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MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer

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BACKGROUND: A major challenge to the development of biomarkers for pancreatic cancer (PC) is the small amount of tissue obtained at the time of diagnosis. Single-gene analyses may not reliably predict biology of PC because of its complex molecular makeup. MicroRNA (miRNA) profiling may provide a more informative molecular interrogation of tumours. The primary objective of this study was to determine the feasibility of performing miRNA arrays and quantitative real-time PCR (qRT–PCR) from archival formalin-fixed paraffin-embedded (FFPE) cell blocks obtained from fine-needle aspirates (FNAs) that is the commonest diagnostic procedure for suspected PC.

METHODS: MicroRNA expression profiling was performed on FFPE from FNA of suspicious pancreatic masses. Subjects included those who had a pathological diagnosis of pancreatic adenocarcinoma and others with a non-malignant pancreatic histology. Exiqon assay was used to quantify miRNA levels and qRT–PCR was used to validate abnormal expression of selected miRNAs.

RESULTS: A total of 29 and 15 subjects had pancreatic adenocarcinoma and no evidence of cancer, respectively. The RNA yields per patient varied from 25 to 100 ng. Profiling demonstrated deregulation of over 228 miRNAs in pancreatic adenocarcinoma of which the top 7 were further validated by qRT–PCR. The expression of let-7c, let-7 f, and miR-200c were significantly reduced in most patients whereas the expression of miR-486-5p and miR-451 were significantly elevated in all pancreas cancer patients. MicroRNAs let-7d and miR-423-5p was either downregulated or upregulated with a significant inter-individual variation in their expression.

CONCLUSION: This study demonstrated the feasibility of using archival FFPE cell blocks from FNAs to establish RNA-based molecular signatures unique to pancreatic adenocarcinoma with potential applications in clinical trials for risk stratification, patient selection, and target validation.

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Pancreatic cancer (PC) is the fourth leading cause of cancer related deaths in the United States (Jemal *et al*, 2010). Mortality from this disease has not changed over the past few decades partly because of lack of screening methods for early detection. Most patients present with advanced disease without any effective systemic therapies. Future advances in targeted therapies must be based on better selection of patients and on the ability to establish molecular signatures from representative tissues. However, progress in tissue-based research is challenged by the paucity of material obtained during diagnostic procedures. The majority of patients with PC are diagnosed by an image-guided fine-needle aspiration (FNA) of the primary or metastatic tumour site (Chen *et al*, 2007; Touchefeu *et al*, 2009). Szafranska *et al* (2008b) reported that miRNA analysis from fresh FNA biopsy samples differentiated malignant from benign PC tissues. Another similar study on FNA samples identified markers in PDAC patients diagnosed with non-resectable tumours (Bournet *et al*, 2012). Despite evidence for miRNAs in tumour tissue obtained at surgery from patients with PC there is little knowledge on miRNA profiling from formalin-fixed paraffin-embedded (FFPE) cell blocks of FNAs material.

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It is well recognised that the development of cancer involves alterations in the expression of multiple genes regulated by transcriptional, post-transcriptional, translational, and post-translational modifications. Therefore, a single gene or protein expression may not reliably predict the biological behaviour of the disease. Nevertheless, traditional approaches to developing biomarkers continue to be based on single-gene assays. MicroRNA expression assays expand the scope to study tumour biology by interrogating multiple target perturbations that are related to key target molecule(s). This may be a better strategy to study PC because of the multiplicity of gene mutations and pathway deregulations that underlie its aggressiveness and resistance to therapy (Sarkar *et al*, 2010; Wang *et al*, 2010). MicroRNAs by virtue of their molecular interactions influence gene and gene products that are amenable to drug effects. Understanding the miRNAs that are involved in gene regulation and others that are 'effectors' in the downstream signalling will provide an opportunity to interrogate these miRNAs to develop biomarkers for the gene in question, and will also provide a means to target the biological effects of the gene(s) of interest and certain undruggable molecules. Establishing signatures that are based on a constellation of upregulated (oncogenic) and downregulated (tumour suppressor) miRNAs that are unique to each patient will risk stratify newly diagnosed patients and provide invaluable tools for personalised therapy.

Experimental evidence supports an increasing role for miRNAs in the molecular biology of PC. Underexpression of miR-200 and let-7 families is associated with shorter survival (Ali *et al*, 2010a; Bao *et al*, 2011a). We and others have shown that miR-200 is a marker of chemo-resistance and that its re-expression or reactivation leads to increased expression of E-cadherin and reduced expression of ZEB1/2 and vimentin that would reverse drug resistance (Hurteau *et al*, 2009; Bao *et al*, 2011b). By its regulation of epithelial to mesenchymal transition (EMT) miR-200 also promotes invasion and metastases (Li *et al*, 2009; Yu *et al*, 2010). Other studies have shown that downregulation of let-7 expression in PC that was inversely associated with worsening tumour and patient survival (Li *et al*, 2009; Torrisani *et al*, 2009; Ali *et al*, 2010b). Interestingly, the frequently mutated oncogene *RAS* in PC is known to be translationally downregulated by the let-7 family (Takamizawa *et al*, 2004; Ali *et al*, 2010b).

The goal of this study was to establish the feasibility of miRNA expression profiling of localised PC using material obtained from diagnostic FNA. In this study, we evaluated the expression profiles of miRNAs in FFPE cell blocks from 29 patients with pancreatic adenocarcinoma and from 15 non-malignant pancreatic tissues. We further validated the expression of selected miRNAs by quantitative real-time-PCR (qRT–PCR) in individual samples to provide quantitative analysis of miRNA expression in real time.

MATERIALS AND METHODS

Tissue collection

Diagnostic FNAs from patients who underwent computerised tomography or endoscopic ultrasound-guided biopsy of a suspicious mass using 20–23 gauge needle were studied (Supplementary Table 1). Two to five needle passes from primary tumour were undertaken per patient with a mean of 3. Diagnostic smears were evaluated using Deff-Quick staining (Mercedes Medical, Sarasota, FL, USA). The remaining needle aspirates were placed in fixative fluid from which pellets were obtained after centrifugation. Cellular pellets were fixed using formalin and embedded in paraffin using standard protocol. Hematoxylin and eosin staining was used for histological confirmation of cancer, to determine the cellularity of representative sections, and to confirm the presence of tumour cells together with lymphocytes and macrophage without any evidence of desmoplastic cells. Minimums of 50 cells were considered necessary to obtain a satisfactory quantity of RNA to perform qRT–PCR, which represented $\sim$80% of the patients. Moreover, this number of cells would also allow for a reliable distinction between benign and malignant histology. The lack of sufficient amount of RNA from the other 20% of the patients could have been due to the lack of sufficient cells but less likelier due to RNA degradation because pre-miRNA template is typically $<$150 nucleotide. We collected FFPE morphologically normal appearing pancreas tissue from 15 patients that were anatomically farther away from the pancreatic tumour to serve as the controls. The institutional human investigation Review Board approved the study. All analyses were performed without knowing the origin of samples.

RNA isolation

Total RNA was isolated from FFPE tissue using the RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol with some modifications. Four freshly cut tissue sections of 10 μm thick and $\sim$1 cm in diameter were placed in micro tubes along with 1 ml xylene. After vigorous shaking for 10 s, samples were centrifuged for 2 min at room temperature. The supernatant was removed, 1 ml of ethanol was added, and centrifuged again for 2 min. The resultant pellet was resuspended in 240 μl of buffer PKD along with 10 μl of proteinase K and incubated at 55 °C for 15 min, and then at 80 °C for 15 min. Approximately 500 μl of buffer RBC was added and transferred to a DNA column, centrifuged, and the flow-through was collected. Ethanol of 1200 μl was added to the aliquot that was subsequently applied directly to the RNeasy column. RNA was washed with buffer solution to remove impurities and eluted in a final volume of 15 μl. RNA was quantified and its purity evaluated by the absorption ratio at 260/280 nm using NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA, USA). The ratio of 260/280 varied from 1.8–2.1. Samples with values less or more were considered to be not usable.

MicroRNA profiling

Purified RNA pooled separately from normal and patient samples were analysed by LC Sciences for miRNA microarray profiling (LC Sciences Houston, TX, USA) as an initial step. The miRNA profiling was performed by miRBase version 16 (LC Sciences). Data were normalised using selected housekeeping genes. Network analysis was performed with the web-based bioinformatics tool Ingenuity pathway analysis software (Ingenuity Systems, Redwood City, CA, USA) functional network analysis.

Real-time reverse transcriptase-PCR (qRT–PCR)

Real time qRT–PCR was conducted on all samples to validate the miRNA profiling results using SYBR Green miRNA-based assay. The Universal cDNA Synthesis Kit (Exiqon, Woburn, MA, USA) was used per manufacturer's protocol. Approximately 10 ng of RNA from tissue was reverse transcribed using 5 μl of master mix containing 5 $\times$ reaction buffer and enzyme mix. The mixture was incubated at 42 °C for 60 min, followed by 95 °C for 5 min. Reverse transcriptase (RT)–PCR reactions were then carried out in a total volume of 10 μl reaction mixture containing 4 μl of RT product mixed with 1.0 μl PCR primer mix, and 5 μl of SYBR Green master mix. All reactions, including controls were performed in triplicate using StepOnePlus Real-Time PCR (Applied Biosystems, Foster City, CA, USA). Relative expression of miRNAs was analysed using the C_t method and was normalised by RNU48 expression.

RESULTS

Expression profiling of miRNAs

RNA from archival FNA cell blocks of PC and control samples ($\sim$5 μl each) were pooled separately. Expression profiling revealed 228 miRNAs that were differentially expressed in subjects with or without PC: 103 were upregulated and 125 were downregulated. The top 10 up and downregulated miRNAs are shown in Table 1. The up or downregulated miRNAs included miR-486-5p, miR-451, miR-423-5p, let-7c, d, f, and miR-200c.

Pathway analysis of expressed miRNAs

Ingenuity modelling of the miRNA profiling was undertaken to better understand the pathways involved and target genes as shown in Figures 1 and 2. Networks involving selected miRNAs were then algorithmically generated based on their connectivity. This analysis showed that many of the miRNAs were regulated through each other either directly or indirectly, and were also regulated by several target genes. We observed upregulation of miR-451, miR-122, miR-150, and downregulation of the let-7 family, miR-200c, and miR-146a, which is a target of E2F1, E2F3, and LIN28A. This was in agreement with our previous observations showing downregulation of the let-7 family and miR-146a in plasma samples from patients with PC (Ali *et al*, 2010b). We also observed that the profiles obtained were within what was seen in 15 bio functional network groups relating to cancer, genetic

disorder, and gastrointestinal disease (Figure 2B). Of the seven miRNAs chosen for further analysis, three were oncomirs (miR-486-5p, miR-423-5p, and miR-451) and four were tumour-suppressor miRNAs (miR-200c, let-7c, let-7d, and let-7f).

Table 1 The top 10 up and downregulated miRNAs by miRNA profiling

	Normal (N)		Tumour (T)			
Reporter name	**Mean**	**s.d.**	**Mean**	**s.d.**	**Log2 (T/N)**	***P*-value**
Top 10 downregulated miRNAs						
hsa-miR-4286	1248	132	36	10	−5.11	2.64E−03
hsa-let-7f	716	90	31	3	−4.54	5.29E−05
hsa-miR-720	13484	514	863	8	−3.97	6.89E−05
hsa-let-7d	815	140	63	7	−3.70	2.29E−04
hsa-miR-1280	7230	1155	575	49	−3.65	1.74E−04
hsa-miR200c	987	146	87	14	−3.51	3.27E−04
hsa-miR-26a	886	18	85	29	−3.38	6.14E−03
hsa-let-7c	982	26	233	46	−2.07	7.09E−03
hsa-miR-146a	24	41	8	4	−1.58	5.42E−01
hsa-let-7b	1212	172	426	39	−1.51	1.91E−03
Top 10 upregulated miRNAs						
hsa-miR-486-5p	11	1	1306	65	6.87	2.65E−04
hsa-miR-451	107	17	5452	344	5.67	6.07E−04
hsa-miR-92a	96	18	1024	27	3.41	1.98E−03
hsa-miR-423-5p	30	2	271	16	3.17	2.40E−05
hsa-miR-124	6	1	57	10	3.15	5.22E−04
hsa-miR-3687	150	15	1251	67	3.06	6.35E−05
hsa-miR-1246	288	30	1847	77	2.68	1.22E−03
hsa-miR-1275	395	60	2264	516	2.52	1.84E−03
hsa-miR-17	15	2	37	8	1.32	7.85E−03
hsa-miR-320a	142	23	329	31	1.21	4.07E−03

qRT–PCR of selected miRNAs

We further analysed the miRNA expression level of seven miRNAs based on the miRNA profiling described above and validated their expression in individual samples using qRT–PCR. Analyses were blinded to source of the samples and performed in parallel to avoid batch effects. The expression level in controls was set at 1.0. The reproducibility of the qRT–PCR assay showed that miRNAs can be efficiently extracted from FNA cell blocks and could be compared across multiple samples. The miRNA expression analysis of four functional tumour-suppressor miRNAs, miR-200c (Figure 3), let-7c, let-7d, and let-7f (Figure 4), showed significant reduction in tumours compared with controls. Moreover, analyses clearly indicated that the expression levels of miR-200c, let-7c, and let-7f were significantly downregulated in almost all the PC patients compared with controls. Conversely, the expression of let-7d was differentially expressed between the samples compared with normal controls (Figure 4). Compared with miRNA levels from controls, the expression levels of oncogenic miR-486-5p and miR-451 were significantly upregulated, and in more than half of the PC specimens the level was increased to >20-fold (Figure 5). On the other hand, the expression levels of miR-423-5p were varied between the samples compared with normal controls (Figure 5), which suggest that some miRNAs can function differently between patients.

Comparative expression of seven miRNAs tested in FNA samples

The expression levels of all seven miRNAs are presented as a box plot in Figure 6. The analysis of the individual samples for miRNA expression level by qRT–PCR revealed that three of the four tumour-suppressor miRNAs were significantly downregulated

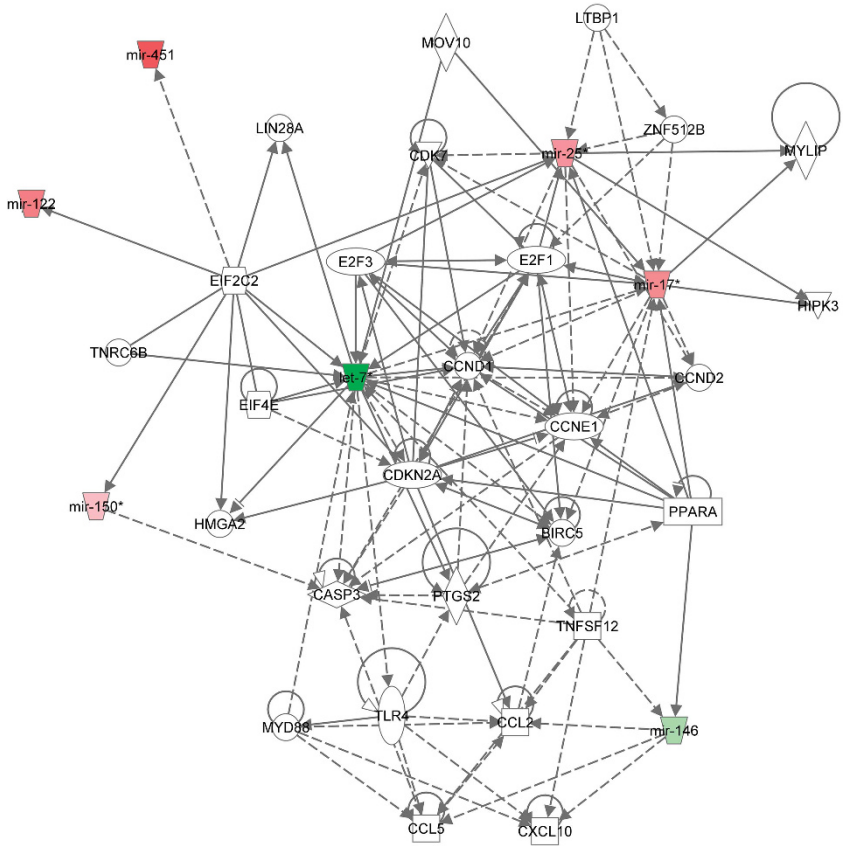

Figure 1 Ingenuity network analysis showing up (red) and downregulation (green) of miRNAs analysed by miRNA profiling in PC and their targeted genes.

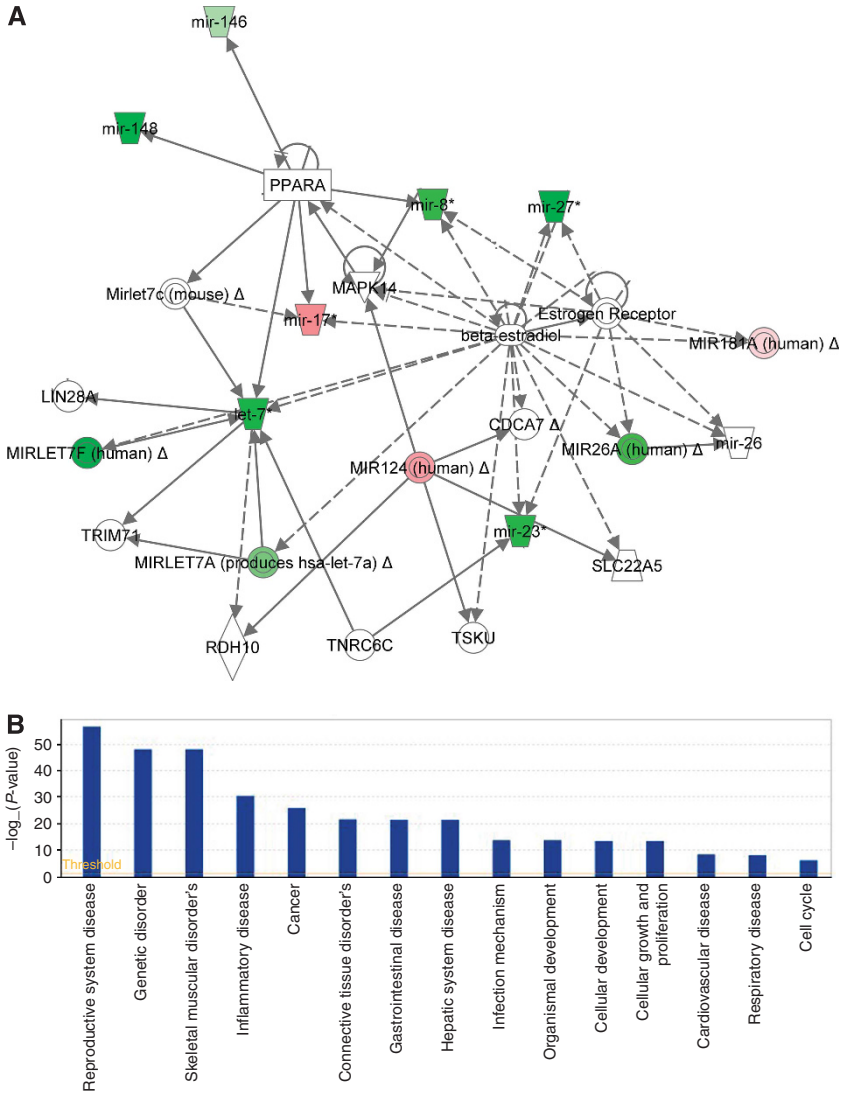

Figure 2 Ingenuity network analysis showing up (red) and downregulation (green) of miRNAs involved in PC and their target genes (**A**). The solid lines connecting genes represent a direct relation and dotted lines indirect relation. We also observed 15 bio functional network groups that included cancer, genetic disorder, and gastrointestinal disease (**B**).

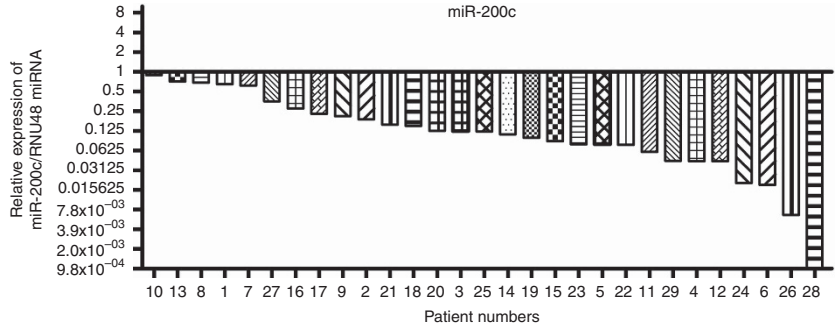

Figure 3 Comparative expression analysis of miR-200c in 29 FNA cell blocks from PC patients analysed individually compared with FNA cell blocks obtained from 15 normal controls by using qRT–PCR. The graph is presented in log2 values and 1.0 represents average of normal subjects ($n = 15$). There was a significant downregulation of miR-200c in all 29 PC patients compared with normal subjects.

compared with controls. Similarly two of the three oncogenic miRNAs were significantly upregulated relative to controls. However, tumour-suppressor miRNA let-7d and oncogenic miRNA miR-423-5p showed differential expression in patients with PC.

DISCUSSION

The understanding of biological functions of miRNAs in patients with PC will significantly help the development of new targeted anticancer drugs and testing of novel therapies. Most investigators

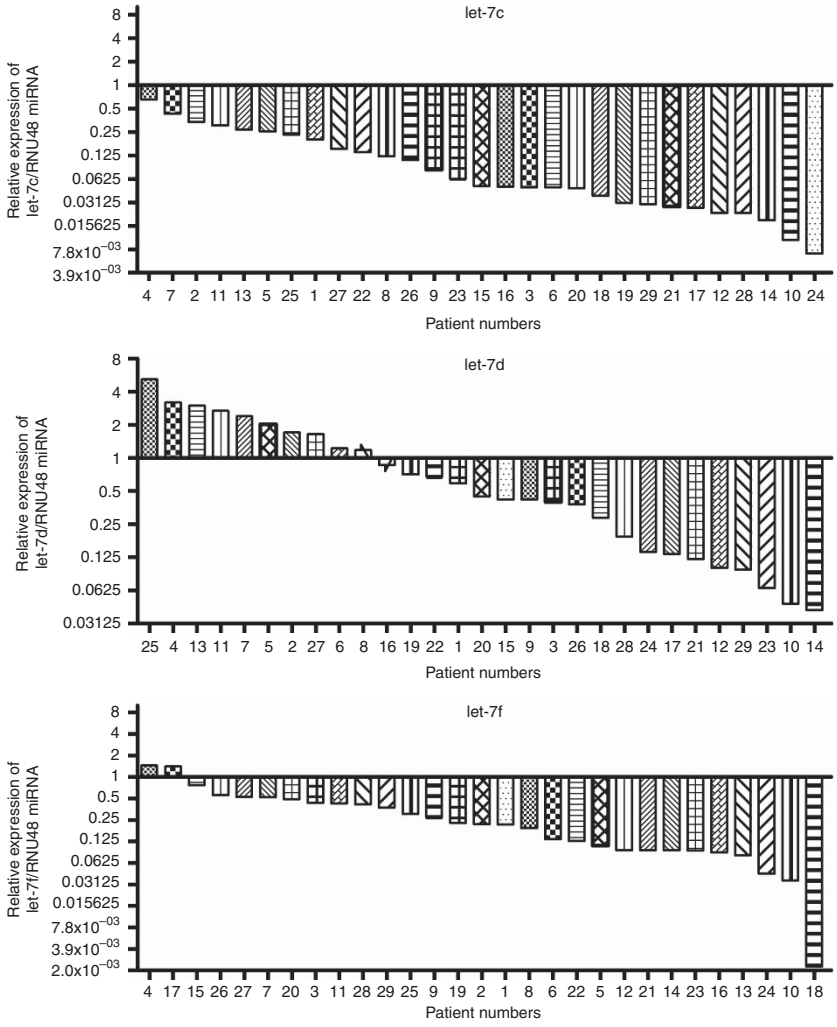

Figure 4 Comparative expression analysis of let-7c, let-7d, and let-7f in 29 FNA cell blocks from PC patients analysed individually compared with FNA cell blocks obtained from 15 normal controls by using qRT–PCR. The graph is presented in log2 values and 1.0 represents average of normal subjects ($n = 15$). The results showed a significant decrease in the expression of let-7c, and let-7f in almost all PC patients compared with normal subjects. In contrast, the expression level of let-7d showed a differential expression between samples in PC patients.

agree that miRNA expression profiling is an accurate method for the analysis of archived surgical specimens from tumours including PC (Li *et al*, 2007; Doleshal *et al*, 2008; Szafranska *et al*, 2008a; Goswami *et al*, 2010). We previously identified miRNA expression from plasma samples that were characteristic of PCs (Ali *et al*, 2010b). The primary objective of this study was to determine the feasibility of performing miRNA arrays and qRT–PCR from FFPE cell blocks obtained from diagnostic FNAs.

To the best of our knowledge this is the first study to determine miRNA expression from FNAs of FFPE cell blocks in patients with PC. We found 228 miRNAs that were differentially expressed in the PC patients compared with normal controls. Consistent with published literature using surgical specimens, we found higher expression of miR-451 and miR-486-5p, and lower expression of let-7c, let-7d, let-7f, and miR-200c in the FNA samples of PC patients. Further analyses revealed marked downregulation of the let-7 family that is well known for its tumour suppressive characteristics and consistent with previous independent studies showing that let-7 is underexpressed in PC (Li *et al*, 2009; Torrisani *et al*, 2009; Watanabe *et al*, 2009; Oh *et al*, 2010; Ngi-Garimella *et al*, 2011). We confirmed the expression of let-7c, let-7d, and let-7f in PC individually and compared with controls by qRT–PCR. We found that the expression of let-7c and let-7f was significantly reduced in all PC specimens, whereas the expression of let-7d was differentially expressed between these samples.

Emerging evidence indicates that EMT has a crucial role in cancer progression and drug resistance and is associated with loss of miR-200c expression (Burk *et al*, 2008; Li *et al*, 2009; Wellner *et al*, 2009; Yu *et al*, 2010). A recent study from our group showed that re-expression of miR-200 suppressed pulmonary metastases of breast cancer cells *in vivo*, whereas anti-miR-200 treatment *in vivo* resulted in increased metastases (Ahmad *et al*, 2011). Here, we report that the expression of miR-200c is significantly reduced in all PC patients tested compared with controls. In addition to downregulated miRNAs in PC, our results also demonstrated a significant upregulation of miR-486-5p, miR-451, and miR-423-5p. These findings are in concordance with the findings of another recent study in gastric cancer where a significant increase in serum miR-423-5p was demonstrated by Solexa sequencing. Furthermore, miR-423-5p expression level showed a substantial increase in patients with metastatic disease compared with those with stages I or II (Liu *et al*, 2011).

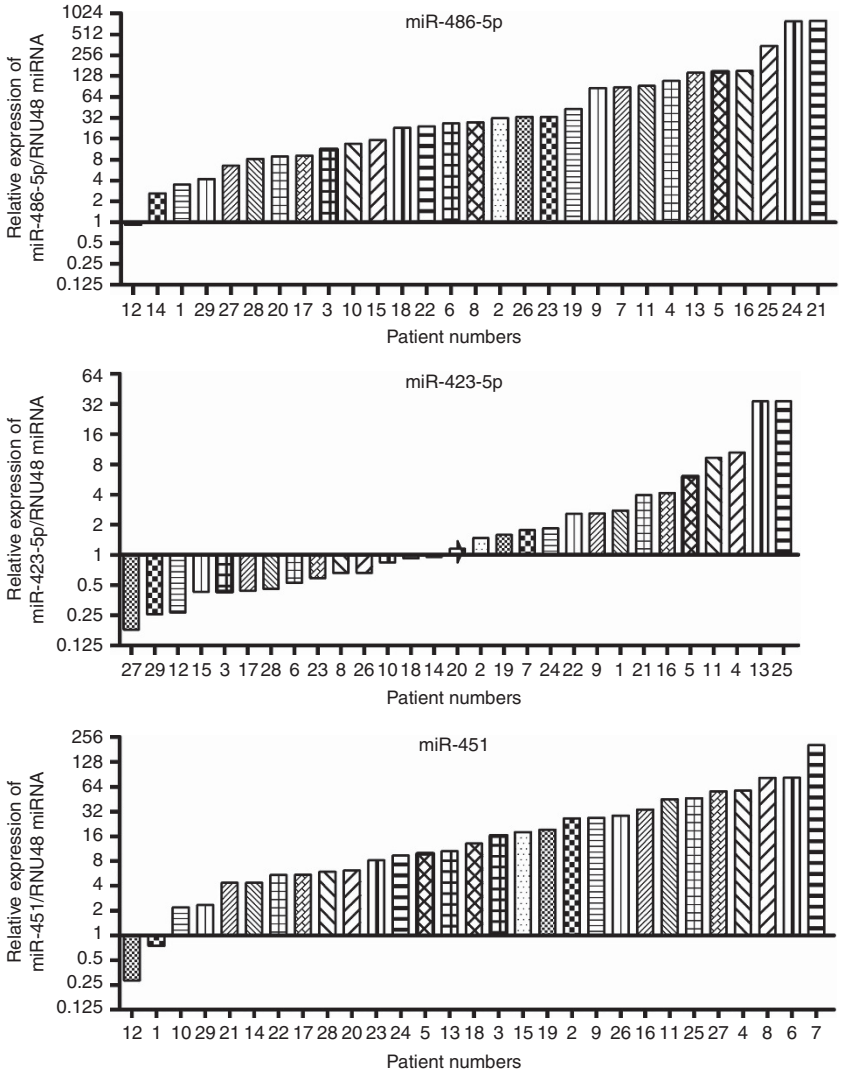

Figure 5 Comparative expression analysis of miR-486-5p, miR-423-5p, and miR-451 in 29 FNA cell blocks from PC patients analysed individually compared with FNA cell blocks obtained from 15 normal controls by using qRT–PCR. The graph is presented in log2 values and 1.0 represents average of normal subjects ($n = 15$). The results showed a significant increase in the expression of miR-486-5p, and miR-451 in almost all PC patients compared with normal subjects. In contrast, the expression level of miR-423-5p showed a differential expression between samples in PC patients.

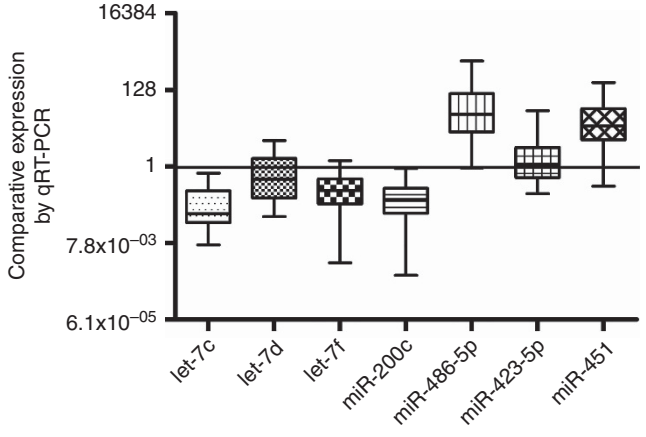

Figure 6 Box plot representing the expression of 7 miRNAs as assessed by qRT–PCR in 29 FNA cell blocks from PC patients analysed individually compared with FNA cell blocks obtained from 15 normal controls by using qRT–PCR. The graph is presented in log2 values and 1.0 represents average of normal subjects ($n = 15$).

It is obvious that with a molecularly very complex disease such as pancreatic adenocarcinoma a single gene product or pathway is unlikely neither to be a robust target for therapy nor to predict biology of disease. A systems biology approach provides a unique strategy to study networks of molecular events that drive various signalling pathways resulting from the deregulations of miRNAs (Azmi *et al*, 2011). The miRNAs and their targets are arranged in complex regulatory networks (Figures 1 and 2), but as the functional roles of miRNAs in PC are better defined, it will improve the sub-classification of PC with respect to prognosis and patterns of response to specific therapies.

In conclusion, this study demonstrates the feasibility of undertaking analyses of miRNAs in very small diagnostic specimens from FNAs of PC that may provide an invaluable research tool to individualise therapy and develop rationally based targeted therapies. Moreover, a unique group of miRNAs were identified that can serve as a tool for investigating the biology of PC with respect to prognosis and response to therapy at the individual level. Prospective clinical trials will be needed to better understand the functions of these miRNAs. The role of miRNAs may also be extended to *in vivo* monitoring of targeted therapies.

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