Author's response to reviews

Title: Peroxiredoxin 2: A potential biomarker for early diagnosis of Hepatitis B Virus related liver fibrosis identified by proteomic analysis of the plasma

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Dear Editor,

Submitted is the revised manuscript of MS-1024691504299574 “Peroxiredoxin 2: A potential biomarker for early diagnosis of Hepatitis B Virus related liver fibrosis identified by proteomic analysis of the plasma” together with supplementary documents.

We have defined HBV in the title according to your instruction.

Concerning Professor Jianjun Shen’s report, we have revised our paper in the following aspects:

1) It is true that pooling samples may mask interesting differences between patients within each patient group. Since the purpose of our study is to identify robust biomarkers related with the progress of liver fibrosis, the differences among various groups are more interesting than the differences between patients within each patient group. We pooled the samples to smooth intrinsic individual differences and enhance common characteristic traits only related to disease status. It is also true that pooling samples may eliminate the number of biological replicates. Therefore, we analyzed protein levels in a larger population by ELISA to make up the disadvantages of pooling samples. We have added discussion of the pro and cons of pooling samples in the revised version (page 15, line 14).

2) In the methods part, we mentioned that we depleted albumin and IgG with the ProteoExtract Albumin/IgG Removal Kit (Calbiochem, Darmstadt, Germany) and desalted with the ProteoExtract Protein Precipitation Kit (Calbiochem, Darmstadt, Germany). All experiments were done according to the manufacture’s instruction. The depletion procedure increased the loading volume from 5 µL to 35 µL (estimated raw plasma volumes with same protein amount), and significantly improved the detection of low abundant proteins. Please refer to the supplementary documents to see the result of depletion. It is true that the depletion of highly abundant proteins may result in the loss of potentially important proteins bound to them at the same time. The more kinds of abundant proteins are depleted, the more unspecific bounded proteins may loose. Therefore, we chose to deplete 2 most abundant plasma proteins, albumin and IgG, to minimize the unspecific protein depletion. We have added discussion of the pro and cons of depletion in the revised version (page 16, line 6).  

3) Both of the two reviewers mentioned the unit mistake in Western blot analysis. It should be 20 µg. We are sorry for the mistake. It has been fixed in the revision.

4) We have gone over the entire manuscript and added a space between a number and its unit.

5) The Mean ± SD in table 1.1 and 1.2 means the age of patients.

6) The “Areas under curve of 25 serological markers” for Table 3 is based on receiver operating characteristic (ROC) analysis. Briefly, the ROC curve is a graphical
plot of the sensitivity vs. (1 – specificity) for a binary classifier system as its discrimination threshold is varied. The best possible prediction method would yield a point in the upper left corner or coordinate (0, 1) of the ROC space, representing 100% sensitivity (no false negatives) and 100% specificity (no false positives). A completely random guess would give a point along a diagonal line. The diagonal line divides the ROC space in areas of good or bad classification/diagnostic. Points above the diagonal line indicate good classification results, while points below the line indicate wrong results. The AUC is the area under the ROC curve. An AUC over 0.5 means the marker can be used for clinical diagnosis. The bigger the AUC is, the more useful the marker may be. We have clarified the meaning of AUC in the revised version.

7) We have re-exported Figure 6 so that both the reviewers and readers can view it clearly. And a more detailed figure legend of this figure is given in the revised version.

Concerning Professor Akhilesh Pandey’s report, we have revised our paper in the following aspects:

1) We have used MALDI-TOF-MS/MS to identify proteins. All the peptides in Figure 2C were used for PMF identification. The 3 red circles in Figure 2C labeled out the peptides further analyzed by MS/MS. All of them are matched. We have provided the result in supplementary documents.

2) It is true that the quality of the western blots is not satisfying. Considering the huge diversity of protein concentration in plasma among individuals, we did not normalize the result of western blot with a house-keeping protein as usual. Instead, we analyzed the expression of Prx II and CLU of the same sample to avoid errors in sample loading and membrane transferring. We cut the areas corresponding to 21kDa and 52kDa to test the expression of Prx II and CLU, respectively. As expected, the changes of both proteins were similar to that of DIGE result. Compared to normal plasma, Prx II was highly present in all stage of fibrosis plasma and CLU was showed to decrease continuously with the progress of fibrosis. Thus, the up-expression of Prx II was reliable.

3) We have provided the raw values of protein levels identified in plasma as supplementary table. It is true that there is variation in Prx II in early fibrosis condition. However, the algorithm used 4 markers, and the combination of the 4 markers leads to an 88.9% success in early fibrosis identification in the training group and a 100% success in the testing group. This may be related to the individual difference and indicate the complexity of liver fibrosis, which disallows any single biomarker to guide the diagnosis, prognosis, and treatment of the disease.

4) We have corrected the plot so that the median for Prx II for early fibrosis condition correspond to the value mentioned in the text.

5) 20 µg protein was used for Western blot.

6) We labeled the same group of sample with either CyDye3 or CyDye5 and ran
the sample in two different gels to eliminate the affect of dyes. Please refer the supplementary documents for sample labeling of the 6 DIGE gels and the dye swap image.

7) We will submit the raw data to public proteomics data repositories for community use as soon as possible.

We hope that the responses could satisfy the raised comments, and the according revision could have improved the paper and will make it acceptable for publication at *BMC Gastroenterology*.

Sincerely yours,

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