Author’s response to reviews

Title: NR2F1 contributes to cancer cell dormancy, invasion and metastasis of salivary adenoid cystic carcinoma by activating CXCL12/CXCR4 pathway

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Author’s response to reviews:

Dear Dr. Catherine Rice,
Thank you for your suggestions to revise our article entitled "NR2F1 Contributes to Cancer Cell Dormancy, Invasion and Metastasis of Salivary Adenoid Cystic Carcinoma by Activating CXCL12/CXCR4 Pathway" (Manuscript ID: BCAN-D-19-00373R1). We have highly regarded the editor’ and reviewers’ insightful suggestions, carefully responded to these suggestions point-by-point in this response letter (see following “responses” part), and revised the manuscript accordingly.

Please contact me with any questions. We look forward to hearing from you.

Thank you for your time and best wishes to you!

Yours sincerely,

Yang-ling Tang

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Editor Comments:

Comment 1: Any manuscript submitted to a BioMed Central journal must be original and the manuscript, or substantial parts of it, must not be under consideration by any other journal. We note that the current submission contains textual overlap with other previously published works. Please re-write these sentences and phrases in your own words to ensure no overlap. There is some overlap in the Methods section; please ensure that you summarize the methods and cite the source.

There is some overlap with other publications.
We recommend the authors use an anti-plagiarism software, such as turnitin or other freely available ones, to access the overlap, and reduce it.

[Author Response] We have rewritten and summarized the Methods section in the revised manuscript. (Please see the following words and sentences).

1) page 7, paragraph 3, line 9: The formalin-fixed, paraffin-embedded specimens from these patients were used to immunohistochemical analysis. → Immunohistochemical analysis for the formalin-fixed, paraffin-embedded specimens from these patients.

2) page 7, paragraph 3, line 10: deleted “A total of 46 tumor samples were graded as T1/T2 and 13 were T3/T4.”

3) page 7, paragraph 5, line 15: The standard streptavidin peroxidase (SP) method was performed for Immunohistochemical staining. The slides were incubated with anti- NR2F1 (1:200, abcam) and proliferative marker Ki-67 (1:400, Cell Signaling Technology) for 90 minutes respectively. → Anti-NR2F1 (1:200, abcam) and Ki-67 (1:400, Cell Signaling Technology) were used for Immunohistochemical staining.

4) page 7, paragraph 7, line 20: Cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) method using In Situ Cell Death Detection Kit, POD (KeyGEN). → Terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) Kit (KeyGEN) was to determine the cell apoptosis.

5) page 8, paragraph 4, line 17: Transient transfection in SACC cells was performed using 20μM of each siRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) using the reverse transfection protocol. → Transient transfection in SACC cells was performed using 20μM of each siRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

6) page 9, paragraph 1, line 1: And then cells were washed in cold PBS, fixed in 4% paraformaldehyde for 20-25 minutes and blocked in 1% bovine serum albumin for 30 minutes at 25°C. → After washed in cold PBS, the cells were fixed in 4% paraformaldehyde for 20-25 minutes and blocked in 1% bovine serum albumin for 30 minutes at room temperature.
7) Rabbit anti-NR2F1 (abcam, 1:200) was used to incubate tumor cells and FITC-conjugated goat anti-rabbit IgG (1:500; Zhongshan Goldenbridge) was followed. Rabbit anti-NR2F1 (abcam, 1:200) and FITC-conjugated goat anti-rabbit IgG (1:500; Zhongshan Goldenbridge) were orderly used to incubate these cells.

8) Cells were counterstained with 4’6-diamidino-2-phenylindole (DAPI; 1 μg/μL), and examined using a fluorescence microscope (Olympus). 4’6-diamidino-2-phenylindole (DAPI; 1 μg/μL) was used to determine the cell nucleus. The results were collected by a fluorescence microscope (Olympus).

9) Real time qPCR was performed using One Step PrimeScript™ RT-PCR Kit (TaKaRa) on an Applied Biosystems ABI PRISM 7300 instrument. One Step PrimeScript™ RT-PCR Kit (TaKaRa) was for Real time qPCR and the results were analyzed by Applied Biosystems ABI PRISM 7300.

10) We designed primers to specifically detect NR2F1/TF-COU1: forward: GCCTCAAAGCCATCGTGCTG; reverse: CCTCACGTACTCCTCCAGTG. NR2F1/TF-COU1: forward: GCCTCAAAGCCATCGTGCTG; reverse: CCTCACGTACTCCTCCAGTG.

11) “Twenty-microgram of total protein was added per lane of 8% SDS-PAGE and transferred to PVDF membranes and blocked for 1 hour in 5% milk in TBS with 0.1% Tween-20 (TBST) at 37°C for 2 hours.”

12) Antibodies as 1:1000 dilution of rabbit anti-NR2F1 and 1:1000 dilution of rabbit anti-Lamin B were applied at 4°C overnight in 5% BSA TBST, washed and visualized with a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. Rabbit anti-NR2F1 (abcam, 1:1000) and 1:3000 dilution of anti-rabbit IgG secondary antibody (ZSGB-BIO, China, 1:1000) were to determine the protein expression. Rabbit anti-Lamin B (ZSGB-BIO, China, 1:1000) was used as an internal control.
13) When cells reached 80% confluence, the individual wells were wounded by scratching with a pipette tip and incubated with medium containing no FBS to 0, 18 h.→ SACC-83 and SACC-LM cells seeded and cultured in a 96-well plate (1000/ml) and were wounded by scratching with a pipette tip when reached 80% confluence, and incubated with medium containing no FBS for 24 hours.

14) page 10, paragraph 7, line 16: added “After 24 hours, the tumor cells were stained by Crystal violet and photographed under microscopy (×100) as previously described.”

Comment 2: If human cell lines are used, authors are strongly encouraged to include the following information in their manuscript:

- The source of the cell line, including when and from where it was obtained
- Whether the cell line has recently been authenticated and by what method
- Whether the cell line has recently been tested for mycoplasma contamination

Further information is available from the International Cell Line Authentication Committee (ICLAC). We recommend that authors check the NCBI database for misidentification and contamination of human cell lines. Please include the sources of all cell lines and their catalogue numbers.

[Author Response] For SACC-83 and SACC-LM cell lines used in the manuscript, we have the cell STR reports and it has been proved that these two kinds of cell lines were not cross-contaminated. Additionally, SACC-83 and SACC-LM cells were purchased from Shanghai life science college cell resource center, Chinese Academy of Sciences and conserved in State Key Laboratory of Oral Diseases, Sichuan University. Hence, in the revised manuscript, we have added “SACC-83 and SACC-LM cell line have been purchased from Shanghai Life Science College Cell Resource Center, Chinese Academy of Sciences and conserved in State Key Laboratory of Oral Diseases.” (Please see Method section, page 8, paragraph 2, line 2 to line 4)

Comment 3: Please confirm whether any of the cell lines required ethics approval for their use and include a statement in the Ethics approval and consent to participate section in the Declarations.

[Author Response] SACC-83 and SACC-LM cell lines in this experiment do not need the Ethics approval and we have described the details related to SACC cell lines in Method section. (Please see page 8, paragraph 2, line 2 to line 4)
Comment 4: Please describe in the Methods the manner by which all animals were sacrificed.

[Author Response] Thanks for your suggestion. The mice were euthanized with a dosage of 150-200mg/kg Pentobarbital Sodium via intraperitoneal injection, and we have added it in the revised manuscript (Please see page 11, paragraph 1, line 4)

Comment 5: Research involving human subjects (including human material or human data) that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html). A statement to this effect must appear in the appropriate Declaration subsection of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.

If the need for ethics approval were waived, then please clearly state this, including the name of the ethics committee that provided the exemption, together with the reasons for the waiver, or a reference to the relevant legislation.

[Author Response] According to this nice suggestion, we have added “And the research involving human data was in accordance with the principles of Declaration of Helsinki.” in the Ethics approval and consent to participate section. (Please see page 19, paragraph 7, line 22)

Comment 6: Please include an Availability of Data and Materials statement in the Declarations and ensure it reflects accurately one of the formats indicated in our submission guidelines for the raw data

[Author Response] Accordingly, we have rewritten the statement of Availability of Data and Materials “Consent for publication of raw data obtained from study participants.”. (Please see page 20, paragraph 5, line 10)

Comment 7: In the Funding section of the Declarations please indicate the role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript. If no specific funding was received for this study, please clearly indicate this in the Funding section.

[Author Response] There is no special funding and we have indicated in the Funding section. (Please see page 20, paragraph 9, line 20)
Comment 8: Please provide specific Authors' contributions for all of the authors; one of the authors appear to be missing.

[Author Response] Thank you! And we further declared the contributions of one of the co-authors, Mei Zhang, who has analyzed the data of the experiment. (Please see page 21, paragraph 1, line 3)

Comment 9: At this stage, please upload your proofread manuscript as a single, final, clean version that does not contain any tracked changes, comments, highlights, strikethrough or text in different colours. All relevant tables and figures should also be clean versions. Figures (and additional files) should remain uploaded as separate files. Should you wish to respond to these revision requests, please include the information in the designated input box only.

[Author Response] We have revised the manuscript accordingly to the requirements you have suggested above and also uploaded the responses separately.