

Plasma MicroRNAs as Novel Biomarkers for Patients with Intraductal Papillary Mucinous Neoplasms of the Pancreas

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal cancers worldwide, partly because methods are lacking to detect disease at an early, operable stage. Noninvasive PDAC precursors called intraductal papillary mucinous neoplasms (IPMN) exist, and strategies are needed to aid in their proper diagnosis and management. Data support the importance of miRNAs in the progression of IPMNs to malignancy, and we hypothesized that miRNAs may be shed from IPMN tissues and detected in blood. Our primary goals were to measure the abundance of miRNAs in archived preoperative plasma from individuals with pathologically confirmed IPMNs and healthy controls and discover plasma miRNAs that distinguish between IPMN patients and controls and between "malignant" and "benign" IPMNs. Using novel nCounter technology to evaluate 800 miRNAs, we showed that a 30-miRNA signature distinguished 42 IPMN cases from 24 controls [area

underneath the curve (AUC) = 74.4; 95% confidence interval (CI), 62.3–86.5, $P = 0.002$]. The signature contained novel miRNAs and miRNAs previously implicated in pancreatic carcinogenesis that had 2- to 4-fold higher expression in cases than controls. We also generated a 5-miRNA signature that discriminated between 21 malignant (high-grade dysplasia and invasive carcinoma) and 21 benign (low- and moderate-grade dysplasia) IPMNs (AUC = 73.2; 95% CI, 57.6–88.9, $P = 0.005$), and showed that paired plasma and tissue samples from patients with IPMNs can have distinct miRNA expression profiles. This study suggests feasibility of using new cost-effective technology to develop a miRNA-based blood test to aid in the preoperative identification of malignant IPMNs that warrant resection while sparing individuals with benign IPMNs the morbidity associated with overtreatment. *Cancer Prev Res*; 8(9); 826–34. ©2015 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths in the United States, with a 5-year survival rate of only 6% (1). Approximately 85% of cases present with metastases, which can be partly explained by a lack of accurate methods to detect disease at an early, operable stage (1). The detection and treatment of noninvasive precursor lesions

may offer the greatest hope in reducing morbidity and mortality. Three noninvasive PDAC precursor lesions (precancers) exist: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN), and intraductal papillary mucinous neoplasms (IPMN; refs. 2, 3). PanINs are microscopic lesions, whereas MCNs and IPMNs are macroscopic cysts accounting for over half of the approximately 150,000 asymptomatic pancreatic cysts detected incidentally in the general population each year by imaging (4). Once detected, endoscopic ultrasound (EUS)-guided fine needle aspirations are often performed to assess the degree of dysplasia, but imaging features and biomarkers obtained from such invasive procedures do not reliably predict disease severity preoperatively (3). Noninvasive approaches are needed to aid in IPMN management and prevent progression to malignancy.

miRNAs are biomarkers that regulate one-third of all protein-coding genes and promote carcinogenesis by regulating tumor suppressors and oncogenes or serving these functions themselves (5). miRNAs are excellent candidate biomarkers of early disease because of their tissue-specific expression patterns (5), their remarkable stability in tissue (6) and biofluids (7) due to their small size and protection from endogenous RNase activity, and their ability to regulate hundreds of genes and biologic pathways (5). Recent studies by our group (8) and others (9–11) have evaluated genome-wide miRNA expression in IPMN tissue, and provide data to suggest that key miRNAs may reliably differentiate low-risk/benign IPMNs (i.e., low- and moderate-grade) that can

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be monitored from high-risk/malignant IPMNs (i.e., high-grade and invasive) that warrant resection. These tissue-based findings combined with discoveries that miRNAs can be readily and reliably detected in circulation (7, 12) raise the possibility that a noninvasive test that measures blood levels of miRNAs may be able to differentiate between individuals with IPMNs and non-diseased controls and between malignant and benign IPMNs. Of only two published studies (13, 14) that have focused on evaluating blood-based genome-wide miRNA expression in early-stage PDAC patients versus controls, one (13) included preoperative serum from 20 patients with IPMNs.

The primary objective of the current study was to measure the abundance of 800 miRNAs in archived plasma obtained preoperatively from individuals with IPMNs and disease-free controls using a novel digital amplification-free quantification method called nCounter technology (Nanostring, Inc.; ref. 15). We then sought to discover circulating miRNAs that may (i) differentiate between patients with IPMNs and non-diseased controls, (ii) distinguish malignant from benign IPMNs, and/or (iii) reflect the paired tumor miRNA expression profile. To our knowledge, this is the first study to conduct genome-wide profiling of circulating miRNAs exclusively among patients with cancer precursors using digital technology.

Materials and Methods

Study population and biospecimens

A prospectively maintained clinical database was retrospectively reviewed to identify individuals who underwent a pancreatic resection for an IPMN between 2006 and 2011 at Moffitt Cancer Center and Research Institute (Moffitt; Tampa, FL) and had provided written consent for blood to be donated preoperatively for research through protocols approved by the Institutional Review Board (IRB) of the University of South Florida (Tampa, FL), including Total Cancer Care (16). IRB approval was granted for the research described herein (IRB#Pro4971). The diagnosis and degree of dysplasia were histologically confirmed using World Health Organization (WHO) guidelines (17). The final diagnosis represented the most severe grade of dysplasia observed in the neoplastic epithelium. None of the cases received preoperative chemotherapy or radiation. Age- and gender-matched healthy controls with no current or prior history of pancreatic disease or symptoms who presented to Moffitt's Cancer Screening and Prevention Center during the same time period and donated blood through a related IRB-approved protocol using the same procedures were also eligible for inclusion.

Blood was collected from consented participants via phlebotomy in a 7-mL EDTA tube and processed for plasma within 2 hours using standard procedures (18). The tube was inverted three times and spun at 3,600 rpm for 8 minutes and then aliquoted into 0.5-mL bar-coded cryovials and banked at -80°C . Demographic, clinical, and epidemiologic data were collected from an electronic questionnaire, the medical record, Moffitt's cancer registry, and other source systems.

RNA isolation from plasma and quality control

One 0.5-mL cryovial of plasma was retrieved and thawed from each study participant. To control for variance in the starting material and the efficiency of RNA extraction, RNA spike-in miRNAs (synthetic control templates) were used. Total RNA isolation was performed on 500 μL of plasma using the Plas-

ma/Serum Circulating and Exosomal RNA Purification Mini Kit (Slurry Format) from Norgen Biotek. After adding lysis buffer, 1,000 attomoles of the synthetic RNA oligonucleotides (spike-in oligos) osa-miR-414, cel-mi-248, and ath-miR-159a (Operon, Inc.) were added. No-template control samples were run to monitor the baseline for spike-ins. RNA was eluted in 100 μL water, concentrated to 20 μL using Amicon Ultra 0.5 mL centrifugal filters (Sigma-Aldrich), and 3 μL was used for each assay.

Total RNA concentration and integrity were assessed using the NanoDrop spectrophotometer (NanoDrop Technologies) and an Agilent Bioanalyzer (Agilent). Since hemolysis (rupturing of erythrocytes) can be a source of variation in studies of circulating miRNAs, we assessed the possibility of hemolysis based on recommended guidelines (18–20): (i) before RNA extraction, samples were visually inspected for a pink/red hue, (ii) oxygen hemoglobin absorbance of isolated RNA was measured at $\lambda = 414$ nm, with values exceeding 0.2 indicative of hemolysis, and (iii) signal levels were evaluated *post hoc* for cellular miRNAs likely to be elevated in the presence of hemolysis (miR-451, miR-16).

High-throughput measurement of miRNA abundance

The nCounter Human v2 miRNA Expression Assay Codeset (Nanostring Technologies) was used to quantify the abundance of 800 human miRNAs and built-in controls: positive controls (spiked RNA at various concentrations to assess overall assay performance, $n = 6$) and negative controls (alien probes for background calculation, $n = 8$). This platform involves direct amplification-free digital measurement of miRNA abundance using color-coded probe pairs (15). Using 3 μL of the extracted plasma RNA as input, preparation involved multiplexed ligation of DNA sequences to mature miRNAs through sequence-specific bridges. Excess tags and bridges were removed via an enzymatic step, and the tagged miRNA probes were hybridized to a color-coded reporter probe pair for data collection. Reporter probes were counted for each miRNA using the nCounter Digital Analyzer at 555 fields of view.

Data processing and quality control

For each sample, background-corrected measures of miRNA expression were estimated by subtracting the negative control average plus two SD cut-point from the raw miRNA counts. miRNAs with less than 20% of samples above the negative control cut-point (i.e., low-expression probes) were removed from downstream analysis. Human mRNA housekeeping genes included in the codeset (ACTB, B2M, GAPDH, RPL19, and RPLP0) were used to evaluate possible sample contamination. Data for each sample were normalized using the geometric mean of the three spike-in oligos. Biologic normalization was then performed using the four most stable/invariant circulating miRNAs as endogenous controls. Normalized data were \log_2 -transformed before signature selection.

Statistical analysis

Descriptive statistics were calculated using frequencies and percents for categorical variables and means and SDs for continuous variables. Data analysis was performed to (i) identify a plasma miRNA signature that differentiates between IPMN cases and non-diseased controls, (ii) identify a plasma miRNA signature that distinguishes malignant (pathologically confirmed as high-grade or invasive) from benign IPMNs (pathologically confirmed as low- or moderate-grade), (iii) discover pathways

controlled by the most deregulated miRNAs, and (iv) explore paired tissue and circulating miRNA expression (Supplementary Fig. S1).

Identification of plasma miRNA signatures. For (i) and (ii), linear models for microarray data (21) were used to identify miRNAs that differentiate between IPMN cases and controls and between malignant and benign IPMNs, respectively. Because miRNAs can be over- or underexpressed, we used principal component analysis (PCA) to combine the most deregulated miRNAs and generate an overall "IPMN-risk score" based on the first principal component (PC1). PC1 accounts for the largest variability in the data and represents the overall combined effect of the IPMN-risk miRNA signatures. Specifically, IPMN-risk score, defined by PC1 as $\sum w_i x_i$, is a weighted average expression among the IPMN-risk miRNAs, where x_i represents miRNA i expression level, w_i is the corresponding weight (PC1's loading coefficient for miRNA i) with $\sum w_i^2 = 1$, and the w_i values maximize the variance of $\sum w_i x_i$. This approach has been used to derive gene signatures previously (22–25). Receiver operating characteristic (ROC) curves were generated to measure the predictive power of the IPMN-risk signatures in discriminating between groups. Estimates of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. For the analysis of malignant versus benign cases, we also conducted multivariable logistic regression analysis to assess whether the identified miRNA signature was associated with malignant IPMN status independent of known prognostic factors (i.e., main-duct involvement, lesion size, serum CA-19-9 level; ref. 26).

Pathway enrichment analysis. To explore the potential roles of identified circulating miRNAs in pancreatic carcinogenesis, pathway-based bioinformatics analysis (iii) was conducted. Using Tarbase 6.0 (27), we obtained a list of genes experimentally validated to target the identified miRNAs. The target genes were then analyzed to identify enriched KEGG pathways (28) predicted to be regulated by the circulating miRNAs.

Exploring paired tissue and circulating miRNA expression. For 12 IPMN cases (4 low-grade, 8 high-grade) that had matching tumor tissue that underwent genome-wide miRNA profiling previously (8), spearman correlation analysis was used to evaluate the relationship between the mean abundance of each miRNA in preoperative plasma compared with paired tissue.

All statistical analyses were performed using SAS version 9.4 and R version 3.11. To visualize miRNA expression patterns, we generated heatmaps and performed unsupervised, hierarchical clustering.

Results

Study population

Preoperative plasma samples were evaluated for 44 IPMN cases and 25 non-diseased controls that were frequency matched to cases on age group and gender. Three samples (2 from cases and 1 from a control) were excluded before normalization and statistical analysis due to presumed cellular contamination characterized by ribosomal RNA bands or high mRNA counts, leaving samples from 66 participants (42 cases, 24 controls) for analysis. Study population characteristics are shown in Table 1. Cases and controls were well matched on age (mean age: 69.0 vs. 69.1 years).

Table 1. Characteristics of the study population ($N = 66$)

Variable	IPMN cases ($n = 42$)	Healthy controls ($n = 24$)
Age at diagnosis/interview, mean (SD; y)	69.0 (10.7)	69.1 (9.6)
Gender, male:female, n (%)	19:23 (45:55)	12:12 (50:50)
Race, n (%)		
White, non-Hispanic	37 (88)	24 (100)
Other	5 (12)	0 (0)
Family history of pancreatic cancer, n (%)		
Yes	4 (17)	1 (4)
No	15 (83)	23 (96)
Ever smoker, n (%)		
Yes	21 (50)	11 (46)
No	21 (50)	3 (13)
Unknown	0 (0)	10 (42)
IPMN grade, n (%)		
Low	4 (9.5)	—
Moderate	17 (40.5)	—
High	13 (31)	—
Invasive	8 (19)	—

NOTE: Data represent counts (percentages) unless otherwise indicated. Counts may not add up to the total due to missing values, and percentages may not equal 100 due to rounding.

Most subjects were white, non-Hispanic. The distribution of low-, moderate-, high-grade, and invasive IPMN cases was 9.5%, 40.5%, 31%, and 19%, respectively.

Exploratory analysis of circulating miRNAs in IPMNs versus non-diseased controls

A total of 558 of the 800 miRNAs evaluated (69.8%) had >80% of signals below background and were excluded, leaving 242 miRNAs for normalization and statistical analysis. This proportion of detectable circulating miRNAs is comparable or better than that of other studies (14, 29–31). No difference in the frequency or the amount of hemolysis was observed in the case versus control samples.

After technical normalization with spike-in oligos and biologic normalization with the most invariant miRNAs in the dataset (miR-378e, miR-579, miR-30e-5p, and miR-570-3p), 30 miRNAs differentiated cases from controls using an FDR < 0.15 (Table 2). The candidate miRNAs had 2.09–4.32 fold higher expression in cases compared with controls (Table 2). The circulating miRNA that was most significantly associated with IPMN case status was miR-145-5p ($P = 8.6 \times 10^{-5}$), with expression levels 3.78-fold higher in cases than controls and an AUC of 0.79 (95% CI, 69.3–90.3) in classifying between groups (Table 2 and Supplementary Fig. S2A and S2B).

The 30-miRNA IPMN-risk signatures, represented by PC1, explained 64% of the variability in the data, suggesting it represents the signature well (Fig. 1A). The overall expression of PC1 was higher in cases than controls ($P = 0.002$, Fig. 1B), was significantly associated with IPMN status [OR; 95% CI, = 1.23 (1.07–1.41), $P = 0.003$], and had an AUC value of 74.4 (95% CI, 62.3–86.5; Fig. 1C). The sensitivity, specificity, PPV, and NPV for IPMN detection were 78.6%, 62.5%, 78.6%, and 62.5%, respectively. A heatmap of gene expression for the 30-miRNA signature in our cohort is displayed in Fig. 2. Pathway enrichment analysis revealed numerous pathways predicted to be regulated by the 30-miRNA IPMN-risk signature (Fig. 3), with "Pathways in Cancer," "p53 signaling," and "PI3K-AKT signaling" comprising the top-ranked pathways with $P = 1.3 \times 10^{-26}$, $P = 1.2 \times 10^{-24}$, and $P = 4.0 \times 10^{-17}$, respectively.

Table 2. The top 30 miRNAs deregulated in plasma of IPMN cases versus non-diseased controls

miRNA ID	Mean normalized counts cases (n = 42)	Mean normalized counts controls (n = 24)	Fold-change	P	FDR	AUC (95% CI)
hsa-miR-145-5p	3.26	1.34	3.78	8.61E-05	0.0208	79.3 (68.3-90.3)
hsa-miR-4454	10.58	9.46	2.18	0.0018	0.1043	72.4 (59.6-85.2)
hsa-let-7f-5p	8.27	6.38	3.69	0.0028	0.1043	73.8 (62.0-85.6)
hsa-miR-146a-5p	8.55	6.55	4.00	0.0032	0.1043	72.4 (60.2-84.6)
hsa-let-7d-5p	7.33	5.42	3.75	0.0043	0.1043	69.1 (55.7-82.4)
hsa-let-7a-5p	11.43	9.83	3.05	0.0044	0.1043	68.6 (55.2-81.9)
hsa-miR-142-3p	12.71	11.29	2.68	0.0060	0.1043	67.2 (53.3-81.0)
hsa-miR-423-5p	8.62	7.04	2.99	0.0061	0.1043	69.4 (56.2-82.7)
hsa-miR-22-3p	10.06	8.93	2.18	0.0072	0.1043	68.8 (55.8-81.7)
hsa-miR-107	7.64	5.84	3.48	0.0073	0.1043	72.3 (59.3-85.4)
hsa-miR-29c-3p	6.97	5.11	3.63	0.0076	0.1043	67.4 (53.6-81.1)
hsa-miR-148a-3p	8.23	6.50	3.31	0.0078	0.1043	70.8 (57.8-83.9)
hsa-miR-340-5p	5.28	3.18	4.32	0.0079	0.1043	70.8 (57.5-84.2)
hsa-miR-181a-5p	5.36	3.34	4.06	0.0079	0.1043	69.6 (56.6-82.7)
hsa-miR-335-5p	3.44	1.92	2.87	0.0083	0.1043	69.4 (56.3-82.6)
hsa-let-7i-5p	8.79	7.31	2.80	0.0092	0.1043	71.0 (58.3-83.7)
hsa-miR-337-5p	3.19	1.71	2.79	0.0092	0.1043	71.4 (58.2-84.7)
hsa-miR-1260b	3.28	1.63	3.15	0.0093	0.1043	67.9 (54.6-81.1)
hsa-miR-593-3p	2.46	1.40	2.09	0.0097	0.1043	68.7 (55.2-82.1)
hsa-miR-27a-3p	4.29	2.61	3.20	0.0097	0.1043	68.8 (55.3-82.2)
hsa-let-7g-5p	11.95	10.70	2.38	0.0099	0.1043	68.5 (55.4-81.5)
hsa-miR-191-5p	10.56	9.29	2.40	0.0100	0.1043	68.2 (54.9-81.4)
hsa-miR-24-3p	8.15	6.21	3.83	0.0100	0.1043	69.4 (56.3-82.4)
hsa-miR-20a-5p+hsa-miR-20b-5p	10.36	9.15	2.30	0.0103	0.1043	68.1 (54.9-81.2)
hsa-miR-26a-5p	10.23	8.91	2.50	0.0114	0.1100	72.4 (60.2-84.6)
hsa-miR-23a-3p	11.16	10.05	2.16	0.0130	0.1206	66.7 (53.2-80.2)
hsa-miR-199a-3p+hsa-miR-199b-3p	10.53	8.95	2.99	0.0150	0.1344	69.5 (56.5-82.6)
hsa-miR-126-3p	13.23	11.90	2.51	0.0168	0.1406	65.8 (52.0-79.5)
hsa-miR-98	4.12	2.51	3.05	0.0174	0.1406	67.4 (54.0-80.8)
hsa-miR-15b-5p	11.24	9.83	2.65	0.0174	0.1406	65.8 (52.3-79.3)

NOTE: Comparisons were conducted with *t* tests.

Exploratory analysis of circulating miRNAs in malignant versus benign IPMNs

We evaluated the ability of the 30-miRNA signature (PC1) to discriminate between the malignant and benign IPMNs and observed that it did not perform well (AUC = 60.8; 95% CI, 42.5-79.1). However, a focused analysis of the 42 IPMN cases showed that five miRNAs (miR-200a-3p, miR-1185-5p, miR-33a-5p, miR-574-3p, and miR-663b) discriminated between malignant and benign IPMNs ($P < 0.05$; Supplementary Table S1). The 5 miRNA IPMN-risk signature characterized by PC1 explained 50% of the variability (Supplementary Fig. S3A). The overall expression of PC1 was lower in malignant compared with benign IPMNs ($P = 0.005$, Supplementary Fig. S3B), was significantly associated with malignant status [OR (95% CI) = 0.36 (0.16-0.83), $P = 0.017$], and had an AUC value of 73.2 (95% CI, 57.6-88.9) in discriminating between groups (Supplementary Fig. S3C). Estimates of sensitivity, specificity, PPV, and NPV of PC1 in detecting malignant IPMNs were 80.9%, 52.5%, 63.0%, and 73.3%. "Cell cycle" ($P = 0.0008$) and "Wnt signaling" ($P = 0.005$) were among top-ranked pathways predicted to be regulated by the 5 miRNA IPMN-risk signature.

Clinical factors associated ($P < 0.05$) with an increased risk of malignancy included cyst size greater >3 cm [OR (95% CI) = 6.4 (1.4-28.5, $P = 0.015$], main duct involvement [OR (95% CI) = 18.4 (2.8-122.4, $P = 0.002$], and lower serum albumin levels [OR (95% CI) = 0.08 (0.01-0.62)]. Cyst size was negatively correlated with the 5 miRNA PC1 score (spearman correlation = -0.53, $P = 0.001$), whereas serum albumin levels were positive correlated with the 5-miRNA signature (spearman correlation = 0.47,

$P = 0.002$). Mean serum CA 19-9 levels were higher in the malignant compared with benign group, but the difference was not statistically significant ($P = 0.130$). The PC1 score for the 5-miRNA signature was not independently associated with malignant status after adjustment for main duct involvement ($P = 0.008$).

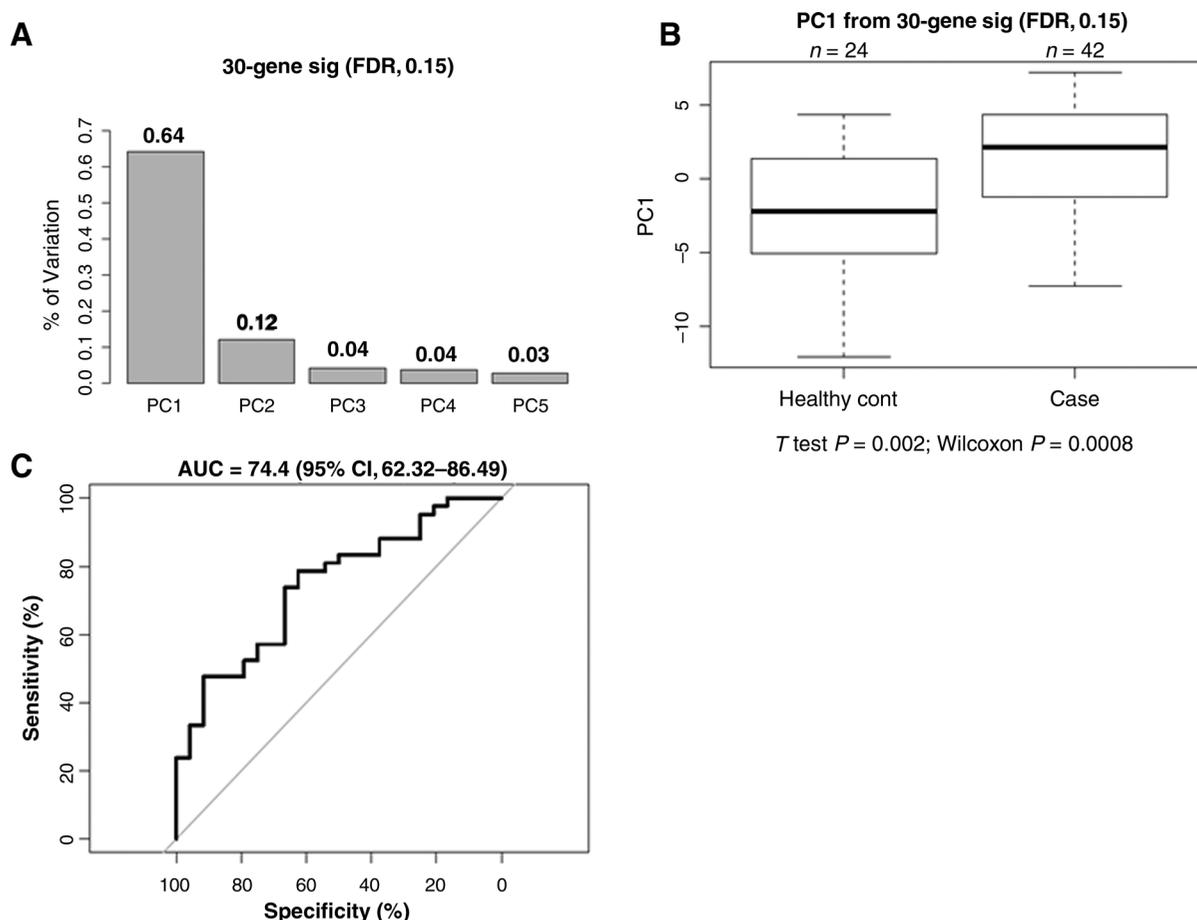
IPMN tumors tissue and paired plasma have distinct miRNA expression profiles

We evaluated matched plasma and tissue specimens from 12 IPMN cases. Among 160 miRNA probes evaluated in both specimen types, expression levels of only 3 (1.9%) were significantly positively correlated ($r > 0.60$, $P < 0.05$), and included miR-484, miR-330, and miR-574-3p (Supplementary Table S2). Of these three miRNA probes, one (miR-574-3p) was represented in the 5-miRNA signature. For the miRNAs that were ranked as the most highly expressed in plasma of IPMN cases versus controls (miR-146a-5p, miR-340-5p, and miR-181a-5p), correlations with tissue miRNA expression were small ($r < 0.60$; Supplementary Table S2). These data suggest plasma and tissue samples from patients with IPMNs can have distinct miRNA profiles.

Discussion

This is the first report to interrogate plasma miRNA expression levels exclusively in individuals newly diagnosed with IPMNs and healthy controls. We used nCounter technology for miRNA quantitation as an alternative to microarray and PCR-based methods to more accurately detect and quantify low

Permeth-Wey et al.

**Figure 1.**

A 30-miRNA signature discriminates IPMN cases ($N = 42$) from healthy controls ($N = 24$). A, percentage of variation explained in the first five principal components using the 30-miRNA signature. B, association of the 30-miRNA signature with case-control status. Box plots were used to display the distribution of the IPMN-risk malignancy score within each group. Two-sample *t* tests were used to determine associations between the continuous PC1 score and case-control status. C, ROC curve analysis using miRNA expression to discriminate IPMN cases from healthy controls. The 30-miRNA signature PC1 yielded an AUC value of 74.4 (95% CI, 62.3-86.5) in differentiating between groups.

miRNA levels present in blood and to eliminate the need for preamplification, which may compromise measurement reliability (32). We also implemented an extensive quality control and data analysis pipeline to account for preanalytical and technical factors that can affect circulating miRNA levels and result in biases that do not reflect underlying biology. This study demonstrates the feasibility of evaluating plasma miRNAs using nCounter technology, and our results suggest that miRNAs in plasma warrant further evaluation as noninvasive biomarkers for the detection of PDAC precursors.

We show that a 30-miRNA gene signature can partially discriminate IPMN cases from healthy controls [(AUC) = 74.4 (95% CI, 62.3-86.5, $P = 0.002$)]. Several of the highlighted miRNAs (let-7a-5p, let-7d-5p, miR-1260b, miR-142-3p, and miR-146a-5p, miR-23a-3p) are unaffected by hemolysis (18, 20), minimizing red blood cell contamination as a potential source of confounding. The identified miRNAs had 2- to 4-fold higher expression in cases compared with controls, and included miRNAs previously shown to be upregulated (miR-107, miR-145, miR-146a, miR-15b, miR-181a, miR-24) or downregulated (miR-142, miR-148a) in PDAC versus normal tissues (33).

Noteworthy, several candidate miRNAs (miR-145-5p and miR-335) may be involved in inhibiting cancer stem cell properties of PDACs by targeting the transcription factor, *OCT4* (34, 35). Candidate miRNAs such as miR-1260b and miR-4454 are novel and also of interest because validated targets include key drivers in pancreatic carcinogenesis, *SMAD4* (36) and *NF- κ B* (37), respectively. The biologic plausibility of findings was enhanced by pathway-based analysis, which predicted that the identified miRNAs may affect critical pathways involved in PDAC initiation and progression.

Although previous studies (32, 38, 39) have evaluated miRNA expression in blood of PDAC patients and healthy controls, the implications for early detection were limited because most cases had locally advanced or metastatic disease. Of two studies (13, 14) that focused on evaluating blood-based genome-wide miRNA expression in early-stage PDAC patients, one (13) included preoperative serum from patients with IPMNs ($N = 20$; 4 low-grade; 16 moderate-/high-grade). Li and colleagues (13) measured 735 miRNAs by qRT-PCR using TaqMan MicroRNA Arrays, and found that miR-1290 had the best diagnostic performance for subjects with PDAC ($n = 41$)

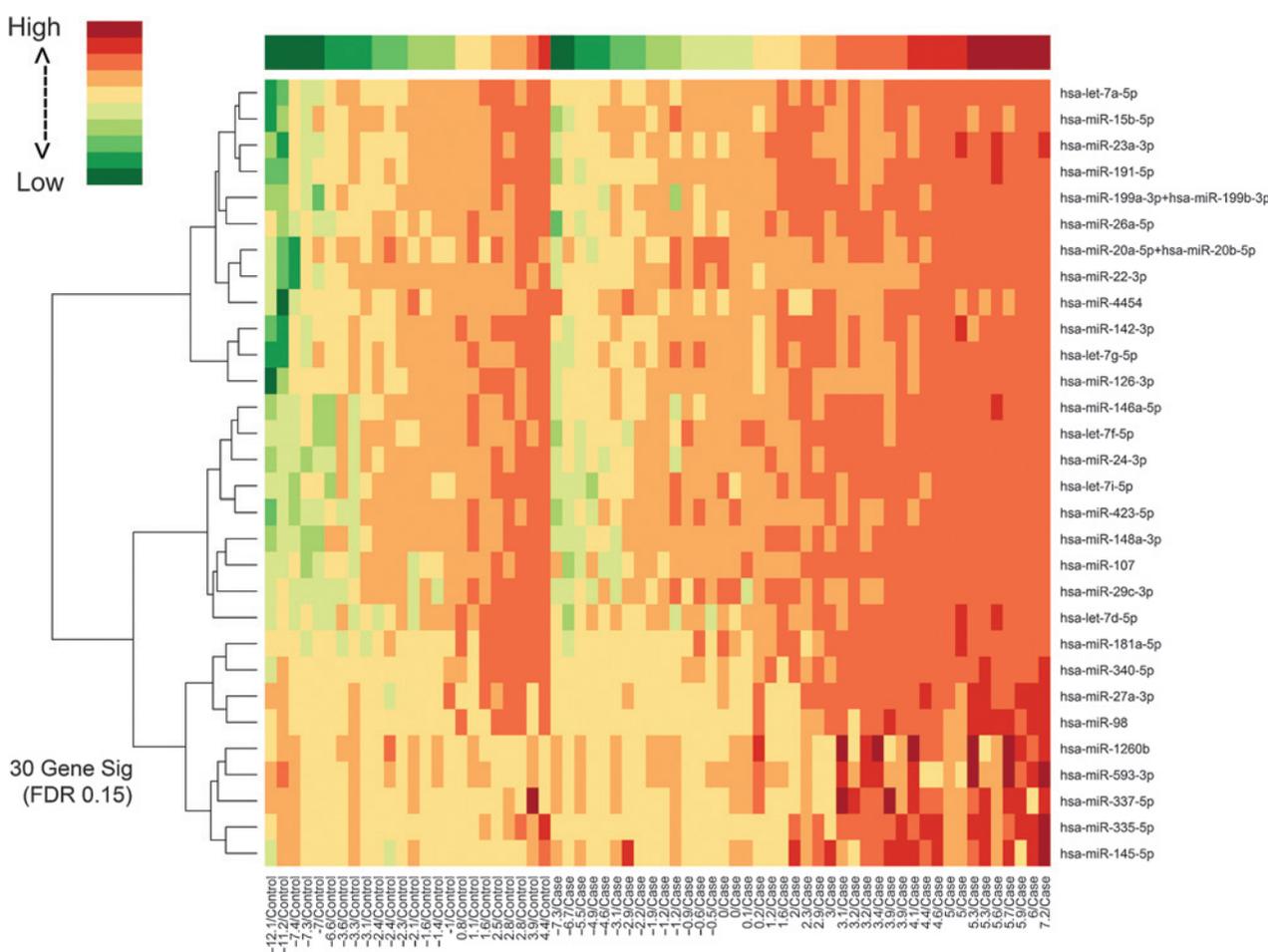


Figure 2. Heatmap of the 30-miRNA signature in IPMN cases and non-diseased controls.

relative to healthy controls ($n = 19$), and that serum miR-1290 levels were higher among the 20 IPMN cases compared with controls (AUC = 0.76; 95% CI, 0.61–0.91). Although miR-1290 was evaluated in the nCounter miRNA codeset, this miRNA was not analyzed because 93% of the 69 samples had values below background. Two miRNAs from our 30-miRNA gene signature, miR-24 and miR-146a, were among the miRNAs that distinguished sera of patients with PDAC from healthy controls with AUCs > 0.70 in the study by Li and colleagues (13), but comparisons of these levels in IPMNs versus healthy controls were not reported. None of the circulating miRNAs identified in our study overlapped with three miRNAs (miR-642b, miR-885,5-p, miR-22) highlighted in a small study of plasma miRNA expression in early PDAC patients versus controls (14).

Analysis revealed a 5-miRNA signature (comprising miR-200a-3p, miR-1185-5p, miR-33a-5p, miR-574-3p, and miR-663b) that partially discriminates between malignant and benign IPMNs (AUC = 73.2; 95% CI, 57.6–88.9). These miRNAs had 2- to -3-fold lower expression in malignant compared with benign cases. Consistent with our findings, miR-200a downregulation has been implicated in epithelial-to-mesenchymal transition and early metastasis (33). On the other hand, miR-200a was shown to be hypomethylated and overexpressed in PDAC (compared with

normal) tissue and in sera of PDAC patients versus controls (40). miR-574-3p can act as a tumor suppressor and regulate cell signaling pathways (41, 42), suggesting that low levels of miR-574-3p may regulate oncogenes that promote malignant IPMN status. It is noteworthy that the candidate miRNAs identified here and in our previous tissue-based study of IPMNs (8) have lower expression in malignant compared with benign IPMNs, supporting a tumor suppressor rather than an oncogenic role. Also consistent with existing data (3), cyst size > 3 cm and main duct involvement were associated with malignant IPMN status in our cohort. We also showed that lower serum albumin levels are correlated with malignant IPMN status, a finding in line with evidence that lower serum albumin levels are associated with poorer survival in PDAC patients (43). Larger studies that account for clinical and radiologic factors are needed to confirm and expand upon these findings.

In the subset of IPMN cases for which paired preoperative plasma and tissue miRNA data were available, plasma miRNA levels were not strongly correlated with paired tissue miRNA expression. This observation suggests that it will be necessary to further explore the origin of circulating miRNAs in IPMN patients. Discrepancies between plasma and tumor miRNA profiles have been reported (29, 44), challenging the hypothesis that the origin

Permeth-Wey et al.

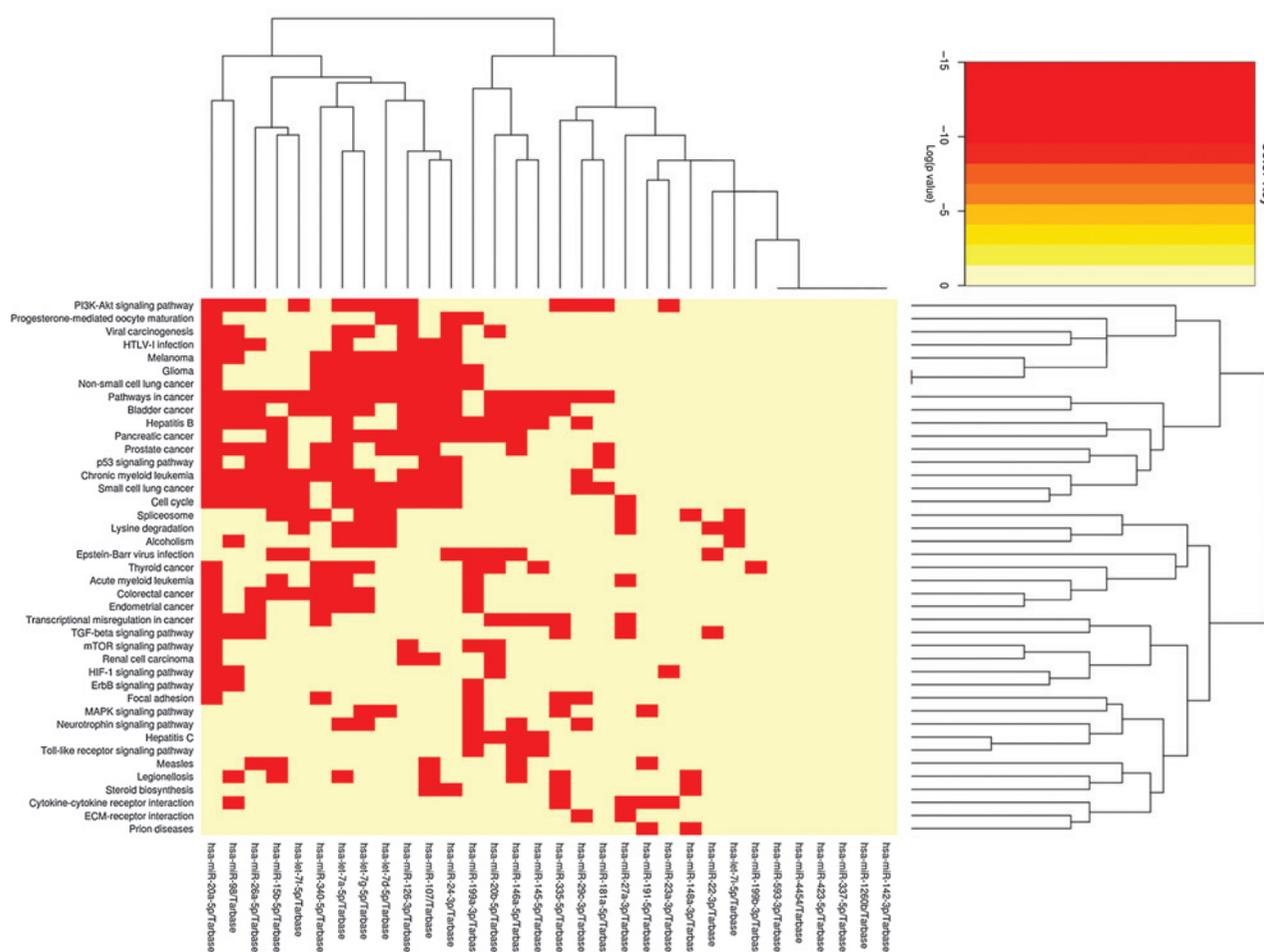


Figure 3. Heatmap of the KEGG pathways enriched for genes targeted by the 30 differentially expressed miRNAs.

of circulating miRNAs is from tumors as a result of cell death and lysis. Alternative explanations for the origin of circulating miRNAs include blood or normal cell contamination, heterogeneity of the primary tumor, dietary sources, and locoregional inflammation reflecting a response of the host microenvironment to disease (19, 45–47).

Despite the strengths and novelty of this study, there are limitations that should be considered. First, the relatively small sample size limits the ability to draw firm conclusions. However, archived plasma from 42 cases and 24 controls provided at least 85% power to detect a miRNA expression difference of 2-fold and above, assuming a set of 800 miRNAs, SD of 1, and a 15% FDR. Technical validation of the Nanostring assay and external validation of findings in a large, multicenter prospective investigation of serial plasma miRNA measurements (pre- and postsurgery or during surveillance) is indicated for individuals newly diagnosed with various types of pancreatic cysts (including IPMNs, MCNs, and benign, nonmucinous cysts) and early-stage PDAC and those at high genetic risk for developing PDAC. To increase diagnostic accuracy and improve outcomes, it may also be necessary to integrate novel classes of molecular markers, imaging features, and clinical characteristics. Future investigations of the most promising miRNAs in animal models may also be instrumental in providing

biologic clues for developing early detection and prevention strategies to impede progression to pancreatic malignancy.

In summary, these data support further development of a plasma miRNA assay for IPMN management. Large-scale studies with rigorous designs and incorporation of epidemiologic and clinical data are needed to further explore the potential for circulating miRNAs to be utilized clinically as novel biomarkers for IPMNs.

Disclosure of Potential Conflicts of Interest

J. Permeth Wey, M. Malafa, and D.-T. Chen have submitted a patent application for a microRNA assay for detection and management of pancreatic cancer precursors. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J. Permeth-Wey, D.-T. Chen
Development of methodology: J. Permeth-Wey, S.J. Yoder, D. Coppola
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Permeth-Wey, S.J. Yoder, D. Coppola
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Permeth-Wey, W.J. Fulp, D.-T. Chen, Y. Zhang, A.M. Magliocco, D. Coppola
Writing, review, and/or revision of the manuscript: J. Permeth-Wey, S.J. Yoder, K. Husain, B.A. Centeno, A.M. Magliocco, D. Coppola, M.P. Malafa

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Permuth-Wey, C. Georgeades, M.P. Malafa
Study supervision: J. Permuth-Wey, M.P. Malafa

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Permeth-Wey et al.

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