A MicroRNA-Based Test Improves Endoscopic Ultrasound–Guided Cytologic Diagnosis of Pancreatic Cancer

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BACKGROUND & AIMS: Endoscopic ultrasound–guided fine-needle aspiration (EUS-FNA) in combination with cytology is the optimal method for diagnosis and staging of pancreatic ductal adenocarcinoma (PDAC) and other pancreatic lesions. Its clinical utility, however, can be limited by high rates of indeterminate or false-negative results. We aimed to develop and validate a microRNA (miRNA)-based test to improve preoperative detection of PDAC.

METHODS: Levels of miRNAs were analyzed in a centralized clinical laboratory by relative quantitative polymerase chain reaction in 95 formalin-fixed paraffin-embedded specimens and 228 samples collected by EUS-FNA during routine evaluations of patients with solid pancreatic masses at 4 institutions in the United States, 1 in Canada, and 1 in Poland.

RESULTS: We developed a 5-miRNA expression classifier, consisting of MIR24, MIR130B, MIR135B, MIR148A, and MIR196, that could identify PDAC in well-characterized, formalin-fixed, paraffin-embedded specimens. Detection of PDAC in EUS-FNA samples increased from 78.8% by cytology analysis alone (95% confidence interval, 72.2%–84.5%) to 90.8% when combined with miRNA analysis (95% confidence interval, 85.6%–94.5%). The miRNA classifier correctly identified 22 additional true PDAC cases among 39 samples initially classified as benign, indeterminate, or nondiagnostic by cytology. Cytology and miRNA test results each were associated significantly with PDAC (P < .001), with positive predictive values greater than 99% (95% confidence interval, 96%–100%).

CONCLUSIONS: We developed and validated a 5-miRNA classifier that can accurately predict which preoperative pancreatic EUS-FNA specimens contain PDAC. This test might aid in the diagnosis of pancreatic cancer by reducing the number of FNAs without a definitive adenocarcinoma diagnosis, thereby reducing the number of repeat EUS-FNA procedures.

Keywords: Pancreatic Cancer; MicroRNA; Cytologic Diagnosis; Fine-Needle Aspirate.

Abbreviations used in this paper: AUC, area under the receiver operating characteristic curve; CP, chronic pancreatitis; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; FFPE, formalin-fixed paraffin-embedded; miRNA/miR, microRNA; NET, neuroendocrine tumor; PDAC, pancreatic ductal adenocarcinoma.
The prognosis for pancreatic cancer remains poor, with 5-year survival rates of 6%. Despite recent genetic evolution studies suggesting that metastatic pancreatic ductal adenocarcinoma (PDAC) takes 15 to 20 years to develop, even those patients undergoing potentially curative pancreatic resection will die of metastatic disease. This is supported by review of recent Surveillance, Epidemiology, and End Results Program (SEER) data showing a 5-year survival rate of 22% in the 8% of patients presenting with localized pancreatic cancer. As a result, a growing number of centers began to use neoadjuvant therapy to identify the subset of patients who clearly do not benefit from surgical resection and to initiate systemic therapy earlier in a patient’s treatment course.

Treatment strategies involving neoadjuvant therapy require a preoperative diagnosis to confirm that the patient has PDAC. Because of the high accuracy rates for diagnosing pancreatic cancer compared with other imaging-guided approaches, endoscopic ultrasound–guided fine-needle aspiration (EUS-FNA) has become the preferred modality for obtaining pathologic confirmation. Studies over the past decade have reported a sensitivity range from 60% to 100% for the diagnosis of PDAC by EUS-FNA. Many factors can impact the diagnostic yield of EUS-FNA including the experience of an endosonographer and a cytologist, availability of on-site cytology, and the inherent limitations of the procedure to identify cytomorphologic features characteristic of well-differentiated cancer, in particular in the setting of chronic pancreatitis (CP). Sensitivity also can be compromised by technical factors such as sampling errors, insufficient cellularity, and the presence of fibrosis or blood.

Recent strategies to improve the diagnostic accuracy of EUS-FNA for PDAC are based on our growing understanding of the pathogenesis of PDAC. MicroRNAs (miRNAs) have unique features, which have made them a particularly promising class of candidate biomarkers for improved management of many human cancers, including pancreatic cancer. miRNAs are stable and easily recovered from formalin-fixed paraffin-embedded (FFPE) tissues as well as from clinical specimens with limited tissue yield, such as FNAs. To date, aberrant up-regulation of specific miRNAs, namely miR-196a, miR-196b, miR-135b, miR-130b, miR-148a, miR-155, miR-210, miR-221, and miR-222, has been associated with a diagnosis of PDAC. Fewer miRNAs were reported to be down-regulated in PDAC, including miR-130b, miR-148a, miR-148b, miR-216, miR-217, and miR-96. Self-normalizing miRNA pairs such as miR-24 and miR-135b or miR-196a and miR-217 have been shown to discriminate between PDAC and CP with a sensitivity and specificity greater than 91% in FFPE specimens. Similar combinations of aberrantly expressed miRNAs also have been applied successfully in serum, plasma, and cystic fluid.

Herein, we propose that the EUS-FNA sampling method may be well suited to molecular diagnostic testing, in which the specimen’s low cellularity or the presence of interfering agents can be compensated for by detection of highly specific and sensitive biomarkers. We report the development and clinical validation of the first miRNA classifier that can aid in the preoperative diagnosis of PDAC in EUS-FNAs with benign, indeterminate, or nondiagnostic FNA cytology.

Materials and Methods

Specimens and Patients

The collection of archived FFPE specimens for classifier training was performed according to a protocol approved by the Ethics Committee of the Ruhr-University Bochum (permission no. 3534-09 and 2392-04). The prospective collection of FNAs for classifier validation occurred between November 2008 and October 2011 following a study protocol approval by the Institutional Review Boards or Ethnic Committees at 8 participating institutions. Study eligibility criteria were as follows: age 21 or older, no previous history of pancreatic cancer or other malignancies, and EUS-FNA indicated for diagnostic work-up of a solid pancreatic lesion. One FNA pass from each study subject was collected in RNASave (Asuragen, Austin, TX) and shipped to a centralized Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory in Austin, Texas, where nucleic acid extraction and molecular testing were performed in a blinded manner. Patient demographics are summarized in Supplementary Table 1.

Molecular Diagnosis

miRNA expression levels in FFPE and FNA specimens were assessed by reverse-transcription and relative quantitative polymerase chain reaction. A miRNA-based predictive model using linear discriminate analysis was parameterized on 95 FFPE specimens to differentiate between PDAC (score, ≥0.5) and CP (score, <0.5). The optimal miRNA classifier then was applied directly to prospectively collected FNAs in a blinded manner to validate its predictive value for the identification of PDAC (score, ≥0.5). Additional information on molecular methods, specimen adequacy criteria, and miRNA model building and selection is presented in the Supplementary Materials and Methods.

Cytology Diagnosis

The diagnosis used for assessing the performance of cytology in prospectively collected FNAs was based on the official cytopathology reports at each study site and was categorized as follows: (1) benign, (2) atypical, (3) suspicious, (4) nondiagnostic, (5) adenocarcinoma, or (6) other malignancy. Categories 2 and 3 are cumulatively
referred to in this article as indeterminate and categories 1, 2, 3, and 4 are referred to as nonmalignant.

**Clinical Diagnosis**

All patients were followed up for the duration of the study (3.5 y) with a required minimum time of at least 12 months after patient enrollment. A final diagnosis of PDAC (n = 184) was determined based on one, or a combination of, the following: (1) pathologic diagnosis after resection of the primary tumor (n = 54), (2) pathologic confirmation of PDAC by biopsy or cytology of the primary tumor or a metastatic site on a subsequent FNA (n = 7), (3) PDAC cytologic diagnosis at time of study, confirmed by clinical follow-up evaluation (n = 102), or (4) clinical progression judged by evolving local invasion or metastatic disease on follow-up imaging, increasing carbohydrate antigen 19-9 levels, or death from apparent disease progression (n = 21). A final benign diagnosis was based on surgical resection or clinical follow-up evaluation (≥18 months without symptoms or imaging findings concerning for an underlying neoplasm).

**Statistical Analyses**

Diagnostic performance of the 5-miRNA classifier was calculated using standard binary statistics for qualitative molecular test results (score, ≥0.5 or <0.5) relative to the histology reference standard for FFPE specimens (PDAC or CP) or the clinical diagnosis reference standard for FNA specimens (PDAC or benign). P values were calculated using the Fisher exact test for categoric variables or logistic regression for continuous variables and multivariate analyses. All miRNA scores for the FFPE and FNA specimens were generated and interpreted in a blinded manner. All corresponding clinical and molecular data are reported in Supplementary Tables 2 and 3.

**Results**

**MicroRNA Model Development in Pancreatic Formalin-Fixed Paraffin-Embedded Specimens**

A total of 95 FFPE specimens, 43 CP and 52 PDAC, were examined for expression levels of 11 miRNAs previously reported to be associated with PDAC (Figure 1).6,8–11,14 The performance of several different models was evaluated using the area under the receiver operating characteristic curve (AUC) and Youden’s index based on replicated 5-fold cross-validation. Initially, a linear discriminant analysis–based classifier consisting of miR-24, miR-96, miR-130b, miR-135b, miR-148a, miR-196a, and miR-375 was selected because it had the top AUC estimate (0.978) and Youden’s index (0.882). Further analysis showed that a classifier without miR-375 and miR-96, two miRNAs with the least significant contribution to the linear discriminant analysis model (<1% score variance), generated scores highly correlated with the initial 7-miRNA classifier (R² = 0.99) (Supplementary Figure 1). For 5-miRNA classifier scores of 0.5 or higher, defined as positive for PDAC, the overall classification agreement was 99% (94 of 95) and 95% (90 of 95) relative to the histology reference standard.

**Validation of the 5-MicroRNA Classifier in Pancreatic Endoscopic Ultrasound–Guided Fine-Needle Aspirations**

To validate the FFPE-trained 5-miRNA classifier in pancreatic EUS-FNAs, 289 specimens from 281 subjects with suspicion of pancreatic cancer were collected prospectively at 8 clinical sites. Fifty-six specimens were determined to be ineligible owing to a protocol deviation (46 specimens were collected without EUS guidance, 8 specimens were a duplicate of the same mass, 2 other: 1 mesenteric cyst and 1 specimen collected from patient with history of lung cancer) and 4 study subjects were lost to follow-up evaluation (Supplementary Figure 2). For the 229 FNAs included in the validation study, molecular testing at a centralized clinical laboratory resulted in a single PCR amplification failure (failure rate, 0.4%). Among the 228 EUS-FNAs with a valid 5-miRNA classifier result, 210 specimens had been collected from study subjects with a final diagnosis of PDAC (n = 184) or a benign pancreatic lesion (n = 26) (Figure 1). The remaining 18 subjects had other neoplasms: 13 neuroendocrine tumors (NET), 2 lymphomas, 1 cholangiocarcinoma, 1 ampullary...
carcinoma, and 1 primary pancreatic squamous cell carcinoma. The correlation between cytopathology diagnoses, molecular results, and final clinical diagnoses for the 228 eligible EUS-FNAs specimens is shown in Figure 2.

Of the 146 study subjects diagnosed with adenocarcinoma by cytology, 145 were determined to have PDAC at final diagnosis, 1 had an ampullary adenocarcinoma, and 130 FNAs had a miRNA score of 0.5 or higher. In the benign cytology category, 9 of 27 study subjects were later diagnosed with PDAC and the miRNA classifier correctly identified 3 of those cases.

There were 40 specimens (17.5%) with indeterminate (atypical or suspicious) and nondiagnostic cytologic diagnoses. Among the 34 indeterminate diagnoses, 19 had a miRNA PDAC score of 0.5 or higher, including 17 with a confirmed PDAC outcome, 1 with a cholangiocarcinoma, and 1 with a benign outcome. Among 6 nondiagnostic FNAs, 2 of 3 study subjects with a PDAC outcome had a miRNA classifier score of 0.5 or higher. Because the study was fully blinded, the 15 specimens with a cytologic diagnosis of other malignancy also were evaluated and analyzed.

**Performance of MicroRNA Classifier and Cytopathology for Diagnosing Pancreatic Ductal Adenocarcinoma in Endoscopic Ultrasound–Guided Fine-Needle Aspirations**

The diagnostic sensitivity and specificity of the 5-miRNA classifier for the 210 specimens with a final diagnosis of PDAC (n = 184) or benign lesions (n = 26) was 82.6% (152 of 184) and 96.1% (25 of 26), respectively, with an AUC of 0.936 (Table 1 and Supplementary Figure 3). In the same set of 210 specimens, 78.8% (145 of 184) of PDAC cases and 69.2% (18 of 26) of benign cases were identified correctly by cytopathology. As expected, age and cytology, but not sex, were associated with a PDAC outcome in univariate analyses (P < .001) (Table 2). However, only cytology and molecular testing remained significant predictors of PDAC when incorporated into multiple logistic regression models. Similarly, the miRNA classifier score was associated strongly with cytology and PDAC outcome in both univariate and multivariate analyses (Table 2).

When combining the cytology and molecular results (cytopathologic diagnosis of adenocarcinoma or miRNA score ≥0.5 = positive for PDAC), the diagnostic sensitivity and specificity were 90.8% (167 of 184) and 96.1% (25 of 26), respectively (Table 1). The positive diagnostic gain of molecular testing compared with cytology alone

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**Table 1. Performance of Cytology and/or Molecular Testing in EUS-FNA Specimens**

<table>
<thead>
<tr>
<th>Diagnostic modality</th>
<th>Specimen, n</th>
<th>Malignant detection rate (95% CI), %</th>
<th>Benign detection rate (95% CI), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology alone</td>
<td>210a</td>
<td>78.8 (72.2–84.5)</td>
<td>69.2 (48.2–85.7)</td>
</tr>
<tr>
<td>Molecular alone</td>
<td>210a</td>
<td>82.6 (76.3–87.8)</td>
<td>96.1 (80.4–99.9)</td>
</tr>
<tr>
<td>Combined molecular cytology</td>
<td>210a</td>
<td>90.8 (85.6–94.5)</td>
<td>96.1 (80.4–99.9)</td>
</tr>
<tr>
<td>Cytology alone</td>
<td>213b</td>
<td>78.1 (71.5–83.8)</td>
<td>69.2 (48.2–85.7)</td>
</tr>
<tr>
<td>Molecular alone</td>
<td>213b</td>
<td>81.8 (75.5–87.1)</td>
<td>96.1 (80.4–99.9)</td>
</tr>
<tr>
<td>Combined molecular cytology</td>
<td>213b</td>
<td>90.4 (85.2–94.2)</td>
<td>96.1 (80.4–99.9)</td>
</tr>
<tr>
<td>Combined molecular cytology</td>
<td>228c</td>
<td>91.1 (86.3–94.6)</td>
<td>96.1 (80.4–99.9)</td>
</tr>
</tbody>
</table>

aIncludes all specimens with a final clinical diagnosis of PDAC (n = 184) or benign (n = 26), independent of initial cytology diagnosis.
bIncludes all specimens with adenocarcinoma, benign, indeterminate, or nondiagnostic cytology (n = 210 plus 1 ampullary carcinoma, 1 cholangiocarcinoma, and 1 NET).
cIncludes all specimens eligible for this study (n = 213 plus 15 non-PDAC malignancies identified by cytology).
was achieved through the correct identification of 22 PDAC cases with a miRNA score of 0.5 or higher in FNAs that were either indeterminate (n = 17), nondiagnostic (n = 2), or benign (n = 3) by cytology (Figure 2). The negative diagnostic gain was obtained in 7 EUS-FNAs with indeterminate (n = 4) or nondiagnostic (n = 3) cytology and a miRNA score of less than 0.5.

Performance of Molecular Cytology in Endoscopic Ultrasound–Guided Fine-Needle Aspiration Including Nonpancreatic Ductal Adenocarcinoma Mass Lesions

Among the 18 specimens with a final malignant diagnosis other than PDAC, 1 ampullary carcinoma had an initial cytologic diagnosis of adenocarcinoma whereas 1 distal cholangiocarcinoma and 1 NET were cytologically indeterminate (Figure 2). When these 3 cases were included in the analysis (n = 213 specimens), the performance of cytology alone, molecular alone, and combined molecular cytology was not affected significantly (Table 1). There were also 15 specimens with a cytologic diagnosis of NET (n = 12), lymphoma (n = 2), or squamous cell carcinoma (n = 1) (Figure 2, “other” category). All cases were confirmed at final diagnosis corresponding to an overall malignancy detection rate of 91.1% for all eligible subjects in our study (n = 228 specimens) (Table 1).

Predictive Value of Molecular Cytology in Endoscopic Ultrasound–Guided Fine-Needle Aspiration

Cytology was highly predictive for adenocarcinoma (146 of 146) (Figure 2) but could not differentiate between PDAC and ampullary carcinoma, two conditions that require the same type of surgical procedure, but potentially different neoadjuvant approaches. The corresponding probability of PDAC in FNAs with a cytopathologic diagnosis of adenocarcinoma was 99.3% (145 of 146) (Table 3). Among the 67 FNAs with a nonmalignant cytologic diagnosis (27 benign, 34 indeterminate, and 6 nondiagnostic), there were 39 subjects ultimately diagnosed with PDAC. The pretest probability of PDAC in this highly selected group of study subjects with sonographic findings concerning for a solid pancreatic lesion was 58.2% (39 of 67). With the inclusion of the single case of cholangiocarcinoma, a condition requiring the same surgical management as PDAC, the probability of PDAC/cholangiocarcinoma before molecular testing in the nonmalignant cytologic group was 59.7% (40 of 67). After molecular testing, the post-test probability of PDAC for FNAs with a miRNA score less than 0.5 was decreased to 39.5% and was increased to 95.8% for scores of 0.5 or greater. Overall, molecular testing correctly predicted 153 PDAC/cholangiocarcinoma cases of the 154 FNAs with a miRNA score of 0.5 or greater (99.4%) (Table 3). The only false-positive result was obtained in a patient who initially was evaluated for a biliary stricture by endoscopic retrograde cholangiopancreatography and was diagnosed clinically as multifocal side-branch intraductal papillary mucinous neoplasm with no signs of cancer during long-term follow-up evaluation.

Discussion

In the present work, we report on the successful development and independent validation of a 5-miRNA classifier for improved cytologic diagnosis of PDAC in EUS-FNAs. The classifier is composed of miRNAs reported by us and others to be expressed aberrantly in PDAC. Expression levels of miR-196a have been shown to increase progressively with the grade of precursor lesion (pancreatic intraepithelial neoplasm-2 and pancreatic intraepithelial neoplasm-3, suggesting that this miRNA may be valuable as an early diagnostic marker.5,14 miR-196a normalized to miR-217 as well as miR-135b normalized to miR-24 have been used previously to distinguish PDAC from CP.6,10,11 The remaining 2 miRNAs, miR-148a and miR-130b, have been shown to be down-regulated in pancreatic cancer.8–10

The use of archived surgical pathology FFPE tissue for classifier training and cross-validation enabled a direct
comparison of the miRNA expression data with the current gold standard—histology. It also presented a potential risk for the subsequent prospective validation study using smaller biopsy specimens collected by needle aspiration. Inadequate sampling or inaccurate needle positioning during the FNA procedure can result in highly variable cellular content, including blood, inflammatory processes, and/or cells of nonpancreatic origin. By selecting appropriate miRNA candidates and carefully designing the classifier, we were able to retain a high specificity and predictive value in FNAs collected in vivo. A principal component analysis using the combined 95 FFPE samples and 228 FNA biopsy specimens further showed a strong separation of the PDAC specimens from all other diagnostic entities independently of the sample types (Supplementary Figure 4).

Our prospective, multicenter validation study in EUS-FNAs showed that the 5-miRNA classifier can improve the performance of cytology alone. It also further expands the concept of risk-based diagnostic stratification in the personalized management of pancreatic cancer. Specifically, we propose to use molecular testing to aid in the evaluation of PDAC in EUS-FNAs with a cytologic diagnosis other than adenocarcinoma or other malignancies (Figure 3). Consistent with previous studies, the false-negative rate of cytology was 20% and the probability or risk of PDAC in FNAs with adenocarcinoma cytology was more than 99%, obviating the need for adjunct miRNA molecular testing in this cytologic group. Among the 67 patients without a definitive cytologic diagnosis of adenocarcinoma (benign, nondiagnostic, or indeterminate cytology), 41 had a final diagnosis of malignancy (39 PDAC, 1 cholangiocarcinoma, and 1 NET), corresponding to a 60% pretest probability of adenocarcinoma. After miRNA testing, the post-test probability of adenocarcinoma for specimens with a classifier score of 0.5 or greater was increased to 96%, thus accurately predicting PDAC. Importantly, those patients with a molecular diagnosis of PDAC would not require a repeat EUS diagnostic procedure. For specimens with a miRNA score less than 0.5, the post-test probability of PDAC was decreased to 40%. Unfortunately, this residual risk of malignancy was still too high to use the miRNA classifier to rule out PDAC. We therefore propose that a score less than 0.5 corresponds to an inconclusive molecular diagnosis and those patients should continue to be managed according to current standards of care based on clinical suspicion of PDAC. It also should be noted that both cytopathology and miRNA testing can be affected by the same inherent limitations of the EUS-FNA procedure and that sampling inaccuracy can contribute, at least in part, to some of the false-negative molecular results.

In our study, 46 FNAs also were collected without EUS guidance (ie, percutaneously), and were not included in the comparative analysis. Exploratory evaluation of these specimens with the 5-miRNA classifier showed a higher technical failure rate (6.5%) and a lower diagnostic sensitivity (58.3%) (Supplementary Figure 5). However, specificity and predictive value remained high at 89.5% and 87.5%, respectively, while the performance of cytopathology was compromised significantly in this FNA set (lower PDAC detection rate and higher nondiagnostic rate, P < .001). The absolute positive diagnostic gain provided by molecular testing therefore was similar to the one obtained with EUS-FNAs (P = .76), suggesting that miRNA-based molecular testing may help to overcome some of the well-documented challenges of percutaneous biopsy collection. Because all percutaneous FNAs originated from 2 non-US study sites, additional prospective validation studies including multiple sites and collection modalities will be required to fully validate the potential clinical utility of the 5-miRNA classifier in percutaneous FNAs.

Another limitation of our study was that cytologic diagnosis and miRNA testing had to be performed on distinct FNA specimens, which could have contributed to some of the observed discrepancies between cytology and molecular results. An additional challenge for data analysis and performance validation was to obtain an accurate final reference diagnosis for all eligible study subjects. Therefore, similar to other studies on EUS-FNAs, we used clinical follow-up evaluation for 18 months to establish the final diagnosis. Finally, our study was designed and powered solely to validate a PDAC-specific diagnostic miRNA signature. Although the results obtained with NET and other rarer clinical presentations are encouraging, additional work is needed to further improve the differential diagnosis of primary pancreatic malignancies and to increase the overall diagnostic yield of molecular testing in pancreatic aspirates.

In summary, our prospective multisite study showed that the use of centralized molecular testing in combination with local cytopathologic evaluation of preoperative pancreatic EUS-FNAs can improve the management
of pancreatic cancer patients. The use of the 5-mRNA classifier in conjunction with cytology (molecular cytology) in FNAs without a definitive adenocarcinoma diagnosis can help to clarify preoperative diagnosis by identifying PDAC and reducing the false-negative rate of FNA cytology. The effective validation of this novel molecular diagnostic tool warrants additional studies to determine whether miRNAs also may be used clinically for the prediction of a patient’s prognosis (eg, early disease progression in patients with resectable disease), or for guiding the choice of therapy.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Clinical Gastroenterology and Hepatology at www.cghjournal.org, and at http://dx.doi.org/10.1016/j.cgh.2014.02.038.

References


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Conflicts of interest
These authors disclose the following: Anna Szafranska-Schwarzbach, Alex Adai, Bernard Andruess, Dennis Wylie, Maura Lloyd, and Emmanuel Labourier are employees of Asuragen, Inc; and Randall Brand and Barbara Centeno are members of Asuragen, Inc’s Clinical Advisory Board. The remaining authors disclose no conflicts.

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