Author's response to reviews

Title: Characterization of genetic rearrangements in esophageal squamous carcinoma cell lines by a combination of M-FISH and array-CGH: Further confirmation of some splitting genomic regions in primary tumors.

Authors:

Jia-Jie Hao (haojj2011@gmail.com)
Zhi-Zhou Shi (zhizhoushi@gmail.com)
Zhi-Xin Zhao (zhaozx2006@gmail.com)
Yu Zhang (zhangyu909@126.com)
Ting Gong (gonqting8@gmail.com)
Chun-Xiang Li (chunxiangl@gmail.com)
Ting Zhan (zhanjing407@163.com)
Yan Cai (caiyan0818@yahoo.com.cn)
Jin-Tang Dong (jin-tang.dong@emory.edu)
Song-Bin Fu (fusb@ems.hrbmu.edu.cn)
Qin-Min Zhan (zhanqimin@pumc.edu.cn)
Ming-Rong Wang (wangmr07@gmail.com)

Version: 2 Date: 11 March 2012

Author's response to reviews: see over
Dear Editor-in-Chief,

Thank you for your letter dated February 09, 2012. We appreciate your careful evaluation of our work and are grateful to the Reviewers for their valuable suggestions. We have made major revisions following your and Reviewers’ advice.

We have revised our manuscript with taking into account all your comments point by point. All amendments have been highlighted by underline in the revised manuscript. Here is how we have addressed each revision request or comment:

Reviewer 1
Major Compulsory Revisions

1. The authors report a tremendous amount of data, but do not systematically describe or discuss many of these data. Thus, this manuscript is incomplete. It could be divided into translocations, breakpoints, and copy number alterations, and each presented clearly and in detail and then discussed in light of the literature on ESCC and then other carcinomas, including lung, head & neck, breast, prostate, bladder, pancreatic, etc. How do these data compare to those available from the International Genome Project or available under NCBI or The Wellcome Trust Sanger Institute Cosmic or Conan sites on the internet?

   We have amended the construction of the article according to the reviewer’s suggestion, dividing it into copy number changes, structural aberrations, and breakpoints. We added more detail descriptions and discussions on these data, and further compared our results with the available online data from the Wellcome Trust Sanger Institute Cosmic site (Paragraph 2 in the “Results”).

2. For example, the authors report many different breakpoints in ESCC, but it is not clear to the reader their implications as delineated below...

2a) It is not clear how they confirmed these breakpoints (the Supplementary Table 5
said to include the BACs/PACs used appears to be missing, not listed in the additional files, and there is a different Supplementary Table 5, and the BACs or PACs used in the FISH figure (Figure 3) are not mentioned in the Figure legends).

We have added the BAC numbers used for dual-color break-apart FISH in the “FISH” part of “Methods” section, and the information of BACs illustrated in Figure 3 have also been appended in the Figure 3 legend.

2b) Further, it is not clear whether the tumors analyzed by FISH in Figure 3, labeled Tumor 1, for example, are all from the same tumor 1 or from different tumors from different patients. Figure 3 legend and other locations in the manuscript mention amplifications, high level or otherwise, but there is no definition of what constitutes an amplification.

The “Tumor 1” used in different panels were from different tumors in different cases, and we have added the annotation in the Figure 3 legend. The definitions of amplifications and high level amplifications have been added in “Results” section (Paragraph 3).

2c) Further, there is no detailed description of copy number gains or losses in these cell lines or tumors in the entire manuscript, only breakpoints. Breakpoints are interesting, but the effects of the breakpoints, gains, losses, and amplifications are usually what are considered to be important in solid tumorigenesis, not the breakpoints themselves, as in hematologic malignancies. Thus, the authors must discuss the effects of the breakpoints, that is, the gains, losses and amplifications adjacent to the breakpoints. Thus, these must be reported in the text and discussed, and should be known from the array CGH studies. They are listed cryptically in Table 2 as G/L, but no amplifications are shown, shown as lines in Figure 2, but amplifications are not distinguished and neither were cell lines, so it is difficult to determine which gains and losses were associated in the same cell line, and not discussed sufficiently in the manuscript. Nor are the gains, losses and amplifications correlated with the clinical parameters like lymph node metastasis and stage. This is more likely what is important clinically, not simply breakpoints. If not, this merits discussion.
According to the reviewer’s recommendation, we have amended the added the descriptions about the copy number alterations (CNAs) (Paragraph 1 to 3 in “Results” section, and Paragraph 2 to 3 in “Discussion” section). In order to reflect the effects of the breakpoints, we have added the description and discussion of the effect of breakpoints adjacent to the CNAs (Table 4, Paragraph 6 in the “Results” section, Paragraph 2 in the “Discussion”).

We have amended the Table 2 and distinguished the gains and amplifications, as well as the losses and deletions, and the letters of “G”, “N” and “L” have been change into the entire word of “Gain”, “Neutral” and “Loss”. In order to clarify the CNAs associated in the same cell line, we have added a new Table S and. More detail descriptions were added in the text (Paragraph 2 in the “Results” section).

Since we have modified the order for presenting the results, “Figure 2” in the last manuscript has been change to “Figure 1”. In order to make the figure clearer to be understood, we have modified the Figure 1 legend.

2d. The recent literature has shown the importance of translocations in carcinomas.

These authors report primarily breakpoints, and also report lists of translocations between chromosomes without reporting chromosome arms or breakpoints in the results, discussion, and Table S1 (e.g., first paragraph of the results and end of second paragraph of discussion) text of the manuscript. Translocations require chromosome arms and breakpoints to be listed according to ISCN 2009. Further, they don’t discuss whether these uninterpretable translocations without breakpoints have been reported in other carcinomas in the literature or what fusion genes at the not listed breakpoints in the translocations might be important.

We have modified the nomenclature and replaced the table of old versions with a new version, and have described and discussed some of these structural aberrations (Table 3). Since the translocations listed in the original Table S1 was not as you mentioned, we have removed the old version of Table S1.

3. It is not clear whether data have been deposited as required in an appropriate public international database.

We have deposited the raw and normalized data of array-CGH results in the Gene
Expression Omnibus (GEO) database.

4. The literature review in the Introduction is woefully inadequate. The authors indicate that the products and implications of chromosomal rearrangements have been described in only a few types of epithelial cancers. Indeed, only a few types of carcinomas have been sequenced extensively to show actual fusion genes. The genomic landscape of head and neck carcinomas was published in Science by Grandis J et al. and should be noted in the manuscript. However, the literature on chromosomal rearrangements in carcinomas is quite extensive, requiring a third edition of the book, Cancer Cytogenetics by Heim and Mitelman in 2009. Thus, in 2012, to cite only a 2007 review that you interpret as saying that the number of rearranged genes in epithelial cancers is limited is terribly inadequate is very inadequate. This reviewer could cite authors of a variety of such papers on various carcinomas without even going to PubMed. The Heim and Mitelman is a good place to start looking for more recent (than 2007) literature reviews before going to the primary literature.

We agree with the reviewer, and have amended the “Introduction” section with more recent literature (Reference 4 to 15).

5. To say that ESCC is one of the most common malignant epithelial cancers without population statistics from the literature is not only wrong, but requires a literature citation(s). Worldwide, in China, where is it one of the most common malignant epithelial cancers? This requires clarification and literature citation.

We have modified the sentence as “Esophageal cancer (EC) is a common malignant epithelial cancer worldwide, causing more than 40,000 deaths each year. The most prevalent type of EC is the esophageal squamous cell carcinoma (ESCC), and China is among the highest risk areas.”, and we have also added relative references (Paragraph 4 in “Introduction”).

Minor Essential Revisions

1. What constitutes a microamplification or microdeletion? How do they differ from
amplifications and deletions/gains and losses or amplifications? How are these defined?

We use “microamplification” and “microdeletion” to interpret the small regions fragments with copy number alterations. In order to make them clear, we have changed them to “small amplification or deletion region”. Table S2 about these alterations in the old version was not necessary for the entire article, so we have removed it from the new version.

2. The language should be edited by a native English speaking scientist familiar with the literature in this field.

The manuscript has been edited by a native English speaking scientist.

Reviewer 2

1. In the first paragraph of “Results” section, authors should clarify that M-FISH was performed on 3 or 4 of ESCC cell lines?

The number of cell lines we used in M-FISH experiment is four, and we have amended the number of cell lines in the first paragraph of “Results” section.

2. In Figure 3C, could authors explain why more copies of Cancer_1D11 were detected in tumor 1 in the left panel compared to the middle panel?

The “Tumor 1” used in the left and middle panels were from different tumors in different cases, and we have added the annotation in the Figure 3C legend.

Reviewer 3

Major compulsory revisions

1. The cell line KYSE180 has been characterized by M-FISH previously as per reference 10. This is not discussed in the introduction where it would be most helpful. Characterization of KYSE450 is said to be described in reference 10 (in Results) but it is not.

According to the reviewer’s suggestion, we have added the information on the
chromosomal alterations in KYSE180 and KYSE450 in the “Introduction” section (Paragraph 4), and we have added the reference for the M-FISH results of KYSE450.

2. Some background information on previously reported findings would be very helpful. Please clearly state which are the novel findings in this paper. The reader is left to go and look up multiple references to find out what is novel here.

We have added some background information about our findings in the “Discussion” sections. To summarize, the novel findings in this study include: 1) providing detail background information on the genomic copy number alterations and structural aberrations for these ESCC cell lines. 2) recurrent breakpoints in ESCC cell lines and primary tumors were discovered, and the genes at or near the boundaries of the breakpoints which may be important. 3) 11q13.3-q13.4 is frequently splitting in ESCC, and it is associated with lymph node metastasis, which has not been previously reported in ESCC.

3. More discussion on the amplification at 11q13 in ESCC and other tumors is warranted. Place your results in the context of previous studies. In particular make it clear that 11q13 amplification is well documented, and discuss what gene(s) may be the target of this.

In view of the reviewer’s suggestion, we have discussed the amplification of 11q13 and its candidate targets (Paragraph 6 in the “Discussion” section).

4. I don’t find the description of the FISH method is adequate to follow. Specifically, the reference for FISH [20] describes only FISH of BAC probes, the composition of the hybridization mixture is not detailed, and protocols for the M-FISH are not described either in the manuscript or reference [20]. It is not explained exactly what is meant by “hybridized twice” (how to remove the first probe for example). This is a useful in-house approach which others might want to use.

We have added the procedures for M-FISH and BAC DNA FISH (see the “FISH” part in “Methods”). The twice hybridization was exactly an in-house method which was developed in our lab, and the detail protocol was introduced in the reference [28], and we have added the instruction at the last paragraph of “FISH” in “Methods”.

5. Detection of recurrent breakpoint regions in primary tumor tissues. The rationale for this section is difficult to understand and needs to be more clearly explained. This is really the only section where clarifying the English will improve meaning. See below.

Many of the rearrangements usually occurred at the boundaries of the breakpoints, resulting in the fusion genes, truncated genes, as well as other structural variants. The recurrent breakpoints are worthwhile to be assessed for identifying the frequent gene rearrangements, which might influence the expression of the oncogenes or tumor suppressor genes at or near the breakpoints. We have added the rationale in the “Introduction” (First two sentences in Paragraph 3) and “Discussion” section (Paragraph 2).

6. There is clearly high level amplification at a common ~800kb region but also the breakpoints cluster at 11q13 (Figure 3A). Can you discuss whether this common amplified region gives any new information which can be used to identify an amplified oncogene? Is the amplified region defined by your results smaller than previously published? Given that these are unbalanced rearrangements with variable breakpoints (not in a single gene) and that there is high level amplification can you give more discussion to the possibility that an oncogene is the target of amplification and how this fits with the other possibilities you mention? Is there a good reason to discount amplification as the oncogenic event as you seem to be suggesting?

We have added the the genes located in the highly amplified region of 11q13 and around the breakpoints (Table 2 and Table 4), and several oncogenes were shown in the results. We have added the amplified regions with the published data and discussed the possible target oncogenes. We have also added the discussion for the possibilities and implications of the breakpoints at the boundaries of CNAs in the “Discussion” (Paragraph 2).

7. The 11q13 breakpoints (Fig 3) occurred on either side of the amplified region. How does this fit with the possibility that you discuss, that the translocations dysregulate another gene? Is the clustering of breakpoints related to the mechanism of translocation (e.g. hotspot of recombination) rather than dysregulation of a specific
gene in addition to amplification?

The genes located inside of the amplification region and those at both sides of the breakpoints may be influenced by different mechanisms, may be affected by amplification, but no breakage. While genes at or near the boundaries of the amplified regions might be one of the rearrangement sites, and gene expression may be further influenced. The genes at the breakpoints may also be rearranged by themselves.

8. Reference 25 is a nice study on the relationship between OSCC and FRA11F and discussion on this aspect would be worthwhile.

We have mentioned the relationship between FRA11F and amplification of 11q13 in the “Discussion”. (The last Paragraph in the “Discussion” section).

9. “Fifty-seven arm breakpoints were located at common fragile sites.” And similar on the next page (“Ten of these … at the common fragile sites”) This is misleading as most of these fragile sites have not been sequenced or accurately localized. The breakpoints have been localized in the vicinity of fragile sites. The significance of this is not discussed.

Accepted the reviewer’s suggestion, we have replaced “breakpoints were located at the common fragile sites” with “breakpoints were localized within the vicinity of fragile sites”. The correlation between the breakpoints of the fusion genes and the fragile sites has been emphasized by previous studies. For example, Burrow et al (2009) analyzed 444 pairs of genes involved in the cancer-specific recurrent translocations using the databases. They found that 52% of the breakpoints in at least one gene of the fusion-gene pairs were localized within the vicinity of the fragile sites, and 12% of the gene pairs were colocalized with the fragile sites. Thus, the breakpoints near the fragile sites may be helpful for further discovering and identifying cancer related fusion genes. We have added the significance to the “Discussion” section (Paragraph 4).

10. You state that splitting of the 11q13 region is an independent predictor of metastasis. Amplification of 11q13 rather than splitting of the region could be the relevant factor here. Has an association between 11q13 amplification and lymph node metastasis been reported? Is there a significant association when there is
amplification but no splitting? Put this finding in the context of other studies linking gene aberrations in ESCC (particularly 11q13 amplification) and other tumors with prognosis, to give authority to your claim. Mention it in your abstract if it is truly novel.

The relation between amplification of 11q13 and lymph node metastasis (LNM) has been studied in several cancers. However, there are still contrary opinions (Paragraph 8 and 9 in “Discussion” section). The correlation between splitting of 11q13.3-q13.4 and LNM in ESCC was first discovered in our study.

11. Discussion paragraph 5, 11q13 is involved in various recurrent translocations but most or all of these are balanced and the genes involved are known, which should be mentioned so as to highlight the difference with the case in ESCC.

According to the reviewer’s recommendation, we have mentioned the differences between ESCC and other malignancies in the “Discussion” (Paragraph 7).

12. Cytogenetic nomenclature is not ISCN compliant e.g. der(1)(1pter-1?p10::9p10->9pter)

We have modified the nomenclature and replaced the table of old versions with a new version (Table 3).

Minor essential revisions
1. Introduction
1) 3rd paragraph – aCGH has been introduced for more than just translocation breakpoints, e.g. copy number aberrations such as monosomy, trisomy, deletion, amplification.

We have added the introduction of array-CGH for detecting the copy number aberrations in the third paragraph of “Introduction” section.

2) 4th paragraph array-CGH references [17-19] presumably was not done on metaphase spreads as stated.

The whole sentence has been modified (Paragraph 4 of “Introduction”).
2. **DOP-PCR is not referenced** DOP-PCR

The cited reference for “DOP-PCR” has been added in the Paragraph 3 of “Methods” section.

3. **BAC/PAC are not defined**

We have removed “PAC”, and added the definition of “BAC” in Paragraph 4 of the “Methods”.

4. **The human genome build is not mentioned. This is a must to allow comparison to the reference map.**

The Build hg18 was used in this study, and we have added the sentence “The annotations for the probes were based on UCSC hg18 (NCBI Build 36).” (“Genomic DNA isolation and oligo array-based comparative genomic hybridization (array-CGH)” in the “Methods”).

5. **I question the use of Metafer (Metasystems) for constructing and analyzing pseudo-color images (as stated) (this is a metaphase finding system).**

The Metafer system consists of several modules, including MSearch, MetaCyte, RCDetect and so on, and all of these modules are based on the Metafer platform. The metaphase finding function was operated by the MSearch module, while the MetaCyte was designed for scanning the images from the interphase FISH slides, and the pseudo-color images could be analyzed by this module. In order to clarify the statement, we amended it as “MetaCyte module of Metafer imaging systems”.

6. **Method for labelling for array CGH is not mentioned (Agilent has more than one).**

The labeling information has been added in the array-CGH methods.

7. **11q is not mentioned in the list of gains (Unbalanced breakpoints, Results section).**

It has been added to the results (Paragraph 1 in the “Results” section).

8. **Methods: FISH – refer to “Split genes were detected…” – refer to split regions, not split genes.**

We have replaced the “genes” with the “regions”.
Discretionary revisions

1. The English is good but could benefit from minor rewording for clarity in the Section: “Detection of recurrent breakpoint regions in primary tumor tissues”. In my opinion substituting “splitting” for “split” except in the 3rd last line (“were split in 16.9%”) improves readability of this section. Change “split occurrence” to “splitting” in the 3rd line. In the rest of the manuscript substitute “splitting” for “split” as appropriate (present tense only).

We accepted that “splitting” is more preferable than “split”, and we have changed them according to the reviewer’s suggestion.

2. Last page, “2) The fragments may be…” (insert “be”)

We have inserted “be” in the corresponding position.

3. Last page, “3) Truncated gene products may result…” (“result” instead of “be resulted”)

The “be resulted” has been changed to “result”.

4. Statistical analysis, line 2 “characteristics” (spelling)

We have corrected the spelling of “characteristics” in the “Statistical analysis”.

Many thanks again for your interest in our work. We hope that in its present form the article will be suitable for publication in BMC Cancer.

Sincerely Yours,
Ming-Rong WANG, Ph.D., Professor
State Key Laboratory of Molecular Oncology
Cancer Institute/Hospital
Peking Union Medical College
Chinese Academy of Medical Sciences