Elevated microRNA miR-21 Levels in Pancreatic Cyst Fluid Are Predictive of Mucinous Precursor Lesions of Ductal Adenocarcinoma

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Key Words

Pancreatic cyst • Intraductal papillary mucinous neoplasm • Mucinous cystic neoplasm • microRNA • Biomarker

Abstract

Background: Biomarkers for the diagnostic classification of pancreatic cysts are urgently needed. Deregulated microRNA (miRNAs) expression is widespread in pancreatic cancer. We assessed whether aberrant miRNAs in pancreatic cyst fluid could be used as potential biomarkers for cystic precursor lesions of pancreatic cancer. **Methods:** Cyst fluid specimens were prospectively collected from 40 surgically resected pancreatic cysts, and small RNAs were extracted. The ‘mucinous’ cohort included 14 intraductal papillary mucinous neoplasms (including 3 with an associated adenocarcinoma) and 10 mucinous cystic neoplasms; the ‘nonmucinous’ cohort included 11 serous cystadenomas and 5 other benign cysts. Quantitative reverse transcription PCR was performed for five miRNAs (miR-21, miR-155, miR-221, miR-17-3p, miR-191), which were previously reported as overexpressed in pancreatic adenocarcinomas. **Results:** Significantly higher expression of miR-21, miR-221, and miR-17-3p was observed in the mucinous versus nonmucinous cysts ($p < 0.01$), with

the mean relative fold differences being 7.0-, 7.9-, and 5.4-fold, respectively. Receiver operating characteristic curves demonstrated the highest median area under the curve for miR-21, with a median specificity of 76%, at a sensitivity of 80%. **Conclusion:** This pilot study demonstrates that profiling miRNAs in pancreatic cyst fluid samples is feasible and can yield potential biomarkers for the classification of cystic lesions of the pancreas.

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Introduction

Pancreatic adenocarcinoma (aka pancreatic cancer) is an almost uniformly fatal disease, which claims the lives of >37,000 Americans each year [1]. The overwhelming majority of patients with pancreatic cancer presents with surgically inoperable disease, such that the only options available remain systemic chemotherapy, which has proven to have minimal efficacy [2]. Therefore, the best hope for ameliorating the dismal prognosis of pancreatic cancer rests on its early detection at an operable, and hence, potentially curable, stage of disease. Recent studies documenting as much as two decades ‘lead time’ from the

initiation of pancreatic neoplasia to the onset of metastatic disease further underscores the window of opportunity for early detection strategies [3].

It is now well established that pancreatic cancer arises through a series of noninvasive precursor lesions, which culminate in invasive neoplasia [4]. The most common precursor lesion is microscopic in nature, and is denoted as pancreatic intraepithelial neoplasia or PanIN [5]. With rare exceptions, PanINs are undetectable by radiological imaging and are only observed histologically in surgical resection specimens, typically in the parenchyma adjacent to invasive adenocarcinoma. In contrast, the two macroscopic cystic precursor lesions of invasive pancreatic cancer, intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) – are detectable with abdominal imaging [6, 7]. Despite clinical and pathological distinctions, both cystic precursor lesions are characterized by a mucin-producing neoplastic epithelium, which distinguishes them from other so-called ‘nonmucinous’ cystic lesions arising in the pancreas that harbor minimal neoplastic potential [7–9]. Once a pancreatic ‘pseudocyst’ has been excluded, the most common nonmucinous cyst of the pancreas is the serous cystadenoma (SCA). Other less common nonmucinous cysts include lymphangiomas and hemangiomas, lymphoepithelial cysts, duplication cysts, and rarely, cystic degeneration within a well-differentiated pancreatic neuroendocrine tumor [8, 9]. Clearly, a biologically and clinically diverse and somewhat confounding array of clinical entities can produce cysts in the pancreas.

The most frequent cystic lesion in the pancreas is a pancreatic pseudocyst (>90%), while SCAs, MCNs and IPMNs account for less than 10%. Of note, most pancreatic pseudocysts are symptomatic; however, and once these are excluded, the majority of asymptomatic cysts of the pancreas are comprised of the remaining three entities listed above. In recent years, the expanded use of abdominal imaging has resulted in the detection of an ever increasing number of asymptomatic pancreatic cysts (incidentalomas) [6, 10]. Histological analyses of surgically resected asymptomatic cysts from several independent series suggest that as many as half are mucin-producing precursor lesions (either IPMNs or MCNs) [11–13]. Thus, detection of an asymptomatic cyst in the pancreas represents a unique opportunity for secondary prevention of an invasive pancreatic cancer. At the same time, the dictum of ‘*primum non nocere*’ mandates that clinicians make every attempt to spare patients with clinically insignificant cysts the rigors of an unnecessary pancreatic surgery. Irrespective of their histogenesis, cer-

tain cystic lesions of the pancreas require surgical resection, either because they become symptomatic, or because of suggestive radiological features of an underlying carcinoma [14, 15]. Nonetheless, a substantial proportion of asymptomatic cysts present a continuing challenge for gastroenterologists and surgeons alike vis-à-vis their best course of diagnosis and management.

The most important clinical tools available for the diagnosis of pancreatic cystic lesions are cross-sectional imaging, endoscopic ultrasound (EUS), and cyst fluid analysis [16]. Even as EUS is rapidly becoming the imaging modality of choice for a variety of pancreatic diseases, this modality may not be sufficiently reliable in differentiating amongst various cystic lesions. The reported accuracy of EUS imaging alone for differentiating benign from premalignant or malignant pancreatic cysts ranges from 40 to 93% [17–19]. In addition to imaging, aspiration of cyst fluid contents can be conveniently and safely performed by EUS-guided fine-needle aspiration, which provides material for cytopathology and the assessment of tumor markers. Unfortunately cystic lesions, in general, are fairly hypocellular, such that the sensitivity of cytology examination remains low (~50%) despite a high specificity [20–22]. Thus, there is an unmet need for ancillary biomarkers in cyst fluid material that can provide additional and reliable distinction between mucinous cystic precursors of pancreatic cancer versus clinically insignificant nonmucinous lesions.

microRNAs (miRNAs) are a diverse class of 18–24 nucleotide noncoding RNA molecules, whose principal function is to regulate the stability and translation of nuclear mRNA transcripts [23, 24]. Aberrant miRNA expression is widespread in human cancers, including pancreatic adenocarcinomas, as we and others have demonstrated [25–28]. Recent studies from our group [29, 30], and others [31], have also established aberrant miRNA expression in precursor lesions of pancreatic cancer, reinforcing the notion that abnormal miRNA expression can be observed early during multistep carcinogenesis. In addition, cancer-associated miRNAs, which are shed by neoplastic cells, are readily detectable in the peripheral circulation of patients [32], and are emerging as candidate biomarkers for the early detection of cancer [33–35]. We postulated that aberrantly expressed miRNAs generated by cystic precursor lesions of pancreatic adenocarcinoma might similarly be released into the cyst fluid, and thus be utilized as ancillary diagnostic markers. In this pilot study, we demonstrate the feasibility of utilizing pancreatic cyst fluid samples as a substrate for miRNA detection, and confirm the presence of aberrantly expressed

Table 1. Clinical and pathological features of pancreatic cystic lesions

Pathologic diagnosis	Subjects	Mean age years	Males: females
Nonmucinous cyst	16	58	3:13
SCA	11	63	3:8
Hemangioma	2	38	0:2
Lymphangioma	1	53	0:1
Lymphoepithelial cyst	1	60	0:1
Duplication cyst	1	53	0:1
Mucinous cyst	24	57	9:15
IPMN	11	66	7:4
MCN	10	42	1:9
IPMN with inv. cancer	3	72	1:2

inv. = Invasive.

miRNAs in these samples. We also identify miR-21 as a promising biomarker for the distinction of mucinous precursor lesions (IPMNs and MCNs) from other non-mucinous cysts of the pancreas.

Materials and Methods

Pancreatic Cyst Fluid Collection

Pancreatic cyst fluid specimens were obtained from a series of 54 patients who underwent surgical resection at the Johns Hopkins Hospital during the period January 2008 to February 2009, with an underlying diagnosis of a pancreatic cyst. Cyst fluid specimens were obtained ex vivo by aspiration in the Surgical Pathology suite, shortly after the surgical removal of cystic lesions. Aliquots were made from the cyst fluid, and were stored at -80°C until further analysis. A minimum of 400 μl of cyst fluid was used for RNA extraction (described below), and thus 14 samples with volumes $<400 \mu\text{l}$ were not included for analysis. Of the remaining 40 cyst fluid specimens, histological validation of the cyst lining was available for each case ('gold standard') using established diagnostic criteria [36]. This sample set included 24 cystic precursor lesions, including 14 IPMNs and 10 MCNs. Of the IPMNs, 11 were noninvasive lesions, while 3 had an associated invasive component; all of the 10 MCNs were noninvasive (table 1). The 16 non-mucinous cysts included 11 cases of SCA, and 5 additional benign cysts (two hemangiomas, and one each of a lymphangioma, lymphoepithelial cyst, and duplication cyst). The physical appearance of the cyst fluid sample is tabulated in online supplementary table 1 (for all supplementary material, see www.karger.com/doi/10.1159/000329183; some of the biospecimens obtained were exhausted, and unavailable for gross characterization). The indications for surgery in the nonmucinous cysts fell into one of two categories: either the patient was symptomatic from the underlying cyst, or preoperative imaging (abdominal computed tomography, CT, scan or magnetic resonance imaging)

could not conclusively exclude a mucinous neoplasm (this was the most common cause for resection of SCAs included in our series).

miRNA Extraction and Quantitative Reverse Transcription PCR

After thawing at 4°C , 400 μl of cyst fluid was subjected to small RNA extraction using the mirVanaTM miRNA isolation kit (Ambion/Applied Biosystems, Austin, Tex., USA) according to the manufacturer's protocol. Quantitative reverse transcription PCR (qRT-PCR) for miRNAs was performed using pre-designed TaqMan[®] miRNA assays (Applied Biosystems, Foster City, Calif., USA) with the 7300 Real-Time PCR system (Applied Biosystems). The assays are two-step protocol, including reverse transcription with human mature miRNA-specific RT primers, followed by real-time PCR with miRNA specific primers, as previously described [29, 30]. These assays target only the mature miRNA sequence and the precursors are not detected. We selected a panel of five miRNAs (miR-21, miR-155, miR-17-3p, miR-191, and miR-221) which were previously described as significantly overexpressed in invasive pancreatic cancers [25–28] as candidates for analysis. The noncoding RNU6B (U6 control) was used as the normalization control. Each cyst fluid sample was assessed in triplicate for any given miRNA. The relative fold expression of the miRNAs was calculated utilizing the comparative C_t (ΔC_t) method [37].

Statistical Analysis

Statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, Ill., USA) and R (<http://cran.r-project.org/>). Continuous variables are presented as mean with standard deviation (SD). The unpaired Student t test was used to compare the ΔC_t values of miRNAs expressed in the mucinous versus nonmucinous cyst fluid samples, and a stringent p value <0.01 was considered statistically significant. The depiction of miRNA differential expression was generated using GraphPad Prism version 5. Receiver operating characteristic (ROC) curves were generated for each miRNA in order to calculate the area under the curve (AUC), a measure of the predictive power for accurately classifying the nature of the cystic lesion. To produce highly stringent (99%) confidence bands for each ROC curve, we implemented a bootstrap resampling procedure. That is, after calculating the mean ΔC_t value for each sample, averaging over all repeated qRT-PCR measurements, we repeatedly simulated new, random datasets by sampling with replacement from these values, so that some were used repeatedly, while others left out at each iteration. The median ROC curve is shown as a solid black line in each plot, while the upper and lower bounds of the 99% confidence bands are represented by broken black lines. Target performance levels of 80% sensitivity and 50% specificity are represented by horizontal and vertical lines, respectively.

Results

A Subset of miRNAs Is Upregulated in the Cystic Precursor Lesions versus Nonmucinous Cysts

Using the ΔC_t method [37], we identified a significantly higher relative fold expression of three miRNAs – miR-21, miR-221, and miR-17-3p – in the mucinous precursor

Fig. 1. Expression profiles of miR-21, miR-221, miR-17-3p, and miR-155 in pancreatic cyst fluid samples. The indicated miRNA target was evaluated by qRT-PCR on small RNAs extracted from 16 nonmucinous and 24 mucinous cysts. Each sample was assessed in triplicate, and the C_t values for individual miRNA biomarkers were normalized to those of U6 noncoding RNA. The horizontal bars represent mean ΔC_t value. The significance level is designated on the panel. Data for miR-191 are not shown.

lesions versus the nonmucinous cysts ($p < 0.01$; fig. 1). In the ΔC_t method, a higher ΔC_t value corresponds to lower expression levels, and vice versa. Thus, the mean ΔC_t values for miR-21 were 9.08 (SD 2.37; 95% CI, 7.82–10.34) and 6.28 (SD 1.61; 95% CI, 5.60–6.95) in the nonmucinous versus mucinous cyst groups, respectively ($p = 0.002$). Similarly, the mean ΔC_t values for miR-221 were 11.37 (SD 2.34; 95% CI, 10.12–12.61) and 8.38 (SD 2.78; 95% CI, 7.21–9.55) in the nonmucinous versus mucinous cyst groups, respectively ($p = 0.006$). Finally, the mean ΔC_t values for miR-17-3p were 12.2 (SD 2.16; 95% CI, 11.05–13.35) and 9.77 (SD 2.64; 95% CI, 8.66–10.88) in the nonmucinous versus mucinous cyst groups, respectively ($p = 0.001$). These ΔC_t values correspond to a relative fold overexpression of 7.0-, 7.9-, and 5.4-fold, respectively, for miR-21, miR-221 and miR-17-3p in the mucinous cysts versus nonmucinous lesions. The expression of miR-155 was not significantly different (mean ΔC_t of 8.15 in the nonmucinous versus 5.92 in mucinous, $p = 0.045$), although there was a trend towards relative upregulation in the mucinous cyst group. In the examined panel, miR-191 demonstrated comparable expression profiles between the two groups ($p = 0.28$; data not shown). Finally, we examined whether there were any significant differences in ΔC_t values between the two mucinous cystic lesions

(IPMNs and MCNs), and none reached statistical significance (p values for miR-21, miR-221, miR-155, miR-17-3p, and miR-191 being 0.73, 0.92, 0.56, 0.65, and 1, respectively).

Performance Characteristics for Cyst Fluid miRNAs in Differentiating between Mucinous and Nonmucinous Pancreatic Cystic Lesions

Since miR-21, miR-221 and miR-17-3p showed significant differences in expression profiles, we generated ROC curves for each of the three miRNAs in order to determine the sensitivity and specificity performance characteristics for the diagnosis of mucinous cystic lesions. As seen in figure 2, both miR-221 and miR-17-3p achieve a sensitivity of 80% at a specificity of 50%, although in each case, the lower confidence bound gives a specificity of less than 20% when the sensitivity is 80%. On the other hand, miR-21 shows very robust performance, with the entire confidence band passing through the performance target represented in the upper, left quadrant of the plot (median AUC of 0.89). In fact, the median specificity is 76%, at a sensitivity of 80%, while at the other end we achieve a remarkable sensitivity of 96% when the specificity is 50%.

Fig. 2. Performance characteristics of miR-21, miR-221 and miR-17-3p in pancreatic cyst fluid. Target performance levels of 80% sensitivity and 50% specificity are represented by horizontal and vertical lines, respectively.

Discussion

Currently, the most accurate and noninvasive visual assessment of the pancreas can be obtained using cross-sectional imaging modalities including contrast enhanced CT and magnetic resonance imaging, as well as by EUS [16, 38]. In particular, EUS not only allows for the evaluation of structural alterations within the parenchyma, but the added possibility of a simultaneous fine needle aspiration biopsy provides a unique opportunity to collect pancreatic cyst fluid, and to subsequently analyze this for cytology and other laboratory tests [39–41]. Cells that are shed from the cystic epithelium (and the nucleic acids and proteins released from these cells) represent a readily accessible proximate source of candidate biomarkers for evaluating the biological potential of a given cyst. Unfortunately, conventional cytology has proved to be rather unreliable in this regard, as the specimens frequently contain scant material or the biopsy samples are contaminated by tissue originating from the route traversed by the EUS needle (such as the gastric mucosa), compromising the sensitivity of the assay [41, 42]. In contrast, the cyst fluid per se is considered to be an extremely promising clinical specimen for biomarker studies.

Both biochemical and molecular cyst fluid analyses are a focus of ongoing research. For example, the diagnostic value of the tumor-specific antigens secreted by the neoplastic epithelium, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 and cancer antigen 125, has been analyzed in numerous studies [42–45]. Of these, cyst fluid CEA appears to have the most promising performance criteria so far in distinguishing mucinous from nonmucinous cystic lesions of the pancreas. In

a cooperative cyst study, a cyst fluid CEA level >192 ng/ml had the highest AUC for differentiating mucinous from nonmucinous cystic lesions (0.79), with an accuracy that exceeded either EUS morphology or cytology [42]. A meta-analysis of 12 pancreatic cyst fluid studies reported that a CEA level >800 ng/ml had a 48% sensitivity, but as much as 98% specificity for diagnosing mucinous lesions of the pancreas [44]. Nonetheless, some limitations in the predictive value of CEA alone have recently been reported by Raval et al. [46], who found significantly elevated CEA levels (>450 ng/ml) in 3 out of 9 lymphoepithelial cysts, which harbor no precancerous potential. Another potential limitation of CEA assessment is that in most clinical laboratories a relatively large volume of cyst fluid is mandated in order to render a reliable analysis. Therefore, this assay is often not feasible in smaller cystic lesions.

Similarly, cyst fluid DNA analysis has emerged as another promising approach for pancreatic cyst classification. For example, Khalid et al. [47] measured amount and quality of DNA, and genetic alterations (somatic *KRAS* mutations and loss of heterozygosity, LOH, analysis with polymorphic microsatellite markers) in order to differentiate benign from malignant pancreatic cysts. In their pilot study, the combination of *KRAS* gene mutations paired with a LOH was highly predictive of malignancy within a pancreatic cyst. The authors validated these initial findings with a subsequent multicenter Pancreatic cyst DNA Analysis (aka PANDA) study [48]. In this trial, cyst fluid *KRAS* gene mutations were predictive of a mucinous cyst, while additional features such as el-

evated amounts of total cyst fluid DNA, and the combination of *KRAS* gene mutations with microsatellite LOH were predictive of malignancy. Other studies have also confirmed that combining molecular analysis with cyst fluid CEA levels has an additive value in distinguishing mucinous from nonmucinous lesions [49, 50].

The objective of this study was to establish the feasibility of performing miRNA analysis in pancreatic cyst fluid specimens, as we postulated that we could discern tangible differences in miRNA expression between mucinous and nonmucinous cystic lesions. Aberrations of miRNA expression are widespread across human cancers [25–28], and as Lu et al. [51] have reported, noncoding RNA profiles can reliably classify cancer subtypes, including outperforming global coding gene expression as classifiers. We and several groups have reported recurrent miRNA alterations in pancreatic cancer, and our group was one of the first to report comparable abnormalities in noninvasive precursor lesions of ductal adenocarcinomas [29–30]. Thus, Habbe et al. [29] assessed the relative expression levels of a panel of twelve miRNAs known to be upregulated in pancreatic cancer in 15 noninvasive IPMNs, and found miR-21 (mean 12.1-fold) and miR-155 (mean 11.6-fold) to be differentially expressed, when compared to normal pancreas. These promising findings in IPMN tissues encouraged us to focus on miRNA expression in cyst fluid samples.

One of the compelling advantages of the relative short mature miRNAs is the lack of propensity for degradation in biospecimens, a feature that plagues coding mRNAs. Weber and colleagues [52] examined the spectrum of miRNAs expressed in twelve varieties of body fluids, such as plasma, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid, and seminal fluid, obtained from normal individuals. They were able to identify differential expression patterns of miRNAs amongst the various biospecimen sources. As a proof of principle, urine samples from individuals with different conditions, including pregnancy, renal cancer, and bladder cancer were analyzed, and the distinct miRNA expression profiles confirmed. Several studies, including one from our group [33–35], have now identified aberrant circulating miRNAs in the serum of patients with cancer. In addition, aberrant miRNAs have been identified in aspiration cytology specimens and shown to be useful in distinguishing pancreatic cancer from chronic pancreatitis [53].

We assessed the relative expression of five miRNAs (miR-21, miR-221, miR-17-3p, miR-155, and miR-191) in

a panel of 40 cyst fluid samples obtained from 24 mucinous and 16 nonmucinous cysts of the pancreas. We used relatively minimal quantities of cyst fluid (400 μ l) for miRNA extraction, a volume that should be amenable to aspiration from most pancreatic cystic lesions. Three of the four miRNAs (miR-21, miR-221, and miR-17-3p) demonstrated highly significant differences in ΔC_t values ($p < 0.007$ for all), which corresponded to a higher relative expression of 7.0-, 7.9-, and 5.4-fold, respectively, for miR-21, miR-221 and miR-17-3p in the mucinous cysts versus nonmucinous lesions. Using ROC curves, miR-21 had the best performance criteria, as assessed by a median AUC of 0.89, and a median specificity of 76% at a sensitivity of 80% in differentiating mucinous versus nonmucinous lesions. Notably, the combination of additional miRNAs with miR-21 did not further improve the AUC, underscoring the ‘primacy’ of miR-21. In light of the very few samples with associated invasive cancer ($n = 3$), we did not attempt to determine if miR-21, or other miRNAs, can further differentiate invasive from noninvasive lesions within the subset of mucinous precursor cysts, and this rather important question will be addressed in future cyst fluid studies. Nonetheless, the emergence of miR-21 as the most promising biomarker candidate in cyst fluid is not surprising, given its preeminent role as one of the original ‘onco-miRs’ in human neoplasia [54], and its well-documented overexpression in noninvasive precursor lesions of pancreatic cancer [29, 30].

A potential criticism of this study is that we preselected four miRNAs essentially based on their aberrant expression in pancreatic adenocarcinomas, rather than by ‘forward genetics’ profiling of IPMN and MCN tissues. This could certainly have impacted the final outcome of our study, since there is no prerequisite that miRNAs altered in ductal adenocarcinomas should *prima facie* be abnormal in cystic precursor lesions. In fact, as prior studies have shown, IPMNs and MCNs harbor certain genetic differences when compared to ‘usual’ ductal adenocarcinomas, prominent amongst them being mutations of the *PIK3CA* and *STK11* genes, and the retention of Smad4 function until fairly late in their multistep progression [55–58]. Thus, an unbiased miRNA profiling study might reveal additional candidates besides miR-21 that could enhance the assay sensitivity and specificity, likely as a multiplex miRNA panel on cyst fluid. Another shortcoming of this study is the absence of concomitant CEA levels in the samples; however, our intent was to maximize available cyst fluid volumes for RNA extraction and the requirements for current CEA assays (~ 1 ml) would have depleted many of the study cases from

miRNA analysis. Finally, this study was conducted on cyst fluid samples obtained from surgical resection specimens, since our intent was to ensure that we have confirmed histopathology on all cysts. A prospective trial of patients with pancreatic cysts where cyst fluid samples are obtained by EUS prior to therapeutic stratification will help cement the role of miRNAs as a diagnostic biomarker.

In conclusion, we present a pilot study demonstrating the feasibility of miRNA analysis using pancreatic cyst fluid as a substrate. Our study affirms that as little as 400 μ l of pancreatic cyst fluid contains sufficient nucleic acids to interrogate at least a limited panel of five miRNAs, and that such analysis can help differentiate mucinous from nonmucinous pancreatic cystic lesions based on the

level of expression for miR-21. Future tissue profiling studies will help optimize the miRNA 'panel' that needs to be selected for improving the performance criteria of a cyst fluid-based assay, and determine how miRNA analysis compares with other modalities such as tumor-specific antigens and mutant DNA.

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