Author's response to reviews

Title: Atrial fibrillation alters the microRNA expression profiles of the left atria of patients with mitral stenosis

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Author's response to reviews: see over
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Dr. Aini Xie
BMC Cardiovascular Disorders
editorial@biomedcentral.com

Re: MS 1251031543111177, “Atrial fibrillation alters the microRNA expression profiles of the left atria of patients with mitral stenosis”

Dear Dr. Xie:

Thank you very much for your letter and advice. We have revised the paper, and would like to resubmit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are in red font in the revised manuscript. We hope that the revision is acceptable, and we look forward to hearing from you soon.

With best wishes,

Yours sincerely,

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We would like to express our sincere gratitude to the reviewers for their constructive and positive comments.

Replies to Reviewer 1

Major Comments:
1. Materials and Methods are vague and this undermines the reliability of the study: reagents that were used and their origin as well as the primers that they were used to determine the expression levels of miRNAs etc.
   We have provided the primer information in the Supplementary files.

2. There is no control group with healthy subjects indicating the basal expression levels of the tested miRNAs.
   We do not think that a control group with healthy subjects was required in our study. The aim of this study was to screen the altered miRNAs potentially associated with AF in LAA from MS patients using miRNA microarray technology and provide direction for further research. The exact mechanism of the differentially expressed miRNAs' involvement in AF was not the objective of the present study. Thus, it is not necessary to indicate the basal expression levels of the tested miRNAs in healthy subjects. In addition, adding a control group with healthy subjects to our study would increase the difficulty of the study, and is really not feasible, as it is well known that obtaining the LAA of healthy subjects in clinical practice is very difficult and almost impossible. Therefore, comparing the miRNA expression profiles in LAA tissues from MS patients suffering from AF with those who remained in NSR was appropriate in our study.

3. The hypothesis on which the study is based is missing. The results of the study do not provide any additional value since there is no sufficient explanation throughout the manuscript regarding why the authors investigated the miRNAs expression profile in the left atria.
   Several sentences have been added in the Abstract (page 2, paragraph 1), Background (page 5, paragraph 2) and Discussion (page 13, paragraph 2; page 14, paragraph 2) in the revised version to address this issue.

   Another study has already adequately examined the miRNAs expression profile in the same pathological context in the right atria (John B, Eur Heart J, 2008).

John et al. (Eur Heart J 2008; 29:2234-2243) examined the electrical remodeling of the RA and LA in MS patients. They found that the RA and LA are electrically remodeled in rheumatic MS and also found differences between the LA and RA. For example, 1) atrial remodeling in MS is characterized by LA enlargement, and 2) both atria in patients with MS possessed a greater percentage of the area below 1.5 mV than controls without structural heart disease; this was observed to a greater extent in the LA (44% cf. 22%; \( P = 0.0009 \)) than in the RA (38% cf. 30%, \( P = 0.2 \)). Areas of electrical silence in the LA were localized to the posterior wall adjacent to the pulmonary veins in 3 patients, anterior LA in 3 patients, and septal LA in 1 patient; while in the
RA it was localized to the lateral RA. Thus, miRNA expression profiles associated with AF in RA and LA from MS patients may be different.

Recently, Xiao et al. (Physiol Genomics 2011; 43:655-664) found 28 miRNAs differentially expressed in the RA between MS patients with AF and those in NSR, while we found in the present study 22 miRNAs differentially expressed in the LA between MS patients with AF and those in NSR. Except for one miRNA (miR-26b), the results of the two studies were completely different. The reason for these significant differences in miRNAs between the RA and LA may, in part, reflect different mechanisms involved in AF after eliminating the influences of the miRNA microarray technologies used in the two studies. Therefore, investigations into the differences in miRNA expression profiles associated with AF in MS patients should focus not only on the RA but also on the LA.

4. The observed alterations in the levels of miRNAs are inadequately associated with the pathophysiological role of them during the atrial fibrillation. No mechanism is suggested regarding the specific differentially expressed miRNAs as well as there are no potential clinical applications mentioned.

As we know, disease mechanisms may alter the expressions of genes or levels of their protein products. Comparing the genetic and protein profiles in different disease states, we can screen for differentially expressed genes and proteins. Of these genes and proteins, some participate in the mechanisms of the disease, while others are the result of the disease. The exact role of these differentially expressed genes and proteins in the disease require further functional study. However, the results of genomic and proteomic studies greatly improve the efficiency of research, providing clues to the functions of differentially expressed genes and proteins. In recent years, more and more studies have focused on the role of miRNAs in cardiovascular disease, and have shown that miRNAs regulate key genetic functions in cardiovascular biology. Some miRNAs are crucial to the pathogenesis of cardiac diseases, such as cardiac development, hypertrophy/heart failure, remodeling, acute myocardial infarction, and myocardial ischemia-reperfusion injury. As in genomic and proteomic studies, those that create miRNA expression profiles have an important role in these studies.

The aim of this study was to screen for altered miRNAs potentially associated with AF in LAA from MS patients, using miRNA microarray technology. The exact mechanism underlying the involvement of differentially expressed miRNAs in AF was not an objective of the present study. The exact targets and pathways by which alterations in miRNAs cause AF in MS patients remain elusive and deserve further work. Because our study was performed with native human tissues, we could not conduct experiments to modulate miRNA levels. Accordingly, the evidence presented here is indirect. Thus, alterations in the levels of miRNAs were detected, but the specific roles of these miRNAs in the pathophysiological of AF was beyond the scope of the study.

However, our study is also very necessary and provides vital direction for further research. In fact, we are conducting functional research now that is based on the present study. We will promptly publish the results of our study.
5. The quantification of miRNAs which are ~22 nucleotides long is a sensitive process and requires a more reliable RT-PCR method like TaqMan real time PCR.

The stem-loop RT-PCR for real-time quantification of miRNAs in our study is reliable and used widely. Please see:


6. The patients sample is very small and this is a major limitation of the study in order any appropriate conclusions to be drawn from it.

The main limitation of this study was the small number of patients included. This was due, in part, to the difficulty of finding MS patients with NSR. We have stated so in the Discussion (page 16, paragraph 4). We set a high criteria in screening differently expressed miRNAs for validation by RT-PCR: $|\log_2(\text{fold change})| \geq 1.5$, and at least one group had a signal intensity $>2000$ units. The aim is to compensate for the small sample size, limiting bias and error.

7. Which are the relative expression levels of the tested miRNAs normalized to the internal control according to the $2^{-\Delta\Delta Ct}$? Please provide a table or a graph with these data.

We have provided a table in the Supplementary files.

Minor Comments:
1. Please indicate the standard deviation of each miRNA expression in figure 2.

We have provided all miRNAs in our analysis in the Supplementary files in the revised version. The purpose of Figure 2 in the original version was to compare other literature, as a reflection of the reliability of our results. In the revised version, we have provided all miRNAs in our analysis and we now think that Figure 2 is not necessary. Therefore, we removed Figure 2 in the revised version.

Replies to Reviewer 2
1. The authors used signal intensities as a filter of microarray data for miRNA inclusion for further studies. The validity of this approach is highly questionable, as microarray results are definitely not quantitative and any difference in signal intensity may merely represent the hybridization efficiency under a given experimental condition but not the quantity of the target genes. The problems associated with your approach may be: (1) misuse of array data as quantitative results, (2) exclusion of some important miRNAs for your analysis in depth and
(3) inclusion of miRNAs that are actually low-abundant ones (I bet miR-466, miR-574, and miR-3613 are all low-abundance miRNAs in heart). Moreover, use of expression level of miRNAs to indicate the relative strength of cellular function is obviously oversimplified; we now know that many extremely low-abundant miRNAs play a big role in certain physiological functions with impressive magnitudes of changes of phenotypes. I suggest the following: (1) remove the intensity filter and include all miRNAs in your analysis and (2) provide the cycle numbers of qPCR for the ones included in quantitative measurement.

We did not use the signal strength as a filter to screen differently expressed miRNAs in LAA from MS patients between the AF and NSR groups. The screening criteria of the microarray analysis in our study is that miRNAs with $P$-values < 0.05 were considered to indicate significant differences in expression between the two groups. This criteria was defined only in Statistical analyses (page 9, paragraph 3) in the original manuscript and have been added in the Microarray processing and analysis (page 8, paragraph 1) in the revised version to address this issue.

We used the signal strength as one of three filters (i.e., $P$-value < 0.05; $|\log_2(\text{fold change})| \geq 1.5$, and at least one group with a signal intensity >2000 units) to select miRNAs for the validation of miRNA microarray data via RT-qPCR. The purpose was to prove the feasibility and reliability of the RT-qPCR experiment. The reasons are the following: firstly, because verifying all the miRNAs is unrealistic; secondly, because of the small number of patients, validation of low-abundance miRNAs with RT-qPCR may produce errors and affect the reliability of the RT-qPCR results.

2. I suggest the authors to give a detailed comparative discussion on the study by Xiao et al (Physiol Genomics 2011, 43(11):655-664) as it is the most relevant study in the literature. In particular, comparison between right and left atrium should be given to highlight the importance of your study. Several sentences have been added in the Abstract (page 2, paragraph 1), Background (page 5, paragraph 2) and Discussion (page 13, paragraph 2; page 14, paragraph 2) in the revised version to address this issue.

3. Another limitation of the study is the small sample size for highly heterogeneous human tissues, as also being well aware of by the authors. I suggest including more clinical information in Table 1, such as blood pressure, other heart diseases, current smoking, etc. The main limitation of this study was the small number of patients included. This was due, in part, to the difficulty of finding MS patients with NSR. We have stated this in the Discussion (page 16, paragraph 4). We set a high criteria in screening differently expressed miRNAs for validation by RT-PCR: $|\log_2(\text{fold change})| \geq 1.5$, and at least one group had a signal intensity >2000 units. The aim is to compensate for the small sample size, limiting bias and error.

There were no significant differences with regard to most other clinical information between the two groups. Thus, we selected the clinical features with relatively strong correlations in MS to show in Table 1.
- Minor Essential Revisions
The manuscript is in general well written, but in many occasions, the descriptions/statements are not sufficiently clear in terms of the expression and style. I suggest the authors to go through carefully the manuscript and make any necessary changes or polishing to improve the work.
Necessary changes and polishing has been made in the revised version.

- Discretionary Revisions
1. Remove the intensity filter and include all miRNAs in your analysis;
   We have provided all miRNAs in our analysis in the Supplementary files.

2. Provide the cycle numbers of qPCR for the ones included in quantitative measurement.
   We have provided a table in the Supplementary files.

3. Give a detailed comparative discussion on the study by Xiao et al (Physiol Genomics 2011, 43(11):655-664) as it is the most relevant study in the literature. In particular, comparison between right and left atrium should be given to highlight the importance of your study.
   Several sentences have been added in the Abstract (page 2, paragraph 1), Background (page 5, paragraph 2) and Discussion (page 13, paragraph 2; page 14, paragraph 2) in the revised version to address this issue.

4. Include more clinical information in Table 1, such as blood pressure, other heart diseases, current smoking, etc.
   There were no significant differences with regard to most other clinical information between the two groups. Thus, we selected the clinical features with relatively strong correlations in MS to show in Table 1.

5. Double check through the manuscript and make any necessary changes of expression and syntax.
   Necessary changes and polishing has been made in the revised version.

Replies to Reviewer 3

The paper “Atrial fibrillation alters the microRNA expression profiles of the left atria of patients with mitral stenosis” evaluated the miRNA expression profiles of left atrial tissues from mitral stenosis (MS) patients with AF or in normal sinus rhythm (NSR). There is a conspicuous question in your paper. Your research was so similar with “MicroRNA expression signature in atrial fibrillation with mitral stenosis”, while results showed that 22 miRNAs differentially expressed in LAA tissues between MS patients with AF or NSR, only’hsa-miR-26a-5p’ was also downregulated among 28 deregulated miRNAs in MS with AF compared with MS without AF in RAA, the others were completely different in two papers. What caused the significant difference between LAA and RAA?
More and more studies indicate that specifically alterations in miRNA expression profiles are associated with specific disease pathophysiologies. Recently, Xiao et al. (Physiol Genomics 2011; 43:655-664) were the first to report miRNA alterations in the RA associated with AF in MS patients; 28 miRNAs were differentially expressed between MS patients with AF and those in NSR. However, miRNA changes due to AF in the LA of MS patients are still unknown. The present study is the first to create and compare miRNA profiles of the LA of MS patients with AF and those without AF. We found that in the LA between MS patients, 22 miRNAs differentially expressed between those with AF and those in NSR. The results of our study and that of Xiao et al. were completely different, except for miR-26b. After eliminating the influences of the miRNA microarray technologies used in the two studies, we conclude that, at least in part, may reflect different mechanisms involved in AF between the LA and RA. In MS patients, electrical remodeling of both the left and right atria (John, B. Eur Heart J 2008; 29:2234-2243) in intrinsic to the initiation, development, and maintenance of AF (Sharma D. Cardiology 2011, 120(2):111-121), and morphological differences have also been demonstrated between the two atria (Chapeau C. J Histochem Cytochem 1985, 33(6):541-550). Thus, it is not surprising that AF also alters the miRNA expression profiles in the LA of MS patients, and that these alterations may differ from those of the RA. These differences may reflect different mechanisms involved in AF between LA and RA. Therefore, investigations into the differences in miRNA expression profiles associated with AF in MS patients should focus not only on the RA but also on the LA.

In addition, you may complete some functional experiments in vitro about at least one microRNA to proof your conclusion and its potential targets.

The aim of this study was to screen for altered miRNAs potentially associated with AF in LAA from MS patients, using miRNA microarray technology. The exact mechanism underlying the involvement of differentially expressed miRNAs in AF was not an objective of the present study. The exact targets and pathways by which alterations in miRNAs cause AF in MS patients remain elusive and deserve further work. Because our study was performed with native human tissues, we could not conduct experiments to modulate miRNA levels. Accordingly, the evidence presented here is indirect. Thus, alterations in the levels of miRNAs were detected, but the specific roles of these miRNAs in the pathophysiological of AF was beyond the scope of the study.

The good news is that we are conducting functional research now that is based on the present study. We will promptly publish the results of our study.