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

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Abstract

Purpose

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

Experimental Design

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

Results

miRNA profiling identified expression signatures associated with PDAC, lymph node involvement, high tumor grade, and 20 microRNAs were associated with overall survival. In the initial cohort of 48 PDAC patients, high expression of miR-21 (Hazard ratio (HR): 3.22, 95% Confidence Interval (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 microRNAs are significantly altered in PDAC. Aberrant expression of a number of miRNAs were independently associated with reduced survival including over-expression of miR-21 and under-expression 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 non-malignant pancreatic tissues as well as molecular signatures that differ according to pathological features. miRNA expression profiles correlated with overall survival of PDAC following resection indicating that miRNAs provide prognostic utility.
Statement of Translational Relevance

The outcome for resected pancreatic ductal adenocarcinoma (PDAC) remains dismal despite improvement in surgical and oncological management strategies. MicroRNAs are small, noncoding regulatory molecules, which have made tremendous impact within cancer biology as well as outcome prediction for many tumors. We hypothesized that PDAC will exhibit a unique microRNA profile associated with tumorigenesis enabling the identification of microRNAs correlating with pathological features and most importantly possess prognostic utility. It was our aim to investigate the microRNA expression profile in 48 resected PDACs using microRNA microarrays. We identified microRNAs associated with pathological features with miR-21, miR-29c, miR-30d, miR-34a, miR-221, and miR-224 having prognostic utility. Validation in a separate cohort of 24 patients confirmed the prognostic utility of miR-21 and miR-34a. These findings have clear implications for prognosis prediction in PDAC and warrant further studies of the role of these clinically relevant microRNAs, particularly within preoperatively collected serum and PDAC cytology specimens.
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-yr survival rate is a dismal 3-5% with the impact of chemotherapeutic options remaining limited (3).

Consequently novel therapeutic routes are required. Staging and prognostic information is provided by standard clinicopathological 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 (4). 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 (5). Although investigation of these known genes and proteins has yielded new information, it has become apparent that non-coding RNA gene products may provide additional insights into PDAC biology (6).

MicroRNAs (miRNAs) are small non-coding 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 (7-10). miRNAs appear to influence various biological processes including cell proliferation, cell death, stress resistance, mainly through negative regulation of gene expression (11). Recently, miRNAs have gained wide attention as a family of molecules involved in cancer development (12). Model therapeutic targets should be causally associated with disease and amenable to therapeutic intervention, while model biomarkers should be easily quantifiable and associate strongly with clinical outcome: miRNAs potentially match both criteria (10).
Our large cohort of patients, who have undergone resection for PDAC (pancreaticoduodenectomy [PD]), with full clinicopathological and follow-up data, was recently used to describe novel prognostic pathological parameters including circumferential resection margin status (13). The aim of the current 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 clinicopathological variables and also with overall survival following resection. Expression of a subset of candidate miRNAs was assessed by RT-PCR within the initial cohort and in a separate validation cohort of 24 resected PDACs.

Results

Clinicopathological characteristics of patient cohort
The initial miRNA profiling was performed on samples from 48 patients with resectable PDAC who underwent PD with curative intent. Table 1 summarizes their clinicopathological 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 37/48 (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 clinicopathological 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) (Log-Rank; \( P = 0.042 \)). All clinicopathological factors with \( P < 0.10 \) 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.

MicroRNA expression profiles differ between PDAC and normal pancreas
We analyzed and compared the miRNA expression profiles in 48 PDACs and 10 paired samples of non-cancerous pancreas tissue. 97 of these miRNAs showed statistically different expression between the two groups ($P < 0.001$). 39 miRNAs were up-regulated and 58 down-regulated in cancer (top 25 up and down-regulated miRNAs are shown in Supplementary Table S3; full list in Supplementary Table S4). miRNAs up-regulated in PDAC compared to normal pancreas included miR-10a, miR-21, miR-143, miR-145, miR-155, miR-222, miR-223, miR-224, and miR-373; miRNAs down-regulated in PDAC compared to 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 performed 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. 1. 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.

**microRNA expression profiles associated with clinicopathological 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, out 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 microRNAs confirms univariate prognostic significance**

Using RT-PCR, for the six 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 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 30.1 months (95%CI:14.2-49.9, \( P = 0.012 \)); miR-224 high expression 17.6 months (95%CI:10.5-24.7) 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).

**microRNA expression associated with site of recurrence**

Of the six miRNAs validated by PCR only miR-30d showed significantly different expression based on recurrence site. 14/21 (67%) patients with distant recurrence showed low miR-30d expression, compared with 5/16 (31%) of those with local recurrence (\( P = 0.047 \)). Likewise, 12/16 (75%) with liver metastases as the primary site of failure showed low expression of miR-30d, by comparison with 8/21 (38%) for patients with recurrence elsewhere (\( P = 0.03 \)).

**Multivariate analysis identifies three independently prognostic microRNAs**

The six univariately prognostic miRNAs were included in a multivariate model (48 patients only) along with prognostic clinicopathological 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, \( P = 0.019 \)), while 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 microRNAs 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 favourable outcome (miR-34a) following resection. The validation group did not differ significantly in terms of clinicopathological features compared to 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 to 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 to 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 performed. 38 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, \( P = 0.05 \)). 34 patients had high and 38 had low expression, with low miR-21 associated with better outcome compared to high miR-21: 11.5 months (95% CI: 8.2-14.8) versus 26.7 months (95% CI: 24.8-28.6, \( P = 0.001 \)).

Of the patients with low miR-21 expression, 21 received chemotherapy and 17 did not, and administration was not associated with improvement in survival: 27.5 months (95% CI: 23.6-31.4) versus 26.6 months (95% CI: 23.1-30.0, \( P = 0.74 \)) without chemotherapy. In contrast, in patients with high miR-21 expression, 16 received chemotherapy and 18 did not. Adjuvant chemotherapy was associated with a significant increase in overall survival, from 7.1 months (95% CI: 1.0-14.3) without chemotherapy to 16.4 months (95% CI: 12.3-18.4) with chemotherapy (Fig. 3C). However
the study is limited by the small sample size and utility as a predictive marker should be tested in an adequately powered, prospective study. Thus in patients with tumors expressing high miR-21 adjuvant chemotherapy resulted in prolonged overall survival ($P = 0.008$); in contrast, for patients with tumors with low miR-21 expression, no survival advantage could be demonstrated, with chemotherapy failing to significantly prolong survival following resection.

Multivariate analysis of the combined cohort demonstrated high miR-21 expression predicted poor prognosis while adjuvant therapy was associated with improved survival (Supplementary Table S6A). Subgroup analysis demonstrated that adjuvant therapy was only an independent predictor of outcome for the low miR-21 group (Supplementary Table S6B, C).

**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 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 over-expression of miR-21 and under-expression 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 (TMA). The miRNA PCR expression levels were correlated with protein expression using IHC (Supplementary Fig. 2).

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 (14), we investigated Bcl-2 expression at the protein level. Our data revealed high miR-21 levels were associated with elevated Bcl-2 expression ($P = 0.001$, Supplementary Fig. 3A). Thus the association of miR-21 in PDAC with poor overall survival may be related to an up-regulation of Bcl-2. A positive correlation between miR-21 expression and maspin protein expression was identified ($P < 0.001$, Supplementary Fig. 3B), whereas miR-21 expression was negatively correlated with PTEN protein expression ($P = 0.004$, Supplementary Fig. 3C). 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%CI: 0.38-0.88, \( P = 0.011 \), Supplementary Fig. 3D, Table S7).

Consistent with \textit{in vitro} study evidence (15), 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. 3E). Furthermore, we have shown that miR-34a down-regulation is associated with increased expression of Bcl-2 (Supplementary Fig. 3F).

\textbf{Regulation of miR-34a expression in PDAC}

\textit{p}53 coordinates cellular response to cellular stresses altering target gene expression culminating in apoptosis, cell-cycle arrest, and increased DNA repair (16). Supporting previous \textit{in vitro} data (17), we identified that miR-34a expression was significantly associated with \textit{p}53 expression in human PDAC specimens (Supplementary Fig. 3G). Although loss of \textit{p}53 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 correlation between complete loss of \textit{p}53 and the magnitude of miR-34a down-regulation (Supplementary Fig. 3G). Therefore, other mechanisms, in addition to \textit{p}53 inactivation likely contribute to the reduction of miR-34a abundance (17). 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 15/37 specimens. Deletion of the genomic interval encompassing miR-34a (1p36) is a common feature in a number of malignancies (18). Previously high-resolution copy number assessment analysis of pancreatic cancer cell lines (19) demonstrated hemizygous loss of the miR-34a locus. Our data therefore support the concepts that gene deletion, lack of activation by \textit{p}53, and possibly other mechanisms contribute to the under-expression of miR-34a in human PDAC.

\textbf{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 up-regulation of 561 mRNA transcripts and down-regulation of 517 (\( P < 0.01 \), Supplementary Table S8A) PDCD4 was notably down-regulated, while MMP7 and MMP9 were up-regulated. For miR-34a,
there was a significant difference between these groups with up-regulation of 389 mRNA transcripts and down-regulation 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 down-regulated genes. For miR-21 over-enriched GO terms among up-regulated genes included cytoskeleton organization (∆P = 1.2x10^{-5}), blood vessel development and angiogenesis (∆P = 4.1x10^{-4}), and regulation of apoptosis (∆P = 3.1x10^{-3}). Genes assigned to the terms MAPK signalling pathway (∆P = 9.2x10^{-3}), regulation of capase activity (∆P = 4.3x10^{-4}), and cell-cycle (∆P = 8.1x10^{-3}) were enriched amongst down-regulated genes. For miR-34a over-enriched GO terms among up-regulated genes included cell division (∆P = 8.1x10^{-6}), response to DNA damage (∆P = 2.9x10^{-3}), and serine/threonine kinase activity (∆P = 5.1x10^{-3}). Moreover, genes assigned to the terms wound healing (∆P = 1.8x10^{-4}), chemotaxis (∆P = 1.2x10^{-4}), and apoptosis (∆P = 7.1x10^{-3}) were enriched amongst down-regulated 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 clinicopathological correlations for PDAC including a signature of prognostic miRNAs. Detailed miRNA profiles have been generated from PDAC cell and animal models (20). Additionally three large-scale profiles of miRNAs in human PDAC have been published (21-23). Initially focussed on tumor versus normal comparison (22, 23), subsequently Bloomston et al. used miRNA microarrays to study 65 PDACs, identifying diagnostic and prognostic candidates (21).

There is considerable overlap between miRNA expression profiles generated in recent PDAC microarray analyses (23) and the current study, far more than evident in mRNA profiling studies (24). 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. Over-expressed miRNAs including miR-21, miR-145 and, miR-155 all commonly associate with malignancy (25, 26), however, miR-23a and miR-103 were novel over-expressed targets that warrant further investigation.
We identified that miRNAs expression correlated with clinicopathological 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 pathological state (27). 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 a number of miRNAs are shared amongst 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 up-regulated in melanoma specimens (28), and in gastric carcinoma, expression was associated with lymph node positivity (29). Notably in a recent neuroblastoma deep sequencing study miR-628 was identified as a putative tumor suppressor gene, being expressed in tumors with favourable outcome (30). miR-21 expression has been correlated with stage and lymph node metastasis in various malignancies (25, 26), including PDAC (31).

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 pathological factors included only miR-21, miR-30d, and miR-125. While many were novel associations, we confirmed high miR-21 expression was independently associated with poor overall survival supporting previous reports (31, 32), including in-situ hybridization assessment in which miR-21 was prognostic in node-negative patients (33). The proposed oncogenic properties of this almost ubiquitously expressed molecule are supported by functional investigations demonstrating that inhibition reduced proliferation of cancer cell lines including breast, hepatocellular, and PDAC (34-36). Conversely, miR-21 precursor transfection enhanced invasion and metastasis in a breast model (37) in addition to pancreatic cancer (36).

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 (38) and in a p53-deficient pancreatic cancer cell model, miR-34a transfection resulted in restoration of the p53 tumor-suppressor function (39). In NSCLC, it was shown to be down-regulated, and low levels of miR-34a expression correlated with a high probability of relapse (40). Our results support a tumor suppressive role for miR-34a as higher than median expression was independently associated with a favourable 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. While not previously implicated in PDAC, over-expression of miR-30d is associated with poor outcome in hepatocellular carcinoma (41). Although miR-29c has not previously been correlated with survival in PDAC, in mesothelioma, miR-29 expression associated with favourable outcome, and overexpression in a cell model resulted in decreased invasion (42). Similarly in mantle cell lymphoma, miR-29 was down-regulated compared to normal lymphocytes with under-expression associating with reduced survival (43).

The miR-221/222 cluster is up-regulated in PDAC cell lines (20) and likely promotes proliferation as in other tumors (44). miR-222 over-expression was previously associated with poorer outcome following PDAC resection (45). While miR-221 has been associated with increasing PanIN grade (46), the current study is the first to associate miR-221 or miR-224 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 demonstrated by Bloomston et al. (21) 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, antagonirs (47) targeting this molecule may yield therapeutic benefits in high expressors. Our assessment of miR-21 predictive utility was confounded by limited sample size and non-standardized chemotherapy regimen.

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 (48), with miR-21 inhibition a potential mechanism by which invasion and metastasis is achieved (49). However, maspin expression has been associated with poor outcome following PDAC resection (50). Our finding of a positive correlation between maspin and miR-21 expression suggests further investigation of this relationship is warranted. miR-34a has been demonstrated to be induced by p53 (17) and subsequently we have evidence supporting this finding in human PDAC. Furthermore, our data suggest that miR-34a down-regulation 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 (15). 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 (39), a finding that our data supports.

Previous analysis of gene expression in pancreatic cell lines according to induced miR-34a status identified up-regulation of cell-cycle, DNA repair, and mitotic checkpoint categories along with down-regulation of angiogenesis related genes (17). Although there was some overlap between the GO identified in the current 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 anti-apoptotic factors amongst the down-regulated targets.

The pathway enrichment analysis conducted for putative mRNA targets of miRNAs associated with poor outcome highlighted established pathways underlying PDAC including Wnt, TGF-β, and MAPK signalling (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 perform 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 appears 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 performed (51), with potential to enhance the clinical management algorithm of borderline resectable cases. The stability and robustness of miRNAs was demonstrated by recent quantification in serum, with miR-196a identifying a poor prognostic group in PDAC (52).

In conclusion, PDAC has extensive alterations of miRNA expression that may deregulate cancer-related genes. The miRNA profiles of PDAC correlated with clinicopathological 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.
Methods

Sample collection
Macrodissected fresh frozen pancreatic tissue samples were used in this study: 48 PDACs and 10 corresponding matched non-tumor 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), U.K. 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, e.g. ampullary, duodenal, distal bile duct adenocarcinomas, mucinous cystadenocarcinomas or intraductal papillary mucinous neoplasms, were excluded. Complete clinicopathological, follow-up and recurrence data were available (13).

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, USA) quantified total RNA, while purity and integrity was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChipVR reagent set (Agilent Technologies, Boeblingen, Germany). Samples with a RNA integrity number (RIN) above 7.0 were deemed suitable for downstream analysis.

microRNA microarray analysis
MiRNA profiling was performed on 48 PDACs and 10 corresponding matched normal pancreas samples. For miRNA expression profiling, 100ng of total RNA were dephosphorylated (Calf Intestinal Alkaline Phosphatase, GE Healthcare, Munich, Germany) and labeled by ligation (T4 RNA Ligase, NEB Biolabs, Frankfurt, Germany) with one Cyanine 3-pCp molecule to the 3\(^{\text{rd}}\) 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, Munich, Germany) as described by Agilent Technologies. Hybridization, microarray washing and detection of labeled miRNA on the microarray were performed according to Agilent Technologies instructions. miRNA expression profiling was performed using Agilent’s Human...
miRNA Microarrays (version 2.0, based on Sanger miRBase version 10.1), carrying 723 human miRNAs (53). Array image acquisition and feature extraction was performed 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 (54). After excluding negative values with hybridization intensity below background, normalization was performed 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 non-tumor 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**

Reverse transcription (RT)-PCR analysis was performed 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, USA) 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 20ng of each total RNA sample. The RT reaction was performed at 37°C for 30 min and then 95°C for 10 min. using the DyNAmo Hot star SYBR Green kit (Finnzymes, Espoo, Finland) 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 min, then 95°C for 15 sec, and 60°C for 35 sec 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 performed 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 performed, 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 hazard ratio (HR) with 95% confidence intervals (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 performed using SPSS 18.0 (IBM®SPSS®, Somers, NY, USA).

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

**Acknowledgements**
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Figure Legend

**Fig. 1. MiRNAs associated with traditional clinicopathological features in PDAC.**

Venn diagram of miRNAs that are associated in PDAC with tumor grade, tumor stage and lymph node status. The numbers are attached to lists of the miRNAs associated with each component of the diagram. miRNAs associated with the presence of venous invasion, resection margin involvement and liver metastases as the site of initial recurrence are listed separately at bottom left: those miRNAs which are common to those in the Venn diagram are highlighted in red. miRNAs in *italics* – down-regulated, the remainder of miRNAs were up-regulated.

**Fig. 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 up-regulation; blue indicates down-regulation). In the survival identifier row, samples colored green indicate survival over 2 years, while red indicates survival below 6 months.

B) Kaplan-Meier analysis of the 20 miRNA predictor demonstrates 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 favourable survival while 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.

**Fig. 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 ($P = 0.32$), while in patients with high miR-21 expression, chemotherapy was associated with significantly prolonged survival ($P = 0.008$). MicroRNA expression levels measured by qRT-PCR were converted into discrete variable by division of samples into two classes (low and high expression) based on median values as the threshold. $P$ values based on Log-Rank test.
References

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>48 Patient cohort</th>
<th>24 Patient cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
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</tr>
<tr>
<td>Gender (M: F)</td>
<td>19/29</td>
<td>10/14</td>
</tr>
<tr>
<td>Age (≤65/ &gt;65)</td>
<td>25/23</td>
<td>10/14</td>
</tr>
<tr>
<td>Pathological</td>
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<td></td>
</tr>
<tr>
<td>Tumor stage (T2/ T3)</td>
<td>4/44</td>
<td>1/23</td>
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<tr>
<td>Lymph node metastasis (no/ yes)</td>
<td>8/40</td>
<td>5/19</td>
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<td>Tumor size (≤30/ &gt;30mm)</td>
<td>28/20</td>
<td>13/11</td>
</tr>
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<td>Tumor grade (low/ high)</td>
<td>32/16</td>
<td>17/7</td>
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<tr>
<td>Perineural invasion (no/ yes)</td>
<td>3/45</td>
<td>0/24</td>
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<td>Venous invasion (no/ yes)</td>
<td>15/33</td>
<td>10/14</td>
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<tr>
<td>Lymphatic invasion (no/ yes)</td>
<td>31/17</td>
<td>15/9</td>
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<tr>
<td>Operative, treatment and outcome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection margin status (R0/ R1)</td>
<td>10/38</td>
<td>6/18</td>
</tr>
<tr>
<td>Adjuvant chemotherapy (no/ yes)</td>
<td>20/28</td>
<td>14/10</td>
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<tr>
<td>Survival (months) (median)</td>
<td>18.0</td>
<td>20.7</td>
</tr>
<tr>
<td>Status at follow up (alive/ dead) ^a</td>
<td>9/39</td>
<td>4/20</td>
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</table>

^a Median follow-up for the cohort was 23.9 months
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 value</th>
<th>Hazard Ratio 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>
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<tr>
<td>miR-29c</td>
<td>0.0055</td>
<td>0.407</td>
<td>0.684</td>
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<tr>
<td>miR-154*</td>
<td>0.0085</td>
<td>4.958</td>
<td>0.708</td>
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<tr>
<td>miR-21</td>
<td>0.0086</td>
<td>2.527</td>
<td>0.883</td>
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<tr>
<td>miR-224</td>
<td>0.0093</td>
<td>2.031</td>
<td>0.947</td>
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<tr>
<td>miR-34a</td>
<td>0.0128</td>
<td>0.395</td>
<td>0.607</td>
</tr>
<tr>
<td>miR-455</td>
<td>0.0146</td>
<td>3.151</td>
<td>0.543</td>
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<tr>
<td>miR-378</td>
<td>0.0152</td>
<td>0.464</td>
<td>0.759</td>
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<tr>
<td>miR-423</td>
<td>0.0178</td>
<td>0.412</td>
<td>0.582</td>
</tr>
<tr>
<td>miR-30a</td>
<td>0.0212</td>
<td>0.506</td>
<td>0.817</td>
</tr>
<tr>
<td>miR-31</td>
<td>0.0221</td>
<td>1.261</td>
<td>2.099</td>
</tr>
<tr>
<td>miR-125b*</td>
<td>0.0222</td>
<td>0.421</td>
<td>0.788</td>
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<tr>
<td>miR-221</td>
<td>0.0232</td>
<td>2.007</td>
<td>0.713</td>
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<tr>
<td>miR-33a</td>
<td>0.0243</td>
<td>0.288</td>
<td>0.482</td>
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<tr>
<td>miR-141</td>
<td>0.0344</td>
<td>0.716</td>
<td>1.336</td>
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<tr>
<td>miR-181b</td>
<td>0.0352</td>
<td>2.166</td>
<td>0.644</td>
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<tr>
<td>miR-193</td>
<td>0.0393</td>
<td>7.083</td>
<td>0.757</td>
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<tr>
<td>miR-223</td>
<td>0.0415</td>
<td>0.257</td>
<td>0.475</td>
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<tr>
<td>miR-186</td>
<td>0.0426</td>
<td>3.344</td>
<td>0.344</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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</tbody>
</table>

a Hazard ratio < 1 miRNA expression associated with good outcome
Hazard ratio > 1 miRNA expression associated with poor outcome
SD - standard deviation
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>Overall survival</th>
<th>Multivariate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>T2/ T3</td>
<td>2.02 (0.36-11.3)</td>
<td>0.425</td>
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<td>Tumor grade</td>
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<tr>
<td>Low/ High</td>
<td>1.60 (0.59-4.29)</td>
<td>0.347</td>
</tr>
<tr>
<td>Venous invasion</td>
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<td>Absent/ Present</td>
<td>2.51 (1.01-6.22)</td>
<td>0.047</td>
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<td>Margin involvement</td>
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<tr>
<td>R0/ R1</td>
<td>6.83 (2.07-22.5)</td>
<td>0.002</td>
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<td>Adjuvant therapy</td>
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<tr>
<td>Yes/ No</td>
<td>0.25 (0.09-0.67)</td>
<td>0.006</td>
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<tr>
<td>miR-21</td>
<td></td>
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<tr>
<td>Yes/ No</td>
<td>3.22 (1.21-8.58)</td>
<td>0.019</td>
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<tr>
<td>miR-29c</td>
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<tr>
<td>Yes/ No</td>
<td>0.53 (0.19-1.47)</td>
<td>0.227</td>
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<td>miR-30d</td>
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<td>0.30 (0.12-0.79)</td>
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<td>miR-34a</td>
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<td>Yes/ No</td>
<td>0.15 (0.06-0.37)</td>
<td>0.001</td>
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<td>miR-221</td>
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<td>Yes/ No</td>
<td>0.92 (0.34-2.54)</td>
<td>0.881</td>
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<td>miR-224</td>
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<td>Yes/ No</td>
<td>0.67 (0.25-1.76)</td>
<td>0.673</td>
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MicroRNA molecular profiles associated with diagnosis, clinicopathological criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma


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