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**2016 Pancreatic Cancer: Global view**

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

Imamura T *et al*. Liquid biopsy of pancreatic cancer

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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

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**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 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 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 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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**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 | N/A | None | RT–PCR: CEA mRNA | 33.3% of PCa |
| Funaki *et al*[29], 1998 | Preoperative | 3 | N/A | 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 | N/A | 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 | N/A | 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 | N/A | 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: α4GnT | 76.4% of PCa, 40.0% of CP, 17.1% of HV |
| Hoffmann *et al*[39], 2007 | All stages | 37 | 16 CP, 15BD | 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  | N/A | 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 | N/A | CELLSEARCH system |  | 11% of PCa |
| Iwanicki-Caron *et al*[43], 2013 | All stages | 40 | N/A | Size-based selection | Cell size and cytopathologic criteria | Sensitivity: 55.5%, specificity: 100%, accuracy: 70% |
| Bobek *et al*[45], 2014 | All stages | 24 | N/A | Size-based selection | DAPI, CK, CEA, vimentin IHC | 66.7% of PCa |
| Sheng *et al*[48], 2014 | Metastatic PCa | 18 | N/A | Multifluidic, "GEM"Chip |  | 94.4% of PCa |
| Rhim *et al*[47], 2014 | All stages | 11 | 19HV | 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 | N/A | 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 | N/A | CELLSEARCH system |  | 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 | N/A | CELLSEARCH® system |  | 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; N/A: Not applicable; PB: Peripheral blood; PCa: Pancreatic cancer; PVB: Portal venous blood; RT–PCR: Reverse transcription polymerase chain reaction.

**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.

**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.