Dear Editor:

Enclosed please find the revised manuscript entitled “Mass spectrometry to profile serum peptides of hepatocellular carcinoma with bone metastasis” with ESPS Manuscript NO: 5204.

We have revised the manuscript carefully according to the reviewers’ comment and answered the question that raised by the reviewers point to point. Professional English language editing of this paper have been finished before submission. Please see the attached invoice.

All authors of this study have read and approved this revised manuscript. The contents of this manuscript will not be copyrighted, submitted, or published elsewhere while under your consideration. There are no directly related manuscripts or abstracts, published or unpublished, by any author(s) of this paper.

Thanks for your hard work on this manuscript. It is our pleasure to publish paper on ***WJG***. Please do not hesitate to contact us if you have any question.

Sincerely yours,

Jian He ，Zhao-Chong Zeng, MD

Department of Radiation Oncology,

Zhongshan Hospital, Fudan University

136 Yi Xue Yuan Road

Shanghai, 200032, China

E-mail: hejian62@163.com

zeng.zhaochong@zs-hospital.sh.cn

Tel: 86-21-64-41990-3699，86-21-64041990;

Fax: 86-21-63-666908， 86-21-64038472;

## Respond to the reviewer

The comments from Reviewer 1:

*The methods of this study appear technically sound but the cross-sectional nature and lack of appropriate controls, specifically HCC patients with non-bone metastases, limit interpretation of the validity of the identified markers. There is no way to conclude that the putative biomarkers are specifically related to bone metastases. There is no way absent a prospective analysis to determine if these are sensitive for early detection of bone metastases. It is unclear what advantage these proteomic markers would have over nuclear bone scans. I'd suggest the authors examine patients with lung metastases but no bone metastases and determine if these markers are specific to bone metastases. If they find a marker that is specific to bone, then I think this is reasonable to publish as long as they don't make any claim that these are valid predictors of bone metastasis development. To make this claim they would need to perform a prospective assessment of patients without initial bone metastases to determine if the marker predicted subsequent development of bone metastases. If so, this would be useful for identifying patients who might benefit from bisphosphonate therapy or increased surveillance.*

**Response:**

Thanks for the reviewer's comments. We agreed the reviewer's comments. The cross-sectional nature is the weak point of this paper. To get the strongest evidence, we need to perform a prospective assessment of patients without initial bone metastases to determine if our marker predicted subsequent development of bone metastases. The prospective study has been launched before the paper submitted. We are recruiting candidates HCC patients without any metastases. We adopted a two-step design in this study. First the cross-sectional study was performed to get the promising markers for bone

metastases and then prospective study was adopted to confirm the results. However, the prospective study is not finished yet because it is a time-consuming process and we need to follow patients for 3 years after recruited. The reviewer is right, we can't make any firm claim that these makers could be used predict subsequent development of bone metastases without prospective study. According to the reviewer's comments, we change the 'Conclusion' to a mild one (please see the last two paragraphs of the Discussion).

Examination of patients with lung metastases but no bone metastases can determine if these markers are specific to bone metastases. This is an excellent suggestion. In this study, we used 72 HCC patients without bone metastasis as control. After one year follow up with MRI/CT/X-rays or SPET-CT/PET-CT, we found 7 HCC patients developed lung metastases in the control group. The model value of the serum peptides in patients with lung metastases were 118.9±47.4. It shows no statistical differences with patients without metastases (123.8 ± 42.7). However, HCC patients with lung metastases shows significant lower expression then patients with bone metastases (222.4 ± 55.5). This result implied that HCC patients would develop lung metastases without increase of the peptide-model value. Therefore, the serum peptides may be specific to bone metastases in HCC patients. However, our results should be validated by prospective study in the future with larger sample size.

The comments from Reviewer 2:

*The study design and labor are well organized and should be admired. This work would be more attractive if the authors more clearly presented the advantage of these new biomarkers as compared with the current diagnostic strategy, such as CT, MRI, and PET-CT. AFP and prothrombin mean that HCC*

*with bone metastasis is at more advanced stage as compared with HCC without bone metastasis. What do the authors speculate biological significance of the other peptides with peak in HCC with bone metastasis? Autophagy related protein is intriguing. The significant peptides may unveil progression of HCC to bone metastasis. If the involvement of these peptides to bone metastasis is revealed, it would be expected to develop a new strategy to suppress bone metastasis. ROC analysis clearly showed that this model is useful for the diagnosis or prediction of bone metastasis of HCC. Where is the description of the diagnostic model? Discussion should be more compact, focusing on significance of the findings.*

**Response:**

Thanks for the reviewer's comments. We have added the description of the advantage of these new biomarkers as compared with the current diagnostic strategy (Please see the Discussion). A timeline model clearly demonstrated that the development of disease is a continuum, with the initial events being molecular perturbations following histomorphological changes and clinical manifestations occurring relatively late in the disease timeline. This theory implied that molecular biomarkers were more likely to be used as early diagnosis/prediction tool than image (such as CT, MRI, and SPECT et al). Furthermore, blood test is convenient and rapid with relative low cost, which also can be used for dynamic monitoring.

Our study is focus on the endogenous polypeptides, a large part of the human serum peptidome is produced ex vivo by degradation of endogenous substrates by endogenous proteases. Polypeptides are generated during the proteolytic cascades that occur in the intrinsic pathway of coagulation and complement activation. Some of these are known bioactive molecules, others represent cleaved propeptides, and still others are seemingly random internal fragments of the precursor proteins.

According to the reviewer's comments, we searched the function

information of the other proteins. All the data were from UniprotKB database (http://www.expasy.org/). The detailed information about protein function are list below:

Serglycin plays a role in formation of mast cell secretory granules and mediates storage of various compounds in secretory vesicles. Required for storage of some proteases in both connective tissue and mucosal mast cells and for storage of granzyme B in T-lymphocytes. Plays a role in localizing neutrophil elastase in azurophil granules of neutrophils. Mediates processing of MMP2. Plays a role in cytotoxic cell granule-mediated apoptosis by forming a complex with granzyme B which is delivered to cells by perforin to induce apoptosis. Regulates the secretion of TNF-alpha and may also regulate protease secretion. Inhibits bone mineralization.

Isoform 2 of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) is an acute-phase protein (APP) involved in inflammatory responses to trauma. May also play a role in liver development or regeneration.

Isoform 1 of autophagy-related protein 16-2 (ATG16L2) may play a role in autophagy, it's detailed functions are not cleared.

Transthyretin is a thyroid hormone-binding protein. Probably transports thyroxine from the bloodstream to the brain. It involved in diseases of amyloidosis, hyperthyroxinemia dystransthyretinemic euthyroidal, carpal tunnel syndrome 1.

Fibrinogen has a double function: yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation. It involved in disease of congenital afibrinogenemia.

However, the peptide sequences identified in our study are protein fragments of molecules, rather than the full protein, therefore the expression levels of peptide may not necessarily reflect the expression of the protein and their biological function may also have nothing to do with their protein. Therefore, the exact biological mechanism of the peptides with peak in HCC with bone metastasis need further study.

We have rearranged the Discussion section and made it focus on the advantage and significance of our findings. We added the description of the diagnostic model in Results section. Briefly, the six selected peptides were detected by MALDI-TOF-MS in every patients and each selected peptides showed a peak after the detection by mass spectrum. The area under the peak were subject to RBFNN and finally a model value was generate for each patients. The software program are as following:

```
>> class=knnclassify(x,x,y,4);
>> [caculate]=knncaculate(y,class,2);
>>    m=[];
    for j=1:50
    b=randfen(y,2);
    for r=1:5
    [Xtr,Ytr,Xte,Yte]=straitsuiji(x,y,b,r,2);
    class=knnclassify(Xte,Xtr,Ytr,4);
    [caculate]=knncaculate(Yte,class,2);
    m=[m;caculate];
    end
    end
```