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Reviews on Current Liquid Biopsy for Detection and Management of Pancreatic Cancers

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Abstract: Pancreatic cancer is the fourth leading cause of cancer death in the United States. Pancreatic cancer presents dismal clinical outcomes in patients, and the incidence of pancreatic cancer has continuously increased to likely become the second most common cause of cancer-related deaths by as early as 2030. One of main reasons for the high mortality rate of pancreatic cancer is the lack of tools for early-stage detection. Current practice in detecting and monitoring therapeutic response in pancreatic cancer relies on imaging analysis and invasive endoscopic examination. Liquid biopsy–based analysis of genetic alterations in biofluids has become a fundamental component in the diagnosis and management of cancers. There is an urgent need for scientific and technological advancement to detect pancreatic cancer early and to develop effective therapies. The development of a highly sensitive and specific liquid biopsy tool will require extensive understanding on the characteristics of circulating tumor DNA in biofluids. Here, we have reviewed the current status of liquid biopsy in detecting and monitoring pancreatic cancers and our understanding of circulating tumor DNA that should be considered for the development of a liquid biopsy tool, which will greatly aid in the diagnosis and healthcare of people at risk.

Key Words: liquid biopsy, early detection, pancreatic cancer, ctDNA, oncogenic mutations

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most devastating cancers with high mortality and poor clinical outcomes.[1] Because of the relative rarity of pancreatic cancer, population-based screening is currently impractical. However, screening of high-risk cohorts may have a role in early detection. Despite considerable advances in imaging, diagnosis of pancreatic cancer has not improved in past decades primarily because of the fact that PDAC patients are asymptomatic in the earlier treatable stages.[2] One of the main reasons for the high mortality rate of pancreatic cancer is the lack of sensitive and specific tools to detect it in early stages. Most of the patients are diagnosed in advanced tumor stages. The overall 5-year survival rate of PDAC is approximately 9%, and the 5-year survival after resection for cancer and chemotherapy has been reported to range between 25% and 35%. Surgical resection is the only curative treatment option, but only 15% of patients present with a resectable tumor at the time of diagnosis.[3] Furthermore, the reason for the focus on early diagnosis is the fact that in patients with localized disease with a tumor size of less than 20 mm and without lymph node involvement there is a 5-year survival rate of 30% to 60%, which further improves to greater than 75% for lesions that are less than 10 mm.[4] Because PDAC is prone to metastasize even when the tumor is very small, it is imperative to diagnose the disease as early as possible.[4] Ideally, it is imperative to diagnose precancerous states such as the pancreatic intraepithelial neoplasia (PanIN) 3 lesions and mucinous cysts with high-grade dysplasia. However, a lack of imaging and tests, which can accurately identify these precancerous states, constitutes a serious problem. Thus, the identification, validation, and application of methods for earlier diagnosis will provide the opportunity to improve survival of patients with PDAC.

Currently, there are several challenges in the early detection of PDAC. Computed tomography (CT) is not sufficiently sensitive to detect pancreatic tumor lesions in asymptomatic patients, and an early detection of PDAC will require screening asymptomatic subjects from high-risk groups with invasive tests such as endoscopic ultrasonography (EUS). In addition, EUS imaging has a decreased sensitivity for finding masses in the setting of chronic pancreatitis. However, EUS with fine-needle aspiration (EUS-FNA) biopsy has a sensitivity of 85% to 92% and a specificity of 96% to 98% for the diagnosis of pancreatic cancer.[5] Therefore, EUS-FNA is the only recommended method of obtaining a biopsy in patients with a resectable cancer. It has been shown to be more sensitive than CT for detection of pancreatic tumors (sensitivity, 85%).[6] Also, because it is an invasive procedure requiring sedation, it may not be adequate for all patients.

The best treatment outcomes for PDAC are noted in patients with early-stage and small pancreatic cancers.[7] Therefore, there is an urgent need for identifying biological clues associated with earlier-stage PDAC to facilitate early diagnosis. Credible screening tests for genetic abnormalities associated with PDAC in high-risk

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populations, such as patients with high-risk genetics, pancreas cysts, diabetes, and chronic pancreatitis will allow for enhanced risk prediction and will guide close follow-up for earlier detection. Currently, the technologies are prohibitively expensive and require extensive sample processing, and point-of-care (POC) implementation is not practical. Development of noninvasive technology with economical application, minimal sample volume requirement, direct detection without sample processing, and potential POC utility will allow it to be used in practice as an initial screening tool. Because of the low prevalence of PDAC cancer, the discovery of a very efficient blood test with high sensitivity and specificity (to avoid multiple false positives) will be clinically useful for potential prognostic evaluation and management of PDAC. Because there are no currently known blood biomarkers that can be used in everyday clinical practice, it is crucial to investigate the oncogenic mutations that can lead to PDAC development.

CLINICALLY IMPORTANT ONCOGENIC MUTATIONS ASSOCIATED WITH PDAC (DIAGNOSIS, PROGNOSIS, AND PREDICTIVE VALUE)

Around 5% to 10% of pancreatic cancer individuals have a predisposing mutation in known susceptibility genes (germline mutations). Development and progression of PDAC involves sequential accumulation of genetic abnormalities. Around 97% of pancreatic cancers are caused by some kind of gene alterations, including amplifications, deletions, translocations, inversions, frameshifts, or substitutions.[8] The most common driver mutated gene in PDAC is Kirsten rat sarcoma viral oncogene homolog (*KRAS*) that affects about 56% of general population, followed by tumor protein P53 (*TP53*) (37%), guanine nucleotide-binding protein G(s) subunit α (16%), mothers against decapentaplegic homolog 4 (*SMAD4*) (13%), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (11%).[9] The percentage of these gene mutations is much higher in PDAC population, where *KRAS* is mutated in almost 95%.[10] In addition, recent reports highlight a clinical significance of breast cancer type 1 susceptibility protein (*BRCA1*) (0.0006%) and breast cancer type 2 susceptibility protein (*BRCA2*) (0.02%) mutations in pancreatic cancer.[9,11] Approximately by the time PDAC patients are diagnosed, the vast majority of patients are detected at an advanced stage of the disease. Thus, it is of great importance to identify individuals at high risk to develop PDAC who can benefit from early diagnostic measures.[12]

Mutations of *KRAS* can also be potentially prognostic for PDAC status and therapy prediction.[13–15] Specifically, modifications in G12 are the most frequent (99%) of all mutations in pancreatic cancer (G12D, 50%), whereas G13 mutations appear much rarer in comparison with other cancers (eg, colorectal cancer 17%).[9] Because conventional medicine has little to offer patients with inoperable PDAC, there are currently 4 chemotherapy drugs approved by the US Food and Drug Administration for the treatment of pancreatic cancer: Abraxane (albumin-bound paclitaxel), Gemzar (gemcitabine), fluorouracil, and Onivyde (irinotecan liposome injection).[16] Actually, studies indicate that PDAC patients with mutations in G12 show a poorer survival after treatment with first-line gemcitabine-based chemotherapy (11.3%) compared with those with wild-type *KRAS* (26.2%). In addition, there is a better survival rate observed in patients treated with a combination of gemcitabine and erlotinib, acting as the epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor ($P = 0.002$), compared with patients treated only with gemcitabine ($P = 0.121$), although the role of *KRAS* as a predictive marker is not routinely used.[17] In another study, treatment of locally advanced pancreatic cancer patients (96% with mutated *KRAS*) with small interfering ribonucleic acid (RNA) against G12D in combination with chemotherapy inhibited tumor progression. Eighty-three percent of patients showed stable disease and median overall survival (OS) of around 15 months.[18] These studies suggest the clinical importance of understanding the *KRAS* status of PDAC patients by showing its predictive value.

Similarly, the *TP53* and *CDKN2A* oncogenes can potentially be used as biomarkers for PDAC prognosis and therapy prediction. Patients with low *TP53* messenger RNA expression are associated with a poor prognosis ($P = 0.032$).[19] Pancreatic ductal adenocarcinoma patients with regular *TP53* expression show a longer progression-free survival period ($P = 0.02$) compared with patients with complete *TP53* loss.[20] In turn, *CDKN2A* deletion was reported in 50% of PDAC patients and was associated with shorter OS rate ($P = 0.002$).[21]

Clinical research studies also show that mutations in *SMAD4* could be used as a prognostic biomarker in pancreatic cancer because it is significantly associated with OS ($P = 0.006$).[22] *SMAD4* gene inactivation can be caused by homozygous deletion (32%) or the Mad homology domain MH2 mutations (20%).[23] Reports show that *SMAD4* genetic alterations are associated with a poor prognosis in PDAC patients (5 months vs 10 months, $P = 0.001$).[23]

Hayashi et al[24] took a holistic approach by examining the profile of mutations in 50 cancer-related genes to identify genomic biomarkers for predicting the outcome of patients with pancreatic cancer. The mutation profile was obtained using a single targeted deep sequencing. They detected mutations in the *KRAS* (96% of cases), *CDKN2A* (42%), *TP53* (13%), and *SMAD4* (7%). Among the patients after a pancreatectomy followed by chemotherapy, the presence of 0 to 2 mutated driver genes served as an independent predictor of a better OS (hazard ratio [HR], 0.20; $P = 0.0040$). Thus, a number of mutated driver genes has a potential to be used as a prognostic biomarker for pancreatic cancer.[24]

In turn, Holter et al[25] reports on a large prospective analysis of the prevalence of germline *BRCA1/2* mutations in a cohort of patients with incident PDAC diagnoses. They identified germline *BRCA* mutations in 4.6% of the patients including *BRCA1* (1%) and *BRCA2* (3.6% of cases). Interestingly, they did not find a statistically significant correlation of *BRCA*-mutation status with a personal history of cancer, family history of PDAC, or family history of breast or ovarian cancer, and none of the *BRCA*-mutation carriers met the criteria for familial PDAC. They also did not detect any *PALB2* (partner and localizer of *BRCA2*) mutations, a gene that had previously been suggested to be associated with PDAC risk.[25,26] In terms of treatment, they suggested that PDAC patients with *BRCA* mutations should be treated using platinum-based regimens (mainly cisplatin, not commonly used in PDAC patients) and PARP (poly (ADP-ribose) polymerase) inhibitors, because according to the literature, they can increase an OS in patients with *BRCA*-mutant PDAC.[27,28] In addition, there is also an early evidence of successful treatment of PDAC patients with germline *BRCA* mutations from phase I/II trials of PARP monotherapy.[28,29] In addition to germline mutations, DNA damage response genes can also be mutated somatically in tumors.[30] Waddell et al[30] found germline and somatic mutations in 8 genes in DNA-damage repair pathways (including *BRCA1*/2). Importantly, they found that the tumors with these mutations were more likely to have an unstable genomic variation (14%) and were significantly associated with response to platinum therapies. Thus, genomic biomarkers of defective DNA maintenance have a potential to be used for identification of *BRCA*ness phenotype patients, who could benefit from DNA damage response gene pathways' therapies.[11] *BRCA2* mutations can also be used as predictive biomarkers for increased sensitivity of pancreatic cancer to the application of DNA-intercalating agents. A *BRCA2* 1153insertionT mutation was successfully treated with combination of cisplatin

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and gemcitabine achieving a complete remission,[31] whereas a 6174delT *BRCA2* mutation in PDAC patient, treated with the combination of docetaxel, capecitabine, and gemcitabine followed by single agent irinotecan, resulted in a prolonged survival.[32]

To explore the spectrum of hereditary pancreatic cancer susceptibility, Slavin et al[12] evaluated germline DNA from pancreatic cancer patients including the Fanconi anemia genes. They identified 30% participants with a pathogenic or likely pathogenic variant that may be associated with PDAC predisposition. Thirteen percent of individuals had mutations in genes associated with well-known cancer syndromes (*ATM* [ataxia teleangiectasia mutated], *BRCA2*, *MSH2* [MutS homolog 2], *MSH6* [MutS homolog 6]). Most importantly, many had also mutations in Fanconi anemia complex genes (*BRCA2*, *FANCF* [Fanconi anemia complementation group F], *FANCM* [Fanconi anemia complementation group M]). In addition, earlier age of pancreatic cancer diagnosis (57.5 years vs 64.8 years) and family history of cancer ($P < 0.0001$) were suggestive of PDAC. Their multigene panel for identifying known cancer predisposing genetic susceptibility in those at risk for hereditary pancreatic cancer can be applied in clinical practice in cases with mutations in actionable genes.[12]

Connor et al[33] investigated the association of distinct mutational signatures with correlates of increased immune activity in PDAC. They evaluated the level of antitumor immunity genes to identify biomarkers predictive of response to systemic therapies. Thus, 4 major PDAC subtypes were reported: age-related, double-strand break repair, mismatch repair, and one with unknown etiology. Forty-five percent of double-strand break repair cases were missing germline or somatic events in canonical homologous recombination genes including *BRCA1*, *BRCA2*, or *PALB2*. Most importantly, double-strand break repair and mismatch repair subtypes were associated with increased expression of antitumor immunity, including activation of CD8-positive T lymphocytes (GZMA [granzyme A gene] and PRF1 [perforin 1 gene]) and overexpression of regulatory molecules (cytotoxic T-lymphocyte antigen 4, programmed cell death 1, and indolamine 2,3-dioxygenase 1), related to higher frequency of somatic mutations and tumor-specific neoantigens.[33]

These studies suggest that understanding the status of molecular alterations (such as *TP53*, *CDKN2A*, *SMAD4*, or exosomal serum carbohydrate antigen 19-9 [CA 19-9]) associated with PDAC can be helpful for assessing their risk and prognosis profiles of PDAC. Identification of an increasingly broad array of PDAC susceptibility genes enables detection of a growing population of high-risk individuals in need of active surveillance and screening. The tools currently available to screen these individuals (eg, cross-sectional imaging such as magnetic resonance imaging/CT and EUS) seem to be not adequate enough, whereas liquid biopsy has the potential to revolutionize their management. However, further research will be needed to validate these findings and introduce the knowledge into standard clinical care.

LIQUID BIOPSY OF PANCREATIC CANCER

Utility of ctDNA and CTCs in PDAC

Currently, the diagnosis of advanced stage PDAC largely relies on imaging modalities.[13,34] However, detection of early-stage pancreatic cancers and very small metastases remains a challenge.[35] Several serum tumor markers have been used as a noninvasive diagnostic approach for the early detection of pancreatic cancer, including CA 19-9 and carcinoembryonic antigen (CEA).[36–38] However, serum tumor markers (eg, CA 19-9, CEA) lack good sensitivity and specificity to be used in clinical practice for diagnosis of PDAC, even in high-risk populations. Their current clinical applications are limited to treatment monitoring and to determining prognosis of previously diagnosed PDAC. Specifically, CA 19-9 performance is promising for advanced and symptomatic tumors (sensitivity of 80%, specificity of 82%, with an area under the curve [AUC] of 0.87), but it is more suitable for diagnosis of small nonmetastatic lesions.[36,39] In turn, the performance of CT and magnetic resonance imaging is generally equivalent for the diagnosis and assessment of pancreatic cancer staging[40] with CT being more effective for the diagnosis of tumor resectability. However, the only clinically available diagnostic modality for PDAC is EUS-guided fine-needle aspiration, regardless of tumor size. Sensitivity of EUS-FNA varies from 65% to 95%. In addition, the negative predictive value is quite high (50%–70%), and the EUS-FNA may be ambiguous in 20% of PDAC cases.[41] Clinical benefit of examining oncogenic mutations in PDAC has been demonstrated in EUS-FNA cytopathology coupled with a *KRAS* mutation[42,43] However, this diagnostic option still presents an issue of invasiveness and also could be limited to tumors that are either symptomatic or apparent on traditional cross-sectional imaging (ie, not early stage). Nevertheless, because the oncogenic *KRAS* point mutation is a frequent event during PDAC, the identification of this gene mutation in tumor tissues may facilitate the clinical diagnosis.[43] Combining results of the *KRAS*-mutation assay with cytopathology can greatly improve the sensitivity and accuracy of diagnoses.[44] In addition, the negative predictive value of cytopathology can be enhanced (67%–88%) if it is combined with a *KRAS* mutation assay.[44]

KRAS mutation has been found in circulating cell-free tumor DNA (ctDNA), circulating tumor cells (CTCs) and in cargo from isolated exosomes.[13] The CTCs are shed from host tumors and circulate in the bloodstream. This process can also occur at an early stage of cancer and metastasis formation.[45] Although solid biopsy–based genotyping is the primary method for categorizing tumors for clinical decisions, tumor tissues provide only a snapshot of the genotyping profile at that time point. Tissue biopsies require an invasive procedure and thus cannot be used to guide treatment over time. In addition, the current treatment options are limited for patients with advanced stage of pancreatic cancer that are not eligible for resection.[35] Current chemotherapy for pancreatic cancer patients (eg, FOLFIRINOX or gemcitabine/nab-paclitaxel for treatment of metastatic PDAC) do not provide optimistic results.[34] Liquid biopsy (ctDNA), as a potential surrogate for the entire tumor genome, can address these concerns by analyzing biomarkers in biofluids such as blood and saliva that can indicate current state of the pancreatic tumor.[46]

Clinically, the ideal liquid biopsy technology needs to be able to capture the signature ctDNA concordant with tissue biopsy genotyping. Previous studies have shown a concordance of the *KRAS* mutation in the primary tumor and ctDNA between 25% and 75%, whereas the sensitivity relied on the nature of the tumor.[47] Buscail et al[13] reports the presence of a *KRAS* mutation in ctDNA in nearly 70% to 80% of locally advanced and metastatic patients and between 30% and 68% in patients with resectable tumors. Similarly, Bettegowda et al[48] showed 48% concordance of oncogenic mutations in plasma in localized pancreatic tumors and >75% in advanced tumors by polymerase chain reaction (PCR) method. However, a study using the next-generation sequencing (NGS) showed the concordance of 90.3% in plasma from advanced pancreatic tumors,[49] whereas a recent study reports a perfect concordance (100%) between oncogenic mutations in plasma and primary PDAC tumor.[50] The heterogeneity of concordance between ctDNA and tissue genotyping highlights the inadequacy of PCR-based and NGS technologies for liquid biopsy.

Similarly, CTCs could be used as markers in the early diagnosis, prediction, and monitoring of treatment response in PDAC patients (Table 1). Circulating tumor cells can be found in blood of patients with PDAC of any stage.[64] The detection rates of CTCs in

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TABLE 1. Cross-Section of the Various Liquid Biopsy Biomarkers (CTCs, Circulating miRNAs, ExoDNA) for PDAC With the Clinical Utility Potential

CTCs	Isolation Method	Clinical Application
	CTC-based CellSearch system[13,51]	PDAC diagnosis, detection of locally advanced or metastatic PDAC disease (11%–48%)
	ISET[52]	PDAC diagnosis (93%)
	Microchip platform (magnetic micropore-based negative immunomagnetic selection with rapid on-chip in situ RNA profiling)[53]	PDAC diagnosis
Circulating miRNAs	**miRNA**	**Clinical Application**
	miR-21[54]	PDAC diagnosis (sensitivity, 0.90; specificity, 0.72; and AUC, 0.91)
	miR-25[55]	PDAC diagnosis (AUC, 0.915)
	miR-196a and miR-196b (increased)[56]	Differential diagnosis between PDAC and multifocal PanIN-2/3 versus PanIN-1, pancreatic neuroendocrine tumors, chronic pancreatitis, or healthy controls
	miR-223[57]	Differential diagnosis between benign IPMN and malignant IPMN
	miR-744 (increased)[58]	Postoperative — poor prognosis (metastasis, recurrences, and chemotherapy resistance) in PDAC
	miR-373-3p (decreased)[59]	Postoperative — poor prognosis (metastasis, recurrences, and chemotherapy resistance) in PDAC
	miR-18a (increased)[59]	Postoperative — poor prognosis (tumor recurrence)
	miR-196a and miR-196b (decreased)[56]	Postoperative prognosis (after resection)
	miR-221 (decreased)[60]	Postoperative prognosis (after resection)
	miR-483-3p (decreased)[61]	Postoperative prognosis (after resection)
ExoDNA	**ExoDNA**	**Clinical Application**
	ExoDNA — *KRAS* mutations[62]	PDAC diagnosis (66.7% of patients with localized disease; 80%, locally advanced disease; and 85%, metastatic disease)
	ExoDNA — *KRAS* mutations (upregulated)[14]	PDAC prognosis (disease progression)
	ExoDNA — *KRAS* mutations[63]	PDAC prognosis (progression in patients with metastatic disease)

PDAC patients vary from 21% to 100% compared with 0% in healthy controls.[37,65] A cohort of patients with early-stage disease found 78% of PDAC,[66] whereas a group of patients with advanced PDAC disease detected 80.5% of PDAC cases.[67] Moreover, a combination of CA 19-9 and CTC detection can even increase the detection rate of pancreatic cancers.[65] Interestingly, monitoring of the CTC burden enables prediction of treatment response in PDAC patients. The CTC count was reduced 3 days after surgery but increased in 10 days after surgery in most PDAC patients.[65] This high number of CTCs in blood after surgery can indicate undetectable metastatic disease[68] as metastatic patients have increased number of CTCs compared with patients with local disease.[69] In addition, a specific phenotype of CTCs (eg, CTCs expressing CD133 and CD44) can be indicative of worse survival.[66]

Currently, the CTC-based CellSearch system (Veridex, LLC, Warren, NJ) claims to detect PDAC in about 11% to 48% of patients in cohorts that include at least 53% of patients with locally advanced or metastatic disease.[13,51] An alternative approach, the isolation by size of epithelial tumor cells method (ISET[52]; Rarecells Diagnostics, Paris, France) presents an even better detection rate of 93% as compared with only 40% for CellSearch. In turn, Ko et al[53] invented a microchip platform that combines fast, magnetic micropore-based negative immunomagnetic selection (>10 mL/h) with rapid on-chip in situ RNA profiling (>100× faster than conventional RNA labeling) of whole blood in PDAC patients, even in those with very low number of CTCs (<1 CTC per mL of whole blood). Overall, CTC-based diagnostic methods of PDAC are highly specific. However, the sensitivity is not high since the number of captured CTCs is quite low.[70]

Because of difficulties in differentiating pancreatic cancer from other conditions (ie, pancreatitis) on the basis of clinical features and imaging investigations, a simple and noninvasive test to detect the mutation profile would be especially valuable. Importantly, because the *KRAS* gene is mutated in >90% of PDAC, a comprehensive analysis of many genes would be unnecessary to detect the majority of cases.[71]

Circulating miRNAs in PDAC

As an important part of liquid biopsy, microRNAs (miRNAs) can also serve as biomarkers for pancreatic cancer detection[68] (Table 1). Li et al[54] reports altered miRNA profiles in early diagnosis of pancreatic cancer with pooled sensitivity of 0.88, pooled specificity of 0.83, and AUC of 0.90, whereas the diagnostic value of a single miR-21 in PDAC blood reached pooled sensitivity of 0.90, specificity of 0.72, and AUC of 0.91. In addition, serum miR-25 has a strong potential to serve as novel biomarker for the early detection of PDAC (AUC, 0.915), which outperformed serum levels of CA 19-9 (AUC, 0.844) and CEA (AUC, 0.725).[55]

Circulating miRNAs could be also used in differential diagnosis (Table 1). Plasma miR-223 tended to discriminate the malignant potential between benign intraductal papillary mucinous neoplasm (IPMN) and malignant IPMN.[57] Patients with pancreatic cancer and multifocal PanIN-2/3 lesions had significantly higher serum levels of miR-196a and miR-196b than patients with PanIN-1, pancreatic neuroendocrine tumors, chronic pancreatitis, or healthy controls.[56] Furthermore, circulating miRNAs have recently been used to predict response to treatments and assess the

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prognosis for pancreatic cancer.[72,73] A high level of plasma miR-744,[58] whereas a downregulated level of miR-373-3p,[59] in postoperative pancreatic cancer patients indicated a poor prognosis, associated with metastasis, recurrences, and chemotherapy resistance.[58] Strikingly, the increased expression of miR-18a can indicate tumor recurrence, even though serum CA 19-9 level remains unchanged. Quite the contrary, the downregulation of miR-196a and miR-196b,[56] miR-221,[60] and miR-483-3p in PDAC patients can be a sign of successful resection.[61] Interestingly, circulating miRNA detection is considered to have a better sensitivity compared with ctDNA in PDAC patients.[74]

Exosomal ctDNA in PDAC

Exosomes are lipid microvesicles (30–100 nm) that are able to migrate systemically through the vasculature of the body promoting intercellular communication.[75] They reside in a multitude of biofluids including urine, blood, breast milk, bronchial lavage fluid, cerebral spinal fluids, and saliva.[76–78] Although the mechanism is not clear yet, exosomes in body fluids are believed to be closely related to cancer development. However, the current state for exosomal oncogene research is limited by (1) low efficient exosome capture method and (2) no real-time exosome assay. Exosomes secrete messenger RNAs, proteins, metabolites, and miRNAs into the circulation, thus resulting in disease progression.[46,79] A panel of pancreatic cancer-initiating cell protein markers (CD44v6, tetraspanin-8, epithelial cell adhesion molecule, and CD104) and miRNAs (miR-1246, miR-4644, miR-3976, and miR-4306) were significantly upregulated in most of pancreatic cancer serum exosomes, but not in healthy controls and patients with nonmalignant diseases.[63] In addition, several other distinct miRNA signatures have been identified in PDAC. MiR-17-5p and -21 are believed to have a high diagnostic value, with a sensitivity and specificity between 72% and 95%.[80] Similarly to proteins and miRNAs, pancreatic cancer–derived exosomal ctDNA has been also used to evaluate for *KRAS* mutations (Table 1). Most importantly, *KRAS* mutations from exosomes (exoDNA) outperformed assessment from ctDNA in detection of PDAC[62] and predicting disease progression.[14] However, *KRAS* mutations in exoDNA were identified in 66.7%, 80%, and 85% of patients with localized, locally advanced, and metastatic disease, respectively. Comparatively, *KRAS* cell-free DNA (cfDNA) mutations were identified in 45.5%, 30.8%, and 57.9% of these patients.[62] Overall, the diagnostic performance of exoDNA ranges between 35% and 69%.[14,62] In addition, for the patients with potentially resectable disease, exoDNA has a good prognostic value because upregulated exoDNA expression after neoadjuvant therapy was indicative of disease progression ($P = 0.003$), whereas ctDNA was not informative.[14] ExoDNA showed also good correlation with progression free survival in patients with metastatic disease.[63] Despite its good prognostic value, exoDNA based on mutant *KRAS* detection may have a limited use for diagnosis of PDAC, as it yields a high rate of false positives.[62]

Ultrashort ctDNA Detection in PDAC

Current approaches to improve sensitivity of ctDNA detection mainly focus on increasing depth of sequencing with limited results because of high false positive rates.[81] Another approach is to consider the biological properties of plasma cfDNA, such as length of ctDNA fragments. Lapin et al[82] demonstrated that a pretreatment cfDNA fragment size of ≤167 bp ($P = 0.002$) and high pretreatment cfDNA levels ($P < 0.001$) can be used to predict disease outcome in patients with advanced pancreatic cancer because they can be indicative of shorter progression-free survival ($P = 0.001$) and OS ($P = 0.001$). Moreover, he observed high concordance between short fragment size (≤167 bp) and high cfDNA levels (>4.66 ng/mL plasma) in 74% of cases of advanced PDAC (κ, 0.475; 95% confidence interval, 0.253–0.696).[82] In addition, Mouliere et al[83] highlighted that enrichment of ctDNA in fragment sizes between 90 and 150 bp can enhance ctDNA detection about 2-fold in >95% of cases and more than 4-fold in >10% of cases. The cfDNA fragmentation features could differentiate between cancer and healthy samples with a high accuracy (AUC of 0.989 for high ctDNA cancers and AUC of 0.891 for low ctDNA cancers). Interestingly, they reported much more efficient detection of cfDNA from patients with cancer types previously observed to have low amounts of ctDNA such as glioma, renal, and pancreatic cancer (AUC, >0.91) compared with those without fragmentation features (AUC, <0.5). Quite the contrary, the cfDNA fragment sizes in plasma of healthy individuals and patients with late-stage glioma, renal, pancreatic, and bladder cancers were significantly longer than in other late-stage cancer types such as breast, ovarian, lung, melanoma, colorectal, and cholangiocarcinoma ($P < 0.001$).[83] Those findings were confirmed in another study, in which it was observed that the small mutant fragments can be specifically found in early-stage cancer patients.[37] Liu et al[37] developed single-strand library preparation and hybrid capture–based cfDNA sequencing called SLHC-seq method, specifically designed for analysis of short degraded cfDNA fragments. They showed much higher sensitivity and accuracy in mutation detection compared with other literature reports, where 791 cancer-specific mutations were identified in the plasma of 88% of patients with *KRAS* hotspots detected in 70% of all patients including 66% of patients with precancerous or early-stage disease.[37] Using the SLHC-seq approach, the detection of *KRAS* mutations served as an efficient marker to distinguish PDAC from healthy individuals (AUC, 0.863). Interestingly, the length of PDAC mutated *KRAS* was nearly 100 bp, whereas the wild-type *KRAS* fragments were about 160 bp in length. It appeared that the short fragments were more prevalent in precancerous IPMN (80 bp) and early-stage PDAC (stage I/II) (140 bp) patients compared with the advanced-stage PDAC (160 bp) ($P < 0.0001$). The concordance improved when a combination of the *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* genes was used for the diagnosis of PDAC (AUC, 0.921; sensitivity, 80%; specificity, 100%). Most importantly, the findings were highly consistent with tissue-based sequencing, with the concordance of 75.3% for *KRAS*, 58.8% for *TP53*, and 41.2% for *CDKN2A*. However, diagnostic accuracy was the highest once the full targeted panel of 62 genes was applied (AUC, 0.951; specificity, 100%; sensitivity, 89%). Lastly, the detection of mutations using the full targeted panel also had the ability to distinguish the early IPMN lesions from PDAC patients (AUC, 0.837; specificity, 87.5%; sensitivity, 66.2%).[37]

Comparison of ctDNA Detection Methods in PDAC

Different methods have been currently applied for *KRAS* mutation analysis and now replace direct sequencing and other methods that require a preamplification step. The new technologies encompass quantitative PCR methods, allele-specific PCR using amplification refractory mutation system technology or coamplification at a lower denaturation temperature, pyrosequencing approaches, and real-time PCR methods.[84] However, detection of ctDNA can be challenging because ctDNA abundance is considered very low (<1.0% in many cases) in total cfDNA.[85] Traditional methods such as Sanger sequencing or pyrosequencing enables identification of mutated tumor-derived DNA fragments only in patients with abundant copies of ctDNA.

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However, recent technological advances, including digital droplet PCR (ddPCR) and NGS, allow the detection of low burden of ctDNA in blood.[48] The ddPCR has demonstrated much higher sensitivity (43%–78%), compared with simple PCR or sequencing (27%–47%).[13] The sensitivity for the detection of *KRAS* mutation using direct sequencing is 10% to 30%, NGS is 10%, whereas by the use of ddPCR is 0.01%.[86] Pécuchet et al[87] compared a microfluidic ddPCR (RainDrop Plus Digital PCR System; Rain Dance Technologies, Billerica, Mass) and NGS analysis (The Ion Proton System; Thermo Fisher Scientific, Carlsbad, Calif) in detecting *KRAS* and *EGFR* mutations and observed the concordance of 97.4% of results. Similarly, Pietrasz et al[88] reported high concordance (odds ratio, 0.94) between the targeted NGS analysis (Ion Proton System) and ddPCR (RainDrop Plus Digital PCR System) in detecting *KRAS* mutant ctDNA. In turn, Takai et al[89] presented a 2-stage approach for detection of *KRAS* mutant ctDNA in PDAC patients, first, using ddPCR (Bio-Rad Laboratories, Irvine, Calif) as a prescreening method, followed by NGS analysis (HiSeq 2000; Illumina Inc, San Diego, Calif). Strikingly, NGS analysis revealed that sequencing of cfDNA identified more somatic mutations related to PDAC than sequencing of tissue samples.[49] As genomic sequencing technologies become less and less expensive, there will be an increased use in clinical applications, as well as whole exome and whole genome sequencing for research purposes.[33]

CLINICAL APPLICATIONS OF LIQUID BIOPSY IN PANCREATIC CANCER

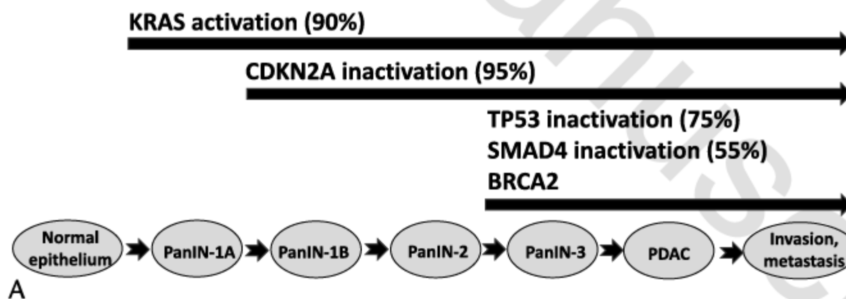
Early Detection, Screening, and Diagnosis of PDAC

Cohen et al[50] recently presented a combination of circulating tumor markers and ctDNA that can be used in early detection of nonmetastatic cancers of the ovary, liver, stomach, pancreas, esophagus, colorectum, lung, and breast cancer. The sensitivities varied from 69% to 98% for ovarian, liver, stomach, pancreas, and esophageal cancer, at >99% specificity.[50] In addition, it is reported that 90% gene mutations in PDAC tumor tissues can be also identified in the cfDNAs.[49] Moreover, ctDNA can differentiate IPMN with malignant potential from other harmless pancreatic tumors.[90] Specifically, the driver gene *KRAS* mutations can be detected in plasma of about 50% of PDAC patients,[91] thus serving as an early diagnostic biomarker.[92,93] Similarly, the involvement of more disrupted genes plays an important role in PanIN grading. Pancreatic intraepithelial neoplasia is a histological precursor to ductal adenocarcinoma in the pancreas. Thus, involvement of only *KRAS* mutations in the carcinogenesis of PDAC is associated with PanIN-1A or PanIN-1B grading, additional presence of *CDKN2* mutations stands for PanIN-1B or PanIN-2, whereas detection of also other genes such as *TP53*, *SMAD4*, and *BRCA1* and *BRCA2* stands already for PanIN-3 grade (Fig. 1).[94]

The diagnosis of PDAC based on ctDNA can also be performed in locally advanced or metastatic PDAC patients, where *KRAS* mutation can be detected in blood of 70% to 80% of patients, but only in 30% to 68% of patients with resectable tumors.[13] Similarly, a ctDNA detection rate of 80% was observed in PDAC patients with advanced cancer, but only in 47% with localized cancer.[48] Interestingly, the sensitivity (67%) and specificity (77%) of serum *KRAS* mutations for the diagnosis of pancreatic cancer can be much improved, when a combination of ctDNA with CA 19-9 levels is used, thus reaching the sensitivity of 98%, and specificity 97%.[95]

Prognosis/Prediction of PDAC

Cell-free tumor DNA can also serve as an independent prognostic marker for monitoring treatment efficacy and disease



Gene	Normal epithelium	PanIN-1A	PanIN-1B	PanIN-2	PanIN-3	PDAC
KRAS	0%	55-60%	> 80%			75-100%
p16	0%	30%	55%	92%		80-95%
p53	0%	0%	0%	0%	12%	50-75%
SMAD4/DPC4	0%	0%	0%	0%	30%	55%
HER2/Neu	0%	82%	86%	92%		70%
BRCA2	0%	0%	0%	0%	0%	7-10%

B

FIGURE 1. A, Schematic diagram for the progression of PDAC and associated oncogenic mutations (adapted from Bryant et al.[14]). The majority of all PDAC progression begins with anomalies in the *KRAS* gene. B, Oncogenic mutations associated with the development of PDAC (adapted from Yonezawa et al., *Gut Liver.* 2008;2:137–154).

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progression in pancreatic cancer patients.[96] Interestingly, recent studies report that tumor-specific mutations in ctDNA can be a better prognostic marker than CTC count.[97,98] In addition, ctDNA can be indicative of shorter survival in resected or metastatic patients when detected after surgery or chemotherapy.[88] Higher levels of plasma ctDNA detected by ddPCR were associated with shorter OS compared with the patients with no detectable ctDNA (60 days vs 772 days, $P < 0.001$).[99] Overall, the presence of a *KRAS* mutation is associated with a poor prognosis for PDAC patients.[13] The survival of patients with *KRAS* mutations in ctDNAs was significantly shorter than that of patients without mutations,[15] specifically in patients with G12V or G12D mutations.[100,101] Hadano et al[102] reports a survival of PDAC patients with mutant *KRAS* in cfDNA of 13.6 months compared with 27.6 months in individuals with wild-type *KRAS* ($P < 0.0001$). Also, in metastatic PDAC, undetectable *KRAS* mutant ctDNA was related to longer survival (8 months vs 37.5 months, $P < 0.004$).[15] Most importantly, *KRAS* mutations in plasma DNA can be considered to serve as a better prognostic factor for OS (HR, 7.39, $P < 0.001$) compared with CA 19-9 levels (HR, 2.49; $P = 0.087$)[103] but worse than longitudinal monitoring through exosome DNA.[14] The concordance rate (tissue vs ctDNA) was 68.2%.[14]

Monitoring Treatment Efficacy and Disease Progression

One of the major potential applications of ctDNA is monitoring treatment efficacy and tumor progression in pancreatic cancer patients. Higher levels of plasma cfDNAs in pancreatic patients may be indicative of metastasis and recurrences.[104] The pretreatment ctDNA level can serve as a predictor of both progression-free survival ($P = 0.014$) and OS ($P = 0.010$). Among ctDNA-positive patients, 90% experienced disease progression, compared with 25% of ctDNA-negative patients ($P = 0.01$).[105] In addition, *KRAS* mutation levels in blood were concordant with both radiological imaging data and CA 19-9 levels.[105] Sausen et al[106] showed that the detection of ctDNA after resection predicted clinical relapse and poor outcomes. Patients with *KRAS* mutant ctDNA after surgery were more likely to relapse than those without *KRAS* mutant ctDNA (9.9 months vs not reached, $P = 0.02$). Furthermore, recurrences were detected approximately 6.5 months earlier by ctDNA compared with CT imaging ($P < 0.0004$).[106] Takai et al[89] also proved that the detectability of *KRAS* mutant cfDNA can be related to the presence of distant organ metastasis, even difficult to detect by routine imaging tests. Interestingly, Conroy et al[107] performed validation of a *KRAS* ctDNA assay in a Clinical Laboratory Improvement Amendments setting using ddPCR. Cell-free tumor DNA was detected preoperatively in 49% patients and was an independent predictor of decreased recurrence-free survival and OS. Persistence of ctDNA in the immediate postoperative period was associated with a high rate of recurrence (sensitivity, 90%; specificity, 88%) and poor median recurrence-free survival (5 months) and short median OS of 17 months ($P = 0.011$).[107] Quite the contrary, Allenson et al[62] and Singh et al[104] were unable to determine a significant difference in survival when comparing patients with cfDNA mutant *KRAS* and those with cfDNA wild-type *KRAS*.

Current Limitations in PDAC Liquid Biopsy

KRAS holds a great premise because it is the most commonly mutated gene in PDAC and the mutations occur at the very early stage of carcinogenesis. However, the current technologies for ctDNA analysis still lack sensitivity and specificity to enable detection of early-stage cancers.[35] Detection of ctDNA is still challenging because of the high background levels of wildtype cfDNA. In particular, in early-stage malignant disease, ctDNA may be presented in low amounts of total cfDNA.[49] Thus, more advanced technologies with better sensitivity are greatly needed. One way to overcome this problem would be to use a combined technological approach, such as exosomics and NGS or miRNA profiling for identification of specific "molecular signatures."[13] In addition, detection of tumor-specific epigenetic alterations in cfDNA could be an alternative approach to improve sensitivity and specificity in the diagnosis of early-stage pancreatic cancer.[35] Another urgent issue that should be addressed is the standardization of methods used for sample acquisition, plasma separation, sample storage, cfDNA extraction, and quantification and for sequencing of cfDNA. All these preanalytical processes for cfDNA analysis require urgent unification.[35] Because there is no current "universal" threshold and quantification norm values, one more limitation is the variability of the detection assay. Therefore, multicenter studies in a larger cohort should be recommended.[108] Furthermore, in case of PDAC, liquid biopsies were performed mainly on total peripheral blood.[109] Thus, other body fluids such as saliva or urine may serve as an additional source of ctDNA. Specifically, pancreatic juice is considered to contain larger amounts of ctDNA.[35] Also, exosomes are a distinct source of tumor DNA that may be complementary to other liquid biopsy DNA sources. However, a substantial minority of healthy samples demonstrated mutant *KRAS* exoDNA in circulation (14.8%–20%). These results insinuate careful consideration and application of liquid biopsy findings.[62]

To summarize, liquid biopsy is the emerging technology that can have a great potential clinical utility in pancreatic cancer patients, specifically at an early stage of the disease. An efficient noninvasive tool to screen a high-risk cohort will significantly facilitate decision making for further diagnosis and therapeutic approaches. Cell-free tumor DNA is expected to provide a minimally invasive approach for PDAC diagnosis, monitoring of chemotherapy-resistant mutations, and overcoming the problem of tumor heterogeneity.[85]

NOVEL ELECTROCHEMICAL LIQUID BIOPSY PLATFORM

As the field of liquid biopsy pushes the boundaries of sensitivity in detecting mutations using NGS- or PCR-based techniques, a point of inquiry is the appropriate workflow for mutation detection in clinical settings. Apart from the ability to sensitively identify mutated ctDNA, even in low abundance relative to wild-type DNA, factors such as clinical benefit, sample volume, test turnaround time, and cost-effectiveness should be considered. Electrochemical (EC) sensors have recently found impactful entries in clinical cancer diagnostics.[110] While offering simplicity in operation and sample manipulation, contemporary EC biosensors also provide highly sensitive and specific measurements of a broad spectrum of biomolecules.[111] The sample volume required for current EC sensors is small, ranging from several microliters to hundreds of nanoliters, including sample pretreatment reagents. In addition, detection time is fast, from minutes to tens of seconds. An important feature of EC sensors is their potential to be easily transformed from a laboratory-based instrument to a POC device or high throughput platform, enabling and advancing laboratory-based technologies into real clinical practices.

The Wong group at UCLA has developed the electric field–induced release and measurement (EFIRM) technology to specifically capture and monitor in biofluids key oncogene mutations in human cancer patients who can be treated with molecular targeted

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therapies. The core technology is an EC platform integrating sensitive and specific multiplex assays, optimized for proteomic, transcriptomic, and genomic biomarkers in biofluids. Core technologies include (1) design of nucleic acid probe to specifically amplify EC signals from low number of targets (<10 molecules) without sample extraction and amplification,[112,113] (2) improvement of the biocompatibility and probe surface density through conducting polymer interface on electrode,[112,114] and (3) enhanced incubation through electric waveform.[112,115]

Specifically, EFIRM is a conducting polymer-based EC plate with an array of 96 bare gold electrode chips as a sensor. This technique is based on the principle that nucleic acid hybridization can be facilitated through applying electric fields selectively.[112,116,117] By applying these electric fields, the mutated ctDNA sequences present in a biofluid can be actively hybridized to an oligonucleotide capture probe immobilized with a conducting polymer to an electrode surface.[113] Following this active hybridization capture of the mutated sequence, a detector probe sequence with a biotin label is hybridized to the remaining portions of mutation sequence that are unbound to the capture probe at the electrode surface. Finally, a reporter enzyme and tetramethylbenzidine-based substrate solution is used to generate oxidation and reduction reactions. These oxidation and reduction reactions that occur at the surface of the electrode are subsequently measured and used for the quantitation of the target sequence present.[112] The total detection time is 30 minutes and requires only 20 to 40 μL of plasma or saliva for direct ctDNA detection.

Exosomal Oncogene for Pancreatic Cancer Research by EFIRM in Saliva: EFIRM Detection of Exosome Communication of Pancreatic Cancer-Associated exRNA in Saliva

Electric field–induced release and measurement is an exosome-specific technology that is capable of, first, selectively capturing CD63 (exosomal specific membrane protein markers) to positive exosomes and then concurrently performing real-time detection for nucleic acids and/or proteins. In a pancreatic cancer animal model, EFIRM was used to study how exosomes, harboring mutated genes, can travel from a distal pancreatic cancer through blood and saliva.[118] In this study, EFIRM was able to assay the contents in exosomes from saliva, serum, and Panc 02 (a pancreatic cancer cell-line) culture media. In addition, all of the 7 exRNAs were detected in exosomes derived from saliva, serum, and Panc 02 cells. Among them, 6 of the 7 genes were found to be upregulated in both saliva and serum-derived exosomes of tumor-bearing mice when compared with control, whereas *Foxp1* was found to be significantly upregulated in saliva-derived exosomes, and *Gng2* was found to be significantly upregulated in serum-derived exosomes of tumor-bearing mice. Aside from whole serum, tumor-derived exosomes, serum-derived exosomes, and saliva and saliva-derived exosomes, all exhibited upregulation of most, if not all, of the 7 validated pancreatic cancer–specific salivary transcriptomic biomarkers.

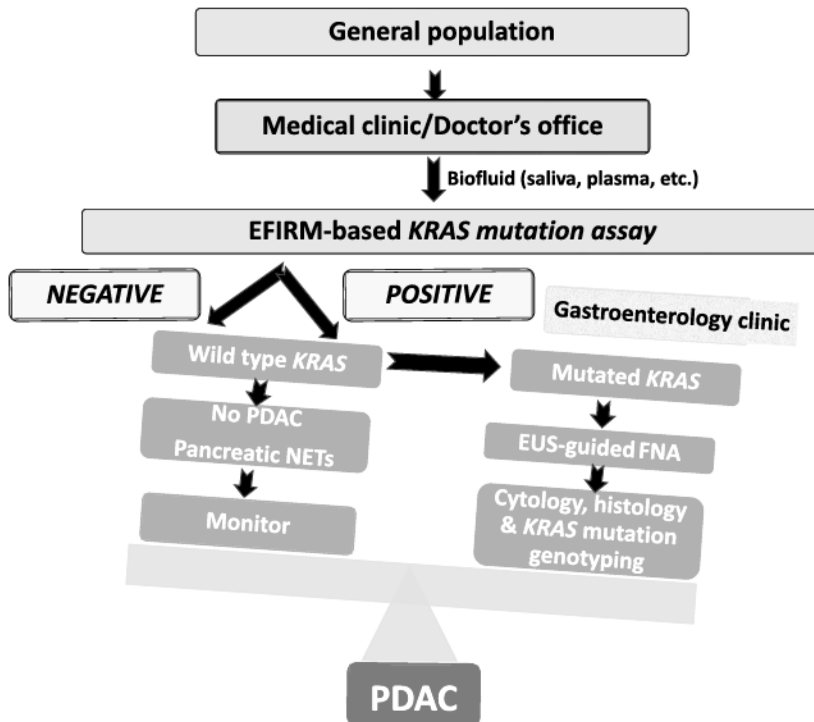

FIGURE 2. A triage map for potential clinical application of EFIRM-based *KRAS* mutation liquid biopsy screening for PDAC. Biofluid will be collected from symptomatic and asymptomatic patients presenting at health provider's office during regular visits. Biofluid will be sent for laboratory EFIRM-based *KRAS* analysis. Patients with mutated *KRAS* will be recommended for tissue biopsy for genotyping for *KRAS* mutation to confirm PDAC. Patients reporting wild-type *KRAS* could be excluded from PDAC diagnosis but may need further diagnosis if other tumor such as NET is suspected. Biomarkers specific for NET can also be evaluated to further differentiate the patient's status. By monitoring high-risk patients for their *KRAS* status, EFIRM may contribute to early detection of PDAC.

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Perspective Utility of EFIRM

We have developed a PDAC-associated *KRAS* mutation assay based on the EFIRM *EGFR* mutation assay, which has previously demonstrated to have 100% concordance with biopsy-based genotyping for *EGFR* mutations associated with non–small cell lung carcinomas in 2 blinded clinical studies.[115] Current oncogene mutation detection technologies are mainly PCR based and require sample pretreatment and several hours of detection. Electric field–induced release and measurement's simplicity and rapid detection time (minutes), while only using a few microliters of the clinical sample, should allow accurate detection of *KRAS* mutations in PDAC patients.

Successful clinical utilization of the liquid biopsy of oncogenic mutations will rely on how efficiently and credibly this information will be captured. Our recent demonstration of EFIRM performance in detecting *EGFR* mutation clearly poises EFIRM assay as a sensitive, specific, and credible tool that will satisfy clinical needs and standards. The clinical utility of EFIRM assay for *KRAS* mutation detection will be to screen at-risk populations and to enrich high-risk cohorts for further diagnosis of PDAC (Fig. 2). Electric field–induced release and measurement will enable the detection of *KRAS* from patients at an early stage. In addition, because the test only takes minutes, it will theoretically allow continuous dynamic monitoring of *KRAS* mutations in PDAC in various biofluids (Fig. 3).

Electric field–induced release and measurement exploits (1) simple and effective biomarker release from body fluids, (2) enhanced sample mixing and accumulation, (3) enhanced hybridization of the oncogene, and (4) suppression of nonspecific interference. Therefore, EFIRM technology is poised to be integrated into PDAC screen programs to augment and enhance the utilities of currently available diagnostic options for PDAC detection. Definitive validation of the EFIRM technology will allow it to be used in practice for screening the population at risk and to develop strategies to use the technology for individualized approaches to treatment. Development of an effective screening tool with credible and validated performance will greatly aid in diagnosis and health care of individuals at risk and more importantly will have a tremendous impact on the improvement of the quality of life, which is the prime goal set to double survival by 2030.

CONCLUSIONS

Pancreatic cancer is a dismal disease with the lowest survival rate among cancers, mainly because of the lack of a diagnostic modality for an early stage of the disease. In this article, we have reviewed molecular entities and alterations associated with pancreatic cancers that serve as scientific foundations for the development of detection modality. We have also reviewed current technology platforms of liquid biopsy that are being used for early detection, screening, and diagnosis of PDAC. We have further discussed current limitations of liquid biopsy solely relying on genetic mutations identified from tissue genotypic analysis.

Recent advances in liquid biopsy field, especially on the nature of cfDNAs, the main analytes in biofluids for liquid biopsy, suggest a potential new paradigm toward necessity of focusing not only on biological/genetic features but also on the physical characteristics of cfDNA to improve specificity and sensitivity of detection. This will in turn require the invention and utilization of a technology platform that will be a best fit for capturing these cfDNA characteristics associated with a specific disease. The

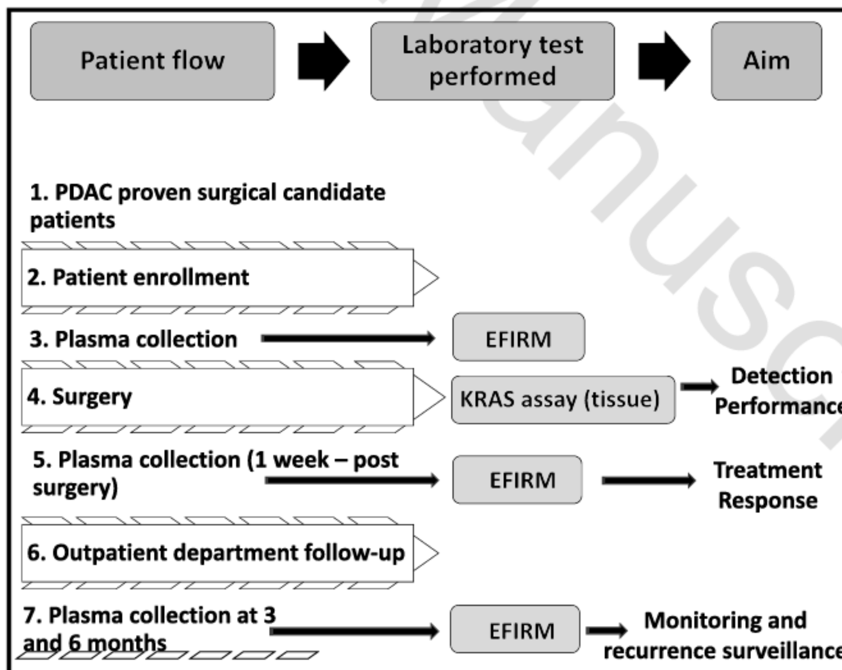

FIGURE 3. Schematic of strategic plan to evaluate EFIRM's application in clinical care. The clinical utility of EFIRM can be tested in the detection, treatment response evaluation, and surveillance of PDAC. Pancreatic ductal adenocarcinoma patients, who are surgical candidates, will be enrolled, and plasma samples will be collected for EFIRM *KRAS* assay before surgery. For detection performance, the concordance will be assessed between the EFIRM assay results and genotyping of tissue acquired during surgery. For treatment response evaluation, plasma collected 1 week after surgery will be evaluated by the EFIRM *KRAS* assay to reveal any residual *KRAS* ctDNA. For PDAC surveillance, during the follow-up at the outpatient department, plasma can be repeatedly collected at 3 and 6 months and analyzed with EFIRM assay to track reoccurrence of PDAC.

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future of liquid biopsy for early detection of PDAC and other cancers in general will be benefited by considering these characteristics for the invention and application of a modality that should be fitted with clinical parameters for achieving the best clinical performance.

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