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The Effect of Pancreatic Juice Collection Time on the Detection of *KRAS* Mutations

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Abstract

Objective: Secretin-stimulated pancreatic juice is collected from the duodenum and analyzed to identify biomarkers of pancreatic neoplasia, but the optimal duration of pancreatic juice collection is not known.

Methods: We compared the yield of *KRAS* mutations detected in pancreatic juice samples aspirated from near the duodenal papilla at 1 to 5, 6 to 10, and 11 to 15 minutes after secretin infusion, and from the third part of the duodenum (at 15 minutes) from 45 patients undergoing endoscopic ultrasound pancreatic surveillance. *KRAS* mutation concentrations were measured by using droplet digital polymerase chain reaction.

Results: Forty of 45 patients had *KRAS* mutations detected in their pancreatic juice, and most patients' juice samples had more than 1 *KRAS* mutation. Of 106 *KRAS* mutations detected in 171 pancreatic juice samples, 58 were detected in the 5-minute samples, 70 mutations were detected in the 10-minute samples, and 65 were detected in the 15-minute samples. Nine patients who did not have *KRAS* mutations detected in their 5-minute sample had mutations detected in samples collected at later time points. Ninety-percent of all pancreatic juice mutations detected in any sample were detected in the 5-or 10-minute samples.

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Conclusions: Collecting pancreatic juice for 10 minutes after secretin infusion increases the likelihood of detecting pancreatic juice mutations over shorter collections.

Keywords

pancreatic juice; secretin; KRAS; pancreatic screening; droplet digital PCR

Pancreatic cancer is projected to be the second leading cause of cancer deaths by 2030 in the United States.¹ Pancreatic screening and surveillance is performed for patients at increased risk for developing pancreatic cancer because of their pancreatic cancer family history, a pancreatic cancer susceptibility gene mutation, or having incidentally detected pancreatic cysts.^{2–12} Endoscopic ultrasonography (EUS) and magnetic resonance imaging/magnetic resonance cholangiopancreatography are accurate tests for detecting pancreatic cysts,^{3,4,9} but better tests are needed for detecting small solid pancreatic cancers, particularly because some patients develop pancreatic cancer despite regular surveillance that reflect missed opportunities for early detection.¹³ Secretin-stimulated pancreatic juice tests are being developed to improve the evaluation of patients undergoing EUS surveillance.^{3,4,9} Pancreatic juice samples collected from the duodenum of patients undergoing pancreatic surveillance commonly have mutations, especially *KRAS* mutations, indicative of pancreatic neoplasia, and these are present in patients with and without pancreatic cysts.^{13–16} Most of these *KRAS* mutations are thought to arise from microscopic pancreatic intraepithelial neoplasia (PanIN).^{14,17} Pancreatic intraepithelial neoplasias are the precursors to most pancreatic ductal adenocarcinomas and are the most common precursor lesion identified at pancreatectomy, at autopsy, and in patients who undergo pancreatic resections for abnormalities identified by pancreatic surveillance.^{18–21} Pancreatic cysts are also commonly identified in patients undergoing pancreatic screening, and most of these are intraductal papillary mucinous neoplasms.^{4,15} The potential diagnostic utility of pancreatic juice tests is evident from studies showing that some patients who have subsequently developed pancreatic cancer have had mutations known to arise in highgrade dysplasia and invasive cancer detected in their pancreatic juice samples collected before their diagnosis of pancreatic ductal adenocarcinoma.¹³ Pancreatic juice samples collected from the duodenum usually have very low biomarker concentrations (~0.1%), raising the concern that the detection of these mutations is very dependent on the adequacy of pancreatic juice collection. Secretin induces a rapid secretion of pancreatic juice within 1 to 2 minutes of infusion, and within 5 minutes of infusion, typically at least 5 mL of pancreatic juice that can be collected for biomarker analysis has been secreted. Pancreatic juice collections for biomarker studies have used short pancreatic juice collections to minimize the burden of performing the test, but it is not known what the optimal duration of pancreatic juice collections should be for identifying biomarkers of pancreatic neoplasia. We hypothesized that biomarker yield would improve with longer pancreatic juice collections. *KRAS* mutations are the most common mutation detected in pancreatic neoplasms^{17,22,23} and are therefore a useful biomarker of pancreatic neoplasia. In this study, we evaluated the yield of DNA biomarkers in pancreatic juice samples collected from patients undergoing pancreatic surveillance in sequential intervals starting 2 minutes and ending 15 minutes after secretin infusion.

MATERIALS AND METHODS

Patients and Specimens

Pancreatic juice samples and clinical information were obtained from participants enrolled at the University of Pittsburgh in the Cancer of the Pancreas Screening-5 study. We evaluated 47 pancreatic juice collections from 45 subjects undergoing pancreatic surveillance between 2015 and 2016 (2 patients underwent a second pancreatic juice collection). Table 1 lists the indications for pancreatic EUS and pancreatic juice collection, which in most cases was for a family history of pancreatic cancer with or without having a pancreatic cancer susceptibility gene mutation. Four patients were undergoing surveillance for incidentally identified pancreatic cysts. All elements of this study were approved by the Johns Hopkins and University of Pittsburgh's institutional review board, and written informed consent was provided from all patients.

Pancreatic Juice Collection

Serial pancreatic juice collections were performed from all participants after infusion of intravenous human synthetic secretin (0.2 µg/kg for 1 minute). Secretin was generously provided by ChiRhoClin Inc (Burtonsville, Md). Pancreatic juice collections were performed during EUS evaluation by aspirating pancreatic juice through the echoendoscopic channel with the tip of the endoscope adjacent to the papilla. The collection of the first pancreatic juice samples began approximately 2 minutes after secretin infusion was completed and pancreatic juice was collected continuously for the next 3 minutes (referred to as the 5-minute sample), the second collection was obtained by aspirating juice continuously over the next 5 minutes (referred to as the 10-minute sample), and the third collection was obtained by aspirating juice continuously over the next 5 minutes (referred to as the 15-minute sample). Many patients also had a fourth pancreatic juice sample collected after the third sample collection was completed, 15 minutes after secretin infusion. This sample was obtained by moving the endoscope tip into the third portion of the duodenum and aspirating pooled pancreatic juice while withdrawing the echoendoscope back to the level of the ampulla (referred to as the 15-minute residual sample). This sample was obtained to determine how helpful it was to collect pancreatic juice from near the papilla compared with simply aspirating pooled contents from the duodenal lumen mostly around the inferior duodenal flexure between the second and third parts of the duodenum.

Pancreatic juice samples were kept on ice and transferred to the laboratory, aliquoted, and stored at -80°C before use. No other sample preparations or modifications were made to the pancreatic juice samples before DNA extraction.

Droplet Digital Polymerase Chain Reaction Methods

DNA was extracted from 450 µL of pancreatic juice using either the DNeasy Blood & Tissue Kit (Qiagen, Germantown, Md) or the circulating DNA isolation kit. We did not find any difference in DNA yields between the 2 kits. The mutational status of *KRAS* codon 12 was investigated using droplet digital polymerase chain reaction (PCR; ddPCR) technology (Bio-Rad Laboratories, Hercules, Calif) as previously described.²⁴ *KRAS* mutations were detected in cell-free DNA.

Analysis was performed blinded to patient and sample collection information. Primers (Integrated DNA Technologies, Coralville, Iowa) and probes (Custom TaqMan Probes; Thermo Fisher Scientific, Waltham, Mass) were designed to detect G12D, G12V, and G12R *KRAS* mutations, which are the usual *KRAS* mutations found (~90% of pancreatic cancers, PanIN, and intraductal papillary mucinous neoplasm).^{17,25} Three separate ddPCR assays were performed, one for each mutant probe. The *KRAS* wild-type probe was run with each assay. The following primers were used for the *KRAS* assay: 5'-GCCTGCTGAAAATGACTGAATATAAACT-3' (forward) and 5'-TTGTTGGATCATATTCGTCCAC-3' (reverse). The following probes were used: *KRAS* wild-type (5'-TTGGAGCTGG TGGCGTA-3') and *KRAS* G12D (5'-TTGGAGCTGATGGCGTA-3'), *KRAS* G12V (5'-TTGGAGCTGTTGGCGTA-3') or *KRAS* G12R (5'-TTGGAGCTCGTGGCGTA-3'). The minor groove binder probes were labeled with FAM or VIC at 5' end and a nonfluorescent quencher at the 3' end. Droplet digital PCR mixes consisted of 10 μ L of 2 ddPCR Supermix for probes (Bio-Rad Laboratories), 900-nM forward and reverse primers, 250-nM probe (FAM for mutant, VIC for wild-type), and 4 μ L of DNA (adjusted to be approximately 10,000 copies/well), with a total volume of 20 μ L. The PCR mix and 70- μ L Droplet Generation Oil (Bio-Rad Laboratories) were loaded into 8-channel cartridge (Bio-Rad Laboratories). A QX200 droplet generator (Bio-Rad Laboratories) created droplets, and the droplets were transferred to a 96-well PCR plate (Eppendorf, Hauppauge, NY). Polymerase chain reaction plates were heat sealed (PX1 PCR Plate Sealer; Bio-Rad Laboratories) with a foil heat seal (Bio-Rad Laboratories) and placed into a Verity thermal cycler (Thermo Fisher Scientific). Polymerase chain reaction conditions were as follows: 95°C for 10 minutes, 39 cycles of 94°C for 30 seconds and 63°C for 60 seconds, and 2 final steps at 98°C for 10 minutes and 4°C hold to enhance dye stabilization.²⁶ Droplets were read by using a QX200 droplet reader (Bio-Rad Laboratories), and results were analyzed using QuantaSoft software (version 1.7.4; Bio-Rad Laboratories). Each assay was conducted with no template, wild-type, and positive control wells.

The fluorescent value thresholds were as follows: *KRAS* wild-type 3500, *KRAS* G12D 4500, G12V 5000, and G12R 5000, and we determined the threshold for calling a mutation to be 2 positive droplets for *KRAS* G12D and 1 positive droplet for *KRAS* G12V and G12R based on the 95 percentile of the false-positive drop distribution as previously described.²⁴ The low-level background detection of *KRAS* G12D may be related to PCR errors in part related to low-level cytosine deamination, as has been observed previously.²⁷

Statistical Analysis

We compared the number of mutations detected in a juice sample at any 1-time interval to the total number of mutations collected in that patients sample. Qualitative variables were compared by using χ^2 tests, and quantitative variables were compared by using Mann-Whitney *U* test. The significance of a correlation between 2 variables was assessed using Spearman rank correlation coefficient. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, Calif). *P* < 0.05 was considered statistically significant.

RESULTS

Patient Characteristics

Of the 45 subjects included in this study, 24 were undergoing surveillance because of being a member of a familial pancreatic cancer kindred, and 16 were carriers of a pancreatic cancer susceptibility gene mutation (8 with FAMMM/ CDKN2A mutations, 5 with *BRCA2* mutations, and 3 were Lynch syndrome mutation carriers), 3 were from families with breast/ovarian cancer syndrome, and 4 had sporadic pancreatic cysts detected incidentally. Overall, 19 patients had pancreatic cysts detected.

KRAS Mutation Detection in Pancreatic Juice Samples Collected at Different Times From the Duodenum Lumen

One or more *KRAS* mutations were detected in 42 (89.3%) of the 47 pancreatic juice collections including G12D in 34 (72.3%), G12V in 39 (83.0%), and G12R in 33 (70.2%) of patient samples. Most mutations were present at very low concentrations (<0.1% of total DNA). One third of patients had 1 or more *KRAS* mutations detected in their pancreatic juice at concentrations greater than 0.1% of total DNA. Eight patients had very low mutation concentrations (<0.05% overall mutation concentration for their samples). Four of the 5 patients who did not have any detectable *KRAS* mutations had normal pancreata by EUS. The patient with the highest concentration of *KRAS* mutations was a patient with FAMMM.

The average total *KRAS* mutation concentration at each time point for each patient is shown in Figure 1. Average mutation concentrations increased modestly with each pancreatic juice collection from $0.04\% \pm 0.06\%$ at the 5-minute sample, $0.048\% \pm 0.05\%$ of total DNA at the 10-minute sample, $0.054\% \pm 0.07\%$ at the 15-minute sample, to $0.059\% \pm 0.07\%$ for the final sample ($P = 0.011$ between the T1 and T4 collection). *KRAS* mutation concentrations were inversely correlated ($r = -0.53$) with the total amount of wild-type DNA (determined by the amount of wildtype *KRAS* DNA by ddPCR) in the juice sample. Pancreatic juice *KRAS* mutation concentrations significantly correlated with patient age ($r = 0.39$, $P = 0.007$; Fig. 2). There was no correlation between mutation concentration and smoking use or alcohol consumption.

Timing of Pancreatic Juice Collection and Mutation Detection

Figure 3 shows the individual *KRAS* mutation concentrations for each patient at each pancreatic juice collection. If each patient had a G12D, G12V, and a G12R mutation detected in his/her pancreatic juice and 47 pancreatic juice collections were analyzed, the maximum number of mutations would have been detected, 141 *KRAS* mutations (47 3). We detected 106 *KRAS* mutations in the pancreatic juice samples collected from the 47 subjects. Fifty-eight of these mutations were detected in the 5-minute sample (and 45 were not detected), 70 mutations were detected in the 10-minute sample, and 65 mutations were detected in the 15-minute sample. Nine patients who had 1 or more pancreatic juice *KRAS* mutations did not have any of their mutations detected in the 5-minute sample. Fourteen mutations (10.5% of all mutations detected) were not detected until the third or fourth pancreatic juice sample. In 3 patients who had mutations detected in their juice, the first sample that had any mutation detected was the 15-minute sample. Among the 14 patients

who had higher levels of mutated *KRAS* (>0.1% mutation concentration) detected in 1 or more pancreatic juice samples, only 1 patient had a mutation first detected in the 15-minute sample that was not detected in the 5- or 10-minute sample.

The fourth pancreatic juice sample (15-minute residual) was collected from pooled pancreatic juice in the duodenal lumen, and the results compared the samples aspirated from near the papilla. In the patients who had this sample collected, there were 47 mutations detected; there were 72 *KRAS* mutations detected when all the *KRAS* mutations detected in these patients' pancreatic juice samples were added together. Four *KRAS* mutations were only detected in the 15-minute residual sample (all at very low concentrations).

Overall, there were significantly more mutations detected in the combined results of the 5- and 10-minute samples compared with the 5-minute sample alone ($P = 0.002$, Wilcoxon signed rank test). Similarly, there were significantly more mutations detected in the combined results of the 10- and 15-minute samples than those in the 10-minute sample alone ($P < 0.001$) as well as in the juice samples from the 15-minute plus the 15-minute residual samples compared with the 15-minute sample alone ($P < 0.001$).

DISCUSSION

In this study, we find that most pancreatic juice mutations can be detected in pancreatic juice samples collected within 10 minutes of secretin infusion, but limiting a duodenal lumen collection of pancreatic juice to the first 5 minutes after secretin infusion will yield samples that lack a lot of mutations. For this study, we measured *KRAS* mutations because they are the most sensitive marker of pancreatic ductal neoplasia. *KRAS* mutations can be considered as representative of DNA shed from neoplastic cells within the pancreatic ductal system. If *KRAS* mutations can be missed, then other mutations can be missed if pancreatic juice collections are not optimal. It should be noted that the effect of pancreatic juice collections may have different effects on the behavior of biomarkers other than DNA (eg, protein). These results have implications for how we interpret the results of pancreatic juice mutation analyses performed to evaluate their utility for patients undergoing pancreatic screening. For the pancreatic juice biomarker studies performed on the Cancer of the Pancreas Screening study population,^{13–16,28} pancreatic juice has been collected from near the duodenal papilla for only the first 5 minutes after secretin infusion, and so the results from these samples may underestimate the potential diagnostic utility of pancreatic juice analysis as a test for pancreatic screening. Other groups have used 10-minute pancreatic juice collections for biomarker studies.^{29,30} Consistent with the results of the current study, we observed a higher prevalence of *KRAS* mutations in this study than in a prior study,¹⁶ although there were also other differences between the 2 studies including the study population and the methods used to detect *KRAS* mutations. We found that 90% of the pancreatic juice mutations detected in our study population were detected within the juice samples collected within the first 10 minutes, and in the few cases where mutations were only detected in samples collected after 10 minutes, the concentration of these mutations was very low. The residual pooled pancreatic juice sample collected after 15 minutes yielded a similar fraction of mutations to that found in the 10- and 15-minute samples. Mutation concentrations were higher in the 15- and 15-minute residual samples than in the 5-minute samples. This raises the question of

whether it would best to simply collect pooled pancreatic juice after 15 minutes from the duodenal lumen as is done for the bicarbonate pancreatic function test. The 15-minute residual samples collected for this study do not include the pancreatic juice aspirated at earlier time points, and so they differ from samples obtained for pancreatic function testing. In theory, a pooled 15-minute sample might be a good representative sample of the pancreatic juice collected at earlier time points. One potential concern about using such a pooled sample is that some pancreatic juice flows further down the intestine during these 15 minutes with the potential loss of biomarkers.

Because it is helpful to optimize the duration of endoscopic juice collections, our results support the routine use of collecting pancreatic juice for 10 minutes after secretin infusion to biospecimens with an optimal yield of biomarkers for pancreatic screening.

In addition to differences in the detection of individual mutations, overall pancreatic juice mutation concentrations are modestly higher at later collection points. This could be due to an increase in the amount of *KRAS* mutations from the pancreas in later collections, or a reduction in the amount of *KRAS* wildtype DNA in the duodenal lumen in later collection points, or a combination of both. Pancreatic juice mutation concentrations are higher in patients with pancreatic cancer than in those under surveillance for their family history or with pancreatic cysts,¹³ but overall pancreatic juice mutation concentrations do not reliably distinguish patients with pancreatic cancer from those without; more important is the detection of mutations that predict the grade of neoplasia (such as *SMAD4* and *TP53* mutations), which are expected to be more likely in patients with pancreatic cancer or high-grade dysplasia.¹³

In conclusion, we find that a 10-minute post-secretin pancreatic juice collection provides a more optimal yield of mutations for pancreatic screening tests compared with shorter collections.

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FIGURE 1.
Pancreatic juice *KRAS* mutation concentrations at each collection point.

FIGURE 2.
Correlation between patient age and pancreatic juice mutation concentrations.

FIGURE 3.

Individual patient pancreatic juice *KRAS* mutation concentrations. *x* Axis: each patient's *KRAS* mutation concentrations at each time point. Time points 1, 2, 3, and R represent the 5-, 10-, 15-, and 15-minute residual samples, respectively. *y* Axis: total *KRAS* mutation concentrations (% of total DNA); blue, yellow, and green bars represent the mutation concentration for *KRAS*G12D, G12V, and G12R mutations, respectively. BRCA2, *BRCA2* mutation carrier; FAMMM, familial atypical multiple mole melanoma syndrome; FPC, familial pancreatic cancer kindred; Lynch, Lynch syndrome. **Editor's note:** A color image accompanies the online version of this article.

TABLE 1.

Patient Characteristics

n = 45	
Sex, male:female	23:22
Age, mean (SD), y	61.7 (8.0)
Race, n *	
White	43
Study group, n	
High risk	41
Control	4
History, n *	
Smoking	18
Drinking alcohol	27
EUS findings, n	
Pancreatic cyst	19

* No information for 2 patients.

SD indicates standard deviation.