PRELIMINARY COMMUNICATION

MicroRNA Expression Patterns to Differentiate Pancreatic Adenocarcinoma From Normal Pancreas and Chronic Pancreatitis

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Context While global microRNA (miRNA) expression patterns of many embryologic, physiologic, and oncogenic processes have been described, description of the role of miRNAs in ductal adenocarcinoma of the pancreas is lacking.

Objective To define the expression pattern of miRNAs in pancreatic cancer and compare it with those of normal pancreas and chronic pancreatitis.

Design and Setting Specimens were obtained at a National Cancer Institute–designated comprehensive cancer center from patients with ductal adenocarcinoma of the pancreas (n=65) or chronic pancreatitis (n=42) (January 2000-December 2005). All patients underwent curative pancreatectomy; those with pancreatic cancer were chemotherapy-naive. RNA harvested from resected pancreatic cancers and matched benign adjacent pancreatic tissue as well as from chronic pancreatitis specimens was hybridized to miRNA microarrays.

Main Outcome Measures Identification of differentially expressed miRNAs that could differentiate pancreatic cancer from normal pancreas, chronic pancreatitis, or both, as well as a pattern of miRNA expression predictive of long-term (>24 months) survival. Significance of Analysis of Microarrays and Prediction of Analysis of Microarrays were undertaken to identify miRNAs predictive of tissue type and prognosis. *P* values were calculated by *t* test, adjusted for multiple testing. Kaplan-Meier survival curves were constructed using mean miRNA expression (high vs low) as threshold and compared by log-rank analysis.

Results Twenty-one miRNAs with increased expression and 4 with decreased expression were identified that correctly differentiated pancreatic cancer from benign pancreatic tissue in 90% of samples by cross validation. Fifteen overexpressed and 8 underexpressed miRNAs differentiated pancreatic cancer from chronic pancreatitis with 93% accuracy. A subgroup of 6 miRNAs was able to distinguish long-term survivors with node-positive disease from those dying within 24 months. Finally, high expression of *miR-196a-2* was found to predict poor survival (median, 14.3 months [95% confidence interval, 12.4-16.2] vs 26.5 months [95% confidence interval, 23.4-29.6]; *P*=.009).

Conclusions Pancreatic cancer may have a distinct miRNA expression pattern that may differentiate it from normal pancreas and chronic pancreatitis. miRNA expression patterns may be able to distinguish between long- and short-term survivors, but these findings need to be validated in other study populations.

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PANCREATIC CANCER IS A LETHAL disease, with annual mortality nearly equaling incidence of approximately 33 000 in the United States.[1] While stage migration is partly to blame for the poor survival, the biology of ductal adenocarcinoma of the pancreas is one of aggressive local invasion, early metastasis, and resistance to chemotherapy and radiation. Known genetic mutations, including *TP53*, *KRAS*, *CDKN2A*, and *SMAD4*,[2] are important in pancreatic cancer but individually do not account for its aggressive behavior.

MicroRNAs (miRNAs) are small noncoding RNAs that are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors in the cytoplasm by RNase III Dicer into their mature form of 19 to 25 nucleotides.[3] Single-stranded miRNAs bind messenger RNAs of potentially hundreds of genes at the 3′ un-

For editorial comment see p 1923.

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translated region with perfect or near-perfect complementarity, resulting in degradation or inhibition of the target messenger RNA, respectively. In humans, aberrant expression of miRNAs contributes to carcinogenesis by promoting the expression of proto-oncogenes or by inhibiting the expression of tumor suppressor genes.[4] Such "oncomirs" have been demonstrated in a variety of hematologic and solid malignancies.[5-7]

In this report, we describe a series of experiments designed to identify the global pattern of miRNA expression in pancreatic adenocarcinoma to accomplish several goals. First, we sought to define miRNAs that can differentiate pancreatic cancer from benign pancreatic tissue. Since pancreatic cancer often occurs in a background of chronic pancreatitis, we also used chronic-pancreatitis specimens as a second control. Next, we hypothesized that a separate pattern of miRNA expression could distinguish patients more likely to achieve long-term survival from those with shorter survival. Finally, we hoped to identify miRNA(s) with expression predictive of survival.

METHODS

Tissue Samples

After exempt status for the study was granted by the institutional review board at the Ohio State University, specimens from 65 consecutive patients who had undergone resection for ductal adenocarcinoma of the pancreas and 42 with chronic pancreatitis from January 2000 through December 2005 were identified from the archival files of the Ohio State University Department of Pathology. All cases were reviewed by a pathologist (W.L.F.) and the diagnoses confirmed. Three 2-mm cores were obtained from the microdissected paraffin blocks for pancreatic cancer and matched benign adjacent pancreatic tissue or for chronic pancreatitis. Benign adjacent pancreas was available from all pancreatic cancer specimens.

miRNA Microarray

Tissue cores were deparaffinized with xylene at 50°C for 3 minutes. Total RNA extraction was undertaken using the RecoverAll kit (Ambion Inc, Austin, Tex) according to manufacturer's instructions. RNA labeling and hybridization on miRNA microarray chips were performed as previously described.[7] Briefly, 5 µg of total RNA from each sample was reverse transcribed using biotin end-labeled random-octamer oligonucleotide primer. Hybridization of biotin-labeled complementary DNA was performed on a new Ohio State University custom miRNA microarray chip (OSU_CCC version 3.0), which contains ≈1100 miRNA probes, including 326 human and 249 mouse miRNA genes, spotted in duplicates. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa647 conjugate and scanned on an Axon 4000B microarray scanner (Axon Instruments, Sunnyvale, Calif).

Statistical and Bioinformatics Analysis

Microarray images were analyzed using GENEPIX PRO 6.0 (Axon Instruments). Average values of the replicate spots of each miRNA were background subtracted, normalized, and further analyzed. Normalization was performed using the per-chip median normalization method and the median array.[8] Finally, we selected the miRNAs measured as present in at least as many samples as the smallest class in the data set (25%). Absent calls were thresholded to 4.5 ($\log_2$ scale) before statistical analysis, representing the average minimum intensity level detectable in the system. More than 95% of blank probes (ie, negative controls) fall below the threshold value of 4.5. miRNAs that are differentially expressed between pancreatic cancer and normal pancreas, pancreatic cancer and chronic pancreatitis, and chronic pancreatitis and normal pancreas were identified using the Significance Analysis of Microarrays (SAM) version 3.0 application with a threshold difference in expression set to 2, s0 percentile set to 0.05 (default), and the number of permutations set to 100 (default).[9] The SAM application calculates a score for each gene on the basis of the change of expression relative to the standard deviation of all measurements. Only mature miRNAs that are differentially expressed are reported. miRNA signatures were determined by the Prediction Analysis of Microarrays (PAM) version 2.1 application, which implements nearest shrunken centroids.[10] The prediction error was calculated by means of 10-fold cross-validation. For hierarchical analysis, we used average linkage clustering of the miRNAs identified by SAM and PAM between normal pancreas and pancreatic cancer (Cluster 3.0). Java Treeview 1.0 (Stanford University School of Medicine, Stanford, Calif) was used for tree visualization.

To perform survival analysis and generate Kaplan-Meier survival curves, miRNA levels measured on the miRNA chips were converted into discrete variables by splitting the samples into 2 classes (high and low expression), using the respective mean level of miRNA expression as threshold. Survival curves were compared by log-rank analysis. Significance was accepted with 95% confidence.

Quantitative Real-time Polymerase Chain Reaction

The single-tube TaqMan miRNA Assay (Applied Biosystems, Foster City, Calif) was used to detect and quantify mature miRNAs on Applied Biosystems real-time polymerase chain reaction (PCR) instruments in accordance with manufacturer's instructions. Normalization was performed with the small nuclear RNA U6 (RNU6B; Applied Biosystems). All real-time reactions, including no-template controls and real-time minus controls, were run in a GeneAmp PCR 9700 thermocycler (Applied Biosystems). Gene expression levels were quantified using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression

was calculated using the comparative C_t method.

Similarly, the TaqMan Gene Expression Assay was undertaken using primers and probes (predesigned, preoptimized) obtained from Applied Biosystems for *KRAS* expression determination. The assays used 150 ng of RNA per sample, and 18S was used to normalize all samples.

miRNA Northern Blots

For miRNA northern blots, 15 µg of total RNA were separated on 15% denaturing polyacrylamide gels, electrotransferred to GeneScreen Plus membranes (PerkinElmer, Waltham, Mass), and hybridized using UltraHyb-Oligo buffer (Ambion). Oligonucleotides complementary to mature *miR-21* were endlabeled with T4 Kinase (Invitrogen Corp, Carlsbad, Calif) and used as probes. Hybridization was performed at 42°C overnight and the membrane washed twice in 0.1× SSPE and 0.1% SDS at 42°C for 15 minutes each. Membranes were then exposed to a storage phosphor screen (GE Healthcare Bio-Sciences, Piscataway, NJ) for 8 hours and imaged using a Typhoon 9410 Variable Mode Imager (GE Healthcare Bio-Sciences). Saved images were cropped using Photoshop 6.0 (Adobe Systems Inc, San Jose, Calif).

Tissue Microarray

Our method for tissue microarray creation has been described.[11] Briefly, 2 tissue cores (2 mm diameter each) were punched out of each paraffin block used to obtain RNA for miRNA analysis and transferred to each of the recipient tissue microarray blocks using a precision instrument (Beecher Instruments, Silver Spring, Md). Paraffin-embedded tissue was cut at 4 microns and placed on positively charged slides, then heated to 40°C for 30 minutes. After leveling paraffin and cores, the array was cooled to 4°C for 15 minutes.

Immunohistochemistry

Our methods for immunohistochemical analysis have been described.[11] Primary antibodies for TP53 (catalog #M7001, clone DO-7; Dako, Carpinteria, Calif), CDKN2 (catalog #CMC802, clone JC2; Cell Marque Corp, Rocklin, Calif), and SMAD4/DPC4 (catalog #sc-7966, clone B-8; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) were used at dilutions of 1:50, 1:20, and 1:100, respectively. Slides were counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions, and coverslipped. The positive and negative controls stained appropriately.

Staining for TP53 was considered positive if nuclear staining in at least 5% of cells was seen. Nuclear and cytoplasmic staining in at least 5% of cells was considered positive for CDKN2 and less than 10% of nuclear and cytoplasmic staining of cells for SMAD4/DPC4 was considered loss of expression. All stains were read by a pathologist (W.L.F.) blinded to tumor stage and clinical characteristics.

RESULTS

Using miRNA microarray,[8] miRNAs were identified that were differentially expressed between pancreatic cancers and matched adjacent benign pancre-

Table 1. Differentially Expressed MicroRNAs and Class Predictors for Pancreatic Cancer, Chronic Pancreatitis, and Normal Pancreas and Their Relative Expression*

MicroRNA	Fold Change†	PAM Score‡	
		Specimen	Control
	Pancreatic Cancer vs Normal Pancreatic Tissue§		
		Cancer	Normal
miR-221	3.42	0.1605	–0.1717
miR-181a	3.01	0.125	–0.1337
miR-155	2.10	0.0232	–0.0249
miR-210	2.97	0.1143	–0.1224
miR-213	2.16	0.0146	–0.0156
miR-181b	2.85	0.1099	–0.1176
miR-222	2.70	0.0751	–0.0804
miR-181b-2	2.20	0.0102	–0.0109
miR-21	3.08	0.0658	–0.0704
miR-181b-1	2.55	0.03	–0.0321
miR-181c	2.36	0.0217	–0.0232
miR-220	2.11	0.0135	–0.0145
miR-181d	2.74	0.0259	–0.0277
miR-223	2.53	0.0219	–0.0234
miR-100-1/2	2.27	0.0111	–0.0119
miR-125a	2.09	0.0037	–0.004
miR-143	2.19	0.0052	–0.0056
miR-10a	2.67		
miR-146	2.44	0.0007	–0.0007
miR-99	2.08		
miR-100	2.49		
miR-199a-1	2.46		
miR-10b	2.40		
miR-199a-2	2.13		
miR-107	2.28		
miR-103-2	2.28		
miR-125b-1	2.03		
miR-205	2.24		
miR-23b	2.00		
miR-23a	2.01		
miR-148a	0.18	–0.189	0.2022
miR-148b	0.31	–0.0676	0.0724
miR-375	0.46	–0.0444	0.0475

(continued)

Table 1. Differentially Expressed MicroRNAs and Class Predictors for Pancreatic Cancer, Chronic Pancreatitis, and Normal Pancreas and Their Relative Expression* (cont)

MicroRNA	Fold Change†	PAM Score‡	
		Specimen	Control
	Pancreatic Cancer vs Chronic Pancreatitis§		
		Cancer	Pancreatitis
miR-96	1.77	0.0115	–0.0212
miR-221	2.48	0.0866	–0.1601
miR-34	1.75	0.0212	–0.0392
miR-497	1.91	0.2434	–0.45
miR-203	4.11	0.0392	–0.0724
miR-155	1.88	0.0133	–0.0245
miR-181a	1.95	0.0422	–0.0779
miR-453	1.64	2.00E-04	–4.00E-04
miR-92	1.86	0.0024	–0.0043
miR-181b	1.67	0.0316	–0.0583
miR-181d	2.17	0.0204	–0.0377
miR-93	1.64	0.0098	–0.0182
miR-181b-1	1.67	0.0211	–0.039
miR-21	1.88	0.0185	–0.0342
miR-181c	1.65	0.0013	–0.0024
miR-494	0.32	–0.1491	0.2755
miR-483	0.51	–0.0598	0.1106
miR-339	0.51	–0.0481	0.089
miR-218-2	0.62	–0.0058	0.0107
miR-148a	0.22	–0.1053	0.1946
miR-375	0.46	–0.0298	0.0551
miR-409-3p	0.62	–0.0055	0.0102
miR-148b	0.33	–0.0318	0.0587
	Chronic Pancreatitis vs Normal Pancreas		
		Pancreatitis	Normal
miR-494	4.67	0.4051	–0.2345
miR-483	1.82	0.0511	–0.0296
miR-383	1.93	0.114	–0.066
miR-197	1.79	.0391	–0.0227
miR-339	1.87	0.0649	–0.0376
miR-194	1.69	0.045	–0.0261
miR-198	1.78	0.0227	–0.0131
miR-409-3p	1.68	0.0285	–0.0165
miR-199b	2.22	0.0658	–0.0381
miR-199a-2	2.59	0.072	–0.0417
miR-199a-1	2.66	0.0538	–0.0311
miR-007-3	2.32	0.0221	–0.0128
mir-128b	2.21	0.0008	–0.0005
mir-100-1/2	3.29	0.0276	–0.016
miR-125a	3.04	0.0153	–0.0088
miR-125b-2	3.16		
miR-195	2.19		
miR-126	2.10		
miR-125b-1	3.06		
miR-100	2.62		
miR-10b	2.37		
miR-99	2.10		
miR-96	0.53	–0.0459	0.0266
miR-497	0.49	–0.4271	0.2473

Abbreviation: PAM, Prediction of Analysis of Microarrays.
*Q = 0% for all rows. Blank cells indicate no PAM score generated because these miRNAs were not predicted as significant classifiers by PAM.
†Presented as actual change in expression.
‡Log ratio of expression relative to the shrunken centroid.[10]
§s0 percentile = 0.05.

atic tissue, between pancreatic cancer and chronic pancreatitis, and between chronic pancreatitis and normal pancreas. The SAM application identified 30 miRNAs that were up-regulated in pancreatic cancers and 3 that were down-regulated compared with normal pancreatic tissue. When pancreatic cancer samples were compared with those from chronic pancreatitis, 15 miRNAs were overexpressed and 8 were underexpressed in cancers. Finally, 22 miRNAs showed increased expression in chronic pancreatitis, compared with 2 that were decreased compared with normal pancreas. TABLE 1 lists differentially expressed miRNAs with at least a 2-fold change in expression by SAM and additional miRNAs identified as class predictors by PAM (see below). One third of miRNAs found to differentiate pancreatic cancer from normal pancreatic tissue also differentiated cancers from chronic pancreatitis (FIGURE 1). No miRNAs were common between all 3 groups of samples.

Cluster analysis based on miRNAs differentially expressed between chronic pancreatitis, normal pancreas, and pancreatic cancer demonstrated a general distinction between each sample type (data not shown). The expression patterns appeared to be most similar between chronic pancreatitis and normal pancreas, with a more clear distinction between these benign tissues and pancreatic cancer. The majority of pancreatic cancers clustered together with some exceptions, including a group of 8 cancers clustering among the normal pancreas and chronic pancreatitis samples. This latter group had clinicopathologic features similar to those of the remainder of the cancers, with survival that was 50% longer but not statistically significant (median, 23.1 months [95% confidence interval {CI}, 19.6-26.6] vs 15.2 months [95% CI, 10.9-19.5]; $P=.15$). This group of 8 tumors had significantly lower levels of *miR-21* expression compared with the other pancreatic cancers (median, 10.9 vs 8.3; $P<.001$).

The PAM application allowed classification of each sample by tissue type based on miRNA expression levels (Table 1). A subset of 21 overexpressed and 4 underexpressed miRNAs were identified that could correctly discriminate pancreatic cancer from normal pancreatic tissue by cross-validation testing in 90% of samples. When comparison was made between chronic pancreatitis and pancreatic cancer, 93% of samples were correctly classified based on 15 overexpressed and 8 underexpressed miRNAs in cancer. Comparison between chronic pancreatitis and normal pancreas identified 15 miRNAs with increased expression and 2 with decreased expression. This pattern of expression correctly differentiated chronic pancreatitis from normal pancreas in all samples. Finally, 95% of pancreatic cancer samples were classified correctly when compared with both chronic pancreatitis and normal pancreas together.

To confirm the microarray findings, quantitative real-time PCR was undertaken in 8 pancreatic cancer samples and 8 matched benign pancreatic tissue controls for *miR-21*, *miR-221*, *miR-222*, *miR-181a*, *miR-181b*, *miR-181d*, and *miR-155*. All of these miRNAs were overexpressed in tumor samples relative to benign pancreatic tissue (FIGURE 2). Northern blot analysis for *miR-21* in 5 additional fresh pancreatic cancer samples also confirmed increased expression compared with 2 unmatched fresh benign-tissue controls (FIGURE 3).

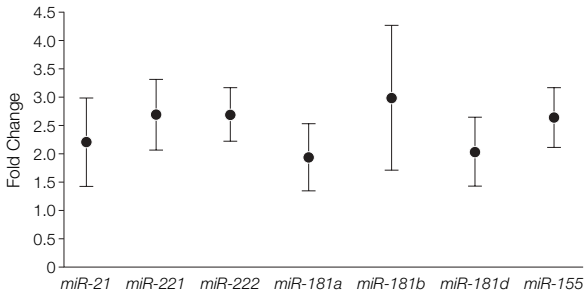
To determine the impact of miRNA expression on survival, we analyzed our microarray data using 2 methods. First, we wanted to determine if the absolute level of miRNA expression could discriminate between short- and long-term survivors with node-positive disease. Given that patients with pancreatic cancer metastatic to regional lymph nodes who are still alive 2 years after resection are often considered long-term survivors, we compared microarray data for node-positive patients having greater than 24-months' survival with data for those dying of disease within 24 months. The SAM application identified 6 miRNAs that were differentially overexpressed in the patients with longer survival (TABLE 2). Next, we wanted to determine the survival based on the relative expression of miRNAs. Kaplan-Meier survival curves were generated and compared by log-rank analysis using the binomial variable of high or low expression relative to the mean expression of each miRNA on the microarray. Based on this, 2 miRNAs of interest were identified: *miR-196a-2* and *miR-219*. Tumors with high expression of *miR-196a-2* had a median survival of 14.3 months (95% CI, 12.4-16.2) compared with 26.5 months (95% CI, 23.4-29.6) for those with low expression ($P=.009$). High expression of *miR-196a-2*, which was seen in 75% of tumors, resulted in 2-year survival of 17% compared with 64% for low expression (FIGURE 4). Median survival in patients with high expression of *miR-219* was 13.6 months (95% CI, 11.8-15.4), compared with 23.8 months (95% CI, 18.7-28.9) for

Figure 1. Matrix of Sets of Differentially Expressed MicroRNAs in Common Between Pairwise Tissue-Type Comparisons

	Pancreatic Cancer vs Normal Pancreas (n=33)	Pancreatic Cancer vs Chronic Pancreatitis (n=23)
Chronic Pancreatitis vs Normal Pancreas (n=24)	*miR-100* *miR-10b* *miR-125a* *miR-125b-1* *miR-199a-1* *miR-199a-2* *miR-99*	*miR-339* *miR-409-3p* *miR-483* *miR-494* *miR-497* *miR-96*
Pancreatic Cancer vs Chronic Pancreatitis (n=23)	*miR-148a* *miR-148b* *miR-155* *miR-181a* *miR-181b* *miR-181b-1* *miR-181c* *miR-181d* *miR-21* *miR-221* *miR-375*	

mi-R designators shown indicate the differentially expressed miRNAs in common between each comparison. Numbers in parentheses indicate the the total number of differentially expressed miRNAs in the indicated pairwise comparison including the number of differentially expressed miRNAs in common.

Figure 2. Relative Expression of MicroRNAs (miRNAs) in Pancreatic Cancer Compared With Matched Normal Pancreas Controls by Real-time Polymerase Chain Reaction

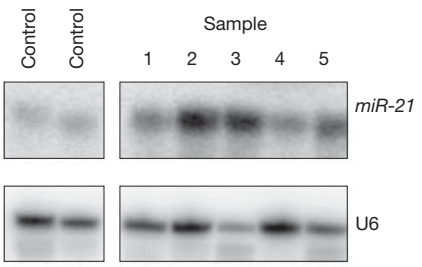


Error bars indicate standard deviation.

those with low expression, with 2-year survivals of 25% and 49%, respectively ($P = .07$). Median survival for all patients was 15.5 months (95% CI, 9.9-21.1), with 2- and 5-year survivals of 33% and 12.5%, respectively. Of note, nodal status, T stage, and histologic grade were not predictive of survival (data not shown).

We next sought to correlate the expression of common genetic abnormalities seen in pancreatic cancer with survival and miRNA expression. Increased TP53 expression was seen in 31 (57%) of 54 tumors. Loss of CDKN2 expression was seen in 49 (88%) of 56 of tumors, while SMAD4/DPC4 expression was lost in 39 (70%) of 56. No correlation was found with survival or with any of the miRNAs listed in Table 2, including *miR-196a-2*. In addition, *KRAS* mutation was identified in 8 (80%) of 10 tumors evaluated by gene expression analysis but did not correlate with miRNA expression.

Figure 3. Northern Blot of 5 Fresh Pancreatic Cancer Samples and 2 Unmatched Benign Pancreatic Tissue Controls for *miR-21*



See "Methods" section for details. U6 indicates small nuclear RNA U6.

Table 2. Differentially Expressed Mature MicroRNAs by Significance of Analysis of Microarrays in Node-Positive Patients With At Least 24 Months' Survival Compared With Those Dying of Disease Within 24 Months*

MicroRNA	Fold Change†	Q Value, %
miR-452	1.81	0
miR-105	1.66	31.0
miR-127	1.37	31.0
miR-518a-2	1.31	31.0
miR-187	1.40	46.5
miR-30a-3p	1.45	46.5

*Variables for significance of analysis of microarrays were set as default (minimum nil fold change, 100 permutations, and s0 percentile of 0.05).
†Presented as actual change in expression.

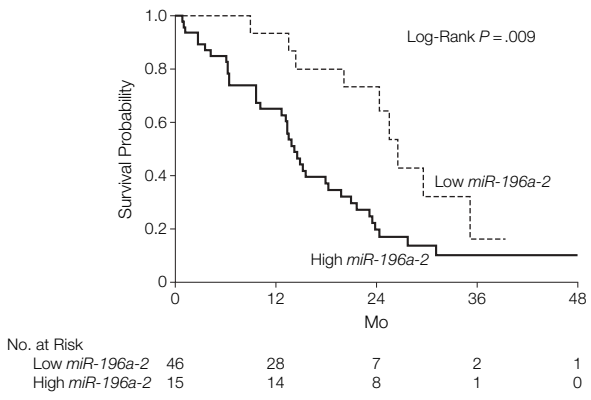
COMMENT

Aberrant miRNA expression patterns have been described in a variety of hematologic and solid-organ malignancies. We have identified—we believe for the first time—a global expression pattern of miRNAs that can differentiate ductal adenocarcinomas of the pancreas from normal pancreas and chronic pancreatitis with 95% accuracy. We also have identified an miRNA, *miR-196a-2*, that may significantly impact survival.

For this series of experiments we chose 2 controls for comparison: adjacent benign pancreatic tissue and chronic pancreatitis. Although our tumor samples were microdissected, contamination with surrounding inflammatory changes common in pancreatic cancers is inevitable. Still, only 7 miRNAs (*miR-99, miR-100, miR-100-1/2, miR-125a, miR-125b-1, miR-199a-1, miR-199a-2*) that were found to be overexpressed in cancers compared with normal pancreatic tissue were also overexpressed in chronic pancreatitis. While the overexpression of these miRNAs in cancers and chronic pancreatitis may suggest a common inciting event for neoplastic growth, the possibility of contamination cannot be excluded. With few exceptions, normal pancreas and chronic pancreatitis tended to cluster together while remaining largely separate from the pancreatic cancers. Interestingly, 1 group of 8 cancers did cluster with the benign-pancreas samples. While this group of patients did not have a significant improvement in survival compared with the others, their nearly 2-year median survival is longer than that in most reports for pancreatic cancer. Noteworthy within this smaller group is the cluster of miRs 23, 103, and 107, which was lower in these 8 cancers than in the remaining cancers or the majority of benign-pancreas samples (data not shown). These miRNAs, among others, have recently been shown to be induced by hypoxia in cancer cells via a hypoxia-inducible factor–dependent mechanism.[12] In fact, the majority of the described hypoxia-related miRNAs are differentially overexpressed in the pancreatic cancers in our study. Given the association between hypoxia-inducible factor and the aggressiveness of pancreatic cancer, decreased expression of these hypoxia-related miRNAs may prove important for survival in a subset of patients.

Several miRNAs commonly associated with malignancy were identified as significantly dereglated in the pancreatic cancers in our study. Most notably, *miR-21* and *miR-155* were uniquely overexpressed in pancreatic cancer vs normal pancreas and chronic pancreatitis. *MiR-21* has been

Figure 4. Kaplan-Meier Overall Survival Curve for Patients With Pancreatic Cancer, Based on Expression of *miR-196-a2*



suggested to play an important role in preventing apoptosis, thus functioning as a proto-oncogene,[13] and has been shown to be overexpressed in cancers of the lung, stomach, breast, colon, and prostate as well as being expressed in pancreatic neuroendocrine tumors.[4,7] While the role of *miR-21* in neoplasia has not been fully elucidated, its inhibition using miRNA-specific antisense oligonucleotides increases in vitro susceptibility of cholangiocarcinoma cells to gemcitabine.[14] *MiR-155* is also overexpressed in solid tumors such as those of the colon, lung, and breast,[4,7] while being associated with the activated B-cell type of diffuse large B-cell lymphoma as well as Hodgkin and Burkitt lymphoma.[15,16] It has also been shown to be involved in leukemogenesis in transgenic mice.[17]

The most consistently highly expressed miRNA in the pancreatic cancers in our study was *miR-221* when compared with normal pancreas and chronic pancreatitis. While this association has not been previously demonstrated in gastrointestinal tract tumors, *miR-221* expression is important in thyroid cancer and is suggested to play a role in angiogenesis.[18,19] Working together with *miR-222*, which was also found to be overexpressed in the pancreatic cancers we studied, *miR-221* targets KIT, the receptor for stem cell factor.

Far fewer miRNAs were downregulated in pancreatic cancer. Notable of these was *miR-375*, which is found in abundance in pancreatic islets but not in the exocrine pancreas.[20] It stands to reason that this miRNA would be significantly underexpressed in our pancreatic cancers because they were all derived from the exocrine pancreas, resulting in obliteration of the intervening islets.

Our laboratory recently reported on the miRNA expression patterns of 40 pancreatic endocrine tumors and 4 acinar carcinomas compared with normal pancreas.[21] In that study, 87 miRNAs were differentially overexpressed in tumors and 8 were underexpressed relative to normal pancreas. This is markedly more than the 30 overexpressed miRNAs and 3 underexpressed miRNAs identified in our pancreatic adenocarcinomas of ductal origin. Given the difference in derivation of pancreatic endocrine tumors compared with ductal adenocarcinomas, this wide variety in miRNA expression is not unexpected. Similar findings have been reported using Affymetrix gene arrays.[22] Similar to our findings in ductal adenocarcinoma, *miR-21* appears to be important in pancreatic endocrine tumors. In the endocrine tumors, however, *miR-21* correlated with more aggressive tumors, as signified by an increased proliferation index by Ki67 and the presence of liver metastases. Similarly, *miR-21* expression was significantly lower in the 8 cancers reported herein that clustered with the benign pancreas specimens, suggesting its role in tumor aggression. *MiR-155*, on the other hand, was underexpressed in pancreatic endocrine tumors relative to benign pancreatic tissue, whereas we found it to be overexpressed in ductal adenocarcinomas. Again, this discrepancy emphasizes the differences in cell origin between the 2 tumor types.

Given the dismal prognosis typically associated with pancreatic cancer, we sought to identify an miRNA expression profile that could discriminate between high-risk patients who could be considered long-term (ie, >24 months) and short-term survivors. A group of 6 miRNAs were identified (Table 2). *MiR-127* is interesting, since it is located within a CpG island on chromosome 14 and has been shown to be silenced in cancers of the prostate and colon.[23] In the pancreatic cancers in our study, *miR-127* expression was increased in nearly half of the tumors, while it was decreased in the other half. Clearly, *miR-127* expression alone does not significantly impact survival but, when taken into acount with the other miRNAs listed in Table 2, may predict long-term survivors. Even less is known about expression of the other listed miRNAs in cancer, making speculation about their role in pancreatic cancer difficult at this time.

Only 1 miRNA was identified that significantly predicted duration of survival, *miR-196a-2*. This miRNA was not identified by SAM to discriminate between the qualitative distinction of long- and short-term survivors. This is not surprising, however, given the different statistical methods used to answer the 2 different questions. While a direct association with malignancy has not been described for *miR-196*, it does appear to perfectly interact with, and degrade, *HOXB8*, a member of the homeobox family cluster involved in various crucial development programs in animals,[24] including the endocrine pancreas.[25] In our patients, 75% of tumors expressed *miR-196a-2* at a level above the mean for the group. Although this miRNA did not help differentiate pancreatic cancers from normal pancreas or chronic pancreatitis, its potential as a predictor for survival warrants further investigation.

Finally, we demonstrated that our samples are representative of typical ductal adenocarcinomas by assaying for the 4 most common genetic abnormalities seen in pancreatic cancer: *TP53, CDKN2, SMAD4,* and *KRAS*. Alterations in these genes of interest seen in the tumors in our study were similar to those described in the literature.[26-28] Similar to earlier reports, these tumor suppressor genes and oncogenes did not correlate with survival in our patients, nor did they correlate with miRNA expression.

The present report contributes to the growing understanding of the role of miRNAs in oncogenesis and describes the global expression patterns of miRNAs in pancreatic adenocarcinoma. As we and other laboratories continue to identify the expression patterns of various solid tumors, the application of this knowledge may be broad. Such patterns may be able to be used to direct therapy in patients with metastatic tumors of unknown primary neoplasms or to help discriminate between benign and malignant neoplasms that

would otherwise be indeterminate by routine histologic and immunohistochemical analysis. More importantly, data such as ours, in which it is possible to begin to differentiate between patients with better or worse prognoses, may help guide the clinician when determining who should or should not receive aggressive therapy. Aside from these diagnostic and prognostic examples of how miRNA expression patterns may be able to be used clinically, the ability of miRNAs to affect multiple genes in various pathways make them a logical target for investigation of novel antitumoral therapies. However, these preliminary data will first need to be validated in other studies.

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Study concept and design: Bloomston, Croce.
Acquisition of data: Bloomston, Frankel, Petrocca, Alder, Liu, Bhatt.
Analysis and interpretation of data: Bloomston, Petrocca, Volinia, Alder, Hagan, Bhatt, Taccioli, Croce.
Drafting of the manuscript: Bloomston, Volinia.
Critical revision of the manuscript for important intellectual content: Frankel, Petrocca, Alder, Hagan, Liu, Bhatt, Taccioli, Croce.
Statistical analysis: Bloomston, Volinia, Hagan, Taccioli.
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