Author's response to reviews

Title: Down-regulation of the expression of CCAAT/enhancer binding protein alpha gene in cervical squamous cell carcinoma

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Author's response to reviews: see over
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Prof Ratna Vadlamudi
BMC Cancer

Dear Prof Vadlamudi,

Revision of Pan et al. (MS: 1013351680111737)

Thank you very much for your email on 27 December 2013 regarding our manuscript, titled “Down-regulation of the expression of CCAAT/enhancer binding protein alpha gene (C/EBPalpha) in cervical squamous cell carcinoma” (MS: 1013351680111737).

On behalf of all authors, I would like to thank you for organizing the peer-review of our manuscript and for the valuable comments from the reviewers. We have revised the manuscript thoroughly according to these comments and reformatted the manuscript to BMC Cancer journal style.

We respond to the comments of the reviewers below, point by point. Our responses are in bold.

Reviewer’s comments
Reviewer #2 (Minoru Tomizawa)
Down-regulation of C/EBPa in carcinogenesis is a classical idea. C/EBPα in cervical cancer has not been analyzed well. The aim and conclusion of this study is reasonable and understandable.

Major compulsory revisions
1. However, staining results of C/EBPa did not seem consistent. This means that the results were not reliable.
Our response:
We detected C/EBPa protein expression using immunohistochemistry method. This method has been used in many published papers and thus is reliable. For instance, a paper published recently below also used this method.

We have addressed the inconsistency issues in detail. See our responses to points 6 and 7 below.

2. Transfection experiments were repeats from many literatures in 1990s. Problem of transient transfection is its efficiency. Non-transfected cells interfere the results. How did the authors think about transfection efficiency? The structure of this
Transfection efficiency is a key issue considered in the design of our study. To ensure the reliability of the transfection results, we set up 2 control groups alongside the experimental group. In the experimental group, we used the plasmid with C/EBPa gene. In one of the control groups, we used the plasmid without C/EBPa gene. In the other control group, the HeLa cells were not subject to transfection.

Minor essential revisions

1. Introduction: First paragraph: Problem should be more clearly explained with cervical cancer. Explain the reason why C/EBPs was analyzed. Were there any suggestive data about the link between cervical cancer and C/EBPs?

   **Our response:**
   Yes, there is suggestive data for the link between cervical cancer and C/EBPs. In our earlier work, we used suppression subtractive hybridization method and found that the expression of C/EBPa and C/EBPβ genes was decreased. In addition, Ko (2008) found that C/EBPβ expression was also reduced in cervical cancer. We have revised the Introduction accordingly.


2. Methods: Information on a kit for cDNA synthesis is absent.

   **Our response:**
   cDNA synthesis kit we used was from Invitrogen.

3. Why did the authors use serum deprived condition?

   **Our response:**
   We used serum deprived condition for transfection because plasmid could enter HeLa cells more easily in this situation.

4. Where did cDNA of C/EBPa come from?

   **Our response:**
   cDNA of C/EBPa gene was from GeneChem Shanghai (China).

5. Regarding luciferase assay. What reporter plasmids used?

   **Our response:**
   We used immunofluorescence luciferase activity detection method. No reporter plasmids were involved in this method.

6. Figure 1. Immunostaining methods appropriate? Was titration of the antibodies same?

   **A:** Collagen fiber is positive. **B,C:** cytoplasm is positive. **C:** Central part is positive, but the peripheral part is negative.
Our response:
As addressed in point 1, we used immunohistochemistry method to detect the expression of C/EBPα protein. This method has been used in many published papers and is an appropriate method for our study. We used the same titration, 1:200, for all our antibodies. The C/EBPα antibody (sc-61, Santa Cruz Biotechnology, USA) we used was polyclonal, which explains the non-specific background staining seen in Fig 1 (A, B, C). Similar non-specific staining was also seen in published studies on skin carcinomas, e.g. Loomis et al (2007), Shim et al (2005).


7. Figure 2. CIN3 should be spelled out. Figure 1 shows C/EBPα was positive in well, moderately, poorly differentiated squamous cell cancer. But figure 2 showed carcinoma in site was negative. Figure 1 and figure 2 should be consistent.

Our response:
CIN3 is the abbreviation for “cervical intraepithelial neoplasia grade 3”. The reviewer is right that Figure 1 and Figure 2 should be consistent. The original CIN3 figure we used was not helpful. We have replaced it with a proper CIN3 figure.

8. Figure 3. How was the expression levels of C/EBPα normalized?

Our response:
C/EBPα gene expression was normalized using GAPDH.

9. Figure 4. It seemed transient transfection. Transfection efficiency might be low. How did the author recognize the low efficiency? How did they overcome it? Evidence is absent to show that transfected plasmid worked.

Our response:
See our response to point 2 above. In addition to the control groups, we used two different methods to confirm the transfection efficiency: 1) immunofluorescence luciferase activity assay, and 2) immunohistochemistry method. We found that C/EBPα gene overexpressed in recombinant plasmid transfected HeLa cells. In the two control groups (empty plasmid group and non-transfected HeLa cells group), however, C/EBPα gene was expressed at very low level.

10. Discussion: Paragraph 1,2, 3 were introduction of C/EBPα. Paragraph 4, 5, 6 were the same as results. What did the authors think about the role of C/EBPα in
carcinogenesis of cervical cancer? Were there any therapeutic applications?

Our response:
We showed that C/EBPa gene expression level decreased in cervical carcinoma. This may be one of the factors that cause cervical cancer. If we can regulate C/EBPa gene expression, we may be able to interfere the carcinogenesis of cervical cancer.

Once again, we thank you and the reviewers for the valuable comments that have greatly improved this manuscript.

Sincerely yours,

Zemin Pan

On behalf of all authors