Author's response to reviews

Title: Somatic mitochondrial DNA Alterations In Esophageal Cancer: Significance of Novel Missense and Frameshift Mutations and Alteration in mtDNA Content

Authors:

Leejun Wong (ljwong@bcm.tmc.edu)
Duanjun Tan (dt33@georgetown.edu)
Julia Chang (67842@cch.org.tw)
Lingling Liu (liull529@yahoo.com)
Ren-Kui Bai (renkuibai@yahoo.com)
Yu-Fen Wang (82587@cch.org.tw)
Kun-Tu Yhe (10159@cch.org.tw)

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Author's response to reviews:

Re: 1005499293890781, somatic mitochondrial DNA alterations in esophageal cancer

Dear Editorial Team:

Below are our point-by-point responses to reviewers' comments. We have revised the manuscript accordingly.

Reviewer 1 Dr. Hibi

1. Dr. Hibi felt that examination of D-loop region is sufficient. There is no need to screening the entire mitochondrial genome for somatic mutations. Our view is different for the following reasons. It is very difficult to correlate functional significance of mutations in the non-coding D-loop region. Whereas, nonsense or frame shift mutations, or some missense mutations in protein coding regions definitely will predict functional impact. As it is the case in this study, we have identified several mutations that for sure have functional significance. Should we looked at the D-loop region only, we would have missed all these important mutations. Numerous reports have focused on the study of mutations in the hypervariable D-loop region, which we cannot explain the functional effect. Indeed, our study is the very first to investigate the somatic mtDNA mutations in the entire mitochondrial genome of esophageal cancer, and we used the cost effective and sensitive TTGE method.

2. Dr. Hibi recommended that we sequence the whole genome directly instead of TTGE analysis. This is impractical and unnecessary particularly if TTGE scanning method is sensitive and cost effective. The validation and advantages of TTGE method have been discussed in detail in several of our previous publications (Wong et al Clin chem. 2002;48:1901-12, Wong et al. Electrophoresis 2004;25:2602-10, Chen et al Clin Chem 1999;45:1162-67, Tan et al Cancer Res 2002;62:972-6).

3. We agree with Dr. Hibi that our sample size was too small to have statistically significant results as we have already mentioned in the manuscript. Although it did not reach the statistically significant level, the trend is there. Dr. Hibi is right that it is possible that the surrounding non-cancerous tissue may already have alterations at the molecular level, and we do not exclude this possibility. As a matter of fact the observation of hetero- to hetero- or hetero- to homoplasmic change from surrounding tissue to tumor may has suggested the change at the molecular level in the surrounding pathologically normal tissue. We have pointed this out in our previous publication (Tan et al Cancer Res 2002;62:972). We agree that microdissection may be a better method to really separate tumor cells from normal cells, but this method still does not exclude the possibility that the mtDNA alteration at the molecular level has occur before the pathological changes can be detected.

Reviewer 2, Dr. Ngan

1. We have trimmed down the manuscript a little bit. The "conclusive statements" in the results section of
old Manuscript have been moved to discussion section.
2. Typing errors as pointed out by Dr. Ngan have been corrected accordingly.

Reviewer 3, Dr. Tan

Major Revisions:
1. The use of surrounding "normal" tissue may not be ideal due to field effects. We have added the following sentences to the second paragraph of discussion. "The observation of heteroplasmic to heteroplasmic or heteroplasmic to homoplasmic changes and back changes from surrounding normal to tumor tissues suggests that mtDNA alterations may have already occurred before the pathological changes can be detected (Tan Cancer Res 8). Thus, histologically normal surrounding tissues may not be normal at molecular level. In this regard, peripheral blood may be a better choice. Our results suggest that mtDNA alterations may potentially be good biomarkers for early detection and/or prognosis of cancer."
2. TTGE is a sensitive method, how are the results compared to sequencing? In general, TTGE can detect low levels of heteroplasmy that cannot be detected by direct DNA sequencing (see second paragraph of discussion and previous publication references 30-32, also response to reviewer 1 point 2).
3. Page 8, text describing Figure 1 has been clarified.
4. page 9 about quantitative heteroplasmic changes have been clarified.
5. Page 10, these particular polymorphisms are mentioned because they occur quite frequently that may imply predisposition to cancer but no proof yet.
6. We believe the numbering is correct
7. page 11, We have clarified that these mutations were studied previously in separate publications.
8. page 12, patient E18 clarified.
9. page 13, we deleted the sentence about large deletion.

Minor changes:
1. All grammatical errors have been corrected.
2. explanation of colors is included in the figure
3. Figure 1C the deletion position is not immediately apparent to the readers. The readers will have to write down the sequences of deletion mutant underneath the sequence of the wild type and compare to the actual data, the heteroplasmic deletion will be quite apparent. Regarding Figure 1D, if the relative peak heights of the mutant "G" and the flanking "A" and "C" are compared, it is apparent.

We hope the response is sufficient.

Sincerely yours,

Lee-Jun C. Wong