Peer Review File

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Reviewer A

This is a well-structured report which uses the case in question to highlight current developments, and the likely direction of travel, of MRD/ctDNA work. I think the background is well supported/appropriate, and the case itself is quite interesting. However, the relevance to the broader themes discussed is couched in very general terms, and there is little or no reflection on how the case could have been handled differently, or would be handled differently should technologies such as panel sequencing of cfDNA become standard. I think it would therefore benefit from substantial revision, to strengthen and clarify the link between case and context. Key points are outlined below:

1. Clarify/discuss choice of monitoring strategy, the possible options now and in the future for cases like this, and include more detailed discussion of how they could be managed better. It is stated that a single Signatera test was used for logistical reasons. What were these – availability of tumour profiles at different times, cost/insurance limitations, something else? If both profiles were available, could 8 drivers have been chosen from each to monitor both signatures? From the existing result, would analysis of any 8 of the 16 targets give a good enough signal? Have you asked the manufacturers if their test can target 2 tumours? I can't think why not, although sensitivity may be affected and validation work may be needed before it can be used clinically.

Reply: A brief explanation of why the assay was chosen as well as clarification regarding the logistics of multiple assays was added to the case description (Paragraph 3). This was a manufacturer limitation, and unfortunately, they do not give details as to why multiple assays cannot be sent simultaneously. We also added potential strategies to avoid false negative ctDNA for MRD assays in the future (Discussion, Paragraph 5).

2. The reader is left to infer that panel sequencing in the future would detect both signatures. Is that so, or are there potential pitfalls with how this technology is used or reported on in this context? For instance, if a tumour signal is already known (in the context of MRD), will it be looked for specifically, or will the entire panel be reported? If the frequency of synchronous tumours is >3%, should MRD panel analysis of ctDNA be considered for use as a check for additional malignancies? Are they already being used in this way? The implications that this case has, for current and future practice, need to be strengthened and reflected upon further. Currently, it comes across as a description of an unusual case facilitating a review of ongoing changing in an important area of disease management, rather than providing any novel insight or perspective relevant to those changes or current practice.

Reply: Additional explanation of tumor-specific assays and their limitations as they apply to this case was provided (Discussion, Paragraph 3). A new paragraph was also added to the discussion specifically regarding the Signatera MRD assay and its relevance to this case and

others with synchronous CRC (Discussion, Paragraph 4). Finally, strategies to overcome these limitations were added to the discussion as mentioned for point #1 above (Discussion, Paragraph 5).

3. Linked to the above, some sections of the report are very vague. The conclusion in the abstract is a good example. How exactly do “the outcomes in this case underscore the ongoing impacts of past and current investigations into MRD on patient outcomes, influencing aspects such as diagnosis, treatment strategies, and prognosis”? This is a case report, not a review. If >3% of cases are multiple primaries, then it is frequent enough to discuss implications in more detail. Conclusions cold also be stronger – if this case highlights are limitation, as stated, can this be overcome easily? In Lynch this is important, as synchronous Lynch tumours in different organ systems is a further possibility that will increase with age. Also, there is some evidence the synchronous CRC is more frequent in Lynch patients – see references cited in https://www.nature.com/articles/ncomms12072

Reply: The conclusion to the abstract was rewritten and concrete examples provided to aid in decision making in this patient population (Abstract, Conclusion). The larger conclusion at the end of the manuscript was also edited to reflect changes made to the abstract. Appreciate the reference for synchronous CRC in LS. There seems to be some conflicting information in the literature. This was added to the discussion (Discussion, Paragraph 2).

Minor points:

A “patient with a history of Lynch syndrome”. A patient with Lynch syndrome, or a Lynch syndrome patient with a history of X and Y tumours....?
Reply: “history of” was deleted. The patient had other cancers as expected but were excluded to avoid identifying the individual.

Highlight box – “What is known” and “What should change now” sections are both very general. As written it could currently be summarised as not enough and more research should be done. More specificity with respect to synchronous cases needed.

Reply: The “what is known” section of the highlight box was edited to include more specific information regarding the prevalence of synchronous CRC and LS. Furthermore, the “what should change now” section was reworded entirely to give specific examples on what could be improved going forward.

Line 72. Lynch syndrome, previously known as HNPCC…… ?

Reply: Sentence edited to “previously known as HNPCC”.

Line 75. EPCAM is not an MMR gene, it is a cell adhesion molecule. Deletions of distal EPCAM exons can result in epigenetic silencing of MSH2. Reword or delete for accuracy. Also suggest clarification that MSI is a molecular phenotype of MMR deficiency, rather than various

gene mutations being associated with MSI.

Reply: EPCAM was deleted. Clarification regarding MMR and MSI was also added to distinguish genetic mutations vs phenotype.

Line 89. MSS and MSI-L have not been defined. Unstable and stable tumours or MSI-H/MSS tumours may be better, as the importance/interpretation of MSI-L is unclear.

Reply: MSI-L was deleted and the comparison made to MSS cancers only. Definition of MSS was left out for brevity as the reader is assumed to have knowledge of MSI-H and MSS.

L150 – There is some additional information below on the frequency of metachronous CRC and synchronous upper tract urothelial cancer:
https://www.sciencedirect.com/science/article/pii/S1078143920300090
https://www.mdpi.com/2673-4095/3/3/20
https://www.mdpi.com/2673-4095/3/3/20
Reply: We appreciate the provided articles. Citation regarding the risk of metachronous CRC in LS was added.

Reviewer B

Authors described a LS case study with two synchronous primary CRCs, which showed distinct genetic profile. ctDNA levels were negative at one month and six-month post-surgery using MRD assay sent from the rectal primary tumor. However, the radiographic recurrence was observed. MRD assay was sent again from the distal transverse primary tumor and showed positive, and the recurrence was confirmed. Anti-PD-1 treatment was able to decrease the ctDNA levels, which was consistent with the small nodes without metastatic disease. It was well-written and easy to follow.
Specific comments:
1. Table 1. How was the tumor DNA sequenced? Sequenced a panel of cancer genes (how many genes in the panel?) or whole exome sequencing? Can authors give more information?

Reply: We have clarified how mutations are initially and subsequently identified – via WES (Case Description, Paragraph 3).

2. Were all the mutations identified in each primary tumors as listed in Table 1 included in the 1st and 2nd custom-built MRD assay? Please clarify this in the text.

Reply: Much of the methodology is propriety according to the manufacturer. According to Signatera, a patient's plasma sample is considered ctDNA positive when at least two individual-specific tumor variants are detected. An additional paragraph was also added to the discussion which gives some more insight into tumor informed assays as they pertain to this case (Discussion, Paragraph 4).

3. Figure 1: Did authors check the ctDNA level pre surgery? In the text, authors stated that Signatera MRD assay sent from rectal tumor primary cancer was negative (undetected) at 1-month and 6-month post-surgery. However, radiographic recurrence was observed at 6-month post-surgery. MRD assay was sent again from the distal transverse colon primary tumor, which was positive. Given two primary tumors showed distinct genetic profile, do authors think that the MRD assay sensitivity is primary tumor-specific (non-mucinous), or advanced grade/stage- or metastasis-dependent? Did authors sequence the metastatic LN to see if the metastasis was mainly from one tumor or both tumors? Please discuss.

Reply: Since the patient had resectable disease, ctDNA levels were only checked post-operatively as part of MRD surveillance (Case Description, Paragraph 3). Additional explanation of tumor-specific assays and their limitations as they apply to this case was provided (Discussion, Paragraph 3). Furthermore, a separate paragraph regarding the specifics of tumor-informed assays was added (Discussion, Paragraph 4).

4. Based on the text, the 1st time point in Figure 1 (1-month post-surgery) was from rectal tumor primary cancer. Please clarify this in the figure legend.

Reply: A legend was added to the figure to clarify ctDNA levels during the post-operative course for the rectal MRD assay and transverse colon MRD assay, respectively.

5. Line 159: Authors stated that "potential limitations specifically regarding the finding of concurrent malignancies with unique genetic profiles". Is it feasible to develop a ctDNA MRD assay using mutations from multiple primary tumors for these patients? Understand the logistical testing limitation for this case. Are there any concerns if only using the mutations from one tumor for ctDNA assay and MRD monitoring for patients with two primary tumors? Please discuss.

Reply: A brief explanation regarding the logistics of multiple assays was added (Case Description, Paragraph 3). This was a manufacturer limitation, and unfortunately, they do not give details as to why multiple assays cannot be sent simultaneously. We also added potential strategies to avoid false negative ctDNA for MRD assays in the future (Discussion, Paragraph 5).