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Integration of *KRAS* testing in the diagnosis of pancreatic cystic lesions: a clinical experience of 618 pancreatic cysts

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With improvements in abdominal imaging, detection of incidental pancreatic cysts are becoming increasingly common. Analysis of pancreatic cyst fluid from fine-needle aspiration is particularly important in identifying intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), which have significant implications in clinical intervention and follow-up. Previous controlled studies have shown that *KRAS* mutations in cyst fluid are highly specific for mucinous differentiation in pancreatic cysts; however, this has not been examined in the clinical setting. Over a 6-year study period, 618 pancreatic cyst fluids obtained by fine-needle aspiration at the time of endoscopic ultrasound were tested for *KRAS* mutations as part of routine evaluation for a cystic neoplasm. Of the 618 specimens, 603 (98%) from 546 patients were satisfactory for molecular analysis. Patients ranged in age from 17 to 90 years (mean, 63.9 years) and were predominantly female (68%). Pancreatic cysts were relatively evenly distributed throughout the pancreas and ranged in size from 0.6 to 11.0 cm (mean, 2.3 cm). Mutations in *KRAS* were detected in 232 of 603 (38%) aspirates. Although sufficient for molecular analysis, 320 of 603 (53%) specimens were either less than optimal (38%) or unsatisfactory (15%) for cytopathologic diagnosis. Surgical follow-up information was available for 142 (26%) patients and consisted of 53 *KRAS*-mutated and 89 *KRAS*-wild-type cysts. Overall, *KRAS* mutations had a specificity of 100%, but a sensitivity of 54% for mucinous differentiation. When stratified by cyst type, *KRAS* had a sensitivity of 67% and 14% for IPMNs and MCNs, respectively. In summary, *KRAS* mutations were highly specific for mucinous differentiation, but were inadequate in identifying MCNs. Future molecular studies and the combination of other fluid markers are required to improve the detection and classification of pancreatic mucinous neoplasms by endoscopic ultrasound fine-needle aspiration.
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With the rapid increase in utilization and ongoing advancements in cross-sectional abdominal imaging, the detection of pancreatic cysts has become increasingly frequent. It is reported that pancreatic cysts are identified in 1.2% to 2.6% of abdominal computed tomography scans.[1,2] The prevalence increases with age and up to 24% of patients at autopsy have pancreatic cysts.[3] Historically, pseudocysts were thought to represent the bulk of pancreatic cysts, but cystic neoplasms actually account for the majority of these lesions. Many of these neoplasms, including serous cystadenomas are benign and can be monitored clinically. In contrast, intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) have the potential to progress to invasive pancreatic adenocarcinoma.[4–7] Consequently, international consensus guidelines for the management of IPMNs and MCNs were established and recently updated.[8,9] Therefore, an accurate diagnosis is critical for proper patient management.

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Currently, a multidisciplinary approach is recommended in the assessment of pancreatic cysts. This includes clinical and radiographic evaluation, endoscopic ultrasound-guided fine-needle aspiration, cytology, cyst fluid analysis (eg, viscosity) and tumor markers (eg, carcinoembryonic antigen (CEA)). Despite a combination of methodologies, the distinction between IPMNs and MCNs from other pancreatic cysts can be challenging. The application of molecular techniques has recently emerged as a promising adjunct to their evaluation. Although the cellular content of pancreatic cyst aspirates is often suboptimal, DNA from lysed or exfoliated epithelial cells shed into the fluid from the cyst lining can be analyzed for genetic abnormalities.[10,11] Previously, the results of a large multicenter study based at our institution (Pancreatic Cyst DNA Analysis Study or PANDA study) of pancreatic cysts with histologic follow-up were published and found that *KRAS* point mutations had a high specificity of 96%, but a low sensitivity of 45% for mucinous differentiation.[12] Elevated CEA (>192 ng/ml) is considered one of the most accurate tests to distinguish a mucinous cyst; however, it had a specificity and sensitivity of 83% and 64%, respectively. Combining *KRAS* and CEA improved the sensitivity from 64% to 82%, whereas maintaining the specificity at 83%. Data from our study and others highlight the importance of interpreting *KRAS* mutations in conjunction with other cyst findings.

Following the PANDA study, we have integrated *KRAS* mutational analysis as part of routine evaluation for pancreatic cystic neoplasms. Herein, we report our clinical and pathologic experience with *KRAS* testing to: (1) identify the prevalence of *KRAS*-mutant cysts in a high-volume pancreatic cyst service; (2) evaluate the role of *KRAS* testing in differentiating mucinous from nonmucinous cysts; and (3) correlate *KRAS* analysis with other accepted diagnostic modalities in the diagnosis of pancreatic cystic neoplasms.

Materials and methods

Cases

Study approval was obtained from the University of Pittsburgh Institutional Review Board. Data were collected on all cases where pancreatic cyst fluid was submitted to the University of Pittsburgh Medical Center, Division of Molecular Anatomic Pathology for *KRAS* analysis. Endoscopic ultrasound fine-needle aspiration obtained specimens were from patients being seen at the University of Pittsburgh Medical Center between November 2006 and October 2012. In all cases, samples were submitted by the endoscopist because of uncertainty as to whether a pancreatic cyst represented a cystic neoplasm. For example, patients with pseudocysts that had documented history of abdominal trauma were often excluded from fluid submission. Further, patients with high clinical suspicion for main duct IPMN by endoscopy and imaging were also refrained from analysis as ancillary testing would not affect patient management. Medical records were reviewed to document patient demographics, endoscopic ultrasound findings, fluid viscosity (as noted by the endoscopist), CEA analysis and cytopathologic diagnoses.

At the time of endoscopic ultrasound fine-needle aspiration, if pancreatic cyst fluid amounted to approximately 750 μl or greater, at least 500 μl was distributed for CEA analysis first, 200 μl for molecular analysis and the remaining for cytology, which includes the needle rinse. In certain instances, amylase was also assessed. In cases, where there was between 200 and 750 μl of cyst fluid, priority was given for cytology, which includes the needle rinse, and 200 μl for molecular analysis; no fluid was submitted for CEA because of insufficient fluid amount for analysis. In cases, where <200 μl of cyst fluid was aspirated, molecular analysis was not performed and thus not included within this study. Of note, secretin stimulation was not used to obtain pancreatic cyst fluid.

For cytology 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.

In all surgical resection cases, patients were first presented at the University Pittsburgh Medical Center Pancreatic Cancer Conference. This is a multidisciplinary conference composed of gastrointestinal endoscopists, surgeons, oncologists, pain management, nursing staff, radiologists and pathologists. Each patient was presented to this group of clinicians, where patient demographics, imaging, cytopathology, fluid characteristics, molecular findings, risks and benefits of surgery were assessed. Furthermore, the 2006 International Consensus Guidelines were also considered including cyst size, presence or questionable presence of a mural nodule by imaging, and a dilated main duct.[9] Owing to the study time frame, the recently published 2012 International Consensus Guidelines were not strictly used when considering criteria for surgery.[8] On resection, hematoxylin and eosin-stained slides were reviewed to confirm the histologic diagnosis, assess grade of dysplasia when appropriate and classify the histologic subtype of resected IPMNs.[13]

CEA Analysis

Pancreatic cyst CEA levels were assessed via a two-site immunoenzymatic 'sandwich' assay using two

monoclonal anti-CEA antibodies, which react with different CEA epitopes. This assay was performed on a Beckman Coulter Unicel DXI 800. Before analysis, specimens were centrifuged for 2 h and at least 500 μl (optimal 1 ml) of a cell-free sample was added to the reaction vessel. In all cases, testing was performed within 24 h of pancreatic cyst fine-needle aspiration. The amount of CEA present within the sample was determined by means of a stored, multipoint calibrator curve.

KRAS Mutational Analysis

Total genomic DNA was isolated from 200 μl of cyst fluid by column separation according to the manufacturer's directions and instructions (Qiagen, Valencia, CA, USA). The quantity of isolated DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For the detection of mutations, 10–50 ng of DNA was amplified with primers flanking exon 2 of the KRAS gene (forward primer 5′-GGTGAGTTTGTATTAAAA GGTACTGG-3′ and reverse primer 5′-TCCTGCACC AGTAATATGCA-3′) and exon 3 of the KRAS gene (forward primer 5′-TGAAGTAAAAGGTGCACTG-3′ and reverse primer 5′-GCATGGCATTAGCAAAGA CTC-3′). Then, PCR products were sequenced in both sense and antisense directions using the BigDye Terminator v3.1 cycle sequencing kit on ABI 3730 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequence electropherograms were analyzed using Mutation Surveyor software (SoftGenetics, LLC, State College, PA, USA). Each case was classified as positive or negative for *KRAS* mutation based on the sequencing results.

Statistical Analysis

Statistical analysis to assess differences between *KRAS*-mutant and *KRAS*-wild-type cysts were compared by the use of Fisher's exact test for dichotomous variables. All the tests were two-sided, and statistical significance was defined as a *P*-value <0.05. Sensitivities and specificities were calculated based on surgical resection material.

Results

Patient Demographics and Cyst Characteristics

Over a 6-year study period, 618 pancreatic cyst fluids were tested for *KRAS* mutations as part of routine evaluation for a cystic neoplasm. Of the 618 specimens, 603 (98%) from 546 patients were satisfactory for molecular analysis. The 15 cases, unable to be tested, were due to either DNA degradation or the presence of polymerase chain reaction (PCR) inhibitors. Repeat fine-needle aspiration and *KRAS* testing was performed on 5 of these 15 patients and was found satisfactory for molecular analysis. In 11 of 546 (2%) patients, 2 separate specimens corresponding to separate pancreatic cysts were submitted for *KRAS* analysis. Further, 41 of 546 (8%) patients had repeat aspiration and *KRAS* testing of their pancreatic cyst on follow-up.

The clinical and pathologic findings of *KRAS* testing are summarized in Table 1. Patients ranged in age from 17 to 90 years (mean, 63.9 years) and were predominantly female (366 of 546, 67%). The pancreatic cysts ranged in size from 0.6 to 11.0 cm (mean, 2.3 cm) and were distributed throughout the pancreas. This included 166 (28%) located in the head of the pancreas, 75 (12%) in the uncinate, 59 (10%) in the neck, 171 (28%) in the body and 132 (22%) in the tail. Although sufficient for molecular studies, the amount of cyst fluid was documented as insufficient for CEA analysis in 121 of 603 (20%) cases. In addition, 320 of 603 (53%) specimens were either less than optimal (38%, $n=229$) or unsatisfactory (15%, $n=91$) for cytopathologic diagnosis. The reason for specimen inadequacy was predominantly because of scant-to-absent cellularity. In these cases, cytologic slides demonstrated mostly histiocytes and other inflammatory cells with no discernible mucin in the background.

KRAS Mutational Analysis and Correlation

In total, 232 of 603 (38%) cases harbored *KRAS* mutations. Among the *KRAS* mutant cysts, point mutations in codon 12 were the most frequent (216 of 232, 93%). The most common mutations were p.G12D (105 of 232, 45%), p.G12V (70 of 232, 30%) and p.G12R (33 of 232, 14%). Other types of mutations seen only in a few cases were p.G12A ($n=3$, 1%), p.G12C ($n=2$, 1%) and p.G12F ($n=1$, 1%). In two (1%) cases, both p.G12D and p.G12V were detected. Mutations in codon 13 were identified in 9 of 232 (4%) cases and corresponded to p.G13D. In addition, 170 of 603 (28%) specimens were tested for *KRAS* mutations in codon 61. Six of 232 (3%) cases were p.Q61H; however, none of these six cases had a concurrent codon 12 or 13 mutation. Considering specimen adequacy may be an issue in the assessment of *KRAS* status, the cytology specimens for both *KRAS*-mutant and wild-type cohorts were compared. Both groups had a similar percentage of less than optimal and unsatisfactory specimens: 123 of 232 (53%) *KRAS*-mutant and 197 of 371 (53%) *KRAS*-wild-type cases.

Univariate analysis showed that *KRAS* mutations were associated with a higher occurrence in males (39% *KRAS*-mutant *vs* 28% *KRAS*-wild type, $P=0.009$), increased mean patient age at diagnosis (69.9 *vs* 60.2 years, $P<0.001$), smaller mean cyst size (2.0 *vs* 2.5 cm, $P<0.001$), the presence of multiple cysts (61% *vs* 35%, $P<0.001$), increased fluid viscosity (74% *vs* 47%, $P<0.001$) and decreased mean DNA concentration (4.32 *vs*

Table 1 Clinical and pathologic comparison of *KRAS*-mutant and *KRAS*-wild-type pancreatic cystic lesions

Patient or tumor characteristics	*Total,* n = *603*		*KRAS-mutant,* n = *232 (38%)*		*KRAS-wild type,* n = *371 (62%)*		P-*value*
Sex							
Male	195 (32%)		90 (39%)		105 (28%)		0.009
Female	408 (68%)		142 (61%)		266 (72%)		
Mean age (range), years	63.9 (17–90)		69.9 (44–90)		60.2 (17–88)		<0.001
Mean size (range), cm	2.3 (0.6–11)		2.0 (0.6–9.8)		2.5 (0.6–11)		<0.001
Location							
Head, neck and uncinate	300 (50%)		114 (49%)		186 (50%)		0.87
Body and tail	303 (50%)		118 (51%)		185 (50%)		
Cyst focality							
Solitary	334 (55%)		91 (39%)		243 (65%)		<0.001
Multiple	269 (45%)		141 (61%)		128 (35%)		
Fluid viscosity							
Thin and watery	258 (43%)		60 (26%)		198 (53%)		<0.001
Slight to marked viscosity	345 (57%)		172 (74%)		173 (47%)		
CEA	*n* = 436		*n* = 158		*n* = 278		
CEA ≤192 ng/ml	271 (62%)		64 (41%)		207 (74%)		<0.001
CEA >192 ng/ml	165 (38%)		94 (59%)		71 (26%)		
Mean DNA concentration (range), ng/μl	7.98 (0.01–263)		4.32 (0.21–36.87)		10.41 (0.01–263)		0.006
Surgical resections	*n* = 142		*n* = 53		*n* = 89		
Adenocarcinoma arising in an IPMN	12 (8%)	75 (52%)	7 (13%)	50 (94%)	5 (6%)	25 (28%)	<0.001
Main duct IPMN	13 (9%)		6 (11%)		7 (8%)		
Branch duct IPMN	44 (31%)		34 (64%)		10 (11%)		
Main and branch duct IPMN	6 (4%)		3 (6%)		3 (3%)		
Mucinous cystic neoplasm	22 (15%)		3 (6%)		19 (21%)		0.015
Cystic PanNET	18 (13%)		0		18 (20%)		<0.001
Solid-pseudopapillary neoplasm	2 (1%)		0		2 (2%)		
Serous cystadenoma	5 (4%)		0		5 (6%)		
Metastatic granulosa cell tumor	1 (1%)		0		1 (1%)		
Schwannoma	1 (1%)		0		1 (1%)		
Pseudocyst	8 (6%)		0		8 (9%)		
Retention cyst	7 (5%)		0		7 (8%)		
Lymphoepithelial cyst	2 (1%)		0		2 (2%)		
Foregut cyst	1 (1%)		0		1 (1%)		

CEA, carcinoembryonic antigen; IPMN, intraductal papillary mucinous neoplasm; PanNET, pancreatic neuroendocrine tumor.

10.41 ng/μl, $P = 0.006$). Cyst fluid CEA levels were available for 158 of 232 (68%) *KRAS*-mutant and 278 of 371 (75%) *KRAS*-wild-type cases. Using a cutoff value of 192 ng/ml, CEA was elevated in 59% of *KRAS*-mutants as compared with 26% of *KRAS*-wild-type cysts ($P < 0.001$). There was no statistically significant correlation between *KRAS* status and pancreatic cyst location ($P = 0.87$).

Follow-Up

Follow-up information was available for 474 of 546 (87%) patients and ranged from 2 to 72 months (mean, 31.3 months). In 41 patients, repeat fine-needle aspiration and *KRAS* testing of their pancreatic cyst was performed on follow-up. Initially, 33 of 41 (80%) patients had a *KRAS*-wild-type pancreatic cyst, whereas 8 of 41 (20%) patients had a *KRAS*-mutant pancreatic cyst. On repeat aspiration and *KRAS* testing, 9 of the 33 (27%) *KRAS*-wild-type pancreatic cysts were *KRAS*-mutant, whereas the remaining 24 (73%) were *KRAS*-wild type. Of the eight *KRAS*-mutant pancreatic cysts, seven (88%) remained *KRAS*-mutant, whereas one (12%) was *KRAS*-wild type.

Surgical follow-up information was available for 142 of 546 (26%) patients and ranged from 2 to 34 months (mean, 14.2 months) from initial endoscopic ultrasound fine-needle aspiration and *KRAS* testing. The surgically resected pancreatic cysts consisted of 53 *KRAS*-mutant and 89 *KRAS*-wild-type cysts. The distribution of *KRAS* mutations in resected specimens was as follows: 25 (47%) p.G12D, 17 (32%) p.G12V, 7 (13%) p.G12R, 1 (2%) p.G12A, 1 (2%) p.G12F and 2 (4%) p.G13D. As summarized in Table 1, *KRAS*-mutant cysts corresponded to either IPMNs ($n = 43$, 82%), adenocarcinomas arising in

association with an IPMN ($n=7$, 12%) or MCNs ($n=3$, 6%). Although *KRAS*-wild-type cysts also consisted of IPMNs and MCNs ($n=44$, 49%); additional cysts included cystic pancreatic neuroendocrine tumors ($n=18$, 20%), solid pseudopapillary neoplasms ($n=2$, 2%), serous cystadenomas ($n=5$, 6%), metastatic granulosa cell tumor ($n=1$, 1%), schwannoma with cystic degeneration ($n=1$, 1%), pseudocysts ($n=8$, 9%), retention cysts ($n=7$, 8%), lymphoepithelial cysts ($n=2$, 2%) and a foregut cyst ($n=1$, 1%).

Intraductal Papillary Mucinous Neoplasms

Of the 142 surgical resections, a total of 75 (53%) were IPMNs (Table 2). At the time of endoscopic ultrasound fine-needle aspiration, patients ranged in age from 44 to 87 years with a slight female predominance ($n=41$, 55%). The cysts ranged in size from 0.6 to 7.2 cm (mean, 2.2 cm). The IPMNs were often identified in the head, neck and uncinate ($n=42$, 56%) and solitary ($n=39$, 52%). Increased fluid viscosity and elevated CEA were noted in 71% and 62% of IPMNs, respectively.

The majority of *KRAS*-mutant cysts (50 of 53, 94%) were IPMNs (Figure 1) or adenocarcinomas arising in association with an IPMN. These were predominantly branch duct IPMNs ($n=34$, 68%) with the remaining consisting of main duct IPMNs ($n=6$, 12%), a mixed main and branch duct IPMN ($n=3$, 6%), and adenocarcinomas arising in association with an IPMN ($n=7$, 14%). When stratified by histologic subtype, 39 (79%) were gastric, 7 (15%) intestinal and 4 (6%) pancreatobiliary. Dysplasia within the *KRAS*-mutant IPMNs was graded as follows: 37 (75%) low, 9 (17%) intermediate and 4 (8%) high.

In all, 25 of 89 (28%) *KRAS*-wild-type cysts were IPMNs or adenocarcinomas arising in association with an IPMN. This included 10 (40%) branch duct IPMNs, 7 (28%) main duct IPMNs, 3 (12%) mixed main and branch duct IPMN and 5 (20%)

Table 2 Clinical and pathologic comparison of *KRAS*-mutant and *KRAS*-wild-type IPMNs

Patient or tumor characteristics	*Total,* n = *75*	*KRAS-mutant,* n = *50 (67%)*	*KRAS-wild type,* n = *25 (33%)*	P-*value*
Sex				
Male	34 (45%)	20 (40%)	14 (56%)	0.22
Female	41 (55%)	30 (60%)	11 (44%)	
Mean age (range), years	67.1 (44–87)	67.5 (44–87)	66.3 (48–85)	0.60
Mean size (range), cm	2.2 (0.6–7.2)	2.2 (0.6–4.7)	2.3 (0.8–7.2)	0.52
Location				
Head, neck and uncinate	42 (56%)	26 (52%)	16 (64%)	0.46
Body and tail	33 (44%)	24 (48%)	9 (36%)	
Cyst focality				
Solitary	39 (52%)	27 (54%)	12 (48%)	0.63
Multiple	36 (48%)	23 (46%)	13 (52%)	
Fluid viscosity				
Thin and watery	22 (29%)	15 (29%)	7 (28%)	1.00
Slight to marked viscosity	53 (71%)	35 (71%)	18 (72%)	
CEA	$n=48$	$n=31$	$n=17$	
CEA $\leq$192 ng/ml	18 (38%)	10 (32%)	8 (47%)	0.36
CEA $>$192 ng/ml	30 (62%)	21 (68%)	9 (53%)	
Mean DNA concentration (range), ng/μl	4.50 (0.03–15.53)	4.47 (0.50–15.35)	4.55 (0.03–15.53)	0.96
Duct involvement				
Main duct	13 (17%)	6 (12%)	7 (28%)	0.11
Branch duct	49 (65%)	37 (74%)	12 (48%)	0.03
Main and branch duct	13 (17%)	7 (14%)	6 (24%)	0.66
Histologic subtype				
Gastric	51 (68%)	39 (79%)	12 (48%)	0.02
Intestinal	16 (22%)	7 (15%)	9 (36%)	0.04
Pancreatobiliary	8 (10%)	4 (6%)	4 (16%)	0.43
Grade of dysplasia				
Low	48 (59%)	37 (75%)	11 (44%)	0.01
Intermediate	17 (27%)	9 (17%)	8 (32%)	0.15
High	10 (14%)	4 (8%)	6 (24%)	0.08

CEA, carcinoembryonic antigen; IPMN, intraductal papillary mucinous neoplasm.

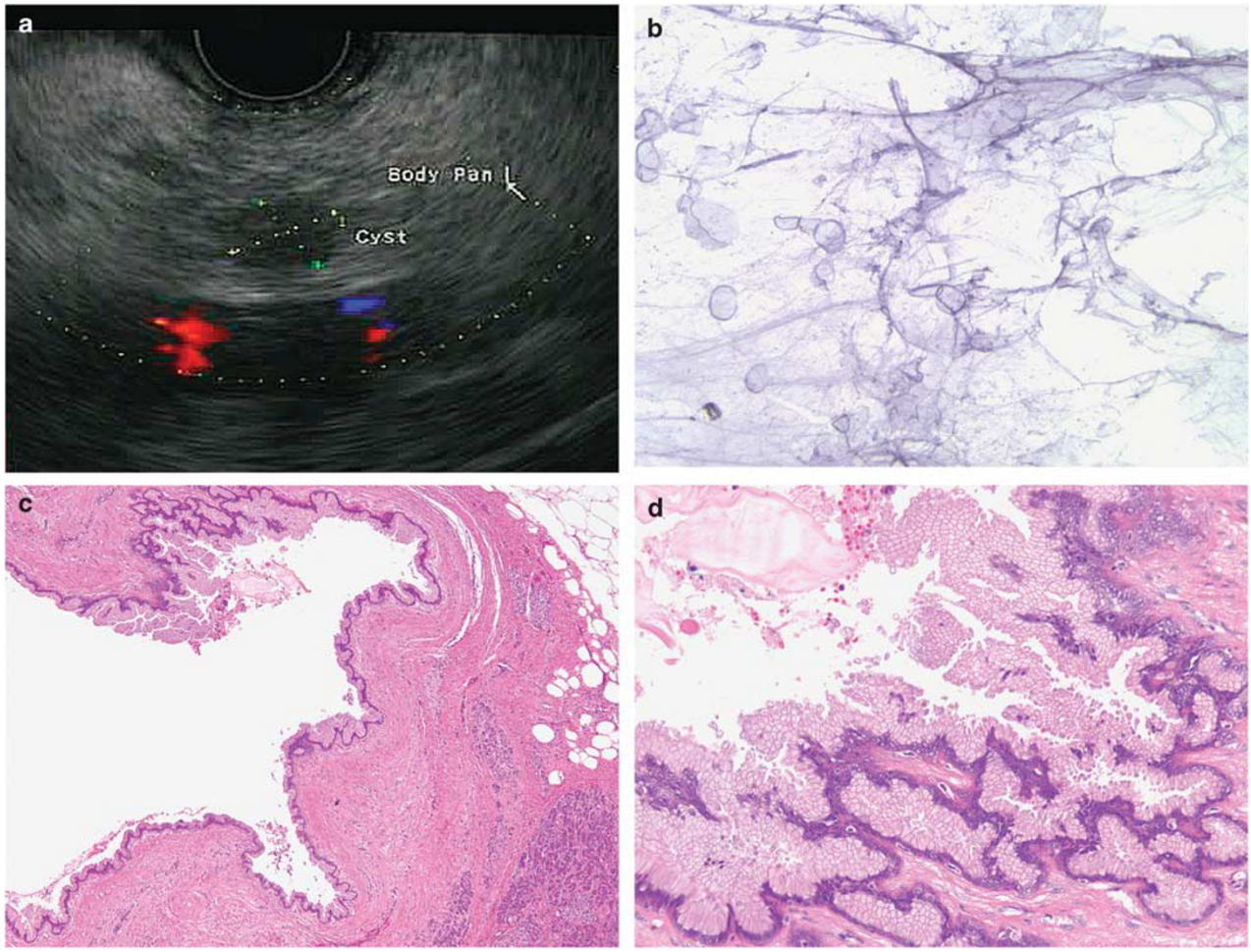

Figure 1 *KRAS*-mutant branch duct, intraductal papillary mucinous neoplasm (IPMN). (**a**) On endoscopic ultrasound, an anechoic and septated cyst was identified within the pancreatic body. The cyst measured 1.8 cm in maximal cross-sectional diameter and did not communicate with the main duct. On fine-needle aspiration, the fluid was noted to be slightly viscous. (**b**) Cytology smears were less than optimal and showed predominantly mucin with rare histiocytes and lymphocytes. The patient underwent a distal pancreatectomy because of an incidental pancreatic neuroendocrine tumor, however, the cyst (**c**, **d**) was consistent with a branch duct, IPMN with low-grade dysplasia and lined by gastric-type foveolar mucosa.

adenocarcinomas arising in association with an IPMN. Based on histologic subtype, 12 (48%) were gastric, 9 (36%) intestinal and 4 (16%) pancreatobiliary. Similar to *KRAS*-mutant IPMNs, low-grade dysplasia (11 of 25, 44%) was more prevalent, followed by intermediate-grade ($n=8$, 32%) and high-grade ($n=6$, 24%). Once again, considering specimen adequacy as a factor in the assessment of *KRAS* mutational status, 12 of 25 (48%) *KRAS*-wild-type IPMNs *vs* 21 of 50 (42%) *KRAS*-mutant IPMNs were less than optimal or unsatisfactory for cytopathologic diagnosis.

Among the resected IPMNs, no correlation between *KRAS* status and patient sex ($P=0.22$), mean age ($P=0.60$), mean cyst size ($P=0.52$), cyst location ($P=0.46$), cyst focality ($P=0.63$), fluid viscosity ($P=1.00$), CEA levels ($P=0.36$) or mean DNA concentration ($P=0.96$) was observed. However, the prevalence of *KRAS* mutations was higher in IPMNs involving the branch duct ($P=0.03$), of the gastric histologic subtype ($P=0.01$) and harboring low-grade dysplasia ($P=0.01$). Furthermore, the absence of *KRAS* mutations correlated with intestinal-type IPMNs ($P=0.04$).

Mucinous Cystic Neoplasms

All 22 MCNs were identified in females that ranged in age from 20 to 75 years (mean, 46.9 years). Cysts measured 2.0 to 9.9 cm (mean, 4.5 cm) and were located within the distal pancreas. Fifteen (67%) were in the tail of the pancreas, whereas 7 (33%) were in the body. By endoscopic ultrasound fine-needle aspiration, cyst fluid was noted to be thin and watery in 10 (45%) cases and viscous in 12 (55%). CEA analysis was performed on 21 of 22 (95%) MCNs and elevated in 12 (55%). On resection, the majority of MCNs had low-grade dysplasia (16 of 22, 73%), whereas the remaining showed intermediate-grade dysplasia ($n=6$, 29%). In all, 3 of 22 (14%) MCNs harbored a

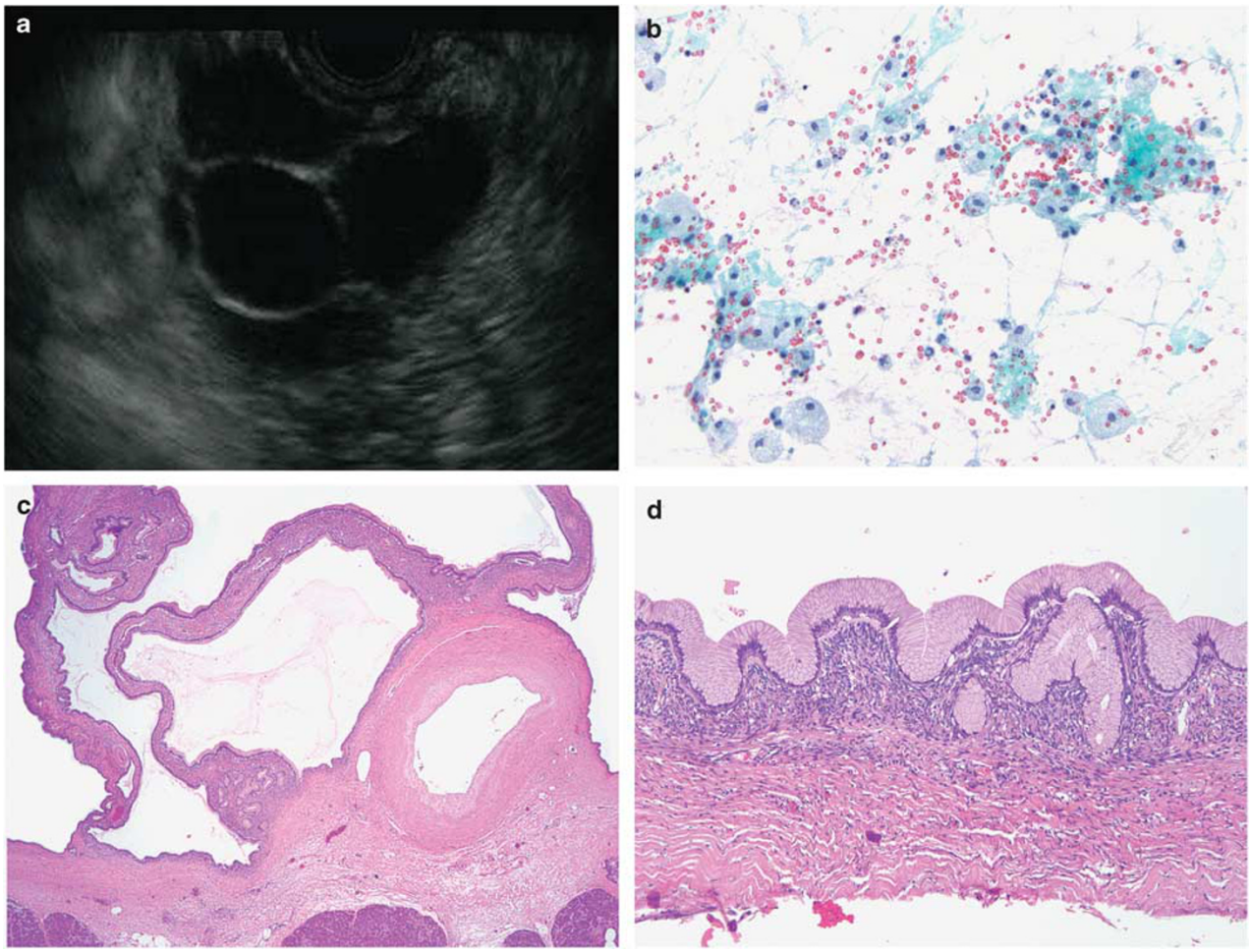

Figure 2 *KRAS*-mutant mucinous cystic neoplasm (MCN). (**a**) An anechoic and distally enhancing cyst was identified in the pancreatic body/tail by endoscopic ultrasound. The cyst measured 6.5 cm in maximal cross-sectional diameter. Multiple thinly septated compartments were identified. Fluid obtained by fine-needle aspiration was noted to be clear and thin. (**b**) Cytology smears were less than optimal, but a cytospin sample showed a moderate number of histiocytes in a background of mucin consistent with cyst contents. (**c**, **d**) A distal pancreatectomy specimen demonstrated a multiloculated cyst with a mucinous epithelial lining. The nuclei were uniform and basally oriented. Beneath the cyst lining was a dense ovarian-type stroma consistent with an MCN.

KRAS mutation (Figure 2). However, no significant differences in patient demographics, endoscopic ultrasound findings, specimen adequacy, cyst fluid analysis, mean DNA concentration or histologic characteristics were seen in comparison with *KRAS*-wild-type MCNs.

Correlation of *KRAS* Status and Other Endoscopic Ultrasound Fine-Needle Aspiration Findings with Surgically Resected IPMNs and MCNs

Overall, *KRAS* mutations had a specificity of 100% for mucinous differentiation in surgically resected pancreatic cysts, however, it only attained a sensitivity of 54%. In comparison, increased fluid viscosity and elevated CEA had a lower specificity (69% and 85%, respectively), but a higher sensitivity (68% and 62%, respectively). A cytopathologic diagnosis of at least suspicious for a neoplastic mucinous cyst had a similar specificity of 73%, but a significantly lower sensitivity of 39%. For IPMNs, the presence of multiple cysts had a specificity of 59% and a sensitivity of 80%. The combination of *KRAS* point mutations and elevated CEA improved the sensitivity of both assays to 83% and maintained a high specificity of 85% for mucinous differentiation. The addition of fluid viscosity to *KRAS* and CEA increased the sensitivity (90%), however, at a loss in specificity (64%). A multimodal approach using *KRAS*, CEA, fluid viscosity and cytology further increased the sensitivity to 91%, but reduced the specificity to 56%.

When stratified by cyst type, *KRAS* had a specificity and sensitivity of 95% and 67% for IPMNs *vs* 58% and 14% for MCNs. Although IPMNs are often multifocal, tend to have an elevated CEA and frequently show increased fluid viscosity, the specificity of each parameter was lower (86%, 69% and 66%, respectively) than compared with *KRAS*. In addition, the sensitivities were either similar or slightly lower (48%, 68% and 63%, respectively). For MCNs, elevated CEA had both a higher

specificity (71% *vs* 58%) and sensitivity (55% *vs* 14%) than *KRAS*. In contrast, increased fluid viscosity had a lower specificity of 48%, but a higher sensitivity of 55%. All MCNs within this study were solitary.

Discussion

To date, the PANDA study was the largest multicenter trial to evaluate molecular analysis of pancreatic cyst fluid in the diagnosis of mucinous cysts.[12] It included 113 patients with pancreatic cysts that underwent surgical resection or had diagnostic aspiration cytology. Cyst fluid DNA was obtained by endoscopic ultrasound fine-needle aspiration and analyzed within a commercial laboratory, Redpath Integrated Pathology (Pittsburgh, PA, USA). Molecular analysis incorporated DNA quantification, *KRAS* codon 12 testing, and multiple allelic loss analysis of a broad panel of microsatellite markers including sequence determination. *KRAS* codon 12 mutations showed the highest odds ratio (20.9) and specificity (96%), but a low sensitivity (45%) for mucinous differentiation. In comparison, elevated CEA had a specificity and sensitivity of 83% and 64%, respectively. *KRAS* mutational analysis improved the sensitivity of CEA analysis from 64% to 82%, while maintaining the specificity at 83%.

Although the PANDA study showed the feasibility of extracting and analyzing DNA from endoscopic ultrasound fine-needle aspiration obtained pancreatic cyst fluid, there were a number of inherent limitations to this study. For example, *KRAS* analysis was performed by a single commercial laboratory and it was unknown whether these results could be reliably reproduced by other laboratories. Further, although DNA analysis is noted to be recommended in centers with expertise in endoscopic ultrasound fine-needle aspiration by the current international consensus guidelines for the management of IPMNs and MCNs, it is uncertain if the high costs of submitting cyst fluid to a commercial laboratory is justified.[8] Another limitation of the PANDA study was the introduction of selection bias. Most of the published studies examining the role of DNA analysis in the diagnosis of pancreatic cysts, including the PANDA study, have a limited sample size and population bias.[14–16] Study cohorts often consist of an enriched population of patients with high-grade cysts than typically observed in the general population. This selection bias may influence the perceived performance of *KRAS* testing. Finally, 11 (9%) cases within the PANDA study were excluded from molecular analysis that corresponded to cystic pancreatic neuroendocrine tumors, mesenteric cyst, mesothelial cyst, solid pseudopapillary neoplasms and schwannoma with cystic degeneration. Although rare, these entities can enter the differential diagnosis of a mucinous cyst, either clinically, radiographically or cytologically.[17] Thus, a larger observation period, accumulation of molecular data and adequate surgical follow-up are needed to draw a definitive conclusion regarding the clinical utility of *KRAS* analysis in pancreatic cyst fluid.

In an effort to address the issues raised from the PANDA study, we have integrated *KRAS* testing as part of routine evaluation of pancreatic cystic neoplasms within our high-volume pancreatic cyst service. The prevalence of *KRAS*-mutant cysts was 38% during a 6-year period. In addition to *KRAS* mutations found at codon 12, we also detected mutations at codons 13 and 61. Although correlative histologic follow-up was present for only 25% of patients, a total of 73 IPMNs and 21 MCNs were resected. Further, the majority of these mucinous cysts (67%) demonstrated low-grade dysplasia. Similar to the results reported by the PANDA study, *KRAS* mutations had a high specificity (100%), but a low sensitivity (54%) for mucinous cysts. CEA analysis had a specificity of 85% and a sensitivity of 62%. In combination, both assays increased the sensitivity to 83%, whereas maintaining a high specificity of 85%. The reproducibility of the PANDA study results with regards to *KRAS* confirms its utility in the identification of mucinous cysts. In conjunction with CEA analysis, *KRAS* testing can improve the diagnostic yield of pancreatic cyst fine-needle aspirates.

Of note, a selection bias still exists within our study. More specifically, *KRAS* testing was performed at the discretion of the endoscopist. Therefore, cyst fluids from pseudocysts in patients with a history of abdominal trauma or straightforward main duct IPMNs by imaging were refrained from *KRAS* analysis. However, an argument can be made that molecular ancillary testing is not necessary in these settings. Of note, previous studies have shown that cyst fluid classification based on *KRAS* mutations alone can lead to false-positive results. Using the same commercial biomarker panel (PathfinderTG) as the PANDA study, Panarelli *et al*[15] identified two pseudocysts harbored *KRAS* mutations. Thus, interpreting *KRAS* results as part of a multidisciplinary approach is imperative.

Somatic mutations in *KRAS* are common in both IPMNs and MCNs with a reported frequency ranging from 30% in low-grade lesions to 80% in high-grade lesions.[18–22] Consistent with previous studies, the prevalence of *KRAS* mutations in IPMNs was 67%. In contrast, only 14% of MCNs harbored a *KRAS* mutation. A lack of sensitivity within our DNA detection technique may account for these findings. However, *KRAS* mutations within IPMNs were consistently identified at a comparable rate as studies using surgical resection material or postoperative cyst fluid. Alternatively, the amount of DNA from lysed and shed epithelium obtained by fine-needle aspiration may be less than that found in postoperative aspiration because of surgical

manipulation. Or, only 14% of MCNs within our study cohort truly have *KRAS* mutations. Regardless of the cause, additional markers are required to improve the sensitivity of cyst fluid DNA analysis.

Recently, deep sequencing using next-generation sequencing technologies and whole-exome sequencing has identified recurrent mutations within the major neoplastic cysts of the pancreas. Using postoperative cyst fluid, Wu *et al*[23] identified a high prevalence of *KRAS* mutations at codon 12 in IPMNs. Similar to our results, *KRAS* mutations correlated with low-grade dysplasia and were more frequently found within the gastric and pancreatobiliary subtypes as opposed to the intestinal subtype. The authors also found a high rate of mutations within the oncogene, *GNAS*. In contrast to *KRAS*, *GNAS* mutations had a higher prevalence in advanced lesions and the intestinal subtype. Further, the presence of either a *GNAS* or *KRAS* mutation was identified in >96% of IPMNs. In another study by Wu *et al*[24], sequencing of cyst epithelium of the four major neoplastic cysts defined a panel of genes that could be used to classify each cyst type. IPMNs were characterized by mutations in *KRAS*, *GNAS* and the E3 ubiquitin ligase, *RNF43*. In addition to *KRAS*, 40% of MCNs also harbored mutations in *RNF43*. The combination of *KRAS*, *GNAS* and *RNF43* assessment would improve the sensitivity, whereas maintaining a high specificity for mucinous differentiation than *KRAS* testing alone. Another E3 ubiquitin ligase, *VHL* was mutated in all serous cystadenomas analyzed; however, wild type in IPMNs and MCNs. Finally, solid pseudopapillary neoplasms were uniquely characterized by mutations in *CTNNB1* and lacked *KRAS*, *GNAS*, *RNF43* and *VHL* mutations. DNA analysis of a five gene panel that includes *KRAS*, *GNAS*, *RNF43*, *VHL* and *CTNNB1* could lead to a highly accurate diagnosis and proper patient management.

An important aspect, not discussed within this study, is the ability of DNA analysis to distinguish mucin-producing lesions with low-grade dysplasia (benign) from those with high-grade dysplasia or an associated invasive carcinoma (malignancy). Notably, *KRAS* mutations alone did not correlate with higher grade in mucin-producing neoplasms. The PANDA study reported that either multiple allelic loss analysis of >82% or the combination of a *KRAS* mutation and high multiple allelic loss analysis was highly predictive of malignancy. These findings were confirmed by Shen *et al*,[14] who analyzed 35 pancreatic cyst fluid specimens using Redpath's PathfinderTG. The authors found an 89% concordance between molecular and clinical consensus diagnoses including 83% for malignant, 87% for benign mucinous and 93% for benign nonmucinous cysts. Conversely, studies by Panarelli *et al*[15] and Toll *et al*[16] reported a concordance rate of 39% and 56%, respectively. The broad variability in agreement between molecular and clinical diagnoses may be explained by the lack of surgical follow-up data in the latter two studies. However, as was with *KRAS* analysis, future studies including a longer observation period and correlative surgical follow-up are required.

In summary, we report the largest series of *KRAS* analysis on endoscopic ultrasound fine-needle aspiration obtained pancreatic cyst fluid. In conjunction with clinical and radiographic findings, *KRAS* was highly specific, but had a poor sensitivity for mucinous differentiation. The addition of CEA analysis, improved the sensitivity of *KRAS* analysis, while maintaining a high specificity. When stratified by mucinous cyst type, *KRAS* testing had a modest sensitivity for IPMNs, but was inadequate in identifying MCNs. Future molecular studies to include additional genes and other fluid markers are required to improve the detection and classification of pancreatic mucinous neoplasms by endoscopic ultrasound fine-needle aspiration.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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