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Prospective, Multi-Institutional, Real-Time Next-Generation Sequencing of Pancreatic Cyst Fluid Reveals Diverse Genomic Alterations That Improve the Clinical Management of Pancreatic Cysts

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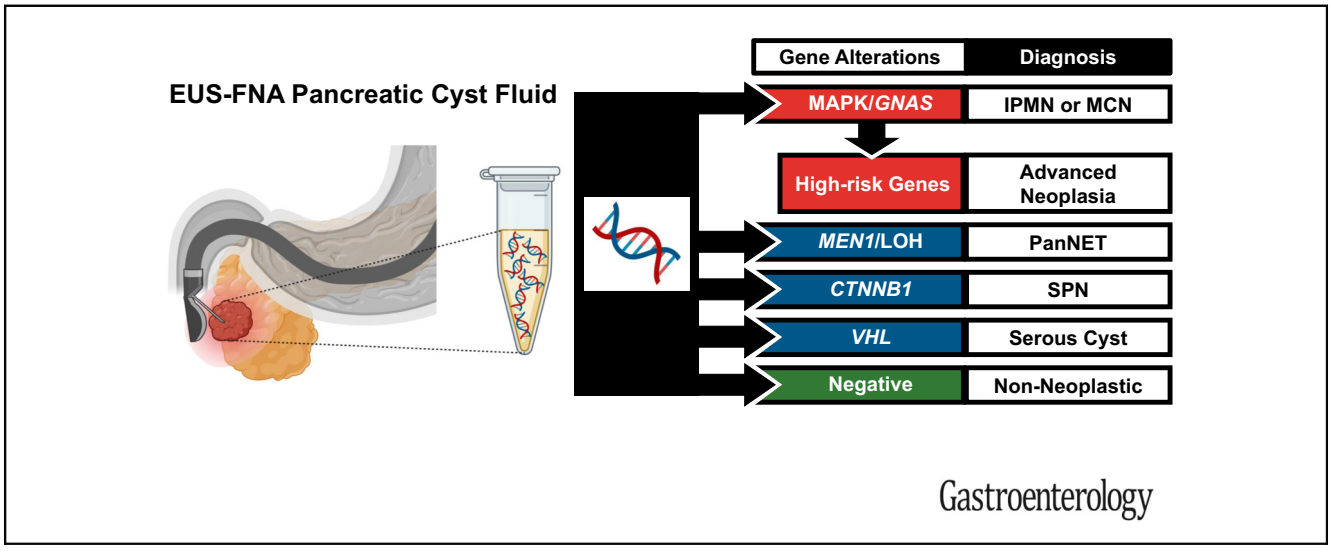

See editorial on page 21.

BACKGROUND & AIMS: Next-generation sequencing (NGS) of pancreatic cyst fluid is a useful adjunct in the assessment of patients with pancreatic cyst. However, previous studies have been retrospective or single institutional experiences. The aim of this study was to prospectively evaluate NGS on a multi-institutional cohort of patients with pancreatic cyst in real time. **METHODS:** The performance of a 22-gene NGS panel (PancreaSeq) was first retrospectively confirmed and then within a 2-year timeframe, PancreaSeq testing was prospectively used to evaluate endoscopic ultrasound–guided fine-needle aspiration pancreatic cyst fluid from 31 institutions. PancreaSeq results were correlated with endoscopic ultrasound findings, ancillary studies, current pancreatic cyst guidelines, follow-up, and expanded testing (Oncomine) of postoperative specimens. **RESULTS:** Among 1933 PCs prospectively tested, 1887 (98%) specimens from 1832 patients were satisfactory for PancreaSeq testing. Follow-up was available for 1216 (66%) patients (median, 23 months). Based on 251 (21%) patients with surgical pathology, mitogen-activated protein kinase/*GNAS* mutations had 90% sensitivity and 100% specificity for a mucinous cyst (positive predictive value [PPV], 100%; negative predictive value [NPV], 77%). On exclusion of low-level variants, the combination of mitogen-activated protein kinase/*GNAS* and *TP53/SMAD4/CTNNB1*/mammalian target of rapamycin alterations had 88% sensitivity and 98% specificity for advanced neoplasia (PPV, 97%; NPV, 93%). Inclusion of cytopathologic evaluation to PancreaSeq testing improved the sensitivity to 93% and maintained a high specificity of 95% (PPV, 92%; NPV, 95%). In comparison, other modalities and current pancreatic cyst guidelines, such as the American Gastroenterology Association and International Association of Pancreatology/Fukuoka guidelines, show inferior diagnostic performance. The sensitivities and specificities of *VHL* and *MEN1*/loss of heterozygosity alterations were 71% and 100% for serous cystadenomas (PPV, 100%; NPV, 98%), and 68% and 98% for pancreatic neuroendocrine tumors (PPV, 85%; NPV, 95%), respectively. On follow-up, serous cystadenomas with *TP53*/*TERT* mutations exhibited interval growth, whereas pancreatic neuroendocrine tumors with loss of heterozygosity of $\geq$3 genes tended to have distant metastasis. None of the 965 patients who did not undergo surgery developed malignancy. Postoperative Oncomine testing identified mucinous cysts with *BRAF* fusions and *ERBB2* amplification, and advanced neoplasia with *CDKN2A* alterations. **CONCLUSIONS:** PancreaSeq was not only sensitive and specific for various pancreatic cyst types and advanced neoplasia arising from mucinous cysts, but also reveals the diversity of genomic alterations seen in pancreatic cysts and their clinical significance.

Keywords: Pancreas; Early Detection; Pancreatic Neoplasm; Diagnosis; Pancreatic Cancer.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

While previous studies have shown targeted next-generation sequencing is a useful adjunct to the preoperative evaluation of pancreatic cysts, these studies have largely been retrospective analyses, single institutional experiences, and focused on intraductal papillary mucinous neoplasms.

NEW FINDINGS

Through prospective, real-time, multi-institutional next-generation sequencing (PancreaSeq) of a large patient cohort, a diverse number of genomic alterations were identified in intraductal papillary mucinous neoplasms (eg, *BRAF*), serous cystadenomas (eg, *TP53* and *TERT*), and pancreatic neuroendocrine tumors (eg, loss of heterozygosity of multiple genes) and are of associated clinical significance.

LIMITATIONS

Considering most pancreatic cysts follow a benign clinical course, diagnostic surgical pathology was available for 14% of tested patients. However, clinical follow-up with a median of 23 months was available for an additional 52% of patients.

IMPACT

The results of this study support the clinical utility of targeted next-generation sequencing in the evaluation of not only pancreatic mucinous cysts, but other cyst types. This study also broadens the number of genomic alterations that characterize pancreatic cysts.

The detection of pancreatic cysts by cross-sectional imaging has become increasingly frequent and represents a significant public health challenge. In the United States, it is estimated that up to 2.5% of the general population harbors a pancreatic cyst.[1,2] The prevalence of pancreatic cysts increases with age and up to 40% of patients who are 70 years and older have a pancreatic cyst.[3] In addition, approximately half of all pancreatic cysts are mucinous cysts, such as intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). IPMNs and MCNs are noninvasive precursor neoplasms to pancreatic ductal adenocarcinoma (PDAC).[4] Consequently, the identification of mucinous cysts is a source of psychological stress for both the patient and the physician, but most mucinous cysts are indolent in nature and only a minority will transform into PDAC.[1,5]

*** Authors share co-first authorship; § Authors contributed equally to this study.**

Abbreviations used in this paper: **AF, allele frequency; ALT, alternative lengthening of telomeres; CEA, carcinoembryonic antigen; EUS, endoscopic ultrasound; FNA, fine-needle aspiration; IPMN, intraductal papillary mucinous neoplasm; LOH, loss of heterozygosity; MAPK, mitogen-activated protein kinase; MCN, mucinous cystic neoplasm; MGP, Molecular and Genomic Pathology; mTOR, mammalian target of rapamycin; NGS, next-generation sequencing; NPV, negative predictive value; PanNET, pancreatic neuroendocrine tumor; PDAC, pancreatic ductal adenocarcinoma; PPV, positive predictive value; SCA, serous cystadenoma; UPMC, University of Pittsburgh Medical Center; WHO, World Health Organization.**

Most current article

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A multidisciplinary approach is currently advocated for the diagnosis and management of pancreatic cysts[6–9]; however, the evaluation of pancreatic cyst fluid is critical to the classification of pancreatic cysts and early detection of PDAC. Among ancillary studies, targeted DNA-based next-generation sequencing (NGS) is a useful tool in the assessment of pancreatic cysts.[10–13] Mutations in the mitogen-activated protein kinase (MAPK) genes and/or *GNAS* are specific for mucinous cysts, whereas alterations in *TP53*, *SMAD4*, and the mammalian target of rapamycin (mTOR) genes are associated with advanced neoplasia (high-grade dysplasia and PDAC arising from a mucinous cyst).[14–17] Targeted NGS can also be used to identify other pancreatic cyst types, such as serous cystadenomas (SCAs), solid-pseudopapillary neoplasms, and cystic pancreatic neuroendocrine tumors (PanNETs) that are characterized by mutations in *VHL*, *CTNNB1*, and *MEN1,* respectively.[10,12,13,18]

To date, several studies have evaluated targeted DNA-based NGS of pancreatic cysts, but published reports have largely been limited to retrospective analyses or single institutional experiences.[10,11,13,19] In addition, most NGS studies have been focused on the assessment of IPMNs and IPMN-associated PDACs. The aims of this study were to (1) develop an expanded, targeted NGS panel (PancreaSeq) that can improve not only the assessment of IPMNs and IPMN-associated PDACs, but also other cyst types; (2) on confirmation of PancreaSeq performance using a retrospective cohort, to prospectively evaluate a multi-institutional cohort of pancreatic cyst patients in real time to determine the diagnostic performance of PancreaSeq testing; and (3) perform repeat PancreaSeq testing and expanded targeted DNA/RNA-based NGS (Oncomine) of paired postoperative specimens to establish concordance rates and identify additional genomic alterations that may further improve the assessment of pancreatic cysts.

Methods

Study Population

Study approval was obtained from the authors' respective institutional review boards and the study design is outlined in Figure 1. For retrospective PancreaSeq testing (Supplementary Material and expected results are summarized in Supplementary Table 1), pancreatic cyst fluid specimens with corresponding clinical, imaging, and diagnostic surgical pathology follow-up were obtained through searching the molecular archives of the Molecular and Genomic Pathology (MGP) laboratory at the University of Pittsburgh Medical Center (UPMC) and cross-referencing the surgical pathology archives of UPMC Department of Pathology. These retrospective molecular specimens were previously reported in 2 large patient cohort studies.[10,15] Prospective PancreaSeq testing was performed between January 2018 and February 2020 and consisted of 1933 pancreatic cyst fluid specimens obtained by endoscopic ultrasound (EUS)–fine-needle aspiration (FNA) that were submitted to the UPMC MGP laboratory from 31 medical institutions. In all cases, the indication for PancreaSeq testing was a clinical concern for a pancreatic cyst. Corresponding patient data were collected to include demographics, clinical presentation, EUS findings, fluid viscosity (as noted by the endoscopist using the string sign), carcinoembryonic antigen (CEA) analysis and cytopathological diagnoses. Endoscopic criterion of main duct dilatation was defined by a diameter $\geq$5 mm. In addition, the presence of a mural nodule was defined as a uniform echogenic nodule of any size without a lucent center or hyperechoic rim. A value >192 ng/mL was used as a cutoff for an elevated pancreatic cyst fluid CEA; however, CEA analysis was not centralized and performed at the submitting institution or reference laboratory. Cytopathologic findings were recorded from the respective submitting institutions and malignant cytopathology was defined as at least suspicious for adenocarcinoma. Diagnostic surgical pathology diagnoses were also obtained from each participating institution and were based on the 2019 World Health Organization (WHO) Classification of Tumors of the Digestive System.[20] Cases diagnostic for a mucinous pancreatic cyst (IPMN and MCN) with high-grade dysplasia and/or an associated invasive adenocarcinoma were interpreted as "advanced neoplasia." In comparison with PancreaSeq testing, absolute surgical resection criteria for the American Gastroenterology Association (AGA) guidelines (cytopathologic evaluation of at least suspicious for adenocarcinoma and/or 2 of the following features: dilated main pancreatic duct, >3.0 cm cyst size, and a solid component) and 2017 revised International Consensus Fukuoka (IAP/Fukuoka) guidelines (high-risk stigmata: jaundice in a patient with a cystic lesion of the pancreatic head, the presence of a mural nodule, main duct dilation suspicious for involvement, and/or cytopathologic evaluation of at least suspicious for adenocarcinoma) were retrospectively applied to the prospectively collected surgical resection study cohort.[7,21]

Nucleic Acid Extraction

Nucleic acid extraction, as well as subsequent DNA- and RNA-based targeted NGS, was performed within the Clinical Laboratory Improvement Amendments– and College of American Pathologists–accredited MGP laboratory at UPMC. Genomic DNA and mRNA were isolated from either pancreatic cyst fluid obtained by EUS-FNA (preoperative specimens) or formalin-fixed paraffin-embedded tissue (surgical resection specimens) using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Indianapolis, IN) on the Compact MagNA Pure (Roche) or the DNeasy Blood and Tissue kit on the automated QIAcube instrument (QIAGEN, Germantown, MD). Extracted DNA and RNA were quantitated on the Glomax Discover using the QuantiFluor ONE dsDNA System and the QuantiFluor RNA system, respectively (Promega, Madison, WI).

PancreaSeq Testing

Amplification-based targeted DNA-based NGS for PancreaSeq was performed with custom AmpliSeq primers for genomic regions of interest within *AKT1, APC, BRAF, CTNNB1, GNAS, HRAS, IDH1, IDH2, KRAS, MEN1, MET, NF2, NRAS, PIK3CA, PTEN, STK11, TERT, TP53, TSC2,* and *VHL* with primer sequences and performance characteristics as previously described to include single nucleotide variants, insertions, deletions, and loss of heterozygosity (LOH)/copy number alteration.[10,12,13,22] Amplicons were barcoded, ligated with specific adapters, and purified. DNA library quantity and quality checks were performed using the 4200 TapeStation (Agilent Technologies, Santa Clara, CA). The Ion Chef was used

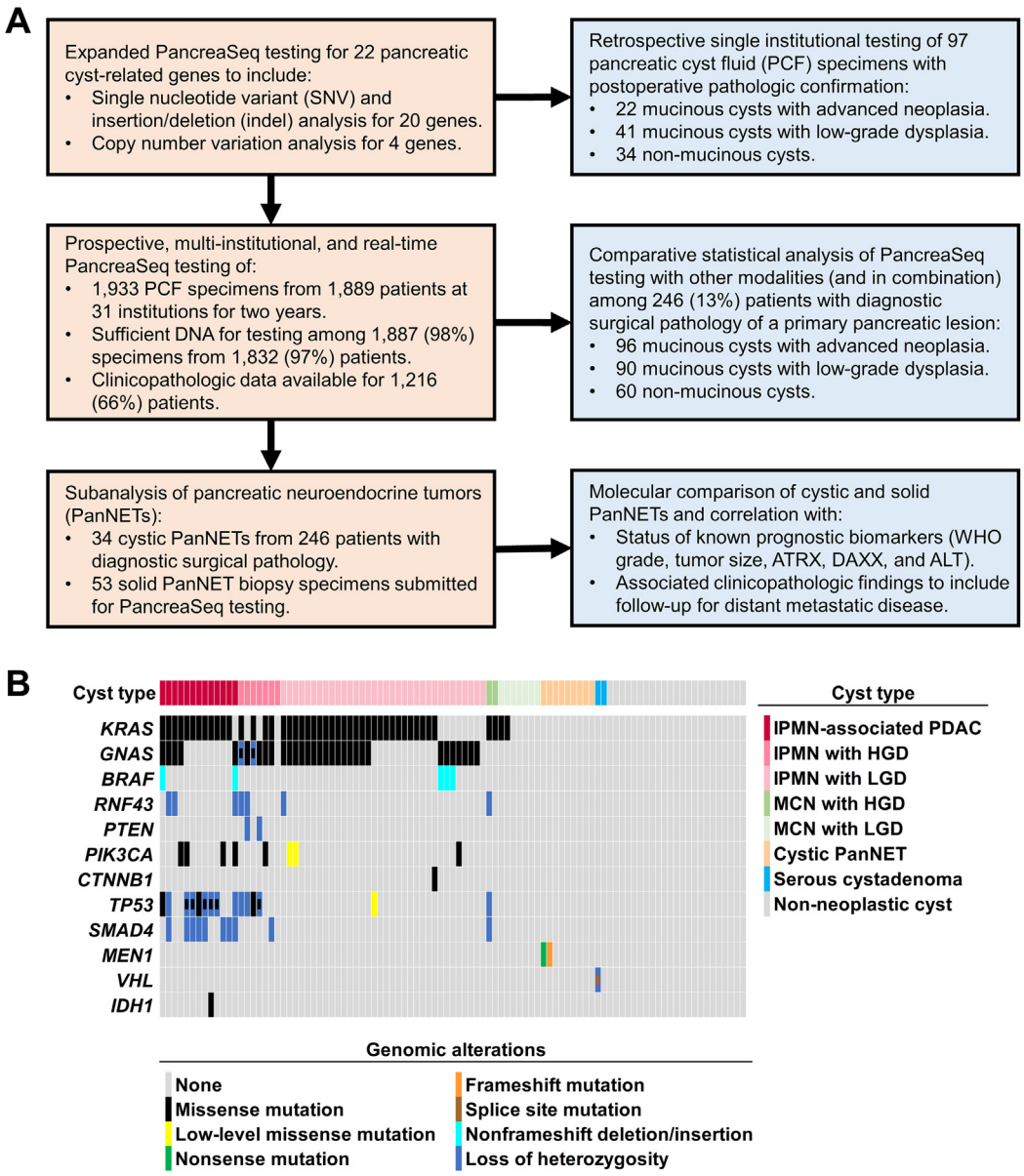

Figure 1. (*A*) A summary of the study design to include details of individual patient cohorts used for PancreaSeq testing (*tan*) and individual analyses performed (*blue*). (*B*) Correlative genomic findings based on retrospective PancreaSeq testing of 97 preoperative pancreatic cyst fluid specimens from 63 mucinous cysts and 34 nonmucinous cysts. Among the 63 mucinous cysts, 22 cysts also harbored high-grade dysplasia and/or invasive adenocarcinoma (advanced neoplasia). Genomic alterations in *KRAS*, *GNAS,* and/or *BRAF* were 100% specific for mucinous cysts, whereas alterations in *TP53*, *SMAD4*, and/or the mTOR genes were preferentially seen in mucinous cysts with advanced neoplasia. Similarly, genomic alterations in *MEN1* and *VHL* were highly specific for cystic PanNETs and SCAs, respectively. The mTOR genes include *PIK3CA* and *PTEN*. HGD, high-grade dysplasia; LGD, low-grade dysplasia.

to prepare and enrich templates and enable testing via Ion Sphere Particles on a semiconductor chip. Massive parallel sequencing was carried out on an Ion GeneStudio S5 Prime System according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA) and data were analyzed with an in-house bioinformatics program, Variant Explorer (UPMC). Each variant was prioritized according to the 2017 AMP/ASCO/CAP joint consensus guidelines for interpretation of sequence variants in cancer using a tier-based system.[23] Tier I, Tier II, and Tier III variants were identified; however, only Tier I and Tier II variants were used for subsequent analysis. The limit of detection of the assay was at 1% mutant allele frequency (AF). The minimum depth of coverage for testing was 1000×. For each mutation identified, an AF was calculated based on the number of reads of the mutant allele versus the wild-type allele and reported as a percentage.[10] A low-level variant was classified based on a 10-fold lower AF as compared with the AF for a MAPK/*GNAS* mutation.[10] LOH analysis was performed as previously described.[24,25]

Oncomine Testing

Expanded targeted NGS-based testing from DNA and mRNA was also performed within the MGP lab at UPMC using the

Oncomine Comprehensive Assay v3 (Oncomine) DNA and RNA primer sets (Thermo Fisher Scientific) according to the manufacturer's protocol. The Oncomine panel evaluates 161 cancer-relevant driver genes to include 760 fusion genes. Briefly, total DNA and mRNA that is reverse transcribed into complementary DNA are subjected to multiplex polymerase chain reaction to amplify the regions of interest. Amplicons were barcoded, ligated with specific adapters, and purified. RNA library quantity and quality check were performed using the 4200 TapeStation (Agilent Technologies, Santa Clara, CA). The Ion Chef was used to prepare and enrich templates and enable testing via Ion Sphere Particles on a semiconductor chip. Massive parallel sequencing was carried out on an Ion GeneStudio S5 Prime System according to the manufacturer's instructions (Thermo Fisher Scientific) and data were analyzed with Variant Explorer (UPMC) for single nucleotide variant, insertions, deletions, copy number alterations, and RNA fusion genes. The limit of detection of this DNA/RNA assay was 1% to 5% neoplastic cells.

Statistical Analysis

χ^2 analysis or Fisher's exact tests were used to compare categorical data, and Mann-Whitney *U* test was used to compare continuous variables. Sensitivity and specificity were calculated using standard 2×2 contingency tables for cases with confirmed diagnostic pathology. All statistical analyses were performed using the SPSS Statistical software, V.26 (IBM, Armonk, NY) and statistical significance was defined as a *P* value of $<.05$.

Results

Retrospective PancreaSeq Testing of 97 Patients With Diagnostic Surgical Pathology

A retrospective diagnostic performance confirmation cohort of 97 patients who underwent EUS-FNA for a pancreatic cyst and had follow-up diagnostic surgical pathology was evaluated using an expanded NGS panel (PancreaSeq) of 22 pancreatic cyst-associated genes (Supplementary Material and expected results are summarized in Supplementary Table 1). The results of retrospective PancreaSeq testing are summarized in Figure 1 (and Supplementary Table 2). Genomic alterations in *KRAS*, *GNAS*, and/or *BRAF* were detected in 56 of 63 (89%) mucinous cysts. Among mucinous cysts with advanced neoplasia, alterations in *TP53*, *SMAD4,* and the mTOR genes were identified in 19 of 22 (86%) cases. Further, 3 of 31 (10%) IPMNs with low-grade dysplasia harbored *PIK3CA* (n = 2) and *TP53* (n = 1) mutations; but, in comparison with *KRAS* missense mutations, alterations in *PIK3CA* and *TP53* were at a lower AF (low-level). Mutations in *VHL* and *MEN1* were also seen, but specific to SCAs (1 of 2, 50%) and cystic PanNETs (2 of 9, 22%), respectively. Twenty-three non-neoplastic cysts were negative for genomic alterations. The sensitivity and specificity of MAPK/*GNAS* alterations for a mucinous cyst was 89% and 100%, respectively. In addition, mutations in *GNAS* and/or *BRAF* were 100% specific for IPMNs. In conjunction with MAPK/*GNAS* mutations, alterations in *TP53*, *SMAD4*, and the mTOR genes had 86% sensitivity and 96% specificity for a mucinous cyst with advanced neoplasia. However, on exclusion of low-level *TP53* and *PIK3CA* mutations, the sensitivity and specificity for advanced neoplasia was 86% and 100%, respectively.

Prospective, Real-Time, Multi-institutional PancreaSeq Testing of 1832 Patients

Prospective PancreaSeq testing was attempted for 1933 EUS-FNA obtained pancreatic cyst fluid specimens from 1889 patients and collected from 31 institutions over a 2-year time frame. Sufficient DNA for PancreaSeq testing was identified in 1887 (98%) specimens from 1832 patients (Supplementary Table 3). Two pancreatic cysts were sampled for 55 (3%) patients at the same EUS-FNA procedure with the clinical indication that the 2 cysts were identified in a different region of the pancreas (head/uncinate/neck versus body/tail). Overall, genomic alterations were detected in 1220 (65%) specimens. Genomic alterations in *KRAS*, *BRAF*, *NRAS*, and *HRAS* were seen in 917 (49%), 91 (5%), 2 (<1%), and 1 (<1%) cysts, respectively (Figure 2 and Supplementary Material). In contrast to other gastrointestinal neoplasms, a minority of *BRAF* alterations were V600E/L/M/R mutations (class I mutations), and instead were predominantly class II and class III *BRAF* mutations (n = 83, 91%) (Supplementary Table 4). The most prevalent *BRAF* alteration was an in-frame deletion involving codon 486. Activating *GNAS* mutations were seen in 569 (30%) cyst fluid specimens, and co-occurred with either *KRAS*, *BRAF,* or both genes in 441 (of 569, 78%), 57 (10%), and 12 (2%) cases. Among *GNAS*-mutant cysts, 510 (90%) harbored a genomic alteration in at least 1 gene involved within the MAPK pathway. In total, mutations in the MAPK genes and *GNAS* were detected in 1050 (56%) cases (Supplementary Table 5). Multiple mutations in *KRAS* and *GNAS* were found in 138 (7%) and 26 (1%) cysts, respectively. In addition, a concurrent LOH in *KRAS* and *GNAS* was seen in 4 and 1 case, respectively.

Among 1050 MAPK/*GNAS*-mutant cysts, 158 (15%) were found to have *TP53*, *SMAD4*, and/or mTOR gene alterations (Supplementary Table 6). With respect to MAPK/*GNAS* AF, low-level point mutations in *TP53* and *PIK3CA* were seen in 18 (of 158, 11%) and 8 (5%) cases, respectively. In addition to *TP53*, *SMAD4*, and the mTOR genes, 11 MAPK/*GNAS*-mutant cysts had *CTNNB1* mutations. Five of 11 MAPK/*GNAS*/*CTNNB1*-mutant cysts had low-level *CTNNB1* missense mutations as compared with the AF for the MAPK/*GNAS* gene(s). Further, none of the MAPK/*GNAS*/*CTNNB1*-mutant cysts had co-occurring *TP53*, *SMAD4*, and/or mTOR gene alterations (Supplementary Table 7).

In the absence of a MAPK/*GNAS* mutation (n = 837), alterations in *VHL*, *MEN1,* or both genes were seen in 125 (15%), 19 (2%), and 11 (1%) cysts, respectively. Co-occurring alterations were identified in 37 of 125 (30%) *VHL*-mutant/*MEN1* wild-type cysts and included point mutations in *TP53* (n = 5), the *TERT* promoter (n = 5), and *PTEN* (n = 1) as well as LOH for *PTEN* (n = 19), *TP53* (n = 18), *SMAD4* (n = 18), and *RNF43* (n = 15). Six of 19 (32%) *MEN1*-mutant/*VHL* wild-type cysts also harbored co-occurring alterations that included a *TP53* missense

mutation (n = 1) and LOH in *SMAD4* (n = 6). Interestingly, the *VHL* alterations in all 11 *VHL*/*MEN1*-mutant cysts consisted of LOH alterations. Further, 9 of 11 (82%) *VHL*/*MEN1*-mutant cysts had co-occurring LOH in *TP53* (n = 6), *SMAD4* (n = 5), *RNF43* (n = 5), and/or *PTEN* (n = 9). In the absence of *VHL* and/or *MEN1* alterations, LOH in *TP53* (n = 5), *SMAD4* (n = 13), *RNF43* (n = 5), and/or *PTEN* (n = 4) was identified in 21 cysts. Point mutations in *TP53* as the sole genomic alteration were seen in 7 cases. Finally, *IDH1* and *IDH2* missense mutations were detected in 1 cyst each without co-occurring alterations.

Clinicopathologic Correlation and Follow-up Information for 1216 Patients

Associated clinicopathologic data were available for 1216 of 1832 (66%) patients (Supplementary Material and Supplementary Table 3) that includes 1253 EUS-FNA obtained pancreatic cyst fluid specimens with genomic alterations detected in 851 specimens, whereas the remaining 402 specimens were negative for detectable mutations. In addition, follow-up information ranged between 2 and 35 months (mean, 20 months; median, 21 months). Diagnostic surgical pathology was available for 251 of 1216 (21%) patients who underwent surgery within 2 to 34 months (mean, 9 months; median, 4 months) from initial EUS-FNA and PancreaSeq testing. This cohort of surgical resected lesions consisted of 246 cysts arising within the pancreas (Figure 3) and 5 metastatic carcinomas involving the pancreas. Alterations in *KRAS*, *BRAF*, and/or *GNAS* were preoperatively detected in 159 of 167 (95%) IPMNs and *KRAS* missense mutations were seen in 9 of 19 (47%) MCNs. In addition to MAPK/*GNAS* mutations, alterations in *TP53*, *SMAD4*, and/or the mTOR genes were identified in 77 of 90 (86%) IPMNs with advanced neoplasia, 6 of 6 (100%) MCNs with advanced neoplasia, and 5 of 77 (6%) IPMNs with low-grade dysplasia (Figure 4 and Supplementary Figure 1). *CTNNB1* missense mutations were also detected in 2 IPMNs with high-grade dysplasia and 1 IPMN with low-grade dysplasia. Both IPMNs with high-grade dysplasia were negative for alterations in *TP53*, *SMAD4*, and the mTOR genes. Low-level point mutations in *TP53*, *PIK3CA, PTEN,* and *CTNNB1* corresponded to either an IPMN with low-grade dysplasia or an MCN with low-grade dysplasia. LOH in *KRAS* or *GNAS* was also observed in 4 IPMNs with an associated adenocarcinoma; however, 1 of 4 IPMNs was preoperatively negative for alterations in *TP53*, *SMAD4*, *CTNNB1*, and the mTOR genes.

All 13 (100%) SCAs harbored *VHL* alterations. In addition to *VHL*, 4 SCAs harbored point mutations in either *TP53* (n = 2) or the *TERT* promoter (n = 2). Before surgical resection, all 4 SCAs with a *TP53* or *TERT* promoter mutation demonstrated an interval increase in cyst size (Supplementary Figure 2). Further, 1 *TP53*-mutant SCA exhibited progressive stricturing of the main pancreatic duct and both acute and chronic pancreatitis. Thirty-four patients who underwent surgery were found to have a cystic PanNET. Genomic alterations found in preoperative cyst fluid specimens from these 34 cystic PanNETs included 7 with *MEN1* mutations and 16, 14, 13, 12, and 11 cases with LOH for *SMAD4*, *VHL*, *TP53*, *PTEN*, and *RNF43,* respectively. Collectively, the presence of an *MEN1* mutation and/or LOH were seen in 24 of 34 (71%) cases.

To further analyze the clinicopathologic features of PanNETs harboring LOH for *SMAD4*, *VHL*, *TP53*, *PTEN,* and/or *RNF43*, 53 preoperative biopsies from patients with a solid PanNET encountered during the study period were tested using PancreaSeq and correlated with surgical outcome and associated follow-up (Supplementary Material and Supplementary Table 8). Based on a total of 87 PanNETs (34 cyst fluid specimens and 53 biopsies), *MEN1* alterations were identified in 21 (42%) cases, whereas LOH of *SMAD4*, *VHL*, *TP53*, *PTEN*, and/or *RNF43* was seen in 51 (59%) cases (Figure 5). The presence of LOH for ≥1 gene correlated with perineural invasion, lymphovascular invasion, regional lymph node metastases, and distant organ metastasis ($P < .012$). LOH for ≥1 gene was also associated with loss of protein expression for ATRX and DAXX, and the presence of alternative lengthening of telomeres (ALT) by telomere-specific fluorescence in situ hybridization ($P < .001$). Of note, within this solid and cystic PanNET study cohort, 21 of 51 (41%) PanNETs with LOH of ≥1 gene were 1.0 to 2.0 cm in greatest dimension.

The remaining 965 patients had clinical follow-up data, but no diagnostic surgical pathology. Of the 965 patients, 2 pancreatic cysts were sampled from 37 patients, and 495 (51%) patients had a pancreatic cyst with a MAPK/*GNAS* alteration. For the 37 patients with 2 pancreatic cyst specimens, both specimens harbored mutations in the MAPK and/or *GNAS* genes. Twelve of the 495 (2%) patients also had mutations in *TP53* (n = 6) or *PIK3CA* (n = 6), but all except 1 case with a *PIK3CA* mutation were low-level point mutations. Co-occurring *CTNNB1* missense mutations were seen in 6 cases, and 4 of 6 cases were low-level alterations. For the 470 patients with a MAPK/*GNAS* wild-type cyst, alterations in *VHL*, *MEN1,* or both genes were seen in 79 (17%), 8 (2%), and 8 (2%) cysts, respectively. Six *VHL*-mutant/*MEN1* wild-type cysts also harbored point mutations in *TP53* (n = 3) and the *TERT* promoter (n = 3). During follow-up, 4 of these 6 *VHL*-mutant/*MEN1* wild-type cysts exhibited an increase in cyst size.

Comparison and Combination of PancreaSeq Testing With Other Diagnostic Modalities

Excluding 5 metastatic carcinomas, preoperative PancreaSeq detection of MAPK/*GNAS* mutations had 90% sensitivity and 100% specificity for a mucinous cyst (Table 1). Increased fluid viscosity and an elevated CEA of >192 ng/mL had lower sensitivities (77% and 73%, respectively) and lower specificities (92% and 94%, respectively). In conjunction with MAPK/*GNAS* mutations, alterations in *TP53, SMAD4*, and/or the mTOR genes had 85% sensitivity and 96% specificity for a mucinous cyst with advanced neoplasia. The sensitivity and specificity for advanced neoplasia increased to 87% and 99%, respectively, on inclusion of MAPK/*GNAS* LOH or *TP53*, *SMAD4*, and/or mTOR gene alterations with equivalent allele

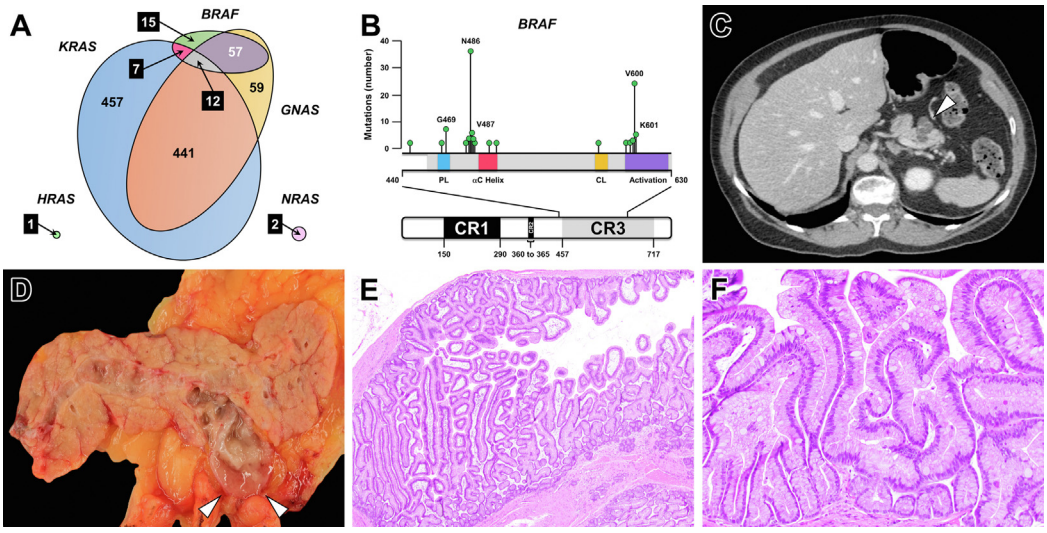

Figure 2. (*A*) An area-proportional Venn diagram demonstrates the distribution of *KRAS*, *GNAS*, *BRAF*, *NRAS*, and *HRAS* mutations identified through prospective PancreaSeq testing of 1887 pancreatic cysts. In addition to *KRAS* and *GNAS*, *BRAF* alterations were often identified in EUS-FNA obtained pancreatic cyst fluid specimens and frequently co-occurred with *GNAS* mutations. (*B*) Most *BRAF* alterations found in pancreatic cysts were non-V600E mutations and were predominantly categorized as class II and class III *BRAF* mutations (n = 83, 91%). (*C*) Based on correlative imaging and pathologic studies, *BRAF*-mutant pancreatic cysts (*white arrowhead*) were commonly found to communicate with the main pancreatic duct, and (*D*) on gross pathology, exhibited abundant, thick mucin (*white arrowheads*). (*E* and *F*) Microscopically, *BRAF*-mutant cysts corresponded to an intraductal papillary mucinous neoplasm with prominent papillary fronds and often lined by both gastric and intestinal epithelium. *(E)* Hematoxylin and eosin stain, magnification 40×. *(F)* Hematoxylin and eosin stain, magnification 200×.

frequencies to MAPK/*GNAS*. Further, the inclusion of *CTNNB1* with equivalent allele frequencies to MAPK/*GNAS* achieved a sensitivity of 89% and a specificity of 98% for advanced neoplasia. In comparison, the presence of associated clinical symptoms, jaundice for pancreatic head cysts, cyst size of >3.0 cm, main pancreatic duct dilatation, a mural nodule on EUS, increasing cyst size, and a cytopathologic diagnosis of at least suspicious for adenocarcinoma were all associated with lower sensitivities and lower specificities. Moreover, combining PancreaSeq testing with the aforementioned parameters improved the overall sensitivity of detecting advanced neoplasia (Supplementary Table 9). The highest sensitivity of 93% while maintaining a high specificity of 95% was attained using both PancreaSeq testing and cytopathologic examination (Supplementary Table 10).

Considering current pancreatic cyst guidelines have primarily focused on detecting advanced neoplasia in IPMNs, a subanalysis of combined PancreaSeq testing and cytopathologic evaluation among the 167 resected IPMNs revealed a sensitivity and a specificity of 88% and 96%, respectively (Supplementary Table 11). A comparison of the absolute criteria for surgical management from the AGA guidelines and the IAP/Fukuoka guidelines showed lower sensitivities (72% and 86%) and lower specificities (66% and 36%) than PancreaSeq and cytopathologic evaluation. Incorporating PancreaSeq testing as another criterion to the AGA guidelines did increase the sensitivity of each alone to 96%, but the specificity was 62%. Similarly, combining PancreaSeq testing to the IAP/Fukuoka guidelines improved the sensitivity to 98%, but at a specificity of 34%. However, in the prospective clinical setting, distinguishing between IPMNs with advanced neoplasia and for that matter mucinous cysts with advanced neoplasia from other neoplastic and non-neoplastic pancreatic cysts can be challenging. Therefore, we evaluated the AGA guidelines, the IAP/Fukuoka guidelines, and PancreaSeq testing in their ability to identify IPMNs and MCNs with advanced neoplasia among the 246 pancreatic cysts with diagnostic pathology. As per the AGA guidelines, the sensitivity and specificity for advanced neoplasia within a mucinous cyst was 72% and 75%, respectively, while the IAP/Fukuoka guidelines yielded a sensitivity of 84% and a specificity of 52%. The addition of PancreaSeq testing to the AGA guidelines and the IAP/Fukuoka guidelines increased the sensitivities of both guidelines to 96% and 98%, respectively, but the specificities remained essentially the same at 73% and 51%, respectively.

Although the number of resected serous neoplasms was limited, the preoperative identification of *VHL* alterations in the absence of other genomic alterations had a sensitivity and specificity of 71% and 100%, respectively. Further, the inclusion of point mutations in *TP53* or the *TERT* promoter increased the sensitivity to 100% and the specificity remained at 100%. In comparison, cytopathology was consistent with a serous neoplasm for only 1 patient, whereas the mixed serous-neuroendocrine neoplasm was misdiagnosed as a PDAC in another patient.

For cystic PanNETs, *MEN1* alterations in preoperative pancreatic cyst fluid were associated with a sensitivity and specificity of 27% and 100%, respectively. However, the inclusion of LOH for *TP53*, *SMAD4*, *PTEN,* and/or *RNF43* improved the sensitivity to 68%, while the specificity remained high at 98%. A preoperative cytopathologic diagnosis of a neuroendocrine tumor had an 85% sensitivity and 100% specificity, and combination of PancreaSeq testing and cytopathology yielded a sensitivity of 97% and a

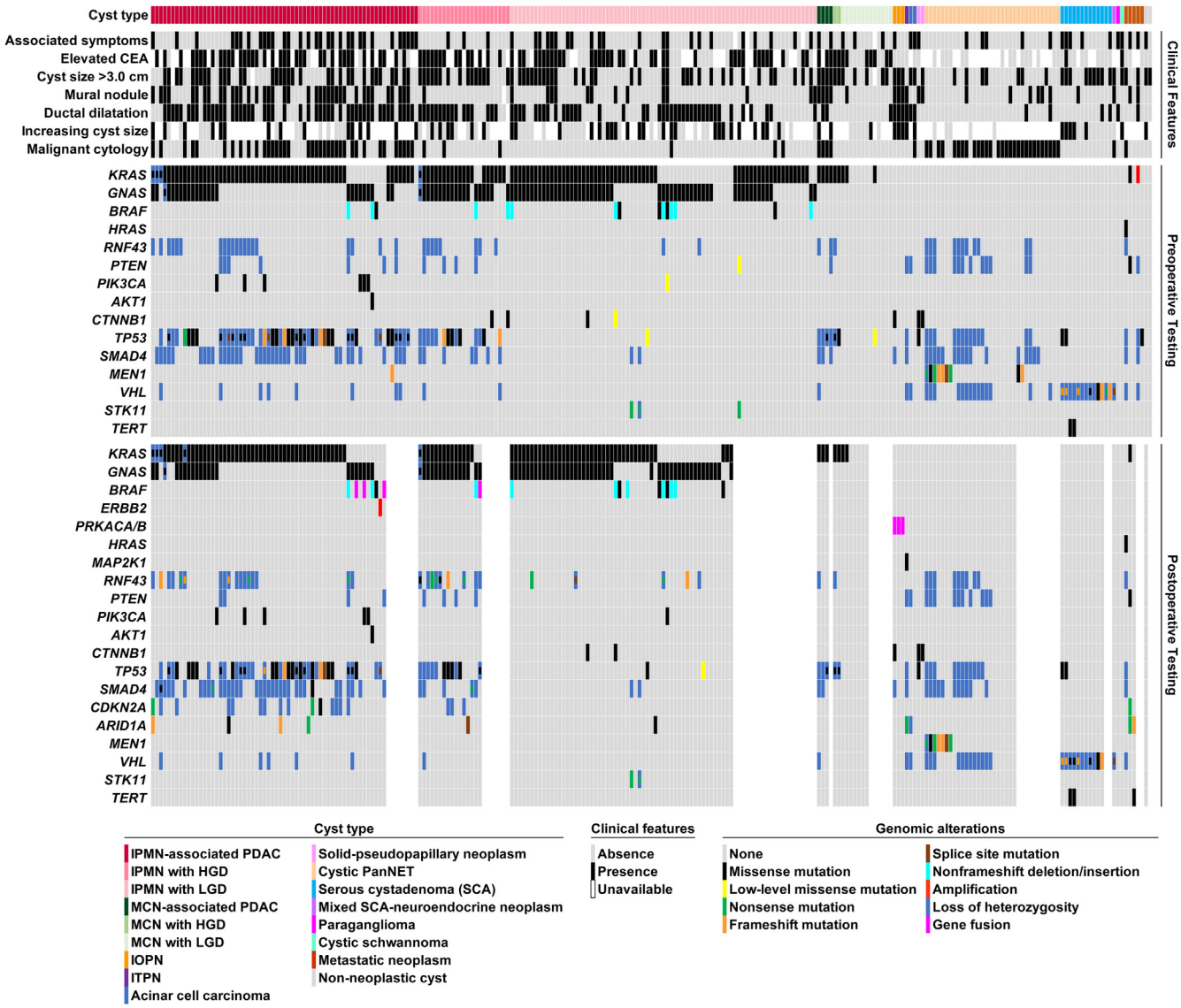

Figure 3. A summary of clinical presentation, imaging findings, pathologic features, preoperative PancreaSeq testing, and postoperative PancreaSeq/Oncomine results for 251 patients with pancreatic cyst with diagnostic surgical pathology. Preoperative genomic alterations involving *KRAS*, *GNAS*, and/or *BRAF* corresponded to the presence of a mucinous cyst, whereas additional alterations in *TP53*, *SMAD4*, *CTNNB1*, and/or the mTOR genes were preferentially found in mucinous cysts with advanced neoplasia. Other key findings were the preoperative detection of LOH for multiple genes that correlated with the presence of a cystic PanNET, and the identification of *TP53* and *TERT* promoter mutations in large SCAs. Postoperative PancreaSeq/Oncomine testing revealed the presence of novel *BRAF* fusion genes and *ERBB2* amplification in *RAS* wild-type IPMNs (Supplementary Figure 3). Moreover, *CDKN2A* alterations were preferentially found in IPMNs with advanced neoplasia. MAPK genes include *KRAS*, *BRAF*, *HRAS*, *ERBB2,* and *MAPK1*, and mTOR genes include *PTEN*, *PIK3CA,* and *AKT1*.

specificity of 98%. Further, the association with metastatic progression increased with the number of genes exhibiting LOH. An LOH of $\geq$3 genes had a sensitivity and specificity of 83% and 76%, respectively, for distant metastasis (Table 2). Comparatively, preoperative tumor size of >2.0 cm and preoperative histologic grade of at least G2 had sensitivities of 92% and 75%, respectively, and specificities of 50% and 74%, respectively, for distant metastasis. Interestingly, among 31 patients with cystic PanNET, 19 patients had tumors of 1.0 to 2.0 cm and only 1 of the 19 patients developed metastatic progression. This WHO grade 1, cystic PanNET harbored LOH for *VHL*, *TP53*, *SMAD4*, *PTEN*, and *RNF43*. Overall, the key genomic alterations detected by PancreaSeq and clinical significance are summarized in Supplementary Figure 3.

Comparative PancreaSeq/Oncomine Testing of Paired Pancreatic Cyst Fluid and Diagnostic Surgical Pathology Specimens

Repeat PancreaSeq and expanded targeted DNA/RNA-based (Oncomine) NGS testing were performed for 192 of 251 (77%) diagnostic surgical pathology specimens (Supplementary Table 12). Discordances between preoperative and postoperative testing were identified in 25 cases

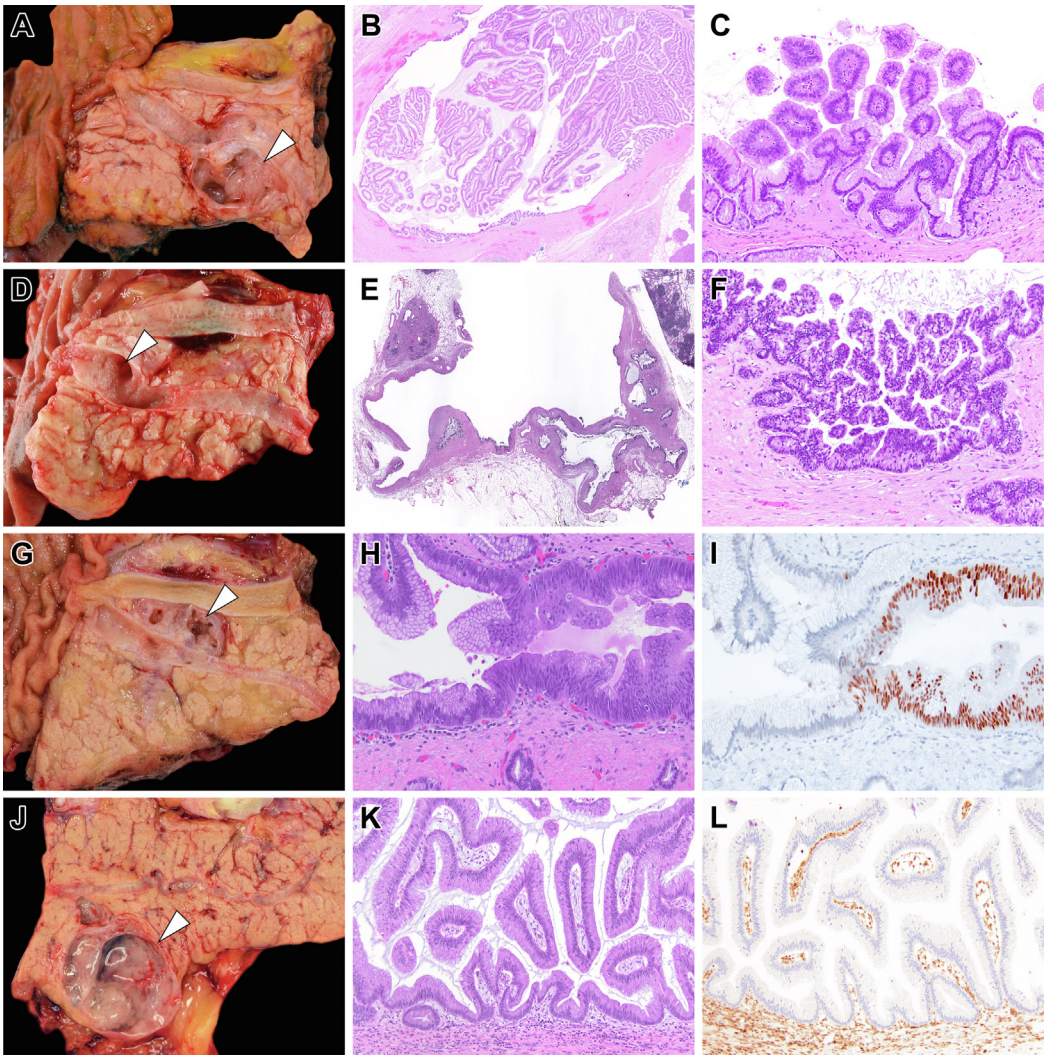

Figure 4. Representative examples of diagnostic surgical pathology for IPMNs that had preoperative PancreaSeq testing. (*A*) A branch-duct IPMN that was resected because of the presence of a mural nodule (*white arrowhead*) detected on preoperative imaging. (*B*) The mural nodule corresponded to collapsed papillary fronds and (*C*) microscopically, correlated with low-grade dysplasia. Preoperative PancreaSeq testing detected the presence of *KRAS* and *GNAS* mutations, but an absence of *TP53*, *SMAD4*, *CTNNB1*, with mTOR gene alterations. (*D*) A branch-duct IPMN (*white arrowhead*) with focal ductal dilation and otherwise no concerning preoperative clinical, imaging, or preoperative pathologic findings. Preoperative PancreaSeq testing identified mutations in *KRAS* and *GNAS,* and LOH for *PTEN* and *TP53*. (*E* and *F*) Diagnostic surgical pathology revealed the presence of high-grade dysplasia. (*G*) A branch-duct IPMN (*white arrowhead*) with focal ductal dilatation and otherwise no concerning preoperative clinical, imaging, or preoperative pathologic findings. PancreaSeq testing detected a *KRAS* mutation and a low-level *TP53* mutation. Although the submitting surgical pathology report documented the presence of an IPMN with low-grade dysplasia, a (*H*) focal area of cytologic atypia was identified and (*I*) corresponded to aberrant nuclear p53 expression. (*J*) A 3.0-cm branch-duct IPMN (*white arrowhead*) with otherwise no concerning preoperative clinical, imaging, or preoperative pathologic findings; however, PancreaSeq testing identified a *KRAS* mutation and *SMAD4* LOH. (*K*) Although histologically consistent with an IPMN with low-grade dysplasia, (*L*) diffuse loss of Smad4 expression was seen throughout the IPMN. The mTOR genes include *PIK3CA* and *PTEN*. *(B)* Hematoxylin and eosin stain, magnification 20×. *(C)* Hematoxylin and eosin stain, magnification 200×. *(E)* Hematoxylin and eosin stain, magnification 20×. *(F)* Hematoxylin and eosin stain, magnification 200×. *(H)* Hematoxylin and eosin stain, magnification 200×. *(I)* p53 immunolabeling, magnification 200×. *(K)* Hematoxylin and eosin stain, magnification 200×. *(L)* SMAD4 immunolabeling, magnification 200×.

and exclusively seen in IPMNs (Figure 3). Of interest, 9 discrepant cases were due to the lack of detectable MAPK/*GNAS* mutations in preoperative pancreatic cyst fluid specimens. For the remaining 16 cases, discrepancies were seen in *RNF43* (n = 8), *TP53* (n = 7), *SMAD4* (n = 2), *CTNNB1* (n = 1), and the mTOR genes (n = 3), but did not affect the overall sensitivity and specificity of PancreaSeq testing. In addition, Oncomine testing found 4 MAPK-negative IPMNs harboring *BRAF* fusions (n = 3) and *ERBB2* amplification (n = 1) (Supplementary Figure 4). To further characterize *BRAF*-mutant IPMNs, whole transcriptome sequencing revealed a similar gene expression profile as *KRAS*-mutant IPMNs (Supplementary Material and Supplementary Figure 5). Additional genomic alterations found among

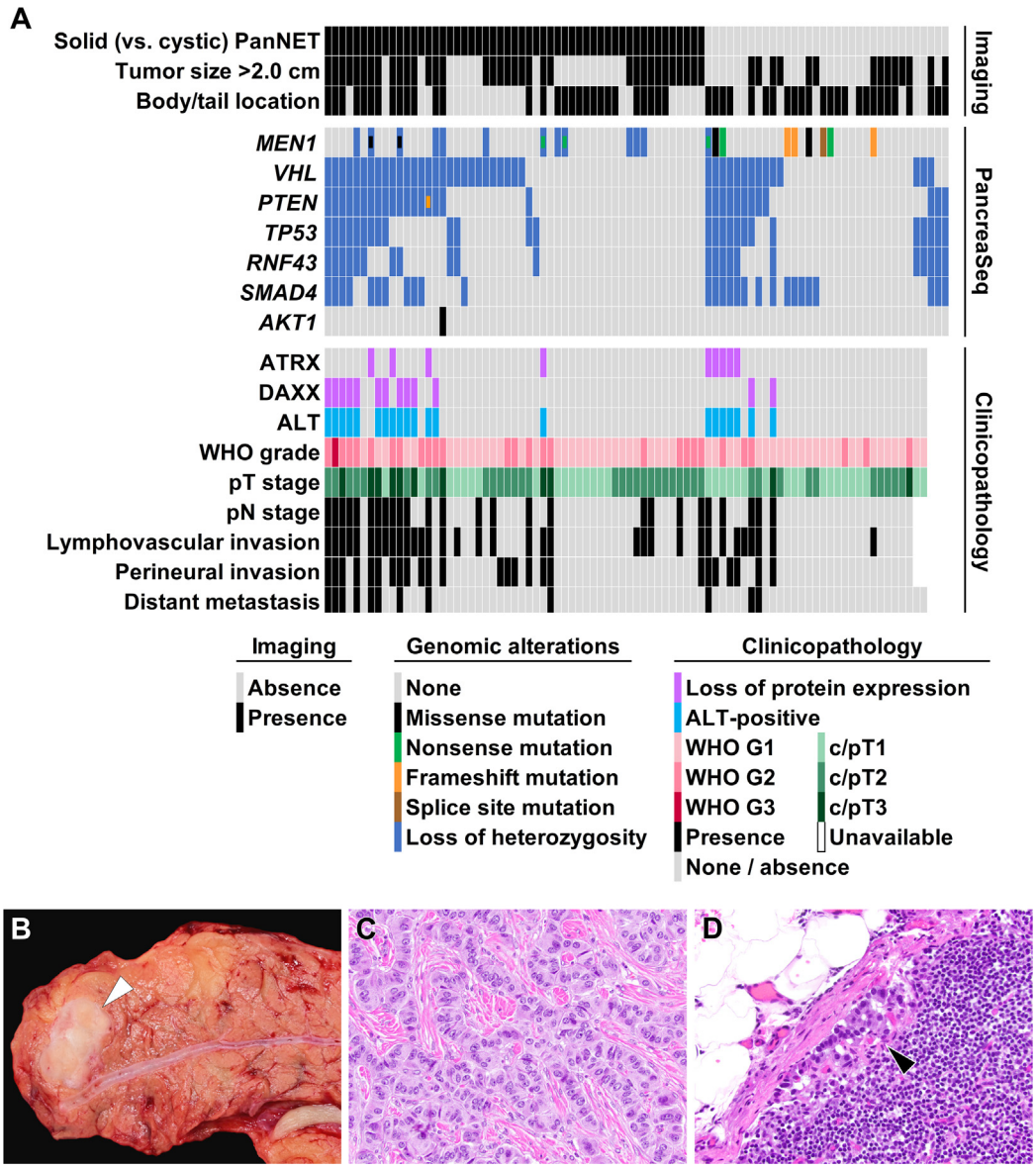

Figure 5. (*A*) A summary of imaging findings, preoperative PancreaSeq testing, and postoperative clinicopathologic features of 87 PanNET patients. Both solid and cystic PanNETs exhibited similar genomic alterations; however, LOH for multiple genes correlated with several adverse clinicopathologic features, such as lymphovascular invasion, perineural invasion, higher T- and N-stage, distant metastases, loss of ATRX/DAXX expression, and the presence of ALT. (*B*) A representative example of a 1.5-cm PanNET (*white arrowhead*) in the pancreatic body that preoperative PancreaSeq testing revealed LOH for 4 genes. (*C*) Microscopically and immunohistochemically, the PanNET was classified as WHO grade 1. (*D*) However, within a single regional lymph node, a metastasis was identified *(black arrowhead)*. In addition, the PanNET exhibited loss of ATRX expression and the presence of ALT. *(C)* Hematoxylin and eosin stain, magnification 400×. *(D)* Hematoxylin and eosin stain, magnification 200×.

IPMNs included those involving *CDKN2A* (18 of 131 IPMNs, 14%) and *ARID1A* (n = 6, 4%). *CDKN2A* alterations were only detected in IPMNs with advanced neoplasia (18 of 75 cases). Two IPMNs with advanced neoplasia that harbored *CDKN2A* alterations also lacked alterations in *TP53*, *SMAD4*, *CTNNB1,* and the mTOR genes.

Discussion

Despite retrospective studies and single institutional experiences, questions remain as to whether DNA-based targeted NGS can improve pancreatic cyst classification and the detection of advanced neoplasia arising in a mucinous cyst.[10–13,19] Based on a multi-institutional, prospectively collected cohort of patients with pancreatic cyst who were evaluated using a centralized molecular laboratory, mutations in the MAPK genes and/or *GNAS* achieved a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for mucinous cysts of 90%, 100%, 100%, and 77%, respectively. Both fluid viscosity and elevated CEA levels demonstrated lower sensitivities and lower specificities. Combining PancreaSeq testing with CEA analysis increased the sensitivity to 99%, but at a loss in specificity of 73%. Similarly, MAPK/*GNAS* LOH or *TP53*,

Table 1. Diagnostic Performance of PancreaSeq Testing and Other Modalities Based on 246 Confirmed Pancreatic Cysts

Parameter	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
IPMN				
MAPK/*GNAS* mutations	95 (0.91–0.98)	89 (0.78–0.94)	95 (0.90–0.97)	90 (0.42–0.66)
Presence of multiple cysts (n = 245)[a]	54 (0.46–0.62)	80 (0.69–0.88)	85 (0.76–0.91)	45 (0.37–0.54)
Increased fluid viscosity (n = 238)[a]	79 (0.72–0.85)	81 (0.70–0.89)	89 (0.83–0.94)	66 (0.55–0.75)
Elevated CEA (n = 173)[a]	74 (0.65–0.81)	73 (0.59–0.84)	86 (0.78–0.92)	54 (0.42–0.66)
IPMN with advanced neoplasia				
TP53, *SMAD4,* and/or mTOR gene alterations	87 (0.78–0.93)	76 (0.69–0.83)	68 (0.58–0.76)	91 (0.84–0.95)
TP53, *SMAD4, CTNNB1,* and/or mTOR gene alterations	89 (0.80–0.94)	74 (0.67–0.81)	67 (0.57–0.75)	92 (0.86–0.96)
MAPK/*GNAS* mutations with *TP53*, *SMAD4,* and/or mTOR gene alterations	84 (0.75–0.91)	92 (0.87–0.96)	86 (0.77–0.93)	91 (0.85–0.95)
MAPK/*GNAS* mutations with *TP53*, *SMAD4, CTNNB1,* and/or mTOR gene alterations	87 (0.78–0.93)	91 (0.85–0.95)	85 (0.75–0.91)	92 (0.87–0.96)
MAPK/*GNAS* LOH or *TP53*, *SMAD4* and/or mTOR gene AFs = MAPK/*GNAS* AFs	86 (0.76–0.92)	95 (0.90–0.98)	91 (0.82–0.96)	92 (0.86–0.96)
MAPK/*GNAS* LOH or *TP53*, *SMAD4, CTNNB1,* and/or mTOR gene AFs = MAPK/*GNAS* AFs	88 (0.79–0.94)	94 (0.89–0.97)	90 (0.81–0.95)	93 (0.88–0.96)
Associated clinical symptoms (n = 244)[a]	38 (0.28–0.49)	71 (0.64–0.78)	44 (0.33–0.55)	66 (0.59–0.73)
Jaundice (n = 131)[b]	31 (0.20–0.44)	89 (0.78–0.95)	70 (0.50 – 0.86)	60 (0.50–0.69)
Index cyst size >3.0 cm (n = 242)[a]	56 (0.45–0.66)	55 (0.46–0.63)	41 (0.32–0.51)	68 (0.59–0.76)
Main pancreatic duct dilatation (n = 244)[a]	71 (0.60–0.80)	65 (0.57–0.73)	54 (0.44–0.63)	80 (0.71–0.86)
Presence of a mural nodule (n = 245)[a]	44 (0.34–0.55)	80 (0.72–0.85)	55 (0.43–0.67)	71 (0.64–0.78)
Increasing index cyst size (n = 125)[a]	50 (0.34–0.66)	54 (0.43–0.65)	36 (0.24–0.49)	68 (0.55–0.79)
Malignant cytopathology[c]	46 (0.35–0.56)	95 (0.90–0.98)	84 (0.70–0.92)	75 (0.68–0.81)
IPMN and MCN				
MAPK/*GNAS* mutations	90 (0.85–0.94)	100 (0.93–1.00)	100 (0.97–1.00)	77 (0.66–0.85)
Increased fluid viscosity (n = 238)[a]	77 (0.70–0.83)	92 (0.81–0.97)	97 (0.92–0.99)	57 (0.47–0.67)
Elevated CEA (n = 173)[a]	73 (0.64–0.80)	94 (0.79–0.99)	98 (0.93–1.00)	46 (0.34–0.58)
IPMN and MCN with advanced neoplasia				
TP53, *SMAD4,* and/or mTOR gene alterations	88 (0.79–0.93)	79 (0.72–0.85)	73 (0.74–0.81)	91 (0.84–0.95)
TP53, *SMAD4, CTNNB1,* and/or mTOR gene alterations	90 (0.81–0.95)	77 (0.70–0.84)	72 (0.63–0.79)	92 (0.86–0.96)
MAPK/*GNAS* mutations with *TP53*, *SMAD4,* and/or mTOR gene alterations	85 (0.76–0.92)	96 (0.91–0.98)	93 (0.85–0.97)	91 (0.85–0.95)
MAPK/*GNAS* mutations with *TP53*, *SMAD4, CTNNB1,* and/or mTOR gene alterations	88 (0.79–0.93)	95 (0.89–0.98)	91 (0.83–0.96)	92 (0.87–0.96)
MAPK/*GNAS* LOH or *TP53*, *SMAD4,* and/or mTOR gene AFs = MAPK/*GNAS* AFs	87 (0.78–0.92)	99 (0.95–1.00)	98 (0.91–1.00)	92 (0.86–0.96)
MAPK/*GNAS* LOH or *TP53*, *SMAD4, CTNNB1,* and/or mTOR gene AFs = MAPK/*GNAS* AFs	89 (0.80–0.94)	98 (0.94–1.00)	97 (0.90–0.99)	93 (0.88–0.96)
Associated clinical symptoms (n = 244)[a]	38 (0.28–0.48)	72 (0.64–0.79)	46 (0.35–0.58)	64 (0.56–0.71)
Jaundice (n = 131)[b]	31 (0.20–0.44)	89 (0.78–0.95)	70 (0.50–0.86)	60 (0.50–0.69)
Index cyst size >3.0 cm (n = 242)[a]	59 (0.48–0.68)	57 (0.48–0.65)	46 (0.37–0.56)	68 (0.59–0.76)
Main pancreatic duct dilatation (n = 244)[a]	68 (0.58–0.77)	65 (0.57 – 0.73)	56 (0.46–0.65)	76 (0.68–0.83)
Presence of a mural nodule (n = 245)[a]	45 (0.35–0.56)	81 (0.74–0.87)	61 (0.48–0.72)	70 (0.63–0.77)
Increasing index cyst size (n = 125)[a]	52 (0.37–0.67)	56 (0.44–0.67)	39 (0.27–0.53)	68 (0.55–0.79)
Malignant cytopathology[c]	46 (0.36–0.56)	97 (0.92–0.99)	90 (0.77–0.96)	74 (0.67–0.80)

NOTE. MAPK genes include *KRAS*, *BRAF*, and *NRAS*; while mTOR genes include *PIK3CA*, *PTEN*, and *AKT1*.
[a]n designates the number of patients with data available for analysis.
[b]Jaundice was evaluated for patients with a cyst in the pancreatic head, uncinate and/or neck.
[c]Malignant cytopathology was defined as at least suspicious for adenocarcinoma.

SMAD4, and/or mTOR gene alterations with equivalent allele frequencies to MAPK/*GNAS* mutations attained 87% sensitivity, 99% specificity, 98% PPV, and 92% NPV for advanced neoplasia. The identification of advanced neoplasia was further improved with the inclusion of *CTNNB1* mutations and yielded a sensitivity, specificity, PPV, and NPV of 89%, 98%, 97%, and 93%, respectively. Moreover, the combination of PancreaSeq testing and cytopathologic evaluation achieved a 93% sensitivity, a 95% specificity, a 92% PPV, and a 95% NPV for advanced neoplasia.

More importantly, the incorporation of PancreaSeq testing to current IPMN-specific guidelines, such as those by the AGA guidelines and the IAP/Fukuoka guidelines, increased the sensitivities of detecting advanced neoplasia from 72% to 96% and 86% to 98%, respectively, whereas the specificities of both guidelines remained essentially the same. Considering the challenges of classifying pancreatic cysts within the preoperative setting, a separate analysis of mucinous cysts (IPMNs and MCNs) with advanced neoplasia also revealed an improvement in the sensitivities of the AGA guidelines (72% to 96%) and the IAP/Fukuoka guidelines (84% to 98%) when applying PancreaSeq testing data, while the specificities of both guidelines once again remained essentially the same. The advantage of PancreaSeq testing is its high specificity for advanced neoplasia. In contrast, the AGA guidelines and the IAP/Fukuoka Guidelines exhibit low-to-moderate specificity, but moderate-to-high sensitivity. The low-to-moderate specificity of both guidelines is not surprising, as they rely on subjective and indirect features of advanced neoplasia, such as large (>3.0 cm) pancreatic cyst size, main pancreatic duct dilatation, and the presence of a mural nodule on EUS. As reported in the AGA technical review, cyst size of >3.0 cm has a pooled sensitivity of 74% for malignancy, but a poor pooled specificity of 49%.[8] Main pancreatic duct dilatation and the presence of a mural nodule have pooled specificities of 80% and 91%, respectively, but poor pooled sensitivities of 32% and 48%, respectively.[16] The sensitivity and specificity of a mural nodule can be highly variable and largely attributable to the challenges in differentiating a mural nodule from adherent mucus within the pancreatic cyst by EUS.[26] The issues with EUS are compounded when factoring interobserver variability and operator dependence.[27] However, the utility of EUS is enhanced when coupled with FNA and cytopathologic evaluation of pancreatic cyst fluid. Cytopathologic evaluation for advanced neoplasia closely approaches 100% specificity, but it is often hampered by the low cellular content of pancreatic cyst fluid.[28] Nevertheless, in the absence of overt malignancy, differentiating high-grade from low-grade dysplasia can be problematic. In addition, distinguishing neoplastic cells from gastrointestinal tract contamination is often problematic, but imperative to establishing a diagnosis. Thus, the reported sensitivity of cytopathology for malignancy can vary widely from 25% to 88%.[8,10,11,19,29,30]

Although this study confirms the diagnostic utility of DNA-based targeted NGS, it also expands the compendium of MAPK alterations among pancreatic cysts. For instance, *BRAF* alterations were found in 5% of all pancreatic cysts and only 8% of *BRAF*-mutant cysts had co-occurring *KRAS* mutations. Most *BRAF* alterations were categorized as class II and class III and included in-frame deletions of codon 486. Previous studies have found class II and class III *BRAF* alterations, especially in-frame deletions, are often mutually exclusive of *KRAS* mutations and activate the MAPK signaling pathway.[31,32] Based on diagnostic surgical

Table 2. Diagnostic Performance of PancreaSeq Testing and Other Modalities for Serous Neoplasms and PanNETs

Parameter	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
Serous cystadenoma/neoplasm[a]				
VHL alteration in the absence of other alterations	71 (0.42–0.90)	100 (0.97–1.00)	100 (0.66–1.00)	98 (0.95–1.00)
VHL alteration w/ or w/o point mutations in *TP53* and *TERT* promoter	100 (0.73–1.00)	100 (0.97–1.00)	100 (0.73–1.00	100 (0.97–1.00)
PanNET[b]				
MEN1 alteration in the absence of other alterations	27 (0.14–0.45)	100 (0.98–1.00)	100 (0.63–1.00)	90 (0.85–0.93)
LOH[c] in the absence of other alterations	59 (0.41–0.75)	98 (0.95–0.99)	83 (0.62–0.95)	94 (0.89–0.96)
MEN1 alteration w/ or w/o LOH[c] in the absence of other alterations	68 (0.49–0.82)	98 (0.95–0.99)	85 (0.65–0.95)	95 (0.91–0.97)
Cytopathology positive for neuroendocrine tumor	85 (0.68–0.95)	100 (0.97–1.00)	97 (0.81–1.00)	98 (0.94–0.99)
MEN1 alteration w/ or w/o LOH[c] and cytopathology	97 (0.83–1.00)	98 (0.95–0.99)	89 (0.74–0.97)	100 (0.97–1.00)
Metastatic PanNET[d]				
LOH of at least 1 gene[e]	92 (0.60–1.00)	49 (0.37–0.61)	23 (0.13–0.38)	97 (0.84–1.00)
LOH of at least 2 genes[e]	92 (0.60–1.00)	68 (0.56–0.78)	32 (0.18–0.51)	98 (0.88–1.00)
LOH of at least 3 genes[e]	83 (0.51–0.97)	76 (0.65–0.85)	37 (0.20–0.57)	97 (0.87–0.99)
LOH of at least 4 genes[e]	58 (0.29–0.84)	88 (0.77–0.94)	44 (0.21–0.70)	93 (0.83–0.97)
LOH of at least 5 genes[e]	33 (0.11–0.64)	93 (0.84–0.97)	44 (0.15–0.77)	89 (0.80–0.95)
Preoperative tumor size >2.0 cm	92 (0.60–1.00)	50 (0.38–0.62)	23 (0.13–0.38)	97 (0.84–1.00)
Preoperative cytopathology WHO grades 2 and 3	75 (0.43–0.93)	74 (0.62–0.83)	32 (0.17–0.52)	95 (0.84–0.99)

[a]Based on 246 diagnostically confirmed pancreatic cysts that includes 13 serous cystadenomas and 1 mixed serous cystadenoma-neuroendocrine neoplasm.
[b]Based on 246 diagnostically confirmed pancreatic cysts that includes 34 cystic PanNETs.
[c]LOH of *TP53*, *SMAD4*, *PTEN*, and/or *RNF43*.
[d]Based on 87 preoperative specimens (34 cystic PanNETs and 53 solid PanNETs) with patient follow-up.
[e]LOH of *VHL*, *TP53*, *SMAD4*, *PTEN*, and/or *RNF43*.

pathology, *BRAF* alterations detected within this study correlated with the presence of an IPMN. Comparative RNA sequencing revealed *BRAF*-mutant IPMNs had similar gene expression profiles as *KRAS*-mutant IPMNs. In addition, through expanded targeted DNA/RNA-based NGS testing of MAPK-negative IPMNs, the spectrum of *BRAF* alterations was expanded to include fusion genes. The relationship between *BRAF* alterations and IPMNs is also interesting. For the entire prospectively collected pancreatic cyst cohort, 77% of *BRAF*-mutant pancreatic cysts harbored *GNAS* mutations, which are known to be specific for IPMNs. Although diagnostic surgical pathology was unavailable, Ren et al[33] reported the association between *BRAF* and *GNAS* mutations for 6 pancreatic cysts that were clinically consistent with IPMNs. Hence, *BRAF* alterations are likely to substitute for *KRAS* mutations as a driver of the MAPK pathway in the pathogenesis of IPMNs.

An unexpected finding from this study was the identification of pancreatic cysts harboring *VHL* alterations and either *TP53* or *TERT* promoter mutations. Consistent with prior studies, alterations in *VHL* alone were specific to serous cystic neoplasms.[12,13,18] In addition, the combination of *VHL* alterations and mutations in *TP53* or the *TERT* promoter correlated with an SCA. However, based on surveillance imaging, SCAs with these additional alterations demonstrated interval growth in size. In fact, the growth of one *VHL*/*TP53*-mutant SCA resulted in progressive stricturing of the main pancreatic duct, and, consequently, the patient developed acute and chronic pancreatitis. Although SCAs are benign and the overwhelming majority are asymptomatic, and slow growing, a subset can demonstrate increased growth and associated symptomatology.[34] Tseng et al[35] reported that patients with SCAs demonstrating a high growth rate (1.98 cm/y) and presented with abdominal pain, fullness and/or jaundice. Similarly, El-Hayek et al[36] found symptomatic patients often exhibited rapid growth of their SCA. In both studies, correlative molecular testing was not performed and, therefore, it is intriguing to surmise that clinically significant growth of an SCA and, consequently, symptomatology due to an SCA, may be associated with the development of a mutation in *TP53* or the *TERT* promoter.

Finally, *MEN1* alterations were highly specific for cystic PanNETs, but the sensitivity was only 27%. The sensitivity for cystic PanNETs improved to 68% on inclusion of LOH at the *TP53*, *SMAD4*, *PTEN*, and/or *RNF43* genomic loci. In comparison, cytopathologic evaluation achieved a sensitivity and specificity of 85% and 100%, respectively. However, the combination of cytopathologic evaluation and PancreaSeq testing yielded a 97% sensitivity and a 98% specificity for a cystic PanNET. To date, available sequencing data for cystic PanNETs are limited, but solid PanNETs are reported to harbor recurrent LOH at multiple genomic loci with a prevalence greater than *MEN1* alterations.[37–39] As described herein, LOH was similarly present in cystic PanNETs and more frequently seen than alterations in *MEN1*. Moreover, within a combined cohort of solid and cystic PanNETs, LOH for at least 1 gene was associated with several adverse prognostic features. Both Pea et al[38] and Lawrence et al[40] published related findings with LOH of multiple genomic loci correlating with an increased risk of distant metastasis. LOH of $\geq$3 genes within the PanNET study cohort had a sensitivity and specificity of 83% and 76%, respectively, for metastatic spread.

Analogous to mucinous cysts of the pancreas, both solid and cystic PanNETs are increasing in prevalence and often incidentally identified by radiographic imaging. While many patients with PanNET develop rapid and widely metastatic disease, other patients may present with indolent and slow-growing disease.[41,42] In fact, the overtreatment of PanNETs has been a subject of debate and an observational approach may be warranted for a subset of patients.[43–46] Despite the development of PanNET prognostic classification systems, such as WHO histologic grading, and tumor staging systems, such as those based on tumor size of >2.0 cm, these parameters do not necessarily reflect the pathobiology of these tumors.[47,48] LOH of at least 3 genes was associated with a higher specificity (76%) for distant metastasis than >2.0 cm tumor size (50%) and advanced WHO grade (grades 2 and 3, 74%). Moreover, LOH was superior in sensitivity (83%) than advanced WHO grade (75%). Interestingly, LOH was also associated with loss of expression of ATRX/DAXX and the presence of ALT. Although the exact mechanism has not been fully elucidated, ATRX and DAXX play an integral role in telomere maintenance, and loss of protein expression coincides with the presence of ALT, a telomerase-independent telomere maintenance mechanism.[49,50] Interestingly, ALT results in broad chromosomal abnormalities, and, therefore, it is plausible that the LOH found at multiple genomic loci in PanNETs is the sequelae of ALT and may reflect a common genomic pathway in the metastatic progression of PanNETs.

We acknowledge that there are several limitations to this study. Although a large number of pancreatic cysts were analyzed, diagnostic surgical pathology was available for only 14% of patients and represents a surgical selection bias. However, clinical follow-up was also obtained for an additional 52% of patients. Our study also suffers from a testing selection bias, as pancreatic cyst fluid specimens satisfactory for targeted NGS were used for analysis. Considering a 2% failure rate of PancreaSeq testing, the effect of this selection bias is likely to be minimal. Nonetheless, molecularly discordant results were identified when comparing preoperative and postoperative specimens. For instance, MAPK/GNAS alterations were not detected in 9 surgically resected IPMNs, but present within the corresponding surgical specimen, which underscores a potential issue of sensitivity for PancreaSeq testing. Alternative explanations for this discordance are the absence of exfoliated neoplastic cells within the pancreatic cyst fluid, degraded mutant DNA within the cyst, and adequate sampling of the pancreatic cyst by the gastroenterologist. In addition, the follow-up period of this study is relatively short to assess the clinical impact of detecting specific genomic alterations, such as *TP53*, *SMAD4*, *CTNNB1*, and the mTOR genes. Although we plan to continue monitoring patients with these genomic alterations, the median duration of follow-up was 23 months or close to 2 years, which by many

pancreatic cyst guidelines is sufficient as the initial time interval for imaging surveillance.[6,7,9,21] Another limitation is the relative paucity of certain genomic alterations to determine their true clinical significance. For example, the inclusion of *CTNNB1* to the assessment of MAPK/*GNAS*-mutant pancreatic cysts improved the identification of advanced neoplasia, but this was based on only 4 diagnostically confirmed IPMNs harboring *CTNNB1* alterations. Moreover, despite PancreaSeq consisting of 22 pancreatic cyst–related genes, it did not include other potentially important genes, such as *CDKN2A*. Several studies have reported recurrent genomic alterations in *CDKN2A* in a subset of mucinous cysts and preferentially those with advanced neoplasia.[12] Similarly, we found *CDKN2A* alterations were detected in only IPMNs and those IPMNs with advanced neoplasia at a prevalence of 24%. In addition, 2 IPMNs with advanced neoplasia that were negative for alterations in *TP53*, *SMAD4*, *CTNNB1*, and the mTOR genes harbored *CDKN2A* alterations. Hence, further studies are required to determine the clinical significance of *CDKN2A* alterations among pancreatic cysts. Moreover, as the identification of *BRAF* alterations to include fusion genes highlights, the full breadth of genomic alterations that characterize pancreatic cysts has yet to be determined. A complicated issue with this study is the incorporation of allele frequencies to improve the performance of PancreaSeq testing. As we reported previously, low-level genomic alterations in *TP53* and *PIK3CA* with respect to MAPK/*GNAS* mutations can be seen in the setting of IPMNs with low-grade dysplasia and it is plausible that these IPMNs are at an increased risk of progression to advanced neoplasia. Admittingly, the current study does not address the malignant potential of this patient population but highlights the increasing complexity of genomic alterations that characterize pancreatic cystic neoplasms. To simplify reporting of key alterations to include allele frequencies, our group is in the process of developing a pancreatic cyst molecular classifier to aid in the interpretation of genomic variants and provide surveillance/treatment guidance to both gastroenterologists and surgeons (Nikiforova and Singhi, unpublished results, 2022). Last, this study does not address the optimal approach of integrating targeted NGS testing to current pancreatic cyst surveillance protocols. As an example, the European evidence-based guidelines could not be applied to this study cohort due to the lack of sufficient data to determine "relative indications" for surgical management. None of the guidelines, however, have sufficient accuracy to dictate appropriate surveillance and management of pancreatic cysts, are admittingly based on "very low quality of evidence," and, not surprisingly, the institutions participating within this study followed different pancreatic cyst guidelines and, in many cases, utilized a personalized approach for their patients.[6,7,9,21,51–53] A major step forward in delineating an optimal pancreatic cyst protocol is the ECOG-ACRIN pancreatic cyst surveillance clinical trial of >4000 patients that will compare the effectiveness between the AGA guidelines and the IAP/Fukuoka guidelines.[54] As a secondary aim of this study, biospecimens will be collected from enrolled patients to assess the utility of promising pancreatic cyst biomarkers.

In summary, we report the results of a large, multi-institutional, prospective, and real-time study that clinically applies targeted NGS testing of EUS-FNA-obtained preoperative pancreatic cyst fluid to the evaluation of pancreatic cysts. Overall, our results underscore the clinical utility of targeted NGS given its high sensitivity and high specificity in the diagnosis of mucinous cysts and the identification of advanced neoplasia within a mucinous cyst. This study also broadens the number of genomic alterations that characterize not only mucinous cysts, but SCAs and cystic PanNETs. Although we recognize that additional studies are required, the data reported herein combined with previous studies support the integration of targeted NGS into the establishment of evidence-based pancreatic cyst guidelines.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2022.09.028.

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Conflicts of interest

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Data Availability

Study data not present within this manuscript to include but not limited to genomic data and other associated clinical and imaging metadata are available on request.

Supplementary Material

Rationale and Design of the PancreaSeq Panel

The PancreaSeq panel used herein was designed in part based on previously published next-generation sequencing testing results for the classification of various neoplastic pancreatic cysts, such as intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), and the identification of pancreatic ductal adenocarcinomas (PDACs) reported to arise in association with mucinous cysts. For instance, mutations in *KRAS, GNAS,* and *RNF43* were included because of their high sensitivity and high specificity for mucinous cysts of the pancreas.[1–11] In rare instances, alterations in *NRAS*, *HRAS*, *BRAF,* and *STK11* have also been reported to be clinically associated with mucinous cysts.[2,5,12,13] *KRAS*, *HRAS*, *NRAS,* and *BRAF* are genes collectively known to be involved in the mitogen-activated protein kinase (MAPK) pathway. Further, the clinical utility of incorporating *TP53*, *PIK3CA*, *PTEN*, and *AKT1* testing in the setting of *KRAS* and/or *GNAS* mutations for the detection of mucinous cysts with advanced neoplasia was previously published in a prospective testing cohort but this cohort comprised only a single institutional study.[5] It is also important to note that other than *PIK3CA*, *PTEN*, and *AKT1*, genomic alterations in the remaining mammalian target of rapamycin (mTOR) genes have rarely been implicated in the molecular pathogenesis of PDAC arising from a mucinous cyst.[14–18] *SMAD4* was included because of its high prevalence in both mucinous cysts with high-grade dysplasia and PDACs associated with a mucinous cyst.[1,2,9,10,19] Specific attention to mutant allele frequencies (AFs) was made considering previously reported results of low-level variants of *TP53*, *SMAD4*, and the mTOR genes with respect to MAPK/*GNAS* alterations corresponding to an absence of advanced neoplasia.[5] However, *CDKN2A* was specifically excluded due its reported detection in both low-grade and high-grade mucinous cysts.[20]

Molecular testing of pancreatic cyst fluid is not only accurate in the identification of mucinous cysts, but also the classification of other neoplastic cysts. Genomic alterations in *VHL* have been identified in serous cystadenomas (SCAs).[1,2,5,7] Similarly, recurrent mutations in exon 3 of *CTNNB1* is highly specific for solid pseudopapillary neoplasms.[21,22] Interestingly, *CTNNB1* mutations have also been reported in mucinous cysts.[20] Mutations in *MEN1* and the mTOR genes have been detected in pancreatic neuroendocrine tumors (PanNETs), but in the absence of *KRAS* and *GNAS* mutations.[23–25] Finally, the absence of genomic alterations in the aforementioned genes is predicted to represent a non-neoplastic cyst with the consideration that false negative results may occur due to insufficient sampling of a neoplastic lesion or potentially an undescribed genomic alteration associated with a subset of pancreatic cystic neoplasms (eg, intraductal oncocytic papillary neoplasm).[26] Expected results based on previously published data are summarized in Supplementary Table 1.

Retrospective PancreaSeq Testing Cohort

The study cohort consisted of 97 endoscopic ultrasound–fine needle aspiration (EUS-FNA) obtained pancreatic cyst fluid specimens that were collected as previously published and had corresponding follow-up diagnostic surgical pathology (Supplementary Table 2). The patients ranged in age from 22 to 83 years (mean, 62.5 years; median, 63.0 years) with a slight male majority of 52%. Based on the patient's electronic medical record, associated clinical symptoms were documented for 47 (49%) patients with jaundice identified for 6 of 42 (14%) patients with a pancreatic cyst involving the head, uncinate, and/or neck. Per EUS reports, most pancreatic cysts within this cohort were seen in the body and/or tail (n = 55, 57%). Further, the pancreatic cysts ranged in size between 1.3 and 9.4 cm (mean, 3.8 cm; median, 3.2 cm) and 53 (55%) patients had a cyst >3.0 cm. Additional imaging findings included the presence of multiple cysts (n = 46, 47%), associated ductal dilation (n = 26, 27%), and a mural nodule (n = 16, 17%). On FNA, increased fluid viscosity was noted for 48 (50%) patients and an elevated CEA for 41 (42%) patients. A cytopathologic diagnosis of at least suspicious for adenocarcinoma was identified in 7 (7%) cases.

On the basis of diagnostic surgical pathology, the retrospective cohort was composed of 13 IPMN-associated adenocarcinoma, 7 IPMNs with high-grade dysplasia, 2 MCNs with high-grade dysplasia, 34 IPMNs with low-grade dysplasia, 7 MCNs with low-grade dysplasia, 9 cystic PanNETs, 2 SCAs, 16 pseudocysts, 2 lymphoepithelial cysts, 2 retention cysts, 1 acinar cell cystadenoma, 1 epidermoid cyst within an intrapancreatic spleen, and 1 squamous cyst of the pancreas. The sensitivity and specificity of MAPK/*GNAS* alterations for a mucinous cyst was 89% and 100%, respectively. In comparison, increased fluid viscosity and an elevated CEA had lower sensitivities (68% and 56%, respectively) and lower specificities (85% and 82%, respectively). In conjunction with MAPK/*GNAS* mutations, alterations in *TP53, SMAD4*, and/or the mTOR genes had 86% sensitivity and 96% specificity for a mucinous cyst with advanced neoplasia. The sensitivities and specificities of individual genomic combinations for advanced neoplasia were as follows: MAPK/*GNAS* and *TP53* alterations were associated with 64% sensitivity and 99% specificity; MAPK/*GNAS* and *SMAD4* alterations were associated with 46% sensitivity and 100% specificity; and MAPK/*GNAS* and mTOR alterations were associated with 32% sensitivity and 96% specificity. Of note, the combination of MAPK/*GNAS* with *TP53* and/or *SMAD4* yielded a sensitivity of 77% and a specificity of 99%. However, on exclusion of low-level *TP53* and *PIK3CA* mutations, the sensitivity and specificity of the MAPK/*GNAS* and *TP53*, *SMAD4,* and/or mTOR gene combination of genomic alterations was 86% and 100%, respectively. The sensitivities and specificities for advanced neoplasia were lower for the presence of associated clinical symptoms (55% and 53%), jaundice for pancreatic head cysts (20% and 89%), cyst size of >3.0 cm (59% and 47%), main pancreatic duct dilatation (45% and 79%), a mural

nodule (27% and 87%), and a cytopathologic diagnosis of at least suspicious for adenocarcinoma (27% and 99%).

Prospective PancreaSeq Testing Cohort

In total, 1993 EUS-FNA–obtained pancreatic cyst fluid specimens from 1889 patients were prospectively analyzed for genomic alterations over a 2-year time frame. Among these cases, 1887 (98%) specimens from 1832 patients were satisfactory for PancreaSeq testing (Supplementary Table 3). The DNA concentration from these specimens ranged between 0.01 and 283 ng/μL (mean, 6.84 ng/μL; median, 4.4 ng/μL). This patient cohort was predominantly female (n = 1048, 56%) and ranged in age from 12 to 80 years (mean, 66.3 years; median, 69.0 years). Associated clinical and imaging data were available for most patients with documentation of associated clinical symptoms (n = 1227, 67%), jaundice for pancreatic head/uncinate/neck cysts (n = 635, 34%), pancreatic cyst location (n = 1225, 65%), pancreatic cyst size (n = 1167, 62%), changes in cyst size (n = 434, 23%), the presence of multiple cysts (n = 1167, 62%), main duct dilatation (n = 1166, 62%), and a mural nodule (n = 1174, 62%). Further, on FNA, increased fluid viscosity by string sign assessment (n = 1119, 59%), pancreatic cyst fluid CEA (n = 712, 38%), and cytopathologic evaluation (n = 642, 34%). Genomic alterations in *KRAS*, *GNAS*, *BRAF*, *VHL*, *TP53*, *SMAD4*, *CTNNB1*, and the mTOR genes and their clinicopathologic correlative findings are summarized in Supplementary Tables 5, 6, and 7.

PancreaSeq Testing of PanNETs

With respect to PancreaSeq testing, a clinicopathologic analysis of cystic (n = 34, 39%) and solid (n = 53, 61%) PanNETs was performed for 87 preoperative specimens (Supplementary Table 8). This study cohort consisted of an equivalent number of female-to-male patients who ranged in age between 25 and 85 years (mean, 61.2 years; median, 65.0 years). PanNETs were predominantly located within the body and/or tail of the pancreas (n = 53, 61%) and ranged in size from 1.0 to 9.3 cm (mean, 2.7 cm; median, 2.2 cm). Most PanNETs were >2.0 cm in greatest dimension (n = 49, 56%). Available surgical pathologic data and follow-up included WHO grade (based on Ki-67 and mitotic index) (n = 84), lymphovascular invasion (n = 82), perineural invasion (n = 82), clinical/pathologic (c/p) T-stage (n = 82), N-stage (n = 82), ATRX/DAXX immunohistochemical expression (n = 84), telomere-specific fluorescence in situ hybridization data to assess for alternative lengthening of telomeres (ALT) (n = 84), and distant metastasis (n = 84).

Comparative Whole Transcriptome (RNA) Sequencing of BRAF-Mutant and KRAS-Mutant IPMNs With Low-Grade Dysplasia

Whole transcriptome (RNA) sequencing and differential gene expression analysis was performed for 18 *GNAS*-mutant, diagnostically confirmed IPMNs with low-grade dysplasia and co-occurring mutations in either *BRAF* (n = 9) or *KRAS* (n = 9). For each cohort, cases consisted of 3 preoperative EUS-FNA specimens and 6 surgical resection specimens obtained from the prospective PancreaSeq testing cohort (Supplementary Figure 4). Although a comparison of *BRAF*-mutant and *KRAS*-mutant IPMNs identified a trend in the differential expression of TERT and SCARNA1, no statistically significant difference was identified. Overall, *BRAF*-mutant and *KRAS*-mutant IPMNs with low-grade dysplasia that also harbored a *GNAS* mutation demonstrated similar gene expression profiles.

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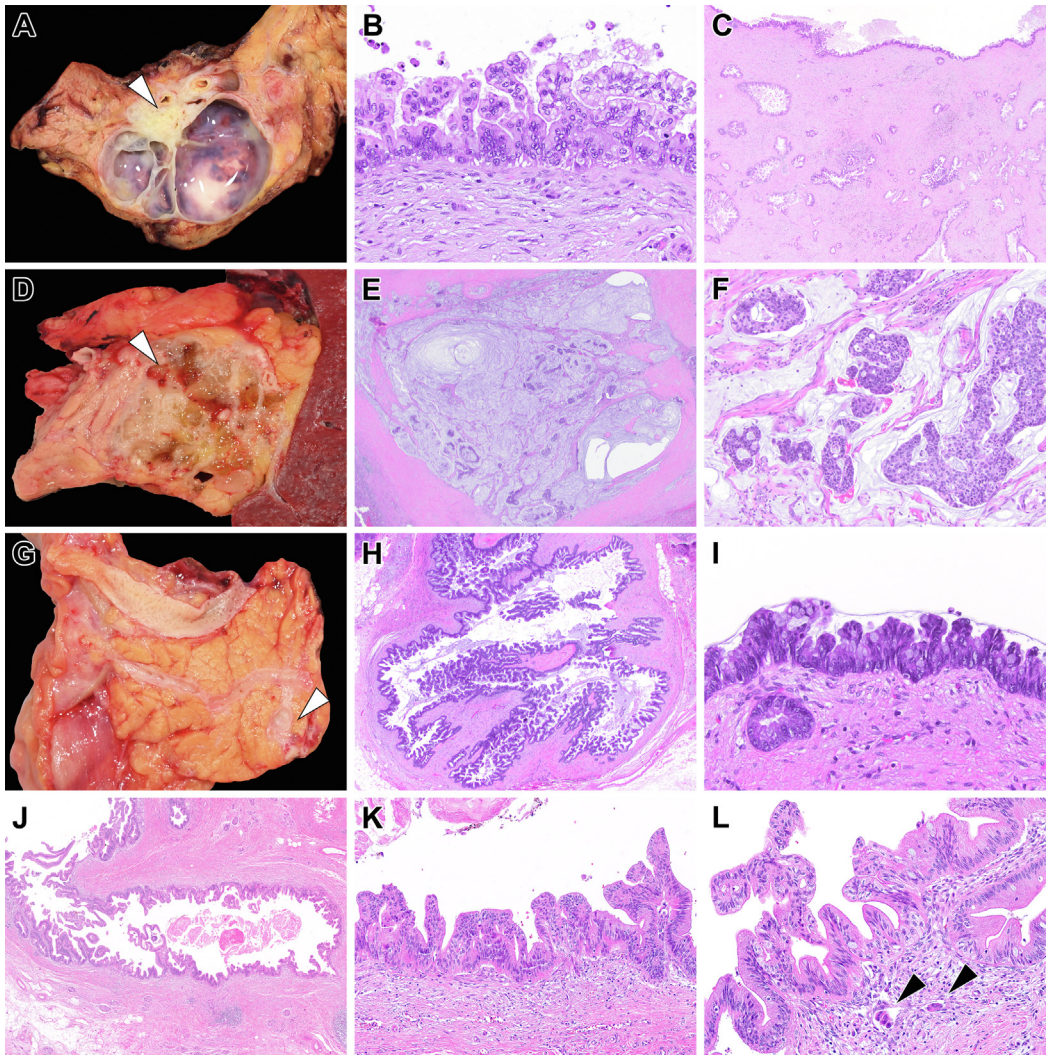
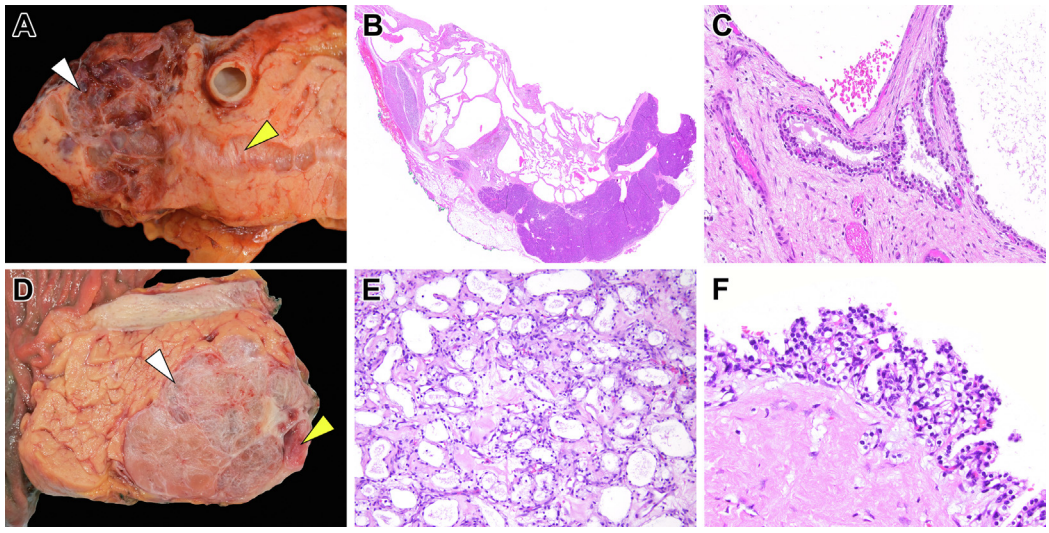
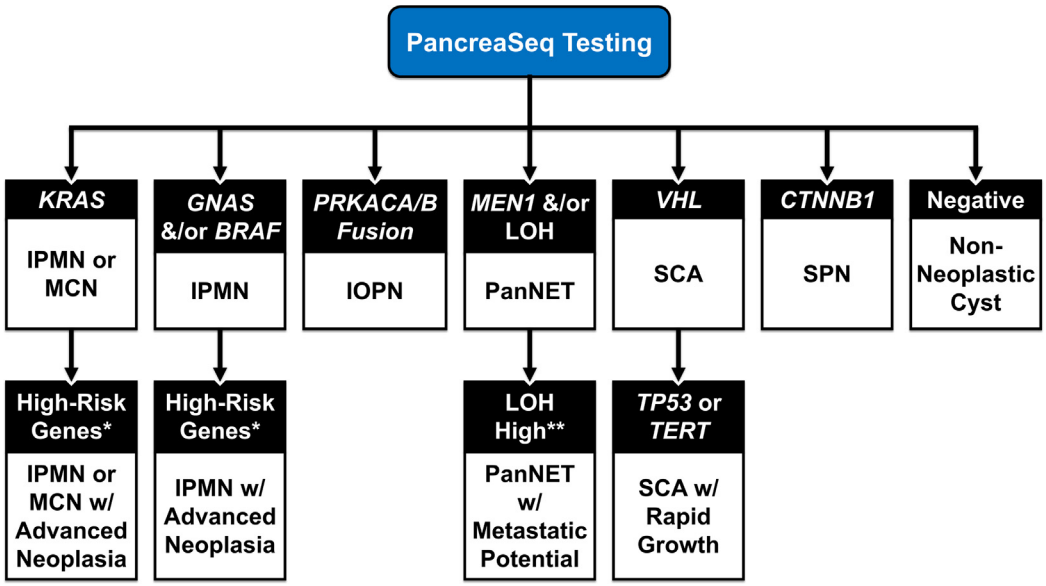


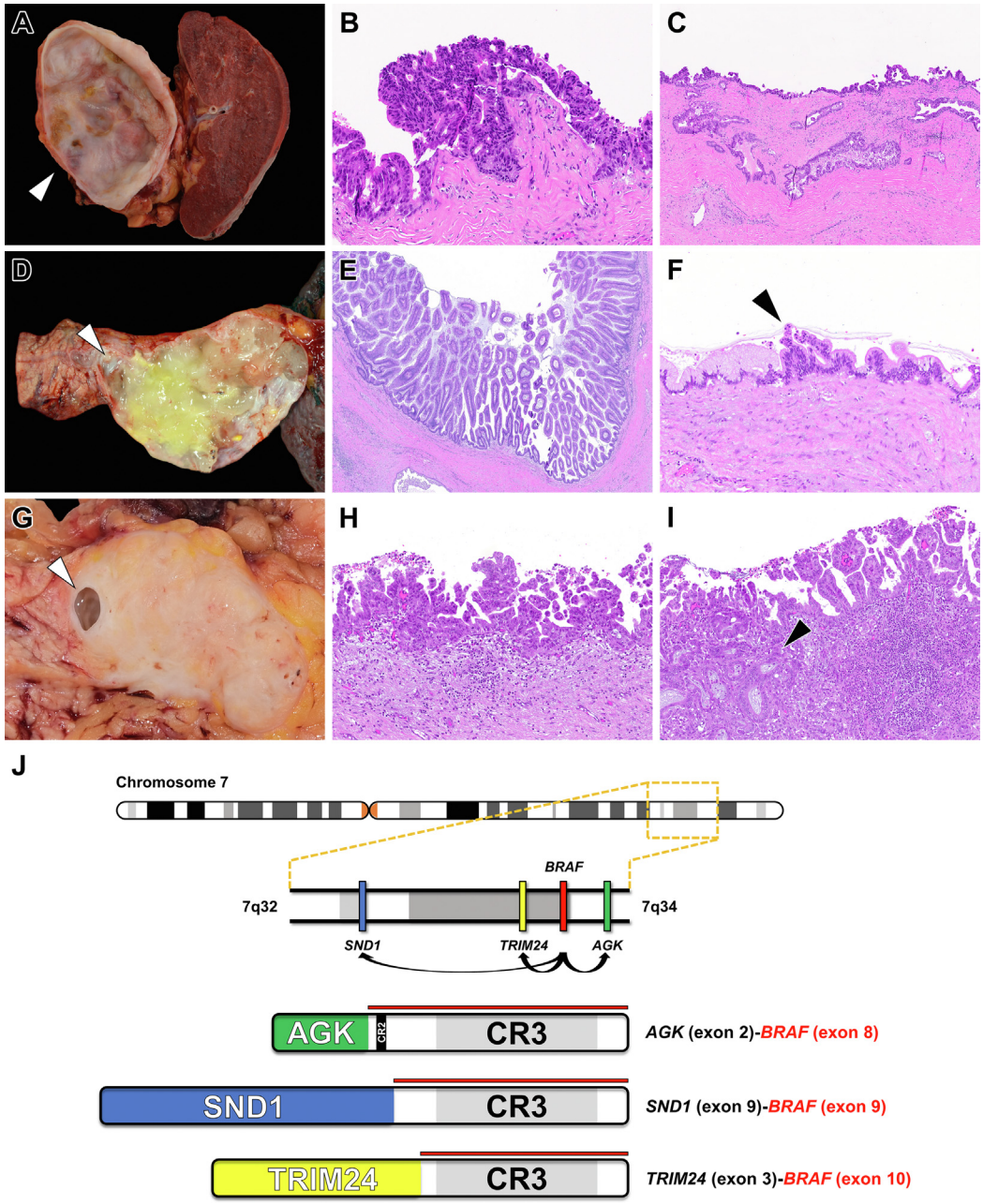
Supplementary Figure 1. Representative examples of diagnostic surgical pathology for IPMNs with advanced neoplasia that had preoperative PancreaSeq testing. (*A*) An IPMN-associated PDAC (*white arrowhead*) in a patient had PancreaSeq testing 1 year prior. One-year prior, other than a 3.1-cm pancreatic cyst, no concerning preoperative clinical, imaging, or preoperative pathologic findings were identified. However, PancreaSeq testing revealed *KRAS* and *GNAS* mutations along with LOH for *RNF43* and *TP53*. The patient deferred surgery and on imaging follow-up a solid lesion was identified and corresponded to (*B* and *C*) a moderately differentiated PDAC in association with an IPMN with extensive high-grade dysplasia. (*D*) A 3.5-cm pancreatic tail cyst (*white arrowhead*) with otherwise no concerning preoperative clinical, imaging, or preoperative pathology findings. Cytopathologic evaluation of EUS-FNA pancreatic cyst fluid only detected acellular mucin, but PancreaSeq testing identified a *KRAS* mutation and LOH for *RNF43* and *TP53*. (*E* and *F*) Microscopically, a colloid carcinoma was identified arising in IPMN. (*G*) A 1.2 cm branch-duct IPMN (*white arrowhead*) with focal ductal dilation, and otherwise no concerning preoperative clinical, imaging, or preoperative pathologic findings; however, PancreaSeq testing revealed mutations in *KRAS, GNAS,* and *CTNNB1* of similar AFs. (*H* and *I*) Histopathologic examination revealed an IPMN with extensive high-grade dysplasia. (*J*) A branch-duct IPMN no concerning preoperative clinical, imaging, or preoperative pathologic findings. PancreaSeq testing, however, detected *KRAS* and *GNAS* mutations and LOH for *SMAD4*. (*K* and *L*) On surgical resection, a small (<0.1 cm), microscopic PDAC (*white arrowheads*) composed of single cells was identified in association with an IPMN with high-grade dysplasia.



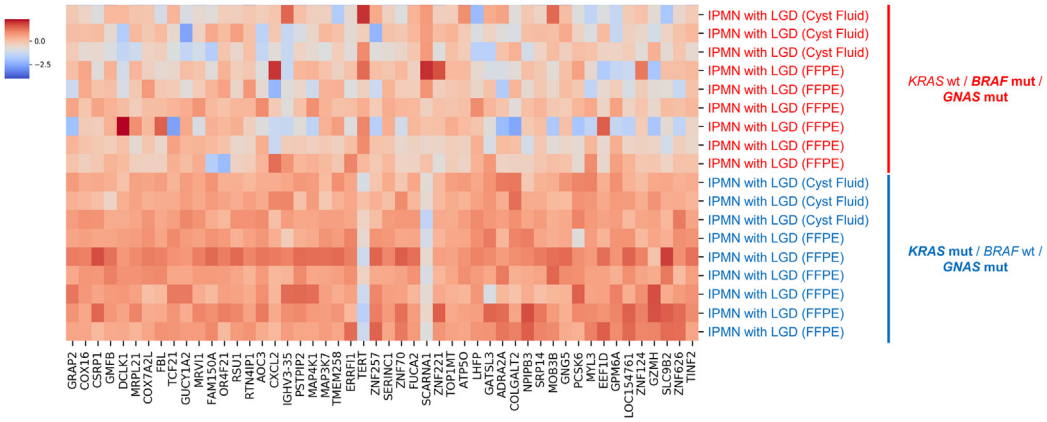
Supplementary Figure 2. SCAs were not only characterized by *VHL* alterations, but also *TP53* and *TERT* promoter mutations. (*A*) A 3.8-cm SCA (*white arrowhead*) of the pancreatic body that was surgically resected due to secondary obstruction of the main pancreatic duct (*yellow arrowhead*). Preoperative PancreaSeq testing revealed *VHL* and *TP53* alterations. (*B* and *C*) Microscopically, the SCA consisted of a multilocular cyst that was lined by glycogen-laden epithelium. (*D*) An 8.0-cm SCA (*white arrowhead*) of the pancreatic head was resected due to main pancreatic ductal obstruction (*yellow arrowhead*) resulting in the patient presenting with chronic pancreatitic symptoms. Preoperative PancreaSeq testing detected *VHL* and *TERT* promoter mutations. (*E* and *F*) The corresponding diagnostic surgical pathology showed a microcystic growth pattern and multiple foci of pseudopapillae of glycogen-laden epithelium.



Supplementary Figure 3. Algorithmic approach to key genomic alterations detected by PancreaSeq testing and their clinical significance. *Refers to high-risk genes that include genomic alterations in TP53, SMAD4, CTNNB1, and the mTOR genes, and **refers to LOH of $\geq$3 genes.



Supplementary Figure 4. Several IPMNs were negative for MAPK mutations by PancreaSeq testing. However, expanded molecular (Oncomine) testing identified alterative MAPK driver mutations for 5 cases. (*A*) An 8.3-cm pancreatic body/tail IPMN (*white arrowhead*) with (*B*) extensive high-grade dysplasia and (*C*) focal invasive PDAC. Oncomine testing detected an *ERBB2* amplification. In addition to *ERBB2*, 4 IPMNs were found to harbor *BRAF* fusion genes. (*D*) A 4.9-cm pancreatic body/tail IPMN (*white arrowhead*) that on preoperative PancreaSeq testing revealed a *GNAS* mutation and LOH for *RNF43* and *TP53*. (*E* and *F*) Microscopically, the IPMN with characterized by papillary and flat architecture, and multiple foci of high-grade dysplasia (*black arrowhead*). Postoperative Oncomine testing of the IPMN found an AGK-BRAF fusion gene. (*G*) A 2.7-cm pancreatic head/uncinate IPMN (*white arrowhead*) was surgically resected due to the detection of a mural nodule and subsequent malignant cytopathology. While preoperative PancreaSeq testing identified *GNAS* and *TP53* mutations of similar AFs, no *KRAS* or *BRAF* mutations were seen. (*H* and *I*) The corresponding surgical pathology was consistent with an IPMN-associated PDAC (*black arrowhead*). In addition, postoperative Oncomine testing showed the presence of an SND1-BRAF fusion gene. (*J*) A total of 4 IPMNs were found to harbor *BRAF* fusion genes and consisted of *AGK* (exon 2)-*BRAF* (exon 8) (n = 1), *SND1* (exon 9)-*BRAF* (exon 9) (n = 2), and *TRIM24* (exon 3)-*BRAF* (exon 10) (n = 1).



Supplementary Figure 5. Differential gene expression analysis was performed for 18 *GNAS*-mutant IPMNs with low-grade dysplasia and co-occurring mutations in either *BRAF* (n = 9) or *KRAS* (n = 9). A trend toward increased expression of TERT and SCARNA1 was identified in *BRAF*-mutant IPMNs as compared with *KRAS*-mutant IPMNs. However, these findings were not statistically significant. Overall, *BRAF*-mutant and *KRAS*-mutant IPMNs with low-grade dysplasia and *GNAS* mutations demonstrated similar gene expression profiles.