Author’s response to reviews

Title: Karyotyping of circulating tumor cells for predicting chemotherapeutic sensitivity and efficacy in patients with esophageal cancer

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Author’s response to reviews:

Dear Editor,

Thank you for your kind letter of “BMC Cancer - BCAN-D-19-00316R1” on 01 May, 2019. First of all, I wish to express my sincere gratitude to you for you kind letter. It really means a great deal to me. It takes your distinguished professor and another reviewer a lot of time to read my first manuscript. Your and your reviewers’ advices are really precious to us and definitely improved my paper. We have revised the paper, and would like to resubmit it for your consideration. Some grammar and spelling errors had also been corrected. Furthermore, the relevant experiments had been made in the manuscript according to the comments of reviewers. Changes to the primary manuscript were red marked. Detailed responses to the reviewers’ comments are listed below. I hope that the revised manuscript will satisfy the requirements for your publication.

If you have any more questions concerning the revised manuscript, please write to me. I will reply to you as soon as possible.

Thanks again for your and all the reviewers’ kind advice.

I am looking forward to hearing from you.

Sincerely yours,
Dear Reviewer 1,

First of all, I wish to express my sincere gratitude to you for your kind advice which definitely improved my paper. It took you a lot of time to read my manuscript. Your timely encouragement means a great deal to me. I have revised the paper according to your suggestion, and answers to your questions are summarized below.

Q: The manuscript lacks a clear introduction of physiology and pathophysiology of multiploidy of CTCs. That greatly weakens the rationale of why the authors read the multiploidy of CTCs instead of cell counts. Please add a brief and essential description in the INTRODUCTION as the background knowledge to support (1) checking the ploidy of chromosome 8, not 9 or 10; (2) checking the ploidy of CTCs, not CTC counts or specific subtype markers of CTCs.

A: Thanks for your suggestion. It really made my paper much more clear. All normal cells in the body have 23 pairs of chromosomes, two for each pair. When a cell becomes cancerous, the earliest and most common abnormalities are the occurrence of aneuploidy on chromosome 8 and specific proteins on the cell surface. It's also accepted. HAO's research showed that the increased copy number of chromosome 8 was significantly correlated with esophageal cancer and lymph node metastasis. CTC count monitoring has been reported to be used for efficacy monitoring and prognosis evaluation of a variety of tumors, but related studies on CTC karyotype subtypes and their clinical significance are rarely reported. This is the purpose of this research.

Q: The clinical information, such as the primary site of the tumor, TNM stages, metastatic sites, radiation dose, chemotherapeutic agents, with or without surgery, and the definition of "benefit number" in the table of major finding were missing. Do the authors mean those patients with PR (n=31) or those who can continue to the second cycle of chemotherapy (n=58)? If the answer is the latter description, the patients who can "tolerate" 2 rounds of chemotherapy can be misclassified as "benefit." Besides, the same question will be raised for the definition of "chemotherapeutic sensitivity" in the manuscript title. These two problems have nearly ruined the major finding of this work.

A: Thanks for your suggestion. Your advices are really precious to us and definitely made my paper much more clear and correct. The patients who benefit from chemotherapy are those we consider to be partial response patients and stable disease patients.

Q: The description of the critical methodology was missing.
In Lines 88-90 in METHOD. The description of the methodology of CTC identification seems to be too brief. Although the methodology has been published, the authors should essentially describe the essential methodology in their words for readers who were not familiar with the device. For that purpose, I recommend the authors to add information containing:
(3-a) The antibodies, kits with their manufacturing information and concentration are all required to describe.
(3-b) The positive and negative controls of experiments. (clinical cases are much better)
(3-c) The definition description of triploidy, tetraploidy or pentaploidy and multiploidy.
(3-d) The definition of CTCs (by marker?) in this study.

A: Thanks for you advice. The testing kit we used was purchased from Cytelligen. This kit has been
commercialized and is easy to operate. We have supplemented the details in the manuscript.

We define the karyotype based on the number of chromosomes 8. The number of chromosome 8 is 3, and the cell is triploidy; the number of chromosome 8 is 4, and the cell is tetraploidy; the number of chromosome 8 is 5, and the cell is pentaploidy; the number of chromosome 8 is more than 5, the cell is multiploidy.

CTCs identified by SET-iFISH are DAPI+ (blue)/FISH+ (aneuploid chromosome 8, range)/CD31-(green) and CD45-(red).

Q: Some critical references seemed to be misplaced, lost and inadequately cited.
(4-a) For example, the author cited reference 1 and 2 to support their description of "the 5-year survival rates of esophageal cancer patients remained as low as only 20-30% due to either chemosensitivity and/or acquired chemoresistance." Reference 1 concluded that CTC could provide important information for patients with metastatic breast cancer and reference 2 was relapse patterns of "early" esophageal cancer who underwent surgical resection. Moreover, the present study was done for patients who received chemotherapy, which means locally advanced stage and unresectable. Reference 3 and 4 were cited to support the sentence, "CTCs measurement as a non-invasive detection method which can monitor therapeutic responses of cancer patients dynamically.". However, reference 3 addressed the issue of prostate cancer. Reference 4 addressed the issue of taking metastasis by CTCs. In my opinion, the first 4 references (the first three at least) seemed inadequately cited. I suggest the authors find other references closely related to esophageal cancer instead of current reference 3. The mistake of citation errors would make readers extremely confusing.

(4-b) The authors cited several very old references for CTC and failed to cite some recent references addressing new insights into clinical values in patients with esophageal cancer. For instance, Reference R1 and R2 were recently published evidence and review of the role of CTC in esophageal cancer. I was wondering the reasons why the author avoid these references in this manuscript?
(4-c) The field of CTC investigation and application evolves very quickly, hundreds of new reference have been published in the literature. Among all the 22 references the authors used, only one reference was published after the year 2017. That implies the citation was too old for readers to catch up with the updated information considering liquid biopsy has been a very hot topic in the most recent decade. I strongly recommend that the authors cite/replace some newer pieces of evidence to support the current work.
(4-d) reference 13 mentioned about circulating "endothelial" cells, not circulating tumor cells, which was epithelial by definition.

A: Thanks for you advice. The reference we quoted were indeed inadequately cited, and we have made modifications and supplements in the manuscript.
Our manuscript was completed relatively early, and some references were newly published, so they were not retrieved at that time. Our references have been updated in the manuscript.
Reference 13 was have made substitutable because we do not inadequately cited.

Q: Table 1 and 2 were crude information. I suggest the authors present them more readable in a statistic way and might consider to move the detailed tables to the appendix.
A: We have made further statistics in table 1. Table 1 was used as supplementary materials.

Thanks again for your kind advice. And I hope my answers will satisfy your requirements.

Sincerely yours,
Dear Reviewer 2,

First of all, I wish to express my sincere gratitude to you for your kind advice which definitely improved my paper. It took you a lot of time to read my manuscript. Your timely encouragement means a great deal to me. I have revised the paper according to your suggestion, and answers to your questions are summarized below.

Q: The methods are not clearly described. For example, the method of CTC isolation is not described. Reagents used in the studies are not identified. The microscope used in the imaging is not described. How to identify patients as triploid is not mentioned. Are there diploid cells? Please add new parts of Methods: Reagents and CTC isolation. The protocol of SET-iFISH should be described. Microscopy imaging should be described. The treatments of chemotherapy should be described.

A: The specific method of CTC isolation was added in the manuscript.

Esophageal cancer patients were classified according to karyotyping of chromosome 8. Patients with number of triploid CTC over or equal to 60% were classified as triploid genotype patients, while patients with less than 60% were classified as non-triploid genotype patients. According to above classified standard, of these 79 patients, 16 were triploid genotype patients, and 63 were non-triploid genotype patients.

Q: Figure 1. Are there diploid cells? How to identify whether they are CTCs or leukocytes.

A: According to the SET-iFISH standard, the number of chromosome 8 is a marker for identifying CTC. Normal cells are diploid, so diploid is not included. CD45 is a surface marker for leukocytes. The cells that express CD45 are leukocytes. CTCs identified by SET-iFISH are DAPI+ (blue)/FISH+ (aneuploid chromosome 8, range)/CD31- (green) and CD45- (red).

Q: Figure 2, is the non-triploid proportion from Table 1 or 3? I assume from table 3. There are 58 patients in Table 3, why only include 17 patients in Figure 2?

A: Figure 2, is the non-triploid proportion from Table 3. Figure 2 shows 58 patients instead of 17. Many data are duplicated in statistics.

Q: It is better to test the correlation of chemotherapeutic efficacy and the non-triploid proportion from table 1 (before therapy) than from Table 3 (after therapy) because you want a parameter to predict the therapeutic efficiency.

A: According to our study, the efficacy of chemotherapy can be predicted based on the ratio of chromosome 8 triploid to triploid in patients undergoing initial chemotherapy. The higher the triploid ratio, the less the benefit of chemotherapy. Our standard is set at 60%. Patients with number of triploid CTC over or equal to 60% were classified as triploid genotype patients, while patients with less than 60% were classified as non-triploid genotype patients. Non-triploid patients are better candidates for chemotherapy.

Thanks again for your kind advice. And I hope my answers will satisfy your requirements.
Sincerely yours,

Prof. Chuanxin Wang