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Mutant *KRAS* and *GNAS* DNA Concentrations in Secretin-Stimulated Pancreatic Fluid Collected from the Pancreatic Duct and the Duodenal Lumen

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OBJECTIVES: The analysis of secretin-stimulated pancreatic fluid is being evaluated as an approach to improve the early detection of pancreatic cancer and pancreatic precursor neoplasms. The method of pancreatic fluid sampling may have a significant impact on tumor marker measurements. The aim of this study was to compare concentrations of mutant DNA in pancreatic fluid from patients who had samples collected from both the pancreatic duct and duodenal lumen.

METHODS: Thirty-six participants enrolled in the Cancer of the Pancreas Screening studies at Johns Hopkins Hospital who had secretin-stimulated pancreatic fluid collected from the duodenum during endoscopic ultrasound (EUS) and from the pancreatic duct during subsequent endoscopic retrograde cholangiopancreatography. Mutant *KRAS* and *GNAS* DNA concentrations were measured in pancreatic fluid samples using digital high-resolution melt-curve analysis and pyrosequencing and were related total DNA concentrations in these samples.

RESULTS: Thirty-four patients had subtle parenchymal abnormalities by EUS; seven had small pancreatic cysts; none had pancreatic cancer. *KRAS* mutations were detected in 29 of 36 (80.6%) pancreatic duct fluid samples. Of these 29 patients, 23 had mutations detected in their duodenal fluid (79.3%). Patients with detectable pancreatic fluid but not duodenal fluid *KRAS* mutations had lower average pancreatic duct fluid *KRAS* mutation concentrations ($P = 0.01$). Patients with *KRAS* or *GNAS* mutations detected in pancreatic fluid but not duodenal fluid had higher total DNA concentrations in their duodenal compared with pancreatic fluid ($P = 0.03$). *KRAS* and *GNAS* mutation concentrations were higher in pancreatic duct fluid samples than in matching duodenal fluid samples (for *KRAS*: 2.62 vs. 0.39%, $P < 0.0001$).

CONCLUSIONS: *KRAS* and *GNAS* mutation concentrations are significantly lower in secretin-stimulated pancreatic fluid samples collected from the duodenum compared with samples collected from the pancreatic duct. Efforts to improve the purity of pancreatic fluid collections from the duodenum could improve the detection of mutations arising from the pancreas.

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INTRODUCTION

Pancreatic cancer is characterized by a late clinical presentation and high mortality and its incidence in the United States is increasing.[1] Only a minority of patients who present with symptoms of pancreatic cancer have resectable disease, and of these, <10% present with stage 1 disease.[2] In an effort to improve the early detection of pancreatic cancer, pancreatic screening has been offered to asymptomatic individuals whose family history or germline gene mutations represent a sufficiently increased risk of developing pancreatic cancer.[3–11] The diagnostic yield of pancreatic screening tests depends on many factors including the age and family history of patients who undergo screening and the accuracy of the tests used for screening.[12] Endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography have been the primary tools used for pancreatic screening[12] and are often used in the surveillance of incidentally identified pancreatic cysts,[13,14] because they can accurately detect pancreatic cysts without the radiation exposure of repeated computerized tomography scanning. Small pancreatic cysts and subtle parenchymal abnormalities are commonly identified by EUS in patients undergoing studies of pancreatic cancer screening,[3–5] but imaging tests are unable to detect microscopic pancreatic intraepithelial neoplasia (PanIN). Patients with a familial susceptibility to pancreatic cancer often harbor widespread PanIN, including PanIN-3.[15] PanIN lesions can harbor the driver mutations of pancreatic ductal adenocarcinoma. For example, over 90% of PanIN-1 lesions harbor *KRAS* mutations and higher grade PanINs can harbor other mutations in other driver genes mutated in pancreatic ductal adenocarcinomas, including *CDKN2A, TP53,* and *SMAD4.*[16,17] Most of the pancreatic cystic lesions identified in patients undergoing pancreatic screening are thought to be intraductal papillary mucinous neoplasms (IPMNs).[4,18] Most IPMNs harbor *GNAS* and *KRAS* mutations[19] and some harbor mutations in other genes, particularly *TP53* and *RNF43.*[18] In an attempt to develop better tests that indicate the presence of pancreatic neoplasia, particularly PanIN and small pancreatic cancers,

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secretin-stimulated pancreatic fluid samples have been collected from subjects enrolled in the Cancer of the Pancreas Screening (CAPS) trials and analyzed to identify accurate markers of pancreatic neoplasia.[3–5] Secretin-stimulated pancreatic fluid analysis serves as a standard pancreatic function test to evaluate patients for the presence of pancreatic insufficiency.[20] Studies using these samples revealed that *TP53* and *GNAS* mutations detected in pancreatic fluid samples collected from the duodenum are highly correlated with the presence of high-grade dysplasia/pancreatic cancer and pancreatic cysts/IPMNs in the corresponding pancreas, respectively.[18,21] Pancreatic fluid is normally collected from the duodenal lumen unless patients are undergoing endoscopic retrograde cholangiopancreatography (ERCP), but pancreatic fluid samples from the pancreatic duct are purer, whereas pancreatic fluid collected in the duodenal lumen is diluted by duodenal contents, potentially obscuring the presence of markers of pancreatic neoplasia. The magnitude of this dilution is not clear.

The purpose of this study was to compare *KRAS* and *GNAS* mutation concentrations in secretin-stimulated pancreatic fluid samples collected from the pancreatic duct and from the duodenal lumen in patients who underwent pancreatic EUS followed by ERCP to investigate abnormalities identified by EUS.

METHODS

Ethical approval. All elements of this study were approved by the Johns Hopkins institutional review board and written informed consent was provided from all patients.

Patients and specimens. This single-center study is part of the ongoing Cancer of the Pancreas Screening (CAPS) studies to evaluate the utility of pancreatic screening and to evaluate markers of pancreatic neoplasia. Specimens and clinical information were obtained from participants enrolled in the CAPS2, CAPS3, and CAPS4 studies (clinicaltrials.gov NCT00438906, NCT00714701).[3,4] For this study, we identified all subjects enrolled in the CAPS studies at Johns Hopkins Hospital (JHH, Baltimore, Maryland) who had undergone both EUS and ERCP (within a few days or weeks of each other) and had sufficient sample available for analysis. Thirty-six subjects were included (31 from CAPS2, 1 from CAPS3, and 4 from CAPS4). All but two CAPS study subjects were asymptomatic subjects undergoing pancreatic screening for their family history of pancreatic cancer, or inherited predisposition to pancreatic cancer. The remaining two patients were enrolled as disease control subjects. A summary of their demographic and diagnostic characteristics is provided in Table 1. Patients enrolled in the CAPS2 study (2002–2004)[4] underwent ERCP to evaluate pancreatic parenchymal abnormalities or pancreatic cysts identified by EUS. At that time, ERCP was used as part of the study protocol to evaluate pancreatic lesions in the familial pancreatic cancer relatives, including parenchymal changes that were thought to be the result of pancreatic neoplasia.[10,22] Patients in the CAPS3 and CAPS4 studies only underwent ERCP for lesions that remained poorly defined after EUS and magnetic resonance cholangiopancreatography (such as suspected pancreatic duct strictures and main duct dilation).[3–5]

Table 1 Patient characteristics and diagnosis

Patient	Sex	Age	Diagnosis
1	Female	42	Familial, parenchymal changes[a]
2	Male	53	Familial, parenchymal changes[a]
3	Male	48	Familial, parenchymal changes[a]
4	Male	32	Familial, parenchymal changes[a]
5	Female	58	Familial, parenchymal changes[a]
6	Female	47	PJS, parenchymal changes[a]
7	Male	54	Chronic pancreatitis
8	Male	52	Familial, parenchymal changes[a]
9	Female	58	Familial, parenchymal changes[a]
10	Male	42	Familial, parenchymal changes[a]
11	Female	65	Familial, parenchymal changes[a], small cyst
12	Male	39	Familial, parenchymal changes[a] (resection; PanIN 2)
13	Female	46	Familial, parenchymal changes[a]
14	Male	53	Familial, parenchymal changes[a]
15	Male	62	Familial, parenchymal changes[a]
16	Male	59	Familial, parenchymal changes[a], small cyst
17	Female	48	Familial, parenchymal changes[a]
18	Female	62	Small cyst
19	Male	53	Familial, parenchymal changes[a]
20	Female	57	Familial, parenchymal changes[a] (small cyst in the future)
21	Female	53	Familial, small cyst
22	Female	58	Familial, parenchymal changes[a], small cyst
23	Male	42	Familial, parenchymal changes[a]
24	Male	58	Familial, parenchymal changes[a]
25	Female	77	Familial, parenchymal changes[a], 18mm cyst (resection: PanIN3 & IPMN)
26	Male	47	Familial, parenchymal changes[a] (resection; endocrine tumor, PanIN2)
27	Female	56	Familial, parenchymal changes[a]
28	Female	50	Familial, parenchymal changes[a]
29	Female	63	Familial, BRCA2 mutation
30	Female	47	Familial, parenchymal changes[a]
31	Female	53	PJS, parenchymal changes[a]
32	Male	56	Familial, BRCA2 mutation, parenchymal changes[a](1 year later, IPMN resected)
33	Female	75	Familial, parenchymal changes[a]
34	Male	62	Familial, 3 small cysts (8 months later, resection; PanIN2 & IPMN adenoma)
35	Female	50	Familial, parenchymal changes[a]
36	Female	72	Familial, parenchymal changes[a] (resection; IPMN adenoma, PanIN3)

IPMN, intraductal papillary mucinous neoplasm; PanIN, pancreatic intraepithelial neoplasia; PJS, Peutz-Jeghers syndrome.
[a]Endoscopic ultrasound changes similar to those found in patients with chronic pancreatitis.

Pancreatic fluid samples were collected from all participants after infusion of intravenous human synthetic secretin (0.2 μg/kg infused over 1 min). Pancreatic fluid samples (~5–15 ml) were collected from the duodenal lumen ("duodenal fluid") as it was secreted from the papilla by suctioning the fluid repeatedly through the endoscopic channel over ~5–10 min with the linear array echoendoscope with the tip positioned near the Ampulla of Vater. ERCP and pancreatic fluid collection were performed separately after EUS and duodenal fluid collections and usually at a later date. The interval between the EUS and ERCP is provided in Table 2. Pancreatic duct fluid (~2–5 ml) was collected through the ERCP catheter over ~5–10 min. This relatively short duration of pancreatic fluid collection is standard for all pancreatic fluid collections for pancreatic screening as the goal of these collections was to

Table 2 KRAS and GNAS mutation analysis for pancreatic duct fluid and duodenal fluid

					KRAS				GNAS			
					Pancreatic duct fluid		Duodenal fluid		Pancreatic duct fluid		Duodenal fluid	
Patient	CAPS	Interval	Color of duodenal fluid	Ratio of duodenal fluid to pancreatic duct fluid DNA concentration	Mutation (%)	Mutation type (estimated %)	Mutation (%)	Mutation type (estimated %)	Mutation (%)	Mutation type (estimated %)	Mutation (%)	Mutation type (estimated %)
1	2	3 days	Serous-pink	25.4	0		0		0			
2	2	3 days	Green	1.4	0		0		0			
3	2	3 days	Serous-pink	1.0	0		0		0			
4	2	3 days	Green	0.4	0		0		0.22	R201C (50%), R201H (50%)	0	
5	2	3 days	Serous	0.3	0		0		0			
6	2	3 days	Serous-pink	0.8	0		0		0			
7	3	Same day	Green	1.1	0		0		0			
8	2	3 days	Serous-pink	7.0	0.22	G12R (100%)	0		0			
9	2	3 days	Serous	0.2	0.22	**G12D (100%)**	0.11	**G12D (100%)**	0			
10	2	3 days	Serous	5.7	0.22	G12D (50%), G12R (50%)	0		0.11	R201H (100%)	0	
11	4	Same day	Serous	0.5	0.22	G13D (100%)	0		0			
12	2	3 days	Pink	8.2	0.33	G12D (66%), G12V (33%)	0		0			
13	2	3 days	Green	2.0	0.33	G12S (33%), **G12R (66%)**	0.11	**G12R (100%)**	0			
14	2	3 days	Serous-pink	3.9	0.33	G12D (66%), **G12V (33%)**	0.11	**G12V (100%)**	0			
15	2	3 days	Serous-pink	21.7	0.56	G12D (40%), G12V (40%),**G12C (20%)**	0.17	**G12C (100%)**	0			
16	2	3 days	Green	1.1	0.67	**G12D (83%)**, G12R (17%)	0.11	**G12D (100%)**	0.33	R201C (100%)	0	
17	2	3 days	Serous-pink	2.1	0.89	G12D (100%)	0		0.33	R201H (100%)	0	
18	4	Same day	Green	2.0	0.89	G12V (56%), G12A (22%), **G12R (22%)**	0.11	**G12R (100%)**	0			
19	2	3 days	Serous-pink	7.8	1.00	G12D (36%), G12V (27%), G12R (36%)	0		0			
20	2	2 months	Serous	1.4	1.22	**G12D (100%)**	0.11	**G12D (100%)**	0.11	**R201H (100%)**	0.11	**R201H (100%)**
21	2	3 days	Green	0.8	1.44	G12D (81%), **G12R (18%)**	0.22	**G12R (100%)**	0.78	**R201C (100%)**	0.06	**R201C (100%)**
22	4	Same day	Serous	10.3	1.67	**G12D (10%)**, G12V (80%), **G12R (10%)**	0.22	**G12D (50%)**, **G12R (50%)**	0.11	R201C (100%)	0	
23	2	3 days	Serous-pink	2.4	1.89	**G12D (40%)**, G12R (60%)	0.11	**G12D (100%)**	0.11	R201H (100%)	0	
24	2	3 days	Serous	2.1	1.89	G12D (10%), G12V (50%), **G12R (40%)**	0.11	**G12R (100%)**	0			
25	2	3 days	Pink	1.0	2.00	G12D (50%), G12V (10%), **G12R (40%)**	0.11	**G12R (100%)**	0.33	R201H (100%)	0	
26	2	3 days	Serous-pink	0.3	2.44	G12V (30%), **G12R (70%)**	0.67	**G12R (100%)**	0			
27	2	3 days	Serous	1.2	2.56	G12D (10%), **G12V (70%)**, G12R (10%), G12A (10%)	0.22	**G12V (100%)**	0			
28	2	3 days	Serous-pink	3.2	2.56	G12D (20%), **G12V (80%)**	0.06	**G12V (100%)**	0			
29	2	3 days	Serous-pink	1.4	4.11	**G12D (43%)**, G12V (14%), G12R (43%)	0.22	**G12D (100%)**	0			
30	2	3 days	Green	1.7	4.78	G12D (38%), G12V (42%), **G12R (20%)**	0.11	**G12R (100%)**	0.11	R201C (100%)	0	
31	2	3 months	Green	13.5	5.56	**G12D (29%),** G12V (29%), **G12R (41%)**	0.22	**G12D (50%)**, **G12R (50%)**	0			
32	2	3 days	Green	5.8	6.67	**G12D (47%)**, G12V (42%), G12R (11%)	0.11	**G12D (100%)**	3.67	**R201C (67%)**, R201H (33%)	0.22	**R201C (100%)**
33	2	3 days	Serous	6.1	7.33	G12D (8%), G12V (8%), **G12R (83%)**	0.11	**G12R (100%)**	0.11	R201H (100%)	0	
34	4	Same day	Serous	0.1	7.44	**G12D (24%)**, **G12V (18%)**, **G12R (58%)**	7.11	**G12D (30%)**, **G12V (12%)**, **G12R (58%)**	0.11	**R201C (100%)**	0.22	**R201C (100%)**
35	2	4 months	Serous	0.8	8.00	**G12D (47%)**, G12V (53%)	0.11	**G12D (100%)**	0			
36	2	3 days	Unknown	6.9	8.56	**G12D (90%)**, G12V (9%)	0.22	**G12D (50%),** G12R (50%)	0			

Bold and underlined mutation types matched pancreatic duct fluid with duodenal fluid.

obtain a lavage of the pancreatic ductal system. The normal positioning of the patient during EUS (left lateral) and ERCP (prone) was not changed to facilitate the collection of pancreatic fluid. Secretin was provided for CAPS3 and CAPS4 by ChiRhoClin (Burtonsville, MD).[3,18,21] Collected fluid ("juice") samples were stored at −80 °C before use. Further details of the CAPS studies and study subjects are provided elsewhere.[3–5]

Digital-high-resolution melt (HRM)-curve analysis and pyrosequencing. Digital-HRM analysis and pyrosequencing were used to evaluate *KRAS* and *GNAS* mutation concentrations in pancreatic fluid samples as described previously.[18] All samples were analyzed for *KRAS* mutations in the same manner and blinded to sample origin. *GNAS* mutation analysis was first determined in pancreatic duct fluid samples and then in the duodenal fluid in samples from patients with *GNAS* detected in their pancreatic duct sample. For digital-HRM analysis, 900 genome equivalents of DNA were dispensed into 90 wells of each 96-well plate of pancreatic fluid DNA analyzed (10 genome equivalents per well), five wells had wild-type DNA and one well had water.[17,18] The primers used for digital-HRM included for *KRAS* (forward 5′-AGGCCTGCTGAAAATGACTG-3′, reverse 5′TTGTTGGA TCATATTCGTCCAC-3′) and for *GNAS* (forward 5′CGGTT GGCTTTGGTGAGATC-3′ and reverse 5′-CAGTTGGCTTA CTGGAAGTTG-3′). Using these assay conditions, the concentration of mutations in a juice sample is represented by the number of wells having a mutation. If the first 96-well plate failed to detect mutations, additional 96-well plates of pancreatic fluid DNA were analyzed for mutations using digital-HRM pyrosequencing analysis. When *KRAS* and *GNAS* mutations were not detected in duodenal fluids but detected in pancreatic duct fluid, deeper sampling was performed by performing digital-HRM. There were no samples excluded for technical reasons such as poor DNA quality. To confirm the digital-HRM results, pyrosequencing was performed on PCR products from representative HRM-positive (up to 10 or more positive wells) and one HRM-negative well and one wild-type well for each plate.[16] The primers used for pyrosequencing were for KRAS (5′-GTGGTAGTTGGAG CT-3′) and for GNAS (5′- AGGACCTGCTTCGCTG-3′).

Figure 1 contains representative pancreatic fluid and duodenal fluid digital-HRM and pyrosequencing results from the same individual. The mutation score was determined by the number of HRM-positive wells with mutations confirmed by pyrosequencing. The mutation concentration (the percentage of wells with mutations) was calculated for each juice sample. A mutation percentage was determined for each type of mutations.

Statistical analysis. Mutation concentrations in pancreatic duct fluid and duodenal fluid samples were compared by Wilcoxon matched-pairs signed rank test. Spearman's rank correlation coefficient was used to evaluate the correlation of the mutation score of paired pancreatic ductal and duodenal fluid samples. Statistical analysis was performed using JMP Pro 11.1.1 software (SAS, Cary, NC). $P<0.05$ was considered statistically significant.

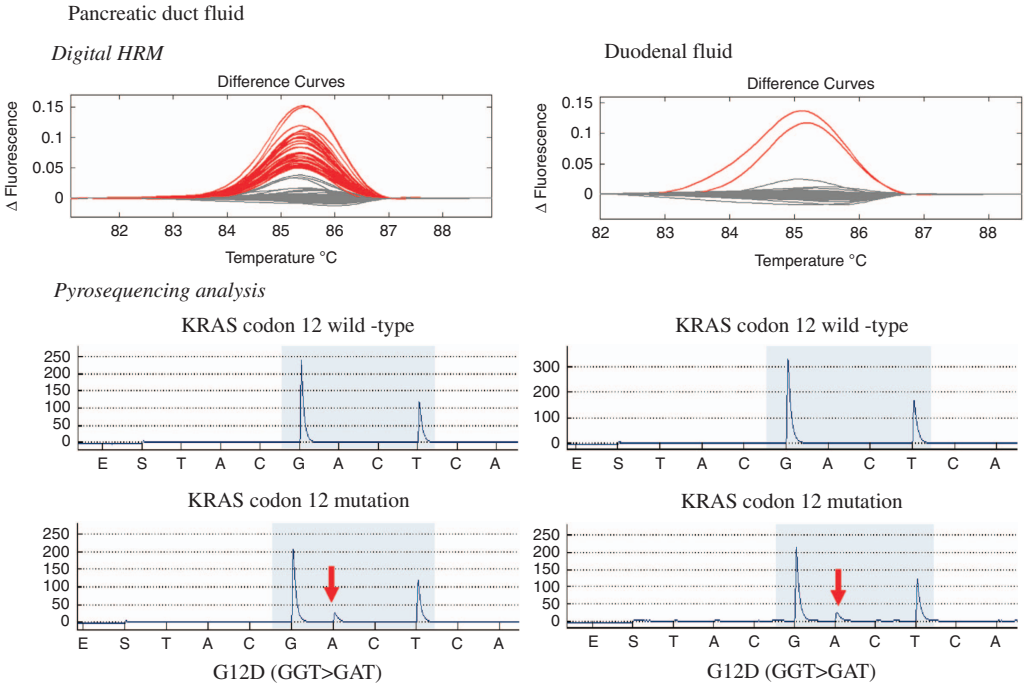

Figure 1 Representative examples of melt-curve analysis and pyrosequencing of pancreatic duct fluid (left) and duodenal fluid (right). The curves in **a** represent the melt curves generated from one 96-well plate of PCR products amplified from pancreatic duct juice DNA. **b** is the same, except that they are generated from duodenal fluid DNA. The gray curves are wild-type; these same curves are generated from melting PCR products amplified from wild-type DNA. The red curves are scored as mutant because similar curves are generated when melting PCR products amplified from DNA samples with KRAS codon 12 mutations. There are many more red curves in **a** than **b** because there were more KRAS-mutant DNA molecules in the duct juice sample compared with the duodenal fluid sample. **c** and **d** are representative pyrosequencing results from PCR wells with normal melt curves (i.e., wild-type for KRAS). **e** and **f** are pyrosequencing results from PCR wells that had mutant melt curves.

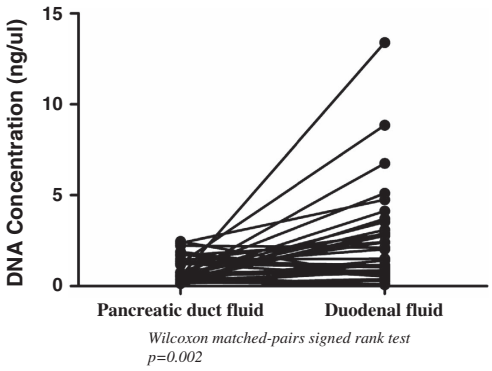

Figure 2 Matching total DNA concentrations in pancreatic duct fluid and duodenal fluid (mean; 0.92 ± 0.65 ng/μl and 2.39 ± 2.70 ng/μl, respectively) ($P = 0.002$). HRM, high-resolution melt-curve analysis.

RESULTS

DNA concentrations of pancreatic duct fluid samples and duodenal fluid samples. Mean total DNA concentrations were significantly higher in duodenal fluid samples (2.39 ± 2.70 ng/μl) than in pancreatic duct fluid samples (0.92 ± 0.65 ng/μl) from the same subjects (Figure 2, $P = 0.002$). Some duodenal fluid samples were bile colored but there was no correlation between DNA concentrations and the color of duodenal fluid samples.

KRAS mutations concentrations in pancreatic duct fluid and duodenal fluid samples. Thirty-six individuals had paired samples of secretin-stimulated pancreatic fluid collected from the duodenum during EUS and from the pancreatic duct subsequent ERCP. The indication for EUS was pancreatic screening for 34 participants, 30 for their family history of pancreatic cancer alone, 2 for Peutz-Jeghers syndrome, and 2 with germline *BRCA2* mutations. For one patient, the indication for EUS and ERCP was suspected chronic pancreatitis and for another, it was to evaluate a pancreatic cyst in a patient without a family history of pancreatic cancer. EUS detected parenchymal abnormalities similar to those found in subjects with chronic pancreatitis in 31 of the 34 screened patients (91.2%, Table 2). Seven patients had pancreatic cysts detected by EUS (average cyst size; 7.6 ± 2.8 mm).

When analyzing 900 g.e. (i.e., one 96-well plate), *KRAS* mutations (almost all codon 12 mutations) were detected in 29 of 36 pancreatic duct fluid samples (80.6%) and in 17 of the 29 corresponding duodenal fluid samples (58.6%). To increase the rate of detection of *KRAS* mutations in the 12 duodenal fluid samples in which mutations were not detected with this initial analysis, we performed additional digital-HRM and pyrosequencing of another aliquot of DNA from these same duodenal fluid samples. With this additional analysis, *KRAS* mutations were detected in six additional duodenal fluid samples so that overall *KRAS* mutations were detected in 23 of the 29 duodenal fluid samples that had *KRAS* mutations in their pancreatic duct fluid sample (79.3%). *KRAS* mutations were not detected in any duodenal fluid samples from patients that did not have *KRAS* mutations detected in their pancreatic duct fluid sample. Most pancreatic fluid samples with mutations had multiple different *KRAS* mutations (24 of 29) (Table 2). Most of the patients with *KRAS* mutations detected in their pancreatic fluid did not have pancreatic cysts, and although some had EUS abnormalities that met criteria for parenchymal changes like those of chronic pancreatitis, others had no such changes. The *KRAS* mutation spectrum observed in pancreatic fluid samples was the same as has been observed previously in pancreatic ductal adenocarcinomas, PanINs, and IPMNs with the most common *KRAS* gene mutations being G12D (43%), G12V (28%), and G12R (27%) (Table 2).[23–25]

The mean *KRAS* mutation concentrations were significantly higher in pancreatic duct fluid than in duodenal fluid samples (2.62 vs. 0.39%, $P < 0.0001$). Despite the low concentration of *KRAS* mutations in duodenal fluid, there was a significant correlation between the mutation concentrations in pancreatic duct fluid and duodenal fluid from the same patient ($P = 0.617$, $P = 0.0004$). Perhaps not surprisingly, *KRAS* mutations were more likely to be detected in duodenal fluid in patients with higher pancreatic duct fluid *KRAS* mutation concentrations. *KRAS* mutations were detected in 17 of 18 (94.4%) duodenal fluid samples when the patient's corresponding pancreatic duct fluid *KRAS* mutation concentration was over 1.0% of total DNA, but in only 6 of 11 duodenal fluid samples (54.6%) when the pancreatic duct fluid *KRAS* concentration was $< 1.0\%$ ($P = 0.01$).

***GNAS* mutations analysis in pancreatic fluid samples and duodenal fluid samples.** *GNAS* mutations were detected in 12 of the 36 pancreatic duct samples by sampling 900 g.e of pancreatic duct fluid DNA. Initial analysis of the same amount of duodenal fluid DNA identified *GNAS* mutations in three samples. Deeper digital-HRM analysis identified *GNAS* mutations in one additional sample. *GNAS* mutation concentrations in pancreatic duct samples were lower than *KRAS* mutation concentrations ($P = 0.001$), which might explain the lower rate of detection of *GNAS* mutations in duodenal fluid. Indeed, only one patient had pancreatic duct fluid *GNAS* mutation concentrations above 1% (Table 2). Pancreatic cysts were identified at the time of their EUS or during a subsequent EUS in 7 of the 13 patients who had *GNAS* mutations in the pancreatic duct samples including the 4 patients who had *GNAS* mutations detected in their duodenal fluid. All six patients with pancreatic duct *GNAS* mutations but no detectable pancreatic cyst had low concentrations of mutant *GNAS* in their pancreatic fluid sample ($< 1\%$ of total DNA) and did not have *GNAS* mutations detected in their duodenal fluid sample. All of the patients who had *GNAS* mutations detected in their duodenal fluid also had *KRAS* mutations detected in their duodenal fluid. The average *GNAS* mutation concentrations in pancreatic duct samples was 10-fold higher than that of duodenal fluid samples (0.17 vs. 0.017%) ($P = 0.01$).

The role of total DNA concentrations in pancreatic and duodenal fluid in mutation detection. We compared concentrations of total DNA in duodenal fluid relative to pancreatic duct fluid (DNA concentration ratio: DNA concentration of duodenal fluid (ng/μl)/DNA concentration of pancreatic fluid (ng/μl)). Eleven of the 13 patients who had *KRAS or GNAS* mutations (or both) detected in their pancreatic fluid but not in their duodenal fluid had higher

total DNA concentrations in their duodenal fluid relative to total pancreatic fluid DNA (concentration ratio >1) compared with only 11 of the 23 cases in which mutations were detected in both samples ($P=0.03$).

DISCUSSION

In this study, we report three main findings. First, we find that the average concentration of *KRAS* mutations and *GNAS* mutations in a patient's secretin-stimulated duodenal fluid sample are considerably lower (7–10-fold) than in their corresponding pancreatic duct fluid sample ($P<0.001$). Second, we find that *KRAS* mutations are more likely to be detected in duodenal fluid samples when patients have relatively high concentrations of mutations ($>1\%$) in their pancreatic duct fluid sample. Third, we find that mutations detected in pancreatic duct fluid were less likely to be detected in duodenal fluid when the concentration of total DNA in these samples is higher than in pancreatic duct fluid suggesting that high DNA concentrations in the duodenal lumen could obscure the detection of low concentrations of mutant DNA in secretin-stimulated pancreatic fluid.

The lower concentration of mutations arising from the pancreas in pancreatic fluid samples collected from the duodenal lumen necessitates these samples undergoing deeper sampling with more sensitive, specific, and expensive assays to detect mutations. An alternative to employing more sensitive mutation detection assays to detect mutations in duodenal fluid collections would be to collect purer samples of pancreatic fluid. The contaminating effect of duodenal contents is not due simply to fluid pooling in the duodenum; there is normally little or no fluid in the duodenal lumen to aspirate prior to secretin-stimulated pancreatic fluid collection. Most of the DNA in the duodenal lumen is probably shed from cells lining the duodenum.[26] As using ERCP to sample the pancreatic duct is not appropriate in the pancreatic screening setting, other approaches are needed. Alternative endoscopic approaches to pancreatic fluid collection at the papilla that did not involve cannulating the papilla of Vater could be very helpful, particularly if secretin-stimulated pancreatic fluid samples proved to be a clinically useful source of markers of pancreatic neoplasia.

We found a high prevalence of *KRAS* mutations (~80% of pancreatic duct fluid samples) in our study population, which consisted mostly of patients undergoing pancreatic screening. We have also observed a similarly high prevalence of *KRAS* mutations in the duodenal fluid samples of other patients undergoing pancreatic screening as part of the CAPS studies (Goggins M, Eshleman J *et al*., unpublished data), often in patients without any diagnostic pancreatic abnormalities by imaging. Although these results suggest that the detection of *KRAS* mutations in pancreatic fluid is not a useful test for the diagnosis of pancreatic cancer, these results do not rule out the possibility that quantifying *KRAS* mutations in pancreatic fluid samples might have diagnostic utility. It should be noted that our study was limited to patients who had undergone EUS and ERCP and was not designed to evaluate the diagnostic utility of *KRAS* mutations. We have maintained long-term follow-up of the patients undergoing pancreatic screening in our study and although several had cystic lesions that required surgery, none developed pancreatic cancer (Table 2). In a separate study, we have found that patients with pancreatic ductal adenocarcinoma have significantly higher concentrations of *KRAS* mutations in their duodenal collections of pancreatic fluid than patients undergoing pancreatic screening and those without evidence of pancreatic disease, but such a test does not reliably distinguish cancer cases from controls (Goggins M, Eshleman J *et al*., unpublished data). Patients with an extensive family history of pancreatic cancer who undergo pancreatic screening commonly have numerous PanINs and IPMNs.[15,27] For example, 39% of patients enrolled in the CAPS3 pancreatic screening study were found to have small, mostly subcentimeter, pancreatic cysts.[3] Most patients who undergo pancreatic resection for enlarging or concerning cystic lesions detected by screening also have PanIN lesions identified in their resection specimen. These PanIN lesions are generally more abundant in the resection specimen than their IPMNs.[15,27] It is also known that most middle-aged and older individuals without any family history of pancreatic cancer have some PanIN-1 in their pancreata.[28,29] For these reasons, it is likely that a lot of the mutant *KRAS* detected in the pancreatic fluid of patients undergoing pancreatic screening arise from multifocal low-grade PanIN lesions. The presence of microscopic PanIN explains why we often detected *KRAS* mutations in pancreatic fluid samples of patients who did not have pancreatic cysts or *GNAS* mutations and why among patients harboring both *KRAS* and *GNAS* mutations in their pancreatic fluid the *KRAS* mutation concentrations are generally higher than GNAS mutations.

In conclusion, we find that the concentrations of *KRAS* and *GNAS* mutations are considerably higher in secretin-stimulated pancreatic fluid samples collected from the pancreatic duct than when pancreatic fluid is collected from the duodenal lumen. The dilution of pancreatic fluid by duodenal lumen contents can limit the detection of pancreatic fluid mutations. Improvements in the endoscopic collection of pancreatic fluid from the duodenum may improve the detection of mutations arising from the pancreas during EUS screening for pancreatic cancer in high risk individuals.

CONFLICT OF INTEREST

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ KRAS mutations arise in most low-grade PanINs and IPMNs.
- ✓ GNAS mutations are a specific marker of IPMNs.
- ✓ Secretin-stimulated pancreatic fluid contains mutations arising from the pancreas.

WHAT IS NEW HERE

- ✓ Mutation concentrations are ~10-fold lower in pancreatic fluid from the duodenum compared with the pancreatic duct.
- ✓ Most patients undergoing pancreatic screening have KRAS mutations in their pancreatic fluid.
- ✓ DNA present in the duodenal lumen can obscure the detection of pancreatic fluid mutations.

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