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Preoperative *GNAS* and *KRAS* Testing in the Diagnosis of Pancreatic Mucinous Cysts

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Abstract

Purpose: Management guidelines for pancreatic intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) are based on the assumption that mucinous cysts can be accurately distinguished from other pancreatic cystic lesions. Previous studies using surgical material have identified recurrent mutations in *GNAS* and *KRAS* in pancreatic mucinous neoplasms. Yet, the diagnostic utility of testing for both genes in pancreatic cyst fluid obtained by endoscopic ultrasound–fine-needle aspiration (EUS–FNA) remains unclear.

Experimental Design: *GNAS* and *KRAS* testing was performed on EUS–FNA pancreatic cyst fluid from 91 pancreatic cysts: 41 IPMNs, 9 IPMNs with adenocarcinoma, 16 MCNs, 10 cystic pancreatic neuroendocrine tumors (PanNET), 9 serous cystadenomas (SCA), 3 retention cysts, 2 pseudocysts, and 1 lymphoepithelial cyst.

Results: Mutations in *GNAS* were detected in 16 (39%) IPMNs and 2 (22%) IPMNs with adenocarcinoma. *KRAS* mutations were identified in 28 (68%) IPMNs, 7 (78%) IPMNs with adenocarcinoma, and 1 (6%) MCN. Mutations in either gene were present in 34 (83%) IPMNs, 8 (89%) IPMNs with adenocarcinoma, and 1 (6%) MCN. No mutations were found in cystic PanNETs, SCAs, retention cysts, pseudocysts, and a lymphoepithelial cyst. *GNAS* and *KRAS* mutations had 100% specificity [95% confidence interval (CI), 0.83–1.00] but 65% sensitivity (95% CI, 0.52–0.76) for mucinous differentiation. Among IPMNs, mutations in either gene had 98% specificity (95% CI, 0.86–1.00) and 84% sensitivity (95% CI, 0.70–0.92).

Conclusions: The combination of *GNAS* and *KRAS* testing was highly specific and sensitive for IPMNs; however, the lack of sensitivity for MCNs highlights the need for additional markers to improve the detection of pancreatic mucinous neoplasms. *Clin Cancer Res; 20(16); 4381–9. ©2014 AACR.*

Introduction

With increased use and improvements in cross-sectional imaging technologies, cystic lesions of the pancreas are becoming frequently encountered in clinical practice (1). Although many cysts, such as pseudocysts and SCA, have a benign clinical course, others, such as intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN), represent precursor lesions to invasive pancreatic adenocarcinoma (2–4). Because of their risk of malignancy, international consensus guidelines for the management of IPMNs and MCNs were developed and recently updated (5, 6). These guidelines were based on the assumption that mucinous neoplasms can be diagnosed correctly on the basis of standard clinical, imaging, and laboratory criteria. Despite these measures, preoperatively distinguishing pancreatic cysts from one another can be challenging and, if incorrect, can pose a significant health risk to the patient. However, changes within the management guidelines recommend cyst fluid molecular analysis in those centers with expertise in endoscopic ultrasound–fine-needle aspiration (EUS–FNA) and cytologic interpretation (6).

Previously, we reported the results of our institution's clinical experience with DNA testing for *KRAS* mutations from pancreatic cyst fluid obtained by EUS–FNA (7). *KRAS* mutations had a specificity of 100% but a sensitivity of 54% for mucinous differentiation. In comparison, carcinoembryonic antigen (CEA), a cyst fluid tumor marker often used to distinguish mucinous from nonmucinous pancreatic cysts, had a specificity and sensitivity of 85% and 62%, respectively, with a cutoff of 192 ng/mL and above. The combination of both assays increased the sensitivity to 83% and maintained a high specificity of 85%. Although *KRAS*,

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Translational Relevance

As a result of widespread use of and advancements in cross-sectional imaging, cystic lesions of the pancreas are increasingly encountered in clinical practice. Although many cysts, such as pseudocysts and serous cystadenomas (SCA), have a benign clinical course, others, such as intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN), represent precursor lesions to invasive pancreatic adenocarcinoma. However, preoperatively distinguishing pancreatic cysts from one another can be challenging and, if incorrect, can pose a significant health risk to the patient. The application of molecular techniques has recently emerged as a promising adjunct to endoscopic ultrasound–fine-needle aspiration (EUS–FNA) in the diagnosis of pancreatic mucinous cysts. Within a large cohort, combined *GNAS* and *KRAS* mutational analysis of pancreatic cyst fluid obtained by EUS–FNA is highly specific and sensitive for IPMNs but lacks sensitivity for MCNs, underscoring the need for additional markers to improve detection of pancreatic mucinous neoplasms.

in conjunction with CEA analysis, improved the diagnostic yield of pancreatic cyst fine-needle aspirates, additional markers are needed to improve the sensitivity of cyst fluid DNA analysis.

Next-generation and whole-exome sequencing technologies have uncovered recurrent mutations within the major neoplastic cysts of the pancreas (8–10). Consistent with our findings, Wu and colleagues (9) identified a high prevalence of *KRAS* mutations in IPMNs. Rather unexpectedly, the authors also found activating mutations within the oncogene, *GNAS*. *GNAS* mutations were highly specific for IPMNs and not seen in the other pancreatic cystic neoplasms (8). Furthermore, the presence of either a *GNAS* or *KRAS* mutation was identified in >96% of IPMNs. However, these studies were based on DNA obtained from postoperative pancreatic cyst fluid or microdissection of the cyst epithelial lining. Thus, the diagnostic utility of testing for *GNAS* and *KRAS* mutations in clinical practice remains unclear. We therefore have analyzed both *GNAS* and *KRAS* mutations within a large cohort of pancreatic cyst fluid obtained by EUS–FNA. In addition, these results were correlated with corresponding clinicopathologic features and other 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. Pancreatic cyst fluids tested obtained by EUS–FNA were consecutively accrued for routine *KRAS* mutational analysis between January 2006 and December 2013 at the University of Pittsburgh Medical Center (Pittsburgh, PA; ref. 7). In all cases, cyst fluid was submitted for molecular analysis by the endoscopist because of uncertainty as to whether a pancreatic cyst represented a cystic neoplasm. In other words, patients with pseudocysts who had documented history of abdominal trauma or high clinical suspicion for main duct IPMN by endoscopy and radiographic imaging were often refrained from ancillary molecular testing. Eighty-three pancreatic cyst fluid DNA specimens were selected for *GNAS* mutational analysis. These cases included 41 IPMNs, 9 IPMNs with an associated invasive adenocarcinoma, 16 MCNs, 10 cystic pancreatic neuroendocrine tumors (PanNET), 3 retention cysts, 2 pseudocysts, 1 lymphoepithelial cyst, and 1 SCA. The diagnoses for all pancreatic cysts were rendered on the basis of standard histomorphologic criteria of corresponding surgical resection specimens (11, 12). Of note, the results of *KRAS* testing from 29 IPMNs, 7 IPMNs with an associated invasive adenocarcinoma, 13 MCNs, and 7 cystic PanNETs were previously reported by Nikiforova and colleagues (7). An additional eight pancreatic cyst fluid specimens were selected that clinically corresponded to SCA based on standard radiographic imaging, endoscopic ultrasound, CEA level of <2.0 ng/mL, and cytopathologic findings (11–13). Furthermore, at the time of *KRAS* testing, the specimens were assessed for loss of heterozygosity (LOH) at the *VHL* locus. In six of eight (75%) cases, *VHL* LOH was identified.

Medical records were reviewed for each case to document patient demographics, radiographic imaging, endoscopic ultrasound findings, fluid viscosity (qualitative assessment noted by the endoscopist at the time of fine-needle aspiration), CEA analysis, and cytopathologic diagnosis. 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 a few histiocytes present. Unsatisfactory specimens were virtually acellular and lacked mucin. A cytopathologic diagnosis of at least suspicious for a neoplastic mucinous cyst was made by individual cytopathologists at the University of Pittsburgh Medical Center based on the presence of extracellular mucin and/or mucinous epithelium with varying degrees of cytologic and architectural nuclear atypia (14–16). Clinical impression, radiographic imaging, endoscopic findings, and CEA levels were available to the cytopathologist before a diagnosis was rendered.

GNAS and *KRAS* mutational analysis

KRAS testing was performed at the time of pancreatic cyst fluid submission as described by Nikiforova and colleagues (7). Briefly, total genomic DNA was isolated from 200 μL of cyst fluid by column separation and according to the manufacturer's directions and instructions (Qiagen). The quantity of isolated DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). For the

detection of mutations, 10 to 50 ng of DNA was amplified with primers flanking exon 2 of the *KRAS* gene (forward primer 5′-GGTGAGTTTGTATTAAAAGGTACTGG-3′ and reverse primer 5′-TCCTGCACCAGTAATATGCA-3′) and exon 3 of the *KRAS* gene (forward primer 5′-TGAAGTAAAAGGTGCACTG-3′ and reverse primer 5′-GCATGGCATTAGCAAAGACTC-3′). The detection of *GNAS* mutations were performed using primers flanking codon 201 at exon 8 (forward primer 5′-TGACTATGTGCCGAGCGA-3′ and reverse primer 5′-AACCATGATCTCTGTTATATAA-3′). PCR products were sequenced in both sense and antisense directions using BigDye Terminator v3.1 cycle sequencing kit on the ABI 3730 (Applied Biosystems) according to the manufacturer's instructions. The sequence electropherograms were analyzed using Mutation Surveyor software (SoftGenetics, LLC).

VHL LOH

At the time of *KRAS* mutational analysis, determination of LOH at the von Hippel-Lindau tumor-suppressor gene, *VHL*, was performed as previously described (17–19). Ten to 50 ng of DNA was amplified with PCR primers based on the use of 2 microsatellite markers: 3p25: D3S2303[L17972] and 3p26:D3S1539[L16393]. Postamplification products were electrophoresed and relative fluorescence was determined for individual alleles (ABI 3100 Genetic Analyzer; Applied Biosystems). The ratio of peaks was calculated by dividing the value for the shorter sized allele by that of the longer sized allele. Thresholds for significant allelic imbalance were determined using normal (non-neoplastic) buccal swabs specimens from each patient. Peak height ratios falling outside of 2 standard deviations beyond the mean for each polymorphic allele pairing were assessed as showing significant allelic imbalance.

Statistical analysis

Statistical analyses to assess differences between *GNAS*- and/or *KRAS*-mutant and wild-type cysts were compared using the Fisher exact test for dichotomous variables using SPSS Statistical software, version 21 (IBM). All tests were two-sided, and statistical significance was defined as a $P < 0.05$.

Results

Pancreatic cyst study cohort

The study cohort consisted of the following: 50 IPMNs, 16 MCNs, 10 cystic PanNETs, 9 SCA, 3 retention cysts, 2 pseudocysts, and 1 lymphoepithelial cyst. In 9 of 50 IPMNs, an associated invasive pancreatic ductal adenocarcinoma was present. All pancreatic cysts except eight of nine SCA were surgically resected and diagnosed on the basis of standard pathologic criteria (12). The remaining eight cysts were diagnosed clinically using accepted radiographic and endoscopic ultrasound findings, a CEA level of <2.0 ng/mL, and cytologic smears consistent with a SCA (13). Furthermore, at the time of *KRAS* testing, these eight SCA specimens were assessed for LOH at the *VHL* locus. In six of eight (75%) cases, *VHL* LOH was identified.

At the time of EUS–FNA, patients ranged in age from 20 to 87 years (mean, 60.2 years) and were predominantly female (55 of 91, 60%). The pancreatic cysts ranged in size from 0.7 to 9.9 cm (mean, 3.0 cm) and were distributed throughout the pancreas. This included 26 (29%) located in the head of the pancreas, 9 (10%) in the uncinate, 4 (4%) in the neck, 26 (29%) in the body, and 26 (29%) in the tail. Although sufficient for molecular studies, the amount of cyst fluid was insufficient for CEA analysis in 21 (23%) cases. In addition, 34 (37%) specimens were either less than optimal (33%, $n = 30$) or unsatisfactory (4%, $n = 4$) for cytopathologic diagnosis. The primary reason for specimen inadequacy was predominantly due to scant-to-absent cellularity.

GNAS and *KRAS* mutational analysis and correlation

Point mutations in *GNAS*, *KRAS*, or both were detected in 18 (20%), 36 (40%), and 11 (12%) cases, respectively. *GNAS* mutations consisted of either p.R201H ($n = 9$, 50%) or p.R201C ($n = 9$, 50%; Fig. 1). Mutations in *KRAS* were predominantly at codon 12 (34 of 36, 94%). These included p.G12D ($n = 17$, 47%), p.G12V ($n = 12$, 33%), p.G12R ($n = 4$, 11%), and p.G12F ($n = 1$, 3%). A mutation in codon 13 of *KRAS* was found in two (6%) cases and corresponded to p.G13D. No *KRAS* mutations in codon 61 were detected.

In total, 16 of 41 (39%) IPMNs and two of nine (22%) IPMNs with an associated invasive adenocarcinoma harbored *GNAS* mutations. *KRAS* mutations were identified in 28 (68%) IPMNs, 7 (78%) IPMNs with invasive adenocarcinoma, and 1 of 16 (6%) MCNs. Mutations in both genes were found in 10 (24%) IPMNs and 1 (13%) IPMN with invasive adenocarcinoma. And, as summarized in Table 1, mutations in either *GNAS* or *KRAS* were present in 34 (83%) IPMNs, 8 (89%) IPMNs with invasive adenocarcinoma, and 1 (6%) MCN. *GNAS* and *KRAS* mutations were absent in cystic PanNETs, SCA, retention cysts, pseudocysts, and a lymphoepithelial cyst. Univariate analysis showed that mutations in either *GNAS* or *KRAS* were associated with higher occurrence in males (58% vs. 23%; $P = 0.001$), increased mean patient age at diagnosis (66.5 vs. 54.5 years; $P < 0.001$), smaller mean cyst size (2.6 vs. 3.4 cm; $P = 0.02$), increased fluid viscosity (79% vs. 25%; $P < 0.001$), and elevated CEA (71% vs. 38%; $P = 0.008$). There was no statistically significant correlation between *GNAS* or *KRAS* status and pancreatic cyst location ($P = 0.14$), focality ($P = 0.13$), or specimen adequacy for cytopathologic diagnosis ($P = 0.52$).

IPMNs

Of the 50 IPMNs within the study cohort, independent statistical correlations between *GNAS* and *KRAS* mutations with respect to patient demographics, endoscopic ultrasound cyst/fluid characteristics, cytologic findings, and histologic features were performed (Table 2). *GNAS*-mutant IPMNs occurred predominantly in male patients (83% vs. 41%; $P = 0.007$); however, no correlation with gender was

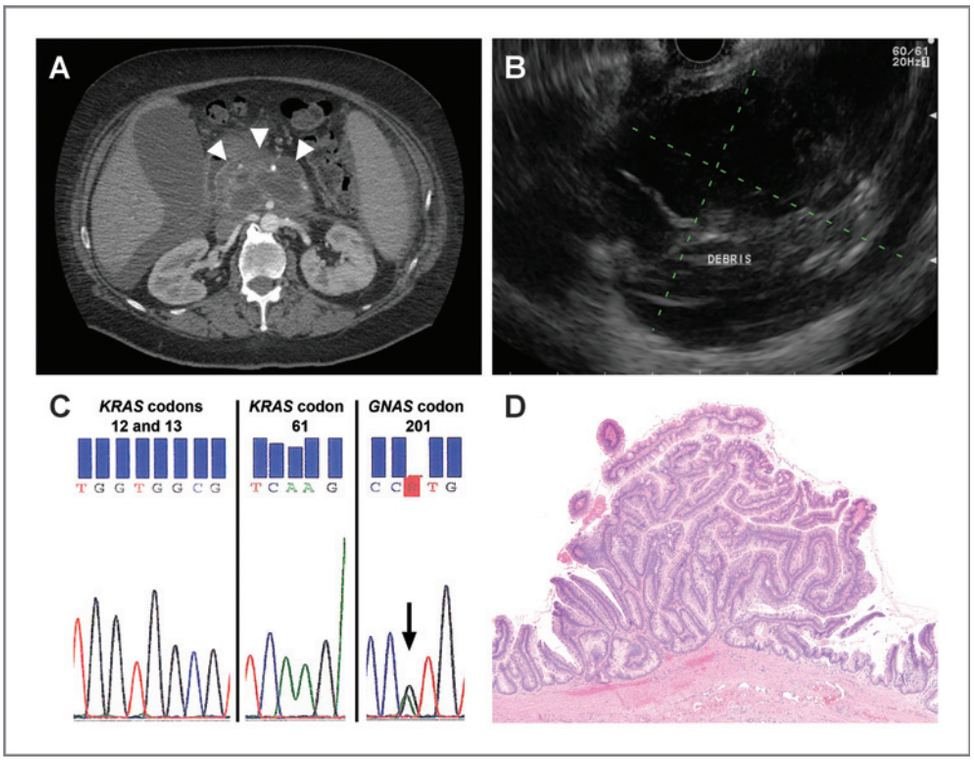

Figure 1. *GNAS*-mutant and *KRAS*-wild-type IPMN. A, contrast-enhanced computed tomography image demonstrating a complex cystic lesion involving the body of the pancreas (arrow heads) that was regarded as a probable pancreatic pseudocyst. B, on endoscopic ultrasound, an anechoic, multicystic, and septated cyst was identified in the body of the pancreas. The lesion measured 7.2 cm × 7.0 cm in maximal cross-section diameter (green dashed lines) and remarkable for internal debris. Fine-needle aspiration revealed a thin, watery, clear fluid. Cytology smears were satisfactory for interpretation and consisted of histiocytes and acute inflammation with a differential diagnosis of primarily a pseudocyst (not shown). C, electropherograms of *GNAS* and *KRAS* mutational status using pancreatic cyst fluid revealed a missense mutation in codon 201 (CGT to CAT) resulting in an amino acid substitution of an arginine with histidine (R201H). Mutations in *KRAS* codons 12 (GGT), 13 (GGC), and 61 (CAA) were absent. D, upon resection, the pancreatic cyst was consistent with a mixed main and branch duct IPMN, displaying intermediate-grade dysplasia and of the intestinal histologic subtype.

seen in *KRAS*-mutant cysts ($P = 0.77$). No statistical differences in mean patient age were observed with either gene (*GNAS*, $P = 0.66$; *KRAS*, $P = 0.36$). With regard to endoscopic ultrasound and radiologic findings, there was no association between *GNAS* or *KRAS* mutations and mean cyst size ($P = 0.43$ and 0.48, respectively), location within the pancreas ($P = 0.77$ and 0.38), focality ($P = 0.77$ and 0.55), main duct dilatation ($P = 1.00$ and 0.11), the presence of a mural nodule ($P = 1.00$ and 0.67), fluid viscosity ($P = 0.07$ and 1.00), or CEA levels ($P = 0.43$ and 1.00).

By cytology, no significant differences between genetic mutation and specimen adequacy were observed (*GNAS* and *KRAS*, $P = 1.00$ and 0.765, respectively). Although this remained an issue for cytopathologic interpretation, a similar number of cases were diagnosed as at least suspicious for a mucinous cyst irrespective of mutational status (*GNAS*, $P = 0.13$; *KRAS*, $P = 1.00$). Upon surgical resection, there was no correlation between *GNAS* mutations and location within the pancreatic duct ($P = 0.92$). However, the prevalence of *KRAS* mutations was higher in IPMNs involving the branch duct (66% vs. 33%; $P = 0.04$) and lower in IPMNs involving the main duct (17% vs. 46%; $P = 0.04$). On the basis of histologic subtype, *GNAS* mutations associated with the intestinal subtype ($P = 0.002$) and, in fact, were found in all (100%) intestinal IPMNs within the study cohort. Conversely, no relationship between histologic subtype and *KRAS* mutations were identified ($P = 0.20$). Of note, oncocytic IPMNs were absent within the study cohort. No statistically significant correlations were seen with grade of dysplasia or association with invasive adenocarcinoma for either gene (*GNAS* and *KRAS*; $P > 0.05$).

Comparison of *GNAS* and *KRAS* testing with other diagnostic modalities

In the preoperative setting, *GNAS* mutations had 100% specificity for IPMNs, but only attained sensitivity of 36% (Table 3). Mutations in *KRAS* had 98% specificity and 70% sensitivity. The combination of *GNAS* and *KRAS* mutations achieved a specificity and sensitivity of 98% and 84%, respectively. In comparison, the presence of multiple pancreatic cysts, a finding on endoscopic ultrasound often associated with IPMNs, had a specificity and sensitivity of 76% and 52%, respectively. In addition, increased fluid viscosity and elevated CEA had both a lower specificity (83% and 69%, respectively) and lower sensitivity (78% and 74%, respectively). A cytopathologic diagnosis of

Table 1. Clinical and pathologic comparison of *GNAS*/*KRAS*-mutant and *GNAS*/*KRAS* wild-type pancreatic cysts

Patient or cyst characteristics	Total, $n = 91$		*GNAS*/*KRAS*-mutant, $n = 43$		*GNAS*/*KRAS* wild-type, $n = 48$		*P*
Gender							
Male	36 (40%)		25 (56%)		11 (23%)		0.001
Female	55 (60%)		18 (44%)		37 (77%)		
Mean age (range), y	60.2 (20–87)		66.5 (44–87)		54.5 (20–85)		<0.001
Mean size (range), cm	3.0 (0.7–9.9)		2.6 (0.8–7.2)		3.4 (0.7–9.9)		0.020
Location							
Head, neck and uncinate	39 (43%)		22 (48%)		17 (35%)		0.144
Body and tail	52 (57%)		21 (53%)		31 (65%)		
Cyst focality							
Solitary	55 (60%)		22 (51%)		33 (69%)		0.132
Multiple	36 (40%)		21 (49%)		15 (31%)		
Fluid viscosity							
Thin and watery	45 (49%)		9 (21%)		36 (75%)		<0.001
Slight to marked viscosity	46 (51%)		34 (79%)		12 (25%)		
CEA	$n = 70$		$n = 28$		$n = 42$		
CEA < 192 ng/mL	34 (49%)		8 (29%)		26 (62%)		0.008
CEA ≥ 192 ng/mL	36 (51%)		20 (71%)		16 (38%)		
Cytologic specimen adequacy							
Satisfactory for evaluation	57 (63%)		25 (58%)		32 (67%)		0.515
Less than optimal or unsatisfactory	34 (37%)		18 (42%)		16 (33%)		
Surgical resections and clinical impression							
Adenocarcinoma arising in an IPMN	9		8		1		
Main duct IPMN	10	50 (55%)	5	42 (98%)	5	8 (17%)	<0.001
Branch duct IPMN	26		24		2		
Main and branch duct IPMN	5		5		0		
MCN	16		1		15		<0.001
Cystic PanNET	10		0		10		<0.001
SCA[a]	9		0		9		
Retention cyst	3		0		3		
Pseudocyst	2		0		2		
Lymphoepithelial cyst	1		0		1		

[a]The diagnosis of 8 out of 9 SCA is based on clinical impression and/or LOH at 3p25–26.

at least suspicious for a neoplastic mucinous cyst also had a lower specificity and sensitivity of 71% and 60%, respectively.

Overall, point mutations in *GNAS* and *KRAS* have a specificity of 100%, but a sensitivity of 65% for mucinous differentiation. Increased fluid viscosity, elevated CEA, and cytology had lower specificities of 96%, 90%, and 76%, respectively. However, increased fluid viscosity and elevated CEA had slightly higher sensitivities of 68% and 69%, whereas cytology was lower at 55%. Although there was insufficient cyst fluid present in 21 cases (23%), the combination of *GNAS* and *KRAS* testing with elevated CEA improved the sensitivity of both assays to 86% and maintained a high specificity of 90% for mucinous differentiation. The addition of fluid viscosity to DNA testing and elevated CEA increased the sensitivity (88%), but at a loss in specificity (86%). Using a multimodal approach, including DNA testing, CEA analysis, fluid viscosity, and cytopathology, further increased the sensitivity to 94%, but reduced the specificity to 67%.

Discussion

Massive parallel sequencing of IPMNs has identified recurrent mutations in *GNAS* at codon 201, resulting in either an R201H or an R201C substitution (9, 10). Among the major pancreatic cystic neoplasms, these activating mutations have been shown to be highly specific for IPMNs (8). Consistent with these studies, we found that *GNAS* mutations had a specificity of 100% for IPMNs, whereas mutations in *GNAS* were absent in MCNs and SCA. In addition, we report, for the first time, the lack of *GNAS* mutations in cystic PanNETs, retention cysts, pseudocyst, and lymphoepithelial cyst. Although infrequent, these entities can enter the differential diagnosis of an IPMN, either

Table 2. Clinical and pathologic characteristics of 50 IPMNs with respect to *GNAS* and *KRAS* status

		GNAS			*KRAS*		
Patient or cyst characteristics	**Total, $n = 50$**	**Wild-type**	**Mutant**	***P***	**Wild-type**	**Mutant**	***P***
Gender							
Male	28	13 (46%)	15 (54%)	0.007	19 (50%)	19 (50%)	0.765
Female	22	19 (86%)	3 (14%)		6 (27%)	16 (73%)	
Mean age (range), y	66.3 (30–87)	65.9 (30–87)	67.2 (44–84)	0.663	64.3 (30–85)	67.2 (44–87)	0.362
Mean size (range), cm	2.5 (0.8–7.2)	2.4 (1.0–6.5)	2.7 (0.8–7.2)	0.433	2.7 (0.8–7.2)	2.4 (1.0–3.8)	0.475
Location							
Head, neck and uncinate	28	17 (61%)	11 (39%)	0.768	10 (36%)	18 (64%)	0.367
Body and tail	22	15 (68%)	7 (32%)		5 (23%)	17 (77%)	
Cyst focality							
Solitary	24	16 (67%)	8 (33%)	0.774	6 (25%)	18 (75%)	0.545
Multiple	26	16 (62%)	10 (38%)		9 (35%)	17 (65%)	
Main duct dilatation							
Yes	14	9 (64%)	5 (36%)	1.000	4 (29%)	10 (71%)	0.114
No	36	23 (64%)	13 (36%)		21 (58%)	15 (42%)	
Presence of a mural nodule							
Yes	6	4 (67%)	2 (33%)	1.000	2 (33%)	4 (67%)	0.667
No	44	28 (64%)	16 (36%)		23 (52%)	21 (48%)	
Fluid viscosity							
Thin and watery	11	10 (91%)	1 (9%)	0.072	3 (27%)	8 (73%)	1.000
Viscous or thick	39	22 (56%)	17 (44%)		12 (31%)	27 (69%)	
CEA	$n = 34$						
CEA < 192 ng/mL	9	5 (56%)	4 (44%)	0.425	3 (33%)	6 (67%)	1.000
CEA > 192 ng/mL	25	18 (72%)	7 (28%)		8 (32%)	17 (68%)	
Cytologic specimen adequacy							
Less than optimal or unsatisfactory	22	14 (64%)	8 (36%)	1.000	6 (27%)	16 (73%)	0.765
Satisfactory for evaluation	28	18 (64%)	10 (36%)		9 (32%)	19 (68%)	
Cytology suspicious for a mucinous cyst							
No	20	10 (50%)	10 (50%)	0.134	6 (30%)	14 (70%)	1.000
Yes	30	22 (73%)	8 (27%)		9 (30%)	21 (70%)	
Duct involvement							
Main duct IPMN	13	9 (69%)	4 (31%)	0.921	7 (54%)	6 (46%)	0.044
Branch duct IPMN	28	17 (61%)	11 (39%)		5 (19%)	23 (82%)	
Main and branch duct IPMN	9	6 (67%)	3 (33%)		3 (33%)	6 (67%)	
Histologic subtype							
Gastric	40	27 (67%)	13 (33%)	0.002	10 (25%)	30 (75%)	0.203
Intestinal	5	0 (0%)	5 (100%)		3 (60%)	2 (40%)	
Pancreatobiliary	5	5 (100%)	0 (0%)		2 (40%)	3 (60%)	
Grade of dysplasia							
Low	22	14 (64%)	8 (36%)	0.856	5 (23%)	17 (77%)	0.646
Intermediate	20	12 (60%)	8 (40%)		7 (35%)	13 (65%)	
High	8	6 (75%)	2 (25%)		3 (37%)	5 (63%)	
Association with invasive carcinoma							
Yes	9	7 (78%)	2 (22%)	0.459	2 (22%)	7 (78%)	0.705
No	41	25 (61%)	16 (39%)		13 (32%)	28 (68%)	

clinically, radiographically, or cytologically. Thus, *GNAS* testing of pancreatic cyst fluid, obtained via EUS–FNA, represents a highly specific diagnostic modality in the identification of IPMNs.

However, within this study cohort, only 18 of 50 (36%) IPMNs harbored *GNAS* mutations. Wu and colleagues (9) identified *GNAS* mutations in 66% of IPMNs. Furthermore, in a follow-up study, Dal Molin and colleagues (20) found that 64% of IPMNs harbored *GNAS* mutations. A lack of sensitivity within our DNA detection technique may account for these discordant results. Indeed, Furukawa and colleagues (10) also performed whole-exome analysis of

Table 3. Comparison of *GNAS* and *KRAS* testing with other diagnostic modalities to identify mucinous differentiation

	IPMNs		IPMNs and MCNs	
	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)
GNAS mutations	100% (0.89–1.00)	36% (0.23–0.51)	100% (0.83–1.00)	27% (0.17–0.40)
KRAS mutations	98% (0.86–1.00)	70% (0.55–0.82)	100% (0.83–1.00)	55% (0.42–0.67)
GNAS and/or *KRAS* mutations	98% (0.86–1.00)	84% (0.70–0.92)	100% (0.83–1.00)	65% (0.52–0.76)
Presence of multiple cysts	76% (0.59–0.87)	52% (0.38–0.66)		
Increased fluid viscosity	83% (0.67–0.92)	78% (0.64–0.88)	96% (0.78–1.00)	68% (0.55–0.79)
Elevated CEA[a]	69% (0.52–0.83)	74% (0.55–0.86)	90% (0.68–0.98)	69% (0.54–0.81)
Cytology suspicious for mucinous neoplasm	71% (0.54–0.83)	60% (0.45–0.73)	76% (0.54–0.90)	55% (0.42–0.67)
GNAS, *KRAS* and increased fluid viscosity	85% (0.70–0.94)	96% (0.85–0.99)	96% (0.78–1.00)	82% (0.70–0.90)
GNAS, *KRAS* and elevated CEA[a]	69% (0.52–0.83)	97% (0.83–1.00)	90% (0.68–0.98)	86% (0.72–0.94)
GNAS, *KRAS*, elevated CEA[a], and increased fluid viscosity	67% (0.49–0.81)	100% (0.87–1.00)	86% (0.63–0.96)	88% (0.75–0.95)
GNAS, *KRAS*, elevated CEA[a], increased fluid viscosity and cytology	47% (0.31–0.64)	100% (0.87–1.00)	67% (0.43–0.85)	94% (0.82–0.98)

[a]On the basis of cases in which sufficient fluid was available for CEA testing.

surgically resected IPMNs, but used Sanger sequencing and found a similar frequency (41%) of *GNAS* mutations within their cohort. Yet, *KRAS* mutations in this study were detected at a comparable rate in IPMNs to those reported by Wu and colleagues (9). An alternative explanation is that 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. Despite evaluating a smaller sample size and lack of surgical follow-up data, Siddiqui and colleagues (21) also identified smaller proportion of *GNAS*-mutant IPMNs (44%) using pancreatic cyst fluid obtained by EUS–FNA. In addition, selection bias may also be a factor in the prevalence of *GNAS* mutations. Pancreatic cyst fluid DNA used for this study was obtained as part of routine clinical evaluation for *KRAS* mutations due to concern for a cystic neoplasm (7). In other words, pancreatic cyst fluid was submitted for DNA testing at the discretion of the endoscopist because of uncertainty as to whether a cystic lesion represented a mucinous cyst. Therefore, straightforward main and/or branch duct IPMNs by imaging were refrained from molecular analysis. Nevertheless, an argument can be made that in this scenario, molecular ancillary testing is not clinically indicated (or, only 36% of IPMNs within our patient population harbor *GNAS* mutations).

Despite the lower prevalence of *GNAS* mutations, the sensitivity and specificity for IPMNs by combining both *GNAS* and *KRAS* analyses was 84% and 98%, respectively. Other standard imaging, laboratory and pathologic techniques had both lower sensitivities and specificities. However, a note of caution should be taken when interpreting these results. As with any test, previous studies have shown that cyst classification based on DNA analysis alone can lead to false-positive results (22). Therefore, the assessment of *GNAS* and *KRAS* mutations should be made as part of a multidisciplinary approach including clinical, radiographic, and cytopathologic findings.

For mucinous cysts (both IPMNs and MCNs), point mutations in *GNAS* and *KRAS* had a sensitivity of 65% and a specificity of 100%. The lower sensitivity of molecular analysis for mucinous cysts as compared with IPMNs alone is largely due to the absence of both *GNAS* and *KRAS* mutations in MCNs. Within this study cohort, only 1 of 16 (6%) MCNs harbored a *KRAS* mutation. The relative lack of *KRAS* mutations identified in MCNs is, once again, contrary to those reported by Wu and colleagues (9) and can be explained by the same aforementioned reasoning for the lower rate of *GNAS* mutations in IPMNs. But more notably, these findings highlight the importance of identifying additional markers to improve the sensitivity of cyst fluid DNA analysis.

Recent whole-exome sequencing of cyst epithelium from the four major neoplastic pancreatic cysts has identified a limited number of genetic mutations that may be used diagnostically to classify each cyst type (8–10). IPMNs were characterized by mutations in *GNAS*, *KRAS*, and the E3 ubiquitin ligase, *RNF43*. *RNF43* mutations were also identified in 40% of MCNs. The combination of *GNAS*, *KRAS*,

and *RNF43* testing would improve the sensitivity, while maintaining a high specificity for mucinous differentiation than *GNAS* and *KRAS* alone. In contrast, SCA had mutations in *VHL* or LOH in or adjacent to *VHL*, and did not contain mutations in *GNAS*, *KRAS*, or *RNF43*. Although not discussed in detail, six of eight (75%) SCA used within this study also demonstrated *VHL* LOH. Finally, solid pseudopapillary neoplasms were characterized by mutations in *CTNNB1* and lacked *GNAS*, *KRAS*, *RNF43*, and *VHL* mutations. A five-gene panel that includes *GNAS*, *KRAS*, *RNF43*, *VHL*, and *CTNNB1* could lead to a highly accurate diagnosis.

Although *GNAS* and *KRAS* testing can be useful in distinguishing mucinous from nonmucinous cysts, a critical role for cyst fluid DNA analysis has been to identify the malignant potential of a pancreatic cyst. Neither *GNAS* nor *KRAS* mutations correlated with grade of dysplasia or the presence of invasive adenocarcinoma within a mucinous cyst. Similar studies have not been performed with *RNF43*. However, Kanda and colleagues (23) reported the presence of mutations within the tumor-suppressor gene, *TP53*, in secretin-stimulated pancreatic juice samples collected from duodena of patients with high-grade lesions (pancreatic intraepithelial neoplasia grade 3 and high-grade IPMNs) and pancreatic cancer. *TP53* mutations were identified in 50% of patients with high-grade lesions and 67% of patients with invasive pancreatic adenocarcinoma. No mutations in *TP53* were detected in patients with low-grade IPMNs. In addition, the authors did not identify *TP53* mutations in subjects with chronic pancreatitis, but prior studies using endoscopic retrograde cholangiopancreatography-collected juice have found *TP53* mutations in a small percentage of individuals (24–26). Although *TP53* mutations may prove to be a useful marker in determining the presence of high-grade lesions and invasive adenocarcinoma, additional studies are required to assess its accuracy in patients undergoing pancreatic cyst evaluation.

In summary, we report the largest series of *GNAS* and *KRAS* analysis from pancreatic cyst fluid obtained by EUS–FNA. Although the combination of *GNAS* and *KRAS* testing was highly specific and sensitive for IPMNs, these results should not be interpreted in isolation. We recommend integrating cyst fluid DNA analysis in conjunction with clinical, radiographic, and cytopathologic data. Regardless, the lack of sensitivity for MCNs underscores the need for additional markers to improve the detection of pancreatic mucinous neoplasms and toward appropriate patient management.

Disclosure of Potential Conflicts of Interest

R.E. Brand is a consultant/advisory board member for Asuragen, Inc. No potential conflicts of interest were disclosed by the other authors.

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References

1. Laffan TA, Horton KM, Klein AP, Berlanstein B, Siegelman SS, Kawamoto S, et al. Prevalence of unsuspected pancreatic cysts on MDCT. AJR Am J Roentgenol 2008;191:802–7.
2. Hruban RH, Maitra A, Kern SE, Goggins M. Precursors to pancreatic cancer. Gastroenterol Clin North Am 2007;36:831–49.
3. Matthaei H, Schulick RD, Hruban RH, Maitra A. Cystic precursors to invasive pancreatic cancer. Nat Rev Gastroenterol Hepatol 2011;8:141–50.
4. Khalid A, Brugge W. ACG practice guidelines for the diagnosis and management of neoplastic pancreatic cysts. Am J Gastroenterol 2007;102:2339–49.
5. Tanaka M, Chari S, Adsay V, Fernandez-del Castillo C, Falconi M, Shimizu M, et al. International consensus guidelines for management of intraductal papillary mucinous neoplasms and mucinous cystic neoplasms of the pancreas. Pancreatology 2006;6:17–32.
6. Tanaka M, Fernandez-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, et al. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. Pancreatology 2012;12:183–97.
7. Nikiforova MN, Khalid A, Fasanella KE, McGrath KM, Brand RE, Chennat JS, et al. Integration of KRAS testing in the diagnosis of pancreatic cystic lesions: a clinical experience of 618 pancreatic cysts. Mod Pathol 2013;26:1478–87.
8. Wu J, Jiao Y, Dal Molin M, Maitra A, de Wilde RF, Wood LD, et al. Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. Proc Natl Acad Sci U S A 2011;108:21188–93.
9. Wu J, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, et al. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. Sci Transl Med 2011;3:92ra66.
10. Furukawa T, Kuboki Y, Tanji E, Yoshida S, Hatori T, Yamamoto M, et al. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. Sci Rep 2011;1:161.

11. Hruban RH, Pitman MB, Klimstra DS. Tumors of the pancreas. Washington, DC: American Registry of Pathology and Armed Forces Institute of Pathology; 2007.
12. Klimstra DS, Pitman MB, Hruban RH. An algorithmic approach to the diagnosis of pancreatic neoplasms. Arch Pathol Lab Med 2009;133: 454–64.
13. Huang P, Staerkel G, Sneige N, Gong Y. Fine-needle aspiration of pancreatic serous cystadenoma: cytologic features and diagnostic pitfalls. Cancer 2006;108:239–49.
14. Pitman MB, Deshpande V. Endoscopic ultrasound-guided fine needle aspiration cytology of the pancreas: a morphological and multimodal approach to the diagnosis of solid and cystic mass lesions. Cytopathology 2007;18:331–47.
15. Pitman MB, Lewandrowski K, Shen J, Sahani D, Brugge W, Fernandez-del Castillo C. Pancreatic cysts: preoperative diagnosis and clinical management. Cancer Cytopathol 2010;118:1–13.
16. Zhai J, Sarkar R, Ylagan L. Pancreatic mucinous lesions: a retrospective analysis with cytohistological correlation. Diagn Cytopathol 2006;34:724–30.
17. Khalid A, Pal R, Sasatomi E, Swalsky P, Slivka A, Whitcomb D, et al. Use of microsatellite marker loss of heterozygosity in accurate diagnosis of pancreaticobiliary malignancy from brush cytology samples. Gut 2004;53:1860–5.
18. Khalid A, Nodit L, Zahid M, Bauer K, Brody D, Finkelstein SD, et al. Endoscopic ultrasound fine needle aspirate DNA analysis to differentiate malignant and benign pancreatic masses. Am J Gastroenterol 2006;101:2493–500.
19. Nodit L, McGrath KM, Zahid M, Jani N, Schoedel KE, Ohori NP, et al. Endoscopic ultrasound-guided fine needle aspirate microsatellite loss analysis and pancreatic endocrine tumor outcome. Clin Gastroenterol Hepatol 2006;4:1474–8.
20. Dal Molin M, Matthaei H, Wu J, Blackford A, Debeljak M, Rezaee N, et al. Clinicopathological correlates of activating GNAS mutations in intraductal papillary mucinous neoplasm (IPMN) of the pancreas. Ann Surg Oncol 2013;20:3802–8.
21. Siddiqui AA, Kowalski TE, Kedika R, Roy A, Loren DE, Ellsworth E, et al. EUS-guided pancreatic fluid aspiration for DNA analysis of KRAS and GNAS mutations for the evaluation of pancreatic cystic neoplasia: a pilot study. Gastrointest Endosc 2013;77:669–70.
22. Panarelli NC, Sela R, Schreiner AM, Crapanzano JP, Klimstra DS, Schnoll-Sussman F, et al. Commercial molecular panels are of limited utility in the classification of pancreatic cystic lesions. Am J Surg Pathol 2012;36:1434–43.
23. Kanda M, Matthaei H, Wu J, Hong SM, Yu J, Borges M, et al. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. Gastroenterology 2012;142:730–3.
24. Lohr M, Muller P, Mora J, Brinkmann B, Ostwald C, Farre A, et al. p53 and K-ras mutations in pancreatic juice samples from patients with chronic pancreatitis. Gastrointest Endosc 2001;53: 734–43.
25. Yamaguchi Y, Watanabe H, Yrdiran S, Ohtsubo K, Motoo Y, Okai T, et al. Detection of mutations of p53 tumor suppressor gene in pancreatic juice and its application to diagnosis of patients with pancreatic cancer: comparison with K-ras mutation. Clin Cancer Res 1999;5: 1147–53.
26. Yan L, McFaul C, Howes N, Leslie J, Lancaster G, Wong T, et al. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. Gastroenterology 2005;128:2124–30.