MicroRNA Molecular Profiles Associated with Diagnosis, Clinicopathologic Criteria, and Overall Survival in Patients with Resectable Pancreatic Ductal Adenocarcinoma


Abstract

Purpose: MicroRNAs (miRNA) have potential as diagnostic and prognostic biomarkers and as therapeutic targets in cancer. We sought to establish the relationship between miRNA expression and clinicopathologic parameters, including prognosis, in pancreatic ductal adenocarcinoma (PDAC).

Experimental Design: Global miRNA microarray expression profiling of prospectively collected fresh-frozen PDAC tissue was done on an initial test cohort of 48 patients, who had undergone pancreatectoduodenectomy between 2003 and 2008 at a single institution. We evaluated association with tumor stage, lymph node status, and site of recurrence, in addition to overall survival, using Cox regression multivariate analysis. Validation of selected potentially prognostic miRNAs was done in a separate cohort of 24 patients.

Results: miRNA profiling identified expression signatures associated with PDAC, lymph node involvement, high tumor grade, and 20 miRNAs were associated with overall survival. In the initial cohort of 48 PDAC patients, high expression of miR-21 (HR = 3.22, 95% CI: 1.21–8.58) and reduced expression of miR-34a (HR = 0.15, 95% CI: 0.06–0.37) and miR-30d (HR = 0.30, 95% CI: 0.12–0.79) were associated with poor overall survival following resection independent of clinical covariates. In a further validation set of 24 patients, miR-21 and miR-34a expression again significantly correlated with overall survival (P = 0.031 and P = 0.001).

Conclusion: Expression patterns of miRNAs are significantly altered in PDAC. Aberrant expression of a number of miRNAs was independently associated with reduced survival, including overexpression of miR-21 and underexpression of miR-34a.

Summary: miRNA expression profiles for resected PDAC were examined to identify potentially prognostic miRNAs. miRNA microarray analysis identified statistically unique profiles, which could discriminate PDAC from paired nonmalignant pancreatic tissues as well as molecular signatures that differ according to pathologic features. miRNA expression profiles correlated with overall survival of PDAC following resection, indicating that miRNAs provide prognostic utility.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive of all malignancies (1). It remains a therapeutic challenge: conventional cancer therapies have little impact on disease course, and patients with potentially resectable disease are in the minority (10%–15%) because of extensive local spread or metastatic disease at presentation (2). The median overall survival of advanced disease is under 6 months and, despite surgery, the 5-year survival rate is a dismal 3% to 5% with the impact of chemotherapeutic options remaining limited (3).

Consequently novel therapeutic routes are required. Staging and prognostic information is provided by standard...
Commonly mutated genes in PDAC include those defining the molecular network of PDAC carcinogenesis. Levels among thousands of genes has contributed to the improved understanding of the disease biology. This would enable tailored treatment as well as improve overall survival. Improved prognostic and predictive biomarkers with similar characteristics have considerable variation in clinicopathologic and imaging information, but patients with similar characteristics have considerable variation in outcome. Improved prognostic and predictive biomarkers would enable tailored treatment as well as improve understanding of the disease biology.

Systematic analysis of mRNA and protein expression levels among thousands of genes has contributed to defining the molecular network of PDAC carcinogenesis. Commonly mutated genes in PDAC include K-RAS, p16, TP53, and SMAD4; these are accompanied by a substantial compendium of genomic and transcriptomic alterations that facilitate cell-cycle deregulation, cell survival, invasion, and metastases. Although investigation of these known genes and proteins has yielded new information, it has become apparent that noncoding RNA gene products may provide additional insights into PDAC biology.

MicroRNAs (miRNA) are small noncoding RNA gene products ranging in size from 19 to 25 nucleotides. miRNAs play important roles in regulating the translation and degradation of mRNAs through base pairing to partially complementary sites, predominately in the untranslated region of the mRNA. miRNAs seem to influence various biological processes including cell proliferation, cell death, stress resistance, mainly through negative regulation of gene expression. Recently, miRNAs have gained wide attention as a family of molecules involved in cancer development. Model therapeutic targets should be causally associated with disease and amenable to therapeutic intervention, whereas model biomarkers should be easily quantifiable and associate strongly with clinical outcome: miRNAs potentially match both criteria.

Our large cohort of patients, who have undergone resection for PDAC [pancreaticoduodenectomy (PD)], with full clinicopathologic and follow-up data, was recently used to describe novel prognostic pathologic parameters, including circumferential resection margin status. The aim of this study was to investigate the genome wide miRNA expression profile in a subgroup this cohort of PDACs resected with curative intent. Subsequently, we correlated this molecular signature with clinicopathologic variables and also with overall survival following resection. Expression of a subset of candidate miRNAs was assessed by reverse transcriptase PCR (RT-PCR) within the initial cohort and in a separate validation cohort of 24 resected PDACs.

Methods

Sample collection

Macrodissected fresh frozen pancreatic tissue samples were used in this study: 48 PDACs and 10 corresponding matched nontumor pancreas in the initial miRNA profiling; plus a further 24 separate PDACs in the subsequent RT-PCR validation. Specimens were obtained from patients undergoing PD with curative intent at the West of Scotland Pancreatic Unit, Glasgow Royal Infirmary (GRI), United Kingdom from July 2003 to December 2008. Tissue was collected prospectively with local ethical approval, fully informed consent, pathology assessment and validation, and storage managed by the GRI Biorepository. Only histologically proven PDACs were included. Other lesions, for example, ampullary, duodenal, distal bile duct adenocarcinomas, mucinous cystadenocarcinomas, or intraductal papillary mucinous neoplasms were excluded. Complete clinicopathologic, follow-up and recurrence data were available.

RNA extraction and analysis

Total RNA was isolated from frozen tissue by standard TRizol (Invitrogen) methodology according to the manufacturer's instructions. A nanodrop spectrophotometer (NanoDrop Tech) quantified total RNA, whereas purity and integrity was assessed on the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChipVR reagent set (Agilent Technologies). Samples with a RNA integrity number (RIN) above 7.0 were deemed suitable for downstream analysis.

miRNA microarray analysis

miRNA profiling was done on 48 PDACs and 10 corresponding matched normal pancreas samples. For miRNA expression profiling, 100 ng of total RNA were dephosphorylated (Calf Intestinal Alkaline Phosphatase; GE Healthcare) and labeled by ligation (T4 RNA Ligase; NEB Biolabs) with one Cyanine 3-pCp molecule to the 3' end of the RNA molecules using Agilent's miRNA Labeling Reagent and Hybridization Kit. Labeled miRNAs were desalted with Micro Bio-Spin Chromatography columns (BioRad Laboratories) as described by Agilent.
Technologies. Hybridization, microarray washing, and detection of labeled miRNA on the microarray were done according to Agilent Technologies instructions. miRNA expression profiling was done using Agilent’s Human miRNA Microarrays (version 2.0, based on Sanger miRBase version 10.1), carrying 723 human miRNAs (14). Array image acquisition and feature extraction was done using the Agilent G2505B Microarray Scanner and Feature Extraction software version 9.5 (Agilent).

An average value of the replicate spots for each miRNA was normalized and uploaded into Biometric Research Branch (BRB)-ArrayTools 3.9 (15). After excluding negative values with hybridization intensity below background, normalization was done by using the median normalization method and normalization to the median array as reference. We then selected 476 miRNAs with consistent log values present in more than 50% of samples. This filtering method was agreed upon a priori to eliminate probes whose miRNA expression were thought to be unreliable. We identified genes that were differently expressed among groups using the class comparison and the serial analysis of microarray (SAM) analysis, with genes considered statistically significant if the \( P < 0.001 \).

Class prediction algorithms in BRB-ArrayTools were used to determine whether miRNA microarray expression patterns could accurately differentiate tumor from nontumor tissue. For these analyses, Bayesian compound covariate and nearest centroid algorithms were arbitrarily chosen and accuracy reports of the percentage of tissues correctly identified.

**PCR analysis**

RT-PCR analysis was done on PDAC samples, from the initial 48 patient cohort (for miR-21, miR-29c, miR-30d, miR-34a, miR-221, miR-224) plus a 24 further PDAC patients (for miR-21 and miR-34a only). RT was conducted with the mirVana quantitative RT-PCR miRNA detection kit (Ambion) according to the manufacturer’s instructions. Briefly, the reaction master mix, containing mirVana 5x RT Buffer, 1x mirVana RT primer, Array-Script Enzyme Mix and nuclease-free water was mixed with 20 ng of each total RNA sample. The RT reaction was done at 37°C for 30 minutes and then 95°C for 10 minutes using the DyNAmo Hot star SYBR Green kit (Finnzymes) and the Opticon 2 DNA Engine (MJ Research). The PCR master mix containing mirVana 5x PCR Buffer (with SYBR Green), 50x ROX, SuperTaq Polymerase, mirVana PCR primers, and RT products was processed as follows: 95°C for 3 minutes, then 95°C for 15 seconds, and 60°C for 35 seconds for up to 40 cycles. All qPCRs were normalized to the small nuclear RNA, U6, as the control. Primers were purchased from Ambion. All assays were done in triplicate.

**Survival analysis**

Within the microarray experiment and based on the dichotomized expression of individual miRNAs using the median value as a cut-off, we identified miRNAs whose expression was significantly related to overall survival following PD. We computed a statistical significance level for each miRNA based on a univariate Cox regression model in BRB-ArrayTools 3.9. These \( P \) values were then used in a multivariate permutation test in which the survival times and censoring indicators were randomly permuted among the arrays. By this means, low- and high-risk groups based on miRNA expression profiles were determined.

Kaplan–Meier survival analysis was used to analyze overall survival time from date of resection. Patients alive at the follow-up point were censored. Survival analysis was done, using a log-rank test, for each clinical covariate to assess their influence on outcome. A multivariate Cox regression model was used for analysis to adjust for competing risk factors, and the HR with 95% CIs was reported as an estimate of overall survival risk. Variables found to be significant on univariate analysis at \( P < 0.05 \) were included in the final multivariate analysis in a backwards stepwise fashion. All statistical analyses were done using SPSS 18.0 (IBM, SPSS).

For additional methods outlining immunohistochemical, gene expression, arrayCGH, and bioinformatics analysis, see the Supplementary Methods.

**Results**

**Clinicopathologic characteristics of patient cohort**

The initial miRNA profiling was done on samples from 48 patients with resectable PDAC who underwent PD with curative intent. Table 1 summarizes their clinicopathologic characteristics. Most patients had stage T3 tumors, with perineural and venous invasion, positive lymph nodes and positive resection margins with 58% receiving adjuvant chemotherapy. At the time of last follow-up, recurrence had occurred in 37 of 48 (77%) of the cohort. We categorized recurrence as local versus distant and liver metastases versus other site of recurrence. The median overall survival was 26.3 months. A further 24 patients were used as a validation set, with their characteristics also described in Table 1.

The prognostic relevance of individual conventional clinicopathologic variables, including tumor grade, T stage, tumor size, lymph node status, perineural, venous invasion, and resection margin status was evaluated (Supplementary Table S1). On univariate analysis, overall survival was prolonged significantly for patients receiving adjuvant chemotherapy \( (n = 28) \), 27.8 versus 11.5 months for those who did not \( (n = 20); \text{log-rank}; P = 0.042 \). All clinicopathologic factors with \( P \) less than 0.10 were included within a Cox regression multivariate model (Supplementary Table S2). In this 48 patient cohort, high tumor grade, lymph node metastases, perineural invasion, venous invasion, resection margin involvement, and no adjuvant therapy remained independently associated with poor overall survival.

**miRNA expression profiles differ between PDAC and normal pancreas**

We analyzed and compared the miRNA expression profiles in 48 PDACs and 10 paired samples of noncancerous...
pancreas tissue. Ninety-seven of these miRNAs showed statistically different expression between the 2 groups (\(P < 0.001\)). Thirty-nine miRNAs were upregulated and 58 downregulated in cancer (top 25 up and downregulated miRNAs are shown in Supplementary Table S3; full list in Supplementary Table S4). miRNAs upregulated in PDAC compared with normal pancreas included miR-10a, miR-21, miR-143, miR-145, miR-155, miR-222, miR-223, miR-224, and miR-373; miRNAs downregulated in PDAC compared with normal included miR-148, miR-216, miR-217, miR-211, miR-345, miR-596, and miR-708. Using the class comparison feature of the BRB-ArrayTools analysis package, the multivariate permutation test was done to control for multiple comparisons. The probability of identifying 97 miRNAs by chance at \(P < 0.001\), if there are no true differences, was 0 as estimated by the multivariate permutation test. Hierarchical clustering of the miRNA profiles for PDAC and normal pancreatic tissue is illustrated in Supplementary Fig. S1. Further analysis of global miRNA profiles enabled PDAC and normal pancreas to be distinguished with 95% accuracy using the Bayesian compound covariate and with 90% accuracy using the nearest centroid class prediction algorithms (10-fold cross-validation). This supports our hypothesis of systematic change in miRNA expression during PDAC formation.

miRNA expression profiles associated with clinicopathologic features and survival

We identified 28 miRNAs differentially expressed based on tumor grade, 23 for tumor stage, 15 for lymph node status, 19 for venous invasion, 11 for resection margin status, and 14 determined by site of recurrence (\(P < 0.001\)). No significant differences were associated with perineural invasion. These miRNA signatures, and their overlap, are illustrated in Fig. 1. Three miRNAs were differentially expressed in association with tumor grade, stage, and lymph node status: miR-21, miR-146a, and miR-628. No miRNA was identified as differently expressed according to patient gender or age.

We investigated the correlation of miRNA expression profiles and patient survival following resection with curative intent. A univariate Cox proportional hazard regression model indicated that, of 476 probes that passed the filtering criteria, 20 miRNAs (Table 2) were associated significantly with overall survival (\(P < 0.05\)). This analysis was visualized by hierarchical clustering, with the highest ranked miRNAs grouping the 48 PDAC specimens according to survival (Fig. 2A). Poor outcome was associated with low expression of 11 miRNAs and high expression of 9 miRNAs. Kaplan–Meier analysis according to expression of the 20 miRNAs shows a significant difference in survival between low- and high-risk groups (Fig. 2B). The poorest PDAC prognosis was associated with low expression of miR-29c, miR-30d, miR-34a, and/or high expression of miR-21, miR-221, and miR-224.

RT-PCR analysis of subset of miRNAs confirms univariate prognostic significance

Using RT-PCR for the 6 individual miRNAs (Fig. 2C), we confirmed high expression of miR-29c, miR-30d, and miR-34a associated with better prognosis. miR-30d high expression associated with a median survival of 30.7 months (95% CI: 15.4–44.7) versus low expression 18.0 months (95% CI: 12.2–23.9, \(P = 0.017\)); miR-34a high expression 43.1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>48-Patient cohort</th>
<th>24-Patient cohort</th>
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<tbody>
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<tr>
<td>Gender (M:F)</td>
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<td>10/14</td>
</tr>
<tr>
<td>Age (&lt;65/&gt;65)</td>
<td>25/23</td>
<td>10/14</td>
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<tr>
<td>Pathologic</td>
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<td></td>
</tr>
<tr>
<td>Tumor stage (T2/T3)</td>
<td>4/44</td>
<td>1/23</td>
</tr>
<tr>
<td>Lymph node metastasis (no/yes)</td>
<td>8/40</td>
<td>5/19</td>
</tr>
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<td>Tumor size (&lt;30/&gt;30 mm)</td>
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<td>13/11</td>
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<tr>
<td>Perineural invasion (no/yes)</td>
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<td>Venous invasion (no/yes)</td>
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<td>6/18</td>
</tr>
<tr>
<td>Adjuvant chemotherapy (no/yes)</td>
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<td>14/10</td>
</tr>
<tr>
<td>Survival, mo. (median)</td>
<td>18.0</td>
<td>20.7</td>
</tr>
<tr>
<td>Status at follow up (alive/dead)*</td>
<td>9/39</td>
<td>4/20</td>
</tr>
</tbody>
</table>

*Median follow-up for the cohort was 23.9 months.
months (95% CI: 20.1–66.1) versus low expression 13.4 months (95% CI: 7.7–19.1, P < 0.001); and miR-29c high expression 39.6 months (95% CI: 15.6–66.1) versus low expression 16.7 months (95% CI: 9.3–23.6, P < 0.001).

High expression of miR-21, miR-221, and miR-224 associated with a poorer prognosis. miR-21 high expression was associated with a median survival of 16.5 months (95% CI: 11.4–21.6) versus low expression of 30.1 months (95% CI: 14.2–49.9, P = 0.012), miR-224 high expression 17.6 months (95% CI: 11.4–24.7) versus low expression 29.8 months (95% CI: 15.1–45.0, P = 0.023), and miR-221 high expression 16.5 months (95% CI: 9.4–23.5) versus low expression 28.3 months (95% CI: 23.9–32.7, P = 0.025, Supplementary Table S5).

miRNA expression associated with site of recurrence

Of the 6 miRNAs validated by PCR only miR-30d showed significantly different expression based on recurrence site. Fourteen of 21 (67%) patients with distant recurrence showed low miR-30d expression, compared with 5 of 16 (31%) of those with local recurrence (P = 0.047). Likewise, 12 of 16 (75%) with liver metastases as the primary site of failure showed low expression of miR-30d, by comparison with 8 of 21 (38%) for patients with recurrence elsewhere (P = 0.03).

Multivariate analysis identifies three independently prognostic miRNAs

The 6 univariately prognostic miRNAs were included in a multivariate model (48 patients only) along with prognostic clinicopathologic factors. Resection margin involvement, venous invasion, and lack of adjuvant therapy remained independent predictors of poor outcome along with high expression of miR-21 (HR = 3.22, 95% CI: 1.21–8.58, P = 0.019), whereas high expression of miR-30d (HR = 0.31, 95% CI: 0.12–0.79, P = 0.014) and miR-34a (HR = 0.15, 95% CI: 0.06–0.37, P < 0.001) independently predicted better survival (Table 3).

Validation of prognostic miRNAs in a separate PDAC cohort

A further validation series of 24 independent PDAC samples was used to evaluate the prognostic significance of a miRNA associated with poor outcome (miR-21) and a
miRNA associated with favorable outcome (miR-34a) following resection. The validation group did not differ significantly in terms of clinicopathologic features compared with the original 48 patient cohort (Table 1). Patients with high miR-21 expression again had a poorer overall survival (13.7 months, 95% CI: 4.7–12.1) compared with low miR-21 expression (25.7 months, 95% CI: 20.2–31.1, \( P = 0.031 \), Fig. 3A). High miR-34a expression was associated again with longer overall survival (26.6 months, 95% CI: 14.9–38.3) compared with low miR-34a expression (6.1 months, 95% CI: 1.75–10.5, \( P < 0.001 \), Fig. 3B).

Predictive utility of miR-21 expression

We subsequently analyzed miR-21 expression along with adjuvant chemotherapy allocation. As the group characteristics were similar, a pooled comparison across the combined 72 patient cohort (48 original + 24 validation PDACs) was done. Thirty-eight patients received chemotherapy and 34 did not. Adjuvant chemotherapy was associated with improved overall survival: 21.8 months (95% CI: 12.6–31.0) versus 13.0 months (95% CI: 8.5–18.0, \( P = 0.031 \), Fig. 3A). High miR-34a expression was associated again with longer overall survival (26.6 months, 95% CI: 14.9–38.3) compared with low miR-34a expression (6.1 months, 95% CI: 1.75–10.5, \( P < 0.001 \), Fig. 3B).

Investigation of potential targets of miR-21 and miR-34a in PDAC

The biological significance of miRNA deregulation is presumed to relate to the effect of miRNAs on their cognate protein-coding gene targets. Detailed analysis of the

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**Table 2. Microarray analysis identified miRNAs univariately associated with overall survival in resected PDAC \((n = 48), P < 0.05\)**

<table>
<thead>
<tr>
<th>miRNA id</th>
<th>Parametric ( P )</th>
<th>( \text{HR}^a )</th>
<th>SD of log intensities</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-30d</td>
<td>0.0008</td>
<td>0.161</td>
<td>0.494</td>
</tr>
<tr>
<td>miR-29c</td>
<td>0.0005</td>
<td>0.407</td>
<td>0.684</td>
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<tr>
<td>miR-154'</td>
<td>0.0005</td>
<td>4.958</td>
<td>0.708</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.0005</td>
<td>2.527</td>
<td>0.883</td>
</tr>
<tr>
<td>miR-224</td>
<td>0.0128</td>
<td>0.395</td>
<td>0.947</td>
</tr>
<tr>
<td>miR-34a</td>
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<td>3.151</td>
<td>0.543</td>
</tr>
<tr>
<td>miR-455</td>
<td>0.0152</td>
<td>0.464</td>
<td>0.759</td>
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<tr>
<td>miR-378</td>
<td>0.0178</td>
<td>0.412</td>
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<td>miR-423</td>
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<td>0.817</td>
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<td>miR-125b</td>
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<td>0.421</td>
<td>0.788</td>
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<td>miR-221</td>
<td>0.0232</td>
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<td>0.713</td>
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<td>miR-33a</td>
<td>0.0243</td>
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<td>miR-141</td>
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<td>miR-181b</td>
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<td>miR-193</td>
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<tr>
<td>miR-30c</td>
<td>0.0495</td>
<td>0.637</td>
<td>0.777</td>
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</table>

\( ^a \text{HR} < 1 \) miRNA expression associated with good outcome.

\( ^b \text{HR} > 1 \) miRNA expression associated with poor outcome.
predicted targets for the top-ranking miRNAs in this study is outlined in the Supplementary Material. To dissect the molecular basis underlying the poor prognosis associated with overexpression of miR-21 and underexpression of miR-34a, we tested gene targets likely involved in PDAC tumorigenesis. Of the 48 patients whose tumors underwent miRNA profiling, 43 had corresponding tissue present on a tissue microarray. The miRNA PCR expression levels were correlated with protein expression using immunohistochemistry (Supplementary Fig. S2).

We first studied miR-21–targeted proteins: Bcl-2, maspin, and PTEN. As previous studies have suggested that miR-21 regulates apoptosis in tumor cells (16), we investigated Bcl-2 expression at the protein level. Our data revealed high miR-21 levels were associated with elevated Bcl-2 expression \( (P = 0.001, \text{Supplementary Fig. S3A}) \). Thus the association of miR-21 in PDAC with poor overall survival may be related to an upregulation of Bcl-2. A positive correlation between miR-21 expression and maspin protein expression was identified \( (P < 0.001, \text{Supplementary Fig. S3B}) \), whereas miR-21 expression was negatively correlated with PTEN protein expression \( (P = 0.004, \text{Supplementary Fig. S3C}) \).

A potential mechanism for the prognostic influence of miR-21 is provided by our evidence that low PTEN protein expression is independently associated with a poor outcome when assessed in the larger cohort \( (n = 117) \) \( (HR = 0.58, 95\% \text{ CI}: 0.38–0.88, P = 0.011, \text{Supplementary Fig. S3D, Table S7}) \). Consistent with in vitro study evidence (17), there was an inverse association between miR-34a expression in the 43 patients and cyclin D1 protein expression potentially impacting on cell-cycle arrest (Supplementary Fig. S3E).

Figure 2. Identification and validation of prognostic miRNAs in PDAC. A, hierarchical clustering of 48 resected PDACs based on the top 20 survival associated miRNAs (miRNA expression by microarray: red indicates upregulation; blue indicates downregulation). In the survival identifier row, samples colored green indicate survival above 2 years, whereas red indicates survival below 6 months. B, Kaplan–Meier analysis of the 20 miRNA predictor shows a significance difference in survival time based on microarray expression values \( (P = 0.02) \) between the low-risk and high-risk groups. C, Kaplan–Meier analysis of the RT-PCR validation. High expression of miR-29c, miR-30d, and miR-34a is associated with favorable survival whereas high expression of miR-21, miR-224, and miR-221 expression is associated with poor survival. Here, miRNA expression levels were measured by qRT-PCR, with high expression levels of miRNA corresponding to a value greater than the median expression. \( P \) values are based on log-rank test.
Furthermore, we have shown that miR-34a downregulation is associated with increased expression of Bcl-2 (Supplementary Fig. S3F).

Regulation of miR-34a expression in PDAC

p53 coordinates cellular response to cellular stresses altering target gene expression culminating in apoptosis, cell-cycle arrest, and increased DNA repair (18). Supporting previous in vitro data (19), we identified that miR-34a expression was significantly associated with p53 expression in human PDAC specimens (Supplementary Fig. S3G). Although loss of p53 would be expected to reduce miR-34a expression, this is unlikely to account entirely for the reduced miRNA expression, as there was not a direct

**Table 3. Multivariate Cox regression analysis including miRNA expression levels and overall survival in 48 patients with PDAC**

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tr>
<td>Tumor stage</td>
<td>T2/T3</td>
<td>2.02 (0.36–11.3)</td>
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<td>Tumor grade</td>
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<td>Absent/present</td>
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<td>Margin involvement</td>
<td>R0/R1</td>
<td>6.83 (2.07–22.5)</td>
</tr>
<tr>
<td>Adjuvant therapy</td>
<td>Yes/no</td>
<td>0.25 (0.09–0.67)</td>
</tr>
<tr>
<td>miR-21</td>
<td>Yes/no</td>
<td>3.22 (1.21–8.58)</td>
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<tr>
<td>miR-29c</td>
<td>Yes/no</td>
<td>0.53 (0.19–1.47)</td>
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<tr>
<td>miR-30d</td>
<td>Yes/no</td>
<td>0.30 (0.12–0.79)</td>
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<td>miR-34a</td>
<td>Yes/no</td>
<td>0.15 (0.06–0.37)</td>
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<td>miR-221</td>
<td>Yes/no</td>
<td>0.92 (0.34–2.54)</td>
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<tr>
<td>miR-224</td>
<td>Yes/no</td>
<td>0.67 (0.25–1.76)</td>
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Figure 3. Further validation of prognostic miRNAs and predictive utility of miR-21. Survival analyses confirming high expression of (A) miR-21 (P = 0.031) and low expression of (B) miR-34a (P < 0.001) was associated with poor survival following resection in a validation cohort of 24 PDAC patients. C, combined analysis of 72 patient cohort examining associations between mir-21 expression and receipt of adjuvant chemotherapy with overall survival. In patients with low tumoral miR-21 expression, adjuvant chemotherapy failed to significantly influence overall survival (P = 0.32), whereas in patients with high miR-21 expression, chemotherapy was associated with significantly prolonged survival (P = 0.008). miRNA expression levels measured by qRT-PCR were converted into discrete variable by division of samples into 2 classes (low and high expression) based on median values as the threshold. P values based on log-rank test.
correlation between complete loss of p53 and the magnitude of miR-34a downregulation (Supplementary Fig. S3G). Therefore, other mechanisms, in addition to p53 inactivation, likely contribute to the reduction of miR-34a abundance (19). Using array comparative genomic hybridization (arrayCGH), we examined the copy number profile of a subset of our cohort (n = 37) and identified loss of copy number for the miR-34a region in 15 of 37 specimens. Deletion of the genomic interval encompassing miR-34a (1p36) is a common feature in a number of malignancies (20). Previously high-resolution copy number assessment analysis of pancreatic cancer cell lines (21) showed hemizygous loss of the miR-34a locus. Our data therefore support the concepts that gene deletion, lack of activation by p53, and, possibly, other mechanisms contribute to the underexpression of miR-34a in human PDAC.

**Gene expression profiles associated with miR-21 and miR-34a expression**

To determine the effects of miR-21 and miR-34a expression on mRNA expression, we compared gene expression profiles of PDACs with low-expression against high-expression for both miRNAs. For miR-21, there was a significant difference between these groups with upregulation of 561 mRNA transcripts and downregulation of 517 (P < 0.01, Supplementary Table S8A). PDCD4 was notably downregulated, whereas matrix metalloproteinase 7 (MMP7) and MMP9 were upregulated. For miR-34a, there was a significant difference between these groups with upregulation of 389 mRNA transcripts and downregulation of 318 (P < 0.05, Supplementary Table S8B).

To better understand the potential global effects of miR-21 and miR-34a expression on the PDAC transcriptome, we examined the Gene Ontology (GO) classifications of the up- and downregulated genes. For miR-21 overenriched GO terms among upregulated genes included cytoskeleton organization (P = 1.2 × 10⁻⁸), blood vessel development and angiogenesis (P = 4.1 × 10⁻⁸), and regulation of apoptosis (P = 3.1 × 10⁻⁸). Genes assigned to the terms mitogen-activated protein kinase (MAPK) signaling pathway (P = 9.2 × 10⁻⁴), regulation of caspase activity (P = 4.3 × 10⁻⁴), and cell cycle (P = 8.1 × 10⁻³) were enriched among downregulated genes. For miR-34a overenriched GO terms among upregulated genes included cell division (P = 8.1 × 10⁻³), response to DNA damage (P = 2.9 × 10⁻³), and serine/threonine kinase activity (P = 5.1 × 10⁻³). Moreover, genes assigned to the terms wound healing (P = 1.8 × 10⁻⁴), chemotaxis (P = 1.2 × 10⁻⁴), and apoptosis (P = 7.1 × 10⁻⁴) were enriched among downregulated genes. Nonetheless, the majority of genes differently expressed between the groups are certainly not related to miR-34a status.

**Discussion**

These data show that miRNA expression profiling can identify novel clinicopathologic correlations for PDAC including a signature of prognostic miRNAs. Detailed miRNA profiles have been generated from PDAC cell and animal models (22). In addition, 3 large-scale profiles of miRNAs in human PDAC have been published (23–25). Initially focused on tumor versus normal comparison (24, 25), subsequently Bloomston and colleagues used miRNA microarrays to study 65 PDACs, identifying diagnostic and prognostic candidates (23).

There is considerable overlap between mRNA expression profiles generated in recent PDAC microarray analyses (25) and in this study, far more than evident in mRNA profiling studies (26). Principally a result of the smaller number of potential targets, this overlap supports the robustness of miRNA methodology, as despite different extraction and analysis techniques, remarkably similar profiles result. Overexpressed miRNAs including miR-21, miR-145, and miR-155 all commonly associate with malignancy (27, 28); however, miR-23a and miR-103 were novel overexpressed targets that warrant further investigation.

We identified that miRNAs expression correlated with clinicopathologic features; notably, tumor grade associated with the greatest number of aberrant miRNAs followed by tumor stage and venous invasion. Resection margin involvement associated with significantly aberrant miRNA expression, suggesting tumor biology variation may underlie this detrimental pathologic state (29). Three miRNAs were commonly differentially expressed in association with tumor grade, stage, and lymph node status: miR-21 emerged once again along with miR-146a and miR-628. This finding suggests that a number of miRNAs are shared among tumors with advanced features. Although this type of analysis is to our knowledge novel in PDAC, our results concur with previous cancer-related miRNA studies. miR-146a has been identified as being upregulated in melanoma specimens (30), and in gastric carcinoma, expression was associated with lymph node positivity (31). Notably, in a recent neuroblastoma deep sequencing study, miR-628 was identified as a putative tumor suppressor gene, being expressed in tumors with favorable outcome (32). miR-21 expression has been correlated with stage and lymph node metastasis in various malignancies (27, 28), including PDAC (33).

Global miRNA profiling with multivariate Cox regression analysis identified numerous miRNAs that significantly associated with overall survival following resection. The overlap with differentially expressed miRNAs based on pathologic factors included only miR-21, miR-30d, and miR-125. Although many were novel associations, we confirmed high miR-21 expression was independently associated with poor overall survival supporting previous reports (33, 34), including in situ hybridization assessment in which miR-21 was prognostic in node-negative patients (35). The proposed oncogenic properties of this almost ubiquitously expressed molecule are supported by functional investigations showing that inhibition reduced proliferation of cancer cell lines, including breast, hepatocellular, and PDAC (36–38). Conversely, miR-21 precursor transfection enhanced invasion and metastasis in a breast model (39), in addition to pancreatic cancer (38).

We identified numerous novel miRNA prognostic associations in this study, in particular, for miR-30d and...
miR-34a. The miR-34 family is strongly implicated as serving a tumor suppressor role in malignancy (40) and in pancreatic cancer cell model, miR-34a transfection resulted in restoration of the p53 tumor-suppressor function (41). In non–small cell lung carcinoma, it was shown to be downregulated, and low levels of miR-34a expression correlated with a high probability of relapse (42). Our results support a tumor suppressive role for miR-34a as higher than median expression was independently associated with a favorable outcome following resection in the test cohort and univariately in the validation set. High miR-30d expression was identified as an independent marker of good prognosis in the test set. Although not previously implicated in PDAC, overexpression of miR-30d is associated with poor outcome in hepatocellular carcinoma (43). Although miR-29c has not previously been correlated with survival in PDAC, in mesothelioma, miR-29 expression associated with favorable outcome, and overexpression in a cell model resulted in decreased invasion (44). Similarly in mantle cell lymphoma, miR-29 was downregulated compared with normal lymphocytes with underexpression associating with reduced survival (45).

The miR-221/222 cluster is upregulated in PDAC cell lines (22) and likely promotes proliferation as in other tumors (46). miR-222 overexpression was previously associated with poorer outcome following PDAC resection (47). Although miR-221 has been associated with increasing PanIN grade (48), this study is the first to associate miR-221 or miR-222 expression with overall survival. The novel survival associations for miR-29c, miR-30d, miR-34a, miR-221, and miR-224 warrant validation and additional studies to investigate potential roles in PDAC tumorigenesis. miR-196a is a notable target previously shown by Bloomston and colleagues (23) that was not identified as prognostic from our microarray analysis. Subsequently, we did not undertake any further investigation and therefore cannot exclude potential prognostic utility. We did however correlate miR-196a with lymph node positivity, T3 tumors, venous invasion, and resection margin involvement supporting a role in disease progression. Expression profile differences may be explained by RNA extraction and analysis platform variation.

Our results suggest that miR-21 had prognostic utility for all patients regardless of adjuvant therapy status, supporting its role as a prognostic marker. However, if miR-21 expression is causal to poor therapeutic outcome, antagonism (49) targeting this molecule may yield therapeutic benefits in high expressers. Our assessment of miR-21 predictive utility was confounded by limited sample size and nonstandardized chemotherapy regimens.

Our data support the suggestion that miR-21 targets genes integral to PDAC tumorigenesis including PTEN, loss of which in turn correlates independently with poor outcome. Maspin has been implicated as a tumor suppressor in malignancies including prostate cancer (50), with miR-21 inhibition a potential mechanism by which invasion and metastasis is achieved (51). However, maspin expression has been associated with poor outcome following PDAC resection (52). Our finding of a positive correlation between maspin and miR-21 expression suggests further investigation of this relationship is warranted. miR-34a has been shown to be induced by p53 (19), and subsequently, we have evidence supporting this finding in human PDAC. Furthermore, our data suggest that miR-34a downregulation is associated with increased expression of cyclin D1; which adds to previous evidence that miR-34a regulates cell-cycle progression in part by targeting G1-phase regulators (17). Restoration of miR-34a in human pancreatic cells has been previously shown to inhibit the expression of Bcl-2, inhibiting growth and invasion, inducing apoptosis, and G1/G2 arrest (41), a finding that our data supports.

Previous analysis of gene expression in pancreatic cell lines according to induced miR-34a status identified upregulation of cell cycle, DNA repair, and mitotic checkpoint categories along with downregulation of angiogenesis-related genes (19). Although there was some overlap between the GO identified in this study, this was certainly not complete, explained by the numerous miRNAs dysregulated in human PDAC. The role of miR-34a in apoptosis is supported by the enrichment of established antiapoptotic factors among the downregulated targets.

The pathway enrichment analysis conducted for putative miRNA targets of miRNAs associated with poor outcome highlighted established pathways underlying PDAC including Wnt, TGF-β, and MAPK signaling (Supplementary Material). Putative target genes commonly targeted by the good prognosis miRNAs were identified by a combination of computational approaches. Clearly additional studies will be required to identify and experimentally validate potential miRNA targets.

Our study was not without limitations, principally our failure to carry out laser capture microdissection, instead using bulk-dissected pancreatic tumor tissue for RNA extraction. This enables the stromal and potentially inflammatory components, which play an increasingly recognized role in carcinogenesis and tumor progression, to be evaluated alongside epithelial components. Despite this benefit, microdissection would have enhanced localization of miRNA expression to individual tissue compartments.

Certainly there seems to be sufficient evidence to assess the prognostic utility miRNAs in prospective trials. The clinical utility of miRNAs may be enhanced by measurement prior to resection with miRNA analysis in PDAC tissue obtained by endoscopic ultrasound-guided fine needle aspirates already done (53), with potential to enhance the clinical management algorithm of borderline resectable cases. The stability and robustness of miRNAs was shown by recent quantification in serum, with miR-196a identifying a poor prognostic group in PDAC (54).

In conclusion, PDAC has extensive alterations of miRNA expression that may deregulate cancer-related genes. The miRNA profiles of PDAC correlated with clinicopathologic features including lymph node status, tumor grade, and subsequently, we identified that various miRNAs possessed independent prognostic utility including miR-21 and miR-34a following resection.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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