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Detection of Circulating Tumor DNA in Patients with Pancreatic Cancer Using Digital Next-Generation Sequencing

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Circulating tumor DNA (ctDNA) measurements can be used to estimate tumor burden, but avoiding false-positive results is challenging. Herein, digital next-generation sequencing (NGS) is evaluated as a ctDNA detection method. Plasma *KRAS* and *GNAS* hotspot mutation levels were measured in 140 subjects, including 67 with pancreatic ductal adenocarcinoma and 73 healthy and disease controls. To limit chemical modifications of DNA that yield false-positive mutation calls, plasma DNA was enzymatically pretreated, after which DNA was aliquoted for digital detection of mutations (up to 384 aliquots/sample) by PCR and NGS. A digital NGS score of two SDs above the mean in controls was considered positive. Thirty-seven percent of patients with pancreatic cancer, including 31% of patients with stages I/II disease, had positive *KRAS* codon 12 ctDNA scores; only one patient had a positive *GNAS* mutation score. Two disease control patients had positive ctDNA scores. Low-normal—range digital NGS scores at mutation hotspots were found at similar levels in healthy and disease controls, usually at sites of cytosine deamination, and were likely the result of chemical modification of plasma DNA and NGS error rather than true mutations. Digital NGS detects mutated ctDNA in patients with pancreatic cancer with similar yield to other methods. Detection of low-level, true-positive ctDNA is limited by frequent low-level detection of false-positive mutation calls in plasma DNA from controls. *(J Mol Diagn 2020, 22: 748—756; https://doi.org/10.1016/j.jmoldx.2020.02.010)*

Pancreatic cancer incidence has been increasing in the United States and although survival rates have been improving slowly, for most patients it is still a deadly disease.[1] Recent improvements in early detection may be beginning to impact survival. For example, pancreatic imaging surveillance of individuals at sufficiently high risk of developing pancreatic cancer is associated with improved survival.[2,3] Further improvements in the early detection of pancreatic cancer likely would occur if accurate blood tests were available to detect early stage disease. Many biomarker blood tests have been evaluated as candidate early detection tests for pancreatic cancer, including circulating tumor DNA (ctDNA). Molecular approaches for detecting ctDNA for patients with pancreatic cancer have focused on identifying *KRAS* hotspot mutations because more than 90% of pancreatic cancers have *KRAS* mutations and more than 95% of these mutations occur at codon 12. Circulating *KRAS* mutation concentrations in patients with pancreatic cancer are generally of low

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abundance and have been detected in only approximately 30% of patients with low-stage disease using existing methods.[4–15] Methods to detect ctDNA include droplet digital PCR, safe-sequencing system (Safeseqs),[16] targeted error correction sequencing (TEC-Seq),[17] simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing (SiMSen-seq),[18] and high multiplex amplicon barcoding PCR,[19] as well as *in silico* error correction strategies[20] and the detection of allelic imbalance after size selection.[21] One method developed to detect low-abundance mutations is digital NGS.[22] Digital NGS involves undertaking discrete NGS analyses on many (eg, 384) individual aliquots of DNA from a single biological sample in which each aliquot contains only a limited number of genome equivalents of DNA so that each aliquot has either zero or one mutation-containing DNA templates at each nucleotide of interest in addition to wild-type templates. The identification of recurrent mutations in more than one aliquot favors a true mutation over NGS-induced error.

Although *KRAS* mutations are the major target of ctDNA detection strategies for pancreatic cancer, some studies have evaluated *GNAS* mutations as a potential ctDNA target because *GNAS* mutations commonly arise early in the development of the pancreatic cystic precursor neoplasm known as intraductal papillary mucinous neoplasm (IPMN). Hotspot mutations in *GNAS* (codon 201) are present in the neoplastic tissue of approximately 60% or more of IPMNs,[23,24] as well as in pancreatic cyst fluid[23] and pancreatic juice from patients with IPMN,[25] raising the possibility that the detection of circulating *GNAS* mutations could have a role in the early detection of pancreatic cancers that arise from IPMNs.

Methods used to detect ctDNA mutations rely on PCR and sequencing; these methods generate rare sequencing errors such as those related to nucleotide incorporation by DNA polymerases that need to be accounted for in the detection of low-abundance mutations. Another source of error with ctDNA detection methods can arise from *ex vivo* chemical modification of DNA such as by cytosine deamination to uracil.[26,27] Enzymatic pretreatment of DNA has been used to try to limit the errors generated by cytosine deamination and other chemical modifications of DNA in formalin-fixed tissues and in forensic samples,[28] but it has not been evaluated extensively for its potential to reduce errors when testing plasma DNA for mutations.

In this study, enzymatic pretreatment of plasma DNA followed by digital NGS was used to detect hotspot mutations in *KRAS* and *GNAS* in patients with pancreatic cancer, including patients with an IPMN-associated pancreatic cancer, and in healthy and disease controls.

Materials and Methods

Patients and Specimens

Plasma samples were obtained either from patients who were enrolled in the Cancer of the Pancreas Screening studies or from patients undergoing a pancreatic resection at the Johns Hopkins Hospital.[2,29] Patients included those with pancreatic ductal adenocarcinoma ($n = 67$), including 10 cases with a co-occurring IPMN lesion, 9 of which were thought to be arising pathologically from the IPMN; and controls ($n = 73$), including healthy laboratory employees ($n = 19$) and disease controls ($n = 54$). The disease controls included patients evaluated by endoscopic ultrasound or endoscopic retrograde cholangiopancreatography for nonpancreatic indications such as abdominal pain or benign biliary disease and found to have normal pancreata ($n = 21$), patients with known or suspected IPMN ($n = 13$), and/or familial/inherited susceptibility to pancreatic cancer ($n = 9$), patients with serous cystadenomas ($n = 8$), patients with acute/chronic pancreatitis ($n = 6$), and pancreatic neuroendocrine tumors ($n = 2$). Disease controls had a similar sex profile and age range to patients with pancreatic cancer, although the average age of the disease controls was somewhat younger (means $\pm$ SD/range, 57.9 $\pm$ 13.2/27 to 84 years versus 67.2 $\pm$ 10.5/44 to 89 years; $P < 0.0001$, unpaired *t*-test). Overall survival from pancreatic cancer was determined from the date of surgery (blood was drawn just before the surgery). Further description of the patient population is provided in Table 1 and Supplemental Tables S1 and S2.

All peripheral blood samples were collected in 10-mL EDTA vacutainers (BD Biosciences, San Jose, CA) and processed within 2 hours of collection. Plasma tubes were first spun at 1200 $\times$ *g* for 10 minutes. The plasma layer then was transferred to a new collection tube and spun at 1500 $\times$ *g* for 5 minutes. Plasma was aliquoted and stored at -80°C until DNA extractions were performed.

Genomic DNA was extracted from approximately 3 mL plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. When available, DNA extracted from archived pancreatic cancer tissues was analyzed to compare mutations found in the plasma with those in the cancer. To isolate tumor tissue, formalin-fixed, paraffin-embedded tumor tissues were cut onto membrane slides for laser capture microdissection to enrich for tumor cellularity, as previously described.[30] Genomic DNA was processed using the DNeasy Blood and Tissue Kit (Qiagen). Extracted DNA was quantified using the Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA). The Johns Hopkins Institutional Review Board approved all elements of this study, and written informed consent was obtained from all patients.

Digital NGS

The Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA) platform was used to to perform targeted NGS for *KRAS* (codons 12 and 13) and *GNAS* (codon 201). PreCR Repair Mix (New England Biolabs, MA) was used to treat DNA before library preparation as recommended by the manufacturer's protocol to limit false-positive detection of

Table 1 Characteristics of Cases Included in This Study

Characteristic	Total (n = 140)	Controls (n = 73)	PDAC (n = 67)
Sex, n (%)			
Male	58 (41.4)	24 (32.9)	34 (50.7)
Female	63 (45.0)	30 (41.1)	33 (49.3)
Unknown	19 (13.6)	19 (26.0)	0 (0.0)
Age, years, mean (range)*	63 (27–89)	58 (27–84)	67 (44–89)
Race/ethnicity, n (%)*			
Caucasian	100 (71.4)	42 (57.5)	58 (86.6)
African American	12 (8.6)	7 (9.6)	5 (7.5)
Hispanic/Latino	2 (1.4)	1 (1.4)	1 (1.5)
Asian	2 (1.4)	2 (2.7)	0 (0.0)
Other/unknown	24 (17.1)	21 (28.8)	3 (4.5)
AJCC stage, n (%)			
IA	5	NA	5 (7.5)
IB	9	NA	9 (13.4)
IIA	1	NA	1 (1.5)
IIB	22	NA	22 (32.8)
III	11	NA	11 (16.4)
IV	19	NA	19 (28.4)
Neoadjuvant chemotherapy, n (%)	34	NA	34 (50.7)

The controls include both healthy (n = 19) and disease controls (n = 54).
AJCC, American Joint Committee on Cancer; IPMN, intraductal papillary mucinous neoplasm; NA, not applicable; PDAC, pancreatic ductal adenocarcinoma.
*Age and race information were not collected from healthy controls.

mutations arising from chemically modified DNA. PCR was performed using the Platinum SuperFi DNA Polymerase after either the Ion Torrent Ion Amplicon Fusion library preparation method or the AmpliSeq library preparation protocol (all from Thermo Fisher Scientific). These two methods were directly compared using 6 reference samples (four disease control and two pancreatic ductal adenocarcinoma patient plasma DNA samples). No significant differences in the detection of mutations between the two methods was observed (Supplemental Table S3). The uracil N-glycosylase enzyme (Thermo Fisher Scientific), which removes uracil residues and results in abasic polynucleotides that are degraded with heat, was added to Platinum SuperFi Taq PCR buffers to limit the carryover of deaminated templates during PCR (uracil N-glycosylase inhibited AmpliSeq PCR and therefore was not used). For digital NGS, multiple (384 of each plasma DNA sample) aliquots were made (100 pg/well) and sequenced separately. If the DNA yield isolated from plasma was not sufficient for 384 NGS reactions (approximately 40 ng total), then fewer aliquots were sequenced (a minimum of 96 NGS reactions were undertaken per sample).

After library preparation, the libraries were cleaned using the Select-a-size DNA clean and concentrator kit (Zymo Research, Irvine, CA), quantified, and loaded into the OneTouch2 (Thermo Fisher Scientific) for emulsion PCR. After enrichment, samples were sequenced on the Ion Torrent PGM using 314v2 chips per the manufacturer's protocol.

Analysis after sequencing included alignment to the human genome assembly 19 human reference genome and variant calling, and was performed using NextGENe software version 2.4 (SoftGenetics, State College, PA). All mutations were verified visually using the Integrative Genomics Viewer version 2.3 (Broad Institute, Cambridge, MA).

A digital NGS score was determined for each hotspot mutation for each sample, with a score of 1 for each independent NGS aliquot with a mutation (up to 384). Background errors tend to arise in proportion to the number of DNA molecules sampled and vary by nucleotide, with more positives at sites of cytosine deamination.[31] The limit of detection for KRAS and GNAS hotspot mutations for the digital NGS assay was calculated by performing eight replicate digital NGS analyses of a wild-type DNA sample (approximately 5000 genome equivalents) on the AmpliSeq platform; the limit of detection (means plus 2 SDs) for the major hotspot mutations ranged from a digital NGS score of 0 for *KRAS* G12R and G12A to a score of 2 of 384 for G12D (approximately 1/5000 genome equivalents), 3 of 384 for G12V and G12C, and for *GNAS* 201C and 5 for *GNAS* 201H. A diagnostic cut-off value for each *KRAS* and *GNAS* hotspot mutation was determined by the means plus 2 SDs of the mean score in the disease control group. Based on this criterion, a positive ctDNA score for *KRAS* G12D was 5 positive wells per 384 tested; for *KRAS* G12V it was 3 positive wells per 384 tested; for G12R it was 2 positive wells per 384 tested; and for *GNAS* 201C and 201H it was 10 positive wells per 384 tested. A few samples were classified as borderline or indeterminate because the mutation score for the sample was just below the cut-off value set for a 384 digital

NGS assay, but fewer NGS reactions were performed (range, 96 to 288). For example, a positive digital NGS ctDNA score for *KRAS* G12D was 5 or more aliquots with mutations detected per 384 tested; an indeterminate result would be 4 positive wells of 288 analyzed.

Cancer Antigen 19-9

Preoperative cancer antigen 19-9 (CA19-9) levels were obtained from the patient's medical record when available ($n = 12$), or were measured in duplicate serum from blood samples obtained before pancreatic resection ($n = 33$) using the CA19-9 enzyme-linked immunosorbent assay kit (DRG International, Springfield Township, NJ). A cut-off value of 37 U/mL was considered increased.

Statistics

Categoric variables were summarized as frequencies (%) and compared with the χ^2 test or the Fisher exact test as appropriate. Sensitivity and specificity were calculated using 2×2 contingency tables. Statistical analysis was performed using GraphPad Prism software version 7 (GraphPad Software, San Diego, CA) and JMP 23 software version 14 (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

Results

Estimating Digital NGS Accuracy

Reference DNA samples developed by the National Institute for Standards and Technology (NIST) that contained *KRAS* (p.G12D) and *GNAS* (p.R201C) mutations spiked into wild-type genomic DNA at a variant allele frequency of either 2% or 0.5% were used to assess the reproducibility of this digital NGS assay.[32] For each sample, 1536 aliquots across six independent NGS experiments were separately sequenced. *KRAS* and *GNAS* mutations were detected at the expected variant concentrations (means ± SD, 2.0% ± 0.3% and 2.08% ± 0.43%, respectively, for the 2% variant allele frequency NIST sample and 0.60% ± 0.24% and 0.54% ± 0.17%, respectively, for the 0.5% variant allele frequency NIST sample).

To address whether DNA pretreatment could interfere with the detection of real mutations, NIST reference DNA samples were pretreated with the DNA repair cocktail (PreCR Repair Mix; New England Biolabs) in three independent experiments, and the variant concentrations for *KRAS* (p.G12D) and *GNAS* (p.R201C) with and without pretreatment were compared. A slight, but not significant, difference in variant allele frequencies for either *KRAS* (means ± SD, 2.12% ± 0.37% versus 1.84% ± 0.53%, untreated versus treated, $P = 0.40$, paired *t*-test) or *GNAS* (means ± SD, 1.91% ± 0.55% versus 1.71% ± 0.28%, untreated versus treated, $P = 0.60$, paired *t*-test) was observed using the 2% mutant DNA reference sample from NIST or the 0.5% sample (*KRAS*: means ± SD, 0.67% ± 0.36% versus 0.55% ± 0.12%, untreated versus treated, $P = 0.50$, paired *t*-test; *GNAS*: means ± SD, 0.55% ± 0.06% versus 0.50% ± 0.15, untreated versus treated, $P = 0.74$, paired *t*-test).

The current study also examined whether pretreatment with the PreCR repair cocktail could induce mutations. A control patient DNA sample was pretreated with the DNA repair cocktail in three independent experiments and observed no induction of either *KRAS* or *GNAS* mutations when compared with the untreated DNA.

Detection of KRAS Somatic Mutations in Patient Plasma Samples

Plasma DNA from 67 patients with pancreatic cancer and from 73 controls was analyzed using digital NGS for *KRAS* and *GNAS* mutations. The cancer cases included 10 with pancreatic cancer associated with an IPMN. Positive digital NGS scores indicating the presence of circulating *KRAS* mutations were detected in the plasma of 23 of 63 (36.5%) patients with pancreatic cancer (Table 2 and Supplemental Table S2). There was no significant difference in the likelihood of having detectable mutant *KRAS* ctDNA among patients who had (14 of 32) versus did not have (9 of 31) neoadjuvant therapy ($P = 0.3$). In addition, four patients with pancreatic cancer had mutation scores that were borderline and therefore classified as indeterminate. Digital NGS scores ranged from 3 to 94, corresponding to a 0.03% to 0.75% mutant concentration range in patients with a positive *KRAS* mutation. Three patients with pancreatic ductal adenocarcinoma had multiple *KRAS* mutations detected in their plasma; all three of these patients had undergone neoadjuvant therapy ($P = 0.24$). Two of 71 (2.8%) controls also had a positive test (Supplemental Table S1). One of these positive controls had a pancreatic lymphoma, the other was under pancreatic surveillance for their familial pancreatic cancer risk and had a small pancreatic cyst, however, importantly, no cancer at follow-up evaluation more than 2 years later.

When analyzed by stage of disease, a positive digital NGS score for mutant *KRAS* DNA was found in 14 of 45 (31.1%) of the resectable pancreatic cancer cases compared with 9 of 18 (50.0%) stage IV patients ($P = 0.16$, χ^2 test). These stage IV cases had small oligometastatic disease.

Although the lack of detection of ctDNA in many pancreatic cancer cases could be considered the result of limited shedding of mutated DNA into the circulation, another factor could be the presence of high concentrations of wild-type plasma DNA that decrease ctDNA to less than detectable levels. Indeed, ctDNA positivity was lowest among pancreatic cancer cases with the highest concentrations of total plasma DNA; among those in the highest quintile of plasma DNA concentration only one of the 15 cases was ctDNA positive versus 21 of the remaining 48 cases ($P = 0.02$).

Table 2 Somatic Mutations Identified in Plasma by Digital NGS

Case	Sex	Age, y	Study group	Stage	dNGS reactions, n	*KRAS* (dNGS score)	*GNAS* (dNGS score)
45	M	62	Disease control	NA	384	p.G12D (7)	p.R201H (11)
49	M	77	Disease control	NA	192	p.G12V (3)	
64	F	66	PDAC + IPMN	IA	384	p.G12D (7)	
65	M	44	PDAC + IPMN	IIB	192	p.G12D (18)	
67	M	64	PDAC + ITPN	IIB	384	p.G12V (94)	
70	M	76	PDAC	IV	384	p.G12V (15)	
73	M	50	PDAC	IV	374	p.G12D (17)	
77	F	66	PDAC	IB	384	p.G12V (4)	
82	M	54	PDAC	IV	384	p.G12R (45)	
84	F	66	PDAC	IV	384	p.G12V (8)	
86	F	75	PDAC	IIB	384	p.G12D (12)	
93	F	48	PDAC	IIB	384	p.G12D (25)	
94	F	66	PDAC	IV	384	p.G12D (5)	
96	M	61	PDAC	IV	384	p.G12D (47)	
98	F	59	PDAC	IV	192	p.G12D (21)	
100	M	89	PDAC	IB	384	p.G12V (4)	
101	M	55	PDAC	IB	384	p.G12D (72)/p.G12V (7)	
105	F	69	PDAC	IIB	241	p.G12D (50)	
115	F	67	PDAC	IV	384	p.G12D (21)/p.G12V (7)	
118	M	80	PDAC	IA	384	p.G12D (8)/p.G12V (8)	
120	F	68	PDAC	IIA	384	p.G12D (7)	
123	F	69	PDAC	IIB	384	p.G12D (9)	
125	F	68	PDAC	IA	384	p.G12D (6)	
126	M	89	PDAC	IV	384	p.G12D (63)	
127	M	65	PDAC	IA	384	p.G12D (5)	

F, female; M, male; dNGS, digital next-generation sequencing; IPMN, intraductal papillary mucinous neoplasm; ITPN, intraductal tubulopapillary neoplasm; NA, not applicable; NGS, next-generation sequencing; PDAC, pancreatic ductal adenocarcinoma.

Matched pancreatic cancer tissue was available for 34 cases. Tumor DNA was isolated from primary or metastatic lesions by laser capture microdissection, and the DNA for *KRAS* and *GNAS* mutations was analyzed. Three patients had a *KRAS* mutation detected in plasma DNA not found in their pancreatic cancer tissue DNA samples (Supplemental Table S4).

Detection of GNAS Somatic Mutations in Patient Plasma Samples

A positive digital NGS *GNAS* mutation score was found in the plasma of 1 of 67 (1.5%) patients with pancreatic cancer and in none of the controls. None of the patients diagnosed with an IPMN, including the nine cases of pancreatic cancer thought to have arisen from an IPMN, had detectable *GNAS* mutations in their plasma (Table 2).

Combined Diagnostic Performance of KRAS Mutations with CA19-9 in Plasma Samples

Next *KRAS* ctDNA status was combined with CA19-9 to compare diagnostic sensitivity versus *KRAS* ctDNA alone. An increased preoperative CA19-9 level was present in 19 of 45 (42.2%; 95% CI, 27.7%−57.9%) patients with resectable pancreatic ductal adenocarcinoma. The combined sensitivity of both ctDNA and CA19-9 was 66.7% (95% CI, 51.1%−80.0%).

Pretreatment of Plasma DNA and Cytosine Deamination

Several *KRAS* and *GNAS* mutation hotspots are susceptible to cytosine deamination *ex vivo*, and this aberrant cytosine deamination is a potentially significant source of background error when attempting to detect the very low concentrations of these mutations in plasma. In an attempt to reduce the background from chemical modifications of DNA, whether pretreating the DNA before sequencing reduces these cytosine deamination events was evaluated. To test the effect of chemical modification of DNA, plasma DNA from eight subjects was split and half were treated with an enzyme to repair damaged DNA [either PreCR Repair Mix (New England Biolabs), uracil N-glycosylase (Thermo Fisher Scientific), or both], the other half serving as the nontreated reference. The prevalence of mutations at the main mutation hotspots, including cytosine deamination events (C:G > T:A mutations) and mutations arising from oxidation in the *KRAS* and *GNAS* hotspots (*KRAS* G12D, G12V, G12R, G12A, G12C, G12S, and G13D, and *GNAS* R201C, R201H), were compared before and after treatment. There was a modest but statistically significant reduction in the mean number of wells called for mutations across these nine mutation hotspots after enzymatic pretreatment (means ± SD, 5 ± 4.3 versus 1.9 ± 1 mutation-

Table 3 Effect of DNA Pretreatment on Background Error in *KRAS* and *GNAS* Hotspot Mutations in Plasma Samples

			Mutation hotspot dNGS score									
Case	Study group	DNA treatment	G12A	G12C	G12D	G12V	G12R	G12S	G13D	R201C	R201H	Overall dNGS score
2	Healthy control	None	0	0	1	0	0	0	0	0	0	1
		UNG	0	0	0	0	0	0	0	0	1	1
3	Healthy control	None	0	0	0	1	0	3	2	5	2	13
		UNG	0	0	1	0	0	0	0	2	0	3
4	Healthy control	None	0	0	0	0	0	0	0	0	1	1
		UNG	0	0	1	0	0	0	0	1	0	2
17	Disease control	None	0	0	3	5	0	0	1	0	0	9
		PreCR + UNG	0	0	1	1	0	0	0	1	0	3
18	Disease control	None	0	0	0	3	0	0	1	2	0	6
		PreCR + UNG	0	0	0	2	0	0	0	0	0	2
33	Disease control	None	0	0	0	1	0	0	0	0	1	2
		PreCR + UNG	0	0	0	1	0	1	0	0	0	2
68	PDAC	None	0	0	0	2	0	0	0	0	0	2
		PreCR + UNG	0	0	0	0	0	0	0	0	0	0
121	PDAC	None	0	0	2	3	0	0	0	0	1	6
		PreCR + UNG	0	0	0	1	0	0	0	0	1	2

The mean number of positive wells calculated for untreated samples was 5 compared with 1.9 for pretreated DNA samples ($P = 0.047$).
dNGS, digital next-generation sequencing; PDAC, pancreatic ductal adenocarcinoma; UNG, uracil N-glycosylase.

positive NGS; $P = 0.047$; paired *t*-test). These mutation calls were found at the hotspots most prone to background error such as from cytosine deamination (Table 3).

Digital NGS Scores in Controls

Many plasma DNA samples from pancreatic cancer cases and controls had low digital NGS scores below the threshold for calling them positive. Many of these low digital NGS scores were considered likely to be a consequence of chemical modifications of DNA occurring before PCR and NGS. Most subthreshold digital NGS scores detected in plasma DNA samples arose at sites of cytosine deamination, with the highest levels found at *GNAS* R201H and R201C. Among the disease controls, the mean digital NGS score was significantly higher at the nucleotide positions subject to cytosine deamination (*KRAS* G12D, G12S, G13D, GNAS R201C, and R201H) than at other mutation hotspots (*KRAS* G12V, G12R, G12A, and G12C) (median digital NGS score, 1 versus 0, Wilcoxon signed rank, $P = 0.0001$). Within the disease controls, there was no significant difference in the subthreshold plasma *KRAS* or *GNAS* digital NGS scores by age, sex, or disease state. For example, there was also no significant difference in the mean levels of subthreshold digital NGS scores in patients with pancreatic cysts than in disease controls without a pancreatic cyst, and the patients with a pancreatic cyst were no more likely than controls without a pancreatic cyst to have a subthreshold digital NGS score (data not shown).

Subthreshold *KRAS* mutation scores were higher in patients with pancreatic cancer than in controls (means ± SD, 2.8 ± 2.1 and 1.8 ± 2.1, respectively; $P = 0.006$) suggesting that some subthreshold digital NGS scores in patients with pancreatic cancer reflect the presence of ctDNA.

Discussion

In this study, digital NGS was evaluated as a method for detecting low-abundance mutations in the circulation. The potential utility of using enzymatic pretreatment of plasma DNA to reduce background errors arising from chemical modification of DNA was also evaluated. Overall, the diagnostic yield of digital NGS for detecting mutant *KRAS* ctDNA is similar to what has been reported recently using other methods.[14] Evidence that enzymatic pretreatment of plasma DNA reduced background errors detected by NGS at mutation hotspots prone to chemical degradation such as by cytosine deamination, although pretreatment did not eliminate such errors and the overall effect was modest. No biological variables were found to predict the presence or absence of a low-level digital NGS score detected in the plasma DNA samples of controls. This supports the hypothesis that many subthreshold digital NGS scores are the result of chemical modifications of plasma DNA and PCR and sequencing errors generated by the assay rather than true mutations.

Two patients with pancreatic cancer had discordant results between their primary tumor (formalin-fixed, paraffin-embedded) and their ctDNA (plasma). This may reflect clonal heterogeneity within the primary pancreatic cancer, or false-positive ctDNA results. Tumor heterogeneity with respect to mutant *KRAS* has identified a small percentage of primary pancreatic cancers.[33] Therapy-induced clonal evolution can induce the emergence of subclones,[34,35] and both of these patients had undergone neoadjuvant chemoradiotherapy. Consistent with this, three patients with pancreatic ductal adenocarcinoma were found to have more than one *KRAS* mutation detected in their ctDNA, all of whom had undergone neoadjuvant therapy.

One notable finding is the almost complete lack of positive digital NGS scores for *GNAS* mutations in patients with pancreatic cancer (1.5% of cases). This low rate of *GNAS* mutations in patients with pancreatic cancer is consistent with the predominant role of the pancreatic intraepithelial neoplasia pathway rather than the IPMN pathway in the development of the overwhelming majority of pancreatic cancers. *GNAS* mutations are not detected commonly in usual pancreatic ductal adenocarcinomas (<5%)[33,36,37] or its most common precursor, pancreatic intraepithelial neoplasm,[38] indicating that many pancreatic cancers that develop in patients with pancreatic cysts arise from their pancreatic intraepithelial neoplasia rather than their IPMN. In patients under surveillance for their IPMN, pancreatic cancers often arise in areas of the pancreas away from IPMN,[2,22] and many pancreatic cancers judged by surgical pathology to involve the IPMN are genetically distinct from the IPMN.[39] A prior study had reported *GNAS* mutations often were present in the plasma of patients with pancreatic cysts.[5] Subthreshold levels of *GNAS* hotspot mutations were found to be no different in controls with pancreatic cysts compared with those without, providing evidence that subthreshold levels likely were not true mutations but background errors.

The low diagnostic sensitivity of mutant *KRAS* ctDNA as a potential test for patients with early stage pancreatic cancer points to the need for better approaches to detect ctDNA. Methods to detect low-abundance mutations need to account for background errors generated either by chemical modification of DNA, or errors generated by PCR/NGS. Digital NGS is one approach to minimize the effect of background errors when attempting to identify low-abundance mutations and chemical pretreatment of DNA is another. Other approaches include Safeseq[16] which uses barcoding of DNA templates to trace errors generated during DNA amplification and Tec-seq,[17] of which the latter combines template barcoding with other steps to minimize other sources of error such as from clonal hematopoiesis that give rise to mutations in *TP53* and other genes. Some approaches to detect ctDNA use the detection of changes in DNA fragmentation,[40] copy number imbalances rather than mutations aided by size selection to enrich for circulating tumor DNA.[21] Head-to-head comparisons of different ctDNA detection methods will help identify the best approaches in different patient populations.

Given the low diagnostic sensitivity of ctDNA detection, alternative markers are needed that can diagnose patients with early stage pancreatic cancer. One approach to improve diagnostic sensitivity is to combine ctDNA and protein markers as performed in CancerSeek[41] or other similar tests. This type of panel test may have utility for the early detection of many cancers. For pancreatic cancer, diagnostic test performance characteristics need to be very high to accurately identify most patients with stage I disease and to do so without generating many false positives. For now, the best approach to identify stage I pancreatic cancers in high-risk individuals under surveillance is to use pancreatic imaging, especially endoscopic ultrasound and magnetic resonance imaging/magnetic resonance cholangiopancreatography.[42] Mutational analysis of pancreatic juice collected from the duodenal lumen during endoscopic ultrasound can identify mutations arising from pancreatic cancers, often at higher concentrations than that found in the circulation, and occasionally have been identified in prediagnostic samples.[22,24] For this reason, pancreatic juice analysis may diagnose pancreatic cancer earlier than ctDNA analysis. Precursor lesions such as pancreatic intraepithelial neoplasia and IPMN also shed mutated DNA into pancreatic juice, and the grade of neoplasia in the pancreas (low-grade neoplastic precursors from high-grade dysplasia and invasive cancer) can be estimated with good accuracy from the overall mutational profile.[22,24]

In conclusion, digital NGS identifies ctDNA in patients with pancreatic cancer with high specificity. Pretreatment of plasma DNA also may help reduce background errors that can lead to false-positive detection of mutations.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2020.02.010*.

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