Author's response to reviews

Title: The expression of Gli3, regulated by HOXD13, may play a role in idiopathic congenital talipes equinovarus

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Author's response to reviews: see over
Dear Dr. Rikki Graham,

Thank you for your kind letter regarding manuscript (No. 4772673862723102) dated 7,23, 2009, and the referees’ remarks. We have revised and modified the MS in accordance with the reviewers’ comments and carefully proofread the manuscript to minimize typographical, grammatical, and bibliographical errors. As you suggested, we have addressed the ethical principles and employed an editing service company to proofread my MS. At this time, we have re-submitted the revised MS and we hope to have an opportunity to publish this paper in BMC Musculoskeletal Disorders

Below, please find our responses to the revisions proposed by the reviewers’ comments.

**Reviewer #1:**

*Comment 1:* The writing is not acceptable in its present form as it includes multiple grammatical errors. It is difficult to read.

Response: I have employed a professional copy editing service to help me copy edit the paper.

*Comment 2:* There are multiple mistakes in the Introduction indicating that the understanding of limb development by the authors is not sufficient. For example, it is unclear the meaning of “segregated expression of transcription factors” and even more in relation to the ZPA. Also, for *Drosophila* should be hh (not Shh) and the explanation of Gli3 Fl versus truncated form is confused.
Response: We have deleted the sentence “This patterning is due to the segregated expression of transcription factors” and changed the Shh gene to Hh in Drosophila. We agree that “Gli3 Fl versus truncated form” was not clearly explained and have made an effort to further clarify the issue.

Comment 3.- The methods are not well described:

- There is no enough information about the experimental and control group of patients. The number of controls is unknown as well as how they were selected and how they match the experimental group regarding age, sex, etc…

Response: We have added the information in accordance with the recommendations of the reviewers. In the experimental group, there were 21 patients (13 males and 7 females), aged 4-12 years old with a mean age of 6.7 years. The control group consisted of five males and four females, aged 5-11 years old with a mean age of 7.5 years.

- The number of ICTEV animal used is unknown, neither the percentage that developed ICTEV after RA treatment. A picture of the morphology of the induced ICTEV in the rats should be shown to appreciate the similarity with the human ICTEV

Response: In accordance with the recommendations of the reviewers, we have added information regarding the model mice induced by RA in the original paper. Pictures of ICTEV patients, model fetal rats and normal control fetal rats have also been included.
- It is unclear what the authors refer as “ankle muscle” because there is no muscle in the ankle, mostly tendons. They should precisely indicate from which muscle or muscles was the biopsy obtained and why the muscle tissue was selected.

  Response: The word “ankle muscle” in the original paper was not precise. It was actually flexor hallucis longus, which is used in the updated manuscript. We selected that muscle tissue because we resected part of the flexor hallucis longus when performing corrective surgery on the patients. Because it was the abnormal growth of this muscle that led to the occurrence of ICTEV, we consider studies on the flexor hallucis longus to be of significance.

- There is no information about how the lung tissue was obtained.

  Response: In the materials section, we have mentioned that the lung tissue was obtained from the Department of Anatomy, China Medical University. The reason why this lung tissue was selected was that the Gli3 gene in the adult human is only expressed in the lung, placenta and testes, therefore, setting the lung tissue as the positive control was appropriate for this study.

- For the Gli3 western blot there is some contradictory information as whether the cytoplasmic or nuclear fraction or both were analyzed. More important, in the western blot (figure 3) only the FL is shown while both the FL and the short truncated form are required.
Response: Previously, it was not made clear whether it was the cytoplasmic or nuclear protein. It was the cytoplasmic protein that was used in the experiment. The nuclear extract kit (Activ Motif, USA) can also be used to extract cytoplasmic protein from tissue. We have modified this statement in the original paper. For the western blot, we had neither transferred the Gli3-83KD fragments to the membrane nor performed ECL color development. We have now performed these experiments according to the recommendations of the reviewers and included the results in the manuscript.

- *The immunos in Fig. 4 are referred either as ankle muscle of ICTEV animals or as hindlimbs?*

Response: Figure 4 shows the hindlimb muscles of ICTEV model rats. The muscle was located in the ankle, which was also the affected site in ICTEV patients. The flexor hallucis longus is located at the same site. Thus, we believe this muscle is significant.

*Comment 4. - The results section is clearly deficient.*

- *It is unclear whether the authors set to analyze Gli3 expression in the “anklemuscle” of patients of unknown age. There is no rational for this.*

Response: We did not analyze Gli3 expression in patients of other ages because all of the patients presented in childhood and surgical corrections were made at that time point. No specimens from older children could be obtained. Moreover, Gli3 is a development-related gene. We consider that Gli3 is expressed on the ankle in the embryo stage, and that Gli3 expression in the ankle of the lower limb may not exist
after birth.

- If they are thinking in a genetic implication of Gli3 in ICTEV, it is unclear whether they set up to study the non-genetic effects of RA. Furthermore, they don’t take into account that RA, at least during limb development, is able of inducing Hoxd13 expression. The relation between the model they use and the human ICTEV is unclear. It is of some interest that they don’t find genetic mutations in Gli3 in patients with ICTEV.

Response: The fact that we do not find genetic mutations of Gli3 in patients with ICTEV does not indicate the lack of a relationship between the Gli3 gene and ICTEV, and we have made additional comments on this topic in the discussion section. There is a clear relationship between HoxD13 and ICTEV (see reference 28). HoxD13 expression level in patients with ICTEV is downregulated, while the HoxD13 binding sites in the promoter region of the Gli3 gene is in a negative control region. We therefore suggest that HoxD13 regulates the Gli3 gene, which leads to the occurrence of ICTEV. We have not yet detected the HoxD13 expression level in model fetal rats with ICTE induced by RA, and further investigations regarding this aspect are planned.

- The results of the Hoxd13 binding sites in the 5’ region of the rat Gli3 are of potential interest but preliminary and the connection with the pathogenesis of the ICTEV unclear.
Response: We plan to perform further experiments regarding this aspect to obtain more convincing results.

Comment 5.- There is almost no discussion or interpretation of the data.
Response: In accordance with the recommendations of the reviewers, we have included additional interpretation and discussion of the data in the discussion section.

Reviewer #2
Comment 1: The manuscript is sound and appropriate. I suggest to spend a few sentences in the discussion to specify that the mouse model induced with retinoic derivatives is not a general model for ICTEV but rather an experimental model. This means that the identified mechanism of Gli3 overexpression might just be restricted to this model.

Response: We have included additional content regarding studies on ICTEV induced by retinoic in the discussion section.

Comment2: The manuscript needs some editing.
Response: We have asked a professional copy editing service to help with the copy editing of the manuscript.

Reviewer #3
Comment 1: Please explain how lung tissue was obtained on clubfoot patients. This is
hard to imagine this getting approved by a local IRB review board.

Response: The lung tissue was not obtained from clubfoot patients, but from cadaver specimens from the Department of Anatomy, China Medical University. The study was approved by China Medical University Ethics Committee.

Comment 2: Where was the muscle biopsies taken from?

Response: The biopsies were taken from the partial flexor hallucis longus resected from patients undergoing surgical correction. Informed consent for participation in scientific research was obtained from all patients.

Comment 3: The animal model used is of questionable value. The model is induced with all-trans retinoic acid.

Response: Indeed, there is some controversy regarding the rat model induced with all-trans retinoic acid, but there is unfortunately no better rat model for ICTEV. Therefore, when selecting specimens for this study, we only selected fetal rats with developmental malformation in the lower limbs, similar to those observed in ICTEV patients, in order mimic symptoms as closely as possible the malformations of human patients.

Thank you in advance for reconsideration of our revised manuscript, and please let us know if there are any additional questions, comments or concerns.
Sincerely yours,

DonHhua Cao