Author's response to reviews

Title: Microarray Analysis of MicroRNA Expression in Peripheral Blood Mononuclear Cells of Critically ill Patients with Influenza A (H1N1)

Authors:

Hao Song (songhao@nwsuaf.edu.cn)
Qi Wang (wangqidl04@126.com)
Yang Guo (guoyangq1024@gmail.com)
Shunai Liu (liusa1031@sina.com)
rui Song (songruii@hotmail.com)
Xuesong Gao (gaohappy@126.com)
Li Dai (biofavor@gmail.com)
Baoshun Li (virus_microrna@163.com)
Deli Zhang (zhangdeli@tsinghua.org.cn)
Jun Cheng (jun.cheng.ditan@gmail.com)

Version: 4 Date: 23 February 2013

Author's response to reviews: see over
Dear Editor:

Thank you very much for the valuable suggestions from the reviewers. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. We have carefully revised the paper base on the reviewer's suggestions. The raised questions were explained as follows:

Reviewer #1:

1. It remains unclear whether just the fever, the induction of cytokines/interferons, inflammation or additional influenza replication in blood cells is causing the discussed miRNA changes. Since influenza virus primarily infects epithelial cells of the respiratory tract, it remains questionable if the miRNA changes result from infection of antigen presenting cells that are additional targets of influenza. The authors should provide in vitro experiments infecting PBMC versus A549 to show differential expression of the validated miRNAs.

Answer:
We agree the reviewer's good advice. It may be very difficult to find all the factors contributing to the changes of miRNAs in PBMC of critically ill patients infected with H1N1 influenza virus because of the complicated mechanisms causing severe disease of influenza virus infection. However, the major factor causing the miRNAs differently expressed is still virus infection, through direct effects(virus itself) or indirect effects(inflammation, et al). H1N1 influenza virus has been found to replicate in both lung epithelial cells and APCs in vitro and in vivo. The in vitro experiments infecting PBMC versus A549 to show differential expression of the validated miRNAs are very useful to find the differences of the two different kinds of cells after infection. However, we apologize that we could not perform this experiment due to virus strain limited and time constraints. Nonetheless, we compared our result with a recent study on miRNA changes in A549 cells infected with swine-origin influenza pandemic H1N1. We found that miR-29a, miR-29c and let-7g were also down-regulated in their result. We discuss it in the revised MS. See Page 17, line 12-20.

2. Does IFN treatment lead to up/downregulation of the validated miRNAs? The authors should
also compare their list of miRNAs to similar studies, is there overlap to any of the cited publications (citation 15-21 e.g.)? In addition, they should compare their list to published data on IFN regulated miRNA patterns. The data need also to be discussed in comparison to similar studies on related (respiratory) diseases.

Answer:

As the Reviewer’s good advices, we compare our result with other studies[1, 2], but there is no overlap concerning the specific miRNAs modulated by IFN. However, we found the down-expressed miR-29a, miR29b could regulate IFN-gamma production. We also compare our result with similar studies on influenza, and we found several overlapping miRNAs including miR-29a, miR-29b, miR-29c and let-7g. We discuss it in the revised MS. See Page 17, line 3-20. In addition, we compare our result with similar studies on respiratory diseases, such as respiratory syncytial virus (RSV). RSV causes substantial morbidity and life-threatening lower respiratory tract disease in infants, young children and the elderly. It has been reported that RSV could modify microRNAs regulating host genes that affect virus replication [3, 4], but there is no overlap between our result and theirs. Therefore, we did not discuss it in the revised MS. However, thanks to Reviewer’s good advices, when we compare changes of miRNAs induced by obesity with our result, we found let-7g and miR-146b-5p were both downregulated. We discuss it in the revised MS. See Page 17, line 21- Page 18 line 18. Further description about relationship among miRNA, obesity and influenza could be seen in our answer to the 4th question.

Reference:


3. A proper control sample group to compare would be same individuals after recovery. Why did the authors not compare miRNAs in the same individuals? The authors should discuss the following: Can the data be used to predict disease stage or progression or differentiate various respiratory diseases? Are the data helpful to understand pathogenicity? Can the data be used to identify novel drug targets to treat influenza?

Answer:
We agree the reviewer's good advice. The comparison between the sample group and the same individuals after recovery is better, However, it is very difficult to revisit all the patients after recovery. Therefore, we chose healthy people as control. As the Reviewer's good advice, Receiver operator characteristic (ROC) curve analysis was conducted and area under the ROC curve (AUC) was calculated to evaluate the diagnostic accuracy of severe H1N1 influenza virus infection. We found that miR-31, miR-29a and miR-148a all had significant potential diagnostic value for critically ill patients infected with H1N1 influenza virus, which yielded AUC of 0.9510, 0.8951 and 0.8811, respectively. These differentially expressed miRNAs could be potential therapeutic target or biomarkers for severe influenza virus infection. See Page 10, line 19 - Page 11 line 2, Page 13, line 10- 22 and Page 16 line 19-23.

4. The patient characteristics need more information (other diseases, use of pharmaceutical drugs..). BMI indicates that all patients were overweight, do any of the patients had metabolic syndrome or diabetes?

Answer:
The critically ill patients in this study all had no underlying diseases including type 2 diabetes, immunodeficiency or cardiopulmonary diseases, but they had comorbidities like pneumonia or acute respiratory distress syndrome (ARDS), which may lead to disease progression. We collected samples as soon as patients were admitted to ICU with confirmed influenza A H1N1 infection, when they were very severe and immediately treated with anti-infective therapy and so on. Interestingly, we found all the critically ill patients in our study were overweight (BMI > 25 kg/m2). Many reports support the view that obesity is associated with higher risks of ICU admission and death in patients.
with H1N1 influenza infection and obese patients with severe infection were more likely to develop pneumonia compared to non-obese patients. A recent study reported that the expression of miR-146b-5p was decreased in monocytes during obesity. Another group found that let-7g was downregulated in the fetal muscle of diet-induced obese ovine compared to control. MiR-146b-5p acts as an inhibitor of NF-κB-mediated inflammation and is necessary for the anti-inflammatory action of high levels of globular adiponectin. The downregulation of let-7g may enhance intramuscular adipogenesis during fetal muscle development in the setting of maternal obesity. Taken together, our findings suggest the downregulation of miR-146b-5p and let-7g were important in further understanding the molecular mechanisms implicated in obese patients susceptible to severe infection of H1N1 influenza virus. See Page 17, line 21- Page 18 line 18.

Reviewer #2:
Thank you for your attention to our manuscript and we appreciate your detailed and professional advices. According to your comments, we revised the manuscript to incorporate your commendations and criticisms. The corresponding explanations of each point which is raised in your comments as follows:

1. Line numbering would have made reviewing easier!
   
   Answer:
   
   We apologize for the mistake. We have added line number in the revised MS.

2. As a general rule, flu should be replaced by influenza.
   
   Answer:
   
   As the Reviewer's good advices, we have corrected it in the revised MS. See Page 4, line 25 and Page 12, line 4.

3. In the Method section P11, I would move the list of primers to a table.

   In the Results section, P13 I would convert table 1 into text here.

   Answer:
   
   As the Reviewer's good advices, we have corrected it in the revised MS. See Page 10, line 13 and Page 12, line 4.
4. *I think the acronyms qPCR and qRT-PCR were not used in a consistent manner.*

**Answer:**

According to the reviewer’s good instructions, we have replaced qPCR into qRT-PCR in the revised MS.

5. *In the results section P15, it is written that the …” increase in the MAPK14 expression level was observed in PBMCs from critically ill patients” … was …“with significant difference” whereas in figure 5 ns is indicated for MAPK14.*

**Answer:**

We apologize for the mistake. We have corrected into “Only a slight increase in the MAPK14 expression level was observed in PBMCs from critically ill patients with no significant difference.”