Author's response to reviews

Title: The correlation of WWOX, RUNX2, and VEGFA protein expression in human osteosarcoma

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Version: 2 Date: 16 September 2013

Author's response to reviews: see over
Dear Prof. Tim Sands,

I would like to thank you and the reviewers for the positive review of our manuscript entitled “The correlation of WWOX, RUNX2, and VEGFA protein expression in human osteosarcoma”. Having carefully addressed all the critique raised by the reviewers, we would like to re-submit our revised manuscript for your consideration as an Original Article in *BMC Medical Genomics*. The new information is marked as blue text in the revised manuscript. Our detailed response to the critiques is listed below.

**Response:**

We have carefully addressed all the critique and suggestions raised by the editor and reviewers and provide a cover letter giving the point-by-point response to the concerns.

Further consideration of your manuscript is conditional on improvement of the English used - please bear in mind that as we are a free-access publisher, we cannot bear the costs of copyediting English ourselves. Please ensure particular attention is paid to the abstract. You should have a native English speaking colleague help you with this, if possible, or you may need to use a professional language editing service. For authors who wish to have the language in their manuscript edited by a native-English speaker with scientific expertise, BioMed Central recommends Edanz (www.edanzediting.com/bmc1). BioMed Central has negotiated a 10% discount to the fee charged to BioMed Central authors by Edanz. Use of an editing service is neither a requirement nor a guarantee of acceptance for publication. For more information, see
our FAQ on language editing services at
http://www.biomedcentral.com/info/authors/authorfaqs#12.

Response:

We have revised the manuscript carefully by native English speaking colleague. Furthermore, we use the professional language editing service, Edanz, which is recommended by BioMed Central.

Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals). It is important that your files are correctly formatted. Please include an 'Authors' contributions' section before the Acknowledgements and Reference list. Please also rename your "disclosure" section "Competing Interests".

Response:

We have revised our manuscript according to the journal style and all our files are correctly formatted. We added "Conclusion" and "Authors' contributions" sections before the Acknowledgements. We also renamed "disclosure" section with "Competing Interests".

Reviewer: Rami I. Aqeilan

Reviewer's report:

Paper by Yang et al investigated the correlation of WWOX, RUNX2 and VEGF in osteosarcoma using aCGH and immunohistochemistry on a relatively small number of samples. They found no correlation between WWOX and RUNX2, WWOX and VEGF but direct correlation between RUNX2 and VEGF.

Overall evaluation. Before even judging this work, it requires extensive English editing to make it clear and readable. It is not understandable due to miss use of improper verb and tense of authors sometime refer to current findings or previous work. When referring to previous findings, authors should present perfect and when referring to new data, they have to use simple past. Several problems hindered this study pre-mature for publication.

Response:

We thank the reviewer for this good suggestion. At his recommendation, we have had the entire revised manuscript reviewed by native English speaker and edited by the professional language editing service, Edanz, which is recommended by BioMed Central. Particularly, we simplified the previous findings in the "Results" section to make data more clearly.

Major points

1. The number of samples for genomic study is at best 10 though I could not see this in Table 2 (see below). This is a relatively low number to make any conclusion especially for correlation studies. This would make the conclusions of this article untrusted.

Response:

We thank the reviewer for raising an important issue. We confess that 10 samples for aCGH is a relatively low number for the correlation between gene copy number alterations and protein
expression. However, osteosarcoma is a rare sarcoma and it is hard to get enough fresh samples to do more aCGH. Due to limited samples and resources we have to analyze their correlation in 10 samples. In the future, we would like to investigate the correlation of these molecules with more osteosarcoma cases.

2. Authors stated in methods that they use 10 fresh osteosarcoma samples for aCGH while in Table 2, WWOX samples are 8; those for RUNX2 are 9 and those for VEGFA are 47!!! How can this be explained??

Response:
We think the reviewer pointed out the confusion of case number in the tables. We explained the case numbers here and also in the text.

In our data, we had overall 10 fresh samples for aCGH. Only 9 of these 10 cases had FFPE tissues for IHC assay and another 1 case did not have FFPET tissue for further assay. Totally we had 54 FFPE samples for FISH and IHC assays, including the 9 cases which their fresh tissues had been analyzed by aCGH.

For the RUNX2 protein expression assay by IHC in total 54 cases, only 9 of the 10 cases which their fresh tissues had been analyzed by aCGH had protein expression results, which was showed in Table 2. We added sentences in the "Methods" section and the Table 2 to make these more clearly.

When we performed IHC assay for WWOX protein expression, the FFPE tissue sections of 1 sample were used up and only 8 cases could be used in IHC assay. So we analyzed the copy number alteration of WWOX gene and its protein expression only in 8 cases, which was showed in Table 2. We added sentences in the "Methods" sections as well as the Table 2 to make these more clearly.

In FISH assay we only got 47 evaluable samples because that FISH results were not available for 7 samples because of no enough tissue and loss of tissue during the pretreatment process. To make clear this issue, we added sentence we added sentences in the "Results" section as well as Table 2 to make these more clearly.

3. A positive correlation was found between VEGFA and RUNX2 only at protein level; VEGFA is already known as a target of RUNX2, however authors do not refer to it. It is to be expected thus to have this correlation.

Response:
We fully agree with the reviewer’s point that VEGFA is already known as a target of RUNX2 and referred in the "Discussion" section. In our analysis showed this data because we wanted to add more in vivo evidence of this correlation in osteosarcoma.

4. Authors do not explain the association between RUNX2 gene amplification and loss of RUNX2 protein expression? This is very unusual!! Is it possible that there is an autoregulatory loop?

Response:
We agree that it need more discussion about the association between RUNX2 gene amplification and loss of RUNX2 protein expression. We went over these cases and found that the FFPE samples of these 3 cases (with loss of RUNX2 expression but harboring RUNX2 gene amplification) were obtained from post-chemotherapy tissues. It might be the chemotherapy affect the RUNX2 expression. We further found evidence from the published paper of Prof. Rami I. Aqeilan (Reference 14: Cancer Res. 2010, 70(13): 5577–5586). In their analysis, immunohistochemical analyses of 56 of osteosarcoma cases revealed that 60% (12/20) of pre-treatment biopsies were positive for RUNX2. However, only 16% (4/25) of post-treatment resections were positive for RUNX2. Paired pre-treatment biopsy and post-treatment resections were available for 12 OS patients. Eight of the biopsies were RUNX2 positive and all (100%) became RUNX2 negative post-treatment. So we hypothesized that the negative expression of RUNX2 protein might due to the chemotherapy. In the future, we plan to obtain pre-treatment tissues to perform aCGH and IHC to validate the hypothesis.

5. Authors stated that the lack of WWOX protein can promote RUNX2 expression, earlier in the text they stated that there is no significant correlation between WWOX and RUNX2 levels protein. They should check at which level RUNX2 is attenuated in RUNX2 gene copy number amplified samples.

Response:

The reported data suggested that the lack of WWOX protein might promote RUNX2 expression (Reference 13 and 14: J Biol Chem 2008; 283: 21629-21639; Cancer Res 2010; 70: 5577-5586). However in our present study we found there was no significant correlation between WWOX and RUNX2 levels protein. It might due to the FFPE tissue sections for IHC assay obtained from post-treatment tissue because pre-treatments such as chemotherapy might affect on the protein expression of RUNX2 (Reference 14: Cancer Res 2010; 70: 5577-5586). We added some sentences in the text to explain this results. In Table 2, it is RUNX2 protein expression (detected by IHC) is attenuated in RUNX2 gene copy number amplified samples. We added notes to make it clearly.

6. There is no in vitro study to assess the association between the three gene to prove that actually high levels of RUNX2 can lead to higher VEGFA expression in osteosarcoma cells.

Response:

We thank the good suggestion of the reviewer to perform vitro study to assess the associations between the three genes to prove that actually high levels of RUNX2 can lead to higher VEGFA expression in osteosarcoma cells. In the future, we would set up such experiments to validate the results.

7. No clinical correlation studies on gene copy number amplification.

Response:

We thank the useful comment of the reviewer. Because only 10 cases were performed aCGH assay, it is hard to analyze the correlations between gene copy number amplification and clinicopathological factors. For VEGFA gene amplification, we had FISH assay data in more than 50 cases and could perform such analysis. The results were published and it showed that
amplification of the VEGFA gene and elevated expression of the VEGFA protein were associated significantly with microvascular density and adverse tumor-free survival in patients with osteosarcoma (Reference 24: Cancer 2011; 117: 4925-4938).

Level of interest: An article whose findings are important to those with closely related research interests

Response:
We thank the reviewer for the positive support and agree with the point that the paper would be useful for readers to get more information about WWOX, RUNX2, and VEGFA in osteosarcoma.

Quality of written English: Not suitable for publication unless extensively edited

Response:
We thank the reviewer for this good suggestion. At his recommendation, we have had the entire revised manuscript reviewed by native English speaker and edited by the professional language editing service.

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Response:
We thank the reviewer for the positive support.

Reviewer: Andrew E Horvai

Reviewer’s report: In this manuscript, the authors investigate the gene copy number changes (by aCGH and FISH) and protein expression (by immunohistochemistry) of three genes (WWOX, RUNX2 and VEGFA) possibly involved in osteosarcoma tumorigenesis. The genomic results and immunohistochemical expression do not correlate for each gene, but the results are nonetheless interesting and worthy of publication. The hypothesis is clearly defined and the methods used are appropriate to answer the questions posed.

Response:
We thank the reviewer for the positive support.

Minor Essential Revisions

1. Although the manuscript is understandable, it might benefit from careful review of grammar by a native English speaker. It contains numerous minor grammatical errors (subject-verb agreement, preposition omitted, spelling etc.).

Response:
We thank the reviewer for this good suggestion. We have had the entire revised manuscript reviewed by native English speaker and edited by the professional language editing service.
2. I notice from Table 1 that 4 osteosarcomas were stage I, which by AJCC/UICC criteria implies they were low grade. Was there any difference in the aCGH pattern of these low-grade osteosarcomas? Since previous studies have suggested 12q13-15 amplification in low grade osteosarcomas, it might be worth mentioning in the discussion how the authors' data on the low-grade tumors relates to those prior findings and to reference the literature on 12q13-15 in osteosarcoma.

**Response:**

We appreciated the reviewer’s good idea to investigate the difference in the aCGH pattern of these low-grade osteosarcomas. However in this study we could not perform such analysis because these 4 cases did not have aCGH data. Both our GSE 19180 and GSE9654 data did not show significant 12q13-15 aberrations, might due to the high grade characteristics of the 20 osteosarcomas (Reference 23 and 24: Cancer Lett 2010; 291: 31-38; Cancer 2011; 117: 4925-4938). Furthermore, MDM2 and CDK4 genes located in 12q13-15 region found no significant aberrance in the 1147 amplified genes list of GSE19180 data (doi:10.1016/j.canlet.2009.09.018; Reference 23: Cancer Lett 2010; 291: 31-38). Further aCGH or Next Generation Sequencing investigation in low grade osteosarcoma might character the12q13-15 amplification.

3. I am confused by Figure 1 A. The legends reads ? line-plots denote the estimated copy number value of 20 specimen? Is Figure 1A an overlay of ALL cases tested by aCGH? Or an average of gains and losses among all the cases? Or is this just a representative genome from one case? Any of the above would be fine, it?s just not clear from the figure legend which one it is.

**Response:**

In **Figure 1A**, the recurrence pattern of copy number alterations (CNAs) in 20 human osteosarcomas are illustrated in 2 microarray-based comparative genomic hybridization (aCGH) datasets (GSE19180 and GSE9654). The x-axis indicates chromosome numbers, and the y-axis indicates the aberration frequency of gains (positive) and losses (negative) for each measured aCGH probe arranged based on their genomic coordinates along the x-axis. Dashed lines indicate the thresholds for significant recurrent aberrations. Measured sequences with aberration frequency that exceeded the thresholds are color coded to emphasize the locations of significantly recurrent aberrations (red indicates significantly recurrent amplification; green, significantly recurrent deletion; gray, nonsignificant recurrence of aberrations).

We changed the legend of **Figure 1A** to make it easier understanding.

**Discretionary Revisions**

1. Figure 2 is not very convincing of amplification. I do not doubt the authors? results, but many cells seem to have equal numbers of orange and green, some have orange but no green (and thus probably should be excluded from analysis). It might be better to just demonstrate one nucleus with clearly increased numbers of orange signals relative to green signals.

**Response:**

We thank the good suggestion on FISH data demonstration. According to the suggestion, we re-organized the **Figure 2** to demonstrate nucleus with clearly increased numbers of orange signals relative to green signals.
2. In the results, the authors state "Interestingly and surprising, in our 10 osteosarcoma samples performed aCGH detection, all 3 osteosarcoma samples with RUNX2 amplification were observed negative protein expression." In the discussion, this is addressed with the following statement: "So our data which demonstrate lack of significant positive relation between RUNX2 gene amplification and frequent protein expression might suggest that in osteosarcoma the increased expression of RUNX2 is likely to be driven by other factors in addition to gene amplification." This discussion point would apply if there was no amplification and the cells overexpressed RUNX2 by immunohistochemistry. But, it does not touch on the disconnect between genetic amplification and undetectable expression by immunohistochemistry. Since this is such an unexpected finding, it would be useful to the readers if the authors proposed some explanation(s).

Response:

We appreciated the good comments on the discussion and agree that it need more discussion about the association between RUNX2 gene amplification and loss of RUNX2 protein expression. Both two reviewers mentioned this issue.

As mentioned above, we tried to explain the results reasonable. We found that the FFPE samples of these 3 cases (with loss of RUNX2 expression but harboring RUNX2 gene amplification) were obtained from post-chemotherapy tissues. It might be the chemotherapy affect the RUNX2 expression. We further found evidence from the published paper of Prof. Rami I. Aqeilan (Reference 14: Cancer Res. 2010, 70(13): 5577–5586). In their analysis, only 16% (4/25) of post-treatment resections were positive for RUNX2. Paired pre-treatment biopsy and post-treatment resections were available for 12 OS patients. Eight of the biopsies were RUNX2 positive and all (100%) became RUNX2 negative post-treatment. So we hypothesized that the negative expression of RUNX2 protein might due to the chemotherapy.

Level of interest: An article whose findings are important to those with closely related research interests

Response:

We thank the reviewer for the positive support and agree with the point that the paper would be useful for understand the correlation of WWOX, RUNX2, and VEGFA in osteosarcoma.

Quality of written English: Needs some language corrections before being published

Response:

We thank the reviewer for this good suggestion. We have had the entire revised manuscript reviewed by native English speaker and edited by the professional language editing service.

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Response:

We thank the reviewer for the positive support and agree with our statistical process.
After completion of these modifications, we feel that we have sufficiently addressed all the critique raised by the reviewers. I hope you will find the revised version suitable for publication in *BMC Medical Genomics*.

Please do not hesitate to contact me if you need additional information.

Sincerely yours,

Jilong Yang, M.D., Ph.D.