Diagnostic Significance of MicroRNA Tumor Biomarkers in Fine Needle Aspirates of Pancreatic Cancer

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SUMMARY POINTS

- Collection of pancreatic fine needle aspirates (FNAs) in RNA*Retain*™ allows stabilization and protection of RNA in tissues containing high levels of nucleases
- Differential microRNA (miRNA) expression in pancreatic FNAs accurately classifies benign and malignant tissue
- Combinations of miRNA and mRNA expression signatures improve the separation between normal tissue and chronic pancreatitis
- miRNAs are suitable analytes for molecular characterization of fine needle aspirates from pancreatic tissues, establishing a potential utility for miRNAs in diagnostic procedures.

INTRODUCTION

Computer tomography (CT) scanning and endoscopic ultrasound (EUS) are standard approaches used in clinical settings to indicate the size and extent of pancreatic tumor. However, these modalities alone can neither discern benign lesions from malignant ones nor determine the type of the neoplasm. Recently, image-guided percutaneous fine-needle aspirate (FNA) has emerged as a very specific modality in the diagnosis of pancreatic adenocarcinoma (PDAC). FNA procedures have been increasingly performed through major medical centers to obtain pre-operative diagnoses and for staging of pancreatic cancer. However, pathological interpretation of FNA samples from pancreas is challenging due to scant tissue, extensive fibrosis and frequent presence of blood following sample collection. Therefore, biomarkers that can be detected in limited cytology samples could become extremely important in diagnosis and management of patients with pancreatic adenocarcinoma.

Over the past few years, several studies have demonstrated that miRNA expression profiles are significantly altered in human cancers and that these small regulatory molecules play an active role in oncogenesis. Recently, we identified specific miRNAs whose expression level can be used to discriminate normal pancreas, chronic pancreatitis and cancerous tissues (Szafranska et al. Oncogene, 2007). The purpose of the study herein was to evaluate the suitability of miRNAs as biomarkers for molecular characterization of pancreatic FNAs. Due to presence of elevated levels of nucleases, isolation of superior quality RNA from pancreas is challenging, and therefore surgically collected FNA samples require immediate stabilization. In our study we used RNA*Retain*™ Pre-analytical RNA Stabilization Solution (Asuragen, Austin, TX) - a tissue storage reagent that rapidly permeates excised tissue leading to inactivation of nucleases, and thereby stabilizes and protects RNA.

MATERIALS AND METHODS

In vivo FNA biopsies for 13 pancreatic cancer patients and an *ex vivo* biopsy for 1 normal pancreas specimen (sampled in triplicate) were performed using a 22-25-gauge needle. Each sample contained 3 needle cores collected in 2 mL of RNA*Retain*™ (Asuragen, Austin, TX) and was stored at -80°C before processing. Total RNA was extracted using the *mir*Vana™ miRNA Isolation Kit protocol (Ambion, An Applied Biosystems Business, Austin, TX) modified to enable efficient processing of low tissue amounts. Concentration and purity of total RNA were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). RNA integrity was assessed with an Agilent 2100 Bioanalyzer and the RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA). Real-time quantitative RT–PCR was performed using TaqMan® miRNA Assays and TaqMan® Gene Expression Assays and TaqMan® 7900 technology (Applied Biosystems, Foster City, CA, USA). qRT-PCR reactions were performed with gene-specific priming for reverse transcription and for PCR using 10 ng (miRNA) or with random priming for reverse transcription and gene-specific priming for PCR using 5 ng (mRNA) of total RNA input.

RESULTS

Pancreatic fine needle aspirate samples

A

Sample type	Sample ID	Sex	Age	Diagnosis
PDAC FNA	FNA AdCa	8F, 2M	57-85	pancreatic ductal adenocarcinoma
Other FNA	FNA Ca*	F	59	suspect for pancreatic ductal adenocarcinoma
		F	46	neoplasm of endocrine pancreas
		F	59	atypical
Normal FNA	FNA NP	M	76	normal pancreas

B

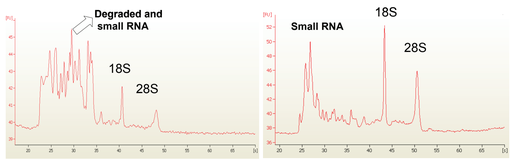

Figure 1:
Characterization of pancreatic FNA samples. **(A)** Description of 16 pancreatic FNA specimens, which comprised 10 pathologically confirmed and 2 unconfirmed carcinomas of exocrine pancreas (pancreatic adenocarcinoma), 1 carcinoma of endocrine pancreas and 1 normal pancreas specimen procured in triplicate. **(B)** Examples of Agilent 2100 electropherogram analyses of total RNA isolated from pancreatic FNA samples preserved in RNA*Retain*™. On average, 4.9 µg RNA was extracted from each sample. Average A260/280 ratio was 1.82.

Differential miRNA expression in FNAs by qRT-PCR

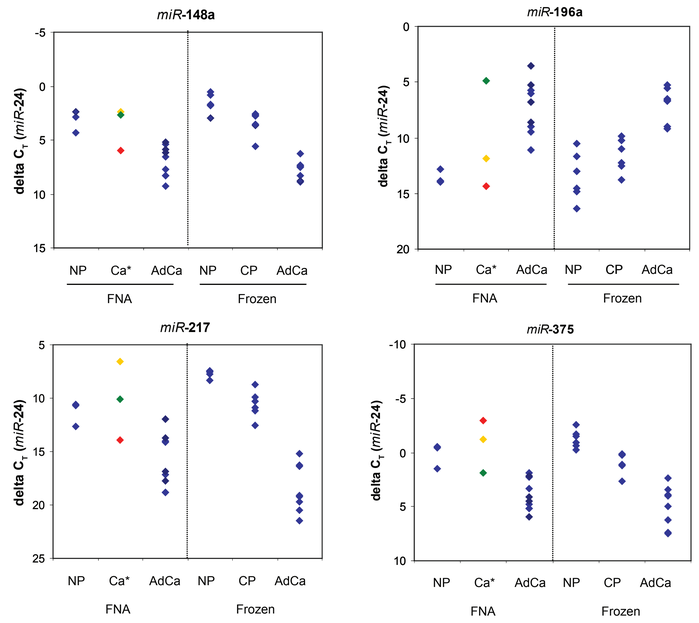

Figure 2:
A correlation of differential miRNA expression data between frozen pancreatic tissue samples and pancreatic FNAs. Differentially expressed miRNAs interrogated in this study were identified previously by Szafranska et al. (Oncogene 2007) through expression analysis of frozen normal pancreas, chronic pancreatitis, and pancreatic adenocarcinoma specimens. In the study herein, the relative miRNA expression levels were determined in 6 normal pancreas (NP), 6 chronic pancreatitis (CP), 8 pancreatic adenocarcinoma (AdCa) tissue specimens, and compared with pancreatic FNA samples. The normal pancreas (NP, n=3) and pathologically confirmed pancreatic adenocarcinoma FNAs (AdCa, n=10) are represented by ◆. FNA diagnosed as neoplasia of endocrine pancreas is denoted as◆, while ◆ and ◆ depict the FNAs with a non-definitive pathological description (Ca*, n=3). The C_T values for individual miRNA biomarkers were normalized to *miR*-24.

Differential mRNA expression in FNAs by qRT-PCR

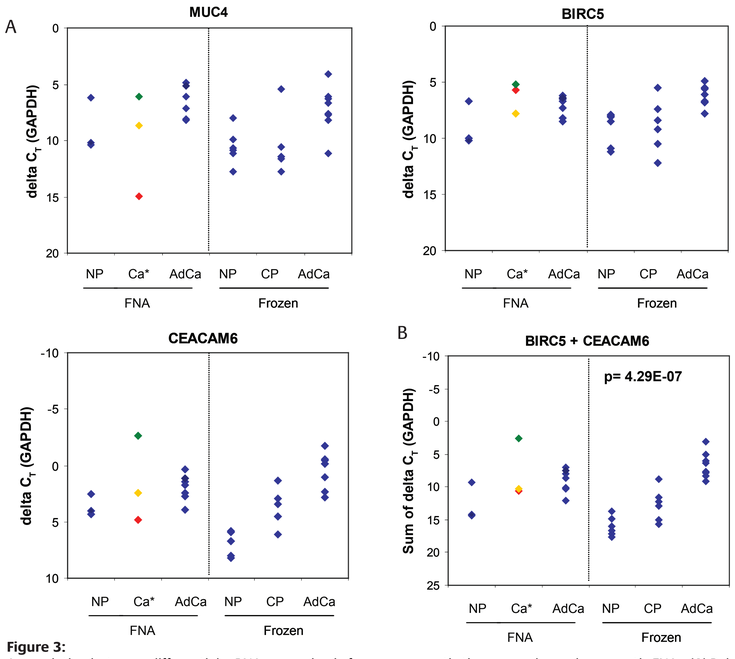

Figure 3:
A correlation between differential mRNA expression in frozen pancreatic tissue samples and pancreatic FNAs. **(A)** Relative expression levels for 3 mRNAs reported in the literature as differentially expressed between benign pancreas and pancreatic cancer tissues were determined in a subset of the sample set from Figure 2 (7 AdCa +3Ca* + 3NP + Frozen). These graphs show separation between the experimental groups achieved through combining the individual C_T values for the selected markers normalized to GAPDH. **(B)** Combination of 2 mRNA biomarkers allows only moderate segregation between diseased and benign pancreas, regardless of the tissue preservation method (frozen vs RNA*Retain*™).

Combinations of multiple classifiers improve separation between benign and diseased pancreatic tissues

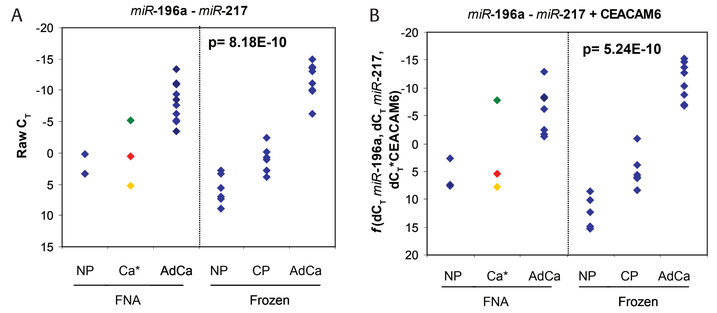

Figure 4:
Expression signatures of individual miRNAs alone or in combination with mRNAs classify pancreatic tissue disease states, regardless of the tissue preservation method (frozen vs RNA*Retain*™). **(A)** Differential expression of *miR*-196a and *miR*-217 identifies normal and diseased pancreatic tissues, regardless of the tissue preservation method (frozen vs RNA*Retain*™). **(B)** Combination of miRNA and mRNA biomarkers may improve separation between normal and chronic pancreatitis samples. The individual miRNA/mRNA expression signatures were normalized to *miR*-24 (miRNAs) or GAPDH (mRNAs).

Analytical and Clinical Assay Sensitivity

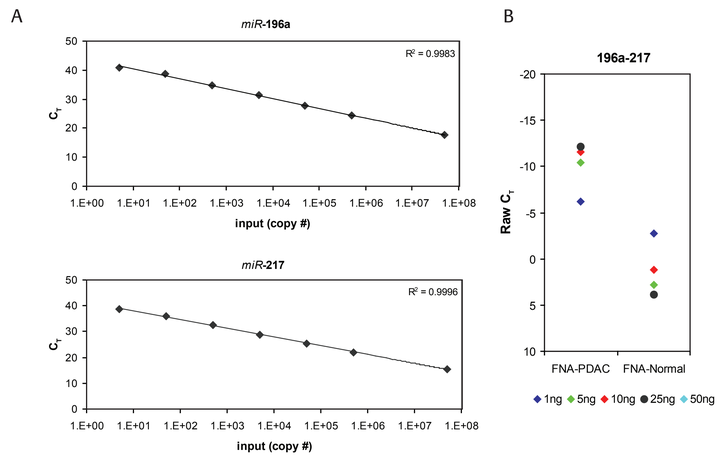

Figure 5:
Preliminary determination of clinical and analytical sensitivity of *miR*-196a and *miR*-217 qRT-PCR assays. **(A)** Replicate 7-point standard curves for TaqMan® miRNA assays were created by spiking the indicated copy number of synthetic *miR*-196a or *miR*-217 oligonucleotides in the background of 5 ng yeast tRNA into independent RT reaction. Each dilution point corresponds to n=80 replicates. qRT-PCR reactions were performed according to the in-house optimized ABI TaqMan® protocol, using ABI 7900HT RT-PCR instrument system. **(B)** Clinical sensitivity of *miR*-196a and *miR*-217 TaqMan® assays was tested at varying inputs of 1, 5, 10, 25 and 50 ng of total RNA using one normal and one PDAC FNA specimens. The separation between cancer and normal pancreatic tissue was comparable between 5 and 50 ng of total RNA input.

CONCLUSION

Collection of FNAs in RNA*Retain*™ allowed us to stabilize RNA and recover intact miRNA fraction from pancreas to perform expression profiling by qRT-PCR. Even in samples where the RNA integrity was severely compromised, miRNA amplification was not affected. miRNAs identified as de-regulated in macro-dissected frozen pancreatic cancer tissues by Szafranska et al. (Oncogene 2007) were also found differentially expressed between pancreatic cancer and normal pancreas FNAs. Furthermore, we showed that relative expression of as few as two miRNAs can distinguish AdCa FNA specimens from benign tissues. We also observed that combinations of miRNA and mRNA expression signatures may increase the separation between normal tissue and chronic pancreatitis in addition to allowing better differentiation of pancreatic cancer from non-malignant tissue. To evaluate clinical utility of our miRNA classifiers, we examined the individual TaqMan® miRNA assays for sensitivity and linearity using synthetic *miR*-196a and *miR*-217. Both assays exhibited 7 log dynamic range with a limit of detection of 5 to 10 copies per RT reaction. The RNA input titration experiment using total RNA from pancreatic FNAs showed that both miRNA assays accurately identify benign and malignant pancreatic tissue between 5 and 50 ng input.