Author’s response to reviews

Title: Comprehensive microRNA profiling reveals potential augmentation of the IL1 pathway in rheumatic heart valve disease

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Author’s response to reviews:

Responses to Editor’s Comments:

Editor’s Comments: This is an interesting study on circulating miRNAs suggesting an involvement of IL1 pathway in rheumatic heart valve disease (RHD). We asked the authors to address all the points mentioned by the reviewers in order to be suitable for publication. BMC Cardiovascular Disorders operates a policy of open peer review, which means that you will be able to see the names of the reviewers who provided the reports via the online peer review system. We encourage you to also view the reports there, via the action links on the left-hand side of the page, to see the names of the reviewers.

Authors’ Response: Thank you for your positive comments. We have carefully revised the manuscript according to the reviewers’ suggestion.

Responses to Reviewer 1 (Paola Rizzo):

Reviewer’s Comments: Paola Rizzo (Reviewer 1): This is a potentially useful study on circulating miRNAs suggesting an involvement of IL1 pathway in rheumatic heart valve disease (RHD).
The authors identified, among others, 2 miRNAs which are downregulated in the serum of RHD patients, compared to normal subjects. They provide in vitro evidence that these miRNAs target IL1 and its receptor and suggest an involvement of IL1, that would be consistent with the already reported inflammatory environment of RDH. To support their findings they show high levels of IL1 and its receptor in diseased valve samples from RHD patients, but not from patients with congenital heart valve disease (CHD).

Before the paper can be considered for publication the author should address the following.

Authors’ Response: Thank you for your comments and valuable suggestions. We have carefully revised the manuscript according to your suggestions.

Reviewer’s Comments: 1) To support the claim of specificity of these miRNAs in RHD, the expression levels of these two miRNAs should be assessed also in serum of CHD.

Authors’ Response: It is very important to assess the level of miR-205-3p and hsa-miR-3909 in CHD as suggested by the reviewer. As it can distinguish whether these two miRNAs and the IL1 signal pathway is specific in RHD. We assessed these two miRNAs in the serum of CHD and found that the level of miR-205-3p in serum of CHD and healthy individual serum is not significantly different. There is no significant difference of hsa-miR-3909 between CHD patient and healthy individual.

The added experimental results are shown in the Results section as follows:

“To further verify whether the altered expression of hsa-miR-205-3p and hsa-miR-3909 were specific to rheumatic heart disease, the expression of these two miRNAs was assessed in the serum of congenital heart disease patients. The results showed that both the hsa-miR-205-3p and hsa-miR-3909 expression levels in the serum of CHD were not significantly altered compared with healthy individuals (Figure 3J-K).”

Please see: Page 8 Line181-186 and Figure 3J-K.

Reviewer’s Comments: 2) Provide Methods for the immunostaining and include a negative control of immunostaining shown in Figure 5. Figure 5 is missing a graph cited in the figure legend. Also the authors should mention how many samples were analyzed by IHC.
“Immunohistochemistry

Mitral valve tissues were fixed in 4% PFA for 24 hours. After fixation, the tissue was dehydrated to enable embedding in paraffin, which is water insoluble. The tissue was dehydrated gently by immersion in increasing concentrations of alcohol. The alcohol was then cleared by incubation in xylene prior to paraffin embedding. The paraffin was typically heated to 60°C and allowed to harden overnight. Finally, the tissue was sectioned into 8-μm-thick paraffin sections using a microtome. Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation for 30 min in 3% H2O2 in methanol at room temperature. Antigen retrieval was performed using microwave treatment for 15 min in citrate buffer (pH 6.0). The sections were blocked with 10% goat serum at 37°C for 1 h and incubated with rabbit polyclonal antibody against human IL-1β or rabbit monoclonal antibody against human IL1RA (both applied at 1:200, Abcam, USA) in a humidified chamber overnight at 4°C. Next, the sections were incubated with HRP conjugated secondary antibody for 1 h at room temperature, developed with DAB chromogen for 10 min at room temperature, rinsed in running tap water for 5 min, and counterstained with haematoxylin-eosin staining. The antibodies used in the present study are as follows: anti-IL1β antibody (ab2105, Abcam, USA) and anti-IL1R1 antibody (ab106278, Abcam, USA).”

Please see: Page 5-6 line 117-133

(2) According to your instruction, we added negative control in Figure 5. The mitral valves were from acquired from the patients receiving valve replacement surgery. We are not able to acquire mitral valves from healthy individuals to represent the true negative controls. As the antibodies against IL-1beta and IL1 receptor are derived from rabbit IgG, the antibodies are replaced with normal rabbit IgG to stain as negative control. The figures are added in Figure 5.

Please see: Revised Figure 5 A

(3) Mitral valves from 6 CDH patients and 6 RHD patients were immunostained. And this was described in the revised Figure legend as follows: “The graph is representative of 6 CHD patients and 6 RHD patients respectively.”

Please see: Page 13-14, Line 359-360.
Reviewer’s Comments: 3) Western blot is cited in the abstract but not shown anywhere

Authors’ Response: I am sorry for missing the Western blot results in the Figure 5. We have added the results in figure 5 and text.

The following descriptions are added:

(1) Figure 5D showed the WB results of IL-1beta and IL1R1.

(2) In the figure legend of fig 5, we added “(D) The expression of IL-1β and IL1R1 was assessed by Western blot as described in the Methods section. β-actin was used as an internal control.”.

(3) We have revised the results section of Figure 5, and the revised text is as follows:

“The results showed that the IL-1β expression is absent in the CHD mitral valve tissue. However, IL-1β was slightly expressed in RHD mitral valve tissue (Figure 5B). IL1R1 was expressed in the CHD mitral valve tissue, but the expression in the RHD valve was much higher (Figure 5C). The upregulated expression of IL-1β and IL1R1 was verified by Western blot of the mitral valve tissues from 6 CHD and 6 RHD patients (Figure 5D).”

Please see: Page 9 line 213-217.

Reviewer’s Comments: 4) the manuscript needs a major revision of English structure and grammar.

Authors’ Response: Thank you for your suggestion. We have had our manuscript reviewed and revised by English language editing service from Nature Research Editing Service according to the suggestion by the editors.
Reviewer’s Comments: 5) Normal control seem to be missing from Figure 1. Please explain.

Authors’ Response: The heat map is drawn based on table 1. Data of LM1,LM2,S1 and S2 represent the fold change of certain miRNAs’ expression compared to NC (Normal control), which means the expression of each miRNA of NC has been adjusted to be 1. The title panel has been changed to Log2Ratio(LM1 /NC), Log2Ratio (LM2/NC), Log2Ratio (S1/ NC) and Log2Ratio (S2/NC). The description in the text was revised as follows:

“miRNAs that were commonly upregulated or downregulated in all four patients were selected and are shown in Table 1. The heat map of the differentially expressed miRNAs based on Table 1 is shown in Figure 1. There were 13 upregulated and 91 downregulated genes. ”

Please see: Page 7 line 159 to 162.

Reviewer’s Comments: 6) Figure 2 A and B are not readable

Authors’ Response: Thank you for this comment. In the revised manuscript, Figure 2A and 2B were uploaded separately. And this will markedly improve the quality of the figure.

Reviewer’s Comments: 7) Title and Discussion should downplay the involvement of IL1/IL1R since the downregulation of their expression by the identified miRNAs is inferred only from Luciferase assay.

Authors’ Response: Thank you for this valuable suggestion. This will make our paper more precise. We have add a word “potential” in the title. And the revised title is “Comprehensive microRNA profiling reveals potential augmentation of IL1 pathway in rheumatic heart valve disease”.

In the discussion section, we discussed the limitations of our present study as instructed by the reviewer. The added sentences are as follows: “However, there are still many limitations to our present study. The downregulation of IL-1β and IL1R1 expression by the identified miRNAs was inferred from the Luciferase assay. Further verification of the involvement of IL-1β and IL1R1 includes the assessment of the expression of IL-1β and IL1R1 in the CHD and RHD mitral valves. Though we have verified the augmented expression of IL-1β and IL1R1 in RHD
valve tissues in comparison with CHD valve tissues, further animal experiments investigating the potential causal role of the IL1 pathway in the progression of RHD are important.”

Please see: Page 10 line 259 to 265.

Responses to Reviewer 2 (Nalini Rajamannan):

Reviewer’s Comments: Nalini Rajamannan (Reviewer 2): This is an interesting and well executed study testing the mechanisms RHD with the use of RNA analysis. This study will provide important information for the field of RHD. One key mechanistic paper is missing from the references: Circulation. 2005 Jun 21;111(24):3296-301. Epub 2005 Jun 13. Calcified rheumatic valve neoangiogenesis is associated with vascular endothelial growth factor expression and osteoblast-like bone formation.

Authors’ Response: Thank you for this valuable suggestion. I have carefully read this paper. And cited this paper in our manuscript. Please see page 8 line 195 and page 12 line 318