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TOPIC HIGHLIGHT

2016 Pancreatic Cancer: Global view

Liquid biopsy in patients with pancreatic cancer: Circulating tumor cells and cell-free nucleic acids

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Abstract

Despite recent advances in surgical techniques and perioperative management, the prognosis of pancreatic cancer (PCa) remains extremely poor. To provide optimal treatment for each patient with Pca, superior biomarkers are urgently needed in all phases of management from early detection to staging, treatment monitoring, and prognosis. In the blood of patients with cancer, circulating tumor cells (CTCs) and cell-free nucleic acids (cfNAs), such as DNA, mRNA, and noncoding RNA have been recognized. In the recent years, their presence in the blood has encouraged researchers to investigate their potential use as novel blood biomarkers, and numerous studies have demonstrated their potential clinical utility as a biomarker for certain types of cancer. This concept, called "liquid biopsy" has been focused on as a less invasive, alternative approach to cancer tissue biopsy for obtaining genetic and epigenetic aberrations that contribute to oncogenesis and cancer progression. In this article, we review the available literature on CTCs and cfNAs in patients with cancer, particularly focusing on PCa, and discuss future perspectives in this field.

Key words: Pancreatic cancer; Biomarker; Liquid biopsy; Circulating tumor cells; Cell-free nucleic acids

Core tip: In the blood of patients with cancer, circulating tumor cells (CTCs) and cell-free nucleic acids (cfNAs), such as DNA, mRNA, and noncoding RNA have been recognized. In the recent years, their presence in the blood has encouraged researchers to investigate their potential use as novel blood biomarkers. This concept, called "liquid biopsy" has been focused on as a less invasive, alternative approach to cancer tissue biopsy for obtaining genetic and epigenetic aberrations that contribute to oncogenesis and cancer progression. In this article, we review the available literature on CTCs and cfNAs in patients with cancer, particularly focusing on pancreatic cancer.

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INTRODUCTION

Pancreatic cancer (PCa) is the fourth leading cause of cancer-related deaths in the United States, and the eighth worldwide[1]. In recent years, as a result of advances in surgical techniques and perioperative management, the perioperative mortality rate has decreased and perioperative chemotherapy and radiotherapy have greatly improved; however, prognostic outcomes for PCa remain poor[2,3]. Even now, the median survival time of patients with PCa is 5-8 mo and their 5-year survival rate is less than 10%[2,3].

Although surgical resection is the only option for macroscopic tumor clearance for PCa, most patients are diagnosed at an advanced and unresectable stage because PCa develops with no symptoms, local invasiveness, and metastases to distant organs in the early stage of its clinical course[1,4,5]. In addition, PCa shows resistance to conventional chemotherapies. Therefore, primary tumors must be detected at an early and resectable stage, whereas patients with far advanced disease must be preoperatively diagnosed to avoid surgical impairments and to select appropriate treatments to improve the quality of remaining life[6]. Consequently, to provide optimal management for each patient, biomarkers are urgently needed that are better than the conventional ones, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), in all phases of management from early detection to staging, treatment monitoring, and prognosis for PCa.

Numerous genetic and epigenetic aberrations contribute to oncogenesis and cancer progression, and the utility of these alterations for diagnostic, prognostic, and therapeutic purposes in various cancers have been investigated. Conventionally, these cancer-related alterations are investigated using tissue samples from surgical or biopsy specimens. These methodologies for pancreatic tissue acquisition cannot always be performed and repeated because of their invasive nature and anatomical difficulties. Thus, conventional examinations may fail to reflect current tumor dynamics and drug sensitivities, which may change during the therapeutic process. Detection and examination of circulating tumor cells (CTCs) and/or cell-free nucleic acids (cfNAs) in the bloodstream by performing a so-called liquid biopsy, which allows repeated sampling, makes it possible to track the current status of a tumor and its heterogeneous characteristics, which single sampling may fail to capture.

In the past decades, numerous studies have shown the potential utility of novel blood-based biomarkers, such as CTCs and cfNAs, for various cancers including PCa[7-10]. These promising markers are considered to possess great potential and could facilitate therapeutic strategies for cancer. In this article, we review the histological backgrounds, characteristics, and developments among CTCs and cfNAs in cancer research and discuss future perspectives, particularly focusing on PCa.

BIOLOGY AND DETECTION OF CTCS

In 1869, Ashworth[11] identified the presence of CTCs for the first time in the blood of a metastatic breast cancer patient in whom cells similar to those in the primary tumors were found in the blood at autopsy. Since then, many groups have challenged and demonstrated the identification and characterization of CTCs in peripheral blood of patients with cancer in several cancers. It has been recognized that CTCs originate from the primary tumor and/or metastatic lesions and are therefore extremely rare in healthy subjects and patients with nonmalignant diseases but are present in various metastatic carcinomas with a wide range of frequencies[12].

CTCs are generally thought to be quite heterogeneous in both phenotype and genotype, and only 2.5% of CTCs develop micrometastases and only 0.01% develop macrometastases[13-15]. During the journey toward the development of a metastatic lesion, some CTCs undergo epithelial-to-mesenchymal transition (EMT), which is characterized by decreased expression of epithelial markers and the acquisition of mesenchymal features[15] that could allow CTCs to escape from epithelial marker-based detection[16]. Furthermore, CTCs are present in peripheral blood at a low density among billions of blood cells in each milliliter of blood[17]. Consequently, accurate detection of CTCs with sufficient sensitivity and specificity has been a major technical challenge for researchers.

Currently, the CELLSEARCH system (Veridex) is

the most widely used CTC platform. In this platform, immunomagnetic beads coated with anti-epithelial cellular adhesion molecule (EpCAM) antibodies capture CTCs, followed by immunostaining with two positive markers: cytokeratins (CKs) 8/18/19 for cytoplasmic epithelium and 4′,6′-diamidino-2-phenylindole hydrochloride for nucleic acids, and a negative marker, leukocyte-specific CD45. The CELLSEARCH system is the only CTC platform to gain the approval of the United States Food and Drug Administration, and its clinical utility has been demonstrated as a diagnostic and prognostic indicator in patients with metastatic breast, prostate, and colon cancer[18-23]. In contrast, EpCAM-based enrichment of CTCs such as the CELLSEARCH system could fail to capture CTCs that have undergone EMT and increase the malignant potential. Several problems still remain regarding the detection and isolation capability and, thus, the clinical utility of CTCs. To improve sensitivity and specificity despite the heterogeneity of CTCs, new technology for the isolation and enrichment of CTCs has been developed. More recently, CTC-Chip[24] was demonstrated to increase the detection of CTCs by using tumor-specific markers, such as PSA in prostate cancer or HER2 in breast cancer, in addition to epithelial markers. Furthermore, Saucedo-Zeni *et al*[25] reported a new technology that enables the capture and enrichment of CTCs *in vivo* using a medical Seldinger guidewire inserted through a standard venous cannula into the cubital veins. Despite these advances, however, the isolation and enrichment of CTCs remains at the development stage.

After the isolation and enrichment of CTCs, identification procedures must be performed to examine their genetic and biological features. Various methods, such as immunocytochemistry and molecular techniques, have been commonly performed for identifying CTCs. Conventionally, immunostaining using 4′,6′-diamidino-2-phenylindole hydrochloride as a nuclear stain, CK as an epithelial marker, and CD45 as a hematopoietic marker have been widely used[26]. Among molecular approaches, quantitative reverse transcription-polymerase chain reaction (RT-PCR) has been generally employed to investigate the molecular characteristics of CKs, CEA, and other driver markers[27].

CTC DETECTION IN PATIENTS WITH PCA AND ITS CLINICAL RELEVANCE

To date, many researchers have tried to detect CTCs in patients with PCa and have demonstrated its clinical utility using various approaches. Table 1 summarizes the previous reports about CTCs in patients with PCa. Early studies of CTCs in PCa employed tumor-specific and/or epithelial-related mRNAs as a molecular target for the detection of CTCs. Among these studies, RT-PCR techniques have been widely used for the detection of mRNAs despite a low concentration. Funaki *et al*[28] first reported the clinical utility of CTCs in patients with PCa using RT-PCR. They demonstrated that the detection of CEA mRNA as a tumor-specific molecule in peripheral blood is useful in finding the hematogenous spreading of adenocarcinoma cells. Three of nine patients with PCa (33.3%) were positive for CEA mRNA, and none of the control patients was positive for CEA mRNA in peripheral blood. Following this study, some groups evaluated the clinical utility of CEA mRNA in the bloodstream for the detection of CTCs in PCa and reported its sensitivity (47.8%-75.0%) and specificity (94.6%-96%) for the detection of PCa[29-32]. Chausovsky *et al*[33] reported that the epithelial-associated molecule CK-20 mRNA is useful for the detection of CTCs in PCa. They successfully demonstrated RT-PCR of CK-20 as a potential biomarker for detecting metastases in blood samples from patients with PCa and in subsequent studies reported data supporting this result[34-36]. Other mRNAs, such as epithelial growth factor receptor mRNA[37], α1,4-*N*-acetylglucosaminyltransferase mRNA[38], and CK-19 mRNA[39] have also been reported as useful targets for the detection of CTCs using RT-PCR in patients with PCa. Regarding these mRNA-based studies, sensitivities varied widely and were relatively low despite high specificity. Furthermore, some of these studies used mononuclear cell fraction from density gradient enrichment or blood samples without enrichment techniques to extract total RNA for the investigation of mRNA expression; therefore, the approach cannot exclude the possible contamination of leukocyte-originated RNAs, an issue that should be considered in interpreting results. More recently, Zhou *et al*[40] suggested that the combined detection of c-Met, h-TERT, CK20, and CEA using RT-PCR following immunomagnetic bead enrichment could be used as an indicator for circulating cancer cells with 100% sensitivity and 100% specificity in patients with PCa.

Meanwhile, an immunocytochemical approach also has been used for identifying CTCs in patients with PCa. Although many studies have investigated the clinical utility of the CELLSEARCH platforms in PCa, a low detection rate ranging from 5% to 42% was reported[12,41,42]. In contrast, some of the studies demonstrated the clinical utility of CTCs as a predictor of disease recurrence and prognosis by reporting that a positive CTC finding was associated with disease recurrence and/or poor survival. To improve the detection rate of the CELLSEARCH system in PCa, several researchers have developed novel technologies for the detection of CTCs, such as size-based isolation[43-46] and microfluidics[47,48], and reported favorable results.

Overall, a certain level of utility of CTCs as a biomarker in PCa may be practically guaranteed; however, there are several problems that remain to be solved before CTCs can be considered useful in a clinical setting. First, CTCs might not be suitable

Table 1 Circulating tumor cells in pancreatic cancer

Ref.	Patient characteristics	Number of patients with PCa	Controls	Enrichment/ isolation method	Detection method	Detection rate
Funaki *et al*[28], 1996	Pre- or post-treatment	9	NA	None	RT-PCR: CEA mRNA	33.3% of PCa
Funaki *et al*[29], 1998	Preoperative	3	NA	None	RT-PCR: CEA-mRNA	
Miyazono *et al*[30], 1999	Intra-operative	21	15 HV	Density gradient enrichment	RT-PCR: CEA-mRNA	61.9% of PCa
Chausovsky *et al*[33], 1999	Metastatic PCa	28	22 BD	Density gradient enrichment	RT-PCR: CK20 mRNA	78.6% of PCa
Z'graggen *et al*[124], 2001	All stages, preoperative	105	66 HV	Density gradient enrichment	ICC: CK-AE1/AE3	Sensitivity: 26%, specificity: 96%
Lukyanchuk *et al*[34], 2003	NA	11	18 HV	Density gradient enrichment	RT-PCR: CK-20 and PSCA	CK-20: 19 of 47 (40.4%), PSCA: 22 of 47 (46.8%)
Clarke *et al*[37], 2003	NA	11	23 HV	Density gradient enrichment	RT-PCR: EGFR mRNA	18% of PCa, 0.0% of HV
Mataki *et al*[32], 2004	All stages	20	15 HV, 15 BD	Density gradient enrichment	RT-PCR: CEA mRNA	Sensitivity: 75.0%, specificity: 94.6%
Zhang *et al*[35], 2005	Stages Ⅱ and Ⅲ	40	5 BD, 5 HV	Not available	RT-PCR: CK20 mRNA	57.5% of PCa
Soeth *et al*[36], 2005	Preoperative	154	NA	Density gradient enrichment	RT-PCR: CK20 mRNA	33.8% of PCa
Ishizone *et al*[38], 2006	All stages	55	10 CP, 70 HV	Density gradient enrichment	RT-PCR: a4GnT	76.4% of PCa, 40.0% of CP, 17.1% of HV
Hoffmann *et al*[39], 2007	All stages	37	16 CP, 15 BD	Density gradient enrichment	RT-PCR: CK-19 mRNA	64% of PCa
Kurihara *et al*[41], 2008	Stages Ⅱ, Ⅲ and Ⅳ	26	11 CP, 10 HV	CELLSEARCH®		42% of PCa
Zhou *et al*[40], 2011	All stages	25	15 BD	Immunomagnetic separation	RT-PCR: C-MET, h-TERT, CK20, and CEA	Sensitivity: 100%, specificity: 93.3%
Khoja *et al*[125], 2012	Stages Ⅲ and Ⅳ	54 PCa	NA	Size-based selection	RT-PCR: EpCAM, CK, vimentin, and CEA	ISET 49/54 (93%) *vs* CELLSEARCH® 21/54 (40%)
de Albuquerque *et al*[126], 2012	Stages Ⅲ and Ⅳ	34	40 HV	Immunomagnetic separation	RT-PCR: KRT19, MUC1, EpCAM, CEACAM5, and BIRC5	Sensitivity: 47.1%, specificity: 100%
Kamande *et al*[127], 2013	All stages	12	5 HV	Microfluidic; ICC	$DAPI^+$, CD45-, CK^+	100% of PCa
Bidard *et al*[42], 2013	Locally advanced PCa	79	NA	CELLSEARCH®		11% of PCa
Iwanicki-Caron *et al*[43], 2013	All stages	40	NA	Size-based selection	Cell size and cytopathologic criteria	Sensitivity: 55.5%, specificity: 100%, accuracy: 70%
Bobek *et al*[45], 2014	All stages	24	NA	Size-based selection	DAPI, CK, CEA, vimentin IHC	66.7% of PCa
Sheng *et al*[48], 2014	Metastatic PCa	18	NA	Multifluidic, "GEM"Chip		94.4% of PCa
Rhim *et al*[47], 2014	All stages	11	19 HV	Geometrically enhanced differential immunocapture	ICC for DAPI, CD45, CK, and PDX-1	73% of PCa
Catenacci *et al*[128], 2015	Stages Ⅱ, Ⅲ and Ⅳ	18	NA	Immune-magnetic separation	CD45-negative and positive for CK8, -18, and/or -19 and DAPI	118.4 ± 36.8 CTCs/7.5 mL PVB, compared with a mean of 0.8 ± 0.4 CTCs/7.5 mL PB
Earl *et al*[81], 2015	All stages	35	NA	CELLSEARCH®		20% of PCa
Cauley *et al*[44], 2015	All stages	105	9 HV	Size-based selection	Cytomorphologic criteria	48.6% of PCa
Kulemann *et al*[46], 2015	Preoperative	11	9 HV	Size-based selection: ScreenCell	Cytologic and detection of KRAS mutation	75% of early PCa, 71.4% of advanced PCa
Zhang *et al*[129], 2015	All stages	32	30 HV	ICC and FISH	$DAPI^+$, CD45-, and CK^+, or CEP8 > 2^+	Sensitivity: 63.6%, specificity: 94.4%
Bissolati *et al*[130], 2015	Intra-operative	20	NA	CELLSEARCH®		PVB: 40%, PB: 20%
Zhang *et al*[131], 2015		15	15 HV	Immunomagnetic separation	BC-15 aptamer or anti-CK staining	73.3% of PCa

BD: Benign disease; CEA: Carcinoembryonic antigen; CK: Cytokeratin; CP: Chronic pancreatitis; CTC: Circulating tumor cell; DAPI: 4′,6′-diamidino-2-phenylindole; EpCAM: Epithelial cellular adhesion molecule; FISH: Fluorescence *in situ* hybridization; HV: Healthy volunteer; ICC: Immunocytochemistry; IHC: Immunohistochemistry; NA: Not applicable; PB: Peripheral blood; PCa: Pancreatic cancer; PVB: Portal venous blood; RT-PCR: Reverse transcription polymerase chain reaction.

in screening tests for early detection (which is desirable in PCa) owing to their low sensitivity. Novel technology with high sensitivity for the detection of CTCs is required to use CTCs in screening tests for early diagnosis. Second, the detection of CTCs has to overcome the challenge of the rarity and heterogeneity of CTCs, and because the methodology for the detection of CTCs remains in a developmental stage, the approach to CTCs and the results of studies have varied widely. Consequently, it is difficult to assess, compare, and interpret the results of multiple studies and establish the evidence and clinical relevance of CTCs. Establishment of a unified methodology and large-scale validation of their utility are required for wider clinical application.

BIOLOGY AND DETECTION OF CFNAS

For many decades, cfNAs have been known to be present in peripheral blood. Mandel and Metais[49] first reported that nucleic acids were detectable in human plasma in 1948. In 1977, Leon *et al*[50] detected cell-free DNAs in the serum of patients with cancer. Since then, several studies have identified tumor-specific and/or tumor-associated alterations in the circulating cfNAs of patients with various cancers. In 1989, Vasioukhin *et al*[51] successfully detected cell-free DNA with neoplastic characteristics, providing the first evidence suggesting that tumors can shed DNA into the circulation. This hypothesis was further supported by a study in which a *NRAS* mutation in the plasma of patients with myelodysplastic syndrome or acute myelogenous leukemia[52] and *KRAS* mutation in the plasma or serum of patients with PCa were detected[53].

Cell-free RNA, which is thought to be more fragile than DNA because RNA is easily degraded by endogenous ribonuclease (RNase) in plasma/serum, has been successfully detected in blood. In 1999, tyrosinase mRNA in the serum of patients with malignant melanoma[54] and Epstein-Barr virus-associated RNA in nasopharyngeal carcinoma[55] was successfully detected. Subsequently, many studies have demonstrated the presence of specific mRNA in plasma/serum and its clinical utility in patients with various cancers[56-58].

Regarding noncoding RNA, Mitchell *et al*[59] firstly demonstrated that circulating microRNAs (miRNAs) had the potential to be novel biomarkers in patients with solid cancers in 2008. Since then, numerous studies examining circulating noncoding RNAs have been performed, with most studies focusing on miRNAs. Although other noncoding RNAs, such as small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), piwi-interacting RNA (piRNA), and long noncoding RNA (lncRNA), have been recognized to have biological functions and may have great potential to be novel blood biomarkers, there are few reports of these noncoding RNAs. Further studies and accumulation of evidence are required.

CIRCULATING CELL-FREE DNA IN PLASMA/SERUM AND PCA

The study of circulating cell-free DNA in the plasma/serum involves two major strategies: the measurement of the amount of cell-free DNA in the circulation and the detection of tumor-derived genetic aberrations such as point mutations, allelic imbalances, microsatellite instability, genetic polymorphisms, loss of heterozygosity, and methylation. Of these, many reports have demonstrated the detection of genetic and epigenetic alterations in circulating cell-free DNA in the bloodstream of patients with cancer[60-63].

Previous reports about circulating cell-free DNA in PCa are summarized in Table 2. In 1983, Shapiro *et al*[64] first reported the presence of circulating cell-free DNA in PCa. This study demonstrated that serum DNA concentration is markedly elevated in patients with PCa compared with normal controls using radioimmunoassay and that an abnormally high concentration of DNA in serum may have diagnostic and prognostic value. Since then, investigations of tumor-derived genetic alterations in plasma/serum have become mainstream. Only a few studies investigating the methylation[65,66], microsatellite instability, and allelic imbalance[67] can be found. In contrast, the detection of K-ras mutation in plasma/serum appears to be the most widely used approach in the diagnosis of PCa. It has been widely recognized that over 90% of pancreatic adenocarcinomas contain mutated K-ras genes[68-70], and the detection of mutant K-ras provides a definitive diagnosis of pancreatic adenocarcinoma in pancreatic tissue[70,71]. Based on the detection of K-ras mutation in tumor tissues, the detection of the tumor-derived genetic alterations in circulating cell-free DNA has been attempted for the diagnosis of PCa. In 1993, Yamada *et al*[72] demonstrated that detection of K-ras mutations in plasma may be clinically useful for evaluating tumor burden and efficacy of treatment for PCa. Subsequently, many groups have reported the possible clinical utility of K-ras mutations in circulating cell-free DNA in PCa[73-80].

PCR has been widely used to detect tumor-derived mutations in genes isolated from the serum and plasma of patients with cancer[51,53]. More recently, droplet digital PCR[81-84] and genome-wide high-throughput sequencing[84,85] has been demonstrated as a potential detection tool for rare mutations and multiple types of mutations in circulating DNA with great accuracy. Using these new technologies in several solid cancers such as colorectal, breast, and ovarian cancer, a correlation has been found between the acquisition of drug resistance and genetic aberrations in cell-free DNA in the blood of patients under treatment[86-88]. It is hoped that the potent utility

Table 2 Circulating cell-free DNA in pancreatic cancer

Ref.	Patients	Controls	Sample	Method	Target candidate	Findings
Shapiro *et al*[64], 1983	201 MD	185 BD	Serum	Radioimmunoassay	Circulating DNA levels	Serum DNA concentration is markedly elevated in 90% of patients with PCa as compared with HV
Yamada *et al*[72], 1998	21 PCa	-	Plasma	Mutant allele-specific amplification method	K-ras mutation	In 9 of 15 (60%) patients with K-ras gene mutation-positive tumors, an identical mutation was detected in the plasma DNA. Detection of K-ras mutations in plasma may be clinically useful for evaluating tumor burden and efficacy of treatment
Giacona *et al*[132], 1998	3 PCa	3 HV	Plasma	Gel electrophoresis and measuring the variation in length by electron microscopy	Length	There are significant differences in non-cell-associated DNA in plasma between patients with PCa and HV
Theodor *et al*[73], 1999	20 PCa	6 CP, 5 HV	Serum	PCR	K-ras mutation	K-ras gene mutations at codon 12 were detected in the sera of 14 of 20 patients with PCa and in none of the 6 patients with CP, or in the 5 HVs
Castells *et al*[74], 1999	44 PCa	37 CP	Plasma	Restriction fragment length polymorphism-PCR and single-strand conformation polymorphism techniques	K-ras mutation	Plasma K-ras analysis is a highly specific, low-sensitivity approach that has diagnostic and prognostic clinical implications in patients with PCa
Zambon *et al*[75], 2000	29 PCa	12 HV	Serum	ME-PCR	K-ras mutation	K-ras was amplifiable in 2 patients with PCa (6.9%), and K-ras was not amplifiable in any of the 12 serum samples obtained from HVs
Maire *et al*[76], 2002	47 PCa	31 CP	Serum	PCR and allele-specific amplification	KRAS2 mutations	KRAS2 mutations were found in 22 patients (47%) with PCa and in 4 controls with CP (13%) ($P < 0.002$)
Melnikov *et al*[65], 2009	30 PCa	30 HV	Plasma	Multiplexed array-mediated analysis of DNA methylation	Methylation	Differential methylation profiling of plasma DNA can detect PCa with 76% sensitivity and 59% specificity
Liggett *et al*[66], 2010	30 PCa	30 CP, 30 HV	Plasma	Microarray-mediated methylation analysis	Methylation	Methylation analysis achieved 81.7% sensitivity and 78% specificity ($P < 0.01$) in the detection of CP (HV *vs* CP) and 91.2% sensitivity and 90.8% specificity ($P < 0.01$) in the differential detection of PCa (PCa *vs* CP)
Chen *et al*[79], 2010	91 PCa	-	Plasma	Direct sequencing	K-ras	K-ras codon 12 mutations were found in 30 of 91(33%) plasma DNA samples and significantly reflected the clinical parameters, including TNM tumor staging and liver metastasis, and independent predict shorter survival time
Wu *et al*[80], 2014	24 PCa	25 HV	Plasma	COLD-PCR combined with an unlabeled-probe HRM approach	K-ras	KRAS mutations were identified in 26 of 36 PCa cases (72.2%), but none were detected in the disease control and/or healthy group
Earl *et al*[81], 2015	31 PCa	-	Plasma	Digital PCR	KRAS	KRAS mutant cfDNA was detected in 26% of patients at all stages, which correlated strongly with OS, 60 d for KRAS mutation-positive *vs* 772 days for KRAS mutation-negative patients
Zill *et al*[85], 2015	26 PCa	-	Plasma	Sequenced on an Illumina Hi-Seq 2500	KRAS, TP53, APC, FBXW7, and SMAD4	The diagnostic accuracy of cfDNA sequencing was 97.7%, with 92.3% average sensitivity and 100% specificity across 5 informative genes
Singh *et al*[133], 2015			Plasma		Levels of ctDNA and K-ras mutation	Higher levels of plasma DNA were significantly associated with lower OS and advanced stage. However, k-ras mutation did not correlate with any of the clinicopathological parameters or survival
Kinugasa *et al*[82], 2015	141 PCa	20 CP, 20 HV	Serum	Digital PCR	G12V, G12D, and G12R in codon 12 of K-ras gene	K-ras mutation rate in ctDNA was 62.6%. The survival of patients with K-ras mutations in ctDNA was significantly shorter than that of patients without mutations
Sausen *et al*[83], 2015	77 PCa	-	Plasma	Next-generation sequencing		The 43% of patients with localized disease had detectable ctDNA at diagnosis. Detection of ctDNA after resection predicts clinical relapse and poor outcome, with recurrence by ctDNA detected 6.5 months earlier than with CT imaging
Takai *et al*[84], 2015	259 PCa	-	Plasma	Picoliter-droplet digital PCR and targeted deep sequencing	KRAS mutation	KRAS mutations were identified in 14 of 48 patients (29.2%) examined by targeted deep sequencing of cfDNA

BD: Benign disease; cfDNA: Cell-free DNA; COLD-PCR: Co-amplification at lower denaturation-temperature PCR; CP: Chronic pancreatitis; CT: Computed tomography; ctDNA: Circulating tumor DNA; HV: Healthy volunteer; ICC: Immunocytochemistry; PCa: Pancreatic cancer; MD: Malignant disease; OS: Overall survival; PCR: Polymerase chain reaction; ME-PCR: Mutant-enriched PCR.

of cell-free DNA for the assessment of residual disease, recurrence, and acquisition of drug resistance as well as for the detection of disease can be proven in PCa.

CIRCULATING CELL-FREE MRNA IN PLASMA/SERUM AND PCA

Although RNA is easily degraded by RNase and the concentration of RNase in blood is high in patients with cancer[89], many groups have demonstrated the stable presence of cell-free mRNAs in the blood of patients with cancer. Recently, it has been considered that these RNAs could be incorporated into exosomes, microvesicles, and multivesicles, which seem to be adequately protected against the degradation caused by the abundant RNases and released from the cellular surface to the bloodstream[90]. There are numerous studies investigating the correlation between cell-free mRNA in the bloodstream and several solid cancers, and these studies are mainly aimed at investigating the mRNAs in plasma/serum that are up-regulated in cancer tissues[56-58,91-93]. Regarding PCa, there are several studies investigating mRNA in peripheral blood mononuclear cells and CTCs[29,32,35,38] as a marker for the detection of CTCs; however, the number of cell-free mRNAs in plasma/serum is extremely small. Kang *et al*[94] recently demonstrated that type IV collagen (COL6A3) mRNA in serum might serve as a biomarker for the detection of PCa according to tumor-specific alternative splicing. Accumulation of evidence and understanding of cell-free mRNA in patients with cancer may have a potential to bring new insights into the field of liquid biopsy in PCa.

CIRCULATING NONCODING RNA IN PLASMA/SERUM

It has been revealed that as much as 80% of genomic DNA is transcribed into RNAs[95]. In contrast, the Human Genome Project discovered that the open reading frames of protein genes constitute less than 2% of the 3.2 billion bases[96,97]. Thus, a large portion of human genomic DNA does not code proteins. It is now becoming evident that a variety of noncoding RNAs (ncRNAs) play important roles in many cellular processes and are not just mere intermediates in the transfer of genetic information from DNA to proteins, which indicates that the ncRNAs expression patterns could be used as molecular markers in specific diagnostic methods[98].

For circulating ncRNAs, almost all of the studies have focused on miRNAs, which are short noncoding RNAs that play important roles in various physiologic and developmental processes. One strand (a guide strand) of mature miRNA is then incorporated into the RNA-induced silencing complex and subsequently hybridizes to the 3′-untranslated region of their target mRNAs to repress translation or degrade these mRNAs. Thus, a single miRNA can influence the expression of hundreds of genes and allow them to function in a coordinated manner. Therefore, miRNAs have been implicated as key molecules in all cellular processes. Numerous studies have shown that alterations in miRNA expression correlate with various diseases, including the development and progression of cancer, and some miRNAs can function as oncogenes or tumor suppressors. Furthermore, several recent studies have demonstrated that some extracellular miRNAs occur not only through cell lysis but also through active secretion[8,99,100]. Cell-derived endogenous miRNAs are present in the blood in a remarkably stable form that is protected from endogenous RNase activity. Kosaka *et al*[100] demonstrated that a subset of miRNAs can be packaged into exosome vesicles and released through a ceramide-dependent secretory mechanism. Furthermore, most miRNAs are stable in plasma owing to their binding to proteins such as Argonaute2 and high-density lipoprotein[101]. All circulating miRNAs, regardless of whether they are incorporated into protein complexes and/or cell-derived microvesicles, seem to be adequately protected against the degradation caused by the abundant RNases in human plasma and serum. These findings have opened up a new and interesting field in the diagnosis of cancer and the treatments of patients with cancer.

Mitchell *et al*[59] first demonstrated that circulating miRNAs have the potential to be novel blood biomarkers in patients with solid cancers in 2008. Since then, numerous studies of circulating miRNAs in cancer have been performed to investigate their potential as candidate novel biomarkers. Regarding PCa, previous reports about circulating miRNAs are summarized in Table 3. Wang *et al*[102] first reported the plasma level of four candidate miRNAs (miR-21, -155, -196a, and -210) that were previously reported to be up-regulated in PCa tissue. Since then, several groups have reported the utility of circulating miRNAs as biomarkers for PCa, and the number of studies and the variety of miRNAs have been increasing. Many studies have demonstrated the clinical significance of deregulated expression of miRNA in plasma/serum, and more than 30 miRNAs have been reported as candidate novel blood biomarkers in PCa. As mentioned before, one miRNA can regulate multiple mRNAs and the numbers of discovered miRNAs and targeted mRNAs are still increasing owing to recent advances in analysis technology. Consequently, more recently, diagnosing PCa with higher sensitivity and specificity has been attempted by employing multiple miRNAs[103,104]. Schultz *et al*[103] reported that two diagnostic panels including four (miR-145, miR-150, miR-223, and miR-636) and 10 (miR-26b, miR-34a, miR-122, miR-126*, miR-145, miR-150, miR-223, miR-505, miR-636, and miR-885.5p) miRNAs based on the expression in whole blood could be used to detect PCa with high sensitivity and specificity. However, it should be considered that the study employed whole

Table 3 Circulating noncoding RNA in pancreatic cancer

Ref.	Sample	Candidate target	Potential value
		miRNA	
Wang *et al*[102], 2009	Plasma	miR-210 (↑), miR-21 (↑), miR-155 (↑), miR-196a (↑)	D
Ho *et al*[134], 2010	Plasma	miR-210 (↑)	D
Li *et al*[135], 2010	Plasma	miR-200a (↑), miR-200b (↑)	D
Ali *et al*[136], 2010	Plasma	miR-21 (↑)	D/P
Kong *et al*[105], 2011	Serum	miR-196a (↑)	D/S/P
LaConti *et al*[137], 2011	Serum	miR-155 (↑)	D
Morimura *et al*[138], 2011	Plasma	miR-18a (↑)	D
Liu *et al*[139], 2012	Serum	miR-16 (↑), miR-196a (↑)	D
Liu *et al*[140], 2012	Serum	miR-20a (↑), miR-21 (↑), miR-24 (↑), miR-25 (↑), miR-99a (↑), miR-185 (↑), miR-191 (↑)	D/P
Li *et al*[141], 2012	Serum	miR-1290 (↑)	D
Wang *et al*[142], 2013	Whole blood	miR-27a-3p (↑)	D
Kawaguchi *et al*[143], 2013	Plasma	miR-221 (↑), miR-375 (↓)	D/S
Zhao *et al*[144], 2013	Serum	miR-192 (↑)	D
Li *et al*[141], 2013	Serum	miR-1290 (↑)	D
Wang *et al*[145], 2013	Serum	miR-21 (↑)	T/P
Carlsen *et al*[146], 2013	Plasma	miR-375 (↑)	D
Que *et al*[147], 2013	Serum	miR-17-5p (↑), miR-21 (↑), miR-155 (↓), miR-196a (↓)	D/S
Schultz *et al*[103], 2014	Whole blood	Multigene index	D
Gao *et al*[148], 2014	Plasma	miR-16 (↑)	D
Chen *et al*[149], 2014	Plasma	miR-182 (↑)	D/S/P
Ganepola *et al*[150], 2015	Plasma	miR-22 (↑), miR-642b (↑), miR-885-5p (↑)	D
Abue *et al*[151], 2015	Plasma	miR-21 (↑), miR-483-3p (↑)	D/S/P
Slater *et al*[152], 2015	Serum	miR-196a (↑), miR-196b (↑)	D
Kojima *et al*[104], 2015	Serum	Multigene index	D
Xu *et al*[153], 2015	Plasma	miR-486-5p (↑), miR-938 (↑)	D
Madhavan *et al*[154], 2015	Serum	miR-1246 (↑), miR-3976 (↑), miR-4306 (↑), miR-4644 (↑)	D
Komatsu *et al*[123], 2015	Plasma	miR-223 (↑)	D/P
Miyamae *et al*[106], 2015	Plasma	miR-744 (↑)	D/S/P/T
		Other noncoding RNAs	
Wang *et al*[117], 2015	Plasma	HOTTIP-005 (↑), RP11-567G11.1 (↑)	D/P
Baraniskin *et al*[155], 2013	Plasma/serum	U2 snRNA (↑)	D

D: Diagnostic marker; miRNA: MicroRNA; P: Prognostic marker; S: Staging marker; T: Treatment marker.

blood as a sample for extracting RNAs; therefore, the miRNAs found obviously included miRNAs derived from blood cells or CTCs other than cell-free miRNAs. Kojima *et al*[104] reported that a combination of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) could achieve high sensitivity, specificity, and accuracy for the detection of PCa.

Furthermore, the studies of circulating cell-free miRNAs in PCa have demonstrated the usefulness of circulating miRNAs as a staging marker, treatment marker, and prognostic marker as well as a biomarker for the detection of PCa. Kong *et al*[105] demonstrated that the serum miR-196a expression level had potential value for predicting median survival time of patients with PCa [high-level miR-196a, 6.1 mo (95%CI: 4.49-7.72), *vs* low-level miR-196a, 12.00 mo, (95%CI: 5.92-18.08), $P = 0.007$). Most recently, our group demonstrated that high expression of miR-744 in plasma might be a useful biomarker for screening PCa, monitoring, and predicting poor prognosis and chemoresistance in patients with PCa[106]. Although more evidence has been accumulating as we have been reviewing previous reports, several problems remain be solved for clinical application. There is no consensus regarding inter- and intra-individual variation, whether plasma or serum is more appropriate, and what molecule is optimal for the most sensitive detection and endogenous controls.

Some kinds of noncoding RNAs other than miRNA have been recognized to have biological functions. Especially in cancers, some noncoding RNAs have been demonstrated to have oncogenic or tumor-suppressive functions and to be deregulated in tumor tissue. Regarding PCa, HOTAIR[107], MALAT-1[108], MEG3[109], Gas5[110], HULC[111], PVT1[112], PPP3CB, MAP3K1, DAPK1[113], BC008363[114], ENST00000480739[115], and HSATII[116] have been reported to have tumor-associated functions and tumor-specific expression. However, there are no studies investigating these cell-free RNAs in the bloodstream of patients with PCa. The noncoding RNAs other than miRNAs, such as long noncoding RNA (lncRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), and Piwi-interacting RNA (piRNA) in the bloodstream of patients with PCa remain largely unexplored. Most recently, Wang *et al*[117] demonstrated that the plasma fragments of lncRNA, HOTTIP-005, and RP11-567G11.1 have the potential to be used as diagnostic biomarkers of PCa. We believe that future studies of circulating noncoding RNAs in PCa will bring new insights to this field.

CURRENT ISSUES AND FUTURE PERSPECTIVE

Blood-based biomarkers, evaluated using liquid biopsy, are attractive as diagnostic, staging, prognostic, and treatment markers for PCa owing to their less invasive nature, and the clinical relevance of using liquid biopsy to identify biomarkers in PCa has become practically guaranteed. However, several problems remain to be solved before application in a clinical setting. One of the important hurdles to overcome is the lack of consensus regarding a technical approach. The

methodology for the detection and assessment of CTCs and circulating cfNAs remains in a developmental stage, and therefore, the techniques used, such as sample type, storage conditions, target molecules, and detection approach, have varied widely among research groups. The standardization and unification of techniques through all processes and the accumulation of many results under the same conditions or in large-scale studies should be emphasized. Meanwhile, recent technical advances allow us to detect a slight amount of circulating cell and nucleic acids even in the body fluid other than blood, and several recent studies have already reported the possible utility of CTC and cfNAs in body fluids other than blood[118-122]. These reports have suggested the possibility of even less invasive and even more effective biomarkers in near future.

For the development of the field, PCa seems to be the ideal cancer to investigate for several reasons. First, the investigation of tumor-associated genetic alterations using tissue samples obtained from surgical or biopsy specimens is costly and difficult owing to anatomical and clinical difficulties. Additionally, the utility of the current serum biomarkers is limited owing to insufficient sensitivity and specificity. Repeatable and less invasive testing with high sensitivity and specificity to obtain information about genetic alterations or tumor dynamics will considerably contribute to the improvement of management for PCa. Second, it has already been revealed that some genetic or epigenetic alterations, such as KRAS, p16, SMAD4, TP53, and CDKN2A are present in most pancreatic tumor cells, which might make it easy to confirm the targets as tumor-derived molecules. Third, a genetic evolutionary model[120] revealed that 10-30 years are required from initiating a mutation until a patient's death. Furthermore, mucinous cystic neoplasms, intraductal papillary neoplasms, pancreatic intraepithelial neoplasia (IPMN), and intraductal tubular papillary neoplasms were identified as premalignant lesions of PCa[121] that develop to invasive PCa through stepwise progression with the accumulation of several genetic aberrations. If these important genetic aberrations could be captured by liquid biopsy, screening and monitoring tests for high-risk lesions or early detection could be realized. To date, there are few reports about the usefulness of liquid biopsy in these premalignant lesions of PCa[122]. Recently our study successfully demonstrated that plasma miR-223 could predict malignant potential of IPMN[123]. We believe that further studies of liquid biopsy in premalignant lesions of PCa could contribute to improve the prognostic outcomes of PCa patients and the biomarker for premalignant lesion is nearing the clinical application.

Overall, liquid biopsy has the potential to allow us to diagnose at an early stage, predict prognosis, track the current status such as therapeutic efficacy or resistance, and provide optimal, individual treatment strategies for patients with cancer, that is, tailor-made treatment. The development of liquid biopsy could provide many benefits for patients with cancer, especially for patients with PCa.

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