaxel (GnP) treatment, suggesting it potential clinical utility as both a diagnostic and prognostic marker[40].

Recent cfDNA-based studies have reported remarkably high diagnostic performance using artificial intelligence (AI)-driven models integrating cfDNA fragmentomics, copy-number variation (CNV), end motifs, and methylation features, with AUC value approaching 0.992 when compared with healthy controls[15]. In addition, methylation-based approaches such as methylated CpG tandem amplification and sequencing (MCTA-Seq) have identified multiple candidate biomarkers with high diagnostic accuracy[41,42]

Tumor-adjacent fluids have also attracted attention as alternative liquid biopsy sources. Yachida et al. reported high diagnostic accuracy for PDA using *KRAS* mutation analysis of DF collected after secretin[43], while analysis of S100P protein expression in DF has shown potential utility as a minimally invasive screening approach[44,45].

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EV-based biomarker studies have further expanded the liquid biopsy landscape. EV surface protein Glypican-1 (GPC1)-positive EVs, in combination with CD82 and CA19-9, have demonstrated high diagnostic accuracy for differentiating PDA from chronic pancreatitis[46]. As summarized in Table 1, Additional studies have identified EV-associated miRNAs, circRNAs, and lncRNAs with diagnostic potential, some achieving performance comparable to or exceeding that of CA19-9 alone[47–52].

Table 1. Recent findings in liquid biopsy research for PDA.

Analysis target	Molecular target	Type of clinical sample	Potential roles for liquid biopsy for PDA diagnosis	Reference
cfDNA	cfDNA fragmentation pattern	plasma	Machine learning models integrating cfDNA fragmentation patterns, including fragment sizes and end motifs.	Yin et al (2025) [15]
cfRNA / EV	13 miRNAs (5 cfRNA and 8 EV RNA)	plasma / serum	The combination of 13-miRNA signature archived an area under the curve (AUC) of 0.98 in training cohort and 0.93 in validation cohort for PDA detection.	Nakamura et al (2022) [29]
EV	EV miRNA-based detection set	urine	Machine learning of urinary extracellular vesicle miRNA combinations showed high diagnostic accuracy for PDA, including early-stage disease.	Baba et al (2024) [30]
EV	MUC5AC	plasma	MUC5AC in plasma EVs was shown to distinguish high-grade IPMNs from low-grade IPMNs with high sensitivity and specificity.	Yang et al (2021) [33]
EV	lncRNA HEVEPA	serum	HEVEPA expression was upregulated in serum EVs from PDA patients compared to healthy controls and IPMN patients, achieving an AUC of 0.86.	Takahashi et al (2024) [33]
protein	APO2-iTQ	blood	APOA2 isoform index (APO2-iTQ) has been implemented as an adjunctive marker for PDA diagnosis in Japan.	Hanada et al. (2024) [39]
protein	EphA2-NF	serum	EphA2-NF is associated with poor prognosis among patients receiving GnP treatment, and has potential clinical utility as both a diagnostic and prognostic marker.	Sato et al. (2023) [40]
cfDNA	methylated CpG tandem amplification	plasma	Methylation scoring and typing system achieved a sensitivity of 97% and 86% for patients in the discovery and validation cohorts, respectively, with a specificity of 100% in both cohorts for PDA.	Hu et al. (2025) [41]
cfDNA	methylated Homeobox A1 (mHOXA1) and methylated somatostatin (mSST)	serum	Analysis of mHOXA1 and mSST combination with CA19-9 showed to be useful to detect early stage of PDA.	Suehiro et al. (2022) [42]
cfDNA	KRAS	DF	KRAS mutation analysis of DF collected after secretin administration showed high diagnostic accuracy for PDA.	Yachida et al. (2025) [43]
protein	S100P	DF	The sensitivity and specificity of S100P protein expression in DF for diagnosing stages 0/IA/IB/IIA PDAC were 85% and 77%, respectively, with an AUC of 0.82.	Ideno et al. (2020) [45]

EV	Glypican-1 (GPC1)	serum	GPC1-positive EVs, in combination with CD82 and CA19-9, have demonstrated high diagnostic accuracy for differentiating PDA from chronic pancreatitis (AUC 0.942).	Xiao et al. (2020) [46]
EV	circRNAs (circ_0006220 and circ_0001666)	plasma	circ_0006220 and circ_0001666) were found to correlate with CA19-9 levels, tumor size, and lymph node metastasis; the combination of these two circRNAs yielded an AUC of 0.884 for PDA diagnosis.	Hong et al. (2022) [47]
EV	10 miRNAs	plasma	Ten miRNAs highly expressed in the body fluids of patients with PDA was selected using public databases; These miRNAs were identified and verified as EV-miRNA candidates for early detection.	Makler et al. (2022) [48]
EV	4 miRNAs	plasma	EV-miRNA panel comprising four miRNAs (miR-93-5p, miR-339-3p, and miR-425-5p/3p) achieved diagnostic accuracy comparable to or greater than CA19-9 (AUC 0.885).	Makler et al. (2023) [49]
EV	molecular clustering of miRNAs	plasma	CT imaging features (radiomics) with the expression analysis of plasma EV-derived miRNAs (e.g., miR-1260b), improved the accuracy of differentiating malignant from benign pancreatic lesions to an AUC > 0.90.	Xu et al. (2025) [50]
EV	miRNAs (e.g., miR-21, miR-10b, miR-451a)	blood	Systematic review demonstrated the utility of plasma and serum EV-derived miRNAs (e.g., miR-21, miR-10b, miR-451a) in the diagnosis of PDA.	Patel et al. (2025) [51]
EV	HULC	serum	EV encapsulated HULC in serum was increased in serum derived from patients with PDA with AUC of 0.92.	Takahashi et al. (2020) [52]

DF; duodenal fluid,

Collectively, these studies underscore the extensive global efforts directed toward biomarker discovery and validation. Rather than relying on a single molecular target, integrating multiple biomarkers across different liquid biopsy platforms may offer a more realistic path toward improved diagnostic accuracy. Accordingly, we propose the development and clinical implementation of a cost-effective "Pancreatic Cancer Diagnostic Panel" that combines complementary biomarkers, as conceptually illustrated in Figure 1.

5. Conclusion and Future Perspectives

Despite substantial advances in liquid biopsy technologies, early detection of PDA remains an unresolved clinical challenge, primarily due to biological constraints such as minimal tumor-derived nucleic acid abundance, marked heterogeneity, and the difficulty in discriminating against indolent precursor lesions from those with malignant potential. Increasing analytical sophistication alone has not been sufficient to overcome these limitations, underscoring the need to reconsider the clinical positioning of liquid biopsy. Rather than serving as a stand-alone screening tool, liquid biopsy is more realistically positioned as a complementary modality that supports diagnosis and risk stratification in well-defined clinical settings. Future progress will likely depend on rationally designed diagnostic panels that integrate multiple molecular features across different biofluids with

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clinical and imaging information, evaluated not only for analytical performance but also for interpretability, feasibility, and cost-effectiveness. Aligning technological innovation with biological insight and clinical need will be essential for the meaningful clinical implementation of liquid biopsy in pancreatic cancer.

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Abbreviations

The following abbreviations are used in this manuscript:

PDA	pancreatic ductal adenocarcinoma
cfDNA	cell-free DNA
cfRNA	cell-free RNA
EVs	extracellular vesicles
CTCs	circulating tumor cells
PanIN	pancreatic intraepithelial neoplasia
IPMN	intraductal papillary mucinous neoplasms
DF	duodenal fluid
PJ	pancreatic juice
CGP	cancer genome profiling
TAT	turnaround time
VAF	frequency
CHIP	clonal hematopoiesis of indeterminate potential
dPCR	digital PCR
ncRNAs	non-coding RNAs
miRNAs	microRNAs
lncRNAs	long non-coding RNAs
AUC	area under the curve
APO2-iTQ	APOA2 isoform index
GnP	gemcitabine plus nab-paclitaxel
AI	artificial intelligence
CNV	copy-number variation
MCTA-Seq	methylated CpG tandem amplification and sequencing
GPC1	Glypican-1

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