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Title: Serum metabolome profiles characterized by patients with hepatocellular carcinoma associated with hepatitis B and C

Author: Saito T, Sugimoto M, Okumoto K, Haga H, Katsumi T, Mizuno K, Nishina T, Sato S, Igarashi K, Maki H, Tomita M, Ueno Y, Soga T

Responses to reviewers:

Reviewer 1

#1 The stage of HCC including number and its size may affect the concentration and variation of metabolites in patient's serum. Hence, we need the information of difference in the number and size of HCC between HCC-B and HCC-C. I think the same stage of HCC is necessary to compare the difference of metabolites directly or indirectly produced by HCC.

We agree with the reviewer's comment. The HCC cases of this study were in early stage of the tumor detection and they had been diagnosed for the first time as having HCC in the clinical setting. Although the number and size of the tumor in most of cases were less than 3 and 3 cm, respectively, it was hard for us to state these correctly in all cases. In this revised version, we have added a statement in the Patients and Methods section that the samples were collected after the first diagnosis of HCC as early as possible with the mean collection date and 78% of them were collected within 30 days after the first diagnosis. In addition, as the limitations of the study, we have stated in the Discussion that many conditions that may affect the concentration and variation of metabolites in patient's serum, such as the stage of tumor, the stage of fibrosis or inflammation in the liver, their received treatment and viral load, were not identical among the cases studied. However, we believe that the concentrations of metabolites and their profiles were valuable to develop a useful biomarker of HCC because they had been diagnosed for the first time during the clinical follow-up in the real-world clinical setting of the chronic liver diseases due to HBV and HCV.

#2 The contents of serum in HCC patients may be affected by the non-cancerous region such as liver cirrhosis because of production of many proteins by hepatocytes. In addition, the degree of inflammation of liver by HBV or HCV may trigger the production of many cytokines etc., so the author should describe the estimation of inflammatory or fibrotic stage of non-cancerous region.

We agree with the reviewer's comment. The stage of non-cancerous region including

fibrosis and inflammation may affect the concentrations of metabolites. The gold standard of such evaluation was the liver histology, but liver biopsy was not always a necessity for the clinical management of HCC patients, and thus it had not been done in all cases. In this revised version, we have added the data of noninvasive markers including Fib-4 index and platelet count in addition to the data of serum transaminase levels in Table 1.

#3 In cluster X specific for HCC-B, why is this profile so specific for HCC-B? Is the stage of HCC-B or the degree of inflammation and fibrosis so focused or may HBV stimulate the production of serum metabolites? The HBV-DNA level may be lower or different in each patients, thus how HBV contributes the focused cluster-X?

Based on the result of heat map, the cluster X represented the concentration pattern of metabolites in HCC-B. Interestingly, although seven of the HCC-B cases (No. 22, 23, 28, 15, 17, 18 and 22) belonging to cluster Y were included, they were likely to be close to cluster X. In addition, the MLR analysis showed that the combination of three metabolites concentrations were accurately discriminated HCC-B from HCC-C.
The reason why cluster X was specific for HCC-B have not been clear in this study, and this is the important research problem to be elucidated in the future. At this time, it is important for us to demonstrate the metabolite profile related to HCC-B and HCC-C.
The HBV-DNA levels were not examined in all cases. We have added a statement that viral load may be different among subjects as a limitation of this study in the Discussion.

#4 The cluster-Y is so wide range, is it possible to divide the cluster-Y to subgroup? #5 Is it possible that the heat map is reconstructed by the level of HBV-DNA or the degree of inflammation or fibrosis in the non-cancerous region?

As we have described in the Discussion, HCV infection causes metabolic disorders associated with glucose and lipid metabolism during the clinical course, and thus the cluster Y may be wider than the cluster X which represented only HCC-B cases. If the number of HCC-C cases is increased, we may be able to divide into smaller cluster. This is a limitation of this study, and we clearly mentioned in the Discussion that further studies are needed to in a larger cohort of patients.

Reviewer 2

Page 6-11. Materials & Methods. What about of anthropometric variables and comorbidities such as type 2 diabetes, etc?

This is an important suggestion. The type 2 diabetes had not been adequately examined in all cases. In this revised paper, we would like to show the levels of fasting blood glucose by adding the data in Table 1.

Page 6-11. Materials & Methods. What about the written consent of each participant? And the approval of ethics committee?

According to the instruction of this journal, ethical statements have been in the top page of the article. In addition, we have added ethical statements in the Materials & Methods section.

Page 6-11. Materials & Methods. The analytical methods sections is too long. I suggest to include a brief section

In accordance with the reviewer's suggestion, we revised as the short analytical methods as is possible. Section 2.2 was deleted and, instead, only modification of the protocol for CE-TOFMS were described just in a sentence at the end of Section 2.1. The general description for cross-validation and bootstrap analyses were also eliminated. Section 2.2 (renumbered from 2.3) was left since this protocol was new.

Page 14. Discussion section. I suggest to include the limitations of this study.

In accordance with the reviewer's suggestion, we have added the limitations of this study in the Discussion as was shown in reviewer 1 comment.

Page 14. Conclusion section. I suggest to rewrite it accordingly with your results.

In accordance with the reviewer's suggestion, we have revised conclusion accordingly with our results.

Page 29. Table 1. What about the albumin, bilirubin, alkaline phosphatase, GGT, platelets and creatinine levels?

We have added these data in Table 1.