

## Review Article

# Circulating biomarkers for early diagnosis of pancreatic cancer: facts and hopes

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**Abstract:** Pancreatic cancer (PC) is characterized by extremely high mortality and poor prognosis, which are largely ascribed to difficulties in early diagnosis and limited therapeutics. Although there is a sufficient window for intervention before preneoplastic lesions progress to invasive disease, effective early detection of PC remains difficult using current biomarkers and imaging techniques. Biomarkers with satisfactory diagnostic efficacy and convenient analysis methods are urgently required. In this review, we summarized recent advances in the identification of biomarkers in circulation for early detection of PC. A number of novel circulating biomarkers, such as metabolites, cell-free DNA (cfDNA), noncoding RNA, and exosomes, that show promising diagnostic value have been discovered using advances in sequencing techniques and “omics” analyses. Panels comprising several biomarkers may also exhibit better diagnostic performance. In the future, we need more efficient circulating biomarkers for the identification of noninvasive precursor lesions and early disease. Collaborative large-scale studies are also required to show the clinical validity and applicability of potential biomarkers.

**Keywords:** Pancreatic cancer, early diagnosis, circulating biomarkers

### Introduction

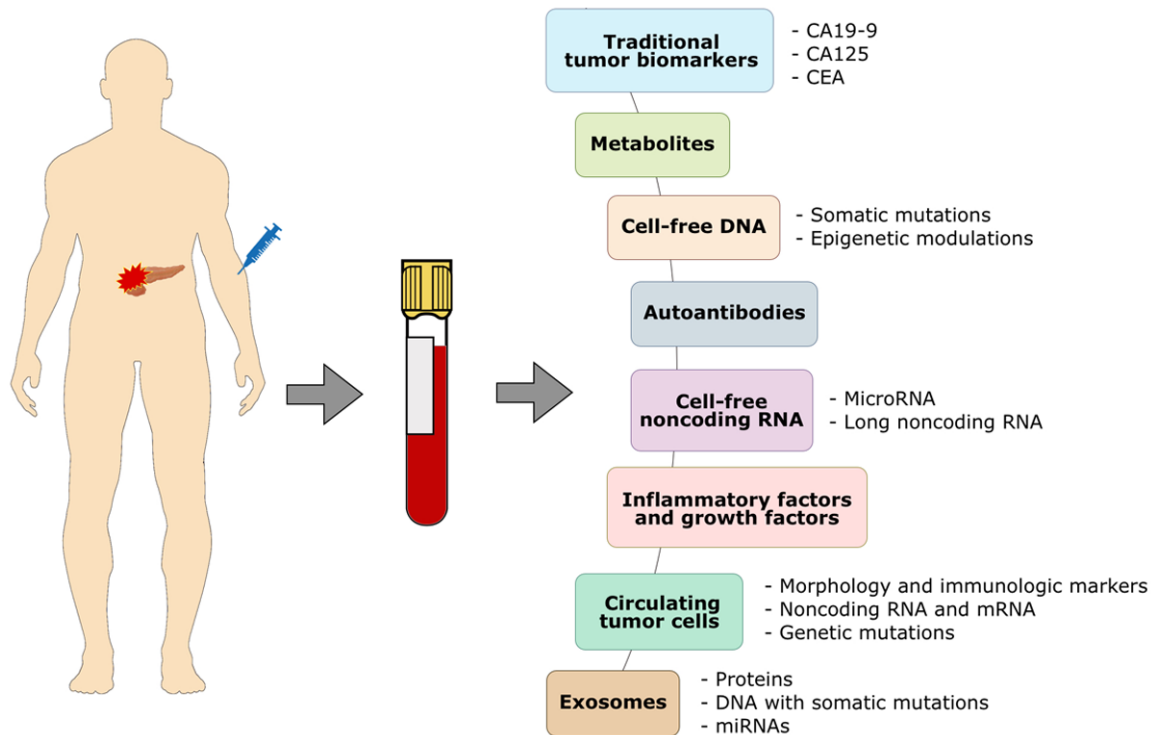
Pancreatic cancer (PC) is characterized by extremely high mortality and poor prognosis and is the 4<sup>th</sup> leading cause of cancer-related death [1]. Unfortunately, compared with other malignancies, there has been little improvement in the survival rate of patients with PC in recent decades.

Surgical resection is currently the only therapeutic that can cure early-stage PC. However, because PC patients mostly present with no clinically informative symptoms or radiologic manifestations at early stages [2], the majority (approximately 80%) of PC patients are diagnosed with locally advanced or metastatic disease and do not have the opportunity to undergo a curative operation [3]. The 5-year survival rate would increase significantly if PC patients were identified at an earlier stage and underwent surgical resection followed by chemotherapy [4-6]. Therefore, the most promising direction for improving the prognosis of PC is to

develop effective screening strategies with which to detect the disease at an earlier stage. There is a possible window of opportunity for the detection of PC from the initial mutations in the pancreas to the development of metastatic sites, suggesting that early detection is feasible in this period, which can span two decades [7].

However, early diagnosis of PC is difficult with the currently available methods [8]. Unlike colonoscopies for colorectal cancer and serum prostate-specific antigen (PSA) levels for prostate cancer, there is currently no standardized PC screening strategy, even for high-risk populations. The diagnosis of PC is usually based on radiology [computed tomography (CT) and magnetic resonance imaging (MRI)] or invasive endoscopic techniques [ultrasound endoscopy-fine needle aspiration (EUS-FNA), endoscopic retrograde cholangiopancreatography (ERCP), and explorative laparoscopy] [9], which are inconvenient for patients and show no superiority over biological markers. Furthermore, PC is characterized by dense desmoplasia surround-

## Circulating biomarkers of pancreatic cancer



**Figure 1.** Overview of major circulating biomarkers for early detection of pancreatic cancer. Various biomarkers can be detected in plasma or serum from PC patients.

ed by a large amount of extracellular matrix, creating a hurdle for pathologic biopsy [10].

Effective biomarkers that can be obtained in a less invasive manner have become a research focus. The ideal biomarkers should be easily detected with satisfactory sensitivity and specificity and should distinguish PC from other benign pancreatic lesions. In the context of early detection, the identification of preneoplastic conditions, such as pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), is of great importance [11].

Blood is easily accessible and relatively stable, making serum an ideal specimen in which to discover biomarkers. However, biomarkers secreted into serum are extremely dilute and probably obscured by other more-abundant serum proteins [12]. Technological advances in the last decade have provided more opportunities to discover circulating biomarkers based on “omics” analyses, including methods focused on proteins, nucleic acids, circulating tumor cells (CTCs), and exosomes. Numerous proteins

of low abundance can be analyzed by mass spectrometry-based approaches and proteomic technologies. Next-generation sequencing techniques provide deeper insight into somatic mutations and epigenetics analysis of the genome and broaden the characterization of circulating tumor DNA (ctDNA) and cell-free RNA. With the development of cell tracking techniques and flow cytometry, it is now possible to capture and analyze CTCs and exosomes.

Thus, in this review, we summarize recent progress in the early detection of PC using various types of circulating biomarkers (Figure 1).

### CA19-9, other carbohydrate antigens, and carcinoembryonic antigen

CA19-9, also called sialyl Lewis a, is the only biomarker approved by the US FDA for monitoring the progression and therapeutic response of PC; it has also been widely used in the diagnosis of PC for a long time [13, 14]. However, the reported sensitivity (ranging from 69% to 98%) and specificity (ranging from 46% to 98%) of CA19-9 are moderate for PC screening [15-18].

## Circulating biomarkers of pancreatic cancer

**Table 1.** Panels of circulating biomarkers for early diagnosis of pancreatic cancer

Classification	Biomarkers in the panel	Participants	Results	Ref.
Concentration measurement	CA19-9, SYCN, REG1B	Early-stage PC vs. NC	AUC=0.87	Makawita et al. [24]
	CA19-9, ICAM-1, OPG	PC vs. NC	Sen=78%, Spe=94%	Brand et al. [21]
	CA19-9, cathepsin D, MMP-7	PC vs. NC	AUC=0.90, Sen=88%, Spe=80%	Park et al. [25]
	CA19-9, IP-10, IL-6, PDGF	PC vs. non-PC	AUC=0.884	Shaw et al. [33]
	HGF, MCP-1, IP-10, Eotaxin	PC vs. NC	Sen=85.7%, Spe=92.3%	Zeh et al. [32]
	Twenty-five types of inflammatory factors and cytokines	PC vs. NC	AUC=0.95, Sen=88%, Spe=85%	Wingren et al. [34]
Metabolomics based on MS	Xylitol, 1,5-anhydro-D-glucitol, histidine, and inositol	PC vs. NC	Sen=84.1%, Spe=84.1%	Kobayashi et al. [48]
	Multi-amino acid-based metabolites	PC vs. NC	AUC=0.891	Leichtle et al. [53]
Methylation status of gene promoters in cfDNA	CCND2, DAPK1, ESR1 promA, HMLH1, MGMT, MUC2, MYOD1, CDKN2B, CDKN1C, PGK1, PGR-proximal, RARb, RB1, SYK	PC vs. CP	Sen=91.2%, Spe=90.8%	Liggett et al. [94]
	CCND2, SOCS1, THBS1, PLAU, VHL	PC vs. NC	Sen=76%, Spe=59%	Melnikov et al. [95]
	BNC1, ADAMTS1	PC vs. NC	Sen=81%, Spe=85%	Yi et al. [96]
Cell-free ncRNA	Panel 1: CA19-9, miR-145, miR-150, miR-223, miR-636 Panel 2: CA19-9, miR-26b, miR-34a, miR-122, miR-126*, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p	PC vs. NC	Panel 1: AUC=0.94, Sen=85%, Spe=85% Panel 2: AUC=0.93, Sen=85%, Spe=85%	Schultz et al. [110]
	Panel 1: miR-486-5p, miR-126-3p, miR-106b-3p Panel 2: miR-486-5p, miR-126-3p, miR-106b-3p, miR-938, miR-26b-3p, miR-1285	PC vs. CP	Panel 1: AUC=0.891, Sen=82.7%, Spe=84.4% Panel 2: AUC=0.889, Sen=82.3%, Spe=81.4%	Cao et al. [111]
Biomarkers in circulating exosomes	PC-initiating cell markers (CD44v6, Tspan8, EpCAM, MET, and CD104) and miRNAs (miR-1246, miR-4644, miR-3976, and miR-4306)	PC vs. non-PC	Sen=100%, Spe=80%	Madhavan et al. [172]

\*PC: pancreatic cancer; NC: normal controls/healthy controls; CP: chronic pancreatitis; MS: mass spectrometry; AUC: area under curve; Sen: sensitivity; and Spe: specificity.

There are some challenges facing the use of CA19-9 alone as a biomarker for the early detection of PC. CA19-9 is an epitope of sialylated Lewis group antigen, and approximately 5-10% of the Caucasian population possesses a Lewis a-/b- genotype and thus does not express CA19-9 [19]. Furthermore, only 65% of patients with resectable PC have elevated serum CA19-9 levels [20]; therefore, CA19-9 testing would result in false-negative results and mislead the diagnosis. Third, an elevated level of CA19-9 has been observed in other gastrointestinal malignancies, such as gastric cancer and colorectal cancer, and in various benign conditions, including chronic pancreatitis and acute cholangitis [13].

Therefore, recent efforts have been focused on improving the diagnostic efficacy of CA19-9 by combining it with a panel of markers [17, 21-23] (**Table 1**). Makawita et al. [24] combined CA19-9 with SYCN, REG1B and AGR2 to produce a better AUC (AUC=0.87) for diagnosing early-stage PC compared with CA19-9 alone (AUC=0.82,  $P < 0.05$ ). After investigating 83 promising proteins in more than 300 PC patients and controls, Brand et al. [21] discovered a panel consisting of CA19-9, ICAM-1 and OPG that could discriminate PC patients from healthy controls with a sensitivity of 78% and a specificity of 94% in a validation set. Furthermore, this panel is highly selective for PC and yields negative results for almost all breast, lung, and colorectal cancers. Park and colleagues [25] also found a panel comprising CA19-9, cathepsin D and MMP-7 with an AUC of 0.900 for discriminating patients with PC from normal controls.

Other conventional biomarkers, such as CA242, CA72-4, CA125 and carcinoembryonic antigen (CEA), have shown limited diagnostic efficacy for early detection of PC [9, 26]; however, in the Lewis-negative population, these biomarkers may have promise. Luo et al. [27] reported that both CEA and CA125 showed high specificity (98.0% and 93.8%, respectively) in Lewis-negative patients with PC and were associated with tumor metastasis and the therapeutic response, which indicates the diagnostic value of elevated CEA and CA125 in patients suspected of having PC.

Additionally, some recent studies have focused on subtypes of carbohydrate antigens. Yue re-

ported that CA19-9 antigenic epitopes on certain mucin backbones, including MUC1, MUC-5AC and MUC16, were directly related to the presence of pancreatic pathologies, with better sensitivity and specificity for PC [28]. A study by Partyka et al. [29] reported antibodies with high specificity against Lewis C glycan, a variation of sialyl Lewis a, in diagnosing PC.

In general, panels combining CA19-9 with other novel biomarkers might represent an ideal strategy for improving the sensitivity and specificity of CA19-9 in detecting PC. Insights into the characteristics and mechanisms of CA19-9 and other conventional cancer biomarkers will lead to the optimization of diagnostics using common assays.

### Inflammatory factors and growth factors

There is a complicated relationship between inflammation and cancer. Various inflammatory factors, including cytokines, chemokines, and growth factors, have been shown to play important roles in tumorigenesis and metastasis [30], indicating that these factors are potential diagnostic markers of PC.

Growth factor stimulation and neovascularization are critical during the development of carcinomas. Similar to patients with other types of cancer, patients with PC have significantly elevated serum VEGF and bFGF levels compared with healthy individuals [31]. A previous study also reported a correlation between the expression levels of these growth factors and tumor size.

Panels combining multiple cytokines, chemokines, and growth factors were superior to a single biomarker for the detection of PC [32-34]. After measuring serum levels of 27 cytokines in 241 participants, including 127 with PC, Shaw et al. [33] proposed a panel of IP-10, IL-6, PDGF and CA19-9 that offered improved diagnostic performance in the differential diagnosis between PC and benign pancreatic diseases.

Wingren et al. [34] constructed a recombinant antibody microarray to identify different inflammatory factor profiles in serum between PC patients and normal subjects. Some key molecules, such as C1 esterase inhibitor, C3, C5, CD40, CD40 ligand, factor B, GLP-1, IFN- $\gamma$ , IgM,

IL-10, IL-11, IL-12, IL-13, IL-16, IL-18, IL-1-ra, IL-1 $\alpha$ , IL-3, IL-5, IL-6, IL-7 and IL-8, integrin- $\alpha$ 11, procathepsin W, sialyl Lewis x, TGF- $\beta$ 1, TNF- $\alpha$  and VEGF, were observed to be differentially overexpressed in a statistically significant manner. Based on these findings, it was concluded that a panel of 25 biomarkers could differentiate patients with PC from normal controls (AUC: 0.95) with a sensitivity and specificity of 88% and 85%, respectively.

### Metabolites

Cancer cells are capable of surviving and proliferating in settings with oxygen and nutrient supply deficiency [35] by a process known as the Warburg effect, which was discovered nearly a century ago. Cancer cells are capable of metabolic rewiring, or metabolic reprogramming, to facilitate survival under these conditions. This strategy is extremely important for PC, since PC cells live in a harsh extracellular environment characterized by hypoxia, considerable desmoplasia and hypovascularization [36-38]. Recent technological advances have attracted more attention and interest in cancer-associated metabolic abnormalities and their potential diagnostic and therapeutic applications [39, 40]. Accordingly, detection of aberrant low-molecular weight products and intermediates from metabolic reprogramming would indicate an abnormal biochemical state in the individual and suggest the presence of a malignancy. Some groups have reported the application of metabolomic biomarkers in detecting various types of cancer [41, 42].

It has been demonstrated that PC cells reprogram intermediary metabolism to meet their metabolic demands, which differ from the demands of normal cells. Until recent years, the distinctive metabolic features of PC were unknown. A large proportion of metabolic rewiring is driven by mutation of the oncogene KRAS, which is almost universally seen in PC cells [43]. To sustain tumor viability, stromal components, including fibroblasts [44, 45] and pancreatic stellate cells (PSCs) [46], play supportive roles by mediating metabolite exchange.

Mass spectrometry-based proteomic analysis of PC revealed alterations in metabolic enzymes and the accumulation of key intermediates [47]. Moreover, some amino acids, lipids, fatty acids, and bile acids in serum have been report-

ed as candidate metabolites that may discriminate PC from benign lesions and non-disease states [48-50]. Iole et al. showed that palmitic acid in serum could distinguish PC patients from healthy controls better than traditional CA19-9 [51]. A combination of four serum metabolites (xylitol, 1,5-anhydro-D-glucitol, histidine, and inositol) proposed by Kobayashi et al. had a higher sensitivity in PC and a lower false-negative rate in chronic pancreatitis than CEA and CA19-9 [52]. Leichtle et al. [53] also reported a panel based on serum amino acids for distinguishing PC patients from healthy controls and pancreatitis patients.

Metabolomics is a sensitive indicator for monitoring precancerous lesions (e.g., PanIN) and identifying early-stage PC, as dramatic changes can be observed during different stages of PC progression [54]. Wen et al. [55] demonstrated that kynurenate and methionine levels were upregulated in PanIN but were decreased in PC at the tissue level. LaConti and colleagues identified at least 50 differences in serum metabolites among early PanINs, late PanINs and invasive PC lesions. The data showed that a panel of metabolites achieved a diagnostic accuracy of 81.5% and 73.2% when distinguishing controls from late PanIN and PC cases, respectively [56].

Although circulating metabolites have shown promising diagnostic value in a series of studies, the application of serum metabolomics for the detection of PC remains to be tested and verified in a larger population including patients with various types of pancreatic disease [57]. However, current approaches for analyzing circulating metabolites are not standardized; thus, there may be high heterogeneity and bias among different studies. Furthermore, intricate connections and regulatory nodes among various metabolic processes form a comprehensive metabolic network. Since the concentration of a single metabolite is determined by a number of enzymes and intermediates and usually exhibit a nonlinear quantitative relationship, a panel of metabolic biomarkers might be necessary to establish an effective and stable diagnostic modality.

### Autoantibodies

Although immune system dysfunction is common in cancer patients, minor immune respons-

es to a developing tumor can be traced, but such responses are not strong enough to produce clinical manifestations [58, 59]. Over the past few years, circulating autoantibodies in the serum of cancer patients have been considered as potential biomarkers for the early detection of cancer. Though little is currently known about the origins and mechanisms of these autoantibodies, data have established that autoantibodies against tumor-associated antigens, such as mutated tumor proteins, misfolded proteins, overexpressed proteins, aberrantly modified proteins and ectopically expressed proteins, are produced in various types of cancer, including PC [30, 60].

Bracci et al. [61] analyzed autoantibodies against carboxy-terminal domain, RNA polymerase II, polypeptide A, small phosphatase 1 (CTDSP1), mitogen-activated protein kinase 9 (MAPK9) and nuclear receptor subfamily 2, group E, member 3 (NR2E3) in PC patients (n=300) and controls (n=300). Significant differences in the expression levels of these autoantibodies were observed between PC subjects and controls. However, their diagnostic value was poor, with AUC values less than 0.70. Anti-mucin 1 (MUC1) antibodies have become one focus of autoantibody research in PC. MUC1 is a membrane-associated glycoprotein that is overexpressed in multiple cancers, including PC, and is associated with CA19-9 antigens. Gold et al. [62] reported that a monoclonal antibody against MUC1 had a sensitivity of 77% and a specificity of 95% for differentiating PC patients from normal controls.

Other autoantibodies have also been discovered in the past 10 years, including autoantibodies to two acidic isoforms of the glycolytic enzyme enolase (ENO1/2) [63], Ezrin [64], a vimentin isoform [65], and calreticulin isoforms [66]. Assisted by the development of mass spectrometry techniques and high-throughput proteomics analysis, a series of autoantibodies, such as those against metabolic enzymes (phosphoglycerate kinase-1, triosephosphate isomerase and isocitrate dehydrogenase) and cytoskeletal proteins, was recently identified [67, 68], but their validity as a diagnostic marker remains to be verified in future studies.

Researchers have observed substantial alterations in malignancies that produce novel and

immunogenic protein sequences called neo-antigens, which are absent from the normal human genome. These neo-epitopes are primarily created by tumor-specific mutations in the genome and become potential targets for cancer detection and immunotherapy [69]. The discovery of additional novel tumor-related autoantibodies is driven by the concept that autoantibodies can indirectly reflect altered genetics or proteomics. However, the frequency at which selected autoantibodies can be detected in cancer patients is low (usually <30%). Tumor heterogeneity complicates the analysis and application of autoantibodies for diagnostic purposes.

### Cell-free DNA (cfDNA) and its epigenetic modifications

Nucleic acids can be released into circulation through cellular apoptosis and necrosis, and the size of such nucleic acids ranges primarily from 70 bp to 200 bp. Compared with the healthy population, patients with solid tumors have significantly elevated levels of cfDNA (180 ng/ml on average) [70]. Although the presence of circulating nucleic acids was discovered in the 1940s [71], it was not until recently that interest in them piqued and rapid advancements in their detection and characterization were made.

With the advent of high-throughput sequencing techniques and droplet digital PCR [72, 73], we now possess a deeper understanding of tumorigenesis and malignant progression through the acquisition of mutations and epigenetic modifications in cfDNA. In contrast to normal cfDNA, circulating tumor DNA (ctDNA) usually carries mutations that have been reported to match somatic mutations in the primary tumor [74]. Moreover, ctDNA is believed to reflect tumor heterogeneity and to correlate with tumor burden [75-77]. Analyzing ctDNA samples from liquid biopsies could provide insight on the tumor state and genetics.

### *Somatic mutation of cfDNA*

In PC, the predominant genetic characteristic is the high rate of KRAS mutations. Analysis of the genomic landscape of PC has revealed that inactivating mutations in tumor suppressor genes (e.g., CDKN2A, TP53, SMAD4, and BRCA2) are also common [43]. Most PC pa-

tients harbor KRAS mutations that can be detected in serum, pancreatic juice, and feces irrespective of clinical stage, even in patients with premalignant pancreatic lesions [78, 79]. KRAS mutations are not specific to PC but are also present in various types of malignancies and in chronic pancreatitis [79].

The role of KRAS mutations in chronic pancreatitis and PanIN before PC development has been explored in the last century. According to a thorough investigation of somatic mutations in PanINs, more than 90% of PanIN cases possess KRAS mutations, and the mutation rate is significantly correlated with PanIN grade [80]. These observations suggest that KRAS mutations are an early event during tumorigenesis. However, whether these mutations can be detected in the circulation and utilized for the diagnosis and follow-up of patients is currently unclear. Mulcahy et al. [81] detected KRAS mutations in the plasma of all four patients with chronic pancreatitis (4/4) and reported that all the patients were diagnosed with PC during follow-up in a prospective study. In contrast, Maire et al. [82] reported that KRAS mutations in cfDNA were detected in only 4 of 31 patients with chronic pancreatitis. None of the four patients with KRAS mutations was diagnosed with PC after a mean follow-up of 36 months. Therefore, KRAS mutations are potential biomarkers with considerable sensitivity for the early diagnosis of PC; however, this finding requires further characterization and verification verified in a large study.

In PC, the presence of mutant KRAS has been shown to be related to clinical stage and metastasis [83]. Uemura et al. [84] reported the detection of KRAS mutations in 35% of plasma samples from PC patients before surgery with a polymerase chain reaction combined with restriction fragment length polymorphism (PCR-RFLP) approach. More recently, Bettegowda et al. analyzed 640 plasma samples from patients with various types and stages of cancer, including 155 PC patients, and showed that the detection rate of ctDNA was 48% in patients with resectable PC. In addition, there was a positive correlation between detection of ctDNA and clinical stage in a subgroup analysis [85]. The author also observed a concordance rate of 95% between plasma and primary tumor mutation status. A similar result was obtained

by Sausen et al., who reported a ctDNA detection rate of 43% (specificity >99.9%) in 51 patients with localized PC [86]. Moreover, correlations have been found between KRAS mutations and clinicopathological features (e.g., metastasis and overall survival) [87, 88].

Because mutations in TP53 and SMAD4 are relatively rare in PC compared with KRAS mutations, few studies have evaluated their diagnostic value in cfDNA. It has been shown that TP53 and SMAD4 mutations are genetic events that occur during the latter stages of disease and are thus most commonly found in PC and grade 3 PanIN [89].

### *Epigenetic modifications of cfDNA*

In addition to genetic abnormalities, aberrant epigenetic modification, especially alterations in the methylation pattern, is notably common in malignancies. Sufficient studies have discovered global hypomethylation of tumor genomic DNA and hypermethylation of tumor suppressor genes. Due to novel methodologies that allow the analysis of epigenetic modifications in DNA from small amounts of sample [90], detection of epigenetic patterns in cfDNA has emerged as a potential biomarker for the early diagnosis of PC.

Previous studies have discovered several targets (UCHL1, NPTX2, SARP2, CLDN5, FOXE1, CDH3, etc.) of abnormal DNA methylation in PC [91]. Similarly, abnormal methylation profiles can be detected in specific regions of cfDNA. Jiao et al. [92] found that the p16 and preproenkephalin promoters were hypermethylated in plasma DNA, with a detection rate of 30% and 25%, respectively, in PC patients. Park et al. [93] reported that the quantitative analysis of plasma NPTX2 by methylation-specific PCR helped differentiate PC from other benign pancreatic lesions, including chronic pancreatitis, with a sensitivity of 80% and a specificity of 75%.

Researchers have also tried to optimize the efficacy of detecting epigenetic patterns in cfDNA by combining several targets. Liggett et al. [94] determined the methylation status of cfDNA in 30 plasma samples from patients with chronic pancreatitis or PC and healthy controls. After statistical analysis, Liggett et al. proposed a panel of 17 gene promoters for dif-

ferentiating chronic pancreatitis patients from controls (sensitivity =81.7%, specificity =78%) and from PC patients (sensitivity =91.2%, specificity =90.8%) based on the methylation status. Another investigation reported a sensitivity and specificity of 76% and 59%, respectively, in distinguishing PC patients from healthy subjects using the methylation profile of a gene set containing CCND2, SOCS1, and THBS1 [95]. Yi et al. [96] also reported that the promoter methylation status of BNC1 and ADAMTS1 in cfDNA is a promising biomarker for detecting early-stage PC, with a sensitivity of 81% and a specificity of 85% when evaluated together.

Although the analysis of epigenetic alterations in circulating DNA is not as widely used as the detection of KRAS mutations in the diagnosis of PC, the former technique offers some advantages over genetic and serum biomarkers [97, 98]. First, the incidence of aberrant DNA methylation at select CpG islands was found to be higher than the incidence of genetic mutations, thereby producing fewer false-negatives. Second, several studies support the concept that aberrant epigenetic alteration is an early event during tumorigenesis that leads to gain-of-function or loss-of-function of critical molecules in cancer cells. Last, the DNA methylation status is relatively stable and can be easily detected with great sensitivity, even when the sample is contaminated.

### Cell-free noncoding RNA (ncRNA)

The use of high-throughput genome-wide sequencing technologies in recent years has revealed that the constitution of the human genome and transcriptome is more complicated than previously considered. The human genome comprises genes that can be translated into proteins, which accounts for only 2% of the whole genome, and a large proportion of sequences (at least 75%) that encode noncoding RNAs (ncRNAs) [99]. ncRNAs form a large family that includes microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), small Cajal body-specific RNAs (sdrRNAs), and long noncoding RNAs (lncRNAs) [100]. Accumulating evidence has indicated that these ncRNAs play regulatory roles by modifying gene expression at multiple levels through interactions with DNA, RNA, and protein during physiological processes and tumor development [101].

A number of studies have discovered that ncRNA profiles, especially miRNA and lncRNA profiles, are tumor (and PC) specific [102] and can be easily detected in the circulation of patients as potential diagnostic biomarkers of malignancy.

### miRNAs

Among circulating ncRNAs, miRNAs have been the subject of the most studies; these small ncRNAs (22-25 nt) play important roles in various physiological processes by binding to complementary mRNA and inhibiting gene expression. Some studies have found evidence that some miRNAs can function as tumor promoters or suppressors. Alterations in miRNA expression have been observed in numerous diseases and in the development and progression of malignancies [103]. Furthermore, miRNAs can circulate in the blood as free RNA bound to hAgo2 and can be incorporated in microparticles such as exosomes [104], which protects the miRNAs from degradation by RNase.

Abnormal expression of certain miRNAs, such as miR-10, miR-21, miR-22, and miR-155, in PC has been demonstrated in disease states according to miRNA profiling studies [105-107]. After several years of exploration, a number of studies have reported that circulating miRNAs or panels of miRNAs identified in the plasma or serum of PC patients show potential diagnostic value, with superiority over CA19-9 [108-110].

Schultz et al. [110] conducted the largest case-control study to date on circulating miRNA in patients with pancreatic diseases, including 409 individuals with PC and 25 patients with chronic pancreatitis, and 312 healthy controls. All the blood samples were collected before the patients underwent chemotherapy or surgery. The authors found nine miRNAs that were consistently dysregulated in the cohort after testing more than 700 miRNAs and identified a panel of miRNAs with diagnostic value that include miR-145, miR-150, miR-223 and miR-636. This diagnostic panel showed an AUC of 0.93, a sensitivity of 0.85, and a specificity of 0.85 but was not superior to CA19-9 (AUC of 0.90, sensitivity of 0.86, and specificity of 0.99). Another multicenter research study conducted by Xu J and colleagues revealed that miR-486-5p exhibits diagnostic potential in discriminating patients with PC from those with



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chronic pancreatitis (AUC=0.693), pancreatic neuroendocrine tumors (AUC=0.660), and other pancreatic tumors (AUC=0.618) and from healthy controls (AUC=0.861); these values indicate a comparable diagnostic value to that of CA19-9 [108]. The research group also discovered two panels composed of 6 and 3 miRNAs and showed that both panels could accurately distinguish patients with PC from those with chronic pancreatitis (AUC of 0.891 and 0.889) [111]. Using microRNA arrays, Li et al. [112] evaluated 735 circulating miRNAs in sera from patients with PC and controls and identified several miRNAs with an AUC>0.7 for distinguishing PC patients from healthy controls. Of these candidate miRNAs, miR-1290 had the best diagnostic performance in discriminating patients with PC from healthy controls (AUC=0.96), patients with chronic pancreatitis (AUC=0.81), and patients with pancreatic neuroendocrine tumors (AUC=0.80). Moreover, miR-1290 performed better than CA19-9 in distinguishing patients with early-stage PC from controls and was elevated in patients with IPMNs compared with healthy subjects. The results suggest that miR-1290 is a promising circulating miRNA biomarker for the early detection of PC. Likewise, multiple studies have discovered various candidate miRNAs, such as miR-21, miR-18a, miR-155, miR-192, miR-196, miR-200, miR-210, and miR-375 [113-117].

For the early detection of PC, identifying alterations in miRNA profiles of PanIN and IPMN, early events in pancreatic tumorigenesis, provides insight regarding these processes [118, 119]. Yu et al. [120] detected 700 miRNAs in PanIN-3 lesions and found that 35 showed altered expression. More attention should be paid to miR-21, miR-155, miR-196 and miR-210 because recent studies have shown their overexpression in PanIN and IPMN lesions [118, 119, 121-123], suggesting that they could be promising biomarkers for the detection of precancerous lesions. Although different studies have reported aberrant miRNA expression in tissue during the early stages of PC development, few studies have discussed the association between preneoplastic conditions and circulating miRNAs [124], probably because the progression of PC is imperceptible and asymptomatic, which increase the difficulty in screening and collecting samples for testing. Further studies are warranted to explore the value of

miRNAs in screening and following patients with PanIN or IPMN.

Above all, circulating miRNAs have advantages, such as small size, stability, convenient extraction and easy detection, but their limitations cannot be ignored. First, it is difficult to quantify and standardize miRNA levels, as there is currently no accepted internal control, making this method unfit for monitoring disease progression. Second, alterations in miRNA profiles are dynamic and easily influenced, leading to potential interference by other diseases or conditions. The origin and mechanisms of circulating RNAs remain unclear, and a panel of miRNAs is recommended in this context to ensure a credible diagnostic result.

### *LncRNAs*

LncRNAs are RNAs longer than 200 nucleotides that are transcribed from multiple locations of the genome with no or limited protein-coding capability [125]. Notably, recent evidence has suggested that lncRNAs are restricted to specific cell types and play crucial roles during tumorigenesis by modulating key pathways at the transcriptional, post-transcriptional, and epigenetic levels [125, 126]. Certain lncRNAs could serve as novel biomarkers for the diagnosis of PC and especially for distinguishing different types and subtypes of malignancies based on tissue specificity [127].

Using a cDNA microarray platform, Tahira et al. were the first to explore lncRNA profiling in PC. The authors found that lncRNAs were differentially expressed in primary and metastatic PC lesions, suggesting that lncRNAs may be an indicator of tumor progression [128]. Moreover, recent studies have reported the dysregulation of several lncRNAs, including H19 [129], HOTAIR [130], HOTTIP [131], MALAT-1 [132], HULC [133], and GAS5 [134], in PC lesions compared with normal pancreatic tissue.

The diagnostic performance of circulating lncRNAs has been demonstrated in prostate cancer [135], hepatocellular carcinoma [136], colorectal cancer [137], and non-small cell lung cancer [138]. However, explorations into the association between circulating lncRNAs and PC remain at the primary stage. Only a few pilot studies have tested the diagnostic value

of lncRNAs in sera of patients with PC. Wang et al. [139] reported that plasma HDRF and RDRF, fragments of the lncRNAs HOTTIP-005 and RP11-567G11.1, are potential biomarkers; HDRF alone, RDRF alone, and a combination of HDRF and RDRF have AUC values of 0.867, 0.770, and 0.862, respectively, in distinguishing PC patients from healthy controls. We believe that circulating lncRNAs will assist in the early diagnosis of PC in the near future.

### CTCs

Although the existence of CTCs was reported by Ashworth nearly one century ago [140], it was not until recently that the selective enrichment and precise analysis of CTCs have become possible, providing a thorough understanding of the underlying mechanisms and associations between CTCs and malignancies. Several studies have shown that CTCs can enter the bloodstream through the shedding of local tumor cells from the primary lesion in the early stages of tumor development and that CTCs can be detected before metastases are established [141-143], suggesting an opportunity for early detection. Moreover, CTCs carry both tumor markers on the surface and somatic mutations [144], thereby enabling “real-time biopsies” of cancer.

Several studies have identified CTCs in 40%-100% of patients with PC [145]. The clinical value of CTCs in predicting PC metastasis and prognosis was recently shown [146]. However, their roles in the early detection and diagnosis of PC have not been extensively explored.

Technological advances have accompanied the discovery of CTCs. Based on the phenomenon that CTCs usually express epithelial cell adhesion molecule (EpCAM), these cells can be selected from peripheral whole blood samples using the CellSearch platform [147]. A study by de Albuquerque et al. [148] reported the presence of CTCs in 47% of PC patients (n=34) and in no healthy controls (n=40), as evidenced by immunomagnetic enrichment using antibodies against EpCAM followed by RT-PCR. However, in another study using the CellSearch system, one or more CTCs were detected in only 5% of locally advanced non-metastatic PC patients (n=79) [149]. Another technique, isolation by the size of epithelial tumor cells (ISET), is a filtration-based, marker-independent method for

CTC capture that has, on average, a higher CTC detection rate than CellSearch (93% vs. 40%) [150]. Using a filtration-based method and KRAS mutational analysis, Kulemann et al. [151] detected CTCs in 73% of 11 patients with PC regardless of tumor stage. The detection rate was 75% (3 of 4) among patients with early PC (American Joint Committee on Cancer stage I-II), and no CTCs were found in blood samples from 9 healthy controls. Recent progress in detection techniques has yielded a higher CTC detection rate. Yang et al. [152] applied a newly developed platform, integrated subtraction enrichment and immunostaining-fluorescence in situ hybridization, to analyze pancreatic CTCs, with a reported sensitivity of 88% and a specificity of 90% at a cutoff value of 2 cells/7.5 ml for the diagnosis of PC. For early PC (AJCC stage I-II), the CTC detection rate was 12/13. In another study, blood samples from PC patients were analyzed using the microfluidic NanoVelcro CTC chip. The investigator reported the presence of CTCs in 54/72 patients with confirmed PC, resulting in a sensitivity of 75.0%, a specificity of 96.4%, and an AUC of 0.867 [153].

In addition to CTC morphology and immunologic surface markers, some studies have focused on substrates, such as mRNA and DNA mutations, inside CTCs. Previous studies have detected CEA mRNA in CTCs with RT-PCR and reported the sensitivity (33.3%-75.0%) and specificity (94.6%-96%) for the detection of PC [154-156]. Epithelial-associated molecule CK-20 mRNA was also shown to be a potential biomarker for the detection of PC-associated CTCs [157]. Ankeny et al. [153] analyzed KRAS mutations in CTCs and primary tumor tissue from five patients with PC and found 100% concordance for KRAS mutation subtype between the primary tumor tissue and CTCs.

Though CTCs show promise in the early detection of PC, the performance of this diagnostic approach relies heavily on detection methodology. Therefore, the establishment of a standardized detection method and large-scale validation are urgently required before clinical application.

### Exosomes

Exosomes, which are small vesicles released from the plasma membrane by almost all cells, including cancer cells, have been shown to

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play an important role in intercellular communication, tumorigenesis and cancer metastasis [158-160]. Structurally, exosomes are enveloped by a lipid bilayer membrane with tissue-specific content instead of cellular organelles, such as pathogenic mRNA, miRNA, DNA fragments, and proteins. After release, exosomes are stable in the extracellular environment or circulation and can be taken up by neighboring or distant cells [161]. During this transfer process, exosomes exchange material and information between cells, inducing gene expression or mediating RNA silencing [162]. Recent studies have implicated PC-derived exosomes in the early development of PC [161] and showed that they are beneficial for establishing a premetastatic niche in the liver [163] and that they promote tumor formation and proliferation [164]. Considering their vital functions and extensive distribution, exosomes are potential circulating biomarkers for the detection of PC.

Exosomes contain a variety of proteins, nucleic acids, and lipids from cancer cells that are candidate diagnostic biomarkers. Several proteins were reported to be highly expressed in PC-derived exosomes [165]. Of these proteins, Melo and colleagues [166] chose glypican-1 (GPC1), a membrane-anchored protein, for further study because of its overexpression in both PC and IPMN and its roles in tumor promotion. This exosomal marker exhibited an essentially perfect performance with an AUC of 1.0 in distinguishing PC patients from healthy controls and from patients with benign pancreatic diseases. GPC1-positive exosomes were also detected at the precancerous lesion stage, such as at the PanIN and IPMN stages. Interestingly, GPC1 from serum exosomes showed superior sensitivity and specificity to freely circulating GPC1 from whole serum. Thus, the diagnostic performance of GPC1 is probably attributed to its enrichment in exosomes, thereby highlighting the potential of exosomes and their contents as biomarkers for the early detection of PC.

Studies thus far have provided sufficient evidence for the diagnostic and prognostic significance of exosomal miRNA in malignancies [167]. miRNAs in circulating PC-derived exosomes have been shown to be differentially expressed compared with non-malignant control exosomes and have been implicated in sev-

eral biological properties of cancer [168-171]. A study conducted by Madhavan and colleagues [172] examined the diagnostic value of a panel of five proteins (CD44v6, Tspan8, EpCAM, MET, and CD104) and four miRNAs (miR-1246, miR-4644, miR-3976, and miR-4306) in circulating exosomes. The combination of these biomarkers could discriminate PC from non-PC cases, including healthy controls, chronic pancreatitis patients, and benign pancreatic neoplasm patients, with a sensitivity of 1.00 and a specificity of 0.80.

Circulating exosomes from PC patients contain a large proportion of tumor DNA, which harbors DNA copy number variations, high-frequency somatic mutations and expressed fusion genes [173]. A recent study by Yang et al. [174] explored the potential clinical utility of circulating exosomal DNA for the identification of KRAS and TP53 mutations. The detection rate of the KRAS<sup>G12D</sup> mutation in exosomal DNA was 39.6%, 28.6%, and 2.6% in patients with PC, patients with IPMN, and healthy controls, respectively; however, TP53<sup>R273H</sup> mutations were detected in 4.2% of the PC cases and none of the healthy subjects. Another study involving 263 individuals evaluated exosomal KRAS mutations in all stages of PC and compared the detection rate in exosomes with that in cfDNA [175]. This previous study reported that more KRAS mutations were identified in exosomal DNA than in cfDNA from PC patients and that fewer such mutations were identified in exosomal DNA than in cfDNA from age-matched controls. Moreover, mutant KRAS in exosomal DNA was detected in 43.6% of early-stage PC patients, suggesting its promising utility in the early diagnosis of PC.

In general, exosomes have some unique advantages. Exosomes are widely distributed in nearly all body fluids, including serum, and are relatively stable when stored long term at -80°C. Second, PC-derived exosomes enter the circulation at an early stage of cancer development and are related to metastasis. These findings are critical for the early detection of PC because PC cells are able to metastasize at an early stage, which greatly influences prognosis. Last, exosomes contain a combination of proteins, DNA, coding and noncoding RNAs, and lipids that can be used as a natural panel of biomarkers for simultaneous evaluation. In summary,

exosomes are promising biomarkers for the early detection of PC, although more evidence from large-scale validation studies are required prior to clinical application.

### Conclusion and perspectives

An ideal diagnostic method for PC should definitively distinguish malignant lesions from benign lesions, provide precise tumor staging, and detect early-stage disease and preneoplastic conditions. Though it takes years or decades for PanIN lesions to progress to PC, thus providing a time frame for diagnosis and an opportunity for early management, there are many challenges in the early detection of PC, including its asymptomatic nature, the lack of a characteristic radiological manifestation, and the absence of specific molecules in body fluid. Therefore, convenient and highly sensitive diagnostic tests for screening PC are probably more important than tests with good specificity but moderate sensitivity. Primary screening using circulating biomarkers followed by confirmative diagnosis based on imaging and pathologic results might be the future strategy for diagnosing PC.

Due to technical advances in recent years, a variety of novel biomarkers from body fluids, including blood, urine, saliva, pancreatic juice, and stool, has been discovered in studies on the early detection of PC [176, 177]. Circulating biomarkers from blood have advantages in terms of stability, convenience, and abundance compared with traditional imaging workups and have exhibited a better diagnostic performance than biomarkers from other body fluids. Moreover, a series of biomarkers can be detected and analyzed efficiently, providing a comprehensive overview of PC.

Various circulating biomarkers have been widely studied, but some limitations are present in most studies. These issues should be handled with caution in future research. First, tumor heterogeneity has been recognized to complicate accurate diagnoses. One or two biomarkers can hardly provide a comprehensive diagnosis of cancer in the context of precision medicine. Additionally, suboptimal sample selection may lead to misinterpretation of the diagnostic value. Most samples in many studies were collected from patients with advanced

disease rather than from those with early disease. Adequate controls, including samples from patients with chronic pancreatitis, autoimmune pancreatitis, pancreatic neuroendocrine tumors, or cystadenoma, should also be collected to evaluate the validity of the differential diagnosis. Third, dynamic changes in biomarkers need to be monitored during follow-up or after treatment, especially in high-risk populations. Last, researchers should not ignore the impact of comorbidities. Obstructive jaundice and diabetes are frequently seen in patients with PC, and these conditions have been shown to influence the performance of biomarkers [178, 179].

Considering these situations, one option is to develop a diagnostic panel based on a series of biomarkers. Although CA19-9 has been reported to have worse diagnostic performance than several circulating biomarkers in small-scale studies, it remains the only circulating biomarker that is widely accepted and used for the diagnosis of PC. New biomarkers discovered in future studies should be compared to and combined with CA19-9 in a panel to improve sensitivity and specificity. The lack of sufficient samples from noninvasive precursor lesions and early-stage PC must be addressed, and animal models are important tools for research. Genetically engineered mouse models harboring key mutations such as those in KRAS and TP53 spontaneously develop PanIN and PC [180, 181]. These models can be used to identify biomarkers in PanIN and early-stage PC, to better understand tumorigenesis and other malignant behaviors, and to test novel therapeutics. In-depth exploration of the fundamentals of PC development and the nature of precursor lesions is definitely a critical step toward discovering and applying novel biomarkers. Collaborative large-scale prospective studies are required in the future to ascertain the reliability of potential biomarkers.

There is still a long journey from bench to bedside. Hopefully, circulating biomarkers will greatly benefit PC patients in the near future by providing the opportunity for early diagnosis and a better prognosis.

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**Disclosure of conflict of interest**

None.

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