Dear Dr. Ma and Reviewers,

We appreciate very much for your comments on our manuscript entitled “N-linked glycoproteomic profiling in esophageal squamous cell carcinoma”. Following the suggestions and requests, we have been extensively revised our original manuscript. Here, we attached a clean copy of our revised manuscript for your evaluation. A point-by-point response to reviewers was also enclosed.

Should you have any questions, please contact us without hesitation.

Kind regards,

Yi-Jun

Reviewer #1:

Specific Comments to Authors: This manuscript by Liu et al profiled N-linked glycoproteins in human esophagus squamous cell carcinoma. Two techniques were utilized to compare cancer and normal tissues. Differential expression of several glycoproteins was validated with Western. Serum samples were also tested. The hypothesis is that glycoproteins are differentially expressed in ESCC vs normal and may potentially contribute to cancer development. Overall, this study is descriptive and hypothesis-generating by nature, but it does provide valuable data to guide future studies. This Reviewer has several comments as follows:

1. Pooling multiple samples together is not a good practice. Because of this, so-called "DEGs" may or may not be true due to a lack of statistical consideration. Technically, those two lists of DEGs discovered with two techniques (2DE and iTRAQ) do not overlap substantially with each other. Even with the same technique (iTRAQ), reproducibility is not that great (Figure 2). Therefore, the list of DEGs is quite shaky. Without a solid list of DEGs it is meaningless to do further bioinformatics analysis (Figure 3 and Figure 4).

Answer 1: We completely agree with the reviewer in this point. For high-throughput experiments, such as PCR, ELISA, IHC, etc., samples are individually examined followed by statistical analysis to identify statistically significant events related to interested features. On the contrary, for low-throughput, time-consuming and cumbersome approaches, sample pools are frequently used in the literature, with the advantage to reduce individual variations as well as the experimental cost. In the discovery phase of biomarker discovery, the sample pools are useful for identification of universal biomarkers. In addition, our study used a large amount of proteins (60 mg) for enrichment of glycoproteins, which also entails pooled samples instead of individual samples. At present, we are developing LECTIN-ELISA using individual samples for expanding validation of the biomarker candidates after preliminary validation in this manuscript.

Two-DE-based and iTRAQ-based proteomic profiling are 2 complementary methods with low overlapping to increase the coverage of profiling. This is one of the strong points of our manuscript.

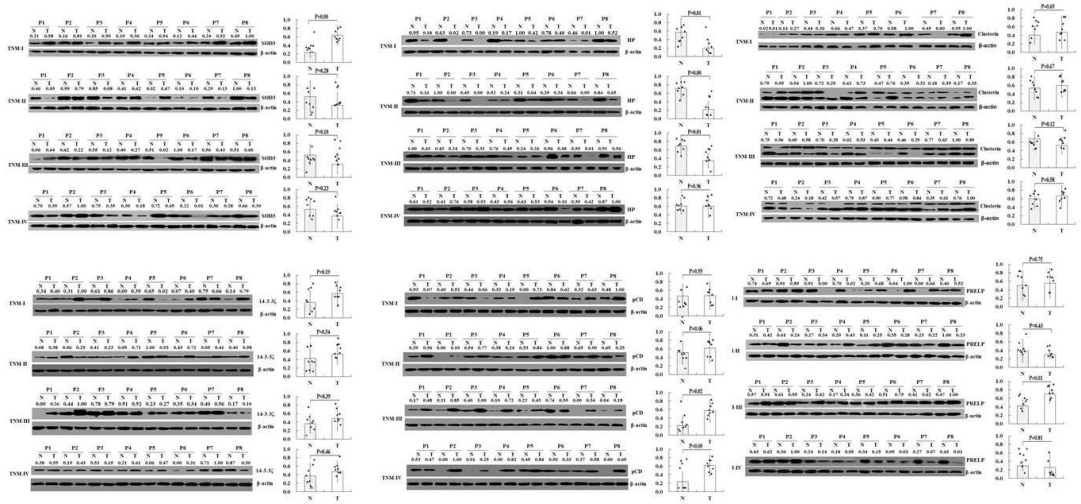
We acknowledge that overlapping of our technical duplicates of iTRAQ pools was moderately low but increased the coverage of glycoproteome. The reason may be that we generated our duplicates from tissue protein extraction in different batches. The tumor/tissue heterogeneity could contribute to the low shared identified proteins as well. In addition, we compared our DEPs identified by iTRAQ with those reported by Pawar etal and found that only 18 DEPs were shared between our 244 DEPs and Pawar's 238 DEPs. In –omic studies, low reproducibility is a major concern. Literature has reported that biological and technical duplicates can increase the reproducibility and coverage of identified molecules. In our study, we used pools prepared in different batches from 10 ESCC and adjacent tissue for iTRAQ quantification, with the intention to increase coverage. Thus, we are confident with our results and conclusions with regards to glycoproteomic profiling in ESCC.

2. As the author indicated, DEGs may result from DEPs or truly differential glycosyltransferase activities. It would be interesting for future studies to generate two lists, DEGs due to DEPs, and true DEGs.

Answer 2: We thank the reviewer for this suggestion. In our study, lectin affinity chromatography can enrich glycoproteins without differentiation between increased expression of proteins and increased level of glycans attached to proteins. In the validation, we used Western blot to interrogate this issue. Among the 6 biomarker candidates, procathepsin D was increased in ESCC tissue as a result of increased levels of both protein and glycosylated proteins. In contrast, haptoglobin only showed reduced total protein but not glycosylated haptoglobin in ESCC tissue, whereas glycosylated haptoglobin was increased in ESCC serum. As suggested by the reviewer, we would clean our data to generate true DEGs in our future studies.

3. Western validation with limited samples (Figure 5) does not really mean too much. Overall, the main drawback of this study is its lack of statistical consideration. The only solid conclusion is that they have developed the techniques to identify DEGs in ESCC, and they are able to differentiate DEGs due to DEPs and true DEGs. I would suggest the authors simplify the manuscript by presenting solid data and removing speculative parts.

Answer 3: We quite agree with the reviewer in this point. Validation using 32 ESCC and adjacent samples is still not enough for biomarker validation in Figure 5 that only showed representative 5 pairs due to large image size (Other more images of Western blot shown below). We will expand our samples to validate the differential expression of glycosylated protein biomarker candidates after establishment of LECTIN-ELISA in our future studies. At this stage, this study provides a resource of glycoproteins for in-depth investigation in future, as stated in the conclusion.



Reviewer #2:

Specific Comments to Authors: The manuscript entitled," N-linked glycoproteomic profiling in esophageal squamous cell carcinoma" by Liu et al exploited mass spectrometry based proteomics for identification of glycoporteomics.

I. Author must summarized studies where iTRAQ has been used for studying ESCC as the study like by Pawar et al (PMID:21743296) on ESCC is missing which is the Ist study utilizing iTRAQ for ESCC profiling.

Answer I: We thank the reviewer for pointing out our negligence. In the revised manuscript, we also compared the functional difference between DEGs and DEPs from Indian ESCC samples and acquire similar findings.

II. Must be changed from "15 ESCC and adajacent non-tumor tissues (5 samples for" to "15 ESCC and adjacent non-tumor tissues (5 samples for").

Answer II: We thank the reviewer for pointing out the errors in our manuscript and we have corrected these errors in the revised manuscript.

III. How far were the tumor samples from the ESCC for paired samples? It must be mentioned.

Answer III: We quite agree with the reviewer in this point. In general, the adjacent non-cancerous samples were at least 3 cm apart from the edge of neoplastic mass, which is added to the part of methods and materials.

IV. For iTRAQ analysis: whether decoy search /FDR was done or not, all that must be mentioned.

Answer IV: We quite agree with the reviewer in this point. "False discovery rate was calculated by a docoy database search strategy" was added to the part of MS identification.

V. Change from "expression ratios caculated by WARP-LC" to "expression ratios calculated by WARP-LC".

Answer V: We thank the reviewer for pointing out the errors in our manuscript and we have corrected these errors in the revised manuscript.

VI. Antibody from which vendor and what dilutions were used must be mentioned.

Answer VI: We thank the reviewer for pointing this out. In the revised manuscript, we have added the details of antibodies used in this study.

VII. Has the data been submitted to public repository? If yes, a link must be provided to have access to the community. If not done yet, data must be submitted to public repository.

Answer VII: Our data has been deposited at https://www.iprox.cn/ with Project ID IPX0004371000 that is added to our revised manuscript.

VIII. English grammer is serious concern in this paper, it must be fixed as you can see some examples already listed.

Answer VIII: We acknowledge that our manuscript still needs language improvement. Our revised manuscript has been edited and polished by Douglas G. Ward, one of our co-authors, a senior scientist with UK nationality.

EDITORIAL OFFICE'S COMMENTS

1. *The author's research design is not very good, and another good research design flow chart should be supplemented.*

Answer: In our manuscript, two methods including 2-DE-based and iTRAQ-based MS/MS proteomic profiling of glycoproteins in ESCC were used to identify differentially expressed glycoproteins followed by validation by Western blot. The flow chart follows the general workflow and protocol reported in literature. In addition, our work described in this manuscript is not implicated mechanistic investigation that usually needs a flow chart/schematic model. Therefore, we did not provide a flowchart for our manuscript.

2. *Self-Citation Count.*

Answer: Four articles published by our group were cited in this manuscript.

3. *It is unacceptable to have more than 3 references from the same journal. To resolve this issue and move forward in the peer-review/publication process, please revise your reference list accordingly.*

Answer: References were revised as per instruction in the revised manuscript.

4. *The article needs a great deal of language polishing.*

Answer: A new language certificate was provided with the revised manuscript.

5. *Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file.*

Answer: Revised as per instruction in the revised manuscript.

6. *Please authors are required to provide standard three-line tables, that is, only the top line, bottom line, and column line are displayed, while other table lines are hidden. The contents of each cell in the table should conform to the editing specifications, and the lines of each row or column of the table should be aligned. Do not use carriage returns or spaces to replace lines or vertical lines and do not segment cell content.*

Answer: Revised as per instruction in the revised manuscript.

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Answer: Revised as per instruction.

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Answer: Highlights are included in the revised manuscript.

10. *It is unacceptable to have more than 3 references from the same journal. To resolve this issue and move forward in the peer-review/publication process, please revise your reference list accordingly.*

Answer: Revised as per instruction in the revised manuscript.

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Answer: Provided as per instruction in the revised manuscript.

Looking forward to hearing from you.

Best wishes,

Yi-Jun Qi