

MicroRNA array analysis finds elevated serum miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls

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Running title: Serum miR-1290 as a marker of pancreatic cancer

Key Words: pancreatic cancer, microRNA, intraductal papillary mucinous neoplasm, pancreatic neuroendocrine tumors, chronic pancreatitis, miR-1290

Statement of Translational Relevance:

Pancreatic cancer is characterized by advanced disease at the time of diagnosis and resistance to most therapeutic treatments. Better diagnostic markers are needed to improve our ability to detect pancreatic cancer early. In this study, we performed comprehensive quantitative analysis of over 700 microRNAs in the serum of cases with low-stage pancreatic cancer, healthy and disease controls and validated 18 miRNAs in an independent set of cases and controls. We identified several miRNAs elevated in pancreatic cancer sera that have not been previously described as pancreatic cancer markers. The most accurate serum miRNA we identified was miRNA-1290. MiR-1290 is overexpressed in primary pancreatic cancer tissues relative to normal pancreas. Serum miR-1290 levels distinguished patients with low-stage pancreatic cancer from healthy and disease controls better than CA19-9 and among patients undergoing pancreatic resection, higher serum miR-1290 levels was associated with a poorer outcome. Serum miR-1290 measurements have potential clinical utility.

Abstract

Purpose: Our goal was to identify circulating miRNA levels that could distinguish patients with low-stage pancreatic cancer from healthy and disease controls

Experimental Design: We measured 735 miRNAs in pancreatic cancer case and control sera by QRT-PCR using TaqMan® MicroRNA Arrays. After array analysis, we selected 18 miRNA candidates for validation in an independent set of cases and control samples.

Results: Of the significantly elevated circulating microRNAs in patients with pancreatic cancer compared to controls, miR-1290 had the best diagnostic performance: receiver operating characteristic (ROC) analysis on miR-1290 serum level yielded curve areas (AUC) of 0.96 (95% CI: 0.91-1.00), 0.81 (0.71-0.91), 0.80 (0.67-0.93), for subjects with pancreatic cancer (n=41) relative to healthy controls (n=19), subjects with chronic pancreatitis (n=35), and pancreatic neuroendocrine tumors (n=18), respectively. Serum miR-1290 levels were also significantly higher than healthy controls among patients with intraductal papillary mucinous neoplasm (IPMN)(n=20) (AUC=0.76, 0.61-0.91). Serum miR-1290 levels distinguished patients with low-stage pancreatic cancer from controls better than CA19-9 levels, and like CA19-9, higher miR-1290 levels predicted poorer outcome among patients undergoing pancreaticoduodenectomy. Greater numbers of miR-1290 transcripts were detected by FISH in primary pancreatic cancer and IPMN than normal pancreatic duct cells. MiR-1290 influenced *in vitro* pancreatic cancer cell proliferation and invasive ability. Several other circulating miRNAs distinguished sera of patients with pancreatic cancer from those of healthy controls with AUCs >0.7, including miR-24, miR-134, miR-146a, miR-378, miR-484, miR-628-3p, and miR-1825. **Conclusions:** The detection of elevated circulating miR-1290 has the potential to improve the early detection of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fourth-leading cause of cancer-related death and the most lethal solid cancer in the USA, with a 5-year survival of ~5% and a median survival of less than 6 months (1). Very few patients are diagnosed with stage I pancreatic cancer (2). Early detection is currently considered the best strategy to improve the outcome of pancreatic cancer, and to accomplish this we need to identify and screen high risk groups using the most effective diagnostic tests. Currently, the best available diagnostic tests are imaging technologies including pancreatic-protocol computerized tomography (CT), endoscopic ultrasound (EUS) and magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP)(3). These tests have been applied to high-risk groups undergoing pancreatic screening where EUS and MRI/MRCP are generally preferred as they identify small pancreatic cysts more readily than current CT technologies and do so without the radiation (4-12). Screening is justifiable only when offered to individuals at sufficient risk of developing pancreatic cancer and when the screening test is safe and effective. An accurate blood test of very early-stage pancreatic cancer could help pancreatic screening efforts. If such a test could be applied to a high-risk population to detect pre-symptomatic precancerous lesions and early-stage cancers, it would have the potential to improve outcome and complement EUS- and MRI-based screening protocols.

Numerous studies have investigated circulating markers as potential diagnostic tests for pancreatic cancer. Most marker studies have investigated protein markers (13-16), whereas DNA-based markers have generally been applied to secretin-stimulated pancreatic fluid (17, 18) and cyst fluids (19). The stability of microRNAs in body fluids (20, 21), and the numerous changes in expression in cancers suggest circulating miRNAs could have useful diagnostic utility. Numerous miRNAs have been described as having altered expression in pancreatic

cancer (22), including miR-21 (23, 24), miR-155 (25, 26), miR-146a (27), miR-196a (28), miR-196b (29), miR-200a/b/c (30-32), and miR-217 (28). Initial studies have found elevated miR-18a (33), miRNA-21, miRNA-155, miRNA-196a, miR-200a/b, and miRNA-210 levels in the circulation of patients with pancreatic cancer relative to healthy controls (21), but differences between cases and controls were not sufficiently discriminating to be useful as a clinical test. Levels of miR-135b in resected pancreatic tissues (34) and miR-196a and miR-217 in fine needle aspirates (FNA) helped differentiate pancreatic cancers from normal pancreata and chronic pancreatitis tissues (28, 35). Several miRNAs show altered expression in precursor lesions (e.g. miR-21 (23, 24), miR-155 (25, 26), miR-196a (24, 28), and miR-200a/b (30, 31)), and miRNA profiles of cyst fluids and pancreatic juice samples have been evaluated for their ability to identify the pathology of pancreatic cysts (36) or to find markers of pancreatic cancer or dysplasia (25, 37).

In this study, to more comprehensively profile circulating miRNAs as potential markers of pancreatic cancer, we quantified 735 circulating miRNA levels using miRNA arrays in patients with pancreatic cancer, disease controls with pancreatic neuroendocrine tumors (PNETs), chronic pancreatitis, and healthy controls.

MATERIALS AND METHODS

Cell lines, tissue and serum samples

Four human pancreatic cancer cell lines, Panc5.04, Panc8.13, Panc10.05, and Panc198, were grown as previously described (29). HPDE, immortalized non-neoplastic human pancreatic ductal epithelium cells, was provided by Dr. Ming-Sound Tsao (University of Toronto).

Fresh-frozen pancreatic ductal adenocarcinoma (PDAC) from 14 patients whose cancers were resected at Johns Hopkins Hospital (JHH), and 7 samples of normal pancreatic ductal epithelial cells (from patients with PDAC, intraductal papillary mucinous neoplasms (IPMN) or serous cystadenoma) were microdissected, by laser capture microdissection, deposited in *RNAlater* (Invitrogen) immediately and stored at -80°C for subsequent analysis as described (38).

Serum samples (n=213) were collected at JHH between 2002 and 2011, including 81 preoperative samples from patients with resectable PDAC, 28 preoperative samples from patients with PNETs, 20 samples with IPMN, 45 with chronic pancreatitis, and 39 healthy controls (Table S1). For the microRNA array analysis, to avoid jaundice confounding our results, we did not include any patients whose pancreatic cancer had caused obstructive jaundice prior to their pancreatic resection (none of the controls had obstructive jaundice). All patients with pancreatic ductal adenocarcinoma underwent pancreatic resection and then underwent a variety of different standard adjuvant (5FU-based) and palliative therapies (including gemcitabine) postoperatively. Patients were not selected with knowledge of their tumor burden at surgery or to their subsequent therapies upon disease recurrence. All sera were collected using standard procedures and stored at -80°C until analysis. Tissue microarrays (TMAs) were used for fluorescent in-situ

hybridization (FISH). All study subjects provided informed consent and specimens were collected and analyzed with the approval of the Johns Hopkins Committee for Clinical Investigation.

RNA isolation

Total RNA was extracted using *mirVana* miRNA isolation kit (Ambion-1560) for cell lines, RNAqueous-Micro kit (Ambion-1931) for microdissected cells, and *mirVana* PARIS kit (Ambion-1556) for serum, according to manufacturer's instructions. Serum samples were processed in a blinded fashion. RNA was treated with DNase (Ambion). Extracted RNA was evaluated by spectrophotometry (NanoDrop ND-1000).

MiRNA profiling

Serum miRNA expression was profiled using TaqMan Array Human miRNA Cards A (v2.1) and B (v3.0) (ABI) as previously described (29). After normalization against miR-16 relative miRNA levels were determined using the $\Delta\Delta C_t$ method. Per the qPCR assays performance characteristics, Ct values ≥ 32 were considered too low for accurate quantification. We evaluated the accuracy of the microRNA array by performing duplicate miRNA arrays on one sample and examining the correlation between the results.

MiRNA expression by real-time RT-PCR

Selected microRNAs were measured using individual TaqMan microRNA assays, with TaqMan® reagents (RT mix, and Universal Master Mix II) as previously described (29). Case

and control samples were analyzed in the same assay in a blinded fashion. For microdissected tissue and serum samples, Megaplex RT and preamplification were performed to increase the limit of miRNA detection. RQ Manager Software 1.2) (ABI) was applied to generate Ct values. Relative miRNA levels were determined by $\Delta\Delta\text{Ct}$ using endogenous controls (cell lines and tissues, U6); serum, miR-16). Cells and serum samples were assayed in triplicate and duplicate, respectively. Ct values ≥ 35 was considered negative amplification. ELISA for CA19-9 was performed as previously described (16).

Locked nucleic acid-FISH

Tissue microarrays (TMA) were constructed from the archival formalin-fixed, paraffin-embedded tissue blocks of surgically resected primary PDAC using a manual Tissue Puncher/Arrayer (Beecher Instruments) as previously described (29). Locked nucleic acid-FISH (LNA-FISH) was performed on two TMA slides containing ductal adenocarcinoma and normal pancreas tissues from 32 patients who had undergone pancreatic resection and also on TMA slides of IPMN and normal pancreatic tissues (39) from 39 patients who had undergone pancreatic resection for an IPMN using LNA oligonucleotide probes against miR-1290 labeled with fluorescein at the 5'-end (Exiqon), according to previously published protocols J (29, 40). The grade of each IPMN was classified based on the highest grade of dysplasia identified in the lesion. The LNA-miR-1290-FISH results were quantified at a single-cell level by counting expression spots per cell as previously described (41).

MiR-1290 mimic and inhibitor

Cells were plated at $1.5-2 \times 10^3$ and 1×10^5 cells per well in 96- and 6-well plates one day before transfection, and transfected with Dharmacon hsa-miR-1290 mimic or hairpin inhibitor with appropriate control miRNAs at 30nM final concentrations using DharmaFECT-2 transfection reagent according to manufacturer's instructions. Cell growth was assessed utilizing a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega), following manufacturer's instructions, with cell-free reactions as controls and assayed at 48, 72, 96, and 120 hours. Measurements were performed in five replicates. Cell invasion was measured using Biocoat matrigel invasion chambers (BD Bioscience, Sparks, MD). First, mimic or inhibitor-treated cells were incubated for 48 hours. Cells were loaded and incubated in matrigel-coated plates in 5% CO₂ at 37°C for 40 h. After non-invading cells were removed from the membrane upper surface, invading cells were fixed (70% ethanol) stained with H&E and counted (~90% of the membrane). All assays were performed on triplicate samples and in triplicate.

Statistics

Principal components analysis was performed using Partek Genomics Suite 6.6. Mann-Whitney was used to compare median miRNA and CA19-9 levels. Since TaqMan arrays do not accurately quantify miRNAs with Ct values >32, we did not evaluate miRNAs commonly below this level in pancreatic cancer sera. Fold change was defined as the ratio of medians between groups. ROC curves were generated to evaluate candidate markers and the area under the curve (AUC) was computed by the trapezoidal method in GraphPad Prism. The receiver operator characteristics (ROC) curve of single markers and marker combinations including adjusted ROCs were calculated in SPSS. ROCs adjusted for differences in disease groups were generated with the predicated probability calculated by binary logistic regression. False discovery rates (FDR) were

calculated to adjust for multiple comparisons (using the “Step-up” method (42); in Partek Genomics Suite v6.6). Power calculations were performed to examine our power to detect differences between cases and controls. For the discovery phase, assuming an alpha error rate of 0.05 and multiple testing of 735 markers we would have a 99% power to detect differences of 10-fold in the mean of a marker in cases vs. controls assuming a standard deviation of half of the mean. For a marker with a 10-fold difference between cases and controls and a standard deviation equal to the mean of the cases, we would have 80% power to identify a significant difference with an alpha of 0.05 but only 7% after adjusting for comparing 735 markers. For the validation phase, for this same marker we would have 92% power to detect a significant difference between cases and controls, after adjusting for multiple testing. Pancreatic cancer patients were stratified into two groups by their median miR-1290 level and the proportion of clinicopathological subgroups compared using *Chi-square*. Overall patient survival, defined as the time from pancreatic resection to death or date of last follow-up, was analyzed using Kaplan-Meier, and differences evaluated using log-rank test. Cox proportional-hazards regression analysis was applied to estimate hazard ratios for survival. A two-tailed *p*-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS Statistics 19.0, Partek Genomics Suite 6.6, GraphPad Prism 5.0, and Excel.

RESULTS

Serum microRNA array profiling

To identify potential serum microRNA markers of pancreatic cancer, we first measured serum levels of 735 microRNAs by real-time PCR, normalized using miR-16, in patients with resectable pancreatic cancer (PC) (n=19; Stage I: 3; Stage II: 16), chronic pancreatitis (CP) (n=10), healthy controls (N) (n=10), and patients with PNETs (n=10) using TaqMan Array MicroRNA Cards after preamplification of serum RNA.

Serum miRNA levels in each diagnostic group were distinct by principal component analysis (Figure S1A). The array results were highly reproducible: Array Ct values for a serum sample (CT8) were compared in two independent arrays and were found to be highly correlated ($r=0.95$ overall and 0.98 using Ct values <32 , Spearman) (Figure S2A).

Serum microRNA levels in patients with pancreatic cancer compared to controls

We compared median miRNA levels in sera from patients with pancreatic cancer and control groups adjusting for false discovery. By microarray analysis, numerous miRNAs were significantly elevated in pancreatic cancer sera compared to healthy controls and subjects with chronic pancreatitis. We provide a list of miRNAs that were significantly different (elevated) or trended towards significance after adjusting for false discovery (Table 1, Figures S1B, and Tables S2-S5). There were no microRNAs whose levels were significantly lower by microarray analysis in all 3 control groups compared to pancreatic cancer cases (data not shown). We therefore attempted to validate the microRNAs identified as elevated in pancreatic cancer sera. Serum levels of eighteen miRNAs (miR-21, miR-22, miR-24, miR-134, miR-146a, miR-200c, miR-210, miR-378, miR-484, miR-486-3p, miR-550, miR-584, miR-625, miR-628-3p, miR-

744*, miR-1285, miR-1825, and miR-1290) were measured with individual Taqman assays for each miRNA (normalized against miR-16) in independent samples; sera from 41 patients with resectable pancreatic cancer (Stage I: 6; Stage II: 28; Stage III: 7) and 72 controls; 35 with chronic pancreatitis, 18 with PNETs, and 19 healthy controls. Among the 18 miRNAs selected for validation, some were selected because they had the most significant differences compared to the disease groups by the microarray analysis (miR-550, miR-584, miR-1285, miR-1825, and miR-1290), three miRNAs were selected because they had previously been found to be overexpressed in pancreatic cancer tissues and elevated in pancreatic cancer sera (miR-21, miR-200c, and miR-210), one miRNA was selected because there was mixed reports as to whether it was over or under-expressed in pancreatic cancer (miR-146a), and the remaining candidates were randomly selected from the list of candidate miRNAs elevated in pancreatic cancer sera by microarray analysis compared to one or more disease control groups.

In this independent sample set, most miRNAs tested (miR-22, miR-24, miR-134, miR-146a, miR-200c, miR-210, miR-378, miR-484, miR-550, miR-625, miR-628-3p, miR-744*, miR-1290, and miR-1825) trended higher in pancreatic cancer sera compared to the sera of one or both control groups (Fig. 1 and Fig. S3), but using a level of significance to account for multiple comparisons ($p < 0.001$), only miR-24, miR-134, miR-146a, miR-378, miR-484, miR-628-3p, miR-1290 and miR-1825 levels remained significantly elevated (at $p < 0.001$) in pancreatic cancer sera compared to one or more control groups.. The microRNA that best discriminated between the pancreatic cancer and control groups was miR-1290. In contrast to our previously reported results (31) we did not find any significant elevation of miR-200a or miR-200b by microarray analysis of the sera of patients with pancreatic cancer compared to controls. One important difference between our prior report and our current results was the use of pre-

amplified miRNAs for the array analysis. We therefore repeated the miR-200a and miR-200b measurements in another set of sera from 21 patients with pancreatic cancer and 10 healthy controls by Taqman assay without pre-amplifying serum miRNA, and again found miR-200a ($p=0.0015$) and miR-200b ($p=0.0334$) were elevated in the cancer compared to control sera. We next examined the influence of miRNA pre-amplification on miR-1290 levels and found pre-amplification had no significant effect on miR-1290 levels (the correlation between pre-amplified and untreated sera was: $r=0.97$; $p < 0.0001$; Spearman) (Figure S2B).

Diagnostic utility of significantly elevated serum miRNAs in pancreatic cancer

We next measured the diagnostic accuracy of the best performing miRNAs (Table 2). Serum miR-1290 had the highest diagnostic utility with receiver operating characteristic (ROC) curve areas (AUC) of 0.96 (95% CI=0.91 to 1.00) against healthy controls 0.81 (95% CI=0.71 to 0.91) against chronic pancreatitis, and 0.80 (95% CI= 0.67 to 0.93) against PNETs. The AUC of serum miR-1290 for pancreatic cancer relative to all controls combined was 0.85 (95% CI=0.78 to 0.92) (Fig. 2A, Table 2). The diagnostic value of miR-1290 was similar in subjects with larger and smaller tumors (≤ 2.0 cm). There was no significant difference in miR-1290 levels among those with smaller vs. larger tumors ($p=0.47$)(Figure 1), perhaps because tumor diameter is not a very reliable estimate of tumor burden in pancreatic cancer,. We next compared the diagnostic performance of miR-1290 to CA19-9 and found miR-1290 had higher overall diagnostic accuracy (Tables 2, S6). To determine if miR-1290 provided independent diagnostic utility beyond CA19-9 we used logistic regression and found both miR-1290 (odds ratio=15.4; 95% C.I., 4.99-47.71; $p < 0.001$) and CA19-9 (odds ratio=5.86; 95% C.I., 2.10-16.32; $p=0.001$) were independent predictors of a pancreatic cancer diagnosis.

CA19-9 levels were measured in all the pancreatic cancer and disease control samples to determine the correlation between CA19-9 and miR-1290 and a significant correlation was observed ($r=0.453$, $p<0.0001$, Spearman). We also evaluated the diagnostic utility of combining miR-1290 with CA19-9. Combining these 2 markers did not improve diagnostic accuracy (data not shown). Since our pancreatic cases and control groups were different by age and gender possible effects of age and gender differences on miR-1290 levels, we adjusted for these potential confounders in a logistic regression model that included age and gender. The age- and gender-adjusted AUCs of ROC curves for miR-1290 in pancreatic cancer cases vs. healthy controls were not significantly different to the unadjusted ROC curves. We did find that an age-adjusted ROC curve was somewhat lower when we compared the pancreatic cancer cases to those with chronic pancreatitis (AUC 0.72 ± 0.06 ; 95% CI: 0.60-0.83).

We also compared the diagnostic performance of the other top candidate miRNAs. Serum miR-146a had the next best performance (AUC=0.78, 95% CI, 0.68-0.89) and 0.82 (95% CI, 0.71-0.92) relative to chronic pancreatitis patients and healthy controls, respectively (Table 2 and S5). Serum miR-22, miR-24, miR-134, miR-210, miR-378, miR-484 and miR-628-3p had AUCs of 0.73-0.82 for differentiating pancreatic cancer patients from healthy controls (Tables 2, S5).

Serum miR-1290 in patients with IPMN

Serum levels of miR-1290 in patients with IPMNs were higher than among healthy controls (fold-change=3.8, $p=0.0052$) (AUC 0.76 (95% CI: 0.61 to 0.91). Patients with invasive pancreatic cancer had higher mean serum levels than patients with IPMNs ($p<0.0001$) (Figure 1). Serum miR-1290 levels trended higher in subjects with IPMNs with intermediate- and high-grade dysplasia than those with low-grade dysplasia ($p=0.098$). MiR-1290 had an AUC of 0.82

(95% CI: 0.68 to 0.96) for differentiating patients with high-grade or intermediate-grade IPMNs from normal controls.

Candidate miRNA expression in pancreatic tissues

We next measured with Taqman assays candidate miRNAs identified from the array analysis (miR-24, miR-134, miR-146a, miR200c, miR-378, miR-484, miR-550, miR-625, miR-628-3p, miR-1825, and miR-1290) in microdissected primary invasive ductal adenocarcinoma cells and normal pancreatic duct cells (Figure 3A). Apart from miR-550, all other miRNAs were elevated in pancreatic cancer tissues (n=14) compared to normal pancreatic duct (n=7) suggesting pancreatic cancer cells as a source of the serum elevation.

LNA-FISH analysis of miR-1290 expression in pancreatic tissues

To evaluate further the source of elevated serum miR-1290 levels, we quantified pancreas miR-1290 expression by LNA-FISH on pancreatic TMAs. LNA-FISH confirmed elevated miR-1290 in primary pancreatic cancer cells; a median of 14.85 spots/cell (95% CI: 12.84 to 15.91) were detected in the cancer cells of 32 primary pancreatic cancers but only 1.9 spots/cell (95% CI:1.90 to 3.04) in tissues cores containing normal pancreatic duct from 31 of the 32 resected pancreata (Figure 3B, 3C) ($p < 0.0001$).

Similarly, primary neoplastic IPMN tissues from 39 individuals had significantly more spots/cell than the evaluable normal pancreatic duct tissues in the resected pancreata of 28 of these ($p < 0.0001$). When we stratified IPMNs by their neoplastic grade, significant overexpression was observed in IPMNs of all grades (Figure 3B, 3D).

Serum miR-1290 and miR-486-3p post-operative survival

To determine if elevated serum miRNA levels predicted outcome after pancreatic resection for pancreatic cancer, we compared post-operative survival in 56 patients with pancreatic cancer according to their serum levels of each of the 18 evaluated miRNAs (follow-up data was not available for 4 cases). We found patients with serum miR-1290 or miR-486-3p levels at or above the median had significantly poorer survival than those with levels below the median ($p=0.0361$ and $p=0.0397$, respectively; log-rank test) (Figure 4). Serum levels of the other 16 miRNAs were not associated with outcome. Univariate Cox analysis revealed T stage ($pT\geq 3$), lymph node metastasis (pN1), overall stage ($> I$), and serum miRNA level (high miR-486-3p and miR-1290 levels) were significantly associated with shorter patient survival (Table S7). Multivariate analysis indicated high serum miR-486-3p, high serum miR-1290, and high stage were independent predictors in each model (Table S7). The survival difference between patients with high and low miRNA levels remained when serum miR-486-3p and miR-1290 levels were combined (high levels of either miRNA vs. low levels of both miRNAs) (Figure 4). There were no significant associations between clinicopathological factors and serum miR-486-3p and miR-1290 levels (Table S8).

MiR-1290 function in pancreatic cancer cells

To explore miR-1290 function, we examined the effects of miR-1290 on pancreatic cancer cell proliferation and invasion. We transfected miR-1290-low-expressing pancreatic cancer cells (AsPC1, Panc5.04) (defined as $<1/5$ the relative expression level in HPDE, data not shown) with miR-1290 mimic and control miRNAs. We also treated miR-1290-expressing pancreatic cancer cells (Panc10.05 and Panc198) with miR-1290 inhibitor and control miRNAs.

The efficiency of miR-1290 treatment and inhibition is shown in Figure S4A. Compared with negative control miRNA, the two miR-1290-low expressing cell lines, exhibited increased cell proliferation 72h after mimic miRNA introduction, whereas miR-1290- expressing cell lines, exhibited growth inhibition when treated with the inhibitor (Figure S4B).

MiR-1290-mimic also significantly increased invasion, relative to mimic control transfected cells, ($p=0.0032$, for both cell lines; Student's test) (Figure S4C, S4D). Similarly, the miR-1290 inhibitor inhibited invasion ($p=0.044$, $p=0.029$; Student's test) (Figure S4C, S4D).

DISCUSSION

We have identified several miRNAs elevated in the sera of patients with resectable pancreatic cancer compared to controls that have not been reported previously. While many of these miRNAs were only modestly elevated in pancreatic cancer sera compared to controls, serum miR-1290 had excellent ability to distinguish serum from patients with low-stage pancreatic cancer from control sera and serum miR-1290 levels discriminated patients with pancreatic cancer from controls better than CA19-9. We found that pancreatic cancer cells overexpress miR-1290 suggesting these cells are the likely source of elevated serum levels, and we also find that patients with the highest levels of miR-1290 (and miR-486-3p) have poorer outcome after resection of their pancreatic cancer. This association could reflect more extensive or more aggressive disease. Serum miR-1290 levels from patients with pancreatic ductal adenocarcinoma were obtained prior to their surgical resection for their disease and their subsequent course and treatment was independent of their preoperative serum level. However, since variation in postoperative treatments within the study population could have affected patient outcome, the prognostic significance of miR-1290 should be evaluated further before being considered a validated prognostic marker.

One potentially important finding was our observation that miR-1290 levels were elevated in the serum of patients with non-invasive IPMNs. The finding that miR-1290 levels are elevated in patients with IPMNs raises the possibility that this marker could be used to monitor patients at risk of developing IPMNs, such as subjects with a strong family history of pancreatic cancer. However, such a possibility requires further study, particularly since highly accurate markers are needed in this setting. The overexpression of miR-1290 in IPMNs suggests that it

also deserves evaluation as a cyst fluid marker to determine if it can aid in the differential diagnosis of pancreatic cysts.

Our results indicate that treating pancreatic cancer cells with miR-1290 can increase growth and invasion, consistent with recent reports about the function and predicted targets of miR-1290 which include *FoxA1* and other genes (43). Variants in *FoxA1* have been associated with breast cancer risk (44), and epigenetic silencing of *FoxA1* in pancreatic cancers has been implicated in epithelial mesenchymal transition (45) suggesting it could be an important target of miR-1290.

Two prior reports have used microarrays to measure circulating microRNAs in subjects with pancreatic cancer (46, 47). These studies utilized pooled serum or plasma and only 2 microRNAs (miR-21 and miR-483-5p) were identified as elevated in pancreatic cancer in both studies. Three miRNAs we identified as elevated in pancreatic cancer sera in training and testing sets (miR-24, miR-134 and miR-378) were identified as elevated in one of these two studies (46, 47). Other studies have evaluated candidate miRNAs. In one study, a 7-miRNA-based marker panel had good accuracy for distinguishing pancreatic cancer sera from healthy control sera (46). Some studies have found that combining microRNAs with CA19-9 improved diagnostic performance (48), although we did not find this to be the case for miR-1290 and CA19-9.

MiR-146, one of the miRNAs we found elevated in pancreatic cancer sera and overexpressed in primary pancreatic cancer cells, has been evaluated as a cancer susceptibility locus and silencing of miR-146a has been reported in several cancers (49).

We employed pre-amplification of microRNA for the miRNA array serum analysis. We found no evidence that pre-amplification affected our serum miR-1290 measurements-results were highly correlated when we directly compared matched pre-amplified to untreated serum

RNA. Furthermore, others have shown that minute levels of miRNAs quantified with or without a pre-amplification step are highly correlated (50). However, we did find evidence that pre-amplification affected serum miR-200a/b levels, so the effects of pre-amplification should be considered when evaluating candidate serum microRNA markers.

In conclusion, using miRNA arrays we have identified multiple elevated miRNAs in the serum of patients with low-stage pancreatic cancer. We find serum MiR-1290 is a highly sensitive and specific marker for pancreatic cancer that has considerable diagnostic potential.

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FIGURE LEGENDS

Figure 1. Serum miRNA-1290 levels by patient group by Taqman real-time PCR; pancreatic

cancer (PC) and intraductal papillary mucinous neoplasms (IPMN) relative to patients with pancreatic neuroendocrine tumor (PNET), chronic pancreatitis (CP), normal controls (N), non-neoplastic controls (CN), and all controls (NCN), PC patients with tumor size of ≤ 2.0 cm and > 2.0 cm, and IPMN patients with intermediate-high-grade dysplasia and low-grade dysplasia. Boxes represent the inter-quartile range and the line indicates the median value. Whiskers indicate 90th and 10th percentiles. MiR-16 was used for normalization. Mann-Whitney was used to evaluate statistical significance.

Figure 2. Receiver operating characteristics (ROC) curve analysis for serum miR-1290 levels (A) patients with pancreatic cancer, (B) patients with smaller tumors (≤ 2.0 cm), and (C) CA19-9 relative to patients with pancreatic neuroendocrine tumor (PNET) chronic pancreatitis (CP), normal controls (N), non-neoplastic controls (CN), and all controls (NCN).

Figure 3. (A). Expression of serum miRNA candidates in microdissected tissues of pancreatic cancer (n=14) relative to normal duct cells (n=7) by Taqman real-time PCR (B) Overexpression of miR-1290 in pancreatic cancer and IPMN tissues by LNA-FISH on tissue microarrays. Arrows indicate LNA-miR-1290 probe signals (green spots) which are circled with white dashed lines. DAPI indicates nucleic acid staining (blue) Original magnification, 40 \times . Quantitative analysis of miR-1290 expression in pancreatic cancer (C) and IPMN (D) by calculating green spots per cell in TMAs. Statistical significance was determined using Mann-Whitney test. Boxes represent the inter-quartile range and the line indicates the median value. Error bars indicate the 90th and 10th percentiles.

Figure 4: Kaplan-Meier overall survival curve of patients with pancreatic cancer based on their expression of miR-486-3p (A), miR-1290 (B) in serum, and combined miR-486-3p and miR-1290.

Figure 1

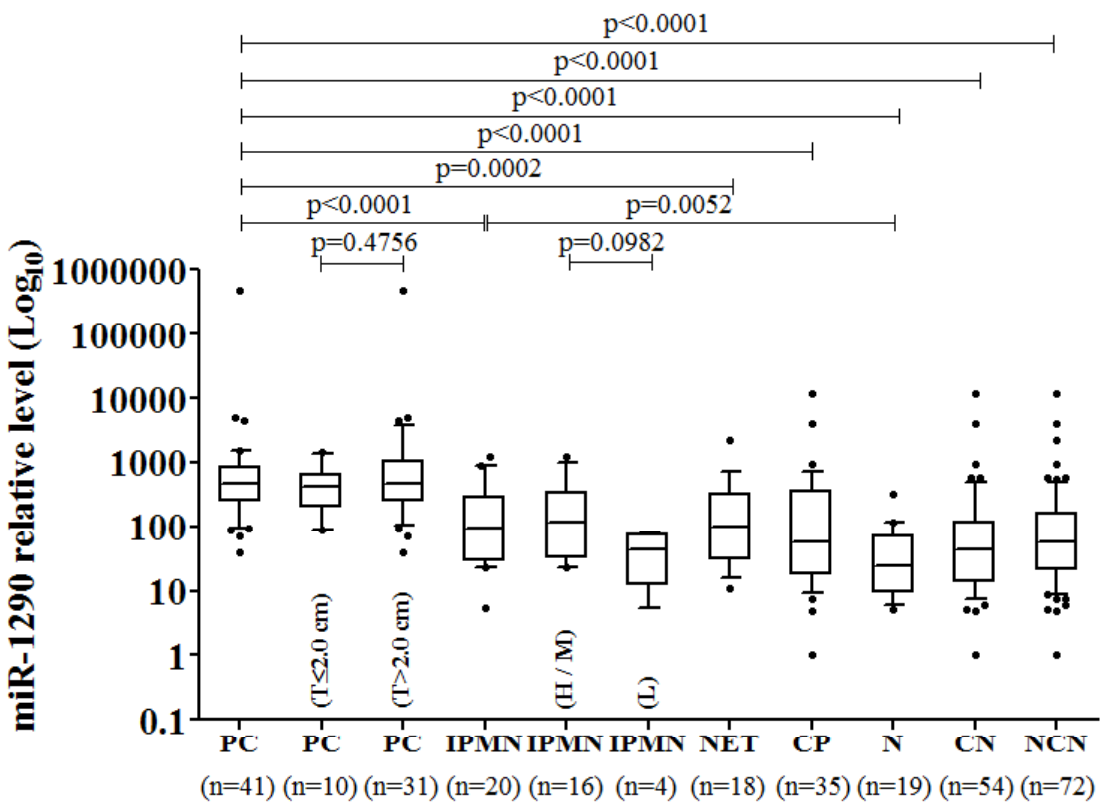


Figure 2

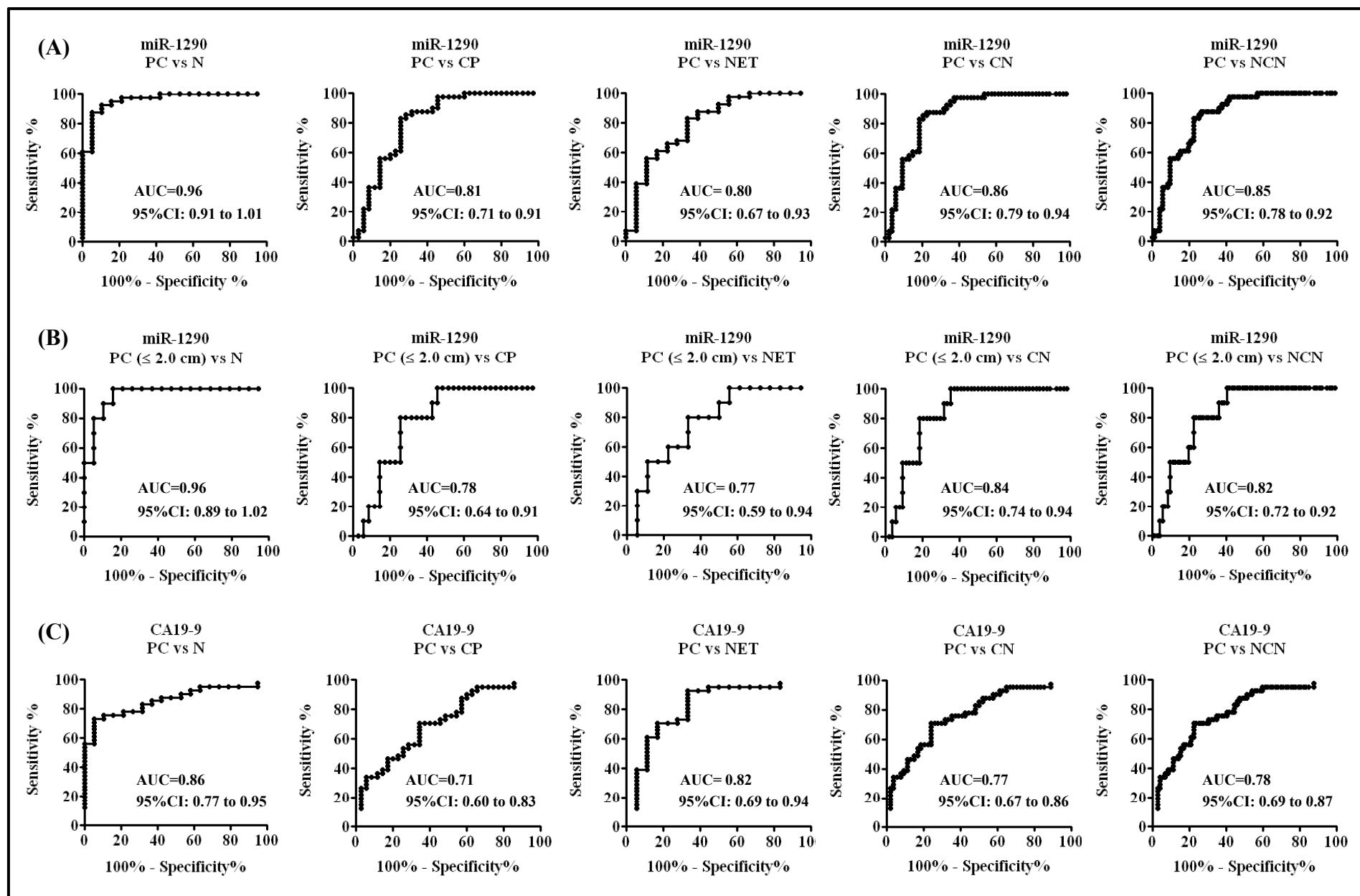


Figure 3

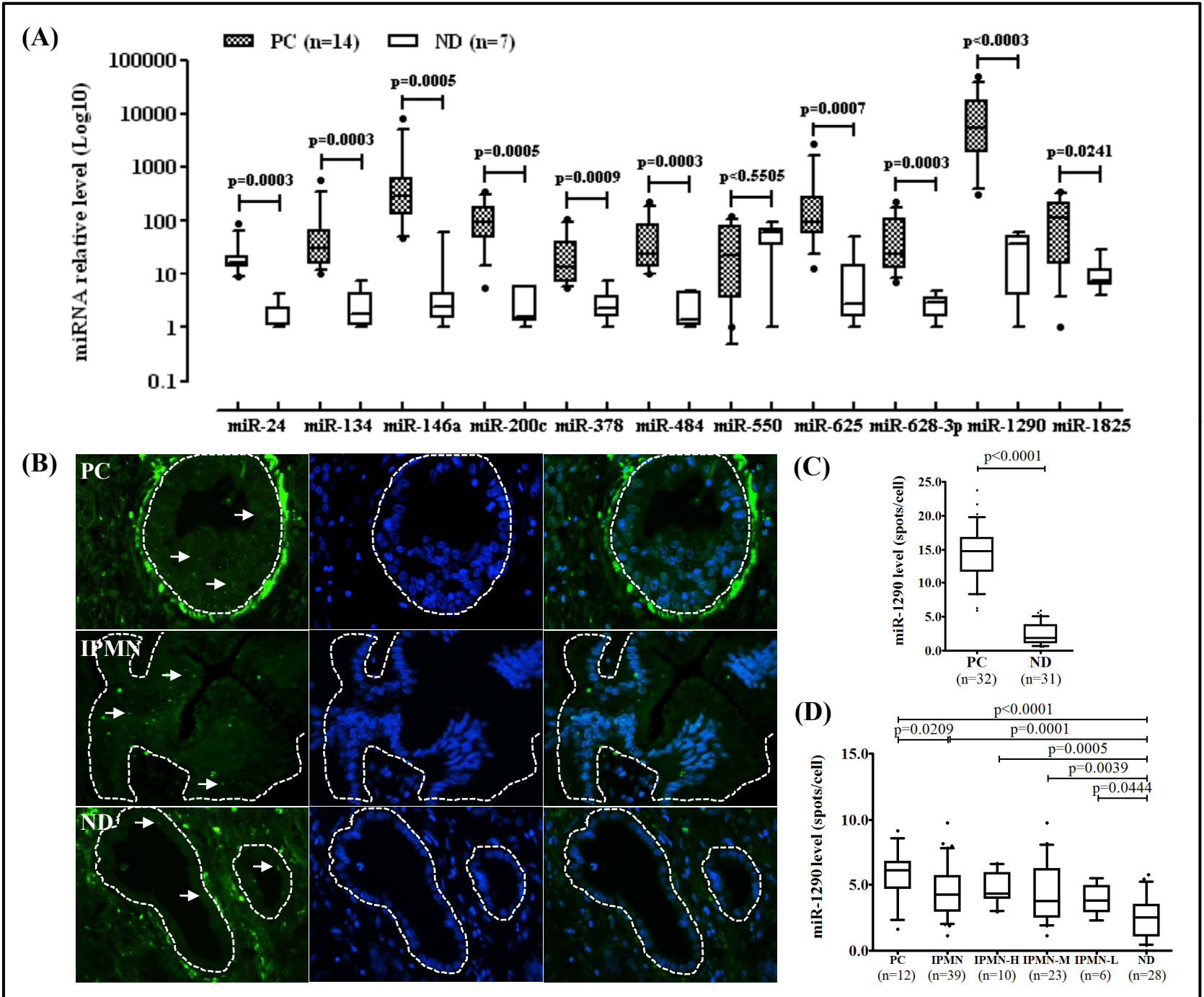


Figure 4

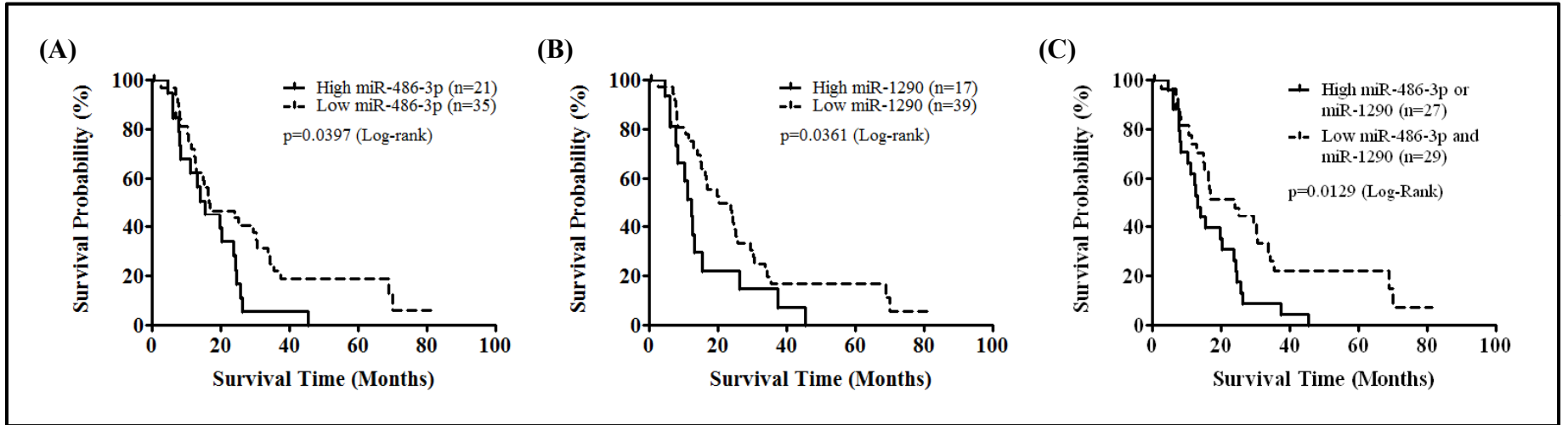


Table 1. MicroRNAs elevated in the serum of patients with pancreatic cancer vs multiple control groups by microarray analysis

No.	miRNA ID	PC vs. N			PC vs. CP			PC vs. NET			No.	miRNA ID	PC vs. N			PC vs. CP			PC vs. NET		
		F.C.	P.	FDR	F.C.	P.	FDR	F.C.	P.	FDR			F.C.	P.	FDR	F.C.	P.	FDR	F.C.	P.	FDR
1	hsa-miR-584	2.9	0.004	0.050	7.1	0.001	0.019	3.6	0.010	0.071	41	hsa-miR-579	3.6	0.004	0.047	13.2	0.012	0.028	ns		
2	hsa-miR-1285	3.1	0.013	0.076	9.9	0.009	0.026	3.7	0.031	0.133	42	hsa-miR-380-5p	126.0	0.019	0.083	489.8	0.012	0.028	ns		
3	hsa-miR-550-002410	2.3	0.025	0.092	3.8	0.009	0.026	314.6	0.002	0.039	43	hsa-miR-93	2.0	0.019	0.083	3.0	0.012	0.028	ns		
4	hsa-miR-1825	2.6	0.048	0.134	5.8	0.017	0.032	1141.9	0.000	0.027	44	hsa-miR-27b	3.3	0.028	0.099	13.3	0.012	0.028	ns		
5	hsa-miR-1290	3.4	0.004	0.047	8.1	0.019	0.034	3.7	0.066	0.205	45	hsa-miR-15b*	793.6	0.002	0.046	7.5	0.013	0.030	ns		
6	hsa-miR-744*	6.7	0.001	0.036	17.7	0.002	0.019	ns			46	hsa-miR-29b	3.6	0.013	0.076	14.0	0.013	0.030	ns		
7	hsa-miR-26b*	3.9	0.001	0.038	10.4	0.001	0.019	ns			47	hsa-miR-1303	628.4	0.004	0.047	15.7	0.015	0.031	ns		
8	hsa-miR-625	4.3	0.003	0.047	8.9	0.001	0.019	ns			48	hsa-miR-130b*	215.3	0.019	0.083	97.6	0.015	0.031	ns		
9	hsa-miR-191*	2.9	0.004	0.047	12.0	0.003	0.019	ns			49	hsa-miR-128	3.1	0.039	0.119	11.5	0.015	0.031	ns		
10	hsa-miR-484	2.0	0.003	0.047	4.1	0.001	0.019	ns			50	hsa-miR-1291	2.4	0.039	0.119	14.9	0.015	0.031	ns		
11	hsa-miR-151-5P	2.7	0.009	0.073	9.9	0.001	0.019	ns			51	hsa-miR-320B	5.1	0.001	0.035	5.4	0.017	0.032	ns		
12	hsa-miR-339-5p	5.2	0.013	0.076	27.8	0.003	0.019	ns			52	hsa-miR-106b*	4.7	0.002	0.046	3.9	0.017	0.032	ns		
13	hsa-miR-340*	4.0	0.012	0.076	16.0	0.001	0.019	ns			53	hsa-miR-34b	4.2	0.008	0.072	9.7	0.017	0.032	ns		
14	hsa-miR-331-3p	2.3	0.012	0.076	7.8	0.001	0.019	ns			54	hsa-miR-572	6.1	0.022	0.087	10.9	0.017	0.032	ns		
15	hsa-miR-151-3p	2.2	0.012	0.076	7.7	0.003	0.019	ns			55	hsa-miR-212	2.6	0.035	0.115	7.2	0.017	0.032	ns		
16	hsa-miR-744	3.1	0.017	0.082	15.9	0.002	0.019	ns			56	hsa-miR-361-5p	4.6	0.010	0.076	14.7	0.019	0.034	ns		
17	hsa-miR-24	2.1	0.019	0.083	9.7	0.001	0.019	ns			57	hsa-miR-134	4.0	0.015	0.080	6.3	0.019	0.034	ns		
18	hsa-miR-199a-5p	3.4	0.022	0.087	76.4	0.003	0.019	ns			58	hsa-miR-363	2.6	0.048	0.134	4.2	0.019	0.034	ns		
19	hsa-miR-181a-2*	2.2	0.028	0.099	9.9	0.001	0.019	ns			59	hsa-miR-370	2.3	0.048	0.134	8.0	0.019	0.034	ns		
20	hsa-miR-200c	2.4	0.035	0.115	7.2	0.002	0.019	ns			60	hsa-miR-130a	3.9	0.002	0.046	7.8	0.022	0.037	ns		
21	hsa-miR-328	2.3	0.039	0.119	14.2	0.003	0.019	ns			61	hsa-miR-130b	4.1	0.003	0.047	9.9	0.022	0.037	ns		
22	hsa-miR-671-3p	2.5	0.044	0.125	12.5	0.002	0.019	ns			62	hsa-miR-136*	350.8	0.007	0.071	32.1	0.022	0.037	ns		
23	hsa-miR-486-3p	2.8	0.044	0.125	4.0	0.004	0.019	ns			63	hsa-miR-20a*	320.9	0.017	0.082	10.5	0.022	0.037	ns		
24	hsa-miR-423-5p	2.2	0.017	0.082	5.4	0.004	0.021	ns			64	hsa-miR-770-5p	263.6	0.000	0.035	280.2	0.028	0.043	ns		
25	hsa-miR-378-002243	4.2	0.009	0.073	8.9	0.005	0.021	ns			65	hsa-miR-661	16.5	0.000	0.035	10.2	0.028	0.043	ns		
26	hsa-miR-769-5p	3.5	0.017	0.082	14.4	0.005	0.021	ns			66	hsa-miR-605	5.4	0.019	0.083	18.4	0.028	0.043	ns		
27	hsa-miR-505	3.6	0.025	0.092	18.6	0.005	0.021	ns			67	hsa-miR-550-001544	174.0	0.008	0.072	4.0	0.031	0.047	ns		
28	hsa-miR-152	3.0	0.013	0.076	14.4	0.006	0.021	ns			68	hsa-miR-30d*	5.1	0.015	0.080	15.8	0.031	0.047	ns		
29	hsa-miR-22*	2.7	0.022	0.087	12.7	0.006	0.021	ns			69	hsa-miR-410	2.8	0.039	0.119	5.6	0.031	0.047	ns		
30	hsa-miR-324-5p	4.7	0.004	0.050	8.2	0.007	0.023	ns			70	hsa-miR-18a*	8.8	0.013	0.076	4.7	0.039	0.055	ns		
31	hsa-miR-144*	3.1	0.025	0.092	6.5	0.008	0.025	ns			71	hsa-miR-652	3.1	0.017	0.082	7.5	0.039	0.055	ns		
32	hsa-miR-501-3p	4.0	0.031	0.108	5.7	0.008	0.025	ns			72	hsa-miR-148a	2.5	0.031	0.108	6.4	0.039	0.055	ns		
33	hsa-miR-21	2.8	0.013	0.076	9.6	0.009	0.026	ns			73	hsa-miR-422a	862.6	0.001	0.035	13.6	0.044	0.060	ns		
34	hsa-miR-210	2.5	0.002	0.046	2.9	0.010	0.027	ns			74	hsa-miR-22	4.9	0.001	0.040	4.3	0.044	0.060	ns		
35	hsa-miR-125a-3p	8.1	0.002	0.047	16.2	0.010	0.027	ns			75	hsa-miR-576-3p	2.7	0.008	0.072	8.0	0.044	0.060	ns		
36	hsa-miR-378-000567	6.8	0.003	0.047	11.1	0.010	0.027	ns			76	hsa-miR-30d	2.6	0.013	0.076	2.4	0.044	0.060	ns		
37	hsa-miR-597	6.1	0.012	0.076	21.4	0.010	0.027	ns			77	hsa-miR-382	7.0	0.012	0.076	20.0	0.048	0.065	ns		
38	hsa-miR-543	4.4	0.022	0.087	16.4	0.010	0.027	ns			78	hsa-miR-30a-5p	2.3	0.015	0.080	2.4	0.048	0.065	ns		
39	hsa-miR-148b*	5.8	0.035	0.115	7.3	0.010	0.027	ns				hsa-miR-628-3p	1.7	0.028	0.099	4.5	0.001	0.019	386.4	0.000	0.027
40	hsa-miR-27a	2.6	0.044	0.125	14.0	0.010	0.027	ns				hsa-miR-146a	1.2	0.271	0.379	7.0	0.006	0.021	ns		

Comparisons performed with Mann Whitney test

Table 2. Diagnostic performance of individual microRNAs measured in serum by Taqman real-time PCR

No.	miRNA ID	PC vs. N			PC vs. CP			PC vs. NET			PC vs. CN			PC vs. NCN		
		AUC	Sensi. (%)	Speci. (%)	AUC	Sensi. (%)	Speci. (%)	AUC	Sensi. (%)	Speci. (%)	AUC	Sensi. (%)	Speci. (%)	AUC	Sensi. (%)	Speci. (%)
1	miR-1290	0.96	88	84	0.81	83	69	0.80	81	61	0.86	83	78	0.85	83	74
2	miR-628-3p	0.82	75	84	0.69	71	57	0.68	73	56	0.74	73	67	0.72	73	65
3	miR-550	0.74	73	58	0.63	51	69	0.70	61	66	0.67	66	56	0.68	49	75
4	miR-1825	0.70	63	79	0.71	76	60	0.72	73	61	0.71	68	65	0.71	66	64
5	miR-24	0.79	73	68	0.70	73	63				0.73	66	76			
6	miR-134	0.80	73	68	0.66	73	51				0.71	66	63			
7	miR-146a	0.82	78	79	0.78	73	80				0.80	78	70			
8	miR-200c	0.64	66	68	0.66	66	57				0.65	66	61			
9	miR-378	0.81	76	79	0.67	68	63				0.72	71	70			
10	miR-484	0.78	76	63	0.75	68	69				0.76	76	63			
11	miR-625	0.66	63	53	0.66	71	63				0.66	71	57			
12	miR-22	0.729	71	79												
13	miR-210	0.727	73	58												
14	miR744*	0.691	68	53												
	CA19-9	0.86	71	90	0.71	71	63	0.82	71	78	0.77	71	74	0.78	71	78

Note: PC: pancreatic cancer; NET: pancreatic neuroendocrine tumor; CP: chronic pancreatitis; N: normal person; CN: chronic pancreatitis and normal person; NCN: pancreatic neuroendocrine tumor, chronic pancreatitis and normal person; AUC: area under ROC curve. Bold text: miRNAs measured in a validation sample set with Taqman assays.

Clinical Cancer Research

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