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Improving pancreatic cancer diagnosis using circulating tumor cells: prospects for staging and single-cell analysis

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Abstract

Pancreatic cancer (PC) is the fourth most common cause of cancer-related death in the USA, primarily due to late presentation coupled with an aggressive biology. The lack of adequate biomarkers for diagnosis and staging confound clinical decision-making and delay potentially effective therapies. Circulating tumor cells (CTCs) are a promising new biomarker in PC. Preliminary studies have demonstrated their potential clinical utility, and newer CTC isolation platforms have the potential to provide clinicians access to tumor tissue in a reliable, real-time manner. Such a 'liquid biopsy' has been demonstrated in several cancers, and small studies have demonstrated its potential applications in PC. This article reviews the available literature on CTCs as a biomarker in PC and presents the latest innovations in CTC research as well as their potential applications in PC.

Keywords

biomarker; circulating tumor cells; diagnosis; liquid biopsy; pancreatic cancer; personalized medicine; precision medicine; single cell sequencing; staging

Pancreatic cancer (PC) is the fourth most common cause of cancer-related death in the USA [1]. It is estimated that 48,960 people will be diagnosed with the disease in 2015 and that 73% of them will die within a year of diagnosis [2]. The 5-year overall survival for PC is largely unchanged at 7% due to the late presentation and lack of effective therapies. Thus, there is a pressing need for better biomarkers to assist in all phases of care from diagnosis to prognosis to treatment monitoring [1].

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Symptoms generally occur late, with the most common being pain, jaundice and weight loss [3]. Unfortunately, the majority of PC patients have metastatic disease at the time of diagnosis. Only 10–15% of patients have local disease, stage I or II by the American Joint Committee on Cancer (AJCC) staging system, at presentation and are considered candidates for surgery. Even with surgery, 5-year survival is only 25–30% for lymph node negative patients and only 10% for those with positive lymph nodes [2]. Thus, the unfortunate truth is that the majority of patients are under-staged by current staging criteria.

Diagnosis & staging of pancreatic cancer

Diagnosis of adenocarcinoma of the pancreas is currently made by pathologic assessment of a tissue biopsy. Given the anatomic location of the pancreas, this presents technical challenges. Acquisition of tissue can be done by surgical biopsy, percutaneous image-guided (CT) biopsy or most commonly via endoscopic ultrasound techniques coupled with fine-needle aspiration (EUS-FNA), the current gold standard. EUS-FNA requires sedation and is associated with low but significant risks of complications such as pancreatitis, bowel perforation and aspiration, all of which can be fatal [4]. An additional confounding issue with needle biopsy is that the majority of the tumor mass is made up of stromal cells, not the epithelial cancer cells, due to the dense desmoplastic reaction that is a hallmark of PC. Thus, false negative results are common, necessitating frequent repeat biopsies. Thus, despite being the gold standard, EUS-FNA only has a sensitivity of 75–94% and a specificity of 78–95% [5]. Furthermore, if the criteria for diagnosis are manipulated to increase the specificity to close to 100%, the NPV drops to as low as 40%. Thus, the current diagnostic paradigm for PC diagnosis suffers from low diagnostic accuracy while also being associated with appreciable risk and significant cost. For the 10–33% of patients who have nondiagnostic FNAs, as well as the significant percentage who cannot undergo endoscopy, clinicians are forced to make treatment decisions based on imaging and CA 19–9 levels alone.

After diagnosis, the patients are staged based on the AJCC 7th edition staging criteria [6]. As mentioned above, the current staging system for PC is based primarily on the sensitivity of cross-sectional imaging (CT or MRI). Current guidelines recommend CT or MRI as the initial staging test for patients with suspected PC, as it also serves to determine the possibility of resection [7]. For the 10–15% of patients who are deemed to have local disease only by imaging, no further staging is required by current guidelines. In practice, most clinicians and patients expect a tissue diagnosis before proceeding to surgery. Among patients deemed resectable by current staging guidelines, only 70–85% of patients will actually be found to have a resectable tumor during the operation, as many patients are found to have metastatic disease or encasement of major vascular structures intraoperatively. This high frequency of aborted operations further emphasizes the need for better diagnostic and staging biomarkers to inform first-line therapy [7,8]. While newer molecular imaging and biomarker assays have demonstrated promise as a means of improved staging of patients, none have translated into clinical practice [9–11].

Treatment of pancreatic cancer

Surgery remains the mainstay of treatment for early disease, defined as AJCC stage I and II, patients. Advances in surgical therapy have decreased perioperative mortality, but, due to the lack of improvement in staging, the high recurrence rates have blunted any gains in patient survival secondary to improved perioperative care. Additionally, due to the morbidity associated with pancreatic surgery, many patients end up having complications that delay the administration of adjuvant chemotherapy [12]. While historically nonsurgical treatment of PC offered patients little benefit, recent breakthroughs have made the first major advances in patient survival in decades. The FOLFIRI-NOX regimen was found to almost double survival in patients with metastatic disease from 6.8 to 11.1 months [13]. Similarly, the nab-paclitaxel and gemcitabine (NG) regimen extended overall survival from 6.8 to 8.5 months [14]. Furthermore, the objective response rates for FOLFIRINOX and NG regimens were 31.6 and 23%, respectively, versus just 7–9% for gemcitabine alone. Currently, preoperative chemotherapy is not routinely recommended; however, this may change as chemotherapy options improve. The treatment of patients with borderline resectable tumors and those with unresectable, nonmetastatic tumors remains controversial. The addition of radiation therapy to chemotherapy has had mixed results, and further studies are needed [15]. As nonsurgical therapies for PC improve, the cost of unnecessary surgeries to patient's survival due to understaging increases significantly, underscoring the need for improved diagnostic and staging methods.

Current screening options for pancreatic cancer

There are currently no screening tests routinely used for PC, despite significant research and numerous trials. This is despite the existence of two known precursors of PC, high-grade pancreatic intraepithelial neoplasia and cystic pancreatic lesions [16]. Potential screening tools studied to date include endoscopy, cross-sectional imaging, serum biomarkers, nomograms and serial biopsies [17]. The location of the pancreas, and the difficulty involved in routine examination of it, combined with the relative rarity of PC, means that it is not amenable to endoscopic screening programs such as those used for other GI malignancies. The utility of imaging studies has been analyzed extensively, and EUS and MRI have consistently demonstrated the highest diagnostic sensitivity and accuracy [17]. Even for high-risk patients, such as those with a genetic syndrome or family history associated with PC, screening studies have not demonstrated a benefit [18]. For example, a 5-year prospective screening study of high-risk patients with annual EUS and MRI found only a single IPMN and no patients with invasive cancer, and similar studies have not demonstrated a survival benefit with screening [11, 18]. This is likely due to the low tumorigenic nature of PC precursor lesions.

Molecular biology of pancreatic cancer

The genetics of PC is defined by a small subset of driver mutations, which are found in the majority of PC cases [19]. These driver mutations, KRAS, p16, SMAD4 and TP53, are mutated in the majority of PCs. Specifically, KRAS mutations are almost ubiquitous, with studies showing nearly 100% of PC tumors have a KRAS mutation [20]. Despite the overall uniformity in the mutational landscape of PC, an inheritable genetic component is only

identified in 10% of cases of PC, and only 20% of these cases involve a known genetic syndrome [17]. This is likely an underestimate though, as a recent meta-analysis found that having a single relative with PC increases the risk of developing PC by 80%. While most of these are hereditary cancer syndromes such as Peutz–Jeghers syndrome, Lynch syndrome, FAP, FAMMM and the BRCA mutations, there are several PC-specific syndromes including cystic fibrosis and the hereditary pancreatitis syndromes [17]. Several modifiable risk factors are also associated with PC including chronic pancreatitis, smoking, obesity and diabetes. In fact, studies estimate that approximately 20% of PC can be associated with smoking [21].

Biomarkers in pancreatic cancer

There are currently over 2000 studies in the literature examining different serum biomarkers for PC [22]. The only US FDA-approved serum biomarker for PC is CA19–9. Unfortunately, it is not tumor specific, being elevated in many hepatobiliary cancers as well as in benign biliary obstruction [7]. Studies on the utility of CA19–9 as a screening test have never demonstrated efficacy, even in high-risk groups [23]. As an adjunct staging and diagnostic biomarker, studies have been mixed. While one study demonstrated the potential utility of CA19–9 as an adjunct diagnostic marker in patients with suspected PC, with a sensitivity and specificity of 70 and 87%, respectively, other studies have not found it to be as accurate [24]. Furthermore, the low specificity means that it will never function as a diagnostic alone. Both carcinoembryonic antigen (CEA) and CA125 have been studied for PC and have not proven useful; however, they are occasionally used by oncologists to track response to chemotherapy [23].

Screening biomarkers would revolutionize the management of PC by allowing more patients to undergo curative surgery, and recent evidence supports the possibility of a screening test in PC. Two studies applied phylogenetic techniques to largescale next-generation sequencing of PC [25,26]. They found that metastases tend to occur late, often 15 or more years after the development of the primary driver mutation, and 5 or more years from the development of an invasive tumor. Despite this long time course, no screening biomarkers are currently available for PC.

Biomarkers are currently used as adjuncts in the diagnosis of PC. While not validated, the CA19–9 assay is routinely obtained in patients without a biopsy-confirmed cancer as an adjunct diagnostic biomarker. Several genetic tests on indeterminate fine-needle aspirates are available; however, none have FDA approval or seen widespread adoption [5,27].

Biomarkers are not currently used in staging PC patients.

Biomarker development

For biomarkers to have clinical utility, they must meet well-defined statistical criteria, and be validated in a large number of patients with different disease to test for false-positive conditions. They must prove that the information they provide to patients and clinicians justifies their cost. Most importantly, they must demonstrate their ability to provide clinically useful information to patients and clinicians. A main factor in preventing the translation of preclinical biomarkers to clinical practice is the failure of research studies to follow established guidelines on the data elements necessary to evaluate a potential

biomarker [28]. Guidelines from the NCI-European Organization for Research and Treatment of Cancer (EORTC) First International Meeting on Cancer Diagnostics criteria, the Reporting Recommendations for Tumor Marker Prognostic Studies and the FDA are all excellent resources [28,29]. By meeting these standards of reporting and statistical analysis, the potential utility of a biomarker can be more accurately assessed by both clinicians and regulatory agencies, facilitating their adoption.

Circulating tumor cells

Thomas Ashworth first identified circulating tumor cells in 1869 during microscopic evaluation of the blood of a metastatic breast cancer patient [30]. Despite being discovered almost a century and a half ago, only recently has their routine identification been made possible. Fueled by these advances, there has been an explosion of research into circulating tumor cells over the past two decades. The first CTC platform to gain FDA approval was the CellSearch platform, which looks at CTC positivity as a prognostic biomarker in metastatic lung, breast and colon cancer. Despite the approval of the CellSearch platform, numerous issues regarding the detection, isolation, enumeration, meaning and clinical utility of CTCs remain to be answered.

Thought to originate from primary tumors or a metastatic site, CTCs have been shown to circulate in the peripheral blood of patients with all solid tumors but only rarely in healthy controls [31]. CTCs are heterogeneous, both between patients and among CTCs of a single patient, expressing markers of both epithelial and mesenchymal origin [32]. While studies have proven the viability of some CTCs through culture, many CTCs appear to be apoptotic, and their half-life varies considerably in blood, from hours to weeks [33,34]. Further functional studies have also demonstrated the metastatic inefficiency of CTCs, with studies suggesting that only 2.5% of CTCs can form micrometastases and only 0.01% can form macrometastases [35,36]. Additionally, the presence of CTCs does not reliably correlate with the presence of metastases, and the isolation of the subset of CTCs with metastatic potential remains an active area of research [37].

The rarity of CTCs, representing just a few cells among billions of blood cells in each milliliter of blood, is the primary difficulty that CTC detection methods must address [38]. Most platforms do so by first enriching the number of CTCs versus other background cells followed by capture or isolation of the CTCs based on unique physical or chemical properties (Figure 1). Once the CTCs are enriched and isolated, they must be distinguished from other mononuclear cells. Some of the properties currently exploited by CTC platforms for enrichment and detection include size, density, stability, viability, charge, cell surface markers, as well as protein, RNA and DNA signatures. A recent review found more than 40 unique technologies for CTC enrichment and detection, with a nearly continuous publication of newer methods [28]. These different techniques result in vastly different samples for analysis and differ considerably in terms of cost. While density gradient centrifugation and RBC lysis are fast and relatively inexpensive, their enrichment is low. Most platforms now use some form of cell surface marker-based enrichment. For most platforms, the cell surface marker is EpCAM, a cell adhesion glycoprotein universally expressed on epithelial cells. However, in many cancers, EpCAM expression is downregulated due to epithelial

mesenchymal transition, resulting in poor detection by EpCAM-based enrichment and detection methods. Increasingly, detection of CTCs using novel tumorspecific or mesenchymal markers has been reported, and these methods have discovered several new classes of CTCs not detected by older EpCAM-based platforms [39,40]. The various methods also result in vastly different numbers of detected CTCs. For example, the CellCollector *in vivo* CTC platform detected between 1 and 5 CTCs in 1.5 l of blood versus 12 and 3167 CTCs/ml of blood reported using the CTC-Chip [41,42].

Circulating tumor cell identification methods rely on many of the same properties used for enrichment and isolation. The two most common methods in use today are immunocytochemistry (ICC) and molecular techniques, especially RT-PCR. ICC uses immunofluorescence to differentiate CTCs from hematopoietic cells. For example, the most common 3-channel ICC definition of a CTC uses DAPI as a nuclear stain, cytokeratins (CK) as an epithelial marker and CD45 as a hematopoietic marker. Thus, a Nuclear⁺/CK⁺/CD45⁻ cell is defined as a CTC, whereas a Nuclear⁺/CK⁻/CD45⁺ cell is a WBC (Figure 2). The detection of Nuclear⁺/CK⁺/CD45⁺ cells by many platforms has been a source of error in many studies and may represent nonspecifically stained hematopoietic cells (macrophages or polymorphonuclear cells) or technical antibody processing errors [43]. Similarly, the presence of Nuclear⁺/CK⁺/CD45⁻ cells in some patients with benign disease may represent endothelial cells, tissue-associated inflammatory cells or true epithelial cells that are released in response to inflammation [44]. The other major method used today is molecular detection of tumor-associated transcripts. Newer molecular techniques have high sensitivity and are able to detect a single mutation among a background of thousands of WBCs. Unfortunately, while rare, studies have demonstrated that illegitimate transcription by WBCs can result in tumor-associated transcripts being found in the blood of normal patients, lowering the specificity of these assays [37]. Therefore, the only truly tumor-specific molecular biomarkers are those looking at gene fusion products or ubiquitous driver mutations peculiar to a particular cancer. As demonstrated below, there is considerable heterogeneity in the definition of a CTC, and the expression of a cancer-associated mRNA is considered by many to be the same as the visualization of an epithelial cell in the blood for defining a CTC. Studies comparing the techniques have generally found a higher sensitivity with RT-PCR-based techniques but a higher specificity with ICC-based ones [45]. Therefore, depending on the purpose of the study and the CTC platform available to them, researchers will adapt their definition of a CTC to meet their needs. For example, due to the excellent specificity of ICC-based methods, they are generally favored by studies looking at using CTCs as a diagnostic biomarker. The same logic applies when choosing cell surface markers and mRNA transcripts: The more tumor-specific ones, such as CEA and CA 19-9, have higher specificity whereas the more general epithelial ones, such as CK, have higher sensitivity.

Studies of CTCs in pancreatic cancer

Circulating tumor cells have been investigated as a biomarker for several indications in PC. While studies have looked at CTCs as a biomarker for the diagnosis, staging, prognosis and management of PC, studies to date have generally been small. Similar to CTC research in general, a large variety of CTC platforms have been used limiting the comparison of the

studies. Furthermore, the data collection and statistical analyses available vary widely between studies, making it difficult to collectively analyze the available data. Therefore, the studies will be discussed individually in the text of this review. Additionally, Tables 1 and 2 provide an overview of the studies that have analyzed CTCs for diagnosis and staging of PC.

RT-PCR-based studies

The earliest studies of CTCs in PC looked at CK-20 mRNA and CEA mRNA. One early study by Chausovsky *et al.* looked at a combination of density gradient enrichment followed by nested RT-PCR amplification of CK-20 mRNA in 28 consecutive PC patients [46]. They were able to amplify CK-20 in 22/28 (78.6%) patients. Soeth *et al.* used the same technique and found CTCs in 52/154 (33.8%) patient's blood samples, with a significant difference between early- and late-stage patients ($p = 0.005$) [47]. After 70 months, they found a significant correlation between CTC positivity preoperatively and overall survival. In a similar study by Hoffmann *et al.*, enrichment by both RBC lysis and density gradient centrifugation was followed by a combination of nested-PCR and quantitative fluorogenic RT-PCR of CK-19 mRNA preoperative, intraoperatively and postoperatively in 37 patients undergoing pancreatic surgery for PC [48]. They found increased CK-19 expression in 24/37 (64.9%) patient's blood samples as well as 11/37 (29.7%) peritoneal lavage samples. However, they did not find a correlation with survival. Overall, RT-PCR studies of cytokeratin alone have only shown a small correlation with survival and have not been looked at for other indications.

Another early study by Uchikura *et al.* looked at CEA mRNA in 67 patients undergoing pancreatic surgery [49]. They looked at preoperative and intraoperative CEA mRNA from peripheral, central and portal venous blood intraoperatively via nested RT-PCR with no enrichment technique. While they did not detect any CEA mRNA in patients preoperatively, they found it in the blood of 32/67 (47.8%) of patients intraoperatively. They found similar rates of CTC detection between portal and peripheral blood. At 2 years follow-up, the rate of recurrence was significantly higher for patients positive for CEA mRNA (37.5%) versus those who were negative (11.4%) ($p = 0.01$). In another study looking at CEA mRNA, Mataki *et al.* used density gradient enrichment followed by nested RT-PCR for 53 patients with PC, 20 of whom had underwent surgery [50]. They measured CEA mRNA expression during follow-up and found that 6/20 (30%) of patients were positive. They found a significant association between CTC positivity and recurrence, with 5/6 positive patients recurring versus just 2/12 CTC negative patients ($p < 0.0001$). There appears to be some clinical utility for CEA mRNA as a CTC marker in PC, similar to results published on other GI cancers.

Recently, multimarker studies have shown better results than single-marker studies alone. Zhou *et al.* used immunomagnetic bead enrichment followed by RT-PCR of human Telomerase reverse transcriptase (hTERT), C-MET, CK-20 and CEA in 25 patients with newly diagnosed PC [51]. Looking at each marker individually, the positive expression rates of C-MET, h-TERT, CK20 and CEA were 80% (20/25), 100% (25/25), 84% (21/25) and 80% (20/25), respectively. It is important to note that among patients with benign pancreatic diseases, 1/15 (6.77%) were positive for CK-20 mRNA. Interestingly, hTERT was found in

all 25 patients with PC, none of the control group, and did not correlate with stage. Further studies would be needed to determine its utility as a screening or diagnostic test. In another multimarker gene panel study, de Albuquerque looked at immunomagnetic enrichment based on MUC1 and EpCAM antibodies followed by RT-qPCR in 34 PC patients [52]. Looking at the combination of *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5* expression, CTCs were found in 16/34 (47.1%) of patients. CTC positivity, as measured by at least one positive transcript, was associated with shorter progression-free survival.

Immunocytochemistry-based studies

There have been several publications looking at the utility of the CellSearch platform in PC. Overall, CTC counts have been low. In fact, in the original publication of the CellSearch platform, the authors comment that CTC counts in PC are the lowest among solid tumors [31]. Allard *et al.* looked at a total of 964 patients with metastatic carcinomas, including 16 patients with metastatic PC. Of the 16 patients, 6/16 (37.5%) had any CTCs, and only one patient had 5 CTCs. A study by Kurihara *et al.* looked at CTC positivity and survival in a group of 26 patients with PC [53]. They found 1 CTC in 11/26 (42%) patients and discovered an association between CTC positivity and shorter overall survival. Bidard *et al.* looked at CTC enumeration during the LAP07 trial of 79 patients with locally advanced PC [54]. They were only able to detect CTCs in 5% of patients before treatment and 9% of patients after 2 months of therapy. CTC positivity was associated with poor tumor differentiation and shorter overall survival. Due to the low enumeration found with the CellSearch system in PC, several groups have explored other methods of detecting CTCs. Methods including size-based isolation, microfluidics and flow cytometry have been used with variable degrees of success.

In one of the first studies using immunocytochemistry, Z'graggen *et al.* used density gradient enrichment followed by ICC in 105 preoperative patients with PC [55]. They found CTCs in 3/32 (9%) of resectable patients and 24/73 (33%) of nonoperative ones. Furthermore, CTC positivity was associated with stage, resectability and disease progression.

Two iterations of the 'CTC-Chip' platform have been studied in PC [56,57]. The platform uses RBC lysis followed by a microfluidic chip covered with microposts that capture CTCs using anti-EpCAM antibodies. Captured cells are then imaged using standard 3-channel ICC for identification, similar to the CellSearch system. Nagrath *et al.* looked at 15 metastatic PC patients and found >5 CTCs in all 15 (100%) patients. Yu *et al.* used the 'HB CTC-Chip' which uses a herringbone modification to increase CTC to chip interactions. They were able to find 3 CTCs in 11/15 (73%) patients. They furthermore isolated the CTCs from the chip and performed RNA sequencing to generate a digital gene expression profile that revealed aberrations in *WNT* signaling in 5/11 patients.

Two studies have used the microfluidic geometrically enhanced mixing (GEM) chip after RBC lysis in PC patients [58,59]. Sheng *et al.* used the platform on an 18-patient sample. Using standard ICC, they were able to detect CTCs in 17/18 (94.4%) patients with metastatic PC. Furthermore, their device allowed them to culture the CTCs they isolated for further analysis. Rhim *et al.* used the same platform on 21 patients with cystic lesions of the pancreas and no diagnosis of cancer (Sendai criteria negative) as well as 11 with biopsy-

confirmed PC. They were able to capture 3 CTCs in 7/21 (33.3%) patients with cystic lesions of the pancreas and 8/11 (73%) of patients with biopsy-proven cancer. Furthermore, 29% of CTCs stained positive for PDX-1, a pancreas-specific transcription factor.

Zhang *et al.* used immunomagnetic CD-45 depletion based enrichment followed by 3-channel ICC with the addition of CEP8 probe FISH to identify CTCs in 22 preoperative patients with PC [60]. They were able to detect CTCs in 16/22 (72.7%) patients preoperatively, and, based on a cutoff of 2 CTCs/3.75 mL, calculated a sensitivity and specificity of 68.2% and 94.9%, respectively. Interestingly, they found a decrease in the CTC count 3 days after surgery but an increase at 10 days after surgery. Additionally, CTC enumeration was associated with worse survival.

Kamade *et al.* used a modular microfluidic device for both CTC enrichment and isolation followed by detection using automated ICC [61]. In a study of 12 patients with PC, five resectable and seven unresectable, they were able to detect CTCs in 100% of patients. They found a significant difference in the number of CTCs between resectable and unresectable patients. Of note, they also found CTCs in some healthy control patients.

Another means of bypassing the low CTC count with the CellSearch system is to capture CTCs based on properties other than cell surface expression. The most common technique for doing so is capturing CTCs based on their relatively large size versus WBCs. Several studies using size-based methods in PC have been performed.

Khoja *et al.* used the ISET platform, which combines size-based filtration with a 5 marker ICC detection panel, and compared the results to that of CellSearch in a study of 54 non-operative PC patients [62]. CellSearch detected 1 CTC in 40% of patients versus 93% for ISET. Additionally, the ISET system captured significantly more CTCs (median CTCs/7.5 ml, 9 (range 0–240) versus 0 (range 0–144) for ISET and Cell-Search, respectively). Furthermore, they analyzed the expression of EpCAM, CK, vimentin and E-cadherin on CTCs captured by the ISET system and found significant heterogeneity in the expression of these epithelial and mesenchymal markers. They concluded that CellSearch likely fails to capture these mesenchymal type CTCs.

Two studies using the ScreenCell size-based platform have been performed in PC [63,64]. The first study compared the results of ScreenCell CTC detection to that of EUS-FNA as a diagnostic test. Of the 40 patients, 27 (67.5%) were successfully diagnosed by EUS-FNA findings. ScreenCell detected CTCs in 15/40 (37.5%) patients, with a sensitivity and specificity of 55.5 and 100%, respectively. Overall, for the diagnosis of adenocarcinoma, the sensitivity, specificity and accuracy of EUS-FNA was 77.8% (CI 95% [65.4%; 90%]), 100% (CI 95% [75%; 100%]) and 85%, respectively. For CTCs, the diagnostic sensitivity, specificity and accuracy was 55.5% (CI 95% [40.1%; 70.9%]), 100% (CI 95% [75%; 100%]) and 70%, respectively. Similarly, Kulemann *et al.* found CTCs in 3/4 (75%) patients with local disease and 5/7 (71.4%) patients with advanced disease using the combination of ScreenCell cytology followed by KRAS mutation detection by RT-PCR.

A study using the MetaCell system, which combines size-based and cell culture-based enrichment followed by ICC, found CTCs in 16/24 (66.7%) patients in the preoperative

setting [65]. Interestingly, CTC positivity was not associated with disease stage, tumor size or lymph node involvement.

Ren *et al.* looked at how CTC counts are affected by chemotherapy by looking at 41 newly diagnosed patients with advanced PC before and 7 days after initiation of a 5-FU chemotherapy regimen [66]. They used a combination of RBC lysis and CD45 depletion for enrichment followed by ICC with CD45, CK, CA19–9 and TUNEL apoptosis staining for identification. They found 2 CTCs in 33/41 (80.5%) patients before therapy and only 12/41 (29.3%) of patients 1 week after the initiation of therapy. Furthermore, they detected significantly higher numbers of apoptotic CTCs by TUNEL staining after initiation of chemotherapy. The clinical significance of these findings was not investigated.

In our own study, we used the Nanovelcro CTC chip platform which uses a combination of density gradient centrifugation enrichment and microfluidic EpCAM-based capture for CTC isolation [67]. CTCs are identified by a tumor-specific ICC method that utilizes CEA and nuclear morphology to increase the specificity of CTC identification. We performed a prospective study in 71 consecutive presurgical patients and detected 1 CTC in 36/48 (75%) of patients with adenocarcinoma and 1/23 (4.6%) patients with non-adenocarcinoma pathology. The specificity of CTCs for the diagnosis of PC was 95.7%, sensitivity 75%, PPV 97.3% and NPV 64.7%. The area under the receiver operating characteristic curve (AUROC) for CTCs in diagnosing PC was 0.860 (95% CI = 0.774 – 0.946, $p < 0.0001$), indicating the overall ability of CTC detection to discriminate PC from non-adenocarcinoma. Additionally, increasing CTC number correlated with clinical stage and PC patients with 2 CTCs per 2 mLs of blood were roughly 23 times more likely to harbor systemic disease.

CTCs as a liquid biopsy: single cell sequencing

The most useful potential application of CTCs in PC would be as a ‘liquid biopsy.’ If realized, it would allow clinicians access to tumor tissue in a real-time, repeatable, safe and cost-effective manner. While true for all cancers, such a liquid biopsy holds particular promise in PC due to the difficulty and cost involved in obtaining tumor tissue by current techniques. As discussed above, the combination of anatomic location and the relative paucity of tumor cells within a desmoplastic stromal background makes PC biopsies difficult, often requiring multiple endoscopies to obtain useful tumor tissue [68].

Furthermore, the separation of the few tumor cells from the dense stromal background for further molecular characterization is difficult and not routinely performed [5]. Therefore, with regard to molecular analysis, CTCs may actually perform better than EUS-FNA due to the enrichment of tumor cells during CTC isolation. Furthermore, the difficulties of performing molecular analysis on the low number of tumor cells obtained by CTC assays have mostly been solved by recent developments in the field of single cell sequencing [69].

The development of single-cell sequencing has realized the goal of having CTCs function as a liquid biopsy due to the increased specificity that molecular analysis can provide. A single cell contains approximately 6 pg of DNA while the input requirements for most next-generation sequencing platforms is on the order of nanograms to micrograms [70]. Techniques for bridging the gap between these amounts are now available allowing for the

whole-genome amplification of single cells to levels required for next-generation sequencing. A number of studies have demonstrated that not only can CTCs act as a liquid biopsy, providing diagnostic accuracy comparable to that of an FNA, but they also have the potential to offer additional biologically relevant information about tumor heterogeneity and metastatic potential [69,71–73]. Furthermore, in some cases, CTCs may actually provide more accurate molecular information than a traditional biopsy due to the enrichment of an important subset of tumor cells against stromal contaminants and less aggressive tumor subclones. Both single-cell RNA sequencing and DNA sequencing have advanced rapidly over the past 4 years, and targeted cancer gene panel sequencing is now routinely used in clinical practice [74,75]. Larger exome and genome sequencing studies that were once prohibitively expensive for even small numbers of patients, let alone the numbers required for a clinical trial, are now possible due to the exponential decrease in the cost of sequencing. At the same time, projects such as The Cancer Genome Atlas and the Catalog of Somatic Mutations in Cancer database provide researchers with the information they need to parse the large datasets that result from next-generation sequencing. Combined, these developments have introduced a vast array of new molecular tests that can increase the diagnostic accuracy of a CTC assay.

Two recent studies offer insight into how single-cell CTC sequencing can be applied to PC [57]. The first study applied single-cell RNA sequencing to a mouse model of CTCs and found that aberrations in the *WNT* pathway might potentially be involved in the metastatic cascade. They then performed single-cell sequencing of CTCs from 15 patients with metastatic PC and found that the majority of them had similar abnormalities in the *WNT* pathway. While only a pilot study, it is representative of both the research and clinical potential of CTCs when combined with single-cell sequencing. In our own research, we have developed a driver mutation-based model for confirming PC diagnosis from CTCs [76]. Because *KRAS* mutations are present in almost all PC tumors, sequencing of a single point mutation can confirm the tumor origin, and thus diagnosis, of PC for the majority of patients. Furthermore, molecular confirmation of tumor origin has allowed us to validate our ICC definition of a CTC, increasing the specificity of our assay. By combining single-cell sequencing and molecular analysis with CTC identification, the full potential of CTCs as a biomarker can be realized.

Performance of CTCs as a biomarker in pancreatic cancer

Overall, the performance of CTCs as a biomarker in PC is similar to the results from studies in other solid tumors. The overall rarity of the cells, and the large number of patients who do not appear to have CTCs despite advanced stage, makes the overall sensitivity of CTCs low. However, the specificity of CTCs is excellent with few studies reporting false positives. Unfortunately, studies to date have used a variety of different platforms and CTC definitions, making comparisons between studies difficult. Thus, larger studies using a single method for CTC capture and identification are needed before the clinical utility of CTCs can be adequately assessed.

No studies to date have looked at CTCs as a screening bio-marker in PC; furthermore, the high cost and low sensitivity of the assay makes it unlikely any will in the future. The only

CTC biomarker that would currently be a possibility is *hTERT* RT-PCR based on the high sensitivity found in a single small study [51]. Preclinical studies have demonstrated that *hTERT* mRNA is often found in the extracellular media around PC cell lines in culture [77]. However, it is also highly expressed by almost all pluripotent cells, lowering its specificity as a biomarker.

Studies looking at CTCs as a diagnostic biomarker have found varied, but promising, results (Table 1). While the sensitivity of CTCs in diagnosing PC is lower than that of EUS-FNA, the specificity, usually 100% and never lower than 95%, is equal to that of a biopsy. The high specificity of the test means that a positive result can be viewed as equivalent to a positive EUS-FNA biopsy, currently the gold standard for confirming the diagnosis of PC. Furthermore, as single-cell molecular techniques improve, CTC enumeration can be combined with molecular analysis to increase the certainty of diagnosis. Given the cost and safety profile of a CTC test versus that of EUS-FNA, CTCs could potentially serve as an initial diagnostic test before EUS-FNA [63]. In the US, the average cost of obtaining a diagnosis of PC by EUS-FNA in 2001 was US\$15,938 [78]. For CTCs, the Medicare reimbursement for the CellSearch CTC assay was US\$371.99 [79]. More importantly, the CTC assay involves virtually no risk and minimal discomfort to the patient, versus the small but considerable risk and significant discomfort of sedation and endoscopy. Thus, despite having a lower sensitivity, if larger studies confirm the high specificity of CTCs, they are likely to enter clinical use as either an initial or adjunct diagnostic test.

The majority of PC patients present with metastatic disease, and the sensitivity of CTCs in these patients is significantly higher, up to 100% in several of the studies reviewed. In the era of precision medicine, in which payment for targeted therapies is often linked to predictive mutational biomarkers requiring tumor tissue, there will be a need for repeat biopsies in metastatic patients. Thus, CTCs may fulfill an important role for metastatic PC patients, offering a safe, repeatable and cost-effective means of providing required 'tumor tissue' for approval of expensive targeted therapies and enrollment in clinical trials.

One other area where CTCs could prove useful is as an adjunct marker in the diagnosis of cancer in patients with pancreatic cystic lesions. Currently, up to 25% of IPMNs that are found to be negative on biopsy contain foci of high-grade dysplasia or invasive carcinoma upon resection [59]. Hopefully, future studies similar to Rhim *et al.* will be able to follow-up with patients following surgery to determine the ability of CTCs to predict invasive cancer within the cystic lesions. However, the use of molecular markers such as *KRAS* may not prove useful in such cases, as studies have shown the presence of these driver mutations in the precursor lesions as well [5]. Overall, CTC tests have a significantly lower sensitivity than EUS-FNA; however, they are cheaper, less invasive and have almost no associated risk. CTCs are therefore an attractive first-line diagnostic test in patients with suspicion of PC based on clinical or imaging findings.

A total of six studies included an analysis of CTCs as a staging biomarker with mixed results (Table 2). Three studies found no correlation between CTCs and stage, while the other three found varying degrees of correlation. However, differences in how staging was performed between the studies limit the applicability. For the three studies that did not find a

correlation, only one confirmed staging by pathology as opposed to clinical staging alone. In contrast, staging was confirmed pathologically in all three studies that did find significance. Therefore, it is possible that those studies that did not demonstrate significance may not have accurate information on the actual stage of the patients. This is likely true given that current guidelines under-stage the majority of patients. The three studies that found a correlation were all conducted in surgical patients, and preoperative CTC positivity was found to correlate with postoperative staging. Furthermore, the results of these staging studies must be considered alongside the large number of studies that have shown an association between CTCs and recurrence. Together, these results make a case for the potential utility of CTCs as a biomarker in the preoperative staging of patients. Larger studies are needed to determine if CTCs have the potential to assist surgeons in balancing the risks and benefits of surgery for PC patients.

Expert commentary

There is a pressing need for new biomarkers in the diagnosis and staging of PC. The advances made in survival for all major solid tumors have yet to be realized in PC, and it remains the only major cancer type with 1-year survival for early-stage tumors below 50% [80]. The lack of progress can be directly attributed to our inability to diagnose patients early, select the appropriate therapy and detect disease progression or resistance to chemotherapy.

Initial studies on CTCs as a biomarker in PC are promising; however, important questions need to be addressed to properly assess the clinical utility of CTCs. First, future studies must follow established guidelines for reporting clinically relevant data points [28]. Currently available studies are underpowered to address diagnostic and staging questions to the level necessary to validate a biomarker for clinical use. Furthermore, the study designs themselves, from lack of follow-up to inadequate blinding, do not allow these questions to be adequately answered. For CTC research specifically, the large variability in CTC platforms makes comparisons between studies difficult. Furthermore, rigorous validation of 'repeatability' is warranted prior to widespread adoption [81]. Following these guidelines will put future studies on the right path to fulfilling the significant analytical and pre-analytical validation required for FDA approval.

Despite these hurdles, PC represents the ideal cancer for developing CTC biomarkers for a number of reasons. Tumor tissue is costly and difficult to obtain due to the combination of anatomic location and the highly stromal background with a relative paucity of tumor cells. The *KRAS* driver mutation is present in over 90% of tumor cells, allowing for easy molecular confirmation of tumor origin and validation of CTC definitions among the different technologies. Perhaps most importantly, existing biomarkers provide little to no useful information, and even the gold standard diagnostic test has relatively low accuracy, making even modest results potentially useful in the treatment of PC patients.

Studies to date on CTCs in PC have demonstrated the potential for clinical applications for two main measures. The first is the potential for CTCs to serve as an initial diagnostic test prior to EUS-FNA. The low cost, high specificity and lack of risk of the assay make it an

ideal initial diagnostic test when compared to the risk and cost of EUS-FNA. While further studies are needed to confirm the tumor origin of identified CTCs on each CTC capture technology platform, initial studies look promising. However, larger studies using established biomarker validation protocols are required before any further conclusions can be drawn. Second, while the application of single-cell sequencing to CTCs for both research and clinical indications is in its infancy, the few small studies available to date demonstrate the potential for CTCs to fulfill the promise of a liquid biopsy in PC.

Five-year view

The pressing need for better biomarkers in PC is likely to continue to drive further research of CTCs in PC. While the studies highlighted above show tremendous potential, they are underpowered, and lack the necessary study parameters, to meet the stringent validation criteria required by the FDA to assess the utility of CTCs in the diagnosis and staging of PC. The encouraging results of these studies have led to considerable interest, and adequately powered studies are likely to occur in the near future. It is very likely that within 5 years CTCs could see use as an initial diagnostic test prior to EUS-FNA. However, due to the low sensitivity of the FDA-approved CellSearch system in PC, these new technologies highlighted above will need to follow the stringent guidelines for biomarker approval before they can enter clinical practice.

The other major advance is likely to come from the application of single-cell isolation and sequencing methods to CTC research. While still in their infancy, these methods are already drastically changing the goals of CTC technologies. In addition to the CTC enumeration data provided by first-generation technologies, second-generation CTC isolation platforms allow for the application of recent advances in molecular biology to augment the performance of CTCs as a biomarker. From single-cell clonal cultures to functional studies to molecular signatures, future studies are likely to incorporate additional information to increase the sensitivity, specificity and clinical relevance of CTCs in PC. Finally, as the era of personalized medicine and targeted therapy is upon us, the need for clinicians to evaluate tumors on a real-time basis becomes more important. CTCs appear to contain many of the necessary elements required to be a key player in the realization of precision, personalized medicine in the care of pancreas cancer patients.

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- of interest
- of considerable interest

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Key issues

- Pancreatic cancer (PC) is the fourth leading cause of cancer-related mortality in the USA, and the only major cancer with a 5-year survival still below 10%.
- One important cause of the dismal prognosis in PC is the lack of effective biomarkers for diagnosis and staging.
- Circulating tumor cells (CTCs) are an emerging biomarker with US FDA-approved uses in several solid tumors including breast, colon and prostate.
- While first-generation CTC technologies suffered from low sensitivity in detecting CTCs in PC, emerging CTC technologies have performed significantly better.
- Initial studies of CTCs as a diagnostic biomarker in PC are promising and demonstrate the potential of CTCs to serve as an initial diagnostic test due to their excellent safety profile and relatively low cost.
- New CTC technologies, combined with advances in single-cell analysis, allow CTCs to function as a 'liquid biopsy,' providing clinicians with safe, repeatable access to patient's tumor tissue.
- The potential of a liquid biopsy is an area of active research, and it holds enormous potential in changing the care and management of patients with PC, especially in the era of personalized medicine.

Figure 1. Methods for the enrichment and isolation of CTCs

Peripheral blood draw: Peripheral blood is obtained from patients, usually 2–20 ml. **RBC lysis:** involves incubation of whole blood with a mixture of ammonium chloride, potassium carbonate and EDTA resulting in lysis of RBCs but not WBCs or CTCs. **Density-based:** density gradient centrifugation uses a density medium to separate mononuclear cells from RBCs and granulocytes based on cell density. **Inertial focusing:** Using a spiral device, cells are separated by size based on different flow patterns due to inertial microfluidics. **Immunomagnetics:** Antibodies are bound to magnetic beads allowing for the capture of CTCs as well as their subsequent manipulation. **Microfluidics:** Antibody-coated nanofabricated microfluidic channels use various methods to ensure cell-antibody interactions, allowing for the capture and manipulation of CTCs. **Size-based:** CTCs are generally larger than RBCs and WBCs and can be trapped by filtration on a micropore membrane.

CTC: Circulating tumor cell; EDTA: Ethylenediaminetetraacetic acid; RBC: Red blood cell; WBC: White blood cell.

Figure 2. CTC identification and enumeration methods

(A) ICC: Representative image of immunocytochemical staining obtained from the Nanovelcro platform from a patient with pancreatic cancer. CTCs are distinguished from other mononuclear cells based on differential immunostaining and cytologic characteristics. The blue nuclear stain (DAPI) identifies all nucleated cells. The epithelial marker cytokeratin is identified by green fluorescence (AlexaFluor 488) on CTCs while the hematopoietic marker CD45 is identified by red fluorescence (AlexaFluor 555) on WBCs.

(B) RT-PCR: The figure shows a representation of a readout from a real-time quantitative RT-PCR study. The PCR reaction is specific for a CTC associated mRNA transcript (e.g., CEA or hTERT). The graph plots the PCR cycle number versus the normalized relative fluorescent signal emitted at a given cycle count. In these types of studies, thresholds are calculated based on the baseline variance, and CTC positivity is defined as fluorescence greater than that threshold at a predetermined cycle count. If the fluorescent signal is greater than the calculated threshold at a cycle count lower than the cutoff, then the sample is positive for a CTC-associated transcript.

CK: Cytokeratin; CTC: Circulating tumor cell; DAPI: 4',6-diamidino-2-phenylindole; ICC: Immunocytochemistry; Nuc: Nuclear stain; RT-PCR: Reverse transcription polymerase chain reaction; WBC: White blood cell.

Table 1

Studies of the utility of CTCs as a diagnostic biomarker for pancreatic cancer.

Study (year)	Pts (n)	Patient type	CTC platform	CTC definition	Findings	Ref.
Iwanicki-Caron <i>et al.</i> (2013)	40	Prediagnosis	Size filter; 'ScreenCell'	Cytology: cell size and cytopathologic criteria	Compared CTCs to EUS-FNA in diagnosing PC. For the diagnosis of adenocarcinoma, sens., spec. and acc. of EUS-FNA was 77.8, 100 and 85%, respectively. For CTCs, the diagnostic sens., spec., and acc. was 55.5, 100 and 70%, respectively	[63]
Rhim (2014)	31	Prediagnosis	Microfluidic; 'GEM' Chip	ICC; DAPI, CD45, CK, and PDX-1	CTCs found in 7/21 (33%) pts with pancreatic cystic lesions. CTCs found in 8/11 (73%) pts with PC and 0/19 (0%) healthy controls	[59]
Ankeny <i>et al.</i> (2014)	71	Prediagnosis	Microfluidic; 'Nanovelcro'	ICC; DAPI, CD45, CK and CEA; nuclear morphology	CTCs detected in 39/52 (75%) pts at diagnosis. The spec. of CTCs for the diagnosis of PC was 95.7%, sens. 75%, PPV 97.3%, NPV 64.7% and AUC = 0.860 ($p < 0.0001$)	[67]
Zhang <i>et al.</i> (2015)	32	Preoperative	Immunomagnetic	ICC and FISH: DAPI, CD45 and CK or CEP8 > 2	CTCs detected in 16/22 (72.7%) pts in the derivation cohort and 7/11 (63.6%) pts in the validation cohort. For the diagnosis of PC, the sens. and spec. of CTCs was 68.18 and 94.9% (AUC = 0.8584), respectively, in the derivation cohort. In the validation cohort, sens. and spec. was 63.6 and 94.4% (AUC = 0.84), respectively. CTCs detected in 1/8 (12.5%) pts with benign disease	[60]
Kamande <i>et al.</i> (2013)	12	All stages	Microfluidic	ICC; DAPI, CD45 and CK	CTCs detected in 100% of pts with both resectable and unresectable PC	[61]
Khoja <i>et al.</i> (2012)	54	Stage III or IV	Size-based; 'ISET' vs. CellSearch	RT-PCR: EpCAM, CK, vimentin, and E-cadherin	CellSearch detected 1 CTC in 40% of pts versus 93% for ISET. ISET found CTCs in higher numbers. ISET isolated additional mesenchymal-type CTCs not captured by CellSearch	[62]
Zhou <i>et al.</i> (2011)	25	All stages	Immunomagnetic	RT-PCR: cMET, hTERT, CK20, and CEA	The sens. for detection of CTCs using C-MET, h-TERT, CK20, and CEA was 80 (20/25), 100 (25/25), 84 (21/25) and 80% (20/25), respectively. CK-20 CTCs were found in 1/25 (6.77%) pts with benign disease. The 4-gene CTC assay had a diagnostic sens. and spec. for PC of 100 and 93.3%, respectively	[51]
Hoffmann <i>et al.</i> (2007)	37	Preoperative	RBC Lysis; Centrifugation	RT-PCR: CK-19 mRNA	CTCs found in 24/37 (64%) preoperative pts. CTCs had a sensitivity similar to CA 19-9 in diagnosing PC	[48]

Study (year)	Pts (n)	Patient type	CTC platform	CTC definition	Findings	Ref.
De Albuquerque <i>et al.</i> (2012)	34	Stage III or IV	Immunomagnetic	RT-PCR: KRT19, MUC1, EpCAM, CEACAM5 and BIRC5	The 5-gene panel has a sensitivity and specificity of 47.1 and 100%, respectively, for the diagnosis of PC in pts with locally advanced or metastatic disease prior to initiation of systemic therapy	[52]

acc.: Accuracy; AUC: Area under the curve; CEA: Carcinoembryonic antigen; CK: Cytokeratin; CTC: Circulating tumor cell; DAPI: 4',6-diamidino-2-phenylindole; EUS-FNA: Endoscopic ultrasound-guided fine-needle aspiration; FISH: Fluorescent *in situ* hybridization; NPV: Negative predictive value; PC: Pancreatic cancer; PPV: positive predictive value; Pts: Patients; n: Number; RT-PCR: Reverse transcription polymerase chain reaction; sens.: Sensitivity; spec.: Specificity.

Table 2

Studies of the utility of CTCs as a staging biomarker for pancreatic cancer.

Study (year)	Pts (n)	Patient type	Platform	CTC definition	Findings	Ref.
Iwanicki-Caron <i>et al.</i> (2013)	40	Pre-diagnosis	Size filter; Screencell	Cytology: cell size and cytopathologic criteria	CTC status did not correlate with metastatic status, lymph node involvement, vascular invasion, size of tumor and CA19-9 serum level	[63]
Rhim <i>et al.</i> (2014)	31	Pre-diagnosis	Microfluidic; 'GEM' Chip	ICC; DAPI, CD45, CK, and PDX-1	CTC status did not correlate with tumor/cyst size, stage, or serum CA19-19 or CEA levels	[59]
Ankeny <i>et al.</i> (2014)	48	Preoperative	Microfluidic; 'Nanovelcro'	ICC for DAPI, CD45, CK, and CEA	CTC enumeration correlated with clinical stage. PC pts with 2 CTCs/2 mL VB were roughly 23 times more likely to harbor systemic disease. CTCs outperformed CA 19-9 in discriminating local/regional from systemic disease	[67]
Zhou <i>et al.</i> (2011)	25	All stages	Immunomagnetic	RT-PCR: C-MET, h-TERT, CK20, and CEA	The expression of C-MET, CK20 and CEA closely correlated with the tumor stage ($p < 0.05$). Positive correlation was also observed between CK20 expression and lymph-node metastasis ($p < 0.05$)	[51]
Soeth <i>et al.</i> (2005)	154	Preoperative	Centrifugation	RT-PCR: CK-20 mRNA	CTCs found in 52/154 (33.8%) preoperative pts. There was a significant difference in the detection rate between stage I–III and stage IV pts ($p = 0.005$)	[47]
Bobek <i>et al.</i> (2014)	24	Preoperative	Size-based; cell culture-based	ICC; DAPI/CK/CEA/Vimentin	CTCs found in 16/24 (66.7%) preoperative pts deemed to have resectable tumors by current staging criteria. CTCs did not predict resectability or presence of metastases prior to surgery	[64]
Z'raggen <i>et al.</i> (2001)	105	All stages	Centrifugation	ICC; CK, AE1/AE3, glycoproteins	CTCs found in 3/32 (9%) of pts with resectable PC and 24/73 (33%) of pts with unresectable PC. The specificity for cancer was 96%. The prevalence of CTCs increased with tumor stage ($p = 0.04$) and decreased with resectability ($p = 0.02$)	[55]

CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; CK: Cytokeratin; CTC: Circulating tumor cell; DAPI: 4',6-diamidino-2-phenylindole; PC: Pancreatic cancer; Pts: Patients; n: Number; RT-PCR: Reverse transcription polymerase chain reaction.