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ORIGINAL RESEARCH—CLINICAL

Blood-Based microRNA Biomarker Signature of Early-Stage Pancreatic Ductal Adenocarcinoma With Lead-Time Trajectory in Prediagnostic Samples

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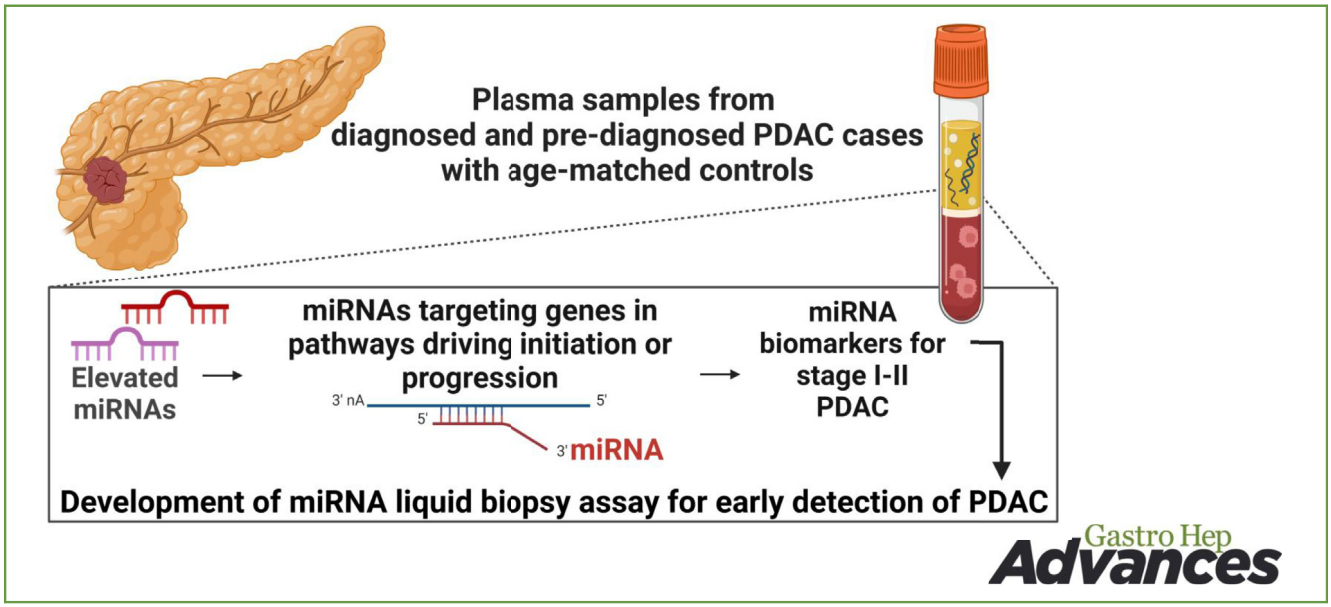

BACKGROUND AND AIMS: Clinically validated biomarker of pancreatic ductal adenocarcinoma (PDAC), carbohydrate antigen 19-9 (CA19-9), has limited sensitivity and specificity for early-stage disease. Circulating miRNAs in plasma associated with cancer relevant pathways were developed as early detection biomarkers. **METHODS:** 2083 miRNAs in 15 μl of plasma from multicenter age-matched cohorts ($N = 203$: healthy controls, $n = 46$; pancreatitis controls, $n = 36$; diagnosed cases: $n = 121$) and a prediagnostic Prostate, Lung, Colorectal, and Ovarian age- and gender-matched cohort ($N = 96$; controls, $n = 48$; prediagnosed cases, $n = 48$) were interrogated. A three-miRNA biomarker signature was developed for early-stage PDAC. **RESULTS:** The three-miRNA signature (let-7i-5p, miR-130a-3p and miR-221-3p)

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Abbreviations used in this paper: AUC, area under the curve; CA19-9, carbohydrate antigen 19-9; CI, confidence interval; ctDNA, circulating tumor DNA; exo, exosomal; GSEA, gene set enrichment analysis; miRNA, microRNA; NES, normalized enrichment score; NPV, negative predictive value; PDAC, pancreatic ductal adenocarcinoma; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; PPV, positive predictive value; ROC, receiver operating characteristic.

Most current article

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detected PDAC from healthy controls independently (area under the curve [AUC] of stage I, II, I-IV = 0.970, 0.975, 0.974) and in combination with CA19-9 (AUC of stage I, II, I-IV = 1.000, 0.992, 0.995). It also discriminated chronic pancreatitis (AUC of stage I, II, I-IV = 0.932, 0.931, 0.929), improving performance of CA19-9 alone (AUC of stage I, II, I-IV = 0.763, 0.701, 0.735) in combination (AUC of stage I, II, I-IV = 0.971, 0.943, 0.951). Blinded validation in prediagnostic Prostate, Lung, Colorectal, and Ovarian cohort revealed lead-time trajectory increase in AUC from 0.702 to 0.729 to 0.757 at twelve-, six-, and three-months before PDAC diagnosis, respectively. The signature also helped stratification of patients with different circulating tumor DNA and imaging subtypes. **CONCLUSION:** Plasma miRNAs associated with oncogenic pathways may serve as PDAC early detection biomarkers.

Keywords: Pancreatic Ductal Adenocarcinoma; Plasma miRNA; Early Detection Biomarkers; Liquid Biopsy; Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial

Introduction

Pancreatic ductal adenocarcinoma (PDAC), the most prevalent type of pancreatic cancer, is currently the third leading cause of cancer-related deaths in the United States with 5-year survival rate of ∼13%.[1] Most patients remain asymptomatic till the disease undergoes local or distal metastasis. The 5-year survival rate surges to 40% if detected early when surgical resection with adjuvant chemotherapy remains an option.[1–3] The Cancer of Pancreas Screening Study 5 data revealed that patients under surveillance with stage I can survive up to 9.8 years in contrast to those outside surveillance with stage IV of about 1.5 years.[4] Survival rates in PDAC patients not significantly improving in the past decade[5,6] underscores the need of developing novel approaches and biomarker assays for early detection of the disease.

A major challenge in early detection of PDAC is the absence of sensitive and specific biomarkers credentialed for detecting asymptomatic patients. The only serum biomarker sialylated Lewis antigen carbohydrate antigen 19-9 (CA19-9) has limited sensitivity for diagnosing patients with early-stage cancer.[7] 15%–25% of PDAC patients have CA19-9 levels below the disease cutoff of 37 U/mL[8,9] while false positives are detected in patients with benign diseases, such as acute and chronic pancreatitis.[10–12] About 5%–10% of the population does not produce the antigen due to germline mutations.[9] CA19-9 is clinically used for screening symptomatic individuals and disease surveillance/therapeutic response.[13,14] Epidemiological studies have indicated association of both acute and chronic pancreatitis with risk of pancreatic cancer. Though the findings from patients with acute pancreatitis have been conflicting, chronic pancreatitis has been reported to increase risk of PDAC.[15,16] Differential diagnosis between PDAC and chronic pancreatitis remains clinically challenging, and developing sensitive and specific blood-based biomarkers for discriminating these patients remains research priority with clinical implications.

The blood-based biomarkers being investigated include tumor-derived mutant/methylated DNA, RNA, proteins, and metabolites either in membrane-free form or encapsulated in extracellular vesicles/exosomes.[17–22] Circulating miRNAs have gained widespread attention as potential liquid biopsy biomarkers due to their frequent dysregulated expression and presence at altered levels in blood and body fluids of patients with diseases, including cancer.[23,24] Prior studies have described circulating miRNAs as candidate biomarkers of PDAC associated with disease stage and prognosis.[25–30] Recently, a 13-miRNA signature comprising exosomal (exo) and total circulating miRNAs with robust diagnostic accuracy was reported for early-stage PDAC.[31] However, clinical relevance of the findings remains uncertain since the control subjects were younger healthy individuals rather than age-matched ones and did not include benign inflammatory pancreatic disease, like pancreatitis.

Here, we describe an adequately controlled study, performed with an extraction-free high-resolution miRNA profiling platform, EdgeSeq[32] for small input samples, that revealed differentially abundant plasma miRNAs associated with cancer-relevant pathways as early detection biomarkers of PDAC. An atlas of 72 significantly elevated plasma miRNAs from stage II PDAC patients compared with age-matched healthy and pancreatitis controls was identified. Elevated miRNAs involved in cancer-relevant pathways were prioritized as candidate biomarkers for panel development in discriminating early-stage disease from controls which led to the development of a three-miRNA signature, which improved diagnostic performance of CA19-9 as a combination panel in detecting early-stage disease. The miRNA signature also performed well in stratifying PDACs with varying content of mutant *KRAS* circulating tumor DNA (ctDNA) and 2 imaging-based disease subtypes. Diagnostic significance of the miRNA signature was further evident from the lead-time trajectory of progressively increasing area under the curve (AUC) prior to PDAC diagnosis.

Methods

Diagnostic PDAC Cohort

A total of 203 individual subjects were enrolled in accordance with approved institutional review board protocols: Lab00-396, PA11-0670 and PA15-0014 of MD Anderson Cancer Center and University of Pittsburgh Medical Center. The multicenter diagnostic PDAC cohort included samples from MD Anderson Cancer Center and University of Pittsburgh Medical Center grouped by final pathologic diagnosis of pancreatitis and PDAC with staging and age-matched nondisease controls (N = 203: age-matched healthy controls, n = 46; age-matched pancreatitis controls, n = 36; stage I-IV PDAC, n = 121). Plasma samples were prepared from peripheral blood collected in purple top ethylenediaminetetraacetic acid coated tubes according to standard operating procedure of the Early Detection Research Network of the National Cancer Institute. Detailed information is presented in Table A1.

Prediagnostic Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Cohort

The PLCO cancer screening trial, a multicenter randomized clinical trial in the United States, comprises plasma samples from 10 US screening centers for about 155,000 men and women aged 55–74 years at their baseline enrollment to evaluate early detection assays for PLCO cancer and disease-specific mortality.[33] This study received approval from the National Cancer Institute under Fred Hutchinson Cancer Center protocol number: 6007; 48 PDAC cases within 24 months prior to diagnosis and 48 age-, race-, and gender-matched controls ($N = 96$) were included in the study. Patients with pancreatic cysts were excluded. All samples were blinded for generating data and only unblinded to the statisticians for analyzing and categorizing the samples with time prior to diagnosis. Sample information is described in Table A5.

miRNA Profiling

15 μL of plasma was profiled for miRNAs by the extraction-free method using the HTG edge miRNA whole transcriptome assay kit (HTG Molecular, Tuscon, AZ, Cat# 916-001-024) according to the recommended vendor's protocol. Samples retrieved from the HTG EdgeSeq processor were molecular barcoded for preparing libraries. The libraries were quantified by quantitative PCR (qPCR) using KAPA Library Quantification kit (Roche, Cat# KK4824) and loaded onto the MiSeq (Illumina, RRID: SCR_016379) together with the MiSeq Reagent Kit v3 (150 cycles) (Illumina, Cat# MS-102-3001). Raw FASTQ file of each sample was parsed, normalized into count per million and transformed to $\log_2$CPM using HTG EdgeSeq Parser and HTG EdgeSeq Reveal software (RRID: SCR_022982).

CA19-9 Measurement

Plasma levels of CA19-9 were determined using a Human CA19-9 ELISA kit (DRG International, Cat# EIA5069R, RRID: AB_3095039) following the manufacturer's protocol. Briefly, samples were sequentially incubated with precoated antibody and HRP-conjugated antibody against CA19-9. Then, substrate was added and followed by stop solution. Absorbance at 450 nm was measured using an ELISA plate reader (iMark Microplate Reader, BioRad) and determined CA19-9 concentrations using four-parameter logistic regression model.

Imaging Characteristics of PDAC

Diagnostic imaging-based contrast CT scans were acquired under an institutional review board–approved protocol PA14-0646 for analyses. The slice thickness for postcontrast scans ranged from 2.5 to 5 mm, which were qualitatively and quantitatively categorized based on conspicuity and shape into low-delta and high-delta subtypes according to the previously published criteria.[34] Imaging subytpes of PDAC are shown in Table A4.

miRNA-mRNA Regulatory Network Analysis and Gene Set Enrichment Analysis (GSEA)

Pathway enrichment analysis using Ingenuity Pathway Analysis (IPA, RRID: SCR_008653) package was performed to interrogate for the top diseases and biological functions of miRNAs in the elevated transcriptome atlas identified in the PDAC plasma samples based on experimentally validated/predicted target mRNAs. GSEA (RRID: SCR_003199) of The Cancer Genome Atlas (TCGA)-PAAD miRNA data was obtained from http://gdac.broadinstitute.org/and mRNA data for GSEA was downloaded from TCGA data portal and normalized using the quantile normalization technique. A total of 178 pancreatic adenocarcinoma cases with the miRNA profiling data were used and calculated for the level of the three-miRNA signature using the average log2 expression of let-7i-5p, miR-130a-3p and miR-221-3p. Then, the samples were categorized into 2 groups by median into 89 samples with high miRNA signature level and 89 samples with low miRNA signature level. The datasets were analyzed using GSEA,[35,36] applied Signal-to-noise and 1000 permutations of the genes. The gene sets for all hallmark and oncogenic signatures were obtained from the Molecular Signatures Database v2023.1 (RRID: SCR_016863) and ranked by normalized enrichment score (NES) and false discovery rate adjusted P value (q-value).

Statistical Analysis

Comparisons of demographic and clinicopathological parameters as well as biomarkers between cancer cases and controls (healthy or pancreatitis controls) were performed by Wilcoxon rank sum test or Kruskal-Wallis test. The Benjamini-Hochberg method was used to adjust the P values for multiple testing.[37] Considering the biological/clinical importance as well as the statistical significance, candidate miRNA biomarkers were selected for panel development. Diagnostic performance was evaluated using the AUC and its 95% confidence interval based on the receiver operation characteristic analysis. The logistic regression model was used to combine the candidate individual miRNA biomarkers, CA19-9, and other patient characteristics for the panel development. We compare the AUCs of the three-miRNA signature with and without CA19-9 to the AUC using CA19-9 alone as reference, and P values were calculated using the bootstrapping method with 1000 bootstrapping samples. Sensitivity, specificity, positive predictive value, negative predictive value, precision, and accuracy of biomarkers were computed using the pROC package (RRID: SCR_024286). All statistical analysis was performed using R statistical software (version 4.1.1; R Foundation for Statistical Computing, Vienna, Austria; RRID: SCR_001905).

Results

Plasma miRNA Profiling for PDAC Early Detection Biomarkers

Circulating plasma miRNAs were profiled to identify candidate biomarkers distinguishing stage II PDAC cases with early-stage resectable tumors, from healthy and pancreatitis controls. 72 miRNAs significantly elevated were identified (Figure 1A and B), which discriminated stage II PDAC patients from both healthy individuals and subjects with pancreatitis (Table A1). IPA analysis revealed involvement of miRNAs in KRAS/EGFR/GRB2, TP53/MDM2, PI3K/AKT1, TGFBR/SMAD pathways regulating cellular development, growth-proliferation, and death-survival functions (Figure A1A and B).

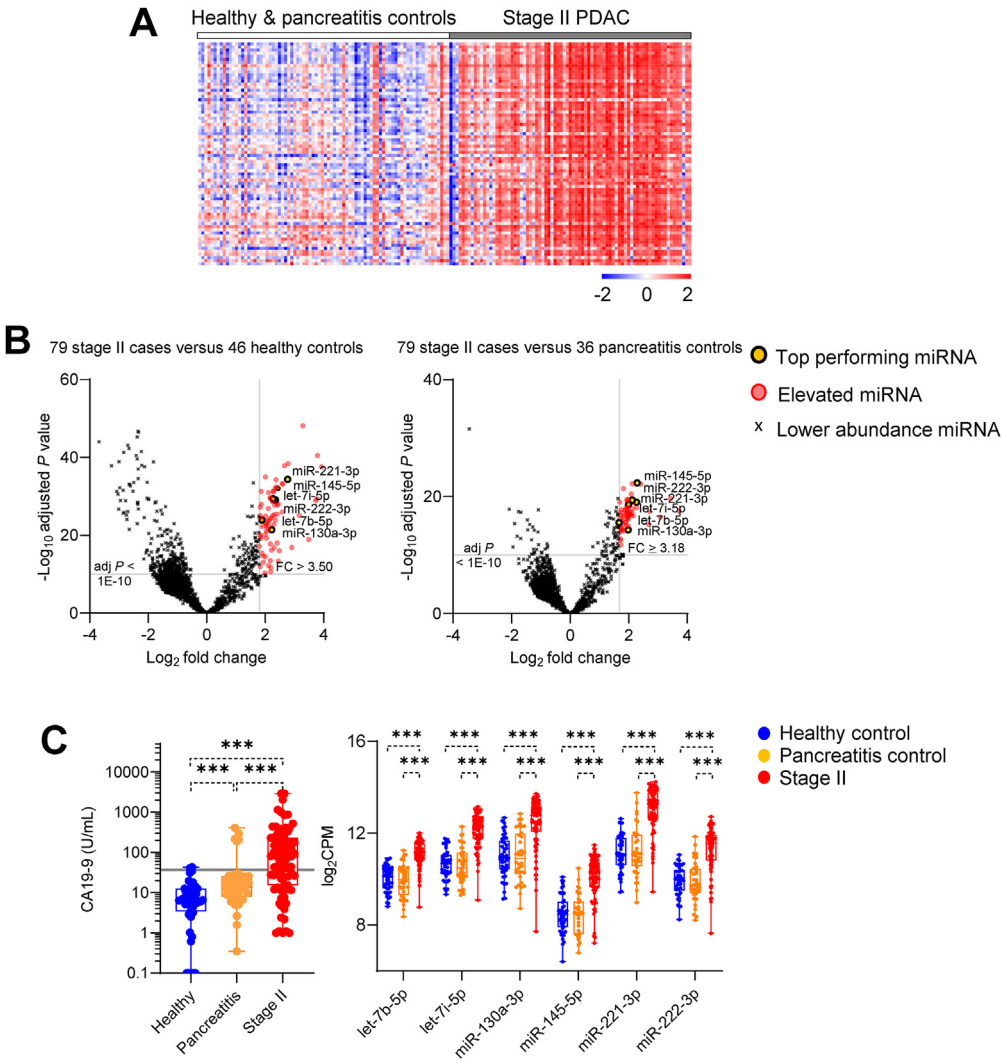


Figure 1. Significantly elevated miRNAs in plasma of stage II PDAC patients identified from an atlas of elevated circulating miRNAs. (A) Heatmap of elevated miRNAs with AUC above 0.8 in identifying stage II PDAC cases ($n = 79$) from healthy and pancreatitis controls ($n = 82$). (B) Volcano plots of differentially expressed miRNAs between stage II PDAC patients vs non-disease controls (left, 79 stage II cases vs 46 healthy controls; right, 79 stage II cases vs 36 pancreatitis controls). Yellow circles represent top-performing miRNAs selected based on cutoff AUC values (>0.8) and pathway involvement; red circles represent elevated miRNAs; black cross signs represent lower abundant miRNAs. (C) Levels of CA19-9 (left) and 6 top-performing miRNAs: let-7b-5p, let-7i-5p, miR-130a-3p, miR-145-5p, miR-221-3p and miR-222-3p (right). Blue circles, healthy controls ($n = 46$); yellow circles, pancreatitis controls ($n = 36$); red circles, stage II PDAC patients ($n = 79$). *, $P < .05$; **, $P < .01$; ***, $P < .001$.

miRNA Biomarkers for Early Detection PDAC

The top 20 performing miRNAs in cancer-relevant pathways were analyzed further for their biological/clinical significance based on published literature, including ours.[25] Of these, 6 miRNAs (let-7b-5p, let-7i-5p, miR-130a-3p, miR-145-5p, miR-221-3p, and miR-222-3p) with seed sequences conserved in 4 miRNA families let-7, miR-130, miR-145, and miR-221/222 were elevated in stage II patients (all adjusted $P < 1E\text{-}10$; Figure 1B and C). In contrast, CA19-9 was elevated in some pancreatitis controls and at lower than the disease cutoff value of 37 U/mL in a subset of stage II patients (Figure 1C). Univariate analyses of miRNAs with AUC values above 0.770 across different comparison groups of stages I/II/I-IV PDAC cases and healthy/pancreatitis controls revealed statistically significant differences (all adjusted $P < .002$; Table 1). Patient demographics and known etiological factors including age, alcohol, diabetes, and smoking statuses were also interrogated for panel development (Table A2).

To avoid model complexity and overfitting, miRNA signature development was restricted to three-miRNA combinations. A three-miRNA signature (let-7i-5p, miR-130a-3p, and miR-221-3p) was identified as the best-performing panel in discriminating cases from both healthy and pancreatitis controls with the highest AUC. The criteria for developing this three-miRNA biomarker

Table 1. Diagnostic Performance of Individual miRNA Biomarkers in Discriminating PDAC Patients Across Different Stages From Healthy (Top), Chronic Pancreatitis (Middle), and Acute & Chronic Pancreatitis (Bottom) Controls

18 stage I cases	vs 46 healthy controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.900 (0.790–0.976)	0.766 (0.906–0.969)	0.548 (0.867–1)	0.683	0.611	0.852 (0.920–1)	<.0001
let-7i-5p	0.906 (0.812–0.977)	0.750 (0.906–0.969)	0.529 (0.923–1)	0.722	0.667	0.852 (0.920–1)	<.0001
miR-130a-3p	0.786 (0.628–0.914)	0.672 (0.828–0.922)	0.452 (0.722–1)	0.500	0.389	0.812 (0.885–0.971)	.0005
miR-145-5p	0.866 (0.740–0.963)	0.719 (0.844–0.938)	0.500 (0.682–1)	0.556	0.444	0.852 (0.936–1)	<.0001
miR-221-3p	0.893 (0.761–0.990)	0.828 (0.922–0.984)	0.654 (0.929–1)	0.778	0.667	0.868 (0.936–1)	<.0001
miR-222-3p	0.836 (0.678–0.957)	0.766 (0.875–0.953)	0.556 (0.800–1)	0.611	0.611	0.837 (0.913–0.979)	<.0001
79 stage II cases	vs 46 healthy controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.925 (0.878–0.966)	0.808 (0.872–0.928)	0.905 (0.958–1)	0.747	0.684	0.662 (0.774–0.880)	<.0001
let-7i-5p	0.931 (0.888–0.968)	0.800 (0.864–0.920)	0.897 (1-1)	0.785	0.747	0.648 (0.730–0.889)	<.0001
miR-130a-3p	0.860 (0.793–0.919)	0.736 (0.808–0.872)	0.855 (0.940–1)	0.637	0.532	0.589 (0.679–0.793)	<.0001
miR-145-5p	0.895 (0.836–0.948)	0.768 (0.848–0.904)	0.852 (0.928–1)	0.658	0.537	0.615 (0.745–0.897)	<.0001
miR-221-3p	0.929 (0.879–0.968)	0.824 (0.888–0.936)	0.928 (0.984–1)	0.810	0.740	0.687 (0.780–0.880)	<.0001
miR-222-3p	0.888 (0.827–0.943)	0.784 (0.848–0.904)	0.912 (0.971–1)	0.754	0.671	0.634 (0.726–0.827)	<.0001
121 stage I-IV cases	vs 46 healthy controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.897 (0.848–0.938)	0.749 (0.826–0.880)	0.933 (0.970–1)	0.691	0.620	0.524 (0.627–0.721)	<.0001
let-7i-5p	0.907 (0.860–0.947)	0.731 (0.808–0.898)	0.915 (0.979–1)	0.719	0.661	0.506 (0.597–0.784)	<.0001
miR-130a-3p	0.816 (0.745–0.877)	0.659 (0.743–0.832)	0.882 (0.949–0.989)	0.540	0.438	0.441 (0.518–0.667)	<.0001
miR-145-5p	0.873 (0.815–0.925)	0.701 (0.820–0.898)	0.880 (0.935–1)	0.587	0.475	0.478 (0.627–0.825)	<.0001
miR-221-3p	0.906 (0.858–0.947)	0.772 (0.850–0.904)	0.942 (0.976–1)	0.744	0.665	0.549 (0.657–0.768)	<.0001
miR-222-3p	0.856 (0.793–0.907)	0.719 (0.790–0.856)	0.931 (0.978–1)	0.691	0.612	0.495 (0.575–0.677)	<.0001
18 stage I cases	vs 23 chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.886 (0.768–0.969)	0.732 (0.854–0.951)	0.643 (0.833–1)	0.556	0.500	0.742 (0.870–1)	<.0001
let-7i-5p	0.885 (0.773–0.971)	0.756 (0.854–0.951)	0.667 (0.917–1)	0.722	0.333	0.742 (0.852–1)	<.0001
miR-130a-3p	0.775 (0.606–0.913)	0.683 (0.805–0.902)	0.600 (0.900–1)	0.556	0.444	0.676 (0.786–1)	.0029
miR-145-5p	0.857 (0.717–0.964)	0.732 (0.854–0.927)	0.654 (0.810–1)	0.556	0.333	0.741 (0.885–1)	.0001
miR-221-3p	0.850 (0.698–0.959)	0.756 (0.878–0.951)	0.714 (0.875–1)	0.667	0.111	0.742 (0.875–1)	.0001
miR-222-3p	0.824 (0.671–0.945)	0.732 (0.854–0.927)	0.682 (0.842–1)	0.611	0.167	0.724 (0.864–1)	.0003

79 stage II cases	vs 23 chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.897 (0.824–0.959)	0.794 (0.873–0.931)	0.910 (0.958–1)	0.559	0.408	0.528 (0.667–0.840)	<.0001
let-7i-5p	0.910 (0.844–0.963)	0.765 (0.843–0.922)	0.935 (0.984–1)	0.785	0.443	0.488 (0.590–0.792)	<.0001
miR-130a-3p	0.853 (0.767–0.919)	0.657 (0.765–0.882)	0.911 (0.981–1)	0.671	0.532	0.396 (0.488–0.727)	<.0001
miR-145-5p	0.883 (0.801–0.947)	0.706 (0.833–0.922)	0.909 (0.960–1)	0.635	0.370	0.432 (0.595–0.818)	<.0001
miR-221-3p	0.900 (0.817–0.962)	0.775 (0.863–0.931)	0.937 (0.983–1)	0.785	0.256	0.500 (0.636–0.808)	<.0001
miR-222-3p	0.867 (0.776–0.937)	0.706 (0.833–0.912)	0.917 (0.967–1)	0.658	0.278	0.429 (0.583–0.759)	<.0001
121 stage I-IV cases	vs 23 chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.873 (0.790–0.941)	0.764 (0.826–0.903)	0.936 (0.971–1)	0.497	0.376	0.383 (0.476–0.645)	<.0001
let-7i-5p	0.888 (0.810–0.951)	0.701 (0.792–0.903)	0.947 (0.987–1)	0.711	0.364	0.339 (0.431–0.647)	<.0001
miR-130a-3p	0.808 (0.721–0.887)	0.576 (0.674–0.861)	0.930 (0.986–1)	0.587	0.446	0.269 (0.324–0.556)	<.0001
miR-145-5p	0.863 (0.783–0.931)	0.674 (0.826–0.910)	0.933 (0.968–1)	0.564	0.332	0.317 (0.476–0.690)	<.0001
miR-221-3p	0.872 (0.784–0.941)	0.715 (0.837–0.903)	0.949 (0.980–1)	0.694	0.200	0.354 (0.495–0.639)	<.0001
miR-222-3p	0.837 (0.746–0.914)	0.639 (0.792–0.875)	0.940 (0.971–1)	0.595	0.215	0.301 (0.429–0.568)	<.0001
18 stage I cases	vs 36 acute & chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.891 (0.788–0.966)	0.704 (0.852–0.944)	0.529 (0.762–1)	0.556	0.500	0.823 (0.914–1)	<.0001
let-7i-5p	0.883 (0.765–0.967)	0.704 (0.852–0.944)	0.531 (0.778–1)	0.556	0.333	0.818 (0.909–1)	<.0001
miR-130a-3p	0.773 (0.606–0.909)	0.630 (0.796–0.889)	0.472 (0.692–1)	0.444	0.278	0.761 (0.857–1)	.0015
miR-145-5p	0.864 (0.719–0.961)	0.722 (0.852–0.926)	0.545 (0.750–1)	0.556	0.333	0.818 (0.917–1)	<.0001
miR-221-3p	0.838 (0.687–0.954)	0.741 (0.833–0.926)	0.571 (0.727–0.933)	0.278	0.111	0.829 (0.917–1)	.0001
miR-222-3p	0.823 (0.660–0.948)	0.722 (0.833–0.926)	0.560 (0.727–1)	0.444	0.167	0.809 (0.903–1)	.0002
79 stage II cases	vs 36 acute & chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.904 (0.845–0.957)	0.791 (0.861–0.922)	0.881 (0.935–0.985)	0.567	0.410	0.615 (0.744–0.868)	<.0001
let-7i-5p	0.907 (0.849–0.957)	0.765 (0.843–0.913)	0.883 (0.952–1)	0.633	0.443	0.581 (0.689–0.871)	<.0001
miR-130a-3p	0.845 (0.773–0.914)	0.687 (0.783–0.870)	0.851 (0.938–1)	0.532	0.456	0.500 (0.600–0.818)	<.0001
miR-145-5p	0.894 (0.832–0.949)	0.765 (0.843–0.904)	0.889 (0.957–1)	0.643	0.372	0.581 (0.680–0.842)	<.0001
miR-221-3p	0.892 (0.821–0.948)	0.783 (0.861–0.913)	0.877 (0.932–0.984)	0.453	0.258	0.608 (0.744–0.875)	<.0001
miR-222-3p	0.866 (0.795–0.933)	0.730 (0.826–0.896)	0.867 (0.929–1)	0.544	0.278	0.538 (0.689–0.833)	<.0001

121 stage I-IV cases vs 36 acute & chronic pancreatitis controls							
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
let-7b-5p	0.880 (0.813–0.935)	0.745 (0.828–0.885)	0.911 (0.953–0.990)	0.502	0.378	0.468 (0.579–0.705)	<.0001
let-7i-5p	0.883 (0.821–0.937)	0.720 (0.818–0.898)	0.906 (0.954–1)	0.537	0.364	0.444 (0.571–0.756)	<.0001
miR-130a-3p	0.802 (0.729–0.874)	0.599 (0.713–0.847)	0.883 (0.946–1)	0.446	0.355	0.358 (0.439–0.646)	<.0001
miR-145-5p	0.871 (0.810–0.925)	0.701 (0.790–0.898)	0.908 (0.965–1)	0.575	0.334	0.430 (0.525–0.771)	<.0001
miR-221-3p	0.864 (0.797–0.927)	0.758 (0.841–0.898)	0.904 (0.944–0.982)	0.379	0.201	0.485 (0.609–0.762)	<.0001
miR-222-3p	0.836 (0.764–0.902)	0.669 (0.803–0.866)	0.900 (0.942–0.989)	0.463	0.215	0.400 (0.550–0.681)	<.0001

P values were computed using the Wilcoxon test and adjusted with the Benjamini-Hochberg method.
95% CI, 95% confidence interval; AUC, Area under the ROC, Curve; NPV, negative predictive value; PPV, positive predictive value; Sens@95%, sensitivity after fixing the specificity at 95%; Sens@99%, sensitivity after fixing the specificity at 99%.

signature included prioritizing the miRNAs targeting PDAC driving *KRAS* pathways and the *KRAS* downstream pathway genes, PI3K/PTEN, MAPK, and SMAD4, which included miR-130a-3p and miR-221/222-3p (Figure A2A and B). To validate the functional significance of the identified miRNA signature in PDAC, we performed GSEA of all hallmark genes for 178 PDAC samples from TCGA. High expression of the miRNA signature was associated with hallmark signatures representing TNFA signaling, G2M checkpoint, E2F targets, interferon alpha and gamma responses, mitotic spindle, p53 pathway, hypoxia, glycolysis, inflammatory response, apoptosis, and epithelial to mesenchymal transition; all NES $>$ 1.3, all q-value$<$0.05 (Figure 2A). Interestingly, PDAC samples with high miRNA signature revealed genes associated with *KRAS* dependency and upregulation of MEK, MYC, AKT, IL15, E2F3, EGFR, SRC, IL2, and TGFB pathways along with downregulation of P53, Polycomb-Repressive Complex 2 and retinoblastoma in oncogenic pathways (all NES$>$1.6, all q-value$<$0.01; Figure 2B).

This miRNA signature (let-7i-5p, miR-130a-3p and miR-221-3p) identified early- and late-stage PDAC patients from healthy controls (stage I: AUC = 0.970 [95% confidence interval 0.908–1], P = .2596; stage II: AUC = 0.975 [0.950–0.993], P = .0003; stage I-IV: AUC = 0.974 [0.953–0.991], P = .0002) independently and in combination with CA19-9 (stage I: AUC = 1 [1-1], P = .0602; stage II: AUC = 0.992 [0.980–0.999], P < .0001; stage I-IV: AUC = 0.995 [0.987–1], P < .0001) compared with CA19-9 alone (stage I: AUC = 0.911 [0.813–0.986]; stage II, AUC = 0.841 [0.771–0.905]; stage I-IV, AUC = 0.866 [0.810–0.915]). Moreover, the miRNA signature revealed statistically robust performance in discriminating stage I, II, I-V PDAC patients from chronic pancreatitis controls (stage I: AUC = 0.932 [0.836–1], P = .0626; stage II: AUC = 0.931 [0.839–0.986], P = .0013; stage I-IV: AUC = 0.929 [0.840–0.984], P = .0035) with significantly higher performance as a combination panel (stage I: AUC = 0.971 [0.913–1], P = .0109; stage II: AUC = 0.943 [0.873–0.988], P = .0003; stage I-IV: AUC = 0.951 [0.889–0.990], P = .0003 than the performance of CA19-9 alone (stage I: AUC = 0.763 [0.601–0.901]; stage II, AUC = 0.701 [0.580–0.815]; stage I-IV, AUC = 0.735 [0.632–0.829]). The miRNA signature also demonstrated similar strong performance in discriminating stage I, II, and I-IV PDAC patients from the combined pancreatitis controls of subjects with acute and chronic pancreatitis. In addition to improved diagnostic performance of the miRNA signature by itself and in combination with CA19-9, diagnostic accuracy, sensitivities at 95% and 99% specificity, positive predictive value and negative predictive value of the miRNA signature without and with CA19-9 were superior to CA19-9 across all stages of PDAC in reference to healthy and pancreatitis controls. The results are summarized in Table 2 and Figure 2C–E.

Incidence of jaundice has been associated with elevated CA19-9.[12] So, we investigated the levels of CA19-9 and the miRNA signature among patients with and without jaundice. Among stage II PDAC patients, CA19-9 levels were significantly elevated in patients with jaundice compared with

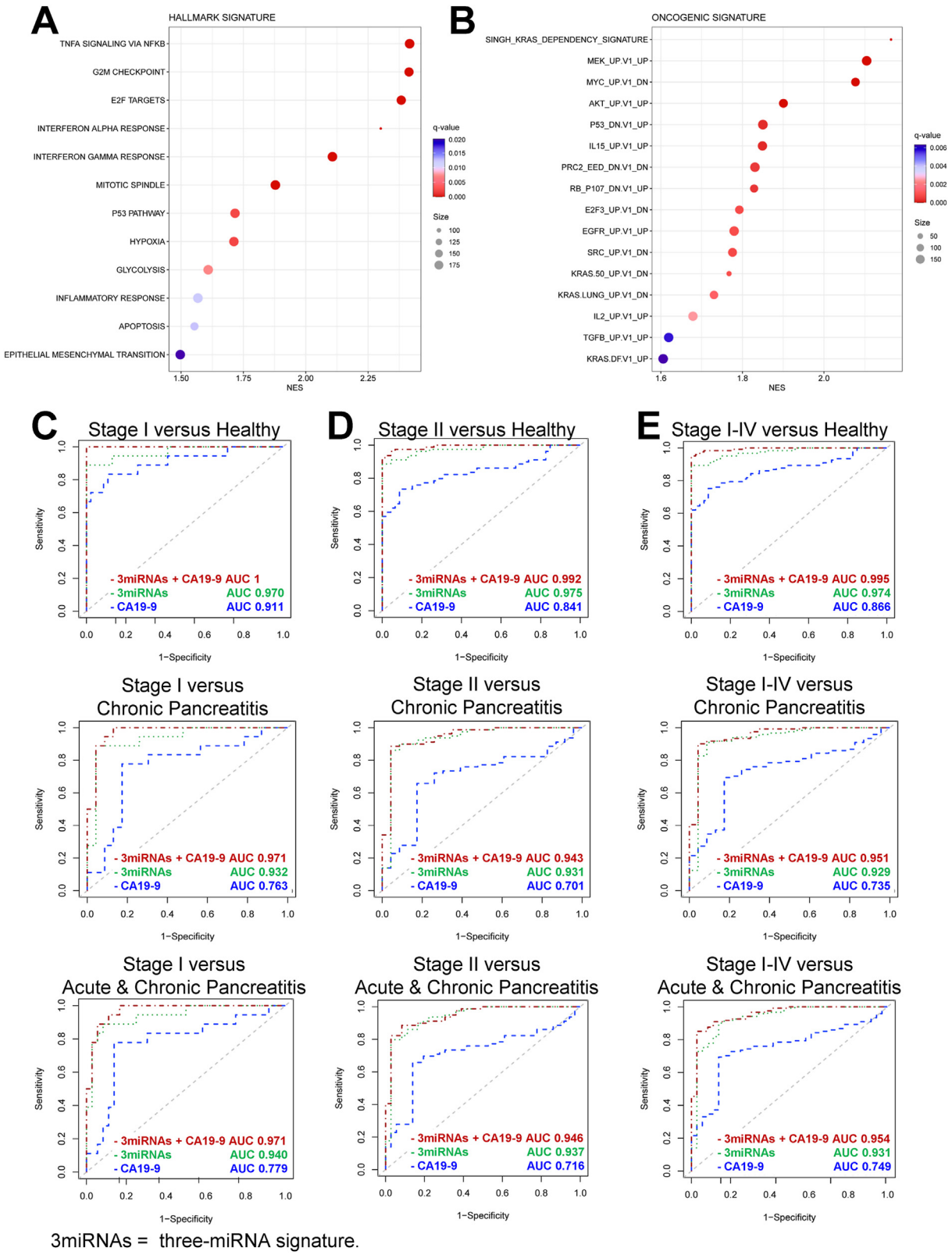


Figure 2. Association of the 3 miRNAs with cancer hallmark pathways and diagnostic performance of the three-miRNA signature with and without CA19-9 in discriminating PDAC cases from healthy and pancreatitis controls. (A and B) GSEA of 178 TCGA PAAD cases with high and low levels of the three-miRNA signature for hallmark signatures (A) and (B) oncogenic signatures ranked by normalized enrichment score (NES) and adjusted *P* value (q-value). (C–E) ROC curve analysis for the miRNA signature, the miRNA-CA19-9 combination panel, and CA19-9 in stage-specific PDAC patients (C, stage I, $n = 17$; D, stage II, n = 79; E, stage I-IV, $n = 121$) vs healthy (top, $n = 46$), chronic pancreatitis (middle, $n = 23$), or combined acute & chronic pancreatitis (bottom, $n = 36$) controls.

those without jaundice (median 175.5 vs 42.80 U/mL, $P <$ .01). A similar trend was also observed in stage I patients (median 141.4 vs 80.70 U/mL, $P = .63$), and III-IV (median 1574 vs 328 U/mL, $P = .92$). For the 3 miRNAs in the signature, levels were comparable among PDAC patients with and without jaundice across all stages (Figure A3A and B). Taken together, the data indicate that while jaundice leads to increased CA19-9 in some PDAC patients, the levels of the 3 miRNAs in the signature do not change in PDAC patients with and without jaundice.

Another panel of miRNAs differentiating late-stage disease from controls (Figure A4A–C) was identified which could serve as candidate biomarkers of disease surveillance/therapeutic response for PDAC patients.

miRNA Signature and Mutant KRAS *ctDNA*

We investigated the performance of the miRNA signature in reference to mutant *KRAS* ctDNA in a subset of PDAC cases, described earlier.[38] Majority of ctDNA-positive patients were diagnosed with late-stage disease at stage IV (Table A3), demonstrating the limitation of a single driver mutant *KRAS* ctDNA allele for early detection of PDAC.[17,38,39] Levels of the 3 miRNAs were significantly higher in both ctDNA-negative and positive PDAC patients compared with healthy and pancreatitis controls (Figure 3A). This miRNA signature by itself displayed superior diagnostic performance in differentiating both mutant *KRAS* ctDNA-negative and positive PDAC cases from healthy individuals (ctDNA-negative: AUC = 0.932 [0.871–0.978], $P = .1892$; ctDNA-positive, AUC = 0.957 [0.878–1], $P = .0315$) and in combination with CA19-9 (ctDNA-negative: AUC = 0.972 [0.930–0.998], $P = .0185$; ctDNA-positive, AUC = 0.976 [0.920–1], $P = .0120$) compared to CA19-9 alone (ctDNA-negative: AUC = 0.867 [0.782–0.937]; ctDNA-positive: AUC = 0.782 [0.638–0.911]). The miRNA signature also discriminated both ctDNA-negative and positive PDAC cases from chronic pancreatitis (ctDNA-negative: AUC = 0.894 [0.792–0.980], $P = .1068$; ctDNA-positive: AUC = 0.943 [0.845–1], $P = .0146$) and combined with CA19-9 (ctDNA-negative: AUC = 0.943 [0.870–0.997], $P = .0158$; ctDNA-positive, AUC = 0.984 [0.935–1], $P = .0029$) compared with CA19-9 alone (ctDNA-negative: AUC = 0.756 [0.616–0.874]; ctDNA-positive: AUC = 0.677 [0.0.467–0.867]). The miRNA signature also discriminated ctDNA-negative as well as ctDNA-positive PDAC patients from the control subjects with acute and chronic pancreatitis taken together (Figure 3B).

miRNA Signature and PDAC Imaging Subtypes

The miRNA signature was next investigated in a subset of PDAC patients characterized for the recently described CT-derived radiographic imaging subtypes.[34,40] Diagnostic performance of the miRNA signature and CA19-9 were analyzed in the 2 imaging subtypes of PDAC, low-delta and high-delta based on quantitative characteristics of the tumor border on baseline CT scans (Table A4). Levels of the miRNAs were significantly higher in both low- and high-delta PDAC subtypes than healthy and pancreatitis controls unlike CA19-9, which was lower in low-delta than high-delta subtype (Figure 4A). The miRNA signature by itself revealed robust diagnostic performance (low-delta: AUC = 0.957 [0.904–0.996], $P = .0622$; high-delta: AUC = 0.978 [0.955–0.994], $P = .0021$) and in combination with CA19-9 (AUC = 1.0 [1-1], $P = .0049$; high-delta: AUC = 0.994 [0.983–1], $P = .001$) compared to CA19-9 alone (low-delta: AUC = 0.844 [0.735–0.942]; high-delta: AUC = 0.888 [0.832–0.937]). in differentiating both low- and high-delta PDAC cases from healthy controls. It also discriminated both low- and high-delta PDAC cases by itself (low-delta: AUC = 0.906 [0.811–0.988], $P = .0242$; high-delta: AUC = 0.933 [0.847–0.986], $P = .0095$) and in combination with CA19-9 (low-delta: AUC = 0.945 [0.864–0.997], $P = .0038$; high-delta: AUC = 0.958 [0.910–0.991], $P = .0007$) compared to CA19-9 alone (low-delta: AUC = 0.700 [0.556–0.843]; high-delta: AUC = 0.766 [0.669–0.858]) from chronic pancreatitis controls. Similarly, the miRNA signature also discriminated low-delta and high-delta PDAC from both acute and chronic pancreatitis (Figure 4B).

miRNA Signature in Early-Stage PDAC Patients with CA19-9 Value Below Disease Cutoff

miRNA signature was analyzed as an independent biomarker among early-stage PDAC patients with CA19-9 levels below the disease cutoff value of 37 U/mL. Levels of the miRNAs were significantly higher in both low- and high-CA19-9 groups than in healthy and pancreatitis controls (Figure 5A). Receiver operation characteristic analysis revealed superior diagnostic performance of the miRNA signature alone in stage I-II PDAC patients displaying CA19-9 below the cutoff (compared to healthy controls, AUC = 0.983 [0.955–0.970], $P <$ .0001; compared to chronic pancreatitis controls, AUC = 0.941 [0.854–0.995], $P <$ .0001) vs CA19-9 alone (compared to healthy controls, AUC = 0.610 [0.484–0.735]; compared to chronic pancreatitis controls, AUC = 0.587 [0.438–0.728]). The miRNA signature also discriminated stage I-II PDAC patients with low CA19-9 from the control cohort comprising of both acute and chronic pancreatitis (Figure 5B).

Lead-Time Trajectory of the miRNA Signature in Prediagnostic PDAC Patients

Sensitive and specific early detection biomarker is expected to detect asymptomatic patients with incipient disease displaying lead-time trajectory prior to diagnosis. The miRNA signature was, thus, analyzed in blinded 48 prediagnostic plasma samples of PDAC cases and 48 matched controls collected in the PLCO cancer screening trial[33] and grouped the prediagnosed PDAC cases by time intervals of blood collection prior to diagnosis with gender, age, race, BMI, and etiology factor-matched controls (Table A5). The miRNA signature AUC values progressively increased to

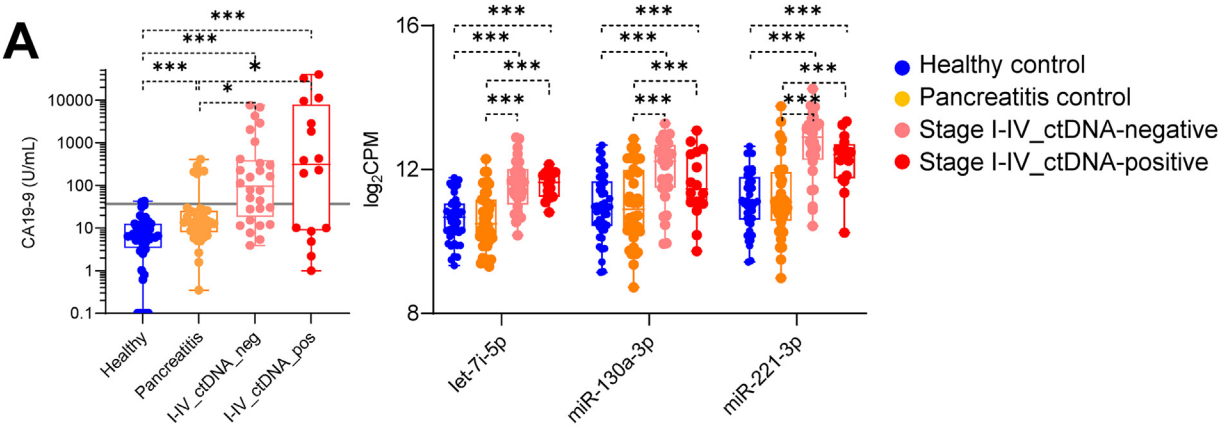
Table 2. Diagnostic Performance of miRNA Signature in Identifying PDAC Patients Across Different Stages From Healthy (Top), Chronic Pancreatitis (Middle), and Acute & Chronic Pancreatitis (Bottom) Controls

18 stage I cases	vs 46 healthy controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.911 (0.813–0.986)	0.781 (0.906–0.969)	0.583 (0.857–1)	0.722	0.667	0.868 (0.936–1)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.970 (0.908–1)	0.891 (0.969–1)	0.750 (1-1)	0.889	0.889	0.920 (0.976–1)	.2596
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	1 (1-1)	1 (1-1)	1 (1-1)	1.000	1.000	1 (1-1)	.0602
79 stage II cases	vs 46 healthy controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.841 (0.771–0.905)	0.728 (0.808–0.872)	0.886 (0.944–1)	0.620	0.570	0.575 (0.672–0.771)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.975 (0.950–0.993)	0.888 (0.936–0.976)	0.952 (1-1)	0.911	0.886	0.767 (0.852–0.957)	.0003
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.992 (0.980–0.999)	0.928 (0.968–0.992)	0.963 (1-1)	0.962	0.924	0.836 (0.936–1)	<.0001
121 stage I-IV cases	vs 46 healthy controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.866 (0.810–0.915)	0.707 (0.796–0.862)	0.929 (0.969–1)	0.661	0.620	0.484 (0.586–0.694)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.974 (0.953–0.991)	0.880 (0.928–0.964)	0.975 (1-1)	0.893	0.893	0.697 (0.793–0.896)	.0002
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.995 (0.987–1)	0.940 (0.976–0.994)	0.984 (1-1)	0.975	0.950	0.821 (0.920–1)	<.0001
18 stage I cases	vs 23 chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.763 (0.601–0.901)	0.683 (0.805–0.927)	0.619 (0.778–0.938)	0.111	0.111	0.720 (0.842–0.958)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.932 (0.836–1)	0.854 (0.927–1)	0.810 (0.941–1)	0.889	0.278	0.821 (0.920–1)	.0626
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.971 (0.913–1)	0.878 (0.951–1)	0.783 (0.900–1)	0.889	0.500	0.913 (1-1)	.0134
79 stage II cases	vs 23 chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.701 (0.580–0.815)	0.618 (0.725–0.814)	0.866 (0.927–0.982)	0.228	0.139	0.340 (0.439–0.559)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.931 (0.839–0.986)	0.833 (0.902–0.961)	0.948 (0.986–1)	0.861	0.139	0.588 (0.719–0.880)	.0013
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.943 (0.873–0.988)	0.843 (0.912–0.961)	0.956 (0.986–1)	0.886	0.342	0.605 (0.731–0.905)	.0003
121 stage I-IV cases	vs 23 chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.735 (0.632–0.829)	0.653 (0.736–0.812)	0.913 (0.955–0.989)	0.273	0.215	0.276 (0.356–0.455)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.929 (0.840–0.984)	0.833 (0.910–0.958)	0.964 (0.990–1)	0.826	0.124	0.488 (0.656–0.822)	.0035
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.951 (0.889–0.990)	0.868 (0.917–0.965)	0.972 (0.991–1)	0.901	0.405	0.550 (0.667–0.822)	.0003

18 stage I cases	vs 36 acute & chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.779 (0.645–0.907)	0.722 (0.833–0.926)	0.560 (0.737–0.923)	0.111	0.111	0.800 (0.892–0.971)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.940 (0.860–0.995)	0.833 (0.926–0.981)	0.692 (0.889–1)	0.778	0.389	0.875 (0.946–1)	.0460
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.971 (0.929–0.998)	0.852 (0.926–1)	0.692 (0.857–1)	0.778	0.500	0.923 (1-1)	.0109
79 stage II cases	vs 36 acute & chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.716 (0.620–0.808)	0.652 (0.730–0.817)	0.843 (0.911–0.969)	0.228	0.139	0.467 (0.550–0.648)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.937 (0.883–0.979)	0.809 (0.878–0.939)	0.914 (0.973–1)	0.797	0.177	0.632 (0.745–0.914)	.0001
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.946 (0.900–0.982)	0.835 (0.896–0.948)	0.934 (0.974–1)	0.823	0.405	0.660 (0.773–0.897)	<.0001
121 stage I-IV cases	vs 36 acute & chronic pancreatitis controls						
Biomarkers	AUC (95% CI)	Accuracy (95% CI)	PPV (95% CI)	Sens@95%Spec	Sens@99%Spec	NPV (95% CI)	*P*
CA19-9	0.749 (0.665–0.827)	0.669 (0.745–0.809)	0.897 (0.944–0.979)	0.273	0.215	0.394 (0.467–0.552)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.931 (0.876–0.973)	0.777 (0.898–0.943)	0.931 (0.965–1)	0.727	0.140	0.507 (0.733–0.871)	.0003
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.954 (0.917–0.984)	0.841 (0.898–0.949)	0.958 (0.990–1)	0.851	0.455	0.590 (0.700–0.850)	<.0001

CA19-9 was used as the reference to assess the improvement in the AUC, of the miRNA, signature and the combination of the miRNA, signature and CA19-9 with 1000 bootstrap samples.
P values were calculated using a bootstrapping method.
95% CI, 95% confidence interval; AUC, Area under the ROC, Curve; NPV, negative predictive value; PPV, positive predictive value; Sens@95%, sensitivity after fixing the specificity at 95%; Sens@99%, sensitivity after fixing the specificity at 99%.

A



B

28 Stage I-IV with ctDNA-negative cases	versus 46 Healthy controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.867	(0.782-0.937)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.932	(0.871-0.978)	.1892
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.972	(0.930-0.998)	.0185

16 Stage I-IV with ctDNA-positive cases	versus 46 Healthy controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.782	(0.638-0.911)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.957	(0.878-1)	.0315
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.976	(0.920-1)	.0120

28 Stage I-IV with ctDNA-negative cases	versus 23 Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.756	(0.616-0.874)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.894	(0.792-0.980)	.1068
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.943	(0.870-0.997)	.0158

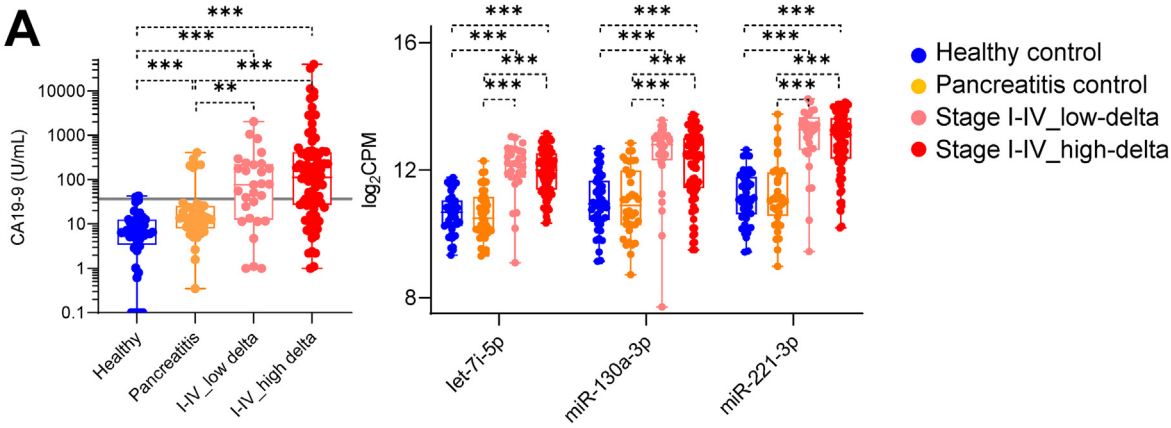
16 Stage I-IV with ctDNA-positive cases	versus 23 Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.677	(0.467-0.867)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.943	(0.845-1)	.0146
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.984	(0.935-1)	.0029

28 Stage I-IV with ctDNA-negative cases	versus 36 Acute & Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.771	(0.639-0.872)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.893	(0.810-0.960)	.0963
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.932	(0.870-0.980)	.0178

16 Stage I-IV with ctDNA-positive cases	versus 36 Acute & Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.680	(0.464-0.877)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.922	(0.840-0.984)	.0296
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.983	(0.946-1)	.0040

*CA19-9 was used as the reference to assess the improvement in the AUC of the three-miRNA signature and the combination of the three-miRNA signature with CA19-9.
***P*-values were calculated using a bootstrapping method with 1000 bootstrap samples.

Figure 3. Diagnostic performance of the miRNA signature and CA19-9 in PDAC patients with and without mutant-*KRAS* ctDNA subsets. (A) Levels of CA19-9 (left) and the 3 miRNAs in the signature (right) in healthy controls ($n = 46$), pancreatitis controls ($n = 36$), and stage I-IV PDAC with *KRAS*-ctDNA negative patients ($n = 28$), and stage I-IV PDAC with *KRAS*-ctDNA positive patients ($n = 16$). *, $P < .05$; **, $P < .01$; ***, $P < .001$. (B) AUC analysis of the miRNA signature and the miRNA-CA19-9 combination panel in mutant-*KRAS* ctDNA-negative ($n = 28$) and ctDNA-positive ($n = 16$) PDAC cases vs healthy (top, $n = 46$), chronic pancreatitis (middle, $n = 23$), or combined acute & chronic pancreatitis (bottom, $n = 36$) controls.



B

29 Stage I-IV with low-delta PDAC cases	versus 46 Healthy controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.844	(0.735-0.942)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.957	(0.904-0.996)	.0622
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	1.000	(1-1)	.0049

87 Stage I-IV with high-delta PDAC cases	versus 46 Healthy controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.888	(0.832-0.937)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.978	(0.955-0.994)	.0021
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.994	(0.983-1)	.0001

29 Stage I-IV with low-delta PDAC cases	versus 23 Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.700	(0.556-0.843)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.906	(0.811-0.988)	.0242
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.945	(0.864-0.997)	.0038

87 Stage I-IV with high-delta PDAC cases	versus 23 Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.766	(0.669-0.858)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.933	(0.847-0.986)	.0095
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.958	(0.910-0.991)	.0007

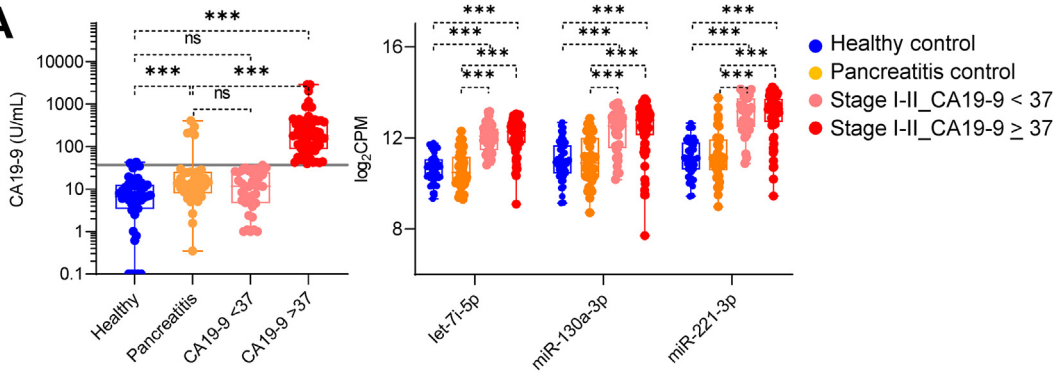
29 Stage I-IV with low-delta PDAC cases	versus 36 Acute & Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.713	(0.584-0.850)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.910	(0.837-0.973)	.0134
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.943	(0.884-0.986)	.0024

87 Stage I-IV with high-delta PDAC cases	versus 36 Acute & Chronic pancreatitis controls		
Biomarkers	**AUC**	**95%CI**	***P***
CA19-9	0.781	(0.690-0.863)	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.937	(0.883-0.979)	.0021
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.964	(0.929-0.989)	.0001

*CA19-9 was used as the reference to assess the improvement in the AUC of the three-miRNA signature and the combination of the three-miRNA signature with CA19-9.
***P*-values were calculated using a bootstrapping method with 1000 bootstrap samples.

Figure 4. Diagnostic performance of the miRNA signature and CA19-9 in imaging-based PDAC subtypes. (A) Levels of CA19-9 (left) and the 3 miRNAs in the signature (right) in healthy controls ($n = 46$), pancreatitis controls ($n = 36$), stage I-IV PDAC cases with low-delta subtype ($n = 29$); stage I-IV PDAC cases with high-delta subtype ($n = 87$). **, $P < .01$; ***, $P < .001$. (B) AUC analysis of the miRNA signature and the miRNA-CA19-9 combination panel in PDAC with low-delta ($n = 29$) and high-delta ($n = 87$) subtypes vs healthy (top, $n = 46$), chronic pancreatitis (middle, $n = 23$), or combined acute & chronic pancreatitis (bottom, $n = 36$) controls.

A



B

36 Stage I & II cases with CA19-9 < 37	versus 46 Healthy controls				
Biomarkers	**AUC**	**95%CI**	**Sens@95%Spec**	**Sens@99%Spec**	***P***
CA19-9	0.610	(0.484, 0.735)	0.278	0.000	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.983	(0.955, 1)	0.944	0.944	< .0001
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.984	(0.958, 1)	0.944	0.889	< .0001

36 Stage I & II cases with CA19-9 < 37	versus 23 Chronic pancreatitis controls				
Biomarkers	**AUC**	**95%CI**	**Sens@95%Spec**	**Sens@99%Spec**	***P***
CA19-9	0.587	(0.438-0.728)	0.139	0.000	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.941	(0.854-0.995)	0.889	0.167	< .0001
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.947	(0.859-0.998)	0.889	0.167	< .0001

36 Stage I & II cases with CA19-9 < 37	versus 36 Acute & Chronic pancreatitis controls				
Biomarkers	**AUC**	**95%CI**	**Sens@95%Spec**	**Sens@99%Spec**	***P***
CA19-9	0.585	(0.452, 0.718)	0.139	0.000	1
let-7i-5p + miR-130a-3p + miR-221-3p	0.947	(0.890, 0.990)	0.806	0.194	< .0001
CA19-9 + let-7i-5p + miR-130a-3p + miR-221-3p	0.947	(0.890, 0.990)	0.750	0.194	< .0001

*CA19-9 was used as the reference to assess the improvement in the AUC of the three-miRNA signature and the combination of the three-miRNA signature with CA19-9.
**P-values were calculated using a bootstrapping method with 1000 bootstrap samples.

C

Time to Diagnosis (months)	12-24	6-12	0-6	0-3
Number of Cases	18	15	15	12
Number of Controls	18	15	15	12
AUC	0.602	0.702	0.729	0.757
(95%CI)	(0.423-0.793)	(0.511-0.889)	(0.524-0.893)	(0.542-0.944)

Figure 5. Diagnostic performance of the miRNA signature in early-stage PDAC patients with low CA19-9 and prediagnosed PDAC patients. (A) Levels of CA19-9 (left) and the 3 miRNAs in the signature (right) in healthy controls ($n = 46$), pancreatitis controls ($n = 36$), stage I-II PDAC patients with CA19-9 < 37 U/mL ($n = 36$), and stage I-II PDAC patients with CA19-9 ≥ 37 U/mL ($n = 61$). **, $P < .01$; ***, $P < .001$. (B) AUC analysis of the miRNA signature in PDAC patients with low CA19-9 ($n = 36$) vs healthy (top, $n = 46$), chronic pancreatitis (middle, $n = 23$), or combined acute & chronic pancreatitis (bottom, $n = 36$) controls. (C) Lead-time trajectory of the miRNA signature AUC values in prediagnosed PDAC cases from the PLCO cancer screening cohort ($n = 96$).

0.702 [0.511–0.889], 0.729 [0.524–0.893], and 0.757 [0.542–0.944] with lead-time for 6–12 months, 0–6 months, and 0–3 months prior to diagnosis, respectively (Figure 5C).

Discussion

The clinically used serum marker for PDAC, CA19-9, with its suboptimal sensitivity and specificity, has been reported to play role in pancreatitis and pancreatic cancer.[41] Published studies also reveal that combined panels of CA19-9 with emerging blood-based biomarkers, ctDNA, proteins, methylated DNA, and miRNAs, may improve early detection of PDAC.[13,17–19,38,42]

We have been investigating circulating miRNAs in blood as early detection biomarkers of PDAC[25,26] in view of their involvement with pathways deregulated in cancer initiation-progression and promising performance in detecting disease reported in prior studies.[18,27,28,43–45] Although different biomarkers, including miRNAs, are at different phases of validation, none has transitioned to clinical diagnostic setting due to concerns about their inadequate sensitivity and specificity as well as varying performances in different studies. It is conceivable that technical biases introduced in different assay methods during analyte extraction[32] and different subtype-specific gene expression profiles underlying PDAC heterogeneity and plasticity[46–48] leads to inconsistent performance of candidate biomarkers in different sample cohorts. To address these confounding pitfalls, we focused on circulating miRNAs in plasma from PDAC patients, involved in cancer-relevant pathways,

utilizing an extraction-free miRNA quantification platform devoid of bias against low-expressing miRNAs in small-volume biological samples encountered in conventional methods.[32] The plasma miRNAs elevated in PDAC cases displaying high AUC values (>0.8), identified candidate biomarker miRNAs associated with the disease. Prioritization of these plasma miRNAs based on their involvement in critical pathways, including those associated with the most frequently mutated oncogene *KRAS*,[49] yielded a panel that discriminated early-stage PDAC cases from healthy and pancreatitis controls with high sensitivity and specificity.

The three-miRNA signature in combination with CA19-9 showed improved diagnostic performance than CA19-9 alone. By using the cutoff of 95% specificity, the miRNA-CA19-9 combination panel yielded a sensitivity of 0.778 compared with 0.111 of CA19-9 alone for stage I cases, 0.823 compared with 0.228 of CA19-9 for stage II cases and 0.851 compared with 0.273 for stage I-IV cases vs pancreatitis, respectively. Importantly, sensitivity of the miRNA signature itself outperformed CA19-9 across all stages of PDAC in discriminating from both chronic pancreatitis as well as chronic and acute pancreatitis cases combined. This is clinically relevant since the risk of PDAC is known to increase over time for patients with chronic pancreatitis and is highest within 1 year of acute pancreatitis episode.[50] Significance of the disease-relevant pathway-focused approach was further affirmed in 36 stage I-II PDAC patients with CA19-9 levels lower than the disease cutoff value (<37 U/mL). In these patients, the miRNA signature detected disease with sensitivity of 0.944 at both 95% and 99% specificities. Interestingly, miRNAs, unlike CA19-9, revealed no association with the incidence of jaundice.[12] These results emphasize the significance of plasma miRNAs as reliable early detection biomarkers of PDAC and reinforces that cancer-relevant pathway-associated miRNAs can be developed as sensitive and specific biomarkers of the disease. A recent report on combined cell-free and exo miRNAs as biomarkers for PDAC with performance superior to CA19-9 partially complement the above findings.[31] The previous study design, however, had limitations of not including age-matched healthy and benign pancreatic disease controls and the 13-miRNA panel described in that study is different from the three-miRNA signature identified in the current study. Nonetheless, 10 cell-free-miRNAs and 3 exo-miRNAs elevated in the early-stage PDAC in the above study[31] were also present among the 72 elevated miRNAs with AUC>0.8 in this study. It is relevant, in this context, that another panel of plasma miRNAs with high AUC values (0.933–0.987) and sensitivities in the range of 0.708–0.917 at 95% specificity for patients with stage III-IV PDAC indicated their plausible role in driving late-stage disease. Pathway analyses of the target genes for the top 10 elevated plasma miRNAs from late-stage PDAC, revealed involvement in epithelial to mesenchymal transition driving cancer progression (Figure A4C).

With molecular analytes being investigated in disease diagnosis and therapy response,[51,52] there is also growing interest in developing multianalyte marker panels in blood[53] and combining these with high-resolution imaging features for multiomics assays to detect early-stage cancers.[54] Development of highly sensitive and specific multianalyte/multiomics assay panels for PDAC is desirable due to its relatively low incidence in the average risk population (~12 per 100,000).[55] We investigated performance of the miRNA signature in reference to ctDNA and high-resolution CT imaging features available for subsets of samples included in the study. Presence of ctDNA with mutant *KRAS* oncogene has gained attention for PDAC detection since >90% patients harbor point mutations in the *KRAS* oncogene.[56] Mutant *KRAS* ctDNA as an independent marker of early-stage PDAC shows modest sensitivity[38] leading to the suggestion that *KRAS* ctDNA could be integrated with CA19-9 and imaging features for early diagnosis of PDAC.[57] Among 44 cases in our sample cohort analyzed for the presence of ctDNA, 5 of 22 stage I-II cases as opposed to 11 of 22 stage III-IV cases were positive for ctDNA,[38] reflecting the different baseline detection rates among patients with localized and metastatic disease. The miRNA signature, however, discriminated ctDNA-negative cases from healthy as well as pancreatitis controls (AUC 0.932 and 0.893) with much better performance than CA19-9 (0.867 and 0.771). Additionally, combination of the miRNA signature with CA19-9 yielded significantly improved performance than CA19-9 alone for both ctDNA-negative and positive PDAC patients in discriminating from healthy (AUC 0.972 and 0.976) and pancreatitis (AUC 0.932 and 0.983) controls (all $P < .02$) controls.

Feasibility of developing multiomics assay for PDAC diagnosis with the three-miRNA signature and the 2 imaging-based disease subtypes revealed that the three-miRNA signature discriminates both “low” and “high” delta PDAC subtypes from healthy as well as pancreatitis subjects with similarly significant sensitivity and specificity. The results suggest that “low-delta” and “high-delta” subtypes sharing identical early detection miRNA signature may help disease stratification among PDAC patients with imaging findings and also predict differential prognosis among those with early-stage disease.

Since it is critical that sensitive and specific PDAC early detection biomarkers be validated for diagnostic accuracy in detecting the disease before cancer is diagnosed, we evaluated time-dependent AUCs for the miRNA biomarker signature in the PLCO sample cohort collected between 3 to 24 months prior to diagnosis. Elevated miRNA levels in these samples were detected up to 12–24 months before diagnosis (AUC 0.602) and progressively increased from 6-12 months (AUC 0.702) to 6 months (AUC 0.729) and 3 months (AUC 0.757) prior to diagnosis. These results indicated that elevated levels of the three-miRNA signature in plasma is, indeed, a promising early detection biomarker signature of PDAC and could provide important lead-time for detection of early-stage disease when multimodality treatment with surgical resection remains a viable curative option.

In summary, this study identified a panel of 72 elevated plasma miRNAs from early-stage PDAC patients

utilizing an extraction-free miRNA quantification platform with minimum technical bias for small-volume samples. By focusing on miRNAs associated with pathways underlying cancer initiation, a three-miRNA biomarker signature was developed for early detection of PDAC. The biomarker signature was validated in multiple sample cohorts that could discriminate not only early-stage disease from healthy individuals and pancreatitis patients with performance superior to clinically used serological marker, CA19-9, but also showed a lead-time trajectory of progressively increasing levels in plasma 12 months before diagnosis. The three-miRNA biomarker signature could detect disease among patients with undetectable or normal levels of CA19-9 and potentially could serve as an anchor marker in multianalyte/multiomics early detection assays for PDAC that would help diagnose asymptomatic cancer among individuals meeting the "risk threshold", of genetic, metabolic and/or familial predisposition. The current study design had some unique strengths, including the use of a reliable assay platform with least technical bias, prioritization of candidate miRNA biomarkers based on their involvement in cancer-relevant pathways, and their validation in multi-institutional sample cohorts with age-matched healthy controls and patients with pancreatitis. The assays were performed in a blinded manner and unblinded for statistical modeling by our biostatistician colleagues. It is, however, important to also acknowledge the limitation of the study, which include the data being derived from a modest sample size of symptomatic and prediagnostic patient cohorts, absence of data on the levels of the candidate miRNA biomarkers from the corresponding tumor and adjacent normal tissues, lack of information on potential confounding factor like abnormal liver function, besides ctDNA and imaging data being available from only a subset of patients. Also, miRNA signatures for different PDAC molecular subtypes could not be developed in the sample cohorts enrolled in the study. It is, therefore, imperative that larger sample cohorts, including prospective "at risk" individuals representing different PDAC subtypes from different ethnicities, be investigated with similar analytical approach to develop and validate disease subtype relevant miRNA biomarkers for translation to clinic.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2024.08.002.

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Conflict of Interest:
Anirban Maitra is a consultant for Tezcat Bioscience and is listed on a patent that had been licensed by ThriveEarlier Detection (an Exact Sciences Company). An Invention Disclosure is being filed by co-inventors Warapen Treekitkarnmongkol, Jianliang Dai, Suyu Liu, and Subrata Sen, for patenting the plasma microRNA panel as early detection biomarkers of pancreatic ductal adenocarcinoma (PDAC). The remaining authors disclose no conflicts.

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Ethical Statement:
Plasma samples for the PDAC diagnostic cohort were collected with approved Institutional Review Board protocols (# Lab00-396, PA11-0670 and PA15-0014) of MD Anderson Cancer Center and University of Pittsburgh Medical Center. Prediagnostic plasma samples from PLCO cancer screening cohort were collected with the approved protocol # 6007 from Fred Hutchinson Cancer Center.

Data Transparency Statement:
Plasma miRNA-seq in this study has been deposited into the NCBI's GEO as GSE259327 which will be available upon publication.

Reporting Guidelines:
None.