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ORIGINAL ARTICLE

Preoperative next-generation sequencing of pancreatic cyst fluid is highly accurate in cyst classification and detection of advanced neoplasia

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ABSTRACT

Objective DNA-based testing of pancreatic cyst fluid (PCF) is a useful adjunct to the evaluation of pancreatic cysts (PCs). Mutations in *KRAS*/*GNAS* are highly specific for intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), while *TP53*/*PIK3CA*/*PTEN* alterations are associated with advanced neoplasia. A prospective study was performed to evaluate preoperative PCF DNA testing.

Design Over 43-months, 626 PCF specimens from 595 patients were obtained by endoscopic ultrasound (EUS)-fine needle aspiration and assessed by targeted next-generation sequencing (NGS). Molecular results were correlated with EUS findings, ancillary studies and follow-up. A separate cohort of 159 PCF specimens was also evaluated for *KRAS*/*GNAS* mutations by Sanger sequencing.

Results *KRAS/GNAS* mutations were identified in 308 (49%) PCs, while alterations in *TP53/PIK3CA/PTEN* were present in 35 (6%) cases. Based on 102 (17%) patients with surgical follow-up, *KRAS/GNAS* mutations were detected in 56 (100%) IPMNs and 3 (30%) MCNs, and associated with 89% sensitivity and 100% specificity for a mucinous PC. In comparison, *KRAS/GNAS* mutations by Sanger sequencing had a 65% sensitivity and 100% specificity. By NGS, the combination of *KRAS/GNAS* mutations and alterations in *TP53/PIK3CA/PTEN* had an 89% sensitivity and 100% specificity for advanced neoplasia. Ductal dilatation, a mural nodule and malignant cytopathology had lower sensitivities (42%, 32% and 32%, respectively) and specificities (74%, 94% and 98%, respectively).

Conclusions In contrast to Sanger sequencing, preoperative NGS of PCF for *KRAS*/*GNAS* mutations is highly sensitive for IPMNs and specific for mucinous PCs. In addition, the combination of *TP53*/*PIK3CA*/*PTEN* alterations is a useful preoperative marker for advanced neoplasia.

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INTRODUCTION

The frequent detection of a pancreatic cyst (PC) by abdominal imaging has created a diagnostic and treatment dilemma. PCs represent a broad and diverse group of lesions that range from benign to malignant entities. For example, pseudocysts and serous cystadenomas (SCAs) do not have malignant potential and can be monitored clinically, whereas mucinous PCs, such as intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), can progress to invasive pancreatic ductal adenocarcinoma.[1 2] However, distinguishing one cyst from another can be challenging on the basis of standard clinical findings, imaging parameters and ancillary fluid studies.[3] Moreover, the rate of progression of mucinous PCs into malignancy is low and difficult to predict. Weighing the risks of cancer development with the risks of surgical intervention, both consensus-based and evidence-based guidelines were developed to aid in the appropriate surveillance and treatment of PCs.[4 5] While these guidelines represent an extrapolation of current data, several studies have found them to be imperfect.[6–8] Hence, the management of PCs is often an individualised approach.

Recently, DNA-based testing has emerged as an adjunct to the assessment of PCs.[9] Although cellular content and fluid volume of PC aspirates are often suboptimal for routine ancillary studies, such as cytopathology and carcinoembryonic antigen (CEA) quantitation, DNA from lysed or exfoliated cyst epithelial lining shed into the pancreatic cyst fluid (PCF) can be analysed for genetic abnormalities.[10 11] Furthermore, sequencing studies have identified distinct mutational profiles of the major PCs as well as those that have progressed to invasive adenocarcinoma.[12–14] For example, mutations in *KRAS* are commonly detected in IPMNs and MCNs and the presence of *GNAS* mutations is highly specific for IPMNs.[15–17] In contrast, *VHL* mutations and/or deletions are characteristic of SCAs and *CTNNB1* mutations in the absence of other genetic alterations are observed in solid-pseudopapillary neoplasms.[13] Additionally, IPMNs with advanced neoplasia (high-grade dysplasia and invasive adenocarcinoma) are reported to harbour mutations in *TP53*, *PIK3CA*, *PTEN* and/or *AKT1*.[18–23] While several studies have evaluated DNA testing of PCs, they have largely been retrospective in design, using postoperative specimens, limited in sample size and/

Significance of this study

What is already known on this subject?

- DNA-based testing has emerged as an adjunct to the assessment of pancreatic cysts (PCs).
- Although cellular content and fluid volume of PC aspirates are commonly suboptimal for routine ancillary studies, such as cytopathology and carcinoembryonic antigen (CEA) quantitation, DNA from lysed or exfoliated cyst epithelial lining shed into the PC fluid can be analysed for genetic abnormalities.
- Several studies have evaluated DNA-based testing of PCs, but they have largely been retrospective in design, using postoperative specimens, limited in sample size, lack adequate follow-up and/or suffer from insensitive detection strategies.

What are the new findings?

- We have prospectively evaluated preoperative DNA-based testing of PC fluid within a large cohort and found mutations in *KRAS* and/or *GNAS* by next-generation sequencing (NGS) are highly sensitive and specific for intraductal papillary mucinous neoplasms (IPMNs), but not mucinous cystic neoplasms (MCNs).
- The sensitivity of preoperative DNA-based testing for IPMNs and MCNs was lower with Sanger sequencing than NGS.
- The preoperative detection of mutations/deletions in *TP53, PIK3CA* and/or *PTEN* with mutant allele frequencies (MAFs) that are equivalent to MAFs for *KRAS* and/or *GNAS* mutations was highly sensitive and specific for IPMNs with advanced neoplasia (high-grade dysplasia and invasive adenocarcinoma).
- Low-level mutations in *TP53, PIK3CA* and/or *PTEN* were detected in IPMNs with low-grade dysplasia and may represent a subset of IPMNs at risk for malignant transformation.
- MAFs for *GNAS* mutations >55% correlated with IPMNs with high-grade dysplasia.

How might it impact on clinical practice in the foreseeable future?

- These results definitively highlight the usage of preoperative NGS in accurately classifying PCs and the detection of IPMNs harbouring advanced neoplasia.

or lack adequate follow-up.[12 14 24 25] Thus, the diagnostic usage of DNA analysis of PCF in routine clinical practice remains unclear.

In this study, we developed a highly sensitive, targeted next-generation sequencing (NGS) assay for genes known to be frequently mutated and/or deleted in PCs and PCs with advanced neoplasia (*KRAS*, *GNAS*, *NRAS*, *HRAS*, *BRAF*, *CTNNB1*, *TP53*, *PIK3CA*, *PTEN* and *AKT1*). Due to technical issues, we were unable to include *VHL* within this panel, but assessed the entire coding sequence of *VHL* by Sanger sequencing with the understanding that the sensitivity of Sanger sequencing is known to be lower than NGS.[26] This test was performed within a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited clinical laboratory using PCF obtained by endoscopic ultrasound-fine needle aspiration (EUS-FNA) for the routine assessment of PCs. Our objectives were to prospectively evaluate DNA-based molecular testing on a large, consecutive cohort of patients to (1) identify the prevalence and distribution of genetic alterations within PCs; (2) determine the accuracy of molecular analysis using both NGS and Sanger sequencing and (3) based on follow-up diagnostic surgical pathology compare these findings with other accepted diagnostic modalities in the preoperative assessment of PCs.

MATERIALS AND METHODS

Cases

Study approval was obtained from the University of Pittsburgh Institutional Review Board (IRB# PRO13020493). Between January 2014 and July 2017, 673 PCF specimens obtained by EUS-FNA were prospectively submitted to the Molecular & Genomic Pathology Laboratory at the University of Pittsburgh Medical Centre (UPMC) for molecular testing. In all cases, the indication for PCF molecular testing was a clinical concern for a mucinous PC. Medical records were reviewed to document patient demographics, clinical presentation, EUS findings, fluid viscosity (as noted by the endoscopist), CEA analysis and cytopathological diagnoses. Endoscopic criterion of main duct dilatation was defined by a diameter ≥5 mm.[4] In addition, the presence of a mural nodule was defined as a uniform echogenic nodule of any size without a lucent centre or hyperechoic rim.[27] A value >192 ng/mL was used as a cut-off for an elevated PCF CEA. For cytopathology specimens, specimen adequacy was assessed in all cases using a three-tiered system: satisfactory, less than optimal and unsatisfactory. Satisfactory was defined as the presence of sufficient epithelial cells and/or mucin representative of the target cyst. Less than optimal consisted of scant epithelium in the absence of mucin, but with at least few histiocytes present. Unsatisfactory specimens were virtually acellular and lacked mucin. Malignant cytopathology was defined as either at least suspicious for adenocarcinoma or positive for adenocarcinoma. Pathology slides were reviewed for each surgical specimen and diagnoses for all PCs were rendered based on standard histomorphological criteria.[28] Cases diagnostic for a mucinous PC (IPMN and MCN) with high-grade dysplasia or invasive adenocarcinoma were interpreted as advanced neoplasia.[7] Pathological staging was performed as outlined by the American Joint Committee on Cancer Cancer Staging Manual (eighth edition).[29]

Molecular testing

Molecular testing was performed prospectively as part of clinical care and within a 14-day (mean, 10 days) turnaround within the CLIA-certified and CAP-accredited Molecular and & Genomic Pathology Laboratory at UPMC at a cost of $750 per PCF specimen. Genomic DNA was isolated from PCF obtained by EUS-FNA using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Indianapolis, Indiana, USA) on Compact MagNA Pure (Roche, Indianapolis, Indiana, USA). Extracted DNA was quantitated on the Qubit V.2.0 Fluorometer using the dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, Maryland, USA). Amplification-based targeted NGS (PancreaSeq) was performed with primers for genomic regions of interest that included *KRAS*, *GNAS*, *NRAS*, *HRAS*, *BRAF*, *CTNNB1*, *TP53*, *PIK3CA*, *PTEN* and *AKT1* with primer sequences and performance characteristics as previously described.[12 30] Amplicons were barcoded, purified and ligated with specific adapters. DNA quantity and quality check was performed using the 2200 TapeStation (Agilent Technologies, Santa Clara, California, USA). The Ion One Touch 2 and One Touch ES were used to prepare and enrich templates and enable testing via Ion Sphere Particles on a semiconductor chip. Massively parallel sequencing was carried out on an Ion Torrent Personal Genome Machine Sequencer or Ion Proton according to the manufacturer's instructions

(Thermo Fisher Scientific, Waltham, Maryland, USA) and analysed with the Torrent Suite Software V.3.4.2. Bioinformatic data analysis is described further within the online supplementary material. The limit of detection was 5% mutant allele frequency (MAF) at 500× or 3% MAF at 1000× coverage for each tested region. The minimal depth of coverage was 500×. For each mutation identified, a MAF was calculated based on the number of reads of the mutant allele versus the wild-type allele and reported as a percentage. Copy number assessment was performed as previously described.[31] The total depth of sequencing coverage at each sequenced region normalised by the normal controls was calculated per sequenced case. A decrease in sequencing coverage below established cut-offs with simultaneous presence of sequence variant at high MAF was considered a biallelic inactivation.

The *VHL* gene was sequenced by Sanger sequencing approach. For the detection of a mutation or deletion, DNA was amplified with primers flanking *VHL* exons 1 (5′-GCG AAG ACT ACG GAG-3′ and 5′-CCG TGC TAT CGT CCC T-3′), exon 2 (5′-GTT TCA CCA CGT TAG CCA-3′ and 5′-TAC AAA TAC ATC ACT TCC A-3′) and exon 3 (5′-CTC TTG TTC GTT CCT TGT-3′ and 5′-AAG CAA TGG TGC CTA TT-3′). The quality of amplified PCR product was evaluated by agarose gel electrophoresis. Then, bidirectional Sanger sequencing was performed using the BigDye Terminator Kit on ABI3730 (Thermo Fisher Scientific, Waltham, Maryland, USA). The detection of mutations was performed with Mutation Surveyor V.3.01 (SoftGenetics, State College, Pennsylvania). The limit of detection was approximately 10%–20% of mutant alleles present in a background of normal DNA. The aforementioned primers include individual exon–intron boundaries, and, therefore, allows for detection of both deletions and insertions within exons or complete loss of an exon by visual inspection of electropherograms. However, a limitation of this method is that it does not detect loss of the entire *VHL* gene.

Statistical analysis

Differences in mutational status were compared using Fisher exact test for dichotomous 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.23 (IBM, Armonk, New York, USA) and statistical significance was defined as a p value of <0.05.

RESULTS

Molecular testing and correlative clinicopathological findings

In total, 673 EUS-FNA-obtained PCF specimens from 642 patients were prospectively analysed for genetic alterations over a 43-month time period (figure 1). Among these cases, 626 (93%) specimens from 595 patients were satisfactory for molecular testing (table 1 and online supplementary data). The remaining 47 cases were unable to be tested due to insufficient DNA for evaluation. For 6 of 595 (1%) patients, two separate specimens corresponding to separate PCs were submitted for molecular testing. Further, 25 of 595 (4%) patients had repeat EUS-FNA and molecular testing of their PC during the study period.

Although sufficient for molecular studies, the amount of cyst fluid was insufficient for CEA analysis in 174 of 626 (28%) cases. In addition, 375 (60%) specimens were either less than optimal (n=297, 47%) or unsatisfactory (n=78, 13%) for cytopathological diagnosis. The primary reason for specimen inadequacy was absent-to-scant cellularity.

The DNA concentration from submitted EUS-FNA-obtained PCF specimens ranged between 0.01 and 248 ng/uL (mean,

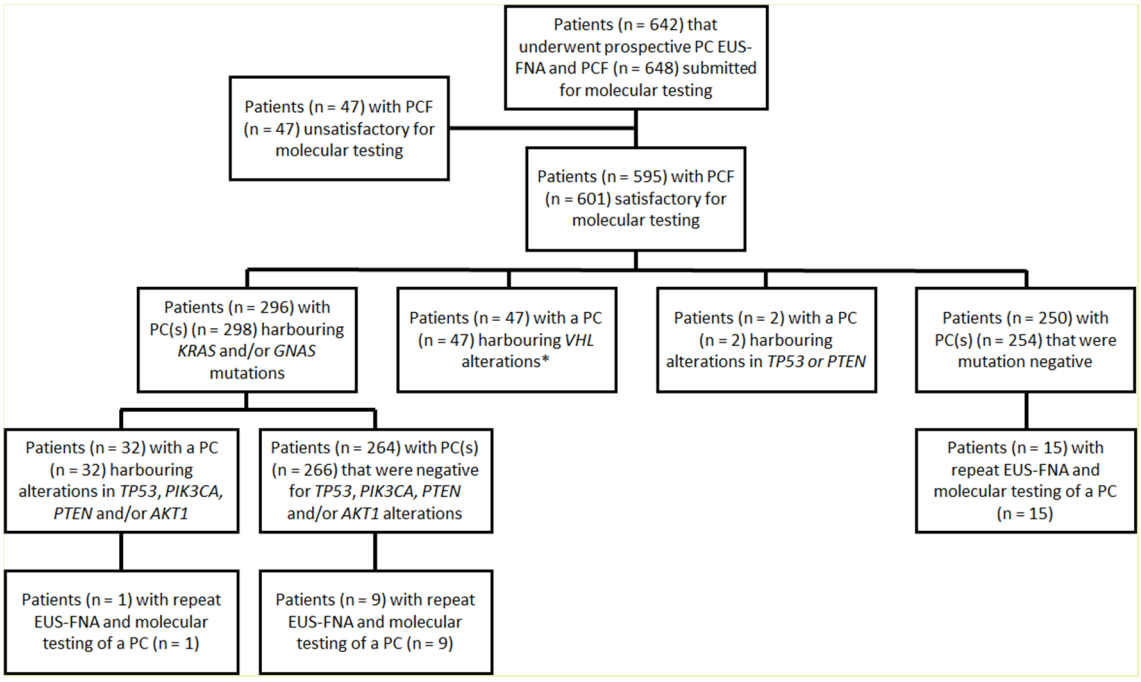

Figure 1 Study cohort. In total, 642 patients with 648 PCs underwent an EUS-FNA and PCF) molecular testing. Forty-seven PCF specimens from 47 patients were unsatisfactory for molecular testing; however, the remaining 601 PCF specimens from 595 patients were satisfactory. Repeat EUS-FNA and molecular testing was performed for 25 PCs from 25 (of 595) patients. (*) Of note, 1 of 47 PCs with a *VHL* alteration also harboured a *TP53* mutation. EUS-FNA, endoscopic ultrasound–fine needle aspiration; PC, pancreatic cyst; PCF, pancreatic cyst fluid.

Table 1 Clinical and pathological characteristics of 595 patients with PCs and correlation with *KRAS*, *GNAS* and *VHL* status

		KRAS and/or *GNAS*			*VHL*		
Patient or cyst characteristics	**Total**	**Wild type**	**Mutant**	**p Value**	**Wild type**	**Mutant**	**p Value**
Gender	*n=595*						
Woman	341	181 (53%)	160 (47%)	0.246	308 (90%)	33 (10%)	0.067
Man	254	122 (48%)	132 (52%)		240 (94%)	14 (6%)	
Mean age (range) (years)	65.0 (15–93)	60.7 (15–90)	69.4 (34–93)	<0.001	65.2 (15–93)	62.3 (33–81)	0.122
Symptomatic presentation	198	112 (57%)	86 (43%)	0.056	189 (95%)	9 (5%)	0.036
Location	*n=626*						
Head, neck and uncinate	320	137 (43%)	183 (57%)	<0.001	299 (92%)	21 (7%)	0.368
Body and tail	306	181 (59%)	125 (41%)		280 (92%)	26 (8%)	
Mean cyst size (range) (cm)	2.7 (0.8–21.0)	3.0 (0.8–21.0)	2.4 (0.8–11.0)	<0.001	2.7 (0.8–21.0)	3.2 (1.0–8.9)	0.060
Cyst multifocality	281	95 (34%)	186 (66%)	<0.001	270 (96%)	11 (4%)	0.002
Increased fluid viscosity	319	85 (27%)	234 (73%)	<0.001	316 (99%)	3 (1%)	<0.001
CEA >192 ng/mL (n=452)*	146	38 (26%)	108 (74%)	<0.001	146 (100%)	0 (0%)	<0.001
Satisfactory cytological adequacy	251	117 (47%)	134 (53%)	0.088	236 (94%)	15 (6%)	0.279
Diagnostic pathology	*n=102*	*n=43*	*n=59*		*n=100*	*n=2*	
Adenocarcinoma arising in an IPMN	13	0 (0%)	13 (100%)		13 (100%)	0 (0%)	
IPMN with low-grade/high-grade dysplasia	43	0 (0%)	43 (100%)	<0.001†	43 (100%)	0 (0%)	
MCN with low-grade/high-grade dysplasia	10	7 (70%)	3 (30%)		10 (100%)	0 (0%)	
Serous cystadenoma	3	3 (100%)	0 (0%)		1 (33%)	2 (67%)	<0.001‡
Cystic PanNET	9	9 (100%)	0 (0%)		9 (100%)	0 (0%)	
Acinar cell cystadenoma	1	1 (100%)	0 (0%)		1 (100%)	0 (0%)	
Pseudocyst	17	17 (100%)	0 (0%)		17 (100%)	0 (0%)	
Retention cyst	2	2 (100%)	0 (0%)		2 (100%)	0 (0%)	
Lymphoepithelial cyst	2	2 (100%)	0 (0%)		2 (100%)	0 (0%)	
Epidermoid cyst	1	1 (100%)	0 (0%)		1 (100%)	0 (0%)	
Squamoid cyst	1	1 (100%)	0 (0%)		1 (100%)	0 (0%)	

*Sufficient PCF for CEA analysis was available for 127 (80%) PCs.
†Follow-up NGS testing for KRAS and GNAS was performed for 24 (15%) PCs.
‡p Value corresponds to SCA versus other PCs.
CEA, carcinoembryonic antigen; IPMN, intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm; PanNET, pancreatic neuroendocrine tumour; PC, pancreatic cyst; PCF, pancreatic cyst fluid; SCA, serous cystadenomas.

6.93 ng/uL; median, 4.7 ng/uL). Overall, genetic alterations using the 11-gene panel were detected in 357 (57%) PCs. NGS revealed activating mutations in *KRAS*, *GNAS*, *BRAF* and *CTNNB1* in 264 (42%), 162 (26%), 5 (1%) and 4 (1%) cases, respectively. No mutations in *HRAS* and *NRAS* were detected. In total, *KRAS* and/or *GNAS* mutations were identified in 308 (49%) PCs with 119 (19%) cases harbouring mutations in both genes (online supplementary table 2). Multiple *KRAS* mutations were present in 10 specimens and included various combinations of codon 12, 13 and 61 substitutions. MAFs for *KRAS* were 3%–55% (mean, 24%; median, 24%). Multiple mutations in *GNAS* were detected in three specimens and consisted of substitutions in codons 201 and 227. *GNAS* MAFs were 3%–92% (mean, 28%; median, 26%). Two PCs had a *GNAS* MAF of >55%. The MAFs for these two cases were 88% and 92%. *BRAF* and *CTNNB1* MAFs were 24%–46% and 6%–46%, respectively. The presence of *BRAF* and *CTNNB1* mutations were only seen in the setting of a *KRAS* and/or *GNAS* mutation. Instead of NGS, exons 1–3 of *VHL* were evaluated by Sanger sequencing. *VHL* mutations and/or deletions were identified throughout the gene coding sequence in 47 of 626 (8%) PCs.

NGS was also used to assess the status of *TP53*, *PIK3CA*, *PTEN* and *AKT1* (table 2). Genetic alterations in *TP53*, *PIK3CA*, *PTEN* and *AKT1* were identified in 24 (4%), 11 (2%), 2 (1%) and 1 (1%) PCs, respectively. Overall, *TP53*, *PIK3CA*, *PTEN* and *AKT1* alterations were present in 35 (6%) cases. For *TP53* and *PTEN*, genetic alterations consisted of mutations and/or deletions throughout the gene coding sequence. Among these cases, MAFs for *TP53*, *PTEN* and *AKT1* were 4%–43%, 11% and 8%, respectively. In addition, homozygous deletions in *TP53* and *PTEN* were detected in 2 (1%) and 1 (1%) cases, respectively. Alterations in *PIK3CA* corresponded to activating point mutations in exon 9 (n=7) and/or exon 20 (n=5) with MAFs of 3%–50%.

PCs with alterations in *TP53*, *PIK3CA* and/or *PTEN* were associated with co-mutation(s) in *KRAS* and/or *GNAS* mutations (p<0.001). However, 3 of 35 PCs that had a *TP53* mutation (n=2) or *PTEN* deletion (n=1) were wild type for *KRAS* and *GNAS*. Among the two *TP53* mutant cases, one harboured a *VHL* deletion and the other was negative for genetic alterations. No other genetic alterations were detected in the single PC with a *PTEN* deletion.

Follow-up information and correlation with diagnostic surgical pathology

Follow-up data were available for 571 of 595 (96%) patients and ranged from 1 to 42 months (mean, 27 months; median, 26 months). Diagnostic pathology was available for 102 of 595 (17%) patients that underwent surgical resection within 1–16 months (mean, 4 months; median, 3 months) from initial EUS-FNA and molecular testing (online supplementary table 2). Except for 2 SCAs, 8 cystic pancreatic neuroendocrine tumours (PanNETs) and 14 pseudocysts, the indications for surgery of

Table 2 Clinical and pathological characteristics of 595 patients with PCs and correlation with *TP53*, *PIK3CA*, *PTEN* and *AKT1* status

		TP53, PIK3CA, PTEN and/or *AKT1*		
Patient or cyst characteristics	**Total**	**Wild type**	**Mutant**	**p Value**
Gender	*n=595*			
Woman	341	328 (96%)	13 (4%)	0.014
Man	254	232 (92%)	22 (9%)	
Mean age (range) (years)	65.0 (15–93)	64.8 (15–93)	68.3 (45–84)	0.107
Symptomatic presentation	198	183 (92%)	15 (8%)	0.267
Location	*n=626*			
Head, neck and uncinate	320	299 (93%)	21 (7%)	0.301
Body and tail	306	292 (95%)	14 (5%)	
Mean cyst size (range) (cm)	2.7 (0.8–21.0)	2.7 (0.8–21.0)	2.8 (0.9–5.2)	0.739
Cyst size ≥3 cm	194	177 (91%)	17 (9%)	0.025
Satisfactory cytological adequacy	251	233 (93%)	18 (7%)	0.213
Main duct dilatation	104	92 (88%)	12 (12%)	0.008
Presence of a mural nodule	35	27 (77%)	8 (23%)	<0.001
Malignant cytopathology*	10	3 (30%)	7 (70%)	<0.001
Mutations in *KRAS* and/or *GNAS*	308	276 (90%)	32 (10%)	<0.001
Diagnostic pathology	*n=102*	*n=83*	*n=19*	
Adenocarcinoma arising in an IPMN	13	0 (0%)	13 (100%)	
IPMN with high-grade dysplasia	4	2 (50%)	2 (50%)	<0.001†
MCN with high-grade dysplasia	2	2 (100%)	0 (0%)	
IPMN with low-grade dysplasia	39	36 (92%)	3 (8%)	
MCN with low-grade dysplasia	8	7 (87%)	1 (13%)	
Serous cystadenoma	3	3 (100%)	0 (0%)	
Cystic PanNET	9	9 (100%)	0 (0%)	
Non-neoplastic cysts	24	24 (100%)	0 (0%)	

*Malignant cytopathology is defined as at least suspicious for adenocarcinoma.
†p Value corresponds to mucinous PCs with advanced neoplasia versus other PCs.
CEA, carcinoembryonic antigen; IPMN, intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm; PanNET, pancreatic neuroendocrine tumour; PC, pancreatic cyst.

the remaining PCs were due to concern for advanced neoplasia within a mucinous PC on the basis of the Fukuoka guidelines and consideration of molecular testing.[7] Mutations in *KRAS* and/or *GNAS* were preoperatively detected in all 56 IPMNs. In addition, *KRAS* mutations were identified in two MCNs with high-grade dysplasia and one MCN with low-grade dysplasia. However, the remaining seven MCNs with low-grade dysplasia were *KRAS*/*GNAS*-negative. The MAFs for *KRAS* and *GNAS* were 3%–47% and 3%–92%, respectively. As previously described, two PCs had MAFs of >55%, and both cases corresponded to IPMNs with high-grade dysplasia. No mutations in *KRAS* and/or *GNAS* were found in the non-mucinous PCs within the resection cohort.

VHL alterations were preoperatively seen in two of three SCAs. Although Sanger sequencing failed to detect a *VHL* alteration in one SCA by EUS-FNA, repeat testing of the corresponding surgical resection specimen identified the presence of a *VHL* frameshift mutation. No alterations in *VHL* were observed in the remaining mucinous and non-mucinous PCs.

Genetic alterations in *TP53*, *PIK3CA* and/or *PTEN* were identified in all 13 IPMNs with adenocarcinoma, 2 IPMNs with high-grade dysplasia, 3 IPMNs with low-grade dysplasia and 1 MCN with low-grade dysplasia (table 3 and online supplementary table 3) The MAFs for *TP53*, *PIK3CA* and *PTEN* were 8%–43%, 3%–50% and 10%, respectively. Except for the one MCN with low-grade dysplasia, co-mutations in *KRAS* and/or *GNAS* were detected in all PCs with alterations in *TP53*, *PIK3CA* and/or *PTEN*. Among the 13 IPMNs with adenocarcinoma and 2 IPMN with high-grade dysplasia, the MAFs for *KRAS* and/or *GNAS* were at least equal to MAFs for *TP53*, *PIK3CA* and/or *PTEN* (figure 2). The three IPMNs with low-grade dysplasia had activating mutations in *PIK3CA*, but the MAFs for *PIK3CA* were less than the MAFs for *KRAS*. While no genetic alterations in *TP53*, *PIK3CA* and/or *PTEN* were detected in two IPMNs with high-grade dysplasia, the MAF for *GNAS* in both cases was >55%. The remaining 2 MCNs with high-grade dysplasia, 36 IPMNs with low-grade dysplasia, 8 MCNs with low-grade dysplasia and non-mucinous PCs were negative for *TP53*, *PIK3CA*, *PTEN* and/or *AKT1* alterations.

Among the remaining 469 patients with follow-up data and no diagnostic surgical pathology, 230 (49%) had PCs with *KRAS* and/or *GNAS* mutations. Fourteen of these 230 (6%) patients also had mutations in *TP53*, *PIK3CA* and/or *AKT1*. However, the MAFs for *TP53*, *PIK3CA* and/or *AKT1* (4%–9%) were less than MAFs for *KRAS* and/or *GNAS* (28%–45%). In addition, two patients harboured a PC with a *TP53* mutation and MAFs of 5%, but wild type for both *KRAS* and *GNAS*. One of these two *TP53* mutant cases also harboured a *VHL* deletion. None of these PCs demonstrated concerning features for advanced neoplasia by both imaging (eg, ductal dilatation or the presence of a mural nodule) and cytopathology (eg, malignant cytopathology). Moreover, all 16 patients are currently alive and well and have not developed pancreatic cancer on follow-up.

As discussed previously, 25 of 595 patients had repeat EUS-FNA and molecular testing of their PC (figure 1). Fifteen of 25 patients harboured a PC with no detectable alterations, while the remaining 10 patients had a *KRAS* and/or *GNAS* mutant PC. Repeat aspiration and molecular testing of all 25 cases continued to identify the same *KRAS* and *GNAS* genetic status as initial

Table 3 Clinicopathological and preoperative molecular findings among prospectively assayed 17 IPMNs and 2 MCNs with advanced neoplasia

Patient	Gender	Age (years)	Primary clinical symptom	Cyst size (cm)	Ductal dilatation	Mural nodule	Malignant cytopathology*	*KRAS* mutation (MAF)	*GNAS* mutation (MAF)	*VHL* alteration	*TP53* alteration (MAF)	*PIK3CA* mutation (MAF)	*PTEN* deletion (MAF)	Diagnostic pathology
1	Woman	77	Asymptomatic	4.6	Absent	Present	Present	p.G12D (40%)	p.R201C (48%)	Absent	Homozygous deletion†	Absent	Homozygous deletion†	AdenoCA arising in an IPMN (pT1bN0)
2	Man	72	Jaundice	4.7	Absent	Present	Present	p.G12V (29%); p.G12D (7%)	Absent	Absent	p.R175H (38%); p.G199L (18%)	p.H1047Y (28%)	Absent	AdenoCA arising in an IPMN (pT1cN0)
3	Man	51	Pancreatitis	3.0	Present	Present	Absent	p.G12D (40%)	p.R201C (53%)	Absent	Homozygous deletion†	Absent	Absent	AdenoCA arising in an IPMN (pT1bN0)
4	Man	82	Asymptomatic	3.7	Present	Absent	Absent	p.G12D (19%)	p.R201H (15%)	Absent	p.R110L (16%)	Absent	Absent	AdenoCA arising in an IPMN (pT1bN0)
5	Man	61	Jaundice	5.2	Present	Present	Present	p.G12D (21%)	Absent	Absent	p.R175H (21%)	Absent	Absent	AdenoCA arising in an IPMN (pT1cN0)
6	Man	48	Asymptomatic	0.9	Absent	Present	Absent	p.G12V (15%)	Absent	Absent	p.R248W (16%)	Absent	Absent	AdenoCA arising in an IPMN (pT1aN0)
7	Man	46	Abdominal pain	3.7	Absent	Absent	Absent	p.G12V (33%)	Absent	Absent	Absent	p.E545K (29%)	Absent	AdenoCA arising in an IPMN (pT1cN0)
8	Woman	62	Asymptomatic	2.7	Absent	Present	Absent	p.G12R (11%)	Absent	Absent	p.R273H (19%)	Absent	Absent	AdenoCA arising in an IPMN (pT1aN0)
9	Woman	56	Pancreatitis	2.3	Absent	Absent	Absent	p.G12R (18%)	Absent	Absent	p.R273H (23%)	Absent	Absent	AdenoCA arising in an IPMN (pT1bN0)
10	Man	77	Asymptomatic	3.0	Absent	Absent	Absent	Absent	p.R201H (51%)	Absent	Absent	p.E545K (50%)	Absent	AdenoCA arising in an IPMN (pT1aN0)
11	Woman	58	Abdominal pain	5.0	Absent	Absent	Present	p.G12V (7%)	Absent	Absent	Absent	p.Y1021C (5%)	Absent	AdenoCA arising in an IPMN (pT1bN0)
12	Woman	72	Asymptomatic	2.0	Present	Absent	Absent	p.G12V (41%)	Absent	Absent	p.D259Y (43%)	Absent	Absent	AdenoCA arising in an IPMN (pT1aN0)
13	Man	74	Abdominal pain	2.7	Absent	Absent	Present	p.G12R (26%)	Absent	Absent	p.R273H (29%)	Absent	Absent	AdenoCA arising in an IPMN (pT1bN0)
14	Man	67	Weight loss	3.5	Present	Absent	Absent	Absent	p.R201C (30%)	Absent	p.R181C (34%)	Absent	Absent	IPMN with high-grade dysplasia
15	Man	72	Asymptomatic	1.5	Present	Absent	Absent	Absent	p.R201C (39%)	Absent	p.R248W (42%)	Absent	Absent	IPMN with high-grade dysplasia
16	Man	67	Abdominal pain	2.8	Present	Absent	Absent	p.G12R (45%)	p.R201C (92%)	Absent	Absent	Absent	Absent	IPMN with high-grade dysplasia
17	Man	72	Asymptomatic	3.0	Present	Absent	Present	p.G12D (41%)	p.R201H (88%)	Absent	Absent	Absent	Absent	IPMN with high-grade dysplasia

Continued

Table 3 Continued

Patient	Gender	Age (years)	Primary clinical symptom	Cyst size (cm)	Ductal dilatation	Mural nodule	Malignant cytopathology*	*KRAS* mutation (MAF)	*GNAS* mutation (MAF)	*VHL* alteration	*TP53* alteration (MAF)	*PIK3CA* mutation (MAF)	*PTEN* deletion (MAF)	Diagnostic pathology
18	Woman	34	Abdominal pain	11	Absent	Absent	Absent	p.G12D (15%)	Absent	Absent	Absent	Absent	Absent	MCN with high-grade dysplasia
19	Woman	83	Abdominal pain	9.8	Absent	Absent	Absent	p.G12R (22%)	Absent	Absent	Absent	Absent	Absent	MCN with high-grade dysplasia

*Malignant cytopathology was defined as at least suspicious for adenocarcinoma.
†Homozygous deletion is based on low sequencing coverage of amplicons for the gene of interest.
AdenoCA, adenocarcinoma; IPMN, intraductal papillary mucinous neoplasm; MAF, mutant allelic frequency; MCN, mucinous cystic neoplasm.

testing. However, among the 10 *KRAS* and/or *GNAS*-mutant PCs, 1 case had a *TP53* mutation on initial molecular testing that was absent on subsequent testing. For this single case, the MAFs for *KRAS* and *TP53* were 39% and 4%, respectively. In contrast, the subsequent specimen had a *KRAS* MAF of only 4%. A comparison of both the initial and repeat EUS-FNA specimens for the remaining six PCs revealed MAFs for *KRAS* and/or *GNAS* that were essentially the same.

Comparison and combination of molecular testing with other diagnostic modalities

Based on 102 cases with follow-up diagnostic pathology, preoperative NGS detection of *KRAS* and/or *GNAS* mutations had 100% sensitivity and 96% specificity for an IPMN (table 4). Further, mutations in *KRAS* and/or *GNAS* mutations had a sensitivity of 89% and a specificity of 100% for both IPMNs and MCNs. Increased fluid viscosity and an elevated CEA had lower sensitivities (77% and 57%, respectively) and lower specificities (89% and 80%, respectively).

In conjunction with *KRAS* and/or *GNAS* mutations, alterations in *TP53*, *PIK3CA* and/or *PTEN* had 88% sensitivity and 97% specificity for IPMNs with advanced neoplasia. Both the sensitivity and specificity increased to 100% by modifying the selection criteria to include cases with either *GNAS* MAFs >55% or *TP53*/*PIK3CA*/*PTEN* MAFs that were at least equivalent to *KRAS*/*GNAS* MAFs. In comparison, main pancreatic duct dilatation and the presence of a mural nodule on EUS had sensitivities of 47% and 35%, respectively, and specificities of 74% and 94%, respectively. A preoperative cytopathological diagnosis of at least suspicious for adenocarcinoma was associated with 35% sensitivity and 97% specificity.

Overall, the sensitivity and specificity of combining *KRAS* and/or *GNAS* mutations with the presence of *TP53*, *PIK3CA* and/or *PTEN* alterations for a mucinous PC with advanced neoplasia was 79% and 96%, respectively. Modification of selection criteria to include the detection of *GNAS* mutations with MAFs of >55% or the MAFs for *TP53*, *PIK3CA*, and/or *PTEN* alterations of either equal or greater to MAFs for *KRAS* and/or *GNAS* mutations had 89% sensitivity and 100% specificity. A diagnosis of at least suspicious for adenocarcinoma on cytopathology had 32% sensitivity and 98% specificity.

Prospective analysis of KRAS and GNAS testing using Sanger sequencing

Prior to this study, prospective EUS-FNA PCF testing of *KRAS* and *GNAS* was performed by Sanger sequencing for 175 PCs from 169 patients over a 12-month time period (online supplementary material and supplementary table 3). Among the 175 PCs, 159 (91%) PCs from 153 patients were satisfactory for molecular analysis. In contrast to a prevalence of 49% by NGS, Sanger sequencing detected mutations in *KRAS* and/or *GNAS* in 39% of PCs (62 of 159). Among this cohort of 159 PCs, 34 cases had diagnostic pathology and included the following mucinous PCs: 5 IPMNs with adenocarcinoma, 1 IPMN with high-grade dysplasia, 12 IPMNs with low-grade dysplasia and 2 MCNs with low-grade dysplasia. By Sanger sequencing, 13 of 18 (72%) IPMNs and 0 of 2 (0%) MCNs were found to harbour mutations in *KRAS* and/or *GNAS*. Overall, the presence of *KRAS* and/or *GNAS* mutations by Sanger sequencing had a sensitivity and specificity of 72% and 100%, respectively, for IPMNs and 65% and 100%, respectively, for both IPMNs and MCNs. Among the 159 PCs tested, 24 cases (8 *KRAS*/*GNAS* mutant and 16 *KRAS*/*GNAS* wild type) underwent repeat testing for *KRAS* and *GNAS*

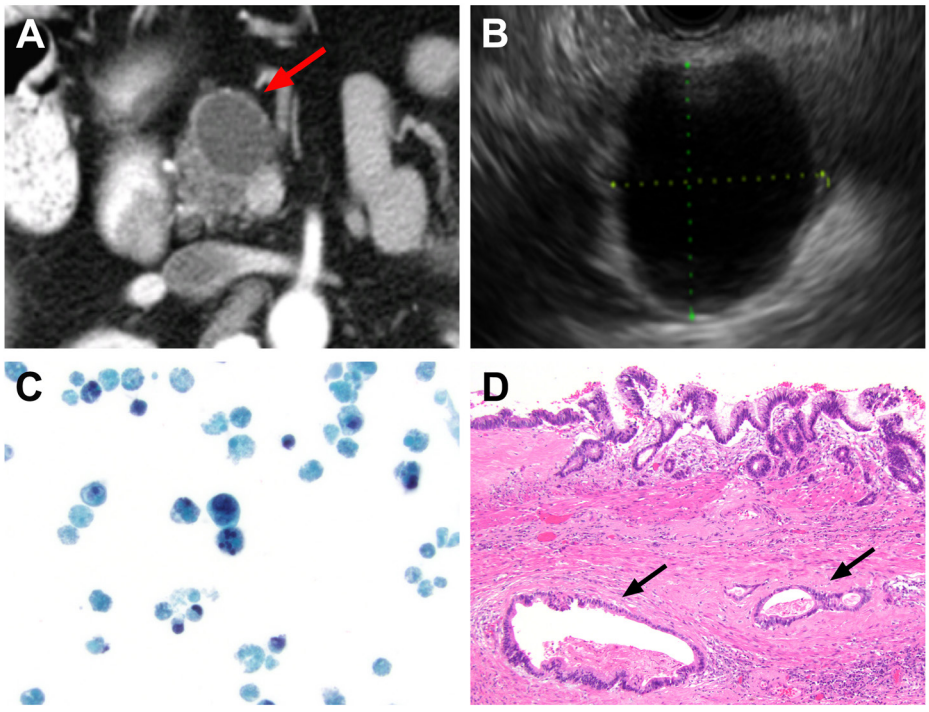

Figure 2 DNA-based molecular testing of a pancreatic head cyst. An incidental 3 cm pancreatic head cyst with no associated main duct dilatation or mural nodule by both (A) CT (red arrow) and (B) endoscopic ultrasound. Fine-needle aspiration and subsequent (C) cytopathology showed atypical cells with no definitive mucin. However, DNA analysis identified mutations in *GNAS* and *PIK3CA* with MAFs of 51% and 50%, respectively. Follow-up surgical resection revealed an (D) invasive moderately differentiated adenocarcinoma (black arrows) arising in an intraductal papillary mucinous neoplasm. MAFs, mutant allele frequencies.

by NGS. The status of *KRAS* and *GNAS* were essentially the same by NGS; however, 3 of 16 *KRAS*/*GNAS* wild type PCs by Sanger sequencing were found to harbour mutations in *KRAS* (n=3) and/or *GNAS* (n=1) by NGS.

DISCUSSION

Although several factors should be considered when evaluating a patient with a PC, key questions need to be answered before continuing further surveillance and treatment. First, what type of PC does the patient have? More specifically, given the malignant potential of mucinous PCs, is the cyst mucinous or non-mucinous? Second, does the mucinous PC harbour malignancy? And, lastly, if not, what is the malignant potential of the mucinous PC within the patient's lifetime?

Similar to previous studies using retrospective cohorts and postsurgical specimens, our prospective evaluation of preoperative DNA-based PC testing identified mutations in *KRAS* and/or *GNAS* to be 89% sensitive and 100% specific for a mucinous PC. Furthermore, the presence of *KRAS* and/or *GNAS* mutations reached 100% sensitivity for IPMNs, and the presence of *GNAS* mutations was 100% specific for an IPMN. However, *KRAS* mutations were detected in only 30% of MCNs. While mutations in *KRAS* are common in MCNs, the prevalence of these activating mutations is reported to increase with the severity of dysplasia.[16 17] Jimenez *et al* identified *KRAS* mutations in 26% of MCNs with low-grade dysplasia and 89% of MCNs with high-grade dysplasia.[32] Among the MCNs within our study cohort, *KRAS* mutations were found in 100% of MCNs with high-grade dysplasia and 13% of MCNs with low-grade dysplasia. Considering the relatively young age of most patients, frequent occurrence within the pancreatic body and tail and unknown risk of progression to adenocarcinoma, surgical resection is typically recommended for patients with MCNs.[4] Therefore, the assessment of *KRAS* alone is inadequate for the detection of MCNs and additional markers are needed to improve the sensitivity of DNA testing. Of note, one MCN with low-grade dysplasia did harbour a *PTEN* deletion and, in the absence of a *KRAS* mutation, may represent a marker for MCNs. Despite the lack of sensitivity for MCNs, the sensitivity and specificity of DNA testing for a mucinous PC were higher than surrogate markers of mucinous differentiation, such as increased fluid viscosity and elevated CEA.

The diagnosis of a mucinous PC is further enhanced by molecular markers to exclude common mimics. Oligocystic and unilocular variants of SCA are often clinically and radiographically indistinguishable from branch duct IPMNs and MCNs. Genetic alterations in *VHL* have been described to be highly specific for SCAs, but could potentially be present in cystic PanNETs.[33] Within our study, the specificity of *VHL* mutations and/or deletions by Sanger sequencing for SCAs was 100%. However, we failed to preoperatively identify a *VHL* alteration in one SCA. Repeat testing of the corresponding surgical pathology specimen revealed a frameshift mutation in *VHL*. The inability to detect a *VHL* alteration within the preoperative PCF may be attributed to the inherent limitations of Sanger sequencing. Prior to this study, we prospectively evaluated *KRAS* and *GNAS* mutations by Sanger sequencing. In contrast to NGS, Sanger sequencing detected *KRAS* and *GNAS* mutations in 39% of PCs and had

Table 4 Sensitivities and specificities of molecular testing and other diagnostic modalities based on 102 surgically resected PCs

Parameter	Sensitivity (95% CI)	Specificity (95% CI)
IPMNs		
KRAS and/or *GNAS* mutations	100% (0.92 to 1.00)	96% (0.84 to 0.99)
Presence of multiple cysts	54% (0.40 to 0.67)	72% (0.56 to 0.84)
Increased fluid viscosity	82% (0.69 to 0.91)	80% (0.66 to 0.90)
Elevated CEA*	57% (0.40 to 0.73)	70% (0.53 to 0.83)
IPMNs with advanced neoplasia		
TP53, *PIK3CA* and/or *PTEN* alterations	88% (0.62 to 0.98)	95% (0.88 to 0.98)
KRAS and/or *GNAS* mutations with *TP53*, *PIK3CA* and/or *PTEN* alterations	88% (0.62 to 0.98)	97% (0.89 to 0.99)
GNAS MAF >55% or *TP53*/*PIK3CA*/*PTEN* MAFs at least equal to *KRAS*/*GNAS* MAFs	100% (0.77 to 1.00)	100% (0.95 to 1.00)
Main pancreatic duct dilatation	47% (0.24 to 0.71)	74% (0.63 to 0.83)
Presence of a mural nodule	35% (0.15 to 0.61)	94% (0.86 to 0.98)
Malignant cytopathology†	35% (0.15 to 0.61)	97% (0.91 to 1.00)
IPMNs and MCNs		
KRAS and/or *GNAS* mutations	89% (0.79 to 0.95)	100% (0.88 to 1.00)
Increased fluid viscosity	77% (0.65 to 0.86)	89% (0.73 to 0.96)
Elevated CEA*	57% (0.42 to 0.71)	80% (0.61 to 0.92)
IPMNs and MCNs with advanced neoplasia		
TP53, *PIK3CA* and/or *PTEN* alterations	79% (0.54 to 0.93)	95% (0.88 to 0.98)
KRAS and/or *GNAS* mutations with *TP53*, *PIK3CA* and/or *PTEN* alterations	79% (0.54 to 0.93)	96% (0.89 to 0.99)
GNAS MAF >55% or *TP53*/*PIK3CA*/*PTEN* MAFs at least equal to *KRAS*/*GNAS* MAFs	89% (0.66 to 0.98)	100% (0.95 to 1.00)
Main pancreatic duct dilatation	42% (0.21 to 0.66)	74% (0.63 to 0.82)
Presence of a mural nodule	32% (0.14 to 0.57)	94% (0.86 to 0.98)
Malignant cytopathology†	32% (0.13 to 0.57)	98% (0.91 to 1.00)

*On the basis of cases in which sufficient fluid was available for CEA testing.
†Malignant cytopathology was defined as at least suspicious for adenocarcinoma.
CEA, carcinoembryonic antigen; MAF, mutant allele frequency; PC, pancreatic cyst.

a sensitivity of 72% for IPMNs. The differences in sensitivity between Sanger sequencing and NGS can be explained by the detection limit for each assay. The lowest limit of detection for Sanger sequencing is approximately 10%–20% of mutant alleles, while NGS as described herein is approximately 3%–5% of mutant alleles. Within this study, 24% of *KRAS* mutant cysts and 22% of *GNAS* mutant cysts had MAFs of <10%. These findings would suggest that Sanger sequencing is insufficient for preoperative *VHL* testing and the prevalence of *VHL* mutations and/or deletions within our study cohort is likely to be inaccurate. Thus, we would discourage the use of Sanger sequencing when evaluating preoperative PCF for molecular alterations.

In addition to accurate cyst classification, PC DNA testing has garnered significant interest due to the genetic differences between mucinous PCs with low-grade dysplasia and those with high-grade dysplasia and invasive adenocarcinoma (advanced neoplasia). Alterations in the gene *TP53* and those within the mTOR pathway have been implicated in the malignant transformation of mucinous PCs. The combination of *KRAS* and/or *GNAS* mutations with *TP53*, *PIK3CA* and/or *PTEN* alterations had 79% sensitivity and 96% specificity for a mucinous PC with advanced neoplasia. Rosenbaum *et al* described their experience with preoperative NGS testing of 113 PCs with corresponding diagnostic pathology available for 38 cases.[25] The authors reported that DNA mutational analysis was associated with 46% sensitivity and 100% specificity for advanced neoplasia. Although the authors did not include *PIK3CA* and *PTEN* within their testing panel, *TP53* was assessed, but only mutated in 17% of cases with advanced neoplasia. Herein, we found that 63% of mucinous PCs with advanced neoplasia harboured *TP53* alterations. While both studies used NGS, the depth of coverage for each assay was significantly different. Coverage refers to the number of times a region is sequenced. The deeper the coverage of a genetic target (eg, the more times a region is sequenced), the greater the reliability and sensitivity of the sequencing assay. Within our study, the minimum depth of coverage for each genetic target was 500×. In fact, we routinely achieved over 1000× depth of coverage. Rosenbaum *et al* aimed for a minimum depth of coverage of 100× with a median of 200× per target.[24 25] Nevertheless, the authors state that MAFs as low as 5% were achieved for individual genes.

Reviewing the results of NGS testing from the entire study cohort revealed two findings that would improve the sensitivity and specificity for detecting mucinous PCs with advanced neoplasia. First, a MAF >55% in *GNAS* was identified in two PCs and corresponded to IPMNs with high-grade dysplasia. Activating mutations in *KRAS* and *GNAS* are typically heterozygous and the MAF is ≤50% due to masking of mutations by contaminating non-neoplastic cells (eg, chronic inflammation and gastrointestinal contamination). In rare instances, the MAF of *KRAS* mutations is >50% through either deletion of the wild-type allele or copy number gain of the mutant allele. This phenomenon is known as mutant allele-specific imbalance (MASI) and reflects increased dosage of the mutant allele by copy-neutral loss of heterozygosity or gene amplification. We previously reported *KRAS* MASI in PCF correlates with mucinous PCs with advanced neoplasia.[11] To date, *GNAS* MASI within PCF has not been described, but based on our findings it seems to be associated with high-grade dysplasia within IPMNs.

A second finding was a MAF for *TP53*/*PIK3CA*/*PTEN* that was at least equal to a MAF for *KRAS*/*GNAS* correlated with the presence of advanced neoplasia within an IPMN. Although the combination of *KRAS* and/or *GNAS* mutations with genetic alterations in *TP53*, *PIK3CA* and/or *PTEN* were frequently

detected in IPMNs with advanced neoplasia, mutations in *KRAS*/*GNAS* and *PIK3CA* were also identified in two IPMNs with low-grade dysplasia. Moreover, 10 PCs without diagnostic surgical pathology were found to harbour mutations in *KRAS*/*GNAS* and *TP53*/*PIK3CA*/*PTEN*, but lacked concerning features for advanced neoplasia by both imaging and cytopathology, and have not progressed to malignancy on follow-up. However, among these cases, the MAFs for *TP53*/*PIK3CA*/*PTEN* were lower than the MAFs for *KRAS*/*GNAS*. By modifying the selection criteria of NGS testing to include a MAF >55% for *GNAS* and a MAF for *TP53*/*PIK3CA*/*PTEN* that is at least equivalent to an MAF for *KRAS*/*GNAS*, the sensitivity and specificity for a mucinous PC with advanced neoplasia was 89% and 100%, respectively. In comparison, a cytopathological diagnosis of at least suspicious for adenocarcinoma within a mucinous PC was associated with 32% sensitivity and 98% specificity. Thus, NGS testing seems to outperform other diagnostic modalities in the detection of a mucinous PC with high-grade dysplasia and invasive adenocarcinoma.

The presence of *TP53*, *PIK3CA* and/or *PTEN* alterations within IPMNs with low-grade dysplasia and IPMNs are clinically not worrisome is an intriguing observation. The traditional thought is mutations in these genes emerge as IPMNs progress from low-grade dysplasia to high-grade dysplasia and invasive adenocarcinoma.[1] Recently, Yu *et al* described their experience with NGS of secretin-stimulated pancreatic juice samples.[22] Analogous to our findings, the authors reported that *TP53* mutations were detected in samples from patients with invasive adenocarcinoma and a minority of patients with low-grade IPMNs. In addition, higher concentrations of mutant *TP53* were present in cases with invasive adenocarcinomas as compared with IPMNs with low-grade dysplasia. The authors also described a case where a *TP53* mutation was identified 1 year prior to the diagnosis of invasive adenocarcinoma and at a time when no worrisome features were evident by imaging. While the natural history of IPMNs with low-level alterations in *TP53*, *PIK3CA* and/or *PTEN* is relatively unknown, it is plausible that these IPMNs are at a high risk for progression to malignancy. Considering 49% of PCs within our study cohort are likely mucinous PCs with majority representing IPMNs, a rational and cost-effective strategy for PC surveillance is needed. The identification of *KRAS* and/or *GNAS* mutations and low-level alterations in *TP53*, *PIK3CA* and/or *PTEN* in PCF may represent a predictive marker of malignant potential within an IPMN.

This study is, however, not without limitations. Although a large number of PCs were analysed, diagnostic surgical pathology was available for a small proportion of patients that represent a surgical selection bias. Our study also suffers from a testing selection bias as PC specimens satisfactory for NGS and Sanger sequencing were used for analysis. Considering 7% of cases failed molecular testing, the effect of this selection bias on our results is likely to be minimal. In addition, the follow-up period of this study is relatively short to assess the clinical impact of detecting alterations in *TP53*, *PIK3CA*, *PTEN* and/or *AKT1*. The results published herein represent our initial findings and we plan to continue monitoring these patients over their lifetime. Further, the molecular panel consisted of 11 genes commonly altered in PCs and present in invasive adenocarcinoma, but did not include *RNF43*, *CDKN2A* and *SMAD4*. Mutations and/or deletions in *RNF43* have been identified in both IPMNs and MCNs, and may improve the sensitivity of detecting MCNs.[13] However, *RNF43* alterations have a prevalence of 8% to 35% in MCNs and, therefore, the addition of *RNF43* may have limited usage in PC testing.[22 34] Deletions in *CDKN2A* and *SMAD4* are associated with IPMNs harbouring high-grade dysplasia and invasive adenocarcinoma.[18 22 25] While these genes may improve the accuracy of detecting molecular testing, using the aforementioned selection criteria to include MAFs for *GNAS* and *TP53*/*PIK3CA*/*PTEN* alterations, the sensitivity and specificity for advanced neoplasia in an IPMN was 100%, respectively. Nevertheless, additional studies are required to identify a minimal set of genes necessary for the assessment of PCs. Lastly, this study does not address the optimal approach of integrating DNA-based molecular testing to current PC surveillance protocols. Previously, we proposed an algorithmic approach to the management of PCs by utilizing molecular testing to stratify patients for appropriate management, but this requires further validation before implementation.[7] Herein, the Fukuoka guidelines were applied to the evaluation of mucinous PCs with consideration of the impact of molecular testing based on prior studies.[7 12–14 16 19–23 25]

In summary, we report the results of a large, prospective study of DNA-based molecular testing of EUS-FNA-obtained preoperative PCF. Overall, our results support the usage of NGS analysis in PCF given the high sensitivity and specificity in classifying PCs, especially IPMNs, and in the diagnosis of IPMNs with advanced neoplasia. Notable limitations of DNA-based molecular testing include the assessment of MCNs using *KRAS* and usage of Sanger sequencing in the evaluation of PCs. Future studies are required to explore the integration of DNA-based molecular testing into current management guidelines.

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