Original Article

Identification of an optimal mutant allele frequency to detect activating *KRAS*, *NRAS*, and *BRAF* mutations in a commercial cell-free DNA next-generation sequencing assay in colorectal and pancreatic adenocarcinomas

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Background: Evaluation for activating mutations in *KRAS*, *NRAS*, and *BRAF* in colorectal cancer (CRC) and in *KRAS* in pancreatic ductal adenocarcinoma (PDAC) is essential for clinical care. Plasma cell-free DNA (cfDNA) next-generation sequencing (NGS) allows convenient assessment of a tumor's molecular profile, however low tumor DNA shedding limits sensitivity. We investigated mutant allele frequency (MAF) of other oncogenic dominant genes to identify a threshold for accurate detection of *KRAS*, *NRAS*, and *BRAF* *(RAS/RAF)* mutations in cfDNA.

Methods: Molecular and clinical data were obtained from the Duke Molecular Registry of Tumors and the SCRUM-Japan GOZILA study. Patients with CRC or PDAC and a *KRAS*, *NRAS*, or *BRAF* activating single

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nucleotide variant (SNV) present on tissue NGS and with available cfDNA assays were included. Recursive partitioning and Wilcoxon-rank statistics methods identified potential cut-points for discriminative MAF values.
Results: One hundred and thirty-five CRC and 30 PDAC cases with 198 total cfDNA assays met criteria. Greatest non-*RAS/RAF* dominant gene MAF of 0.34% provided maximum discrimination for predicting *RAS/RAF* SNV detection. Sensitivity for *RAS/RAF* SNVs increased with dominant gene MAF, with MAF ≥1% predicting sensitivity >98%, MAF between 0.34 and 1% predicting sensitivity of 84.0%, and MAF ≤0.34% predicting sensitivity of 50%. For 43 cfDNA assays that did not detect *RAS/RAF* SNVs, 18 assays detected 34 other oncogenic variants, of which 80.6% were not also detected on tissue.
Conclusions: Non-*RAS/RAF* dominant oncogenic mutation MAF ≥1% on cfDNA NGS predicts high sensitivity to detect *RAS/RAF* oncogenic SNVs in CRC and PDAC. MAF ≤0.34% indicates an assay may not reliably detect *RAS/RAF* SNVs, despite detection on tissue testing. Most variants from assays that did not detect *RAS/RAF* had MAF <1% and were not detected on tissue, suggesting potential confounding. These data suggest a practical approach to determining cfDNA assay adequacy, with implications for guiding clinical decisions in CRC and PDAC.

Keywords: Cell-free DNA (cfDNA); colorectal cancer (CRC); pancreatic cancer

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Introduction

In colorectal cancer (CRC), activating single-nucleotide variant (SNV) mutations in *KRAS*, *NRAS*, and *BRAF* are common (1-3), present in 39–44%, 4–8%, and 5–9% of patients, respectively. Therapy with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (mAb) alone or in combination with chemotherapy is an important therapeutic option in *RAS/RAF* wild-type (WT) CRC (4,5). Retrospective analyses comparing anti-EGFR therapy with the anti-vascular endothelial growth factor (VEGF) mAb bevacizumab in combination with chemotherapy have suggested superior efficacy for anti-EGFR therapy in left-sided metastatic *RAS/RAF* WT CRC as first line therapy (6), which has recently been confirmed in results from the prospective randomized PARADIGM trial (7). In contrast, the presence of activating SNVs in *KRAS/NRAS* and $BRAF^{V600}$ predict resistance to anti-EGFR mAb (8,9) and alternative targeted therapies for $BRAF^{V600}$ (10-12), and $KRAS^{G12C}$ (13-15), are now available for patients progressing on first-line therapy. These data and the increasing availability of targeted treatments solidifies the importance of accurate ascertainment of *RAS/RAF* status prior to first line therapy in CRC, supported in national guidelines (16).

In pancreatic ductal adenocarcinoma (PDAC), 85–90% of patients harbor *KRAS* activating mutations (17), with a small fraction harboring targetable $KRAS^{G12C}$ mutations (13,18,19). Patients with *KRAS* WT PDAC often have actionable fusion alterations (20-24), providing additional therapeutic options and spurring new treatment strategies. In addition, there are several novel molecular and immune-based approaches targeting *KRAS*-mutated cancers in

Highlight box

Key findings

- Increasing mutant allele frequency (MAF) in cell-free DNA (cfDNA) sequencing predicts sensitivity for *KRAS*, *NRAS*, and *BRAF* mutations in colorectal and *KRAS* mutations in pancreatic adenocarcinoma.

What is known and what is new?

- Sequencing of cfDNA offers a convenient, safe, and relatively rapid method to assess a tumor's molecular profile. However, sensitivity may be limited by low tumor DNA shedding into the plasma.
- This analysis uses MAF as a surrogate for tumor DNA content in a commercial cfDNA assay to predict sensitivity for clinically important mutations in colorectal and pancreatic ductal adenocarcinoma.

What is the implication, and what should change now?

- These data demonstrate practical and easily applied MAF thresholds to determine cfDNA assay adequacy to detect *KRAS*, *NRAS*, and *BRAF* mutations that may affect treatment selection. If an assay is deemed adequate, a patient may proceed to appropriately selected treatment more quickly or avoid an invasive tissue biopsy.

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development that will be of particular interest in pancreatic cancer given limited therapeutic options in this malignancy (25,26). Finally, *KRAS* mutational status carries prognostic significance in PDAC (27). Thus, accurate molecular testing to determine *KRAS* mutational status (although not *NRAS* or *BRAF*, as in CRC) is increasingly important in PDAC, both to allow access to novel targeted therapies and also because a negative *KRAS* result should prompt an extensive evaluation for alternative driver events, including rare fusions.

Sequencing of cell-free DNA (cfDNA) derived from plasma provides a convenient method to assay a tumor's molecular profile (28). In comparison to tissue biopsies, advantages of sequencing cfDNA include avoidance of invasive procedures, speed to rapidly assess a tumor when urgent treatment is required, ease of sequential repeat assays over time, and assessment of the full spectrum of a cancer's molecular profile. However, the sensitivity of cfDNA assays for actionable alterations is limited by the proportion of circulating tumor DNA (ctDNA) in the plasma (29). ctDNA content and thus sensitivity is affected by many factors, including volume of disease, location of metastases, and tumor specific factors (30). Overall, metastatic CRC exhibits high rates of detectable ctDNA in clinical practice, while PDAC has among the lowest rates of detectable ctDNA (31). However, CRC patients with metastases to either only lung or to the peritoneum have lower ctDNA content compared with liver metastases or more extensive multi-focal disease (32). Relatively low ctDNA shedding in PDAC is thought to be related to several factors, including low neoplastic cellularity and a highly desmoplastic stromal environment that restricts shedding (33).

In addition, cfDNA sequencing may detect mutations from sources other than the target malignancy. The detection of mutations in peripheral blood leukocytes, i.e., clonal hematopoiesis (CH), is a now well-documented phenomenon (34). Because the majority of cfDNA is derived from leukocytes, even very small CH clones may be detected on cfDNA next-generation sequencing (NGS) testing (35). While the most common CH mutations, such as *DNMT3A* and *TET2*, are specific to CH and thus easily recognized as myeloid in origin, mutations in *TP53* and more rarely *KRAS* and *BRAF*, among others, are also seen in CH and may be difficult to distinguish from solid tumor-derived variants (35-39). The large majority of these confounding mutations are seen at a mutant allele frequency (MAF) <1% (35). Both low ctDNA shedding and potential confounding mutations present a challenge to assessing cfDNA assay adequacy.

To address this issue, Meador *et al.* examined sensitivity in cfDNA for *EGFR* and *KRAS* mutations in non-small cell lung cancer (NSCLC) based on the maximum allele frequency of other dominant variants in the cfDNA (40). Their data showed that cfDNA with dominant gene maximum allele frequency of >1% predicted 100% sensitivity for *EGFR* and *KRAS* mutations. Greater resolution was limited by small patient numbers (n=29) with maximum allele frequency below 1% and the assay itself was institution-specific, limiting broad applicability. Nonetheless, the idea of a simple quantitative threshold to predict assay sensitivity is appealing for the practicing oncologist. Similarly, Nakamura *et al.* proposed an identical threshold of >1% as sufficient to assess microsatellite instability in Guardant cfDNA assays (41).

Defining both mutated and WT status for *RAS/RAF* mutations is important for appropriate care in CRC and PDAC, however the sensitivity for oncogenic *RAS/RAF* SNVs in the cfDNA of these cancers based on non-*RAS/RAF* oncogenic variant MAF is not known. Thus, we explored a clinically applicable MAF threshold in the Guardant360 cfDNA commercial assay for detection of *KRAS*, *NRAS*, or *BRAF* oncogenic mutations in CRC and PDAC tumors. We present this article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-114/rc).

Methods

Patient populations

Patient molecular and clinical data were obtained from an internal database of patients with CRC and PDAC undergoing treatment at Duke Cancer Institute (DCI) who received cfDNA NGS testing and from the SCRUM-Japan GOZILA study (42). Only patients with metastatic disease at the time of cfDNA testing were included. GOZILA is a plasma genomic profiling study involving 31 core cancer institutions in Japan. Patients with metastatic gastrointestinal cancers were eligible for GOZILA; patients with CRC and PDAC were selected for this study. Eligible patients for GOZILA provided written informed consent, including publication of any materials. To avoid the suppression of ctDNA shedding because of chemotherapy, the GOZILA patients were required to have disease progression following systemic chemotherapy

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and to have not yet started subsequent therapy at the time of blood sampling. For GOZILA, patients were enrolled and plasma genotyping took place between January 2018 and February 2021.

All comprehensive genomic profiling (CGP) tests performed at DCI were ordered at the discretion of oncology providers as a component of routine cancer care and were stored in the electronic medical record as well as an internally developed data warehouse solution called the Duke Molecular Registry of Tumors (MRT) (43). Approval for the establishment of MRT as a clinical and research data repository was granted by the IRB of the Duke University Medical Center (DUMC) (No. Pro00085260). Specific approval for this research collaboration and waiver of consent was also granted by the DUMC IRB (No. Pro00109863).

All included patients underwent tissue CGP with commercially available assays. Duke patients underwent tissue testing with FoundationOne CDx (Foundation Medicine). The GOZILA cohort underwent tissue testing with the Oncomine Comprehensive Assay (ThermoFisher). These tissue-based assays are multi-gene panels that cover and report genes of interest to this study, specifically activating mutations in *KRAS* and *NRAS* exons 2, 3, and 4, and *BRAF*. Guardant360 (Guardant Health) was the sole cfDNA assay used for both Duke and GOZILA patients.

Patients with CRC or PDAC with both tissue and cfDNA assays available were identified in the GOZILA and MRT databases, and tumors with oncogenic activating SNVs in *KRAS*, *NRAS*, or *BRAF* identified on tissue-based sequencing were selected for further analysis. Activating mutations in *KRAS* and *NRAS* exons 2-4 (codons 12, 13, 34, 61, 117, and 146), *BRAF* codon V600, and oncogenic, non-*BRAF* V600 Class II and Class III variants were included (see Table S1 for all included variants). Although *BRAF* Class II and Class III variants do not currently guide clinical decision making for anti-EGFR therapy, these variants have been shown to be biologically relevant in CRC and could potentially guide clinical trial enrollment, and were thus included in this study (44,45). In two instances in which an *NRAS* or *BRAF* non-V600 mutation was found with a *KRAS* mutation on the tissue sample, only the *KRAS* mutation was included in the cfDNA sensitivity analysis in order to avoid redundancy.

These studies were conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects. All study protocols were approved by the institutional review board of each participating institution and the GOZILA study was registered at the University Hospital Medical Information Network Clinical Trials Registry (No. UMIN000042612).

cfDNA assay characteristics

Guardant360 CDx is a cfDNA NGS assay that reports SNVs and insertions/deletions (Indels) for 74 genes, in addition to 18 copy number alterations and 6 fusion products not relevant to the current study (46). Guardant reports analytical sensitivity for SNVs and Indels of 99.7–100% based on MAF of ≥0.5%, and 70.6–86% based on MAF <0.5% with 30 ng of input DNA. Guardant360 CDx limit of detection (LoD) is reported at 0.8–3% MAF per copy number at 5 ng input DNA and 0.1–0.8% MAF per copy number for 30 ng of input DNA.

Variant analysis

All variant analysis was performed by Guardant Health. Variants of uncertain significance (VUS) were excluded from the analysis. All clinically reported, non-VUS alterations were considered oncogenic and included in the analysis. Comprehensive germline variant testing was not available and so germline variants could not be definitively excluded. In addition, mutations in *JAK2* were excluded as this gene is strongly associated with CH but lacks a known biologic role in CRC or PDAC. Other similar genes commonly associated with CH, such as *DNMT3A* and *TET2*, are not reported in the Guardant360 CDx assay (46).

Analytic plan

Each available cfDNA assay was treated independently. On each cfDNA assay, detection of the tissue *RAS/RAF* mutation was noted, and the non-*RAS/RAF* oncogenic mutation ("dominant" mutation) with the greatest MAF was identified. Assays with no detected non-*RAS/RAF* oncogenic alterations were considered MAF =0. The assays were then analyzed for sensitivity based on MAF. We also selected cfDNA NGS assays that did not successfully detect *RAS/RAF* mutations for further analysis. Of these, we identified assays with oncogenic variants present, and compared these variants to results from tissue NGS assays. Concordant and non-concordant variants were noted for each individual patient.

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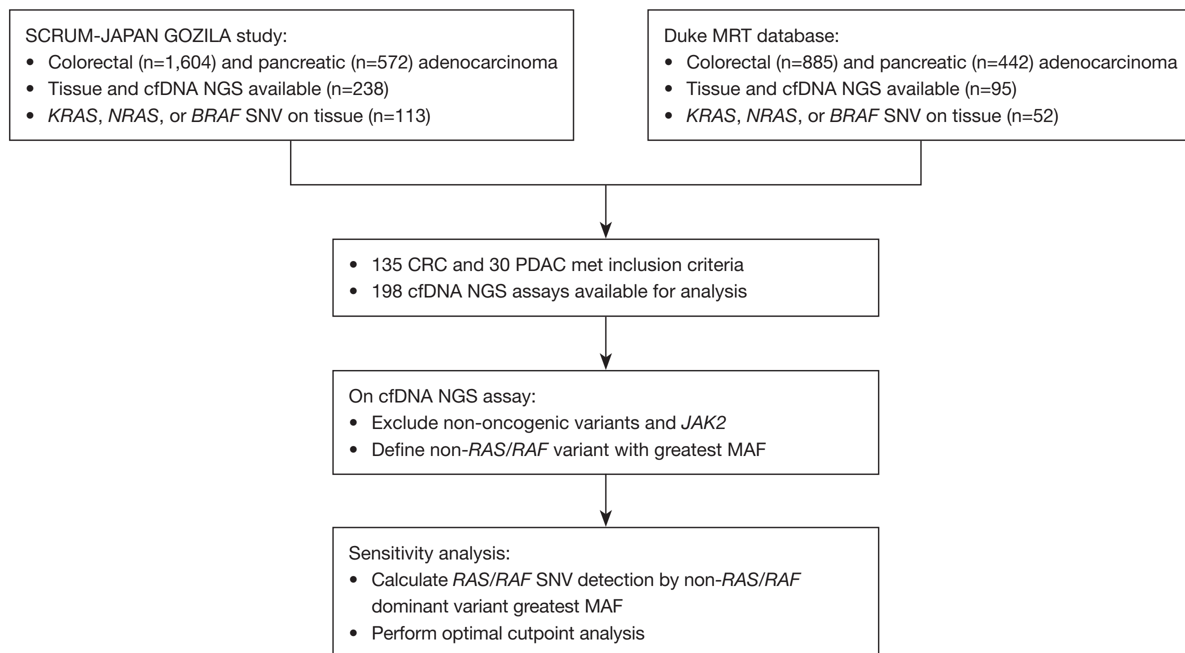

Figure 1 Workflow schematic for patient selection and *RAS/RAF* SNV sensitivity analysis. Patients with CRC and PDAC with activating SNVs in *KRAS*, *NRAS*, or *BRAF* and both tissue and cfDNA NGS assays available were selected for analysis. Predicted benign variants and *JAK2* were excluded. The non-*RAS/RAF* dominant mutations with greatest MAF were identified and *RAS/RAF* SNV detection by greatest MAF was calculated. cfDNA, cell-free DNA; NGS, next-generation sequencing; SNV, single nucleotide variant; MRT, magnetic resonance tomography; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; MAF, mutant allele frequency.

Statistical analysis

We employed recursive partitioning and a maximally selected rank statistic to identify optimal cutpoints of MAF for sensitivity. We then used the resulting optimal cutpoint to select ranges of dominant oncogenic mutation greatest MAF below 1% to calculate sensitivity for *RAS/RAF* SNVs, and predefined intervals of 1–5%, 5–10%, and >10% MAF for ranges above 1%. Recursive partitioning (https://cran.r-project.org/web/packages/rpart/index.html) splits the data by the MAF values and the proportion of the group where *RAS/RAF* SNVs are detected (47). Each cutpoint maximizes the number of detected *RAS/RAF* SNVs in one group *vs.* the group below the cutpoint. The maxstat package (https://cran.r-project.org/web/packages/maxstat/index.html) tests every possible cutpoint where reasonable sample sizes exist in each group and identifies the cutpoint at which the probability of *RAS/RAF* SNV detection is maximized using a Wilcoxon rank statistic (48). All analyses were done using R version 4.1.3 (49) (2022-03-10) within RStudio 2022.7.1.554 (50).

Results

Patient characteristics

Of 1,604 CRC and 572 PDAC patients enrolled on the GOZILA study, and 885 CRC and 442 PDAC patients from the Duke MRT, 165 total patients with 198 cfDNA assays met criteria and were included in the analysis (*Figure 1*). Clinical characteristics of this cohort, overall and by cancer type, as well as primary tumor site within the colon or pancreas, are shown in *Tables 1,2*. The GOZILA study contributed 108 of 135 CRC and the Duke MRT contributed 25 of 30 PDAC cases. Patients in the GOZILA cohort were more likely to have undergone resection of the primary tumor. Number and distribution of metastatic sites at time of initial cfDNA NGS testing is shown in Table S2.

Twenty-two point two percent of patients with CRC received anti-EGFR mAb therapy at some point in their clinical course (Table S3), however all patients had comprehensive testing on tissue obtained prior to anti-EGFR therapy. Most of these patients had *BRAF* mutations and received anti-EGFR therapy either as part of a clinical

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Table 1 Patient characteristics

Characteristic	CRC + PDAC	CRC	PDAC
Full cohort, n	165	135	30
Duke MRT	53	27	25
GOZILA-SCRUM	113	108	5
Age at 1st treatment, median [range]	60 [30–83]	60 [30–83]	62 [36–82]
Female, n (%)	86 (52.1)	72 (53.3)	14 (46.7)
Race/ethnicity, n (%)			
Asian	115 (69.7)	109 (80.7)	6 (20.0)
Caucasian	38 (23.0)	17 (12.6)	21 (70.0)
African-American	11 (6.7)	8 (5.9)	3 (10.0)
Native American	1 (0.6)	1 (0.7)	0
Hispanic ethnicity	0	0	1 (3.3)

CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; MRT, Molecular Registry of Tumors.

Table 2 Cancer primary site

Primary site	Patients, n (%)
Colorectal primary	
Cecum	18 (13.3)
Ascending	25 (18.5)
Transverse	11 (8.1)
Descending/sigmoid	42 (31.1)
Rectal	36 (26.7)
Unknown	3 (2.2)
Primary resected, full cohort	96 (71.1)
Duke MRT	14 (51.9)
GOZILA-SCRUM	82 (75.9)
Pancreatic primary	
Head	15 (50.0)
Body	7 (23.3)
Tail	8 (26.7)
Primary resected, full cohort	4 (13.3)
Duke MRT	1 (4.0)
GOZILA-SCRUM	3 (60.0)

MRT, Molecular Registry of Tumors.

trial, as SOC in combination with BRAF/MEK inhibitors, or harbored a *BRAF* Class II or III mutation that was not considered a contraindication to anti-EGFR therapy. Five patients received anti-EGFR despite predicted resistance, though the clinical reasoning was not readily discernable; in at least one instance CGP on a past tissue sample revealed a previously undetected *KRAS* mutation.

Patient molecular testing

Among CRC patients, *KRAS* SNV mutations were most common (98/135, 72.6%) followed by *BRAF* (35/135, 25.9%) and *NRAS* (4/135, 3.0%) (*Table 3*, Table S1). For the 2 patients with a concurrent *KRAS* and either *BRAF* or *NRAS* mutation, only the *KRAS* mutation was considered for the purposes of the primary analysis. All 30 patients with PDAC harbored *KRAS* mutations.

One hundred and ninety-eight total cfDNA assays were included in the analysis. Twenty patients contributed 2 or more assays to the analysis (*Table 3*). Median time from reference tissue NGS to cfDNA NGS was 293.5 (range, −712 to 2,512) days for all assays, 315 (range, −316 to 2,512) days for CRC assays, and 10.5 (range, −712 to 1,403) days for PDAC assays. CRC, PDAC, and all assays combined had tissue and cfDNA NGS obtained within +/− 90 days in 15.3%, 42.3%, and 19.3% of assays respectively. The large majority of patients received systemic therapy between tissue and cfDNA assays.

No oncogenic, non-VUS alterations were detected in 9.0% of CRC and 32.3% of PDAC cfDNA assays (*Table 3*). *TP53* variants were the most frequently detected mutations

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Table 3 cfDNA NGS assay summary

Characteristic	CRC + PDAC (n=165)	CRC (n=135)	PDAC (n=30)
Total cfDNA assays, n	198	167	31
Patients with 2+ assays, n (%)			
2	8 (4.8)	7 (5.2)	1 (3.3)
3	11 (6.7)	11 (8.1)	0
4	1 (0.6)	1 (0.7)	0
Time from tissue NGS to cfDNA NGS specimen collection			
Complete data, n (%)	176/198 (88.9)	150/167 (89.8)	26/31 (83.9)
Median days (range)	293.5 (–712 to 2,512)	315 (–316 to 2,512)	10.5 (–712 to 1,403)
n within 90 days (%)	34/176 (19.3)	23/150 (15.3)	11/26 (42.3)
Systemic therapy between tissue and cfDNA NGS, n (%)			
Complete data	172/198 (86.9)	146/167 (87.4)	26/31 (83.9)
Treatment	143/172 (83.1)	126/146 (86.3)	17/26 (65.4)
RAS/RAF SNV on tissue, n (%)			
KRAS	128 (77.6)	98 (72.6)	30 (100.0)
NRAS	4 (2.4)	4 (3.0)	
BRAF	35 (21.2)	35 (25.9)	
RAS/RAF SNV present on cfDNA NGS, n (%)	155/198 (78.3)	138/167 (82.6)	17/31 (54.8)
No oncogenic variants detected in cfDNA, n (%)	25/198 (12.6)	15/167 (9.0)	10/31 (32.3)
Dominant mutation with greatest MAF, gene (% of detected)		TP53 (53.9)	TP53 (80.0)
		APC (21.1)	

cfDNA, cell-free DNA; NGS, next-generation sequencing; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; SNV, single nucleotide variant; MAF, mutant allele frequency.

with greatest MAF. The *RAS/RAF* SNV detected on tissue testing was also detected in 82.6% of CRC cfDNA assays and 54.8% of PDAC cfDNA assays (*Table 3*). Number of metastatic sites and solitary site of metastasis stratified by successful detection of *RAS/RAF* SNV in cfDNA is shown in Table S2. Twenty point seven percent of CRC had only 1 site of metastatic disease at time of the cfDNA assay, and of these only 50% had successful detection of *RAS/RAF*. For CRC with 2 or sites of disease, detection of *RAS/RAF* SNVs was numerically greater at 86.8%. Forty-three point three percent of PDAC had 1 site of metastatic disease, with 61.5% successful detection of the *KRAS* SNV.

Sensitivity for RAS/RAF mutations by MAF

Oncogenic, non-*RAS/RAF* dominant oncogenic variants with the greatest cfDNA MAF were identified and patients were stratified by detection of the *RAS/RAF* SNV (*Figure 1*). Pairs of *RAS/RAF* and dominant mutations with associated MAF are shown in Table S1. The distribution of dominant variants with greatest MAF stratified by cancer and detection of *RAS/RAF* SNVs is shown in *Figure 2A*. Recursive partitioning and maximally selected rank statistic methods were employed to identify the optimal MAF discrimination for sensitivity for *RAS/RAF* SNVs while excluding the fewest assays, with both methods yielding an MAF of 0.34%. Cutpoints above 0.34% improve sensitivity marginally but exclude a greater portion of total assays. For example, 76 of 198 total assays (38.4%) fell below the MAF threshold at an MAF of 1% compared with 50 of 198 assays (25.3%) at MAF 0.34% (Figure S1). In contrast, cfDNA assays in which the *RAS/RAF* SNV was not detected rise sharply with dominant gene MAF below 0.34%, but exclude significantly fewer assays (Figure S1). Ninety-six percent of

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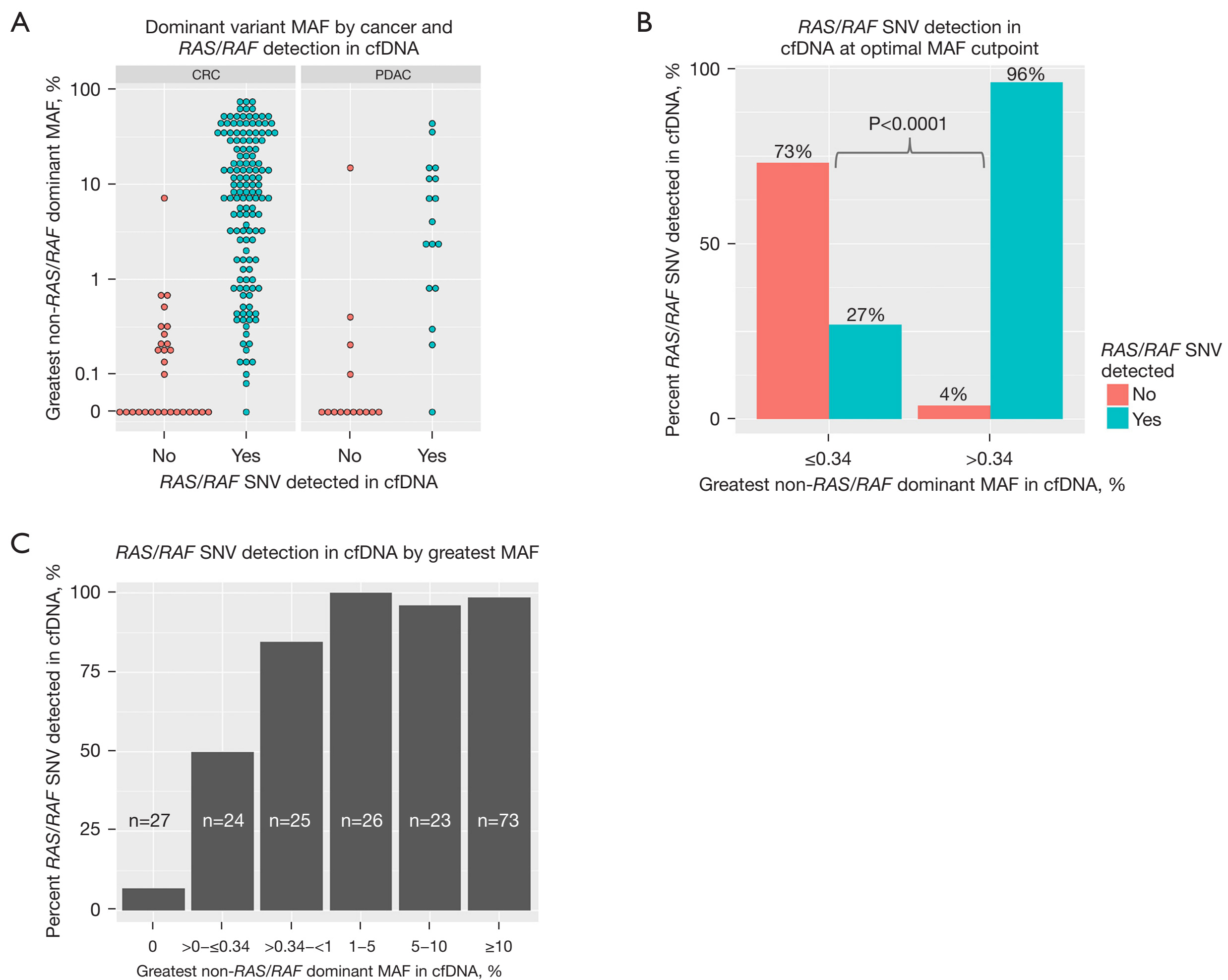

Figure 2 Analysis of CRC and PDAC patients with matched tissue and cfDNA NGS. (A) Distribution of MAF of greatest dominant mutation of each assay, stratified by cancer and detection of *RAS/RAF* SNVs. Y-axis is log10. (B) Percent *RAS/RAF* detected versus not detected with greatest dominant MAF below or above the optimal cutpoint of 0.34%, determined by maximally selected rank Wilcoxon rank statistic (P<0.0001) and recursive partitioning. (C) Sensitivity for the *RAS/RAF* SNV increases with greatest MAF of the dominant oncogenic mutation. Assays were binned by ranges of greatest dominant MAF. Percent *RAS/RAF* SNV detected (sensitivity) in each bin is shown. For dominant MAF >0% and ≤0.34%, sensitivity for *RAS/RAF* SNVs was 50%. For dominant MAF >0.34% and <1%, sensitivity was 84.0%. For dominant MAF ≥1%, sensitivity was >98%. MAF, mutant allele frequency; SNV, single nucleotide variant; cfDNA, cell-free DNA; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma.

RAS/RAF SNVs were detected with MAF >0.34%, while only 27% were detected ≤0.34% (significant at P<0.0001 by Wilcoxon rank statistic, *Figure 2B*). Sensitivity analysis by ranges of greatest dominant MAF (*Figure 2C*) confirmed increasing sensitivity with increased dominant MAF; *RAS/RAF* sensitivity was >98% with MAF greater than 1%, fell to 84.0% for MAF between 0.34 and 1%, and was only 50.0% for MAF >0 and ≤0.34%.

Analysis of false negative cfDNA assays

Next, the 43 cfDNA NGS assays in which the *RAS/RAF* SNVs were not detected (i.e., false negatives) were examined. Oncogenic variants were detected in 18 of these 43 cfDNA assays while the remaining 25 had no oncogenic variants detected (*Figure 3A*). For the 18 patients with detected oncogenic variants, the individual assay with the

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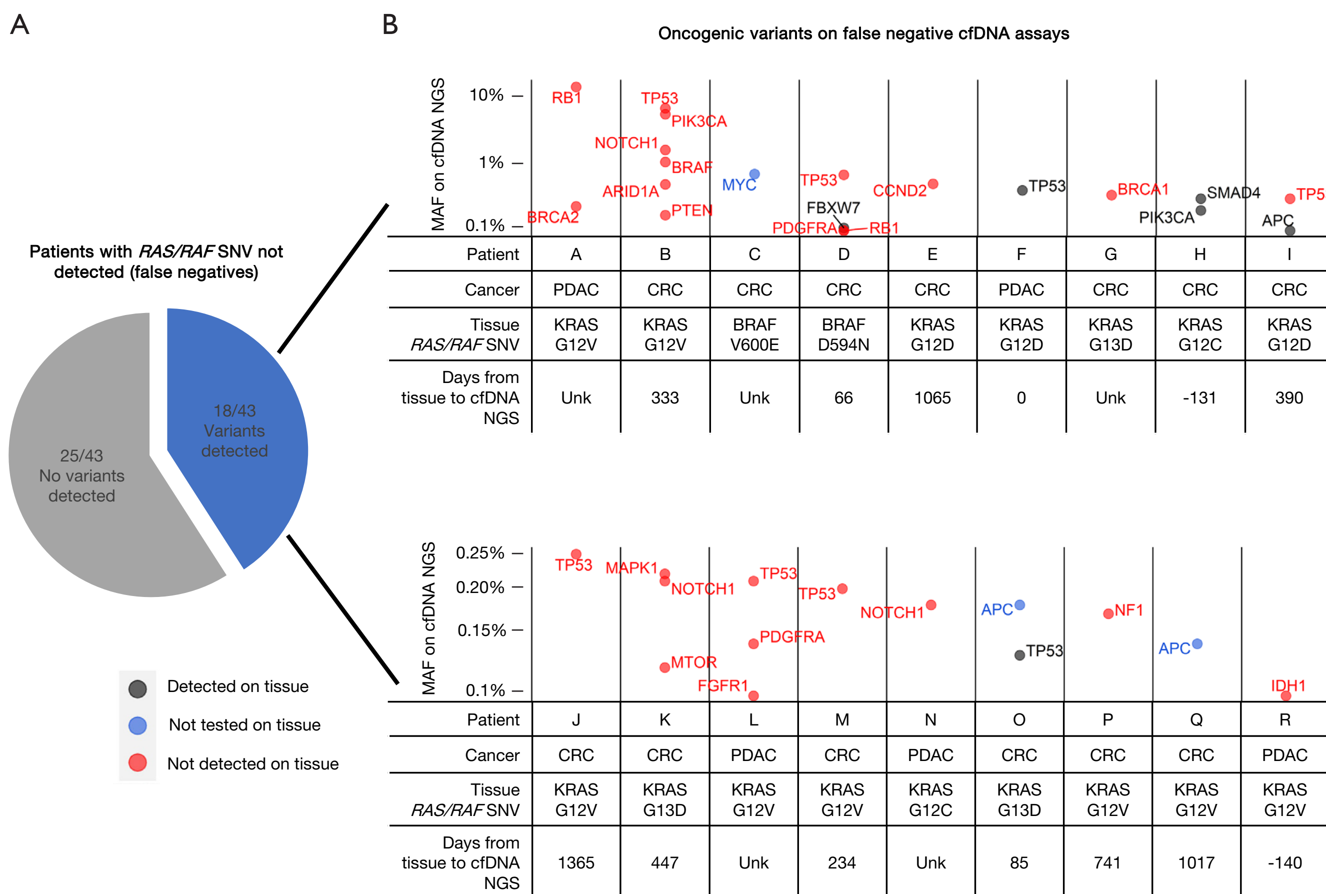

Figure 3 Analysis of cfDNA assays that did not detect the tissue *RAS/RAF* SNV (false negatives). (A) Pie chart showing proportion of false negative assays in which no oncogenic variants were detected versus assays in which at least one oncogenic variant was detected. (B) Panels show the detected oncogenic variants and associated MAF for each patient and whether the variant was also detected on tissue testing. 3 of 34 variants were not included on the tissue assay. Cancer type, *RAS/RAF* SNV detected on tissue, and time in days between tissue and cfDNA NGS are shown below each assay and individual patient with detected variants. Only 2 patients (A and B) showed variants with MAF >1%. Days between tissue and cfDNA assay varied widely. Of note, each panel is on a different log scale. "Unk" indicates time between tissue and NGS assays was not available. MAF, mutant allele frequency; SNV, single nucleotide variant; cfDNA, cell-free DNA; NGS, next-generation sequencing; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma.

detected cfDNA variants, associated cancer, tissue *KRAS*, *NRAS*, or *BRAF* SNV, and time between tissue and cfDNA NGS are shown in *Figure 3B*. From these 18 cfDNA assays, 25 of 34 total detected variants and 13 of the 18 variants with the greatest MAF were not detected on tissue NGS (*Figure 3B*). Only 6 of 34 detected variants were also found on tissue NGS, while 3 variants (1 *MYC* and 2 *APC* mutations) seen on cfDNA were not tested on the tissue assay (*Figure 3B*). Only 5 mutations had MAF >1%, 4 from one patient, none of which were detected on tissue. *TP53* was the most frequently mutated gene not also detected on tissue, however >1 mutation in *RB1*, *NOTCH1*, and *PDGFRA* were also observed.

Discussion

Despite the several advantages of cfDNA NGS, assay sensitivity represents a practical challenge for a clinical oncologist. In particular, defining mutational status of a gene, such as *KRAS*, is important for clinical decision making, however the absence of a mutation raises the question of whether the mutation was present but not detected. In this study, we define thresholds of greatest MAF of dominant non-*RAS/RAF* mutations for sensitivity

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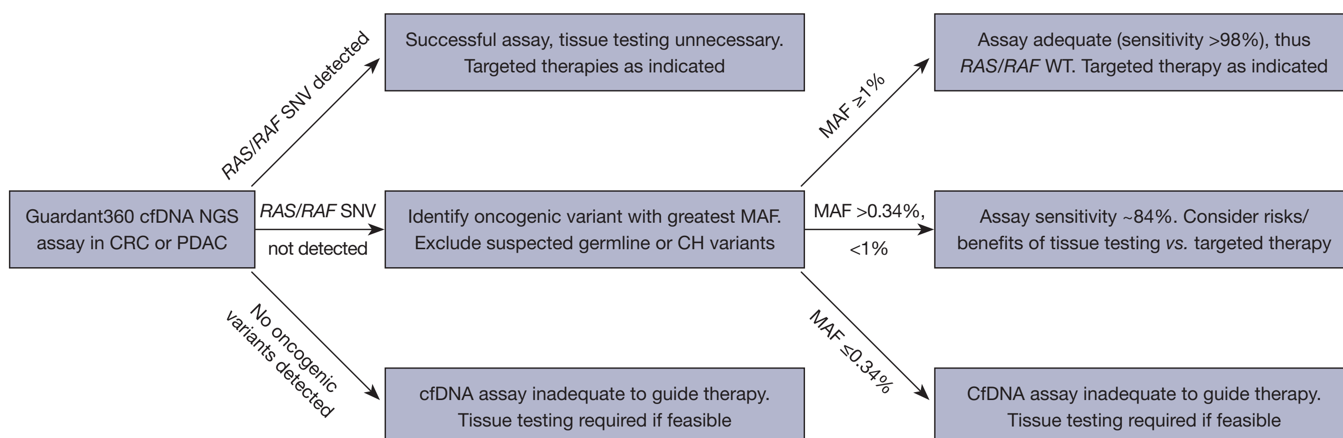

Figure 4 Framework for a practical approach to determining cfDNA NGS assay adequacy to detect *RAS/RAF* SNVs in CRC and PDAC. This proposed framework suggests that, in patients with CRC or PDAC, if a *RAS/RAF* SNV is detected, the assay is sufficiently sensitive. If no variants are detected, the assay is inadequate and alternative testing is needed. If variants are detected but no *RAS/RAF* SNV of interest (i.e., the patient appears wild-type), greatest MAF may be used to predict assay adequacy. Dominant MAF >1% predicts very high sensitivity, MAF between 0.34% and 1% requires clinical judgment regarding the utility of further testing, and MAF below 0.34% suggests an assay is insufficiently sensitive for clinical decision making. cfDNA, cell-free DNA; NGS, next-generation sequencing; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; SNV, single nucleotide variant; MAF, mutant allele frequency; CH, compound heterozygous; WT, wild-type.

for *RAS/RAF* SNVs in CRC and *KRAS* SNVs in PDAC. Based on these findings, we propose a practical approach to predicting the adequacy of the Guardant360 CDx cfDNA assay in CRC and PDAC (*Figure 4*). If a *RAS/RAF* SNV is detected, the assay may be considered accurate. If no oncogenic variants are detected, the assay is inadequate and alternative or repeat testing may be needed. If oncogenic variants are detected, but no *RAS/RAF* mutation, the oncogenic variant with the greatest MAF may predict if the assay is sufficiently sensitive. Specifically, dominant gene MAF >1% suggests very high sensitivity, while MAF >0.34% and <1% also retains moderately high sensitivity. Below MAF of 0.34%, the assay should be considered insufficiently sensitive to determine WT status.

These thresholds may grant increased confidence around the sensitivity of the Guardant360 CDx assay and may have significant implications for anti-EGFR therapy or rechallenge in CRC, targeted therapies against *KRAS*^{G12C} or *BRAF*^{V600E}, and the necessity of additional biopsy. In PDAC, high confidence that a patient is truly KRAS WT may prompt more extensive testing, especially to evaluate for rare but often actionable fusions. In addition, this analysis may inform use of cfDNA NGS assays to define inclusion and exclusion criteria for clinical trials. Relying solely on cfDNA may provide the most rapid assessment of a tumor to minimize time to treatment and avoids the need for invasive biopsy. However, in the case of a negative or inadequate cfDNA assay, the utility of repeat cfDNA NGS is unknown and best answered in the context of a trial.

An important limitation of our study is the use of a sole commercial cfDNA assay. Other assays and vendors have different testing characteristics and the technology itself may evolve over time, limiting lessons drawn specifically from this study. An advantage of this commercial assay is broad availability and use in the community; in contrast some prior studies (40) have used institutional assays or technologies associated with clinical trials that the clinicians may not access. In addition, while the technical specifications of cfDNA NGS assays may vary, we believe we have demonstrated important inherent characteristics of these assays.

Interestingly, among cfDNA assays that failed to detect the RAS/RAF SNV, we found that 25 of 31 (80.6%) of oncogenic variants were unique to the cfDNA assay and not detected on tissue testing. In addition, the MAF was <1% for nearly all of these variants. We speculate that this observation may be due to detection of variants arising from intra-tumoral heterogeneity, clonal evolution of the tumor, secondary malignancy, or CH. Of these, the contribution of CH is the most intriguing and problematic for interpretation of cfDNA assays. Leukocytes are thought to contribute the largest fraction of plasma cfDNA and

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detection of even low-level clonal variants in leukocytes is well-described and unsurprising (35). The most common variants associated with CH such as *DNMT3A*, *TET2*, and *JAK2* are easily recognized and excluded (34). However, mutations in many genes important in solid malignancies such as *TP53* are also regularly observed in leukocytes (36,37) and may confound accurate assessment of a tumor.

Unfortunately, our study did not have access to leukocyte sequencing to confirm the degree of confounding from CH. We suspect that sensitivity assessment and thus assay reliability may be improved with leukocyte variant subtraction but would add greater cost and greater burden of interpretation. Whether a strategy of leukocyte sequencing and subtraction is cost-effective or clinically meaningful requires further study. This finding further suggests that these assays may not be limited by poor sensitivity inherent to the assay itself at MAF <1%, but rather confounding from low level variants. Instead, many of the patients in our study with greatest MAF <1% may actually have levels of ctDNA below the limits of the assay. An important implication of this confounding is that improving the technical limits of a cfDNA assay, whether the Guardant360 assay used in this study or an alternative, for example by increasing read depth or DNA input, is also more likely to identify very small clonal variants. These low MAF variants may then be difficult to interpret for clinical actionability, given the increased likelihood these are leukocyte-derived. Our data does show that sensitivity for clinically relevant tumor-derived mutations improves with increasing greatest dominant gene MAF and provides a practical rule for assessing assay adequacy despite the potential presence of confounding mutations.

Conclusions

In this study, we show that greatest MAF of an oncogenic variant is a useful proxy for ctDNA content and to assess that an assay is sufficiently sensitive to detect clinically important mutations in *KRAS*, *NRAS*, and *BRAF*. In cases where tissue-based molecular testing is unavailable or difficult to obtain, cfDNA assay with adequate ctDNA may allow more rapid clinical decision making and help guide or avoid the need for invasive biopsy. In addition, low concordance between variants detected on false negative assays and tissue assays in our study suggest increased risk of confounding or heterogeneity when ctDNA content is low, though further research is needed to adequately explore this finding. These data, building on prior studies, increase the practicing oncologist's confidence to interpret and effectively use cfDNA NGS assays in clinical care.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects. All study protocols were approved by the institutional review board of each participating institution and the GOZILA study was registered at the University Hospital Medical Information Network Clinical Trials Registry (No. UMIN000042612). Approval for the establishment of the Duke MRT as a clinical and research data repository was granted by the Duke University Medical Center (DUMC) IRB (No. Pro00085260). Specific approval for this research collaboration and waiver of consent was also granted by the DUMC IRB (No. Pro00109863).

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