Cyst Fluid Biosignature to Predict Intraductal Papillary Mucinous Neoplasms of the Pancreas with High Malignant Potential

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BACKGROUND: Current standard-of-care technologies, such as imaging and cyst fluid analysis, are unable to consistently distinguish intraductal papillary mucinous neoplasms (IPMNs) of the pancreas at high risk of pancreatic cancer from low-risk IPMNs. The objective was to create a single-platform assay to identify IPMNs that are at high risk for malignant progression.

STUDY DESIGN: Building on the Verona International Consensus Conference branch duct IPMN biomarker review, additional protein, cytokine, mucin, DNA, and microRNA cyst fluid targets were identified for creation of a quantitative polymerase chain reaction-based assay. This included messenger RNA markers: ERBB2, GNAS, interleukin 1β, KRAS, MUCs1, 2, 4, 5AC, 7, prostaglandin E2R, PTGER2, prostaglandin E synthase 2, prostaglandin E synthase 1, TP63; microRNA targets: miRs 101, 106b, 10a, 142, 155, 17, 18a, 21, 217, 24, 30a, 342, 532, 92a, and 99b; and GNAS and KRAS mutational analysis. A multi-institutional international collaborative contributed IPMN cyst fluid samples to validate this platform. Cyst fluid gene expression levels were normalized, z-transformed, and used in classification and regression analysis by a support vector machine training algorithm.

RESULTS: From cyst fluids of 59 IPMN patients, principal component analysis confirmed no institutional bias/clustering. Lasso (least absolute shrinkage and selection operator)-penalized logistic regression with binary classification and 5-fold cross-validation used area under the curve as the evaluation criterion to create the optimal signature to discriminate IPMNs as low risk (low/moderate dysplasia) or high risk (high-grade dysplasia/invasive cancer). The most predictive signature was achieved with interleukin 1β, MUC4, and prostaglandin E synthase 2 to accurately discriminate high-risk cysts from low-risk cysts with an area under the curve of up to 0.86 ($p = 0.002$).

CONCLUSIONS: We have identified a single-platform polymerase chain reaction-based assay of cyst fluid to accurately predict IPMNs with high malignant potential for additional studies. (J Am Coll Surg 2019;228:721–729. © 2019 by the American College of Surgeons. Published by Elsevier Inc. All rights reserved.)

Disclosure Information: Nothing to disclose.

Support: Dr Maker is supported by a grant from NIH/National Cancer Institute (K08-CA190855), Drs Scarpa and Lawlor are supported by a grant from the Associazione Italiana Ricerca Cancro (#12182) and an FP7 European Community Grant Cam-Pac (#602783).

Abstract presented at the Americas Hepato-Pancreato-Biliary Association Annual Meeting, Miami, FL, April 2017.

Received December 31, 2018; Revised February 1, 2019; Accepted February 2, 2019.

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https://doi.org/10.1016/j.jamcollsurg.2019.02.040
ISSN 1072-7515/19

Abbreviations and Acronyms

AGA = American Gastroenterological Association
IL = interleukin
IPMN = intraductal papillary mucinous neoplasm
miRNA = microRNA
mRNA = messenger RNA
PCR = polymerase chain reaction
PTGES = prostaglandin E synthase

Intraductal papillary mucinous neoplasms (IPMNs) are pancreatic cysts with adenomatous proliferation of ductal epithelium causing mucin production and dilation of the pancreatic ductal system. The incidence of asymptomatic pancreatic cysts, including IPMNs, is 3% to 15% of the population and is increasing secondary to ubiquitous cross-sectional imaging and advancements in imaging quality.[1,2] Up to 24% of patients at autopsy will have a pancreatic cyst, of which 20% might contain atypia or high-grade dysplasia.[3] As a result, IPMN has become the most common cystic precursor lesion of pancreatic adenocarcinoma, representing 10% to 25% of resected pancreatic neoplasms.[4,5]

The main challenge in treating IPMNs is in accurately predicting malignant potential and determining the risk to benefit of a surgical resection. Studies on the clinical signs and imaging characteristics of the disease have evolved to form the basis of multiple clinical consensus guidelines.[6,7] Although the sensitivity of the current treatment guidelines is satisfactory, the specificity remains poor. As a result, the vast majority of resected IPMNs will be low risk and, as we have reported, often contain only low- or moderate-grade dysplasia on final pathology.[5,8] Surgical intervention often involves major pancreatic resection, which carries significant risk of mortality and morbidity.[9] Particularly for small branch duct IPMNs, enhanced tests with improved negative predictive value are desired to avoid the potential complications of surgical resection for the cysts that will prove to have low malignant potential and might otherwise have been treated with surveillance alone.

Current clinical guidelines lack accuracy in determining the level of cyst dysplasia,[6,10-12] and additional molecular diagnostic data to distinguish high- from low-risk cysts are desperately needed. We have previously shown that endoscopic ultrasound fine-needle aspiration cytology has limited use in surgical decision making for IPMN, and we have identified multiple prognostic molecular biomarkers within the cyst fluid.[13-17] Cyst fluid is easily and safely accessible preoperatively and contains shed genetic material from the cyst wall that is representative of the entire cyst.[13] Although previous biomarker studies have focused on proteins, RNA, DNA, microRNA (miRNA), cytokines, glycoproteins, or mucins in the cyst fluid, we endeavored to combine the most predictive markers from each of these classes using the coding genes in one comprehensive assay. The objective of this study was to create a gene expression-based assay incorporating multiple IPMN biomarkers into a signature that could accurately stratify IPMNs as low or high risk for malignant progression.

MATERIALS AND METHODS

Biological samples

The International IPMN Cyst Fluid Collaborative was created of groups from high-volume pancreatic surgery centers with an expertise in IPMN across Europe and the US.[10,13] Intraductal papillary mucinous neoplasm cyst fluid samples were obtained from prospectively maintained institutional databases and repositories after approval by the IRB of the University of Illinois at Chicago. Only samples with a confirmed diagnosis of IPMN on final pathology and with the specific grade of dysplasia determined by an expert pancreatic pathologist were included in the study. Analysis evaluated samples by low-risk (low- and moderate-grade dysplasia) or high-risk (high-grade dysplasia and invasive cancer) pathology for the purpose of risk stratification, as has been used in multiple other biomarker studies in this field due to its clinical applicabiity.[14,15,18]

Quantitative analysis of messenger RNAs and microRNAs

Total RNA was extracted from 100 to 400 μL IPMN fluid using Quick-RNA MicroPrep R1050/R1051, implemented on a Maxwell16 instrument. DNAse treatment was performed according to the manufacturer's instructions. Subsequently, total RNA was split into 2 paths for messenger RNA (mRNA) and miRNA analysis using quantitative polymerase chain reaction (PCR).

For analysis of mRNA, total RNA was reverse transcribed using random primers and the High Capacity complementary DNA reverse transcription kit (#4368814; ThermoFisher Scientific), according to manufacturer's instructions. Complementary DNA was prepared for quantitative PCR using a pre-amplification step, with the Taqman PreAmp master mix kit (#4384267; ThermoFisher Scientific). Taqman gene expression assays were pooled to serve as primers for the pre-amplification step according to the manufacturer's instructions. Assays included IL1b(Hs01555410_m1), muc-1(Hs00159357_m1), muc-2(Hs00894025_m1),

muc-4(Hs00366414_m1), muc-5ac(Hs01365616_m1), muc-7(Hs00379529_m1), PTGER2(Hs04183523_m1), PTGS1(Hs00377726_m1), prostaglandin E2-R(Hs00168755_m1), KRAS(Hs00364282_m1), GNAS(Hs00255603_m1), GAPDH(Hs99999905_m1), RPLP0(Hs99999902_m1), TP63(Hs00978341_m1), ERBB2(Hs01001580_m1), and prostaglandin E synthase (PTGES) 2(Hs00228159_m1). Quantitative PCR reactions were performed using Taqman Fast Advanced master mix (#4444556; ThermoFisher Scientific) in 384-well plates using a ViiA7 real-time PCR instrument (Life Technologies). All reactions were performed in triplicate and in volumes of 10 μL. Real-time data were processed using the comparative C(t) method.[19] The chosen endogenous control gene was RPLP0, based on performance across the entire data set.

Reverse transcription of miRNA was performed using the Taqman miRNA reverse transcription kit (#4366596), with Taqman miRNA assays in place of random primers. The assays used for this study included miR17-3p, miR142-3p, miR532-3p, miR342-3p, miR30a-3p, miR21, miR155, mir101, mir10a, miR106b, miR18a, miR217, miR24, miR92a, miR99b, and RNU6B. Real-time data were processed using the comparative C(t) method, using the RPLP0 gene as an endogenous control.[19]

Polymerase chain reaction amplification and sequencing of GNAS and KRAS mutation sites

Genomic DNA was extracted from IPMN fluid using the Maxwell16 Tissue DNA kit (AS1030; Promega). Mutation analysis of codons 12 and 13 in KRAS and codon 201 in GNAS were performed by PCR, followed by Sanger sequencing. Each 50 μL PCR reaction contained 1× PCR buffer with 1.5 mM $MgCl_2$, 0.5 μL HotStarTaq DNA polymerase (203203; Qiagen), 0.2 mM dNTP mix (D7295; Sigma-Aldrich), 20 pmol of forward and reverse primers, and 5 μL DNA template. The KRAS PCR reaction in addition contained 25 pmol of a LNA oligo (5′ GC+T+G+G+T+G+G+C+GTA/3′invdT 3′) to suppress wild-type amplification (Exiqon). Amplification products were purified and bi-directionally sequenced on an ABI3130XL genetic analyzer using the PCR primers and the BigDye 3.1 terminator cycle sequencing kit. Sequence chromatograms were visualized manually to determine if a mutation was present. The analytical sensitivity is 1% mutant sequence for KRAS codons 12 and 13 and 15% mutant sequence for GNAS codon 201. Appropriate positive and contamination controls were included. Mutation nomenclature was according to standard guidelines (http://varnomen.hgvs.org/recommendations/DNA/variant/substitution/). Sample workflow is outlined in Figure 1.

Statistical analysis

Relative quantification values were z-transformed, log2 transformed, and scaled (X-mean/SD). Pearson correlation coefficients were used to remove highly correlated variables with a cutoff of 0.7. Principal coordinate analysis was then performed. Models were run adding sequencing data from KRAS and GNAS mutational analysis and evaluated as +kras mutation, +gnas mutation, +gnas/+kras mutation, or 0, 1, or 2 mutations. Mutational analysis as an independent variable was appended to the data matrix with 22 markers for learning and used in classification and regression analysis by a support vector machine training algorithm. The R-package Glmnet, a package that fits a generalized linear model via penalized maximum likelihood, was used together with logistic regression.[20] Batch effect correction was performed. Highly corrected markers were removed.

Lasso (least absolute shrinkage and selection operator)-penalized logistic regression with binary classification and 5-fold cross-validation used area under the curve (AUC) as the evaluation criterion to create the optimal signature. A machine learning algorithm identified markers significantly related to the level of dysplasia/risk of pancreatic malignancy. In N patient cyst fluids, each of which consists of p predictive genes and a level of dysplasia as single outcome; y_i is the classification of dysplasia and $x_i = (x_1, x_2, \ldots, x_p)^T$ the gene expression (covariate vector) for the i^{th} case. Where the aim is to identify the least number, but optimal subset, of markers that minimize the classification error between high- and low-risk IPMNs,[20,21] the objective of lasso was to solve the following equation:

$$\min_{\beta \varepsilon R^p} \left\{ \frac{1}{N} \| y - X\beta \|_2^2 + \lambda \| \beta \|_1 \right\}$$

RESULTS

Selection of targets and cyst fluid

Specific protein, cytokine, mucin, DNA, and miRNA cyst fluid targets were identified from primary research and an extensive literature search of proposed biomarkers in IPMN, as published previously.[13-15,17] This included 14 mRNA markers, 15 miRNA targets, as well as GNAS codon 201 and KRAS codons 12 and 13 point mutational analysis. A total of 134 cyst fluid samples were evaluated for inclusion. Sufficient fluid volume of samples with IPMN grade of dysplasia (low n = 18, moderate n = 16, high n = 12, and invasive n = 13) was confirmed for 59 cyst fluid samples. Ninety-five percent of samples contained sufficient genomic material for additional analysis.

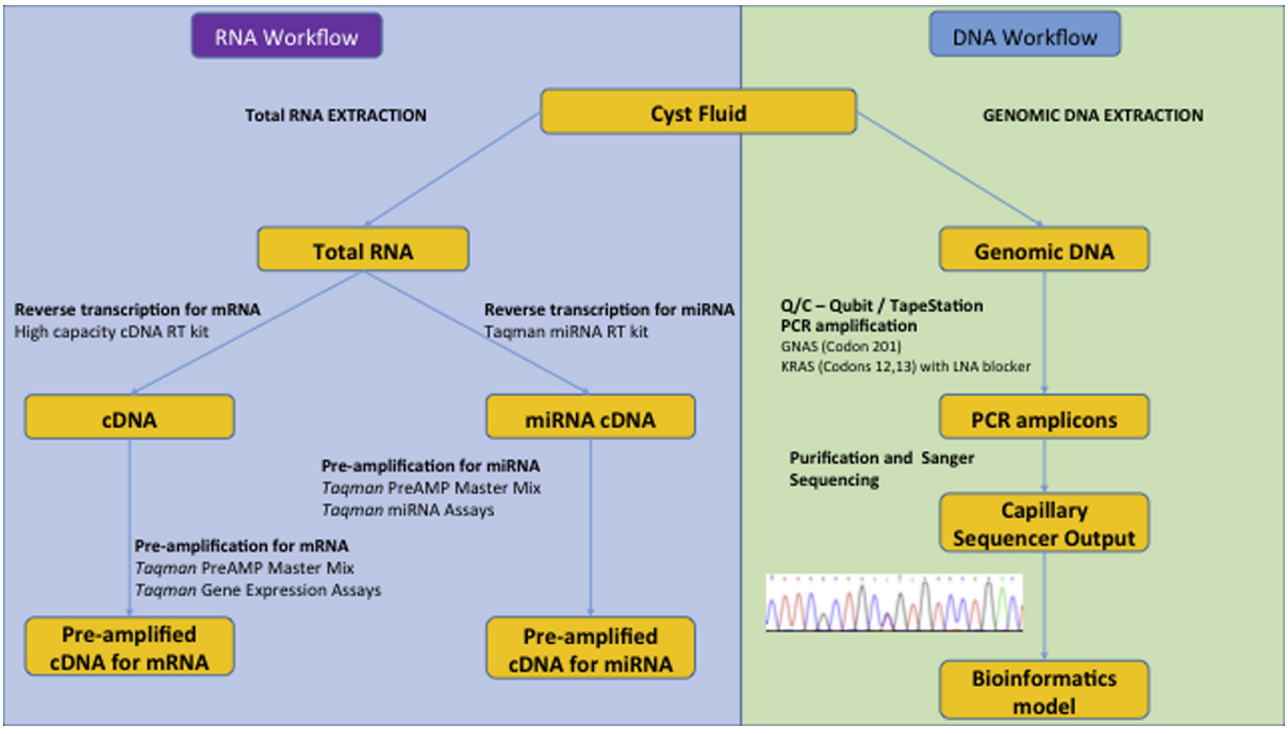

Figure 1. Workflow of cyst fluid preparation for input into the bioinformatics model to predict level of cyst dysplasia.

Principal component analysis, batch effect correction, and removal of confounders

Principal component analysis demonstrated minimal institutional bias/clustering, which was batch effect-corrected. As highly corrected markers will cause difficulties in machine learning algorithms to identify individual features for the signature, within each group of highly correlated markers (Pearson correlation > 0.7), one representative marker was kept for additional analysis using R package caret (https://github.com/topepo/caret/). Confounding genes were removed from the analysis (Fig. 2).

Specific mutational analysis

Based on previous data,[13,22-24] GNAS codon 201 and KRAS codons 12 and 13 were sequenced for mutational analysis. Of 49 samples with sufficient DNA harvested from the cyst fluid to reliably sequence, 30 (61%) contained a point mutation in GNAS (13 of 49 [43%]) or KRAS (26 of 49 [53%]). For GNAS, 7 of 13 (54%) samples had p.R201H mutations and 6 (46%) had p.R201C mutations; and for KRAS, 7 (27%) had a p.G12R mutation, 14 (54%) had a p.G12V mutation, 8 (31%) had a p.G12D mutation, 1 (4%) had a G12F mutation, 1 had a p.G12A mutation, and 1 had a p.G13D mutation. Three of 49 (6%) samples each had 2 KRAS codon 12 point mutations. Nine of 49 (18%) samples contained both GNAS codon 201 and KRAS codon 12 mutations, of which 1 also contained the KRAS codon 13 mutation.

Lasso regression results

In a binomial logistic regression model with AUC as the objective function, the maximum AUC was achieved with miR21, miR342, interleukin (IL) 1β, KRAS, MUC4, and PTGES2, resulting in an AUC of 0.82 ($p = 0.003$) to differentiate low-risk from high-risk cysts. Subset analysis including iterations involving GNAS and KRAS point mutation analysis were performed to determine the most accurate predictive biosignature. When a mutation in either GNAS or KRAS was considered, the most predictive signature was achieved with IL1β, MUC4, and PTGES2 to construct the equation of: $y = 0.37 + (-0.06\text{IL1}\beta) + (-0.01\text{MUC4}) + (-0.50\text{PTGES2})$, AUC = 0.86, $p = 0.002$ (Fig. 3). Using PCR data, the level of expression of IL1β, MUC4, and PTGES2 in IPMN cyst fluid can be entered into this equation and enabled accurate discrimination of IPMNs with low- or moderate-grade dysplasia (low-risk) from high-grade dysplasia or invasive cancer (high-risk).

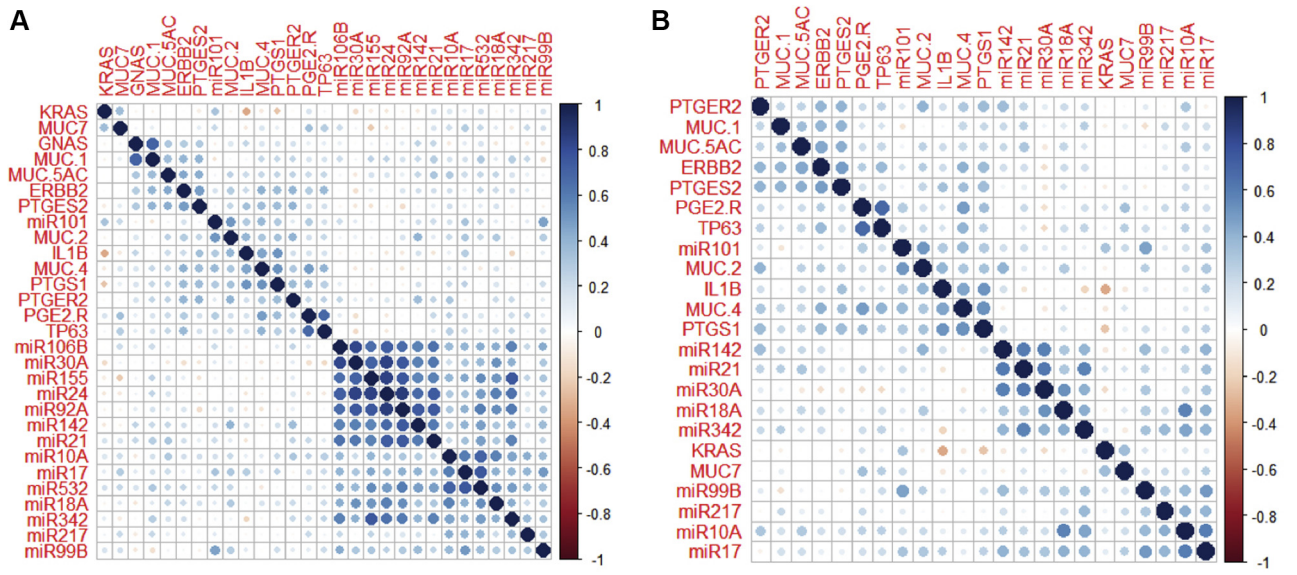

Figure 2. Removal of confounders. A Pearson correlation matrix was constructed between each pair of gene markers. GNAS, miR106B, miR155, miR24, miR92A, and miR532 were removed from the analysis due to high correlation of gene expression that confounded machine learning algorithms in identifying predictive markers for the biosignature.

DISCUSSION

Evidence supports a progression model for IPMNs from low-grade dysplasia to adenocarcinoma, however, the time frame for this transformation is unknown.[25,26] Currently, many cysts at low risk of malignant transformation are being removed at the expense of mental, physical, and financial costs for patients and society, with the added risk of up to 2% mortality and approximately 40% morbidity postoperatively. Balancing the risks and benefits of resection is the crux of the challenge in surgical decision making for this disease, where the ramifications of missing an occult pancreatic adenocarcinoma, or delay in resection that allows progression, can result in significant cancer-related mortality. In the US, the vast majority of patients currently undergoing surgical resection for IPMNs will have low-risk cysts determined on final pathology, despite multiple US, European, and international guidelines intended to direct patient selection toward high-risk lesions.[5] It has been demonstrated that up to 65% of lesions predicted by the guidelines to be high risk for high-grade dysplasia or invasive cancer are found to be low risk on final pathology,[27] and other small branch duct IPMNs predicted to have a low risk of malignancy with the same guidelines will demonstrate high-risk pathology up to 25% of the time.[28]

The 2 most commonly used guidelines for clinical decision making in the US are the revised Sendai (Fukuoka) and American Gastroenterological Association (AGA) guidelines. The Fukuoka guidelines have been found to have a high false-positive rate, with 21% specificity for malignancy. The same study found the AGA guidelines to have a lower false-positive rate, with 44% specificity, but with a higher false-negative rate and 12% more of malignancies overlooked.[29] Similar analysis supported that the Fukuoka guidelines had a 65% to 72% false-negative rate to identify high-risk cysts, and the AGA guidelines misidentified 45% of high-risk IPMNs.[30,31] The field is in need of novel and reliable biomarkers that will be able to differentiate between cysts with minimal risk of malignant transformation and those with high-risk pathology or occult malignancy.[32] For this reason, a biosignature that uses the inherent molecular makeup of the cyst as opposed to only size or radiographic findings alone, has great practical and clinical value.[33] Preoperative next-generation sequencing of cyst fluid has shown the ability to differentiate mucinous from nonmucinous cysts through mutational analysis of KRAS and GNAS, and in the same study, a combination of mutations or deletions in TP53/PIK3CA/PTEN served as a marker of advanced neoplasia.[34] Additional studies evaluating cyst fluid for subtle mutations, loss of heterozygosity, and aneuploidy in 11 genes aided in identification of pancreatic cyst type and histologies for which operation might be recommended.[23] DNA-based testing of genetic material shed into the cyst fluid is a reliable tool through which to study the biology of IPMN disease.

In response to this need, an IPMN cyst fluid gene biosignature was shown to have the ability to discriminate

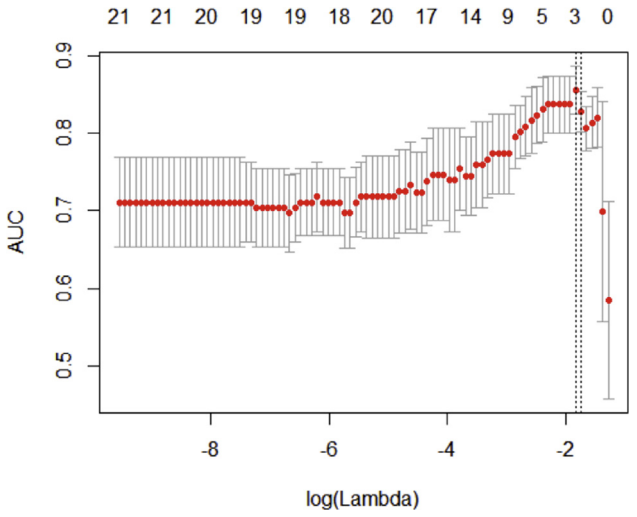

Figure 3. Intraductal papillary mucinous neoplasm (IPMN) cyst fluid biosignature can differentiate high-risk from low-risk cysts. Lasso (least absolute shrinkage and selection operator)-penalized logistic regression with cross-validation identified a 3-gene cyst fluid signature with optimal accuracy to predict the risk of pancreatic malignancy in IPMNs. In this model, low-risk (low and moderate grade dysplasia) vs high-risk (high-grade dysplasia and invasive cancer) cysts were predicted with an accuracy, as measured by area under the curve (AUC), of 86%; y = 0.36+ (-0.06IL1β) + (-0.01MUC4) + (-0.50PTGES2); AUC = 0.86, p = 0.002.

high-risk from low-risk IPMNs with up to 86% accuracy. This is compared with the 50%, 76%, and 60% accuracies of the Fukuoka, AGA, and American College of Radiology criteria, respectively, in the multi-institutional 5-year study reported by Xu and colleagues.[35] The current study uses a unique gene panel not evaluated previously as a biosignature and that requires only a single PCR platform to quantify.

A combination of IL1β, MUC4, and PTGES2 were consistent across the predictive models. Interleukin 1β is secreted into the extracellular space, where it can be measured in pancreatic cyst fluid.[36] We previously determined that IL1β was nearly undetectable in the cyst fluid of low-grade IPMNs and serous cystadenomas and that its presence in dysplastic cysts reflected an inflammatory microenvironment,[15] with a likelihood ratio of 17× to distinguish low-risk from high-risk cysts. In addition, IL1β is a known mediator of pancreatic cancer cell invasion.[36-41] MUC4 is implicated in IPMN development, and increased expression can transform borderline cysts to a malignant phenotype.[42] Our previous data identified high cyst fluid expression with high-risk IPMNs.[14,43] Prostaglandin E synthase 2 is an enzyme that is encoded by the PTGES2 gene. It catalyzes the conversion of prostaglandin H2 to prostaglandin E2, which, in excess, is known to contribute to inflammatory diseases and cancer. Elevated prostaglandin E2 has been implicated in distinguishing IPMNs from other mucinous pancreatic cysts and trends to increase with higher levels of IPMN cyst dysplasia and pancreatic cancer.[44,45]

There are limitations to the study. Bias was minimized as much as possible by including an international multi-institutional cohort, running samples in large batches, preselecting candidate biomarkers,[13,17] and through robust statistical methods with 5-fold cross-validation. Nonetheless, the samples were collected prospectively, but compiled and evaluated retrospectively. To become a routine study in this disease, it is vital that validation with additional sample sets be performed. In addition, as the value of this test is in accurate prediction of the level of IPMN dysplasia preoperatively, additional validation using prospective collection of endoscopic ultrasound fine-needle aspiration cyst fluid compared with final surgical pathology of resected specimens will be necessary to change practice. Little is known about the genetic features of IPMNs undergoing surveillance, therefore, there might be a bias in selection, as the lesions for operative intervention likely contained high-risk or worrisome features. Future analysis will include IPMN phenotype, including duct type and size, patient demographics, and features that led to surgical intervention; information not included in the current data set. However, in other excellent analyses of IPMN cyst fluid where clinical nomograms were included in a predictive model, the accuracy was not significantly higher than what was achieved in this analysis.[18] Regardless, future validation sets will include prospective collection of detailed cyst phenotype variables and patient demographics in the equation, which, when combined with the correct biosignature, can enhance the selection of high-risk lesions over clinical nomograms alone, and will also allow for determination of additive predictive value over Fukuoka guideline characteristics alone.[18,23,46] Certainly, a strength of the current study was inclusion of the gene expression, mutational analysis, and epigenetics of targets selected for the biosignature. Interestingly, when KRAS and GNAS mutational analysis were added to the model, they were not selected as contributing features to the predictive value, possibly because of the high prevalence of these mutations in IPMNs overall.[47,48] Although previous studies have focused on signatures made from groups of proteins, RNA, DNA, miRNA, cytokines, glycoproteins, or mucins individually, we endeavored to combine the best predictive markers from each category using only the cyst fluid into one rapid comprehensive assay. Proteins predictive of phenotype that had been identified in our previous analyses[13,17] were represented in this analysis by expression of their coding gene. A strength of the analysis was building the model around samples with pathologically confirmed

IPMNs, which allowed the signature to focus specifically on low-risk compared with high-risk IPMNs, although future validation sets might also evaluate whether the signature can also discriminate between other non-IPMN histologies or levels of dysplasia. In addition, the number of genes evaluated was limited and highly selected based on our comprehensive review of the literature to decrease model overfitting, however, there might be other genes that, if included, could have contributed to the model.

Using a single, easily accessible platform, such as PCR, is extremely cost-effective, practical, and proven to be reproducible. This technology is in place in most every institution and accessible for outpatient use throughout the world without requiring complex or expensive equipment and reagents, sequencing technology, or tests of heterozygosity. Signatures using this technology have precedent in other cancer histologies for widespread use and application in patient treatment decision making.[49-53] DNA is a very stable molecule for transport and easy to collect because it is shed into the IPMN cyst fluid. Cyst fluid is accessible by endoscopic ultrasound fine-needle aspiration and often collected as part of a standard IPMN clinical workup. The clinical utility of creating such an assay would ultimately be to evaluate small IPMNs that do not meet current clinical criteria for resection to provide additional quantitative data that can potentially be used for early detection and to better inform the patient and treating physician of the risk of malignant transformation.

CONCLUSIONS

Using one of the largest multi-national IPMN cyst fluid banks, a biosignature has been identified that predicts IPMNs with high-malignant potential using a PCR-based assay.

Author Contributions

Study conception and design: Maker

Acquisition of data: Maker, Hu, Kadkol, Hong, Brugge, Winter, Yeo, Hackert, Büchler, Lawler, Salvia, Scarpa, Bassi, Green

Analysis and interpretation of data: Maker, Hu, Kadkol, Green

Drafting of manuscript: Maker, Hu, Green

Critical revision: Maker, Hu, Kadkol, Hong, Brugge, Winter, Yeo, Hackert, Büchler, Lawler, Salvia, Scarpa, Bassi, Green

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