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ORIGINAL ARTICLE

Novel blood-based microRNA biomarker panel for early diagnosis of pancreatic cancer

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Abstract

AIM: To develop a panel of blood-based diagnostic biomarkers consisting of circulating microRNAs for the detection of pancreatic cancer at an early stage.

METHODS: Blood-based circulating microRNAs were profiled by high throughput screening using microarray analysis, comparing differential expression between early stage pancreatic cancer patients (n = 8) and healthy controls (n = 11). A panel of candidate microRNAs was generated based on the microarray signature profiling, including unsupervised clustering and statistical analysis of differential expression levels, and findings from the published literature. The selected candidate microRNAs were then confirmed using TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to further narrow down to a three-microRNA diagnostic panel. The three-microRNA diagnostic panel was validated with independent experimental procedures and instrumentation of RT-qPCR at an independent venue with a new cohort of cancer patients (n = 11), healthy controls (n = 11), and a group of high risk controls (n = 11). Receiver operating characteristic curve analysis was performed to assess the diagnostic capability of the three-microRNA panel.

RESULTS: In the initial high throughput screening, 1220 known human microRNAs were screened for differential expression in pancreatic cancer patients versus controls. A subset of 42 microRNAs was then generated based on this data analysis and current published literature. Eight microRNAs were selected from the list of 42 targets for confirmation study, and three-microRNAs, miR-642b, miR-885-5p, and miR-22, were confirmed to show consistent expression between microarray and RT-qPCR. These three microRNAs were then validated and evaluated as a diagnostic panel with a new cohort of patients and controls and found to yield high sensitivity (91%) and specificity (91%) with an area under the curve of 0.97 (P < 0.001). Compared to the CA19-9 marker at 73%, the three-microRNA panel has higher sensitivity although CA19-9 has higher specificity of 100%.

CONCLUSION: The identified panel of three microRNA biomarkers can potentially be used as a diagnostic tool for early stage pancreatic cancer.

Key words: MicroRNA; Diagnosis; Biomarkers; Pancreatic cancer; Blood plasma; Circulating

Core tip: This study employed high throughput screening as a screening tool to identify blood-based circulating microRNA markers for detection of early stage pancreatic cancer. Two levels of confirmation were performed to ensure the validity of the identified microRNA targets. First, a panel of potential microRNA

markers was generated and confirmed using a more specific and sensitive secondary assay, real-time quantitative reverse transcription polymerase chain reaction. Second, the confirmed panel of microRNA markers was independently validated with different experimental procedures and instruments, by independent personnel, and at a different institution, to diagnose a new cohort of patients and controls.

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem[1]. It has a five-year survival rate of only 6% and is estimated to have 43920 new cases and cause 37390 deaths in 2012 in the United States, a number that has been steadily increasing for more than a decade[2,3]. Conventional treatment approaches such as surgery, radiation, chemotherapy, or a combination thereof have had little impact on the course of this aggressive cancer. Collective studies from Japan indicate that those patients who were incidentally found through other imaging modalities to have early stage carcinoma have an improved five-year survival rate of 30% for those with a 2 cm carcinoma, 57% for those with a 1 cm or less "minute" carcinoma, and 100% for patients with a ductal epithelium tumor measuring less than 1 cm[4-6]. Therefore, these studies emphasize that the hope for better control of this disease is through diagnosis at its earlier stages when surgical resection may be curative.

The current most widely used biomarkers for pancreatic cancer are CA19-9, and, to a lesser degree, carcinoembryonic antigen (CEA)[7] and genetic markers such as *K-RAS* and p53[8]. Whereas clinicians may rely on CA19-9 levels as a prognostic tool when managing patients with late stage disease, or in determining operability or monitoring patients for recurrence, these markers have generally inadequate specificity and unreliable sensitivity to pancreatic cancer and are not recommended for screening and diagnosis of early disease[9,10]. Consequently, there is an urgent, unmet need for development of valid, reliable biomarkers for early detection and monitoring of pancreatic cancer.

MicroRNA (miRNA) are small non-coding RNA about 18-24 nucleotides in size[11]. A large body of evidence indicates that miRNAs regulate gene expression at the post-translational level in almost every biological event and play important roles in tumorigenesis, cancer development, migration and metastasis[12]. The differential expression of miRNAs has been related to various cancers, and efforts have been made to profile the global miRNA expression patterns associated with these cancers[13]. Numerous investigations have evaluated the miRNA signature of pancreatic cancer, utilizing pancreatic tumor tissue and cell lines, searching for biomarkers and their association with tumorigenesis and patient survival[14-20]. However, tissue-based miRNA signature profiling is limited to the availability of tissue specimens. Therefore, it would be technologically challenging to detect cancer at its earlier stages when the tumor size is still small and proper tissue procurement may be difficult. Thus, a simple, noninvasive procedure, such as blood-based signature profiling, would be ideal for detecting pancreatic cancer at earlier stages.

Recent studies have shown that miRNAs are relatively stable and can be readily extracted and detected in bodily fluids such as blood plasma[21]. Therefore, the presence of circulating extracellular miRNAs can potentially be used as markers for cancer detection in a noninvasive way[22]. Studies investigating the potential role of circulating miRNAs as pancreatic cancer markers have shown some promise although the findings have been limited to a small number of predefined target miRNAs[23-25]. High throughput screening studies of known circulating miRNAs as biomarkers of pancreatic cancer have only recently begun to emerge, and by combining the efforts from all researchers in this area, a final panel of ideal markers can be developed to combat this dreadful disease[26-28].

This is a pilot study to employ high throughput screening microarray technology to screen for 1220 known human miRNAs (based on the miRBase version 16.0 database released in 2010)[29], comparing the differential expression signature among eight early stage pancreatic cancer patients and eleven healthy controls. The identified panel of miRNAs were subsequently confirmed, and also validated for their diagnostic potential in an independent cohort of eleven early stage pancreatic cancer patients.

MATERIALS AND METHODS

Participant population

This study was reviewed and approved by the Valley Hospital Institutional Review Board. Written informed consent was obtained from all study participants. The pancreatic cancer patient group included stage ⅡA/ⅡB patients whose stage was confirmed post-operatively by pathologists from the Valley Hospital. Eight patients diagnosed with ductal adenocarcinoma were used in the microarray screening and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) confirmation study. A second group of eleven ductal adenocarcinoma patients was used in the validation study. The control group for all studies was comprised of eleven healthy participants with no family history of pancreatic cancer. The high risk group included eleven healthy participants who had a strong family history of pancreatic cancer, including ten participants with at least two "first degree relatives" and one participant with two "second

Table 1 Participant demographics

	n	Median age (IQR), yr	Female gender
Microarray analysis and RT-qPCR confirmation			
Patients	8	64 (57-65)	38%
Controls	11	46 (42-49)	46%
RT-qPCR validation			
Patients	11	68 (62-79)	46%
Controls	11	46 (42-49)	46%
High risk	11	48 (46-50)	73%

IQR: Interquartile range. RT-qPCR: Real-time quantitative reverse transcription polymerase chain reaction.

degree relatives" having a pancreatic cancer diagnosis. The participant demographics are shown in Table 1.

Patients were excluded from the study if they had prior pancreatic cancer surgery, had other concomitant cancers other than non-melanoma basal cell skin cancer, or had a history of HIV infection.

Blood specimen collection and processing

Patient blood was drawn by peripheral venipuncture into BD Vacutainer® CPT™ (Cell Preparation Tubes) with Sodium Citrate (Becton Dickinson, Franklin Lakes, NJ). Blood processing was typically done within two hours of collection and performed according to the manufacturer's protocol. Harvested plasma was stored at -80 ℃.

MiRNA microarrays and analysis (performed at Ocean Ridge Biosciences, FL)

Plasma samples were processed at Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL) for analysis using custom multi-species microarrays containing 1209 probes covering 1220 human mature miRNAs present in the miRBase version 16.0 database released in 2010. The sensitivity of the microarray is such that it could detect as low as 20 amoles of synthetic miRNA being hybridized along with each sample. The microarrays were produced by Microarrays Inc. (Huntsville, Alabama), and consisted of epoxide glass substrates that have been spotted in triplicate with each probe.

Sample processing: Samples were isolated from 0.7 to 1.0 mL of plasma using TRI Reagent® BD (Molecular Research Center, Cincinnati, OH) as per manufacturer instructions. For quality control, a mixture of 10 synthetic miRNAs were added (spike-in) at a mass of 12.5 fmoles/mL of plasma to each plasma sample during isolation and one miRNA was added at 200 amoles per sample prior to labeling and hybridization. Total RNA was 3'-end labeled with Oyster-550 fluorescent dye using the Flash Tag RNA labeling Kit (Genisphere, Hatfield, PA). Labeled RNA samples were hybridized to the miRNA microarrays according to conditions recommended in the Flash Tag RNA labeling Kit manual. The microarrays were scanned on an Axon Genepix 4000B scanner, and data was extracted from images using GenePix v4.1 software.

Data pre-processing: Spot intensities were obtained for the 8816 features on each microarray by subtracting the median local background from the median local foreground for each spot. The 95th percentile of the negative control spots was also calculated for each array. The spot intensities and 95th percentile of negative controls (TPT95) were transformed by taking the Logarithm base 2 (indicated as $\log_2$) of each value. The normalization factor (N) for each microarray was determined by obtaining the 20% trimmed mean of the human probe intensities that were detected one $\log_2$ unit above TPT95 (TPT95 + 1) in all samples and with standard deviation of probe intensities among all samples less than 1.25. The $\log_2$-transformed spot intensities for all 8816 features were normalized, by subtracting N from each spot intensity, and scaled by adding the grand mean of N across all microarrays. The mean probe intensities for each of the 1209 human probes on each of the 20 arrays were then determined by averaging the triplicate spot intensities. Spots flagged as poor quality during data extraction were omitted prior to averaging. The 1209 human non-control $\log_2$-transformed, normalized, and averaged probe intensities were filtered to obtain a panel of 290 human miRNA probes showing probe intensity greater than one $\log_2$ unit above TPT95 (> TPT95 + 1) in at least 10% of the samples.

Quality control: Sensitivity of the microarray hybridization was confirmed by detection of hybridization signal for all 11 spikes that were added during isolation and labeling well above TPT95. The array also contains a set of specificity control probes complementary to three different miRNAs. Each specificity control includes a perfect match, single mismatch, double mismatch, and shuffled version of the probe. Specificity of the hybridization was confirmed by detection of hybridization signal on the microarray for the perfect match probes and not the double mismatch and shuffled version of the probes. Reproducibility of the arrays was determined by monitoring the hybridization intensity for the triplicate human spots on each array.

Differential expression analysis: For statistical analysis, samples were binned in two groups: Healthy Controls and Pancreatic Cancer. The $\log_2$-transformed and normalized spot intensities for the 290 detectable human probes were examined for differences between the groups by 1-way ANOVA using National Institute of Ageing Array Analysis software[30]. This ANOVA was conducted using the Bayesian Error Model and 50 degrees of freedom. A total of 116 probes exhibited significant differences between the healthy controls and the pancreatic cancer patients. Statistical significance was determined using the False Discovery Rate (FDR) method[31]; an FDR < 0.15 was considered significant in this study.

Hierarchical clustering of miRNA array data: Data for the 290 detectable human probes were clustered using Cluster 3.0 software[32]. Genes were median-centered prior to hierarchical clustering. Hierarchical clustering

was conducted using Centered Correlation as the similarity metric and Average Linkage as the clustering method. Intensity scale shown is arbitrary.

Confirmation with quantitative real-time RT-PCR (RT-qPCR, performed at ORB)

Total RNA was extracted from 0.9 to 2.1 mL of plasma using TRI Reagent® BD (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions with minor modifications. For down-stream quality control monitoring, a mixture of 10 synthetic miRNAs were added at a mass of 12.5 femtomoles/mL of biofluid following homogenization of the samples in Trizol-BD.

RNA isolated from plasma was diluted and used in 10 μL reverse transcription (RT) reactions using ABI (Applied Biosystems by Life Technology) miRNA-specific RT primers (Life Tech, Carlsbad, CA). For each miRNA and sample, RNA equivalent to 50 μL of biofluid was used in each RT reaction except for the probe sets for miR-642b-3p and miR-7, in which case RNA equivalent to 100 μL was used. The cDNA product was diluted 1:10 in water and 4.5 μL of the diluted product was combined in triplicate PCR-plate wells with 1 × ABI Universal PCR amplification mix and ABI miRNA-specific Taqman PCR primers in a final volume of 10 μL/well. The PCR plate was subjected to thermal cycling in an ABI StepOne Plus real-time PCR instrument. The cycling conditions were an initial incubation for 10 min at 95 ℃, followed by 40 cycles of: 15 s at 95 ℃, 1 min at 60 ℃. The Cq (Quantification Cycle according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines[33], also known as threshold cycle (C_T), or number of cycles required for the well to reach a specified threshold of fluorescence intensity) was determined for each well using ABI software and a threshold setting of 0.05. In the initial pilot experiment, only miRNA with Cq value at 36 or above were used for confirmation study. Triplicate Cq values were averaged to obtain the "mean Cq value."

Independent validation RT-qPCR (performed at the Valley Hospital, NJ)

MiRNA was extracted from 2.0 to 4.0 mL of plasma using QIAamp circulating Nucleic Acid Kit (Qiagen, Germantown, MD) with QIAvac Connecting System (Qiagen), following manufacturer instructions. The concentration of extracted miRNA was determined using Agilent Small RNA kit with 2100 Bioanalyzer (Agilent, Santa Clara, CA), and was in the range of 10 to 100 ng.

RT-qPCR experiments were performed using TaqMan MiRNA Assays (Life Tech, Carlsbad, CA), for each miRNA, following manufacturer instructions. The TaqMan miRNA assays used were: hsa-miR-885-5p (ID#002296), hsa-miR-22-3p (ID#000398), hsa-miR-642b-3p (ID#462949_mat), and hsa-miR-3196 (ID#241941_mat). For RT, 1 to 10 ng RNA isolated from plasma was diluted and used in 5 μL of RT reactions with each miRNA-specific RT primers and MiRNA Reverse Transcription kit (Life Tech), without pre-amplification. The cDNA product was diluted 1:3 in water, and 5 μL of diluted cDNA was used in triplicate PCR-plate wells with miRNA-specific TaqMan PCR primer and Universal Master mix Ⅱ (Life Tech). The qPCR reaction was performed on 7500 real-time PCR system (Life Tech). The Cq value required for the well to reach a specific threshold of fluorescence intensity was determined for each well using 7500 Software (Life Tech) with threshold setting at 0.05. All Cq values were 36 or less and were included for subsequent calculation. Triplicate Cq values were averaged to obtain the "mean Cq value".

RT-qPCR and microarray data analysis using the comparative C(T) Method

The normalization control used for the RT-qPCR experiments was hsa-miR-3196. The endogenous control has been selected based on both microarray and pilot RT-qPCR experiments. MiR-3196 demonstrated relatively consistent and stable expression in microarray analysis and also in a series of pilot RT-qPCR. The use of the same extracted plasma miRNA (patients and controls, $n = 19$), with the same miRNA input, yielded consistent Cq values at 29.33 ± 0.50.

Fold change calculation was performed following the Comparative C(T) Method[34]. The "mean Cq value" of hsa-miR-3196 was subtracted from the mean Cq value for each miRNA to obtain a "normalized mean ΔCq value" for each miRNA. The "normalized mean ΔCq value" for each miRNA was then converted to $2^{-(\Delta Cq)}$. Fold change of each miRNA was determined by calculating the ratio of the mean of $2^{-(\Delta Cq)}$ of all pancreatic cancer patients to the mean of $2^{-(\Delta Cq)}$ of all healthy controls; and the mean of $2^{-(\Delta Cq)}$ of all high risk controls to the mean of $2^{-(\Delta Cq)}$ of all healthy controls. The fold change values were transformed to a $\log_2$ scale for the purpose of plotting the values on a continuum.

In order to further evaluate the RT-qPCR experimental results against those observed from the microarray analysis, microarray data for eight miRNAs (miR-642b-3p, miR-762, miR-4253, miR-885-5p, miR-18a, miR-486-5p, miR-7, and miR-22) were re-analyzed following the Comparative C(T) Method[34]. The spot intensities of all data points (in $\log_2$) were normalized by subtracting the values for each miRNA from miR-3196 (as normalization control) to generate "normalized $\log_2$ values", which were used as the equivalent of "normalized mean ΔCq value" in RT-qPCR for further calculation. The fold change values are then calculated as stated above for RT-qPCR data analysis.

CA19-9 test

The CA19-9 serum marker test was performed by the Valley Hospital Histology Laboratory following the routine diagnostic laboratory testing protocol using Tosoh A1A-360 Immunoanalyzer (Tosoh Bioscience, Inc.). CA19-9 range above 47 U/mL is considered to be a positive test result.

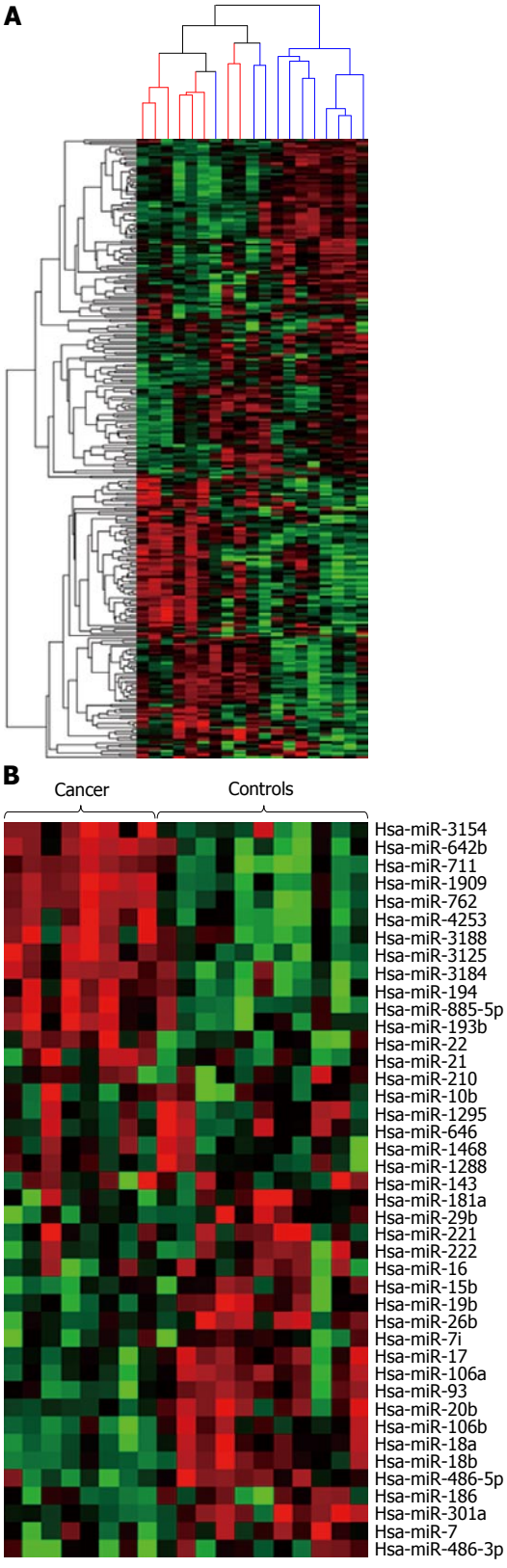

Figure 1 The microarray signature profile of circulating microRNA in pancreatic cancer patients and healthy controls. A: The unsupervised hierarchical clustering of 290 miRNAs that are differentially expressed among pancreatic cancer patients (n = 8) and healthy controls (n = 11). The dendrogram on top indicates the hierarchical clustering relationship between pancreatic cancer patients (in red) and healthy controls (in blue); B: Heat map depicting the subset of 42 miRNAs chosen for confirmation RT-qPCR study, arranged as indicated. Heat map color scale represents fold increase (red) or decrease (green) from baseline. MiRNA: MicroRNA.

Statistical analysis

The Wilcoxon-Mann-Whitney test was used to compare the fold changes observed among the patient groups for each miRNA. Receiver operating characteristic (ROC) curves were generated to evaluate the sensitivity and specificity of predicting cancer cases and control cases for each miRNA and for the combination of miRNAs. Area under the curve and their respective 95%CI were calculated for all ROC curves. P-values $\leqslant 0.05$ were considered to be statistically significant. All analyses were done using IBM-SPSS software (Version 19).

RESULTS

Circulating miRNA profiles revealed putative candidate miRNA markers

The miRNA expression signature was profiled using custom miRNA microarray chips covering 1220 human miRNAs derived from the miRBase database, version 16, released in 2010[29]. The unsupervised hierarchical clustering of the 290 miRNAs with acceptable detection intensities is shown in Figure 1A. Remarkably, as depicted in the hierarchical dendrogram, the clustering pattern clearly separates the pancreatic cancer from most healthy controls.

To compile a set of targeted miRNA markers for further investigation and confirmation, the list of 290 miRNAs was narrowed down to 31 miRNAs with significant differential expression and combined with an additional 22 miRNAs, which have been shown to be candidate biomarkers for pancreatic cancer by other investigators[14,17-20,25]. The latter criterion was added to ensure the inclusion of miRNAs which may not have exhibited significant differential expression in this experimental setting but have otherwise been shown by others to be good candidates. As shown in Table 2, among the final set of 42 miRNAs, 11 miRNAs exhibiting the most significantly different expression levels (FDR $\leqslant 0.15$) in this study (miR-194, miR-18a, miR-7, miR-26b, miR-301a, miR-106b, miR-16, miR-93, miR-106a, miR-19b, and let-7i) as well as in the previously published literature. The heat map of the 42 miRNAs in Figure 1B demonstrates a differential expression signature between pancreatic cancer patients and controls similar to the results observed from the unsupervised clustering in Figure 1A.

RT-qPCR confirmed three potential miRNA diagnostic markers

The panel of 42 miRNAs were subjected to further investigation using real time quantitative RT-PCR (RT-qPCR). In the initial pilot experiment using available miRNA TaqMan probes and testing the relative abundance of expression, 8 of the 42 miRNAs gave acceptable and most consistent signals (data not shown) and therefore were chosen for the subsequent confirmation study.

As shown in Figure 2, when comparing microarray and TaqMan RT-qPCR results side-by-side after normalizing both data sets with the same control, miR-3196,

Table 2 The 42 microRNAs for confirmation study

MiRNA	Fold change	FDR	Ref
Up-regulated in Pancreatic Cancer			
MiR-3184	4.5	0.005	
MiR-642b	3.3	0.028	
MiR-1909	3.0	0.030	
MiR-3154	3.5	0.064	
MiR-711	3.1	0.064	
MiR-3125	5.2	0.069	
MiR-4253	2.8	0.082	
MiR-762	2.4	0.082	
MiR-885-5p	3.0	0.103	
MiR-3188	2.4	0.154	
MiR-194	2.1	0.154	[19]
MiR-193b	2.4	0.155	
MiR-22	1.4	0.706	[19,20]
Up-regulated in Healthy Controls			
MiR-486-5p	3.0	0.064	
MiR-18b	2.9	0.064	
MiR-1288	2.6	0.064	
MiR-486-3p	2.3	0.082	
MiR-18a	2.7	0.082	[19,25]
MiR-7	2.5	0.091	[19]
MiR-26b	3.9	0.112	[19]
MiR-646	2.5	0.131	
MiR-1295	2.4	0.131	
MiR-301a	2.4	0.131	[18]
MiR-106b	2.3	0.151	[19]
Let-7i	4.0	0.154	[20]
MiR-16	3.7	0.154	[17,19]
MiR-20b	2.9	0.154	
MiR-93	2.6	0.154	[19]
MiR-106a	2.6	0.154	[17,19]
MiR-17	2.5	0.154	
MiR-19b	2.5	0.154	[19]
MiR-1468	2.2	0.154	
MiR-29b	2.1	0.171	[20]
MiR-15b	2.3	0.172	[19]
MiR-186	1.8	0.196	[19]
MiR-10b	1.9	0.250	[19]
MiR-143	2.3	0.323	[19,20]
MiR-181a	1.7	0.361	[14,19]
MiR-222	1.6	0.546	[14,17-19]
MiR-221	1.6	0.546	[14,18,19]
MiR-210	1.2	0.813	[17,19]
MiR-21	1.4	0.866	[14,18,19]

The 42 final target list based on the 290 miRNAs identified by miRNA microarray. The fold change shown is calculated as the ratio of pancreatic cancers over healthy controls (under "Up-regulated in Pancreatic Cancer") or healthy controls over pancreatic cancers (under "Up-regulated in Healthy Controls"). MiRNA: MicroRNA; FDR: False discovery rate.

the relative expression level of each miRNA varied due to the differing nature of the experimental technology. However, three of the eight miRNAs, miR-642b-3p, miR-885-5p, and miR-22-3p, demonstrate consistent outcomes across the two methodologies with higher expression seen in pancreatic cancer patients than in healthy controls. Therefore, they were chosen for further validation study as a potential diagnostic panel.

The validation study was performed on a new independent cohort of 11 pancreatic cancer patients, 11 healthy controls and 11 high risk controls. As shown in Figure 3A, the three miRNAs, plotted using the raw data

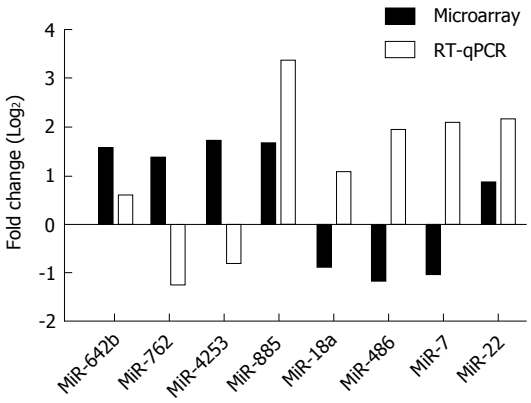

Figure 2 Confirmation study of the expression profile of eight microRNAs. Microarray (solid bar) and RT-qPCR (open bar) data sets were analyzed based on the "Comparative C(T) Method", normalized to miR-3196, and calculated as the ratio of mean fold change of pancreatic cancer patients (n = 8) to healthy controls (n = 11). MiRNA: MicroRNA.

of "normalized mean ΔCq value", differentiate pancreatic cancer patients from both the healthy and high risk controls. Likewise, as shown in Figure 3B, when determining the relative fold expression change by calculating the ratio of pancreatic cancer to healthy controls, expression of the three miRNAs is clearly up regulated in pancreatic cancer patients. For the high risk controls, on the other hand, the expression level is either comparable or lower when compared to the healthy controls. Furthermore, the relative expression levels of all three miRNAs are remarkably consistent in direction and magnitude between confirmation RT-qPCR (Figure 2) and validation RT-qPCR (Figure 3B), despite the use of different patient samples and independent experimental procedures. This lends support to the suggestion that they could play potential roles as diagnostic biomarkers for pancreatic cancer.

Assessing diagnostic potential of the three-miRNA panel

To assess the potential use of the three miRNA as a diagnostic panel for pancreatic cancer, ROC analysis was performed on the validation data set for each of the individual miRNAs and the combination of the three. As summarized in Table 3 and shown in Figure 4B and 4C, miR-885-5p and miR-22-3p each demonstrated high sensitivity of 82% for cancer case identification and relatively high specificity of 73% and 82%, respectively, for identifying healthy controls. For miR-642b-3p (Figure 4A), a high sensitivity of 82% for identifying cancer cases was demonstrated, but a lower specificity for identifying healthy controls of 55% was observed. When using the optimal cut-point, as shown in Figure 4D, the composite of all three miRNAs yielded both a sensitivity and specificity of 91%.

Given that the CA19-9 serum marker is the only marker currently available for routine diagnostic testing, we sought to compare CA19-9 results with the three-miRNA panel for predicting pancreatic cancer. The CA19-9 test has been performed on our cancer patient

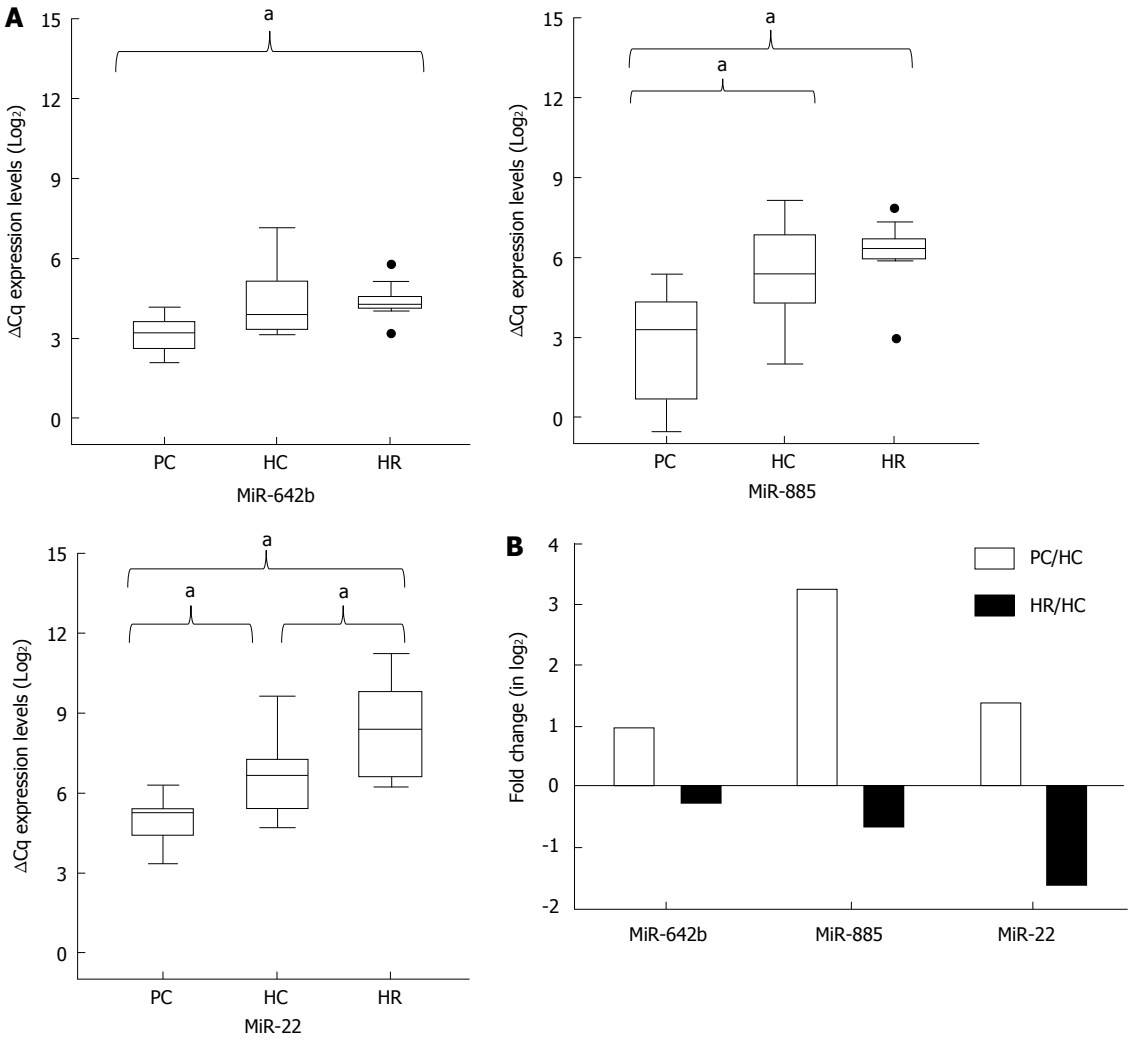

Figure 3 Validation of three-microRNA panel using quantitative real-time reverse transcription polymerase chain reaction. A: Box plot of relative expression of three miRNAs, based on normalized mean ΔCq values [mean quantification cycle (Cq) normalized to miR-3196] of pancreatic cancer patients (PC, *n* = 11), healthy controls (HC, *n* = 11), and high risk controls (HR, *n* = 11). The whiskers extend to the observations which are no more than 1.5 times the length of the box (interquartile range) away from the box. More extreme observations are considered outliers and are indicated as dots. "a" indicates *P* value ≤ 0.017 (0.05/3, a Bonferroni-adjusted α-level based on the 3 multiple comparisons performed among patient groups); B: Fold change expression levels of three miRNAs calculated as the ratio of mean fold change of PC/HC and HR/HC. MiRNA: MicroRNA.

Table 3 Receiver operating characteristic curve analysis of the 3 microRNAs validated by quantitative real-time reverse transcription polymerase chain reaction for diagnosing pancreatic cancer

MiRNA	Sensitivity	Specificity	AUC (95%CI)	*P* value
MiR-885-5p	82%	73%	0.84 (0.68-1.00)	0.006
MiR-22-3p	82%	82%	0.86 (0.70-1.00)	0.004
MiR-642b-3p	82%	55%	0.79 (0.59-0.98)	0.02
Composite of 3 miRNAs	91%	91%	0.97 (0.90-1.00)	< 0.001

AUC: Area under the curve; MiRNA: MicroRNA.

sample (*n* = 11) and non-cancer patient samples (healthy and high-risk controls, *n* = 22), and the sensitivity was observed to be 73% (8 out of 11 patients), and the specificity was observed to be 100% (all controls were below the reference point).

DISCUSSION

Early cancer detection remains a major challenge in pancreatic cancer but holds promise of resulting in a more favorable disease outcome. In light of the fact that current research progress into early detection of pancreatic cancer has resulted in limited actual clinical applications using various biomarkers such as tissue nucleic acid, proteins, tumor cells, and plasma proteins, we elected to focus on a relatively new source of potential biomarker, blood-based circulating miRNA. Given this test is blood-based, it would be noninvasive and ideal for diagnosing asymptomatic cancer. We performed an array-based high throughput screening process for all known human miRNA species (released by miRBase in 2010). We employed two levels of confirmation with RT-qPCR, using two independent samples of pancreatic cancer patients studied under two different sets of experimental conditions. We have

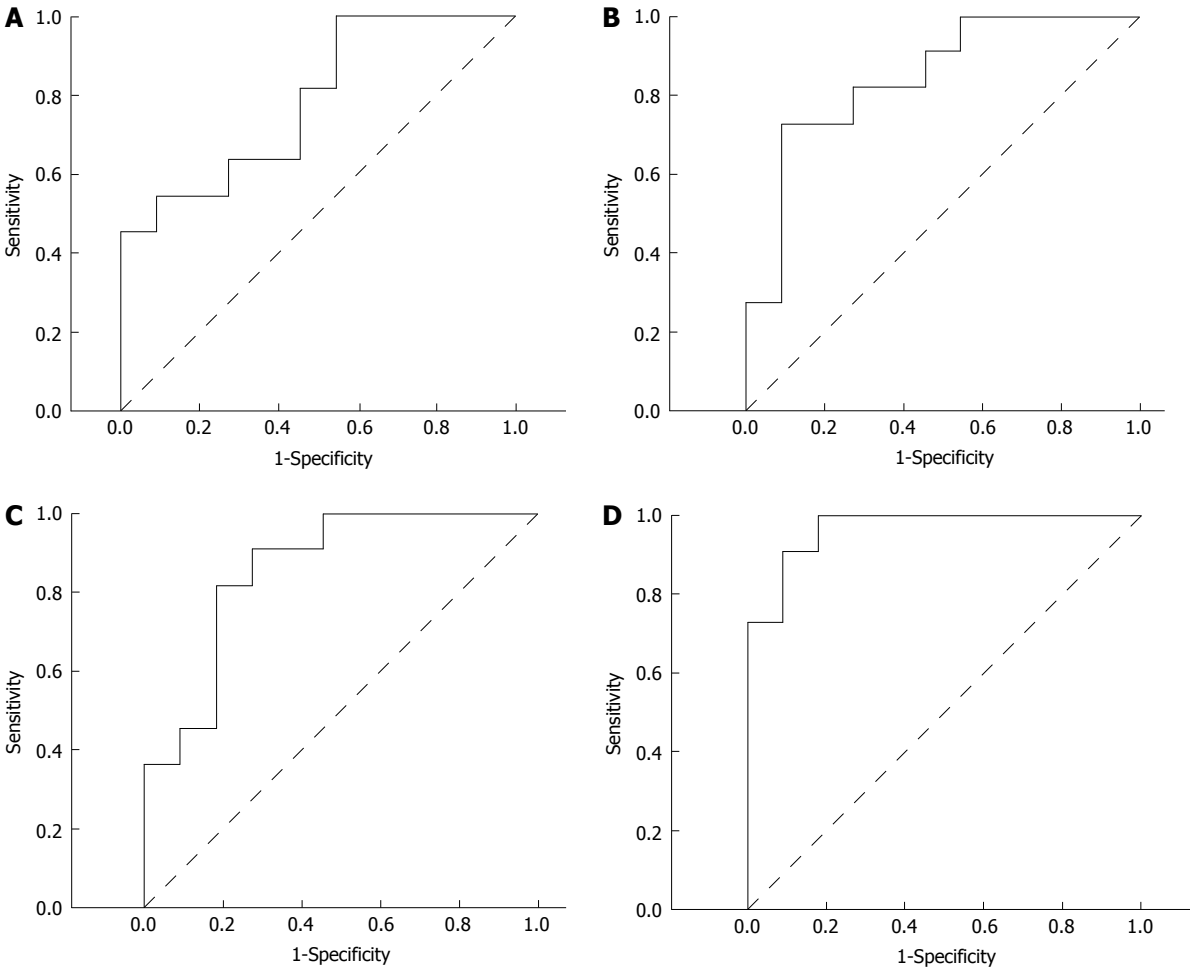

Figure 4 Receiver operating characteristic curve analysis of three microRNAs. Validation study with 11 stage ⅡA/ⅡB pancreatic cancer patients and 11 healthy controls. A: Receiver operating characteristic (ROC) curve for the miR-642b-3p data alone [area under the curve (AUC) = 0.79]; B: ROC curve for the miR-885-5p data alone (AUC = 0.84); C: ROC curve for the miR-22-3p data alone (AUC = 0.86); D: ROC curve for all three miRNAs (miR-642b-3p, -885-5p, 22-3p) as a composite panel (AUC = 0.97). MiRNA: MicroRNA.

identified and validated a panel of three miRNAs (miR-642b-3p, miR-885-5p and miR-22-3p) with high combined sensitivity of 91% and specificity of 91%.

Three prior studies have performed screening of circulating blood miRNAs for pancreatic cancer. Ali *et al*[28] profiled plasma miRNAs based on a pooled plasma specimen from 50 newly diagnosed pancreatic cancer patients (without specifying cancer staging). They identified miR-21 to be significantly higher and the expression of the let-7 family (especially let-7d) and miR-146a to be significantly lower in cancer. Liu *et al*[26] used Illumina Sequencing by Synthesis (SBS) technology and identified seven miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191) as a potential panel of biomarkers, with high sensitivity of 83.6%. However, surprisingly, the SBS technology employed did not identify any new, as yet unknown miRNA markers, considering that more mature human miRNA continue to be identified and updated yearly (a total of 2578 mature human miRNA are included in miRBase version 20 issued in 2013)[29]. Also, Liu *et al*[26] screened for pancreatic cancer patients from all stages (13.2% stage Ⅰ, 24.4% stage Ⅱ, 22.8% stage Ⅲ, 33.5% stage Ⅳ, and 6.1% stage unknown). Therefore, it is unclear whether the seven-miRNA panel should be used as pan-pancreatic cancer prognostic markers or as early stage cancer screening markers, considering the gene expression profile can change dramatically from early to late stages[35,36]. Carlsen *et al*[27] used the same strategy as presented in our study and identified circulating miR-375 as the sole potential marker although with relatively low accuracy (70%), and it did not outperform CA19-9 as a diagnostic marker. However, Carlsen *et al*[27] used chronic pancreatitis patients as controls instead of normal healthy donors. It is uncertain why chronic pancreatitis patients were used as controls considering only a small population (8%) of pancreatic cancer patients have concomitant chronic pancreatitis[37]. There is a weak link between pancreatitis and pancreatic cancer[38], and only 4% of patients within 20 years of chronic pancreatitis diagnosis have developed into pancreatic cancer[39].

The research presented here focuses solely on stage Ⅱ pancreatic ductal adenocarcinoma patients, of which the gene expression profile should resemble early stage pancreatic cancer more closely than advanced stage cancer. The validation experiment was done by comparing pancreatic cancer patients to healthy controls, and in par-

ticular to high risk controls who might have inherited genetic susceptibility[40]. Our three-miRNA panel can differentiate pancreatic cancer patients from healthy controls with high sensitivity and specificity. Nevertheless, the 11 miRNAs, as presented by the three prior studies mentioned above, were also found in our 290-miRNA panel to have acceptable detection intensities (presented in Figure 1A). It would be of great interest to conduct a future larger scale clinical trial comparing the three-miRNA panel identified here to the eleven-miRNAs identified previously to see how they fare in predicting pancreatic cancer, or if the combination of all 14 miRNAs could be developed into a diagnostic test for cancer.

Of the three miRNAs identified in this study, two miRNAs (miR-642b-3p and miR-885-5p) were shown to be significantly up-regulated in cancer patients by our screening process while the third miRNA (miR-22-3p) was shown to be up-regulated in cancer patients in the literature[19,20]. It should be noted that miR-642b is a relatively novel miRNA marker with no prior publication about its potential functional role or utility as a marker. However, miR-885-5p has been shown to be a potential serum marker for liver pathologies, including hepatocellular carcinoma, liver cirrhosis, and chronic hepatitis B[41]. Functionally, miR-885-5p is found to be located in the 3p25.3 genomic region and is known to have a tumor suppressive function by triggering cell cycle arrest and senescence and/or apoptosis[42]. MiR-885-5p activates the p53 pathway, causes down-regulation of cyclin-dependent kinase and mini-chromosome maintenance protein, and suppresses matrix metallopeptidase 9 expression and Caspase genes[42-44]. MiR-22, on the other hand, is one of the most common miRNAs in the colorectal cancer transcriptome and has been studied for its critical role in breast cancer and bone metastasis[45,46]. It is known for directly targeting the estrogen receptor α mRNA[47] and is proposed to be a putative tumor suppressor by repressing the EVI1 oncogene expression[48]. It inhibits cell cycle progression by repressing Max and ErbB3 expression post-transcriptionally, mediates the effects of the tumor-suppressor p53, and suppresses interferon gene expression by blocking interferon regulatory factor-5[49-51]. MiR-22 has been proposed as a potential serum marker for non-small cell lung cancer[52], esophageal squamous cell carcinoma[53], and nasopharyngeal carcinoma[54].

Our experimental strategy in this study focused on using hybridization-based microarray technology as the means to screen thousands of genes simultaneously, but it is also severely limited due to its issues with reproducibility and its tendency to produce a high rate of false positive and false negative results. This restricts its potential use as a reliable diagnostic tool for cancer. RT-qPCR, on the other hand, is based on sequence-specific amplification, which is highly specific and sensitive for individual testing targets. It has been developed for use in diagnostic/prognostic tests such as the Oncotype DX test for breast cancer and the Cervista® HPV HR assay. Furthermore, our approach using two layers of confirmation with RT-qPCR, utilizing different sets of patients, independent experimental procedures and instrumentation, still showed a remarkable consistency that suggests the potential future application of RT-qPCR-based diagnostic tests using circulating miRNA markers.

Although CA19-9 is not considered an ideal biomarker for the early diagnosis of pancreatic cancer, data from this study demonstrated a relatively high sensitivity of 73% and specificity of 100%. It should be noted, however, that all patients recruited for this study were "confirmed cancer cases" by pre-surgery imaging [magnetic resonance imaging (MRI) and computed tomography (CT)] and post-surgical examination by pathologists. Therefore, even with 100% confirmed cancer cases, CA19-9 has only a 73% sensitivity to detect them. The three-miRNA panel, on the other hand, exhibited a 91% sensitivity. It would be of great interest to test the three-miRNA panel, alongside CA19-9, in a future large scale clinical trial of suspected cancer cases to see how the two-marker system fares when they are compared to one another or when they work together.

In summary, we have identified three blood-based circulating miRNA targets, miR-642b-3p, miR-885-5p and miR-22-3p, which, when combined, provided a high level of diagnostic accuracy for early stage pancreatic cancer. Our plan is to study an expanded sample of patients to further develop and refine the diagnostic miRNA panel based on RT-qPCR. This new panel may work alone or in conjunction with other known immunoassays, such as CA19-9 and CEA, as a diagnostic test for early stage pancreatic cancer. We envision that the future miRNA biomarker panel can immediately apply to the category of patients at high risk for pancreatic cancer before more expensive and invasive modalities like CT, MRI, endoscopic ultrasound and endoscopic retrograde cholangiopancreatography are used. A similar strategy can also be utilized to identify miRNA panels for other cancer types where early detection is crucial for a favorable disease outcome.

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COMMENTS

Background

Pancreatic cancer is one of the most lethal human cancers with a mere 6% 5-year survival rate. Studies have shown that early detection is the best option available for controlling this disease. The goal of this study, therefore, is to explore and compile a diagnostic biomarker panel, based on microRNA (miRNA) in the circulating blood, for detection of pancreatic cancer at earlier stages.

Research frontiers

A diagnostic panel of biomarkers for cancer is highly desirable because it would help to fight cancer at the earliest possible stage when the disease is still curable. However, for the past fifteen to twenty years, despite numerous studies and publications, this goal remains elusive with only scanty numbers of risk assessment and prognostic panels eventually developing into clinical tests. One of the major problems is study reproducibility. The results of one study cannot be readily reproduced by another study. In addition, gene expression profiles can vary greatly among the different stages for each type of cancer, as well as between blood-based markers and tissue-specific markers. Therefore, it is critical for each investigator to be clear on their general experimental strategy which can, in turn, address issues pertaining to the development of future clinical tests.

Innovations and breakthroughs

The current study focuses on identifying a panel of blood-based biomarkers for pancreatic cancer. If it is effective, pancreatic cancer detection can be performed by a simple non-invasive blood draw instead of an invasive procedure. The approach used in this study is innovative due to the fact that, in addition to the general strategy of microarray screening followed by polymerase chain reaction-based confirmation, the authors employed a second layer of validation experiments, using different experimental procedures, instrumentation, and lab personnel at an independent location. The results of the two experimental strategies are remarkably similar even with a new cohort of patient specimens, suggesting high validity of this diagnostic panel. Furthermore, the authors have included a group of high risk individuals as controls. High risk controls are subjects with a strong family history of pancreatic cancer (at least two first degree relatives with the disease). Therefore, they have inherited genetic susceptibility to developing pancreatic cancer and hence are genotypically closer to individuals with disease than to normal healthy controls. Remarkably, the three-miRNA panel identified in this study can differentiate pancreatic cancer patients from both normal and high risk controls, demonstrating its high sensitivity and specificity for pancreatic cancer.

Applications

The identified panel of three miRNAs can potentially be used as a diagnostic detection set for early stage pancreatic cancer.

Terminology

MiRNA are small non-coding RNA approximately 18-24 nucleotides in size. The abnormal expression of miRNA found in patient blood is known to be associated with cancer progression. Hence, the utilization of miRNA biomarkers was proposed as a way to potentially detect pancreatic cancer.

Peer review

This is a well written paper examining a potentially useful means of screening for pancreatic carcinoma. The authors would recommend publication of the manuscript.

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