utmb Health

DEPARTMENT OF PHARMACOLOGY AND
TOXICOLOGY

October 23rd, 2014

Dear Editor,

We would like to thank you and the reviewers for the time and effort in the review of our article. We have carefully considered the comments and hope that our responses address the concerns. We truly appreciate the feedback and now provide you with an improved resubmission.

Please find enclosed the edited manuscript in Word format (file name: 13275-review.doc).

Below is an abridged summary of each of the reviewer's comments with a detailed description of the changes made to the article

Title: Targeted Proteomics for Biomarker Discovery and Validation of Hepatocellular Carcinoma in Hepatitis C Infected Patients

Author: Mustafa G.M†, Larry Denner#, John R. Petersen* and Cornelis Elferink†

Name of Journal: World Journal of Hepatology

ESPS Manuscript NO: 13275

The manuscript has been improved according to the suggestions of reviewers:

1) The format has been updated as per requirements and core tips have been included (change of title, key words added).

2) Revisions have been made according to the suggestions of the reviewer

(1) **Reviewer # 2937214**

" Similar unmet medical needs abound in most fields of medicine and require novel methodological approaches. Proteomic profiling of body fluids presents a sensitive diagnostic tool for early cancer detection. Here the authors introduce "A Proteomics Pipeline for Biomarker Discovery and Validation". The presentation reflects the present state of knowledge and the figures are attained from experimentation/literature supported by the line of reasoning. The paper is written in clear language. I recommend to consideration for the publication of this manuscript as a Review paper. Maybe, it requires minor revision for theme of this manuscript as a review paper prior to its publication."

We are grateful to hear the manuscript is written in clear language. We also have made many revisions to strengthen this as a review paper.

(2) **Reviewer # 2911666**

" For publishing this paper must be more detailed to make clear understanding and enable reproduction for other researchers, once many approaches for pipeline are proposed. My comments are in attachment.

Q1) The title do not reflect the major content. The main approach is about Bottom up proteomics (characterization of proteins by analysis of peptides released from the protein through proteolysis). The term proteomics is too broad and could refer to another strategy type – Top down proteomics (is used to characterize intact proteins). This must be specified in the title.

We appreciate this comment regarding the specificity of the title and have changed it to "targeted proteomics" to eliminate the confusion of a broad term like proteomics.

Q2) This article suggests a number of approaches that could be used to detect biomarkers. Although it is unclear which type of sample must be used. The authors give only the example of serum analysis. Saliva or another fluids could be used? This topic must be more discussed in final version.

This is an excellent point, which we have now discussed on page 4, line 20-25 . We have also added references 3 and 4.

Q3) In Page 8: In spite of many recent technological advances in methods for the separation and analysis of proteins, two-dimensional gel electrophoresis (2D-PAGE) is widely used for proteomics research and is still the ''gold standard'' technique in this area. This assertion is questionable, because some proteins could be in the same spot, when they have the same mass/charge and in a first observation cannot be "separated" also has limited dynamic range capacity, thus can not be "gold standard" method. This assertion must be reviewed by the authors.

This is an excellent point. We understand that "gold standard" is an oversimplification and have removed this terminology (page 7, line 33). We also understand that are typically several protein "hits" present in one spot in the discovery phase. This requires that all proteins need to be

independently verified and validated. However when we validated the proteins in spots by MRM the highest scoring protein was responsible for the differential expression in 23 out of 28 cases.

Q4) In page 9: "…This protocol can be completed for 2 sets of 6 patient samples/set (e.g., 6 HCV and 6 HCC) in 3-5 weeks. We used 18 HCV and 18 HCC samples for 2D-DIGE and identified 43 significantly differentially expressed proteins." There is knowledge that verification of biomarkers can require 100−1000 samples, whereas validation of biomarkers requires analysis of even larger numbers (thousands to tens of thousands). Your experiment has few samples to determine and validate the proposed approach. This should be discussed.

This is another excellent point. Clearly the number of samples required is related to the power required to identify a difference between groups. Dr. Steven Carr, a world leader in biomarkers, has published a revealing study on the number of samples required in these studies. We have included this reference and a discussion of the relevant variables for biomarker studies from our perspective. Furthermore, using machine learning approaches to a biomarker panel reduces the number of samples even more because it is not based on a single target that does, indeed, require far more patients. We have addressed this on page 11, lines 5-19. We have also added reference (75-77).

Q5) In Page 11 "However advances in the form of high-resolution mass spectrometers allow the quantitative analysis of thousands of proteins, these techniques still do not allow definitive identification of the entire proteome of complex mixtures, such as serum." This problem is also not solved by 2D gel technique. Example: 2-DE is incapable of analyzing low abundant proteins in a high dynamic range sample such as plasma and 2-DE gel has a limited resolution for a large number of proteins which comprise the whole proteome. Nevertheless is important to enhance the MS-based proteomics technologies like LTQ Velos Orbitrap or LC-QTOF, with high resolution and dynamic range, enable identification of a large number of proteins with high throughput.

The reviewer is correct. We have changed our description because identification of the entire proteome is not possible even using a diversity of technologies. In order to analyze lower abundance proteins, we used proteominer technology to increase the dynamic range of proteins in the serum to allow enrichment of low abundance proteins. Of course, the use of high-resolution instruments with greater dynamic range are important approaches that can be used as alternate approaches. We have included discussion of this point on page 10, lines 1-17. We have also added references (65-68).

Q6) In Page 8 and 17: "…desalted (using 2D-cleanup kits or desalting columns)" "…To remove any salts, desalting of sample is also very important." How this step can be done? ZipTip? Spin Columns? This step must be well described.

We have added the reference (50) where these steps are described in detail.

Q7) I would like to know if there some possibility to perform this pipeline with in solution hydrolysis?

Yes, in fact we are using solution-phase hydrolysis in other studies and included mention of this on page 12 under "Assay Development"

Q8) Bioinformatics tools must be more explored and detailed, make it clear how analysis should be done.

We have discussed one example of machine learning approaches that we use, Multivariate Adaptive Regression Splines (MARS; page 15, lines 17-23). We have also added the references (97-100).

Q9) For publishing this paper must be more detailed to make clear understanding and enable reproduction for other researchers, once many approaches for pipeline are proposed

We have referenced a previous manuscript that includes extensive details of the methods (50) and discussed other approaches on page 10, lines 1-17.

(3) **Reviewer # 70055**

In the current form, this paper is not a research paper or review paper suitable for publication.

We appreciate this comment and have now focused on the review perspective throughout the manuscript.

(4) **Reviewer # 505946**

"This review provides a brief overview on the strategy of comparative proteomics to identify potential serum-based biomarkers distinguishing high-risk chronic HCV infected patients from HCC patients. It is a neat review, publication can be considered with the following comments.

Q1) As HBV is more well associated with hepatocellular carcinoma, the proteomics pipelines should also touch on this important aspect.

Although HBV continues to be the most common HCC risk factor worldwide, its importance is likely to decrease during the coming decades due to the widespread use of the HBV vaccine in newborns. We have added references (8-10) to expand on this idea.

Q2). It would be nice to have a Table or two for putting different these approaches together and compare their pros and cons.

We have now included a discussion comparing the pros and cons (page 10, lines 32-34 and page 11, lines 5-19) since we thought a table would be rather cumbersome for the required details.

Q 3) How about the turnaround time?

The overall time is about 2 years although it took nearly 4 years for us to do all the independent validation required for us to learn this new approach to have high confidence in the results. Having built this confidence, we no longer need to do all of the studies it took in our first study.

Q4). In the Concluding Remarks: "However there are still some limitations that must be overcome before they are put into clinical applications." The authors better to delineate these limitations in details and how to overcome.

We are grateful for this comment and have added a description on page 15, lines 32-34 and page 16, lines 1-3. We have also added a reference (101).

Q5) Edition of English is needed.

We hope the reviewers find the improved manuscript of sufficiently high quality.

Q6). Some more relevant references can be considered in this review as listed below but not exclusive:-

We have added more than 60 references.

3) References and typesetting were corrected.

Thank you again for considering our review for publication in the World Journal of Hepatology.

Sincerely yours,

Gul Mehnaz Mustafa

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