Supplemental Materials

Supplemental Methods

Pancreatic fluid collection

Pancreatic fluid is aspirated through the echoendoscope channel into a sample trap. The trap is placed on ice immediately after collection until it is aliquoted in the laboratory and placed in a -80^{o}C freezer. No other handling or processing of the pancreatic fluid is required to preserve the pancreatic fluid DNA for subsequent analysis. DNA is extracted from one aliquot of pancreatic fluid (400ul). The concentration of DNA in duodenal fluid is typically ~5 ng/ul (range 1-10 ng/ul) and we required ~ 50 ng of DNA for the digital-HRM and pyroseqeuncing assays for this study. Although very little pancreatic fluid is required for these assays, ~5mls or more of pancreatic fluid is collected from the duodenum because pancreatic fluid mixes with duodenal contents and collecting 5mls after 5 minutes helps to ensure that the sample collected reflects a good lavage of the pancreatic duct system.

When secretin function tests are performed to evaluate patients for pancreatic insufficiency pancreatic fluid is collected over 45 minutes and reduced volumes may be evident in the presence of pancreatic ductal obstruction or pancreatic insufficiency. In this study, we did not encounter any patients with complete or near complete pancreatic ductal obstruction from pancreatic cancer and there was no difference in the volume of fluid collected from the duodenum from patients with pancreatic cancer compared to other patients. However, because only 5-10mls of fluids is collected, the volume of fluid collected may not be a reliable indicator of pancreatic fluid secretion because some of the fluid collected from the duodenal lumen represents duodenal contents. The presence of a pancreatic, biliary or duodenal stent could

potentially interfere with pancreatic fluid collection but none of the patients in this study had any of these stents.

KRAS and GNAS mutation detection

DNA was quantified using Q-PCR (Quantifiler, ABI). In each 96-well plate, 10 genome equivalents (g.e.) (60 picograms) of pancreatic juice DNA or control DNA were dispensed. We chose 10 g.e. per well because serial dilution experiments demonstrated that control samples with 10% concentrations of mutant DNA were reliably detectable by melt-curve analysis, and the detection of 1 mutant template in 10 g.e. of DNA is well within the 5% limit of detection of pyrosequencing [1]. Assays of 96-well plates of samples with 10% mutant DNA produced expected numbers of positive wells consistent with Poisson statistics (data not shown).

High-resolution digital HRM analysis was performed to detect KRAS and GNAS mutations. For *KRAS* mutation detection, each pancreatic juice DNA was sample subjected to high-resolution digital HRM analysis in 350 to 360 PCR reactions. Typically, pancreatic juice DNA was added to 88 wells per 96-well plate; two additional wells each were filled with the following: water (no template control), wild-type *KRAS* DNA (HT29), 20% Hct116 DNA diluted in HT29 (containing 10% concentrations of *KRAS* G13D mutation), and 100% Hct116, all at 10 g.e. per well. Four plates were run per sample (approximately 3520 genomes, 88 wells x 4 plates x 10 g.e. per well). We also calculated the effective DNA concentration in each sample by determining the efficiency of PCR amplification of the *KRAS* amplicon (the number of wells amplified) in 96 well plates by Poisson statistics.[2] Most samples, including all of the CAPS3 samples, were analyzed for *KRAS* mutations using a digital HRM analysis adapted from Wallen et al.[3] *KRAS* exon 2 was amplified using LightCycler FastStart DNA Master Hybridization Probes Mix (Roche, Indianapolis, Indiana), 500 nM Forward Primer (5'-

AAGGCCTGCTGAAAATGACTG-3'), 100 nM Reverse Primer (5'-GGTCCTGCACCAGTAATATGC-3'), 400 nM Sensor Probe (5'-Rox-TGCCTACGCCACCAGCTCCAA-Phos-3'), and 200 nM Anchor Probe (5'-CCACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACT-FAM-3'), including a PNA NH2-CTACAGTGAAATCTCG-COOH HPLC purified (Panagene, Daejeon, Korea). Reactions were amplified in a Veriti Thermal Cycler as follows: 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, 61°C for 10 seconds, and 72°C for 15 seconds. Melting curve analysis was carried out on the StepOne Plus instrument (AB) through one cycle of 95°C for 20 seconds, 45°C for 60 seconds, and ramp to 78°C at 0.4°C/sec. Juice samples from the CAPS2 and CAPS4 study were analyzed for *KRAS* mutations using a similar digital HRM method (four 96-well plates, 10 g.e. pancreatic juice DNA per well in 90 wells, 5 wells with wild-type DNA and 1 well with water) using PCR and melting conditions described previously[4] (and by Sadakari et al, in submission). The same pyrosequencing assay was used to confirm all mutations detected by both digital HRM methods.

Pancreatic ductal adenocarcinomas, PanINs and IPMNs have a characteristic spectrum of *KRAS* mutations with ~95% of mutations G12D, G12V or G12R, and most of the remaining mutations G12C or G12A.[4-7] Furthermore, in other studies we have also found that *KRAS* mutations can be generated *ex vivo* due to spontaneous cytosine deamination that can result in spurious G12S, G13S and G13D mutations.[8, 9] Therefore, we only included the pancreatic cancer-associated mutations (G12V, G12D, G12R, G12C and G12A) in our analysis.

KRAS mutation concentrations were compared to *GNAS* mutation concentrations. Most of the *GNAS* results were reported previously.[10] *GNAS* mutations were detected using a similar digital melt-curve pyrosequencing method to that described above to detect *KRAS* mutations.[10]

Pyrosequencing was performed on digital-HRM-positive wells per juice sample as well as one HRM-wild-type and one HRM-negative control well. Identified wells were re-amplified using primers listed above with the exception that the reverse primer was biotinylated. Reactions were amplified on GeneAmp9700 Thermal Cycler (AB) as follows: 95°C for 15 minutes, 41 cycles of 95°C for 20 seconds, 53°C for 30 seconds, 72°C for 20 seconds, and a final extension at 72°C for 5 minutes. The amplicons were sequenced using the PyroMark Q24 (Qiagen) with PyroMark Gold reagents (Qiagen, Valencia, CA)) containing 0.3µmol/L sequencing primer (5'-TGTGGTAGTTGGAGCT-3') and annealing buffer. The nucleotide dispensation order for codons 12 to 16 was: 5'-TCAGACTACGTACTAGACTATCGTACAAGAGT-3′. To ensure assay specificity, a juice sample was only deemed as having a mutation when the mutation was confirmed by pyrosequencing.

Estimating the false-positive rate of digital HRM/pyrosequencing for mutant KRAS

We obtained DNA wild-type for *KRAS* codons 12 and 13 from a healthy control's peripheral blood and performed digital HRM as described above on six 96-well plates (564 wells). Each positive well by digital HRM was subjected to pyrosequencing. A second digital HRM/pyrosequencing experiment was performed using similar PCR conditions (described in [4]) with twenty 96-well plates (1920 wells).

Statistical questions

The prevalence of mutations and mutation scores was compared among the five different diagnostic groups (pancreatic cancer, pancreatic screening, sporadic pancreatic cyst, pancreatitis and normal pancreata). The main questions were to determine; if patients with pancreatic cancer

had a higher prevalence and higher levels of mutations than other groups; if patients undergoing pancreatic screening had higher levels than patients without pancreatic cancer; and what factors were associated with mutations detected in patients undergoing screening.

Supplemental Results

The diagnoses of the "Normal Pancreas control" subjects are listed in Supplemental Table 1. These were patients who were referred for endoscopic ultrasound that had no evidence of exocrine pancreatic neoplasia.

Because our previous studies found mutation concentrations in duodenal juice collections in the 0.1% concentration range[10], we assayed ~3500 genome equivalents of pancreatic juice DNA per sample to ensure sufficient sampling depth to detect *KRAS* mutations at this concentration.[10]

We determined the specificity of our *KRAS* assay by analyzing control DNA wild-type for *KRAS* and detected *KRAS* codon 12 mutations in 3 of ~2500 wells (0.122%). To account for the false positive rate of our assay, we set a threshold of requiring at least 2 wells with mutant *KRAS* (out of the ~350 average number of wells analyzed per juice sample) to call the juice sample positive for mutant *KRAS*. It was considered unlikely that our assay would generate 3 or more false-positive mutations of the same type in one sample, so when determining the score of an individual *KRAS* mutation (e.g. G12D), if a sample had a 3 or more wells having the same *KRAS* G12D mutation, it was given a score of three.

There was no significant difference in the average *KRAS* mutation score among subjects in the screening group with more than one cyst compared to those with 1 cyst, or between those with cysts above vs. below the median cyst size (5mm) (data not shown). There was also no

significant difference in the number of unique *KRAS* mutations among patients undergoing screening with one or more cysts compared to those without cysts (data not shown).

In patients undergoing pancreatic screening, subtle parenchymal changes similar to those observed in patients with chronic pancreatitis[11] are often detected using EUS.[12] These changes are associated with the presence of focal lobulocentric atrophy in areas affected by PanIN[12], but these EUS changes are not specific; they are also observed in individuals without suspected pancreatic disease.[13] Information about EUS-defined pancreatic parenchymal abnormalities was available for 136 patients in the screening group, 28 of whom (21%) met EUS criteria used to diagnose chronic pancreatitis: These 28 subjects had higher mean pancreatic juice *KRAS* mutation concentrations (4.1+/-7.4) than the 108 patients without these criteria (1.3+/-2.7, p=0.06), although there was no significant difference in the overall prevalence of *KRAS* mutations between these two groups (53% vs. 45%). The percentage of subjects who met these EUS criteria was similar in those with pancreatic cysts (21.2%) and those without (20%). As reported previously[14], within the pancreatic screening group the average age of subjects with pancreatic cysts was significantly older than those without cysts (Table 2).

Seven of the 194 individuals in the pancreatic screening group underwent pancreatic resection for their pancreatic cysts, all of which were IPMNs, and six of these seven had detectable *KRAS* mutations in their duodenal fluid samples. The one exception was a 46 year old with Peutz-Jeghers syndrome. Duodenal fluid *KRAS* mutation concentrations in the seven patients who underwent pancreatic resection were not significantly different from the rest (n=187) of the pancreatic screening population (data not shown). The normal pancreas control with an individual *KRAS* mutation with a score of >3 had a diagnosis of recurrent papillary stenosis.

Supplemental Table 1: Indications for pancreatic evaluation in "normal pancreas cont

1. celiac disease, gastric wall thickening, rule-out GIST (none found)
2. sub-xiphoid pain, eosinophilic esophagitis
3. dyspepsia
4. abdominal pain, possible pancreatic lesion on CT (none found)
5. Primary sclerosing cholangitis
6. dilated common bile duct post-cholecystectomy, no mass found on EUS
7. peripancreatic lymph nodes, considered non-neoplastic, no pancreatic lesion
8. nausea and abdominal pain (normal EUS)
9. papillary stenosis
10. abdominal pain of unknown etiology
11. common bile duct stones
12. common bile duct stones
13. previous distal pancreatectomy for solid pseudopapillary tumor
14. abdominal pain, elevated liver enzymes
15. abdominal pain, questionable pancreatic lesion on CT (none found)
16. abdominal pain (normal EUS)
17. screening for esophageal CA because of a strong family history (normal EUS)
18. small well differentiated pancreatic neuroendocrine tumor
19. gastric GIST, no pancreas abnormalities
20. weight loss of unknown etiology
21. common bile duct stone
22. ampullary stenosis

Supplemental References

1. Tsiatis AC, Norris-Kirby A, Rich RG, et al. Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications. J Mol Diagn 2010;12:425-32.
2. Furth EE, Thilly WG, Penman BW, et al. Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates. Anal Biochem 1981;110:1-8.
3. Wallen M, Tomas E, Visakorpi T, et al. Endometrial K-ras mutations in postmenopausal breast cancer patients treated with adjuvant tamoxifen or toremifene. . Cancer Chemother Pharmacol 2005;55:343-346.
4. Kanda M, Matthaei H, Wu J, et al. Presence of Somatic Mutations in Most Early-Stage Pancreatic Intraepithelial Neoplasia. Gastroenterology 2012;142:730-733.e9. Epub 2012Jan 5 PMC3321090.
5. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. 2008;321:1801-6. Epub 2008 Sep 4.
6. Wu J, Matthaei H, Maitra A, et al. Recurrent GNAS Mutations Define an Unexpected Pathway for Pancreatic Cyst Development. Sci Transl Med 2011;3:92ra66.
7. Biankin AV, Waddell N, Kassahn KS, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature 2012;491:399-405.
8. Chen G, Mosier S, Gocke CD, et al. Cytosine Deamination Is a Major Cause of Baseline Noise in Next-Generation Sequencing. Mol Diagn Ther 2014:in press.
9. Lin MT, Mosier SL, Thiess M, et al. Clinical validation of KRAS, BRAF, and EGFR mutation detection using next-generation sequencing. Am J Clin Pathol 2014;141:856-66.
10. Kanda M, Knight S, Topazian M, et al. Mutant GNAS detected in duodenal collections of secretin-stimulated pancreatic juice indicates the presence or emergence of pancreatic cysts. Gut 2013;62:1024-33 epub 8/2/2012 PMCID: Journal in process.
11. Catalano MF, Sahai A, Levy M, et al. EUS-based criteria for the diagnosis of chronic pancreatitis: the Rosemont classification. Gastrointest Endosc. 2009;69:1251-61. Epub 2009 Feb 24.
12. Brune K, Abe T, Canto M, et al. Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer. Am J Surg Pathol 2006;30:1067-76.
13. Rajan E, Clain JE, Levy MJ, et al. Age-related changes in the pancreas identified by EUS: A prospective evaluation. Gastrointest Endosc 2005;61:401-6.
14. Canto MI, Hruban RH, Fishman EK, et al. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. Gastroenterology 2012;142:796-804.