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Investigating MicroRNA Expression Profiles in Pancreatic Cystic Neoplasms

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OBJECTIVES: Current diagnostic tools for pancreatic cysts fail to reliably differentiate mucinous from nonmucinous cysts. Reliable biomarkers are needed. MicroRNAs (miRNA) may offer insights into pancreatic cysts. Our aims were to (1) identify miRNAs that distinguish benign from both premalignant cysts and malignant pancreatic lesions using formalin-fixed, paraffin-embedded (FFPE) pathology specimens; (2) identify miRNAs that distinguish mucinous cystic neoplasm (MCN) from branch duct-intraductal papillary mucinous neoplasm (BD-IPMN).
METHODS: A total of 69 FFPE pancreatic specimens were identified: (1) benign (20 serous cystadenoma (SCA)), (2) premalignant (10 MCN, 10 BD-IPMN, 10 main duct IPMN (MD-IPMN)), and (3) malignant (19 pancreatic ductal adenocarcinoma (PDAC)). Total nucleic acid extraction was performed followed by miRNA expression profiling of 378 miRNAs interrogated using TaqMan MicroRNA Arrays Pool A and verification of candidate miRNAs. Bioinformatics was used to generate classifiers.
RESULTS: MiRNA profiling of 69 FFPE specimens yielded 35 differentially expressed miRNA candidates. Four different 4-miRNA panels differentiated among the lesions: one panel separated SCA from MCN, BD-IPMN, MD-IPMN, and PDAC with sensitivity 85% (62, 97), specificity 100% (93, 100), a second panel distinguished MCN from SCA, BD-IPMN, MD-IPMN, and PDAC with sensitivity and specificity 100% (100, 100), a third panel differentiated PDAC from IPMN with sensitivity 95% (76, 100) and specificity 85% (72, 96), and the final panel diagnosed MCN from BD-IPMN with sensitivity and specificity approaching 100%.
CONCLUSIONS: MiRNA profiling of surgical pathology specimens differentiates serous cystadenoma from both premalignant pancreatic cystic neoplasms and PDAC and MCN from BD-IPMN.

INTRODUCTION

Mucinous pancreatic cysts have malignant potential, whereas nonmucinous cysts are benign.[1–3] Therefore, accurate characterization has important clinical implications. Current methodologies fail to differentiate mucinous from nonmucinous pancreatic cystic lesions with a high degree of accuracy with accurate diagnosis in only 50–70% of cysts.[4] Cytology from endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) has <50% sensitivity for diagnosis of the cyst.[5] Few cyst fluid markers have proven valuable with many mucinous lesions missed using a cutoff for a carcinoembryonic antigen (CEA) <192 ng/ml.[6,7] DNA mutation analyses from cyst fluid has low sensitivity for mucinous and malignant lesions ranging 37–45%.[8,9] Therefore, further research is necessary to explore new and more accurate diagnostic biomarkers for pancreatic cystic lesions.

MicroRNAs (miRNAs) are small noncoding RNAs (18–25 nucleotides) that regulate gene expression post-transcriptionally.[10,11] miRNAs bind to messenger RNA (mRNA) and prevent gene expression by inhibiting translation or inducing mRNA cleavage. MiRNA expression profiling has shown great promise in multiple cancers including pancreatic adenocarcinoma (PDAC), where changes in miRNA expression levels correlate with diagnosis and prognosis.[12–14] A recent multi-center clinical investigation reported that a 7-miRNA classifier (miR-196a, -130b, -135b, -148a, -375, -96, and -24) can be used to improve the sensitivity and specificity for the diagnosis of PDAC in the setting of nondiagnostic or indeterminate EUS-FNA cytology.[15] A clinically available classifier was developed using formalin-fixed, paraffin-embedded (FFPE) specimens, which differentiates PDAC from normal pancreas and chronic pancreatitis with sensitivity and specificity of 95%.[16] Differential expression of miRNA panels has also been reported in pancreatic cystic lesions.[17–19]

The aim of this exploratory investigation is to perform a comprehensive analysis to identify miRNA signatures that accurately distinguish the various pancreatic lesions using micro-dissected FFPE-archived surgical pathology specimens.

METHODS

This investigation was approved by the Partners Institutional Review Board 2009P002606. We used sensitive and high throughput miRNA survey techniques on pathology tissue of pancreatic cystic lesions and employed bioinformatics analyses to identify miRNA biomarkers that distinguish benign,

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nonmucinous (SCA) from both premalignant, mucinous (MCN, branch duct IPMN (BD-IPMN), main duct IPMN (MD-IPMN)), and malignant (PDAC) lesions in FFPE specimens.

Archived pathology samples. FFPE specimens from adult patients (age > 18 years) who underwent surgical resection at Brigham and Women's Hospital were identified from a surgical pathology database spanning 1995–2012. The following specimens were chosen based on the WHO classification:[20] SCA, BD-IPMN with low-grade dysplasia, MD-IPMN with moderate dysplasia, MCN, and PDAC. All pathology slides were reviewed by two gastrointestinal pathologists (A.B., L.D.) to confirm the final diagnosis.

Database development. The Partners Healthcare electronic medical record was used to develop a password-protected, electronic database recording individual patient information including age, gender, race, history of acute or chronic pancreatitis, history of cigarette smoking or alcohol use, presence of abdominal pain and/or weight loss, EUS morphology, EUS-fine needle aspiration (FNA) cytology, cyst fluid chemistry (CEA, amylase), DNA mutation analysis for k-*ras* and loss of heterozygosity (LOH) (RedPath Integrated Pathology, Pittsburgh, PA, USA), and computed tomography (CT) and/or MRI of the pancreas.

MiRNA experimental workflow. Once the archived pathology samples were identified and diagnosis confirmed, the workflow included (a) specimen processing, (b) total nucleic acid extraction, (c) miRNA expression profiling, and (d) statistical analysis plan. Detailed methodology is outlined below for each step of the experiment.

(a) Specimen processing. Manual microdissection was performed from each FFPE block to enrich for epithelial lesional tissue before total RNA extraction. In brief, one hematoxylin and eosin (H&E) slide and up to 10 unstained slides were generated from each FFPE block. The target lesion was marked on the H&E slide for each case, which was then used to guide the removal of non-target tissues (e.g., non-neoplastic pancreatic acinar, ductal, and endocrine tissue) from unstained slides. The tissue area of interest was scraped off the slide into an eppendorf tube to facilitate total RNA extraction.

(b) Total nucleic acid extraction. Total RNA and total nucleic acid from the target tissue area were extracted using internally developed, validated procedures optimized for recovery of miRNA fraction from FFPE tissues based on the RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Life Technologies, Austin, TX, USA).

(c) MiRNA expression profiling. Differentially expressed miRNAs among the diagnostic groups were identified using a high throughput screening platform (TaqMan MicroRNA Arrays Pool A; Applied Biosystems, Grand Island, NY, USA), which contained probes for 378 known mature miRNAs (Sanger version 14.0). From this high-throughput miRNA screen, 35 miRNA candidates were selected via exploratory bioinformatics analyses for verification on FFPE specimens by singleplex TaqMan quantitative reverse transcriptase polymerase chain reaction (qRT–PCR). Candidates were selected by the following criteria: highly significant *P*-value,

strong expression levels, and high effect sizes; the detail specifications of these selection criteria are described in the statistical analysis plan below. The resulting miRNA biomarkers were refined through bioinformatics analysis to generate classifiers that allow differentiation among the various pancreatic cystic neoplasms.

(d) Statistical analysis plan

(a) *Specimens and power calculation*: The sample size estimates/power calculations for archived FFPE specimens used in this study are based on published microarray classifier literature using standardized fold change of 2.0.[21] A sample size of at least 17 per diagnostic group would yield 80% power with an alpha of 0.000133.

(b) *DiffPair biomarker selection*: Candidate miRNAs were selected in pairs (miR-X, miR-Y), with the delta-Ct calculated as Ct(miR-X) − Ct(miR-Y) used as the expression measure for each pair. These "DiffPairs" were used as biomarkers for differential expression analysis instead of the raw Ct values of individual miRNAs in order to normalize the data by removing the influence of gross differences in RNA quantity or quality between samples. Because these differences are expected to have similar impact on all the measured Ct values of all miRNA, the delta-Ct expression measured for a given DiffPair would be expected to be approximately independent of such factors. Unlike more traditional normalizer-based approaches, DiffPair analysis does not require any assumptions about the stability of biological expression of either miRNA in the pair; in fact, the most useful DiffPairs for two-group classification problems are likely to consist of one miRNA that is upregulated in the condition of interest paired with another miRNA that is downregulated in the same condition.

(c) *MiRNAs included in DiffPair analysis*: Only miRNAs with strongly expressed Ct levels (mean Ct across all samples < 30) were included in DiffPair analysis. Those miRNAs that survived this mean-expression filter were then included in DiffPairs if they met either one of two criteria:

1. A sample-grouping-independent variance filter: the s.d. across all samples of all groups for a marker had to exceed 1 Ct (such variance-based filtering has been shown to increase the power to detect differential biomarker expression),[22] or
2. selection as one of the five highest ranking potential normalizer miRNA species by the concordance correlation method described in Wylie *et al.*[23]

(d) *DiffPair selection criteria*: DiffPairs were selected for four distinct comparisons on the basis of overall expression ANOVA or *t*-test Benjamini–Hochberg-FDR-adjusted *P*-value and minimum effect size magnitude. The four comparisons consisted of three pairwise comparisons:

1. SCA vs. MCN: FDR < 0.00125, log-ratio magnitude > 6.5,
2. SCA vs. BD-IPMN: FDR < 0.00125, log-ratio magnitude > 6.5,
3. MCN vs. BD-IPMN: FDR < 0.01, log-ratio magnitude > 4.5,

4. PDAC vs. SCA vs. mucinous (where "Mucinous" is defined as MCN and all IPMN specimens): $FDR < 1.25 \times 10^{-7}$ with no additional effect size requirement. The FDR and effect size thresholds were chosen in order to obtain similar numbers of miRNA candidates for further investigation on the basis of each comparison, as all four of these comparisons were considered approximately equi-relevant.

(a) *Classifier development*: An L2/ridge-penalized (with the penalty parameter fixed at 2.5, based on optimization of cross-validated model likelihood in previous unrelated projects) logistic regression modeling strategy was employed for feature selection using markers selected by a penalized linear regression forward stepwise feature selection process. It is critical for the application of this modeling strategy that the qRT–PCR data be suitably normalized. The DiffPair strategy employed during biomarker selection achieves such normalization by considering only differences in expression between two different miRNA species; this idea can be extended to linear models involving more than two miRNAs by constraining the sum of the model coefficients to be equal to 0. (Note that, for a model based on only two miRNAs, this constraint forces the model to be a DiffPair, as the coefficient assigned to the first miRNA must be exactly -1 times the coefficient assigned to the second miRNA for the coefficients to sum to 0.).

The selected classification strategy (including feature selection) was then evaluated under bootstrap-case cross-validation with bias reduction (BCCVPBR)[24] for diagnostic performance metrics including area under receiver operating characteristic curve (AUC), accuracy, sensitivity, and specificity (BCCVPR is a method allowing calculation of confidence intervals for cross-validation-based performance estimates).

RESULTS

Study cohort. The demographics and clinical characteristics of the 69 patients included in the study are summarized in Table 1. Mean age was 61.4 ± 12.8 years; 64% were female. PDACs were more likely to be symptomatic and associated with a previous history of cancer and/or a smoking history when compared with the other types of pancreatic cystic neoplasms. CEA levels were markedly elevated in mucinous cystic lesions. As shown in Figure 1, 35 differentially expressed miRNAs were identified from all 69 FFPE-archived specimens.

MicroRNA expression profiling showed marked differences among the pancreatic cystic neoplasms and PDAC. As previously reported,[12–15] our data confirm that PDAC has a different miRNA expression profile when compared with nonmalignant pancreatic cystic neoplasms. In addition, our data facilitated development of four different miRNA classifiers that could distinguish between and among the various pancreatic cystic neoplasms and PDAC.

A SCA classifier consisting of the following miRNAs: miR-31-5p, miR-483-5p, miR-99a-5p, and miR-375, distinguished SCA from all mucinous cystic lesions (MCN, BD-IPMN, and MD-IPMN) and PDAC with 90% (73, 100) sensitivity and 100% (98, 100) specificity (Figure 2).

Similarly, an MCN classifier was developed that distinguished MCN from other pancreatic cystic neoplasms and PDAC. The four miRNAs in this classifier included miR-10b-5p, miR-202-3p, miR-210, and miR-375, and these accurately differentiated MCN from SCA, IPMN and PDAC with a sensitivity of 100% (100, 100) and specificity of 100% (100, 100) (Figure 3).

Furthermore, differential miRNA expression profiles were observed within the mucinous group. A panel consisting of miR-192-5p, miR-202-3p, miR-337-5p, and miR-130-3p diagnosed MCN from BD-IPMN with a sensitivity and specificity approaching 100% (Figure 4, Mucinous classifier). A PDAC classifier (miR-21-5p, miR-485-3p, miR-708-5p, and miR-375) was also developed that distinguished PDAC from IPMN with a sensitivity and specificity of 95% (76, 100) and 85% (72, 96), respectively (Figure 5).

Table 1 Demographic and clinical description of study cohort

|	SCA (n = 20)	MCN (n = 10)	BD-IPMN (n = 10)	MD-IPMN (n = 10)	PDAC (n = 19)	P value
Age (years)	57.8	49.2	68.7	66.4	65.1	<0.05
Female gender[a]	14 (70%)	10 (100%)	8 (80%)	3 (30%)	9 (47%)	<0.001
Symptoms[b]	8 (40%)	5 (56%)	1 (10%)	2 (20%)	17 (89%)	<0.005
Family history pancreatic cancer	0	0	0	0	2	>0.1
Personal history of other cancers[c]	3 (15%)	0	0	1 (10%)	5 (25%)	<0.05
Smoking[b]	7 (35%)	1 (11%)	6 (60%)	6 (60%)	15 (75%)	<0.05
Alcohol use	0	0	1 (10%)	2 (20%)	2 (11%)	>0.1
CEA (ng/ml)[d]	0.83 ± 0.42	9,495.50 ± 13,091.80	3,464.10 ± 3,893.16	NA	NA	<0.05
Amylase (U/l)	NA	33,527.50 ± 46,545.30	50,762.20 ± 68,185.68	2,370.50 ± 3,338.25	N/A	≥0.6
k-ras	0/2	NA	1/5 (20%)	1/2 (50%)	N/A	>0.9
LOH	2/2	NA	1/4 (25%)	2/2 (100%)	N/A	≥0.4

BD-IPMN, branch duct-intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm; MD-IPMN, main duct-intraductal papillary mucinous neoplasm; NA: not available; PDAC, pancreatic ductal adenocarcinoma; SCA, serous cystadenoma.
[a]PDAC vs. MCN or MD-IPMN.
[b]PDAC vs. SCA, MCN, BD-IPMN, MD-IPMN.
[c]PDAC vs. MCN, BD-IPMN, MD-IPMN.
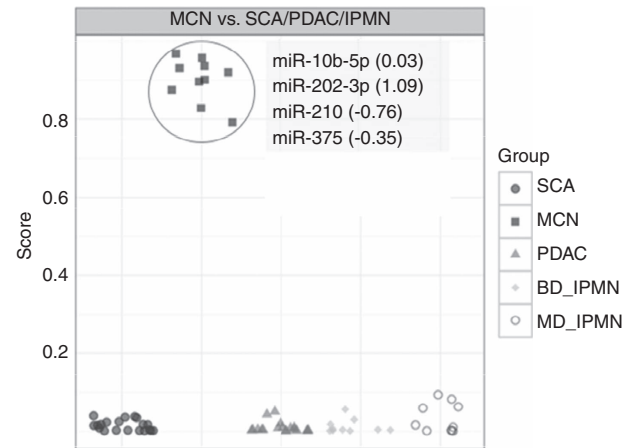[d]SCA vs. MCN and BD-IPMN.
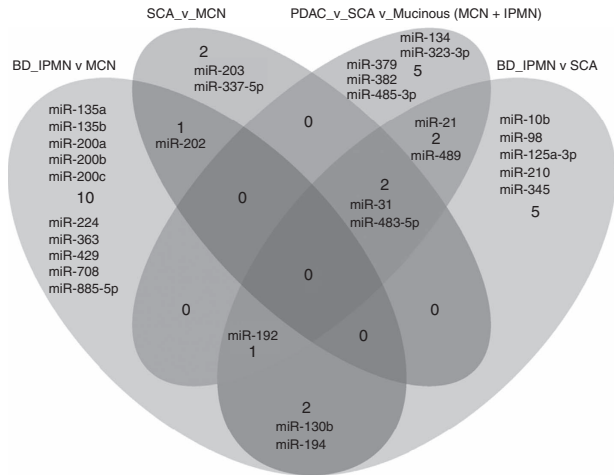
Figure 1 Venn diagram built based on the DiffPair significance results from the original Megaplex discovery set. A total of 30 miRNAs were significant. This set of 30 was modified to achieve the final 35 candidates as follows: three miRNAs (miR-194, miR-200a, and miR-200b) were removed because of redundancy of expression pattern with other miRNAs from the set of 30; two additional miRNAs (miR-181a-5p and miR-324-5p) were chosen as normalizers; six more miRNAs (miR-24, miR-30a-3p, miR-93, miR-99a, miR-342-3p, and miR-375) were added based on other considerations. miRNA, microRNA.
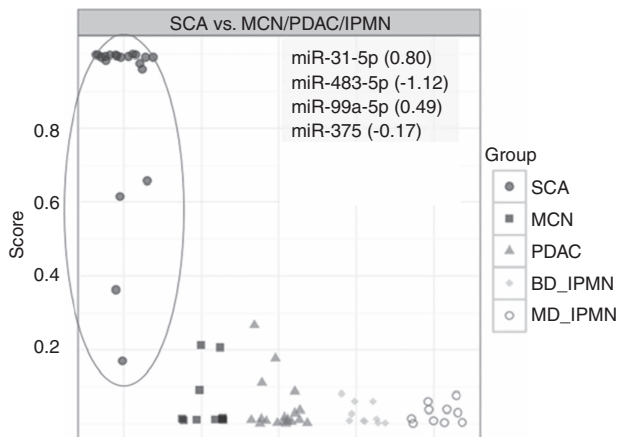
Figure 2 Serous cystadenoma classifier. The "score" for each specimen is the probability that the specimen is a SCA and not a MCN, IPMN, or PDAC. The values next to the listed miRNAs represent the coefficients from the logistic regression model. IPMN, intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm; PDAC, pancreatic ductal adenocarcinoma; SCA, serous cystadenoma.

Figure 3 Mucinous cystic neoplasm classifier. The "score" for each specimen is the probability that the specimen is a MCN and not a SCA, IPMN, or PDAC. The values next to the listed miRNAs represent the coefficients from the logistic regression model. IPMN, intraductal papillary mucinous neoplasm; miRNA, microRNA; MCN, mucinous cystic neoplasm; PDAC, pancreatic ductal adenocarcinoma; SCA, serous cystadenoma.
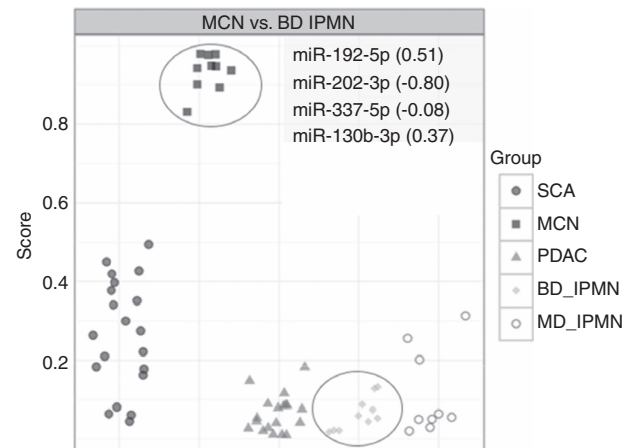
Figure 4 Mucinous classifier. The "score" for each specimen is the probability that the specimen is a MCN and not BD-IPMN. The values next to the listed miRNAs represent the coefficients from the logistic regression model. BD-IPMN, branch duct-intraductal papillary mucinous neoplasm; miRNA, microRNA; MCN, mucinous cystic neoplasm.

DISCUSSION

In this study, we investigated miRNA expression patterns in archived surgical pathology specimens from pancreatic cystic neoplasms and pancreatic cancer. This is the largest study to date interrogating miRNA expression in resected pancreatic cystic neoplasms. We identified differentially expressed miRNAs that can serve as components of a highly accurate disease classifier panel to distinguish among various pancreatic cysts. Specifically, our miRNA classifiers distinguished nonmucinous cysts (serous cystadenoma) from mucinous cysts (BD-IPMN, MD-IPMN, MCN) and pancreatic cancer (PDAC) with diagnostic accuracies over 95% (Figures 2 and 3).

Improved diagnostic tools are needed to aid in the management of pancreatic cystic lesions by distinguishing malignant, premalignant and benign disease, as well as determining which cystic neoplasms are likely to progress to malignancy. A surgical pathology study demonstrating 68% agreement between preoperative and postoperative diagnosis highlights the need for more conclusive tests in the preoperative setting. A pilot study of five miRNAs in cyst fluid demonstrated that miR-21 had 80% sensitivity and 76% specificity for mucinous compared with nonmucinous cysts.[18] Interestingly, a surgical pathology study of pancreatic cysts did not identify miR-21 as differentially expressed between SCA and PDAC, but found that it expressed at higher levels in both compared with normal pancreas tissue.[25] Recently, cyst
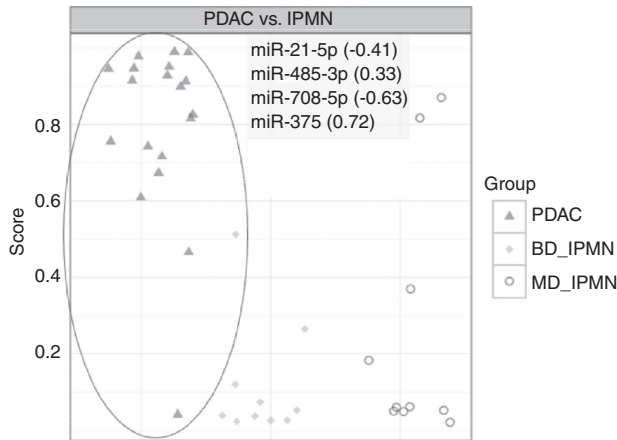
Figure 5 PDAC classifier. The "score" for each specimen is the probability that the specimen is a PDAC and not an IPMN. The values next to the listed miRNAs represent the coefficients from the logistic regression model. IPMN, intraductal papillary mucinous neoplasm; miRNA, microRNA; PDAC, pancreatic ductal adenocarcinoma.

fluid specimens collected during surgery were used to build a 9-miRNA classifier (miR18a, -24, -30a-3p, -92a, -342-3p, -99b, -106b, -42-3p, and -532-3p), which predicted degree of dysplasia within IPMNs and identified cysts that likely needed surgical resection.[19]

We identified a miRNA classifier distinguishing between SCA and mucinous pancreatic cystic neoplasms. SCA were distinguished from mucinous lesions with 90% sensitivity and 100% specificity using a miRNA panel composed of miR-31-5p, miR-483-5p, miR-99a-5p, and miR-375. Accurate diagnosis of SCA is important in management, as these patients only undergo surgical resection if the cyst causes symptoms or enlarges rapidly.[26]

In addition, a clinically relevant miRNA classifier composed of miR-192-5p, miR-202-3p, miR-337-5p, and miR-130-3p differentiated between the mucinous cysts, MCN and BD-IPMN (Figure 4). As current management of mucinous cysts follows the International Association of Pancreatology (IAP) consensus guidelines and requires surgical resection for MCN and MD-IPMN, but only a certain subpopulation of BD-IPMN,[27] this classifier may be helpful in improving differentiation between MCN and BD-IPMN. These findings are very exciting as to date, no other available test including imaging, cytology, CEA, and DNA markers allows separation of these two mucinous cystic lesions. Again, the clinical implications, if validated, are significant as IAP consensus guidelines recommend surgical resection of all MCN while surveillance is reasonable for most BD-IPMN.[27]

The use of archived surgical pathology tissue provides a rich reservoir of specimens to increase the power and validity of our findings but also presents a limitation when trying to extrapolate these findings to more pertinent clinical samples in the preoperative setting, such as cyst fluid. These same miRNA classifiers may not be observed in cyst fluid; however, we chose to initially analyze the readily available "gold standard" of surgical resection specimens to determine if differential miRNA expression could be observed among the various pancreatic cystic neoplasms. While our data are very

promising, future investigations will seek to investigate differential miRNA expression in pancreatic cyst fluid samples. In addition, our analysis was limited to BD-IPMN with low-grade dysplasia and MD-IPMN with intermediate-grade dysplasia, as these represented the most common subtypes in the surgical pathology files. Future studies will need to explore the ability of miRNA to differentiate BD-IPMN and MD-IPMN with varying degrees of dysplasia.

In conclusion, we report the largest, most comprehensive study to date of surgical pathology specimens demonstrating differential miRNA signatures among pancreatic cystic neoplasms. Specifically, we identified several miRNA biomarker classifiers that accurately diagnosed serous cystadenoma, mucinous cystic neoplasm, and IPMN in addition to discriminating mucinous cystic neoplasm from BD-IPMN.

CONFLICT OF INTEREST

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- A clinically available micro RNA (miRNA) classifier diagnoses pancreatic ductal adenocarcinoma (PDAC) from chronic pancreatitis and normal pancreas.
- MiRNA classifiers may allow the identification of intraductal papillary mucinous neoplasm (IPMN) with differing degrees of dysplasia.

WHAT IS NEW HERE

- Comprehensive miRNA profiling of pathology specimens revealed a 4-miRNA panel that differentiates serous cystadenoma from mucinous cysts and PDAC.
- Mucinous cystic neoplasms (MCN) are distinguished from serous cystadenoma, IPMN, and PDAC with a different 4-miRNA panel.
- MCN is separated from branch duct IPMN by a 4-miRNA panel including miR-192-5p, miR-202-3p, miR-337-5p, and miR-130-3p.

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