Peer Review File

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Reviewer A

Comment 1:
The authors should clarify the definition of non-driver mutation. It seems that the authors are defining non-driver mutation as non-RAS/RAF (including non-V600E) mutations, but I believe this can be misleading as some of non-RAS/RAF mutations can be also driver. I suggest that the authors may include MAF of RAS and RAF and change the greatest MAF including all non-VUS mutations, or may just go as greatest non-RAF/RAF MAF.

Reply 1:
Thank you for the insightful comment. We agree that the use of "driver" may create some confusion as this term may be used in many different ways in different contexts. To increase clarity, we have changed "driver" to *RAS/RAF* SNV or similar throughout the text. We will also define the non-RAS/RAF variant with greatest VAF as the "dominant" mutation rather than "non-driver". We have made these edits throughout the abstract, text, figures, and figure legends.

Changes in the text 1:

- We have changed all instances of "driver" to "*RAS/RAF* SNV" or occasionally *KRAS, NRAS, and BRAF*, or *KRAS* in instances where PDAC is discussed specifically. We will omit each instance here for brevity.
- We have similarly changed "non-driver" to "non-RAS/RAF dominant" mutation or variant throughout the text. We have defined this as "*other oncogenic dominant genes"* in the last sentence of the abstract background, and again in the main text in the Methods Analytic plan and analysis as "*non-RAS/RAF oncogenic mutation ("dominant" mutation)"*

Comment 2:
Abstract should be revised. In background, the authors investigated the greatest MAF not just MAF, and in Results section, the authors are testing sensitivity for detecting RAS or RAF SNV, not driver SNV. The current descriptions are unclear and could be potentially misleading. These changes should also be made in the main text. This research is showing the value of greatest MAF to evaluate adequacy of ctDNA data to evaluate presence of KRAS/NRAS/BRAF mutations in CRC and PDAC, and this should not be extrapolated.

Reply 2:
Thank you for the valuable comment. As with comment 1 above, we have changed "driver" to be "RAS/RAF SNV" or similar in the abstract and throughout the paper and figures for the purpose of greater specificity and clarity. In addition, we have clarified that we are using greatest MAF of non-RAS/RAF mutations to evaluate sensitivity. With these changes we make more clear that our claims are valid for KRAS/NRAS/BRAF mutations in CRC and KRAS in PDAC and not for other mutations or cancers.

Changes in the text 2:

- Throughout the text, the term driver has been changed to "*RAS/RAF SNV*" and "non-driver" has been changed to "non-RAS/RAF dominant" mutation or gene.

- In the abstract, last sentence of Background has been changes to "Mutant allele frequency (MAF) *of other dominant oncogenic genes* to identify a threshold for accurate detection of *KRAS, NRAS, and BRAF (RAS/RAF)* mutations in cfDNA".
- In the abstract, the first sentence of Conclusions has been changed to "*Non-RAS/RAF dominant oncogenic mutation* MAF ≥1% on cfDNA NGS predicts high sensitivity to detect *RAS/RAF* oncogenic SNVs *in CRC and PDAC*."

Comment 3:
In the introduction, the authors state that detection of KRAS or BRAF mutations are important in pancreatic cancer. Although, the CODEBREAK trial showed some responses of KRAS G12C mutant PDAC to sotorasib, it is only phase 1/2 trial and only 0.38% of PDAC patient harbor BRAF V600E mutation. I think the authors need to explain the necessity of adding PDAC population in this study to convince the readers.

Reply 3:
Thank you for the insightful comment. We will clarify in the abstract, introduction, and discussion that evaluation for KRAS/NRAS/BRAF is valuable for colorectal cancers and KRAS alone is valuable for pancreatic cancer. In addition, we will add to the discussion to emphasize the clinical utility of cfDNA evaluation for KRAS and inclusion in this study.

We agree with the reviewer that there is currently greater utility in evaluation for *KRAS/NRAS/BRAF* mutations in colorectal cancer than in pancreatic cancer, as actionable mutations (KRAS G12C or BRAF V600E) or wild-type status allowing anti-EGFR therapy are more common than in pancreatic cancer. However, we believe there is a strong and increasing clinical rationale for accurate comprehensive genomic profiling and particularly evaluation for KRAS status in pancreatic cancer, and that evaluation with cfDNA assays has a strong role to play. As the reviewer notes, KRAS G12C is rare, but actionable, and we want to ensure we do not miss it when present given lack of available therapies for this disease. Other KRAS inhibitors or immune based strategies are also in development and trials; accurate KRAS mutation ascertainment will be important to identify patients for these trials and eventually for standard therapy. Furthermore, our study in truth is more focused on predicting when a patient is truly RAS/RAF wild-type in both colorectal and pancreatic cancer. In pancreatic cancer, as laid out in the second paragraph of our introduction, 85-90% of patients have KRAS mutations, leaving a significant minority of 10-15% who are wild-type. Among these patients, there is a high frequency of actionable alterations and fusions; while individually rare, cumulatively they add up to a non-trivial fraction of all patients. In our experience in the practical application of molecular testing, a finding of KRAS WT should prompt a very extensive search for an alternative target, often requiring specialized extended RNA-based fusion panels or NTRK IHC, for example. We note as well that pancreatic cancer often has limited tissue availability for NGS testing, given the low cellularity and high stromal content of the tumor and limited accessibility requiring advanced endoscopic approaches for biopsy. While less sensitive, cfDNA is often the only practical available method of CGP prior to starting therapy in pancreatic cancer. Thus, a finding of KRAS wild-type on cfDNA may prompt the challenging decision of attempting another biopsy versus proceeding with therapy immediately, or repeating biopsy at a later time. We believe therefore that a guideline such as greatest MAF can provide assurance that a cfDNA assay is adequate in order to assist with this kind of decision making.

Changes in the Text 3:

- In the Abstract, changed the first sentence of the background to: "*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"

- In the Introduction, paragraph 2, added "*In addition, there are several novel molecular and immune-based approaches targeting KRAS-mutated cancers in development that will be of particular interest in pancreatic cancer given limited therapeutic options.*"
- In the Introduction, paragraph 2, added "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 and extensive evaluation for alternative driver events, including rare fusions".
- In discussion paragraph 1, removed "the RAS and BRAF" and changed to "defining mutational status *of a gene, such as KRAS*, *is* important for clinical decision making".
- In discussion paragraph 1, changed 3rd sentence to "In this study, we define thresholds of greatest MAF of *dominant non-RAS/RAF* mutations for sensitivity for *RAS/RAF SNVs* in CRC and *KRAS SNVs* in PDAC."
- In discussion paragraph 2, added: "*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.*"

Comment 4:
In the methods, the contents in the patient selection section should be moved to the patient population section. Also, analytical plan should be more elaborated.

Reply 4:
Thank you for the helpful comment. We have moved the patient selection section to the patient population section and left the variant analysis as its own section. In the analytic plan section, we have further elaborated on the application of the optimal cutpoint analysis to the sensitivity analysis. Finally, we have added a paragraph to the analytic plan section describing the analysis of the "false negative" cfDNA assays.

Changes in the text 3:
- We have moved the paragraph "Patients with CRC or PDAC with both tissue and cfDNA assays available…" to become the 4th paragraph of the Methods patient population section.
- We have changed the "patient selection and variant analysis" section title to simply "Variant analysis"
- We have added to the Methods analytic plan and analysis section by adding to paragraph 1: *"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%."*
- We have added a second paragraph to the Methods analytic plan and analysis section: *"We 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."*

Comment 5:
In Figure 3, some patients with 'false negativity' had several mutations detected in ctDNA only. This may be mostly explained by the inter-tumoral heterogeneity within a patient. In discussion, the authors interpretates that this may be owing to CH or secondary malignancy. However, I cannot agree as most CH associated variants are excluded during ctDNA analysis as the authors mentioned in the methods and we may not conclude like this in a targeted NGS.

Reply 5:

Thank you for the insightful comment. In the discussion, we have de-emphasized the likely role of clonal hematopoiesis and emphasized the likely contribution of intra-tumoral heterogeneity. We agree with the reviewer's overall observation, as we cannot ascertain the origin of these mutations definitively and we are only speculating based on other publications. We note that Gaurdant's bioinformatic process excludes reporting variants that are highly specific to clonal hematopoiesis, such as DNMT3A, TET2, and ASXL1 (JAK2 is a notable exception that is retained). Unfortunately, this process cannot easily exclude variants that may be both solid tumor derived or arise from clonal hematopoiesis, with *TP53* being the prime example. There are several studies (cited in our article) that point to the disturbing degree of confounding that can arise from clonal hematopoiesis. For example. in the paper from Leal et al (PMID: 31988276), in a study of gastric cancers, nearly 50% of TP53 mutations detected in cfDNA were actually leukocyte derived. Finally, while tumor heterogeneity likely explains some of the observed discordant variants, the degree of discordance is very high for these selected patients that failed to detect RAS/RAF mutations, which is why we emphasized alternative sources. Generally, the degree of concordance between tissue and cfDNA is quite high, especially for truncal or founding mutations such as TP53, APC, or KRAS.

Changes in the Text 5:

- In the Discussion, third paragraph, we have added: 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 clonal hematopoiesis. Of these, the contribution of clonal hematopoiesis is the most intriguing and problematic for interpretation of cfDNA assays*."
- In the Discussion, 4th paragraph, we changed the first sentence to "Unfortunately, our study did not have access to leukocyte sequencing to confirm *the degree of confounding from clonal hematopoiesis."*
- In the Discussion, 5th paragraph, we have added "These *low MAF* variants may then be difficult to interpret for clinical actionability, *given the increased likelihood these are leukocyte-derived."*

Comment 6:
The Figure 4 may be an exaggerated implication of this study. This study only deals with certain types of driver mutation in certain solid tumors without validation. This should be clearly shown. Also, considering the inter-tumoral heterogeneity within a patient which may be overcome by liquid biopsy, there may be some patients with negative RAS/RAF from a single tissue NGS and positive results from ctDNA. Hence, the authors should discuss the usefulness of repeated ctDNA analysis.

Reply 6:
Thank you for the helpful comment. We agree with the reviewer that the study findings apply only to KRAS/NRAS/BRAF SNVs in CRC and KRAS SNVs PDAC. Reflecting changes throughout the paper based on Comments 1 and 2, we have also changed language in Figure 4 and the Figure 4 legend to be more specific to RAS/RAF SNVs and in CRC and PDAC only. There is currently no clinical guidance for repeat ctDNA analysis in the event of assay failure; we will note that the utility of repeated assays are best answered in the context of a clinical trial. We agree with the reviewer that cfDNA assays are excellent at assessing tumor heterogeneity and may overcome the sampling bias of tissue biopsy. This approach is particularly valuable when assessing for development of subclonal resistance mutations. Unfortunately, our study was not designed to address this question, as we selected patients specifically based on the presence of RAS/RAF mutations on tissue.

Changes in the Text 6:

- In the Discussion, we have added to the end of paragraph 2: "*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*."

- The Figure 4 legend has been updated to: "This proposed framework suggests *that, in patients with CRC or PDAC*, if a *RAS/RAF SNV* is detected, the assay is sufficiently sensitive."
- Figure 4 has been updated to reflect changes from "driver" to "RAS/RAF" as per comments 1 and 2.

Comment 7:
The clinical characteristics of the patients should be given, at least as supplement (demographics, disease setting..)

Reply 7:
Thank you for the helpful comment. We have added Race/ethnicity data to Table 1 as well as the primary site of each cancer (ascending colon, rectum, pancreatic head etc). In a new Supplementary Table 2 (for clarity), we have added the number of sites of metastatic disease and if only 1 site, which solitary site, stratified by disease and whether the RAS/RAF SNV was successfully detected. Additional language in the text describes Supplementary Table 2. The prior Supplementary Table 2 has become Supplementary Table 3 based on reference order in the text.

Changes in the Text 7:
- Table 1 has been updated to include Race/ethnicity data
- Table 1 has been updated with a new section "Cancer Primary Site" to include data for primary site of disease, including cecum, ascending colon, transverse colon, descending/sigmoid colon, rectum, and unknown for CRC, and Head, Body, and Tail for PDAC. Previously included "Primary resected?" data has been moved to this section as well.
- We have added supplementary Table 2, which includes number of sites of metastatic disease stratified by cancer and detection of the RAS/RAF SNV
- The prior Supplementary Table 2 has become Supplementary Table 3 based on reference order in the text.
- Referring text has been updated:
    - In Results Paragraph 1: "*Number and distribution of metastatic sites at time of initial cfDNA NGS testing is shown in Supplementary Table 2.*"
    - In Results Paragraph 5: "*Number of metastatic sites and solitary site of metastasis stratified by successful detection of RAS/RAF SNV in cfDNA is shown in Supplementary Table 2. 20.7% 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%. 43.3% of PDAC had 1 site of metastatic disease, with 61.5% successful detection of the KRAS SNV.*"

Reviewer B

Comment 1:
For Table 1, there is not much patient-level data provided.

Reply 1:
Thank you for the helpful comment. As above for Reviewer A Comment 7, we have added race/ethnicity data to Table 1 as well as the primary site of each cancer (ascending colon, rectum, pancreatic head etc). We have also added metastatic sites in in Supplementary Table 2, with corresponding additional text in the results.

Changes in the Text 1:

- Table 1 has been updated to include Race/ethnicity data
- Table 1 has been updated with a new section "Cancer Primary Site" to include data for primary site of disease, including cecum, ascending colon, transverse colon, descending/sigmoid colon, rectum, and unknown for CRC, and Head, Body, and Tail for PDAC. Previously included "Primary resected?" data has been moved to this section as well.
- We have added supplementary Table 2, which includes number of sites of metastatic disease stratified by cancer and detection of the RAS/RAF SNV
- The prior Supplementary Table 2 has become Supplementary Table 3 based on reference order in the text.
- Referring text has been updated:
    - In Results Paragraph 1: "*Number and distribution of metastatic sites at time of initial cfDNA NGS testing is shown in Supplementary Table 2.*"
    - In Results Paragraph 5: "*Number of metastatic sites and solitary site of metastasis stratified by successful detection of RAS/RAF SNV in cfDNA is shown in Supplementary Table 2. 20.7% 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%. 43.3% of PDAC had 1 site of metastatic disease, with 61.5% successful detection of the KRAS SNV.*"

Comment 2:
It would be important to know the sites of metastatic disease in the patients included given potential impact of disease volume on ability to detect ctDNA (PMID: 28445469). Patients with mCRC and lung-only and peritoneum-only metastatic disease have reportedly lower levels of ctDNA (PMID: 35544728).

Reply 2:
Thank you for the helpful comments and references. We agree with the reviewer that sites of metastatic disease and overall volume of disease affects ctDNA content and overall assay sensitivity. As referred to above, for the reader's reference, in a new supplementary Table 2 we have added the number of metastatic sites and, if only one site of disease, the location of the solitary site of metastasis. We have stratified the metastatic sites by cancer and by successfully detection of the RAS/RAF SNV. Additional language in the text has been added to discuss and reference this new table. We have also added discussion of the effect of tumor volume and site on ctDNA content and sensitivity, while adding literature references in support.

Changes in the Text 2:

- We have added supplementary Table 2, which includes number of sites of metastatic disease stratified by cancer and detection of the RAS/RAF SNV

- The prior Supplementary Table 2 has become Supplementary Table 3 based on reference order in the text.
- Referring text has been updated:
    - In Results Paragraph 1: "*Number and distribution of metastatic sites at time of initial cfDNA NGS testing is shown in Supplementary Table 2.*"
    - In Results Paragraph 5: "*Number of metastatic sites and solitary site of metastasis stratified by successful detection of RAS/RAF SNV in cfDNA is shown in Supplementary Table 2. 20.7% 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%. 43.3% of PDAC had 1 site of metastatic disease, with 61.5% successful detection of the KRAS SNV.*"
- We have added to Introduction Paragraph 3: *"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 mestases 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)."*
    - We have added 4 references to the literature in support of the above addition to the text, including the 2 referenced in comment 2.

Comment 3:
It would also be helpful for readers to summarise the driver mutations from Table S1 in an extra row of Table 1, similarly to how they are described on p8 paragraph 3 in the text. Readers can still refer to Table S1 for more details.

Reply 3:
Thank you for the valuable comment. We agree and have taken your suggestion, except that we have added the summary of the RAS/RAF mutations detected to Table 2 rather than Table 1, along with the other data summarizing the cfDNA results, as we believe this will offer greater clarity within the other data offered in those tables.

Changes in the Text 4:
- We have added a summary of *KRAS, NRAS*, and *BRAF* mutation distribution by cancer in Table 2.

Comment 4:
Reference 7: the PARADIGM study has now been published. Consider citing the full publication - PMID: 37071094.

Reply 4:
Thank you for the helpful comment. We have updated the reference to the recently published paper for the Paradigm Study.

Changes in the Text 4:
- We have changed reference 7 to the recently published article on the PARADIGM study (PMID: 37071094).

Comment 5:
In Figure 2A, there is no statistical comparison between non-driver MAF and driver detection. On p8 the authors have described as association between the two. Consider providing a statistical comparison or removing the word "associated" from the text (p8, line 283).

Reply 5:
Thank you for the insightful comment. Figure 2A iss meant as an intuitive visualization of the data; the data is highly non-parametric and more appropriate statistical evaluation of the relationship between MAF and RAS/RAF detection using non-parametric tests is depicted in Figures 2B and 2C and Supplementary Figure 1. We will remove the word "associated" from the text and clarify the text in the results with more appropriate language.

Changes in the Text 5:

- In the Results, Paragraph 6, we changed the sentence describing Figure 2a to "*The distribution of dominant variants with greatest MAF stratified by cancer and detection of RAS/RAF SNVs is shown in Figure 2a.*"

Comment 6:
In figure 2B, the percentage of driver detection is described as 96%, while in the text on p9 this is listed as 97%.

Reply 6:
Thank you for the helpful comment. We have corrected this discrepancy; the correct number is 96%.

Changes in the Text 6:

- In the Results, Paragraph 6, changed "97%" to "*96%"* to correct this error.

Comment 7:
In Table 2, I believe it would be helpful for readers to list the number of lines of treatment between tissue and cfDNA analysis given that the ctDNA landscape may change as resistance to sequential lines of therapy occurs.

Reply 7:
Thank you for the insightful comment. We agree that this information would be useful to the reader. Unfortunately, we do not have this data comprehensively available and cannot provide it here.

Changes in the Text 7:
No change

Comment 8:
It would be helpful for the authors to discuss why they think the yield of cfDNA was much lower in the PDAC group compared to the CRC group e.g. whether this may be due to differences in disease volume, TME, neoplastic cellularity or other factors between the two cancer types.

Reply 8:
Thank you for the valuable comment. We agree with the reviewer that PDAC has much lower ctDNA shedding than CRC. This phenomenon has been previously well documented. We will add to the introduction to refer to differences in shedding based on metastatic site, tumor volume, and tumor factors suggest as neoplastic cellularity and stromal content.

Changes in the Text 8:

- As discussed above in Reviewer B Comment 2, We have added to Introduction Paragraph 3: *"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 mestases 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)."*
    - We have added 4 references to the literature in support of the above addition to the text, including the 2 referenced in comment 2.