Cell-Free Plasma MicroRNA in Pancreatic Ductal Adenocarcinoma and Disease Controls

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Objectives: There are no tumor-specific biochemical markers for pancreatic ductal adenocarcinoma (PDAC). Tissue-specific gene expression including microRNA (miRNA) profiling, however, identifies specific PDAC signatures. This study evaluates associations between circulating, cell-free plasma-miRNA profiles and PDAC in a disease and disease-control cohort.

Methods: We performed a microarray profiling of 847 different mature miRNAs from plasma in an exploratory cohort of 20 patients with PDAC or other pancreatic diseases, profiling of 45 miRNAs in plasma samples from PDAC (n = 48) and disease controls (n = 47), and evaluation of associations of data with diagnosis, survival, and CA-19-9.

Results: We find 7 significantly deregulated miRNAs in PDAC using univariate statistics. At a false-discovery rate of 5%, miRNA-375 remained significantly elevated in PDAC. MicroRNA-375 did not improve diagnosis of PDAC in this cohort (70% accuracy) and did not correlate with survival. However, 3 controls (other gastrointestinal cancers) with increased CA-19-9 did not show increased miRNA-375.

Conclusions: In the plasma-miRNA population, we find miRNA-375, which is selectively expressed in the endocrine pancreas under normal conditions, increased in PDAC cases compared with patients with other pancreatic or gastrointestinal diseases. The miRNA-375 does not outperform CA-19-9 diagnostically in the present cohort. However, it shows promising specificity and should be examined in larger prospective studies.

Key Words: MicroRNA, pancreatic ductal adenocarcinoma, CA-19-9, miRNA-375

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Pancreatic cancer, with ductal adenocarcinoma (PDAC) constituting about 95% of the cases, is the fourth leading cause of cancer death in the world and is characterized by delayed diagnosis, high metastatic potential, and resistance to therapy. Together, these factors lead to a very high mortality. The diagnosis still relies on histology. Imaging techniques have improved PDAC detection, but better biochemical markers are much in demand. The best known marker currently is CA-19-9. An important differential diagnosis is chronic pancreatitis. Biomolecular markers are attractive in comparison with invasive and imaging techniques requiring specialized equipment, trained operators and having potential risks for the patients. Gene expression and mutational analyses of PDAC tissues have repeatedly demonstrated multiple alterations including distinct clonal populations in primary tumors, deregulated signaling pathways, and cancer-related alterations in tissue and cellular microRNA (miRNA) profiles.[1–4] Indeed, a fine-needle aspirate-based miRNA test (using the miR-196a/217 expression difference for diagnosing PDAC) was the first marketed miRNA test for a disease.[5] MicroRNAs are short, single-stranded RNA molecules that act as posttranscriptional regulators of gene expression.[6] Many studies have demonstrated that miRNA expression profiles in malignant tissues are distinctly different from the profiles in corresponding normal tissues.[7,8] The extent to which this is reflected by circulating miRNA profiles is not settled, but owing to the stability[9] and the availability of quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) methods for their assessment, the study of circulating miRNAs as biomarkers for disease diagnosis and progression is now being applied widely in exploratory studies. The surprisingly stable, cell-free miRNAs circulate (as RNA-protein complexes and/or encapsulated in exosomes) in plasma in concentrations much lower than in cells and show some promise as diagnostic molecules for PDAC in preliminary studies comparing small numbers of specific miRNAs in plasma from pancreatic cancer patients and healthy controls.[10,11] A recent publication deals with miRNA measurement in blood cells and pancreatic tissues comparing PDAC and chronic pancreatitis and finds no significant profile.[3] There has been no study, however, investigating cell-free miRNAs in plasma comparing PDAC and non-PDAC pancreatic disease cases, especially chronic pancreatitis. Here, we study global miRNA expression in plasma in a limited number of PDAC cases and disease controls followed by quantitative profiling of 45 miRNAs in 95 cases and controls. A 7-miRNA signature specific for PDAC was found using univariate analysis, and after correction for multiple testing, a more than 3-fold elevation of miR-375 remained a significant characteristic of PDAC cases.

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MATERIALS AND METHODS

Patients and Samples

Samples were collected prospectively from patients referred to endoscopic ultrasonography of the pancreas or endoscopic retrograde cholangiopancreaticography because of suspicion of pancreatic cancer (mass in the pancreas shown by radiological investigations, pain, and weight loss). Final disease status was determined after 12 months of follow-up. Venous blood was sampled uniformly at the first patient visit in EDTA after an overnight fast between 8 AM and 1 PM. Within 2 hours, the samples were centrifuged (2608*g*, 7 minutes), and the

supernatant plasma stored at −80°C until analysis. Storage time ranged from 10 months 17 days to 3 years 2 months 20 days. We included 48 cases with PDAC and strongly suspected of having PDAC (typical clinical and endoscopic findings and a rapid demise but no confirmative histology) and 47 disease controls without PDAC but with other gastrointestinal (GI) conditions mimicking PDAC enough to warrant endoscopic examination of the pancreas, mostly patients with chronic pancreatitis but also other types of GI cancers. In total, 103 different patients were included. Of these, 95 cases and controls were included in the dynamic array analyses (48 cases with PDAC and 47 controls without PDAC [Table 1]).

Quantitative Immunoassay

CA-19-9 was measured in sera by an immunofluorescent assay (Brahms, Hennigsdorf, Germany) run on a Kryptor analyzer using time-resolved amplified cryptate emission technology. The cutoff value is 37 U/mL. The coefficient of variation for the CA-19-9 measurement was 6.7%.

RNA Purification

RNA was purified from plasma (100 or 300 μL for the array) using a total RNA purification kit (Norgen Biotek Corp, Thorold, Ontario, Canada), according to the instructions of the manufacturer with small modifications: 10 mM dithiothreitol and *Caenorhabditis elegans* synthetic miR-39, miR-54, and miR-238 (Tag Copenhagen A/S, Denmark), each at 1.7 pM, were added to a volume of kit lysis buffer sufficient for all the samples. This volume was then aliquoted out into 2-mL portions and kept at −20°C until used. Also, 1 μL of RNAse inhibitor (20 U/μL; Applied Biosystems [ABI], Foster City, Calif) was added to every elution tube before elution of RNA. Purified RNA was kept at −80°C before being used for reverse transcription.

Affymetrix GeneChip miRNA Arrays

A total of 20 plasma-miRNA samples were applied to 20 Affymetrix GeneChip miRNA Arrays (Affymetrix, Inc, Santa Clara, Calif). For the array analysis, total RNA was purified from 300 μL of each plasma sample as described above. The purified RNA samples were concentrated by vacuum evaporation in a SpeedVac centrifuge to 5 to 10 μL. RNA concentrations were determined by 260/280 nm spectrophotometry using a NanoDrop 1000 instrument (Thermo Fisher, Inc, Wilmington, Del). A total of 150 ng RNA was used for each Affymetrix GeneChip miRNA Array. The samples were processed and hybridized to Affymetrix GeneChip miRNA Arrays using Genisphere 3DNA Array Detection FlashTag Biotin HSR kit (Genisphere LLC, Hatfield, Pa).

Various normalization and background correction methods were tested, and the best result was obtained by doing total RNA normalization and robust multichip average background correction.[12]

Dynamic Microfluidic Array Real-Time PCR

Reverse Transcription

Reverse transcription was performed by the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) according to the instructions of the manufacturer with modifications. The RT-primer mix consisted of equal volumes of each of 48 different 5× RT miR-specific stem-loop primers (Applied Biosystems) (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/MPA/A234). Reverse transcription

TABLE 1. Patient Characteristics

	Cases		Controls		*P*
No. (%) patients	48 (50.5)		47 (49.5)		
Sex, male:female, n (%)	27:21 (56.3:43.7)		26:21 (55.3:44.7)		0.55
Age at inclusion, median (95% CI)	65.49 (46.18-79.25)		59.53 (40.14-77.65)		0.01*
Histopathology, staging, and final diagnosis	PDAC verified by histology	40 (83.3)	Chronic pancreatitis	21 (44.7)	
	TMN		No pathology	10 (21.2)	
	1	0	Benign cysts	2 (4.3)	
	2	6 (12.5)	Gall stones	2 (4.3)	
	3	21 (43.75)	Adenocarcinoma metastasis from colon	1 (2.1)	
	4	21 (43.75)	Cholangiocarcinoma	2 (4.3)	
	PDAC not verified by histology	8 (16.7)	Periampullary cancer	2 (4.3)	
			Sequelae after acute pancreatitis	2 (4.3)	
			Suspicion of mucinous cystic neoplasm	1 (2.1)	
			Metastasis from lung cancer	1 (2.1)	
			Primary sclerosing cholangitis	1 (2.1)	
			Epithelioid sarcoma	1 (2.1)	
			Vascular malformation	1 (2.1)	

*Statistically significant differences between groups are indicated.

TABLE 2. Exploratory Study

Probe Name	*P*	FDR
HBII-52-4_x_st	1.00E−04	0.1794
hsa-miR-548b-5p_st	**4.00E−04**	**0.3133**
hsa-miR-548h_st	**0.0016**	**0.7998**
14qII-3_st	0.0036	0.7998
ENSG00000199633_st	0.0037	0.7998
14qII-6_st	0.0039	0.7998
HBII-85-4_x_st	0.0044	0.7998
hsa-miR-186-star_st	**0.0048**	**0.7998**
hsa-miR-25-star_st	**0.0052**	**0.7998**
U32B_x_st	0.0066	0.7998
ENSG00000212139_st	0.0069	0.7998
ACA42_st	0.0074	0.7998
hsa-miR-208a_st	**0.0078**	**0.7998**
ENSG00000199783_st	0.0084	0.7998
hsa-miR-513a-3p_st	**0.009**	**0.7998**
HBII-420_st	0.0094	0.7998
HBII-166_st	0.0095	0.7998
U69_st	0.0095	0.7998
hsa-miR-511_st	**0.0096**	**0.7998**

List of 19 most significant ($P < 0.01$) probes. MicroRNAs are indicated in bold font.

FDR indicates significance after a false discovery rate based correction for multiple testing.

reaction volume was 10 μL using 1 μL of Multiscribe reverse transcriptase, 3 μL of RT-primer mix, 1 μL of 10× RT buffer, 0.2 μL of 100 mM dNTPs, 0.15 μL of RNase inhibitor, and 4.65 μL of RNA purified from plasma. Reverse transcription was performed on an ABI 2720 Thermal Cycler (Applied Biosystems) using a standard protocol (16°C, 30 minutes; 42°C, 30 minutes; 85°C, 5 minutes; hold at 4°C). Reverse-transcribed samples were kept at −20°C until used.

Preamplification

Specific target amplification of the cDNA was accomplished using the TaqMan PreAmp master mix (Applied Biosystems) and a mix of the TaqMan miRNA Assays (Applied Biosystems) consisting of equal volumes of the 48 different 20× assays diluted with 1× TE buffer to a final concentration of 0.2×. Preamplification mixtures (10 μL) contained 2.5 μL of cDNA (diluted 1:3 with H_2O), mixed with 5 μL of 2× TaqMan PreAmp master mix and 2.5 μL of 0.2× TaqMan miR-assay mix. Preamplification was performed on an ABI 2720 Thermal Cycler (Applied Biosystems) with a program at 95°C for 10 minutes, 16 cycles of (95°C, 15 seconds; 60°C, 4 minutes), then hold at 4°C. Preamplified samples were diluted 1:5 with H_2O before the next step (qPCR).

Quantitative PCR

Ninety-five preamplified samples (diluted 1:5 with H_2O) and 48 (20×) TaqMan miRNA assays (see Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/MPA/A234) were applied to a primed 96.96 dynamic array chip using loading and assay reagents according to the manufacturer (Fluidigm Corp, South San Francisco, Calif). All miR assays were performed in duplicates. After loading the reaction chambers using the integrated fluid circuit HX controller from Fluidigm the real-time PCR including image capture after each cycle was performed in a BioMark real-time PCR system (Fluidigm Corp) using single-probe (FAM-MGB, reference: ROX) settings and GE 96 × 96 standard v1 protocol with 40 cycles. Data processing took place using the Fluidigm real-time PCR analysis software (v. 3.0.1).

Data Handling and Analysis

In the 96.96 chip array analysis, the auto(detectors) setting was chosen for data handling. Average raw Cq scores greater than 30 were excluded from all data sets. Each remaining

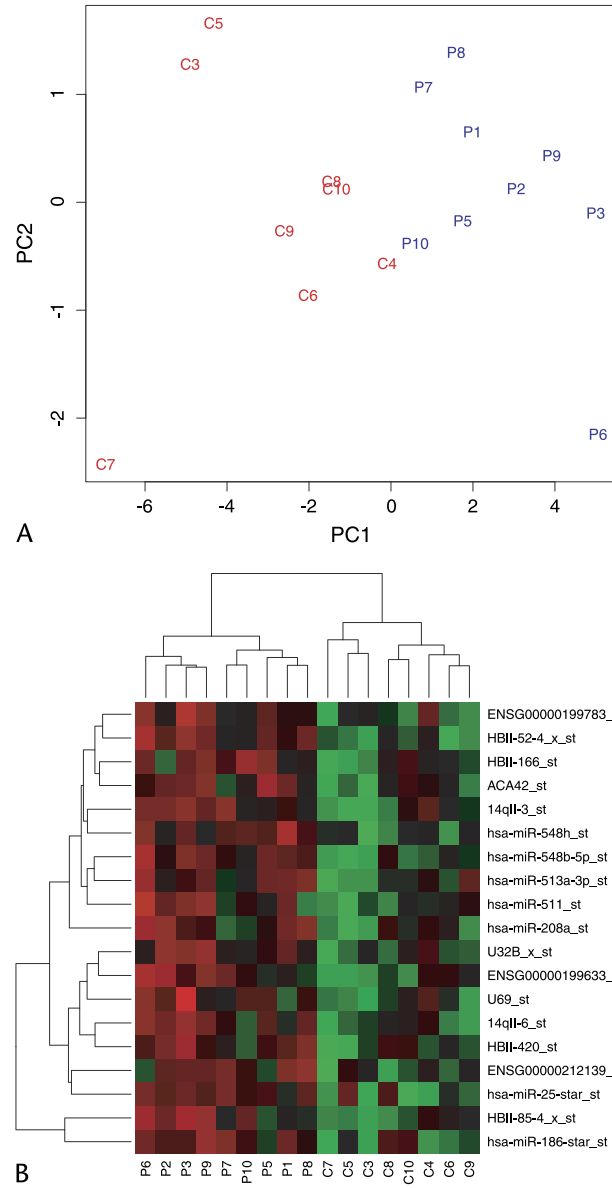

FIGURE 1. Top 19 classifiers (Table 2) from microarray analysis of plasma RNA from PDAC and chronic pancreatitis patients. A, Principal components analysis of PDAC (blue, P) and chronic pancreatitis patients (red, C). B, Hierarchical clustering performed on the subset representing the top 19 most differentially expressed probe sets comparing PDAC patients (P, n = 9) with chronic pancreatitis patients (C, n = 8).

average Cq value was then subtracted from the average Cq of the 2 Cel-miRs (Cel-miR-54 and Cel-miR-238) for that particular sample yielding the ΔCq values used in the further analyses. In this way, samples with high cycle numbers (low expression of a miRNA) get lower numbers than samples with low cycle numbers (more highly expressed miRNAs). All ΔCq values then were row normalized to correct for variations in total input RNA. For each sample, we used the average ΔCq of 13 miRNAs detected in all samples (miR-16, 30a-3p, 92a, 99a, 141, 146a, 155, 181b, 184, 196a, 196b, 222, 513a-3p) to subtract from ΔCq values of all miRNAs. These row-normalized expression values were used for the statistical analyses.

Statistical Analysis

Sample size was estimated in accordance with Flahault et al.[13] Graphs of miR-expression data, receiver operating characteristic (ROC) curves, and statistical analyses of ΔCq values in pancreatic cancer and controls were performed using GraphPad Prism v. 5.04 (GraphPad Software Inc, San Diego, Calif). Statistical significance between groups was determined by unpaired *t* tests or Mann-Whitney *U* tests. $P < 0.05$ was considered significant. Principal components analysis (PCA) and heat maps with unsupervised hierarchical clustering were created using Heatplus library from www.bioconductor.org with the default settings that use Euclidian distance with average linkage and scaling of rows (genes) to the standard score: $(x - \text{mean}) / \text{SD}$.

RESULTS

Exploratory Microarray Analysis of Circulating miRNAs in PDAC

Expression values for 847 human mature miRNAs and 1882 other probes were determined in 20 samples by Affymetrix arrays. After preliminary data analysis, which showed no obvious grouping by PCA (data not shown), 3 samples (1 sample from a suspected PDAC case and 2 samples from patients with normal findings) were excluded to allow comparison between 2 well-defined groups (confirmed PDAC cases and chronic pancreatitis cases). This data set consists of 8 chronic pancreatitis and 9 PDAC cases. A total of 44 miRNAs are included among probes with $P < 0.05$ in the univariate test, and a set of 19 probes (all with univariate $P < 0.01$, Table 2), of which 7 were miRNAs, was able to separate the PDAC patients from the patients with chronic pancreatitis in a PCA plot and in a heat map arranged by unsupervised clustering (Fig. 1). None of the probe sets, however, remained significant after a false-discovery rate–based (FDR = 0.05) correction for multiple testing. All of the 7 miRNAs with univariate $P < 0.01$ were included in the 45-miRNA panel used for the next step of our study.

TABLE 3. Expression Values of 25 miRNAs From Plasma of PDAC Cases and Controls (Table 1)

Rank	miRNA	Cases	Controls	Ratio	*P*	FDR
1	hsa-miR-375	0.0633	0.0203	3.1093	0.0005	0.0125
2	hsa-miR-141	89.5940	57.6725	1.5535	0.0098	0.1025
3	hsa-miR-548b-5p	0.0014	0.0005	2.5469	0.0123	0.1025
4	hsa-miR-221	0.0824	0.1878	0.4389	0.0204	0.1275
5	hsa-miR-92a	0.0254	0.0393	0.6452	0.0307	0.1535
6	hsa-miR-513a-3p	106.8210	77.6406	1.3758	0.0401	0.1671
7	hsa-miR-222	0.0895	0.1518	0.5896	0.0497	0.1775
8	hsa-miR-181b	0.2561	0.1961	1.3057	0.0861	0.2475
9	hsa-miR-17 -5p	0.0052	0.0119	0.4315	0.0943	0.2475
10	hsa-miR-29a	0.0115	0.0186	0.6163	0.0990	0.2475
11	hsa-miR 30a-5p	0.0225	0.0341	0.6611	0.1401	0.2950
12	hsa-miR-15a	0.0006	0.0012	0.5581	0.1416	0.2950
13	hsa-miR 146a	0.3986	0.5877	0.6781	0.1663	0.3198
14	hsa-miR-423-5p	0.0013	0.0022	0.5999	0.1811	0.3234
15	hsa-miR-99a	0.0857	0.0642	1.3345	0.2024	0.3373
16	hsa-miR-148a	0.0050	0.0068	0.7341	0.2588	0.4044
17	hsa-miR 24	0.0728	0.1068	0.6816	0.3010	0.4426
18	hsa-miR 106a	0.0100	0.0149	0.6668	0.4227	0.5608
19	hsa-miR-155	0.7546	0.6667	1.1318	0.4262	0.5608
20	hsa-miR-100	0.5779	0.3862	1.4965	0.4496	0.5620
21	hsa-miR-125b	0.0003	0.0003	0.8511	0.4753	0.5658
22	hsa-miR-16	2.6853	2.9440	0.9121	0.5457	0.6201
23	hsa-miR 196b	6.3183	5.8729	1.0758	0.6982	0.7443
24	hsa-miR-21	0.0389	0.0318	1.2247	0.7433	0.7443
25	hsa-miR-196a	31.5681	33.3177	0.9475	0.7443	0.7443

Included are the geometric means of the normalized expression values as well as the ratio between the means in cases and controls for each miRNA. Data are ranked according to significance, and yellow marks the 7 miRNA with $P < 0.05$ in the univariate *t* test as well as the remaining significant miRNA after correction (FDR) for multiple comparisons.

Profiling of 45 miRNAs in 48 PDAC and 47 PDAC Control Plasma

A total of 38 different miRNAs chosen from previous reports about PDAC tissue-expressed miRNAs in addition to the 7 most significant miRNAs suggested by the array experiments (see Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/MPA/A234; for a complete list of miRNAs) were measured in a total of 95 PDAC and PDAC controls using a microfluidic dynamic array for simultaneous qPCR batch analysis of all samples.[14] Of the 45 assayed miRNAs, 25 could be consistently detected at levels corresponding to cycle thresholds of 30 or less. The case/control expression ratios and statistical significance are listed in Table 3. In summary, 7 (4 up-regulated and 3 down-regulated) of 25 miRNAs were significantly differently expressed in PDAC cases versus controls in a one-by-one (univariate) analysis. The performance of these miRNAs in an unsupervised clustering analysis is shown in Figure 2A, and graphs of the individual values of these miRNAs are included in the Supplemental Figure 1, Supplemental Digital Content 2, http://links.lww.com/MPA/A235). The data show that miR-221, miR-222, and miR-92a were down-regulated, and miR-141, miR-375, miR-513a-3p, and miR-548b-5p were up-regulated in pancreatic cancer cases compared with the disease controls. MiR-513a-3p and miR-548b-5p were among the 19 most significantly ($P < 0.01$) increased probes in the array analysis (Table 2), whereas miR-375 was not significant in the exploratory array analysis. After correction for multiple testing, only miR-375—that on the average was more than 3 times elevated in the PDAC cases—remained statistically significantly different ($q = 0.0125$).

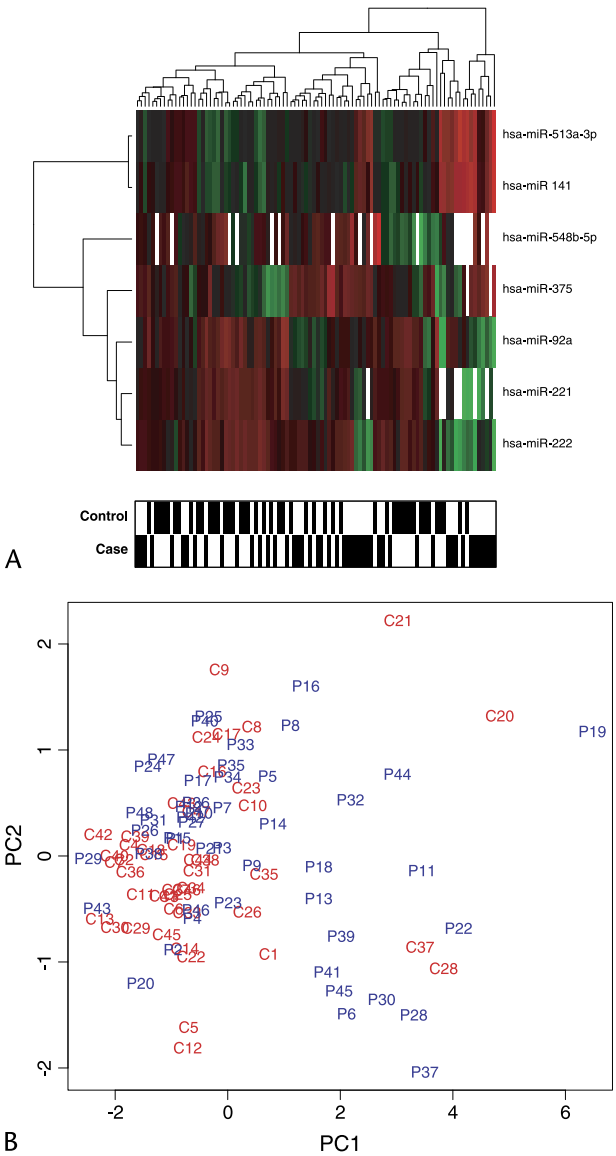

FIGURE 2. Performance of top 7 miRNAs from 45-miRNA panel test (Table 3) in diagnostic classification. A, Unsupervised hierarchical clustering of the 7 significantly deregulated miRNAs. Expression values are given a standard score by scaling each row ([x − mean] / SD) to enable comparing across the miRNAs. B, The PCA (PC1 and PC2 together account for 95% of the variation) of the case (blue) and control (red) samples using the top 4 miRNAs that were detectable in all samples (ie, miR-513a-3p, miR-141, miR-92a, and miR-222).

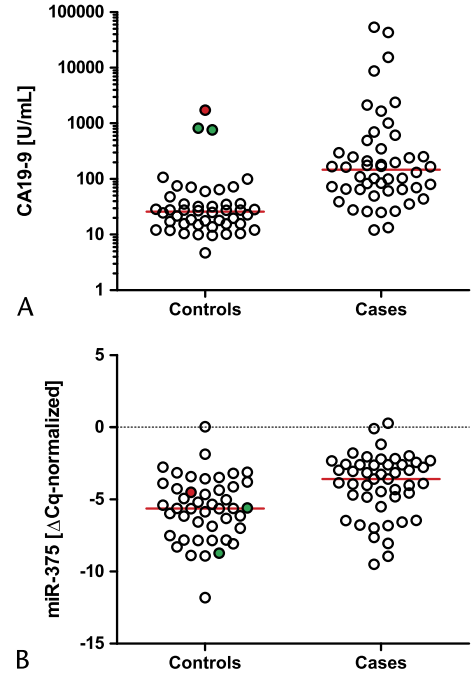

FIGURE 3. CA-19-9 (A) and miR-375 (B) data for all 95 samples. Red lines mark medians. The differences between controls and cases are highly significant in both cases as indicated (Mann-Whitney). Also indicated are 2 cases of cancer of the gallbladder (green) and 1 case of duodenal cancer (red).

MiRNA diagnostic performance

To explore the main contributors to variation between the 2 groups of patients among the miRNAs, we performed a PCA of the top miRNA expression values (Fig. 2B). Because of missing values, only 4 miRNAs could be used for input in the PCA. This did not separate the groups, and thus no combinations of the 4miRNAs contribute to differentiating between the 2 patient groups better than miR-375 alone.

We next compared the diagnostic performance of miR-375 with the established serological marker CA-19-9 (Fig. 3).

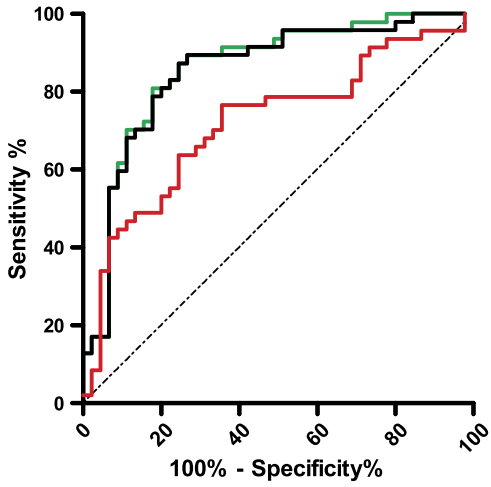

FIGURE 4. Receiver operating characteristic curves for the 92 samples (47 PDAC and 45 PDAC controls) with CA-19-9 and miR-375 data. Red line, miR-375; black line, CA-19-9; green line, miR-375 and CA-19-9 combined.

For CA-19-9, the whole group of PDAC cases had a highly significantly ($P < 0.0001$, Mann-Whitney) increased value (166.3 U/mL; 95% confidence interval [CI], 84.4–216.9 U/mL) compared with the PDAC control group (25.9 U/mL; 95% CI, 17.7–30.4). In Figure 4, the performance of this serological marker is illustrated by the black ROC curve (excluding the 8 samples [3 PDAC and 5 PDAC controls] that were left out from the miRNA panel analysis and 3 samples [1 PDAC and 2 PDAC controls] that did not yield measurable miR-375). The AUC for the CA-19-9 ROC curve is 0.85. For comparison, the red curve (AUC = 0.72) illustrates the performance of the miR-375 values in the same patients. As illustrated by the green curve, the combined CA-19-9 + miR-375 score did not result in better diagnostic performance than CA-19-9 alone. No correlation between CA-19-9 and miR-375 values was observed. Three samples (duodenal and gallbladder cancer) from the PDAC control population that were clearly positive in the CA-19-9 test (colored in Fig. 3) were located close to or below the median of the miR expression values.

Overall, no correlation with survival and miR-375 or miR-196a[15] expression was found.

DISCUSSION

None of the 4 miRNAs (miR-21, miR-210, miR-155, and miR-196a) previously reported to be differently expressed in plasma in PDAC patients compared with healthy controls[10] were confirmed in the present study. Three (miR-21, miR-155, and miR-196a) of the 4 miRNAs were detectable in our cohort, but we were not able to detect any difference between PDAC cases and disease controls, and one (miR-210) was not detectable. The previous study used heparinized plasma (requiring treatment of purified RNA with heparinase to enable miRNA detection by RT-qPCR[16]), and healthy controls were used. In the present study, we analyzed EDTA plasma and diseased controls. Also, the methods for total RNA purification are different in the 2 studies. Another recent study[11] reports the increase in plasma-miR-18a in 36 pancreatic cancer patients compared with 30 healthy controls. This miRNA ranked ($P = 0.059$) as the 124th miRNA on the exploratory array and was not included in the 45-miRNA panel used in the second stage of our study. It is part of the polycistronic oncogenic miR-17-92 cluster consisting of 7 miRNAs, and we here find one member of this cluster, miR-92a, differentially regulated in PDAC plasma—however, it is 1.6-fold down-regulated. Thus, an up-regulation in the circulation of the miR-17-92 cluster in PDAC compared with disease controls cannot be confirmed in our larger study in an ethnically different population. Also, although miR-196a was detectable, the other part of the tissue-based diagnostic test, miR-217,[17] could not be detected in the circulation in our material.

In tissues and cell lines, a number of studies have reported specific PDAC miRNA signatures.[4,15,17–20] These studies find profiles of miRNA that mostly consist of up-regulated miRNAs in microdissected tumor tissues or formalin-fixed, paraffin-embedded tissues that differentiate them from adjacent normal tissues with some consistent alterations but also many unique miRNAs reported in each study. Some studies are focusing on precursor miRNAs, but it has been shown that in many cell types there is a poor correlation between the number of transcribed and mature miRNAs.[21] For mature miRNAs that differentiate PDAC from chronic pancreatitis in tissues, a consistent finding is decreased expression of miR-375 and increased expression of miR-155, miR-196a, miR-203, and miR-221.[4,11,15,17,19,20] We do not find these alterations in the profile of circulating miRNAs in our study. Indeed, we find miR-375 significantly increased in PDAC samples and miR-221 decreased, whereas miR-196a and miR-155 are not statistically significantly different between groups, and miR-203 is not detectable. Thus, the relative contribution of cancer tissue to the circulating miRNA population may be small compared with other tissues. The increase in circulating miR-375 may be due to an increased expression when neighboring tissue is malignant—it could still be decreased in the PDAC cancer tissue itself because this tissue would be expected to contain very few endocrine cells.

A limited number of studies deal with blood-borne miRNAs for PDAC diagnosis[3,10,11,22] but vary greatly regarding sample types (heparinized plasma, serum, whole blood), RNA purification and amplification techniques, and regarding the controls used (mostly healthy controls). Accordingly, different results are reported. Unlike in previous studies, we here use EDTA plasma and a cohort of well-characterized PDAC cases and controls with other conditions, chiefly chronic pancreatitis, that represent the clinical challenge, and all are referred under the suspicion of PDAC. The differentiation between nonmalignant pancreatic inflammation and PDAC by blood cell miRNA analysis was not possible in a previous study using whole blood[3] but was possible in the present study focusing on cell-free miRNA. This miRNA population is a complex mixture of protein-bound and exosomal miRNA.[23–25] The type of fluid (eg, plasma or serum), the way it is isolated, and the ethnicity of the patients significantly affect circulating miRNA profiles.[16]

The miR-375 is selectively expressed in the endocrine pancreas under normal conditions under control of the *Pdx1* transcription factor.[26–28] MiR-375 may also be found in the brain,[29] is induced in some GI tumors,[30] is down-regulated in hepatocellular carcinoma, and acts as an apoptosis regulator.[31] Also, miR-375 is a regulator of the interleukin 13 transcriptome activated in allergic inflammation and is by itself regulated by interleukin 13.[32] Targets of miR-375 include the exocytotic protein myotrophin and phosphoinositide-dependent kinase 1 (PDK1) transcripts,[26,28] and thus miR-375 is a potential tumor suppressor with effects on cellular growth including antiproliferative effects,[17,33] in addition to having multiple targets that are associated with immunoinflammatory conditions.[32]

Conclusively, our study found that miR-375, while being inferior to CA-19-9, is the statistically most significant top

candidate for separating PDAC cases from controls based on profiling of EDTA plasma. The future role of this miRNA as a noninvasive diagnostic aid for early detection and tool to guide prognostic evaluation and treatment in PDAC will await new sets of independent data obtained in larger series of patient samples.

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