Author's response to reviews

Title: Targeted sequencing of circulating cell-free DNA in stage II-III resectable oesophageal squamous cell carcinoma patients

Authors:

Pei Meng (p.meng@umcg.nl)
Jiacong Wei (j.wei@umcg.nl)
Yiqun Geng (yqgeng@126.com)
Shaobin Chen (Chensb535176@hotmail.com)
Miente Terpstra (m.m.terpstra.cluster@gmail.com)
Qiongyi Huang (huang_qiong_yi@126.com)
Qian Zhang (qianzi4706@163.com)
Zuoqing Su (forever.szq@163.com)
Wanchun Yu (904923208@qq.com)
Min Su (minsu@stu.edu.cn)
Klaas Kok (k.kok@umcg.nl)
Anke van den Berg (a.van.den.berg01@umcg.nl)
Jiang Gu (2523381625@qq.com)

Version: 2 Date: 12 Oct 2018

Author's response to reviews:

Point-by-point response to the reviewers’ comments
Dear Editor:

We would like to resubmit the revised version of our manuscript (BCAN-D-18-00953R1) entitled “Targeted sequencing of circulating cell free DNA in stage II-III resectable oesophageal squamous cell carcinoma patients” to BMC cancer. All comments raised by reviewers have been addressed in the revised version. In addition, we deleted one patient sample because of technical issues and made some corrections of our inconsistence filtering criteria as we mentioned in the previous email which result in changes of our initial results. All changes are indicated with highlighting in the manuscript text for ease of reference. The specific answers to the reviewers’ comments are indicated in our response to the reviewers. In addition, we made some small textual changes to improve English language. Minor textual changes were not highlighted. Moreover, we asked our editor to check the English throughout the manuscript.

We hope that our revised manuscript is acceptable for publication.

Yours sincerely,

Maria Lung (Reviewer 1):

Comment #1: The quality of sequencing data is unknown, although the average coverage was described by the authors. The detailed NGS data statistics is missing in the manuscript

Response: We apologize for not including a detailed overview of the NGS quality data. We have now presented the sequencing quality data in Supplementary table S2. In the results section (2nd paragraph) we briefly described these data.

Comment #2: The robustness of their NGS benchwork and analysis pipeline for detecting mutations in cfDNA is not clear. What method is used for mutation calling? Can the authors catch the mutations with allele frequency as low as 0.5% accurately?

Response: We apologize again for the incomplete description of our data analysis pipeline. In the methods section of the revised manuscript, we have now added the approach of our data analysis workflow and the applied filtering criteria (5th paragraph of methods section, data analysis part).
As presented in Supplementary table S2, the average mismatch rate observed in this study is 0.43%, which is about 0.14% for each alternative nucleotide. The MAF of 0.5% as applied for cfDNA samples in this study is well above this average mismatch rate. To further demonstrate robustness of our data, we also checked all reported cfDNA mutation positions manually in IGV and determined the number of altered reads observed for the two nucleotides not detected in the primary tumour (so the non-REF and the non-variant nucleotides). The nucleotide with the highest read count is shown in the supplementary figure 1 to illustrate the mismatch error rate at the positions mutated in primary tumour samples. All these background variants (shown in red) were filtered out by our custom filter criteria and all variants listed as true cfDNA mutations were above these background levels.

Comment #3: Have the authors validated the mutations detected by cfDNA by other approach? There is a large variation in the sequencing depth in cfDNA. Some sample has average coverage as low as 154X. Six patients have no mutation detected in cfDNA before surgery. It is highly possible that it is caused by the low sequencing coverage in their study.

Response: Due to lack of material we could not do an independent validation of the mutations detected in cfDNA. The second part of this comment is related to the low average coverage of 154x in one sample. The reviewer probably misunderstood our description of the median coverage data. The low coverage of 154x was observed for a single variant position in one of the patients. In the revised version, we rephrased this sentence to clearly indicate the coverage of the reported mutations (result section, the third last paragraph). Moreover, we carefully checked our entire manuscript and changed a few additional unclear descriptions.

In contrast to the remark of the reviewer, we only had 3 patients without mutations in pre-surgery cfDNA (for the other three we did not have sufficient cfDNA to do the analysis). We do not think that this lack of finding mutations is related to the coverage. To demonstrate this, we have now added 2 additional figures (figure.3) in the revised version of our manuscript. The sequencing coverage of the variant positions in pre-surgery cfDNA of the 3 patients without mutations is within the same range as those of the other patients. For completeness we also made a similar figure for post-surgery samples. These figures illustrate that sequencing depth is not the reason that we did not detect cfDNA mutation in part of the cfDNA samples.
Comment #4: Suggest the authors to demonstrate the reproducibility of their workflow, and evaluate the sensitivity and specificity for detecting the mutations with low allele frequency by the spike-in experiments.

Response: Good suggestion for future work, but regretfully cannot be implicated in this study. We tried to show reliability of our data by additional analysis shown in figures and comments listed above for the previous comments of this reviewer.

Comment #5: The information about how to define the harmful mutation should be further clarified in the manuscript.

Response: We define the detected variants as harmful when Combined Annotation Dependent Depletion (CADD) score \( \geq 20 \). CADD values are listed in the supplementary table S3. In the methods section (data analysis part, the fourth last sentence) of the revised manuscript we now included the CADD score.

Comment #6: The sample size of the study is rather small. Increase of sample size with robust bench work and analysis workflow will strengthen the conclusion in the manuscript.

Response: We are aware that the sample size is a limitation of our study. It is not easy to collect 4 matched samples per each patient and to follow up these patients for 2 years in a relatively undeveloped region.

Reviewer 2 (Reviewer 2):

Comment #1: In this view, the authors should specify how in 3 out of 18 cases there was not enough circulating tumour DNA: is that an expected rate? Were those the first samples studied before perfecting the technique?

Response: These three samples were the last three to be included in our study making it unlikely that technical issues explain the low yield of cfDNA. We do not have an explanation for the low DNA yields in these three samples.
Comment #2: The data are well presented and although not novel, the study presents solid results. The authors should clarify if the post-surgery quantitative data on DNA are expected, if they are dependent on the day after surgery the blood samples are taken and make a comment about a standardization of those times. No obvious correlation with the type of gene or mutation seem to emerge and the authors should be more clear about it.

Response: We agree with the reviewer that this finding is unexpected. The amount of cfDNA was reported significantly reduced three to six months after tumour resection. A potential explanation might be that normal cells die due to the surgical procedures and lead to release of normal DNA. We compared cfDNA in blood samples obtained 3-4 hours after surgery to those obtained 2-9 days after surgery. No obvious difference between the two groups was observed. We now added description about this observation to the result part (Result section, first paragraph, the last sentences).

Comment #3: As stated before, Justify the ccfDNA failure rate and the increase of the tumor DNA after surgery (if time-dependent or other explanation)

Response: Please see our response to points 1 and 2 above.

Comment #4: Few mistakes in the English language in the Discussion section (are lack, more optimal, etc..). The discussion is too long, can be cut in the parts referring to other neoplasms

Response: Language mistakes were revised in the manuscript (highlighted with blue), and we also reduced our discussion as proposed by this reviewer.