Peer Review File

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Reviewer A

Comment 1: In short, I think that the authors have done very good description of the problem, novelty and analysis of the situation. There is not much adding to this paper. Well done!

Reply 1: thank you.

Reviewer B

I read your case report with great interest. As you state in your report, it is well known that TP53 is often mutated in white blood cells due to clonal hematopoiesis (CH) and it is also known that smoking and radiotherapy (which your patient received) can enhance CH (your ref 14). Also, CH has been demonstrated in cfDNA in many patients with cancer prior to your study. In fact your ref 15 states that "The vast majority of cfDNA mutations (81.6% in controls and 53.2% in patients with cancer) had features consistent with clonal hematopoiesis" and several authors have advocated confirming cfDNA findings by also analysing DNA from leukocytes. Others have suggested that constant low level VAFs in repeated cfDNA samples might be indicative of CH e.g. Maron 2019 PMID: 31427281 in their studty on cfDNA in gastroesophageal cancer patients: "Three patients with detectable mutations after surgery, each at similar low maxVAFs prior to treatment/surgery, have not recurred to date, and none of these mutations were identified by tissue sequencing, which suggests that they may not be tumor-derived at all".

Comment 1:Therefore, I do not agree that your case report "unveils" a potential pitfall of cfDNA diagnostics. Your patient might be the first with esophageal cancer to have proven CH, but, others (Maron) have suggested CH.

However, the strength of your case report lies in the long-time follow-up and the fact that you have confirmed your findings using ddPCR. I assume that you also have detailed clinical information (?) and I suggest you emphasis these points instead.

Reply 1: We agree with the reviewer that we are not the first ones who unveiled false-positive cfDNA analyses due to clonal hematopoiesis and that the strength of this manuscript lies within the unique clinical aspects.. In our introduction we had already

introduced clonal hematopoiesis as known pitfall for cfDNA analysis. According to the reviewer's suggestions, we revised the manuscript to emphasize the clinical strengths.

Changes in the text:

- 'Our case unveils a potential pitfall in cfDNA diagnostics' was changed to 'Our case illustrates a number of challenges currently associated with cfDNA diagnostics in esophageal carcinoma'(page 9, line 200).
- 'This is the first case in which false-positive plasma genotyping due to clonal hematopoiesis is described in a patient with esophageal cancer' was changed to 'In this case report we describe the long term follow-up of an esophageal carcinoma patient with false-positive plasma genotyping due to clonal hematopoiesis' (page 8, line 173)

Comment 2: Indeed, you show that the V216M was constant between baseline and 21wks although the patient had complete response, which would suggest CH. Of interest, the VAF of V216M doubled at 27mon and increased further at 30 mon, which would have made it difficult to exclude as CH if this was the only data available. Also, it was detected at low levels in the tumour (likely due to blood cells in the tumour) which might be worth commenting on.

Reply 2: We agree with the reviewer that this is worthwhile mentioning in the manuscript.

Changes in the text: Added the above comment to the manuscript: 'The VAF of the p.V216M variant was constant between baseline and 21 weeks while the patient developed a clinically complete response, which would suggest a clonal hematopoietic origin. Of interest, the VAF of *TP53* c.646G>A; p.V216M doubled at 27 months and increased further at 30 months. This would have made it hard to ascribe it to CH if leukocyte analysis had not been performed. Also, *TP53* c.646G>A; p.V216M was detected at low levels in the metastasis resection specimen, probably due to infiltration of blood cells in the tumor tissue.' (page 9, line 188)

Comment 3: The true tumour TP53 variant was only detected at VAF 0,2% in cfDNA even when the patient had multiple soft tissue and skeletal metastases. It would therefore be of interest if you had information on the total tumour burden, the number and sites of metastases (liver and bone marrow have been suggested to be correlated to higher cfDNA levels), proliferation index (rapid turnover might be correlated to higher cfDNA levels), other cancer markers in the blood etc etc.

Reply 3: Additional clinical information has been added to the manuscript.

Changes in the text: We added to the case description:

- ‘Imaging studies showed a total of 12 skeletal- and soft tissue metastases located in the vertebral column, the dorsal musculature, the pelvis and the bones and muscles of the right lower extremity.’ (page 7, line 136)
- ‘Pathological analysis of metastatic tissue did not reveal remarkable mitotic activity.’ (page 6, line 129)
- The patient received palliative treatment with analgesic medication and localized external beam radiation therapy on symptomatic bone metastases.’ (page 7, line 139)
- We also added images of PET-CT results. (figure 2)

Comment 4: Also, did the tumour NGS analysis only identify a single TP53 mutation at diagnosis and follow-up and no other aberrations? Add the total NGS-results to the report. If other aberrations were identified, could they also be detected in cfDNA? Which mutations had the highest VAF in the tumour, was it TP53? (If other mutations had higher VAFs, did you analyse these with ddPCR or a gene panel i n plasma?)

Reply 4: In the present case-report we focused on TP53 mutation analysis. Our panel also identified other tissue mutations within the exonic region or the splice site. However, these mutations were not of interest for cfDNA analysis because either the coverage for these mutations was below the quality threshold or these mutations were not covered by the Oncomine panel used for cfDNA analysis. The complete NGS results have been added as supplementary data.

In the tumor, CLEC10A c.103T>C; p.C35R had the highest VAF (97.6%). However, the functional consequences of this mutation are unknown and the mutation is not covered by the Oncomine panel we have used for cfDNA analysis. Given the unknown significance of this tissue mutation, we did not further evaluate this mutation. This was also the case for SYTL1 c.1331A>G; p.Q444R (VAF 48.9%). On the basis of these VAFs it most probably concerns polymorphisms and not tumor-specific mutations. Diagnostic NGS on germline DNA could have confirmed this however we chose not to further investigate this since it was beyond the scope of the article. We chose not to report these findings.

Changes in the text:

- To the discussion was added: ‘In the present case-report we focused on TP53 mutation analysis.’ (page 9, line 197)

Comment 5: Also, I suggest that you provide some annotation for the TP53 variants. Are they known to be pathogenic somatic variants? (See e.g. iarc, cosmic, phantm: http://mutantp53.broadinstitute.org)

Reply 5: We provided some remarks regarding pathogenicity with reference to the CLINVAR database.

Changes in the text:

- We added about R175H: 'This is a well described pathogenic variant associated with a variety of malignancies(citation).' And 'Mutant p53 has been demonstrated to result in enhanced integrin and epidermal growth factor receptor (EGFR) trafficking, and by that way promote cell scattering and invasion .' (page 8 line 176)
- We added about V216M: '*TP53* c.646G>A; p.V216M variants have been associated with various malignancies. Although pathogenicity has not as yet clearly been established, it has been ‚mentioned as a recurrent clonal hematopoietic variant' (page 9, line 185)

Comment 6: It is also important to add information on which tubes were used for blood collection and how was plasma extracted? (page 6, line 135) as this can greatly influence the results.

Reply 6: Information about collection of blood samples is now provided in the text.

Changes to the text: To the paragraph on analyses the following was added: 'Blood samples drawn at t=0, t=2 and t=3 were collected using EDTA tubes. Plasma was separated, isolated and frozen within one hour after collection in order to provide stable cfDNA levels. All other samples were collected using CellSave tubes in which cfDNA levels are stable up to 96 hours after collection and were isolated and frozen within 48 hours.'(page 7, line 151)

Comment 7: With a slightly different angle to your text, I believe that your case report would be of interest and would further highlight the challenges with cfDNA analysis even in patients with metastatic disease.

Reply 7: With the above additions and changes we hope to have turned the focus of the text towards highlighting the challenges of cfDNA analysis in the esophageal cancer patient

Reviewer C

Comment 1: Would suggest changing annotation of timepoints from Tx to Timepointx (or alternative) to clearly distinguish from T-stage (particularly T1-4)

Reply 1: applied this comment in the text

Changes in the text: Everywhere in the text T1-11 was changed to t=0-11

Comment 2: What was the timing of liquid biopsies with respect to surgery (was each biopsy taken pre-op) - could the authors please clarify in the manuscript

Reply 2: Liquid biopsies were obtained at baseline (t=0) as well as fourteen (t=2) and twenty-one weeks after baseline (t=3). Surgery was performed at t=4. At t=8, t=9 and t=10 more sets of liquid biopsies were collected. All of this information is in the text.

Changes in the text: To clarify this chronology in the manuscript the following sentence was transferred from the middle of the paragraph to the end: 'Twenty-two weeks after baseline (t=4) the patient underwent a standard transhiatal esophagectomy. Resection specimen showed ypT1bN0 (all 19 removed lymph nodes negative) with Mandard's Tumor Regression Grade 2 (1-10% residual vital tumor cells). The resection specimen did not contain sufficient residual tumor tissue for NGS.' (page 6, line 114)

Comment 3: Table 1 is untidy, but that may be adjusted with formatting.

Reply 3: Table 1 gives an overview of all cfDNA levels at all time points. The new line graph (figure 2) provides a more tidy overview of cfDNA levels. Table 1 may be moved to the supplementary material, but we would like to leave this decision with the editors.

Minor suggestions:

Comment 4: Line 58-60: change to: However the critical limitation of this approach is the 10% risk of subclinical residual disease, and risk of patient non-compliance due to the high burden of invasive diagnostics

Reply 4: accepted this suggestion

Changes in the text: changed 'missing one out of ten residual tumors'to 'a 10% risk of subclinical residual disease'(page 3, line 57)

Comment 5: line 63: add comma after tissue

Reply 5: accepted this comment

Changes in the text: added a coma (page 3 line 61)

Comment 6: line 91: add "other" prior to malignancies

Reply 6: accepted this comment

Changes in the text: added other prior to malignancies (page 4, line 89)

Comment 7: line 99: add finding of endoscopy to biopsy

Reply 7: endoscopy findings have been added

Changes in the text: Changed this line to 'Fourteen weeks (t=2) as well as twenty-one weeks after baseline (t=3), post-nCRT endoscopy was performed during which no macroscopic tumor could be found. At these points in time, biopsies were taken of the tumor site, showing no residual tumor cells.'(page 5, line 97)

Comment 8: line 99: could the authors explain why surgery was undertaken at 22 wks (unusual timing) - was there clinical evidence of recurrence

Reply 8: This patient participated in the preSANO trial (Noordman BJ et al. Lancet Oncol. 2018 Jul;19(7):965-974). The start of neoadjuvant chemoradiotherapy according to CROSS regimen started 4 weeks after diagnosis at the latest and this treatment regimen takes 5 weeks. The preSANO trial, in which two response evaluations after neoadjuvant chemoradiotherapy were performed, takes 12 weeks to complete. This has led to surgery being performed at 22 weeks after diagnosis. However, regardless of participation in this trial, surgery at 12-14 weeks after completion of neoadjuvant chemoradiotherapy is not an unusual timing anymore these days (Shapiro J et al. Ann Surg. 2014 Nov;260(5):807-13).

Changes in the text: We added:

- We added: 'The patient participated in the preSANO trial, which aimed to assess the accuracy of diagnostic response evaluations after nCRT. In context of this trial,..' (page 5, line 95)
- For the time points prior to esophagectomy, we also added additional indications for the time points with reference to completion of chemoradiotherapy (page 5, line 109-115). *E.g.:* 'Twenty-two weeks after baseline (t=4; i.e. 14 weeks after completion of nCRT) the patient underwent a standard transhiatal esophagectomy.'

Comment 9: line 112: remove "of progression towards". The patient has metastatic disease

Reply 9: accepted

Changes in the text: Rephrased this sentence to 'Twenty-one months after initial diagnosis (t=5), biopsy from a solitary mass in the distal semimembranosus muscle revealed adenocarcinoma proving the patient had metastatic disease.' (page 6, line 122)

Comment 10: line 114: please reword; surely the metastatic character was confirmed on the finding of adenocarcinoma in a muscle

Reply 10: accepted

Changes in the text: rephrased this sentence to:. Tissue mutation analysis revealed a *TP53* c.524G>A; p.R175H mutation, confirming it being a metastasis from the forementioned esophageal carcinoma.(page 6, line 124)

Comment 11: line 114: add timepoint 6 to chemotherapy

Reply 11: accepted

Changes in the text: added '(t=6)' (page 6, line 125)

Comment 12: line 115: change to " PET-CT imaging post chemotherapy continued to show the solitary soft tissue metastasis only, so the patient underwent surgical resection of this mass (Time point 7)

Reply 12: accepted

Changes in the text: changed this sentence to 'PET-CT imaging post chemotherapy continued to show only this solitary soft tissue metastasis, therefore the patient underwent surgical resection of this mass (t=7).' (page 6, line 127)

Comment 13: line 155 change to " identified in the primary tumour tissue via endoscopic biopsy"

Reply: accepted

Changes to the text: removed 'tissue obtained from' (page 8, line 176)

Comment 14: line 159: change to "by clinical symptoms and standard imaging and biopsy techniques"

Reply 14: accepted

Changes in the text: changed this sentence to: 'This mutation became detectable only after distant metastases had already been confirmed by clinical symptoms and standard imaging- and biopsy techniques.' (page 9, line 183)

Reviewer D

The manuscript by Spoor and colleagues presents the case of a patient with esophageal adenocarcinoma who had false-positive results in the circulating tumor DNA (ctDNA) analysis owing to clonal hematopoiesis. The number of ctDNA tests performed for patients with cancer has been increasing worldwide. The false-positive

result of the ctDNA analysis may lead to inappropriate treatment by misidentification of the tumor burden and/or actionable mutation. This case report may give an insight into the decision-making of treatment based on the ctDNA test results in patients with cancer as well as esophageal carcinoma. The reviewer has raised the following points:

Comment 1: Clonal hematopoiesis of indeterminate potential is a common aging-related phenomenon in which outgrowths of hematopoietic progenitor cells with somatic mutations occur. Moreover, it appears that there is no association between clonal hematopoiesis and cancer types. Is it meaningful to emphasize the appearance of clonal hematopoiesis in one cancer type, such as in esophageal adenocarcinoma?

Reply 2: The aim of our paper was not so much to emphasize the presence of clonal hematopoiesis but rather the challenges this poses to cfDNA diagnostics in esophageal carcinoma. In accordance with the comments of reviewer B, several changes were made to emphasize this case report.

Changes in the text: Please see our replies to comments 1-3 of reviewer B.

Comment 2: In Table 1, it is difficult to understand the dynamics of circulating mutant DNA level from both tumor cells and white blood cells (i.e., clonal hematopoiesis). The reviewer recommends showing them in a line graph. In addition, CT or PET-CT images during the course of treatment may help us understand the discrepancy between the dynamics of tumor burden and circulating mutant DNA level.

Reply 2: We recognize this problem. We have designed a new image showing a line graph and with corresponding PET-CT images per timepoint illustrative of tumorload during follow-up

Changes in the text: Added a linegraph illustrating the cfDNA level dynamics and images showing PET-CT diagnostics during follow-up (figure 2)

Comment 3: The authors described that “We recommend the correlation of mutations in cell-free DNA with mutations in tumor biopsies.” An advantage of liquid biopsies, including ctDNA analysis, is the elimination of the influence of tumor heterogeneity. It appears that clonal hematopoiesis cannot be excluded by the analysis of tumor and cell-free DNA alone; thus, genomic analysis of white blood cells is also needed.

Reply 3: In theory this is correct. However we know that in practice TP53 and P16 mutations are almost always clonal. This means that tumor biopsies and liquid biopsies suffice. For further information see: van Nistelrooij AM, van Marion R, Koppert LB, Biermann K, Spaander MC, Tilanus HW, van Lanschot JJ, Wijnhoven BP, Dinjens WN. Molecular clonality analysis of esophageal adenocarcinoma by

multiregion sequencing of tumor samples. BMC Res Notes. 2017 Apr 4;10(1):144. doi: 10.1186/s13104-017-2456-5. PMID: 28376920; PMCID: PMC5379534.

Changes in the text:-