Author's response to reviews

Title: A novel biomarker TERTmRNA is applicable for early detection of hepatoma

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Author's response to reviews: see over
Dear Dr. Hans Zauner,

Thank you very much for your letter of Nov. 4, 2009, with regard to our manuscript (MS: 7290476972927319) together with the comments from the five reviewers. We appreciate your comments and deeply consider your suggestions in the revision of our manuscript. I am sending herewith a file of our revised manuscript. (Pages 1-32 have been retyped.)

Our incorporation of the reviewers’ suggestions is as follows:

Reviewer: Dr. Giovanni Tarantino

Reviewer's report:
Major Compulsory Revisions
The AA are kindly requested

1) to improve the statistical presentation, better describing the used tools and giving appropriate outputs, i.e., ORs, r or rho and so on.
Answer: We have changed the statistical presentation in Table 1 and added the data of OR to Table1-(b), as suggested.

2) to set the criteria for the gold standard.
Answer: Regarding gold standard, we already have described the details in the published report [4,5,9,10]. We have treated this matter as references.

3) to discuss other findings present in literature,
Answer: We are terribly sorry for this mistake. We erased such a discussion about small HCC according to the comments we received from reviewer of previously submitted journal. However, we thought that the discussion is indispensable. We briefly added the comments to Discussion section (p.15, L22-p16, L3) as “Furthermore, we could detect
the serum hTERT mRNA even in HCC patients with less than 10 mm moderate-differentiated tumor, indicating that hTERT are upregulated during rapid proliferation of tumor at the early phase of oncogenesis, de-differentiation.”

4) to pinpoint the limitations of this study and perform a cost/benefit analysis of the propose test, and finally,

Answer: We have added the limitations of this method to Discussion section (p16, L12-17) as “This method depends on RNA stability in each process of RNA purification, storage, and quantification. In the light of its superior positivity to other markers, the assay will be applied for clinical use in the strict condition because it is required to keep the serum RNA as it is in blood and avoid the degradation of quality. Now we are improving RNA stability and PCR condition to better cost/benefit of this assay.” In Japan, we are performing clinical trial study (large scale, multi-facilities) of HCC markers using hTERT mRNA. Furthermore, we are now comparing hTERT mRNA quantification with the findings of PET-CT. It costs 20USD per sample to do this assay. We are trying to control the cost per one tube up to 10USD, resulting in the improvement of balance of cost/benefit.

5) to cut down a lot of words in the Abstract, Introduction and Discussion sections.

Answer: We have corrected the abstract, introduction, and discussion, except for the indispensable content, resulting that several overlapping expressions and words were corrected and manuscript was shortened. We erased 21 and 72 words from background sand Introduction.

Reviewer: Dr. Helen Reeves

Reviewer's report:
This is an interesting and thorough research study, which includes large numbers of patients and appropriate controls. It is generally well written and represents an important contribution to the search for diagnostic biomarkers for early HCC in patients with chronic viral hepatitis.

Major Compulsory Revisions / Minor Essential Revisions:
The authors demonstrate very clearly that serum hTERTmRNA is superior as a biomarker, both in terms of sensitivity and specificity, and positive and negative predictive values, to other commonly used biomarkers. However, although the authors comment, for example, on the numbers of patients negative for one biomarker who were positive for another, they do not present data analyzing the success of combinations of
biomarkers. eg. what is the level of improvement in sensitivity and specificity when combining hTERT with AFP ? or with DCP ? This would be particularly interesting for the early/smaller tumours. Really, I would have liked to see more of the data for different size and differentiation grade tumours. Figure 5 is excellent in this regard for size, and a similar figure for grade may further enhance the manuscript, but do not reveal additional value of biomarker combinations.

**Answer:** We have added the sensitivity improved by combining hTERTmRNA and AFP to Result section (p.10, L2-3) as “Combinations of hTERTmRNA with AFP level improved the sensitivity/specificity up to 96.0%/87.2%“. Also, I prepared for the dot blot that shows the correlation of hTERTmRNA with tumor differentiation. It was provided as supplementary figure 3 because biopsy samples are performed in about 1/3 of HCC patients. The result was described in Result section (p.11, L13-14) as “Dot blot regarding the correlation of hTERTmRNA quantification with tumor differentiation was shown in Supplementary Figure 3.” Also, we added “Serum hTERTmRNA quantification in HCC patients (n=101) diagnosed by liver biopsy was shown, categorized by tumor differentiation. The quantification in serum of HCC patients with well-/moderately-/poorly-/un-differentiation was 4.4±1.4/5.4±2.0/6.3±3.3/5.9 (mean ±SD).” to the explanation in the additional file 3 (p.32, L1-4)

**Discretionary revisions -** The authors imply in their abstract, introduction and early discussion that it is primarily because HCC is a complex disease, involving many cellular signaling pathways, that a novel biomarker is required. While this may well be true, it is primarily because the presently used biomarkers are not sufficiently good enough, especially in early disease, to be used as surveillance or diagnostic tests. I would suggest this be introduced at an earlier stage.

**Answer:** We are now launching the comparison of hTERTmRNA with PET-CT for primary care. As described in last sentence of discussion, we definitely aim the induction into primary care and are developing both wet and hard part of assay with Japanese company (KonicaMinolta) because hTERTmRNA is detectable in other cancers such as lung, ovary, GI, etc.

**Reviewer:** Dr. Patrizia Pontisso

**Reviewer's report:**
The authors describe the diagnostic performance of serum human telomerase reverse transcriptase mRNA for hepatocellular carcinoma. The results indicate that this approach is highly sensitive and specific, allowing the conclusion that hTERT mRNA is
superior to conventional tumor markers in the diagnosis and recurrence of HCC. The availability of useful diagnostic markers for primary liver tumors is still an unmet clinical need and the study has addressed such an important issue. To better define the clinical relevance of the study, the following points should be addressed:

1. HCC occurs in the majority of the cases on a cirrhotic liver, and cirrhosis is the population at risk, where other tumor markers, such AFP, are frequently positive, lowering the specificity of the biomarker, when compared to this population. On the basis of these considerations, it would be interesting to calculate the diagnostic performances of hTERT mRNA, including ROC curve analysis, using cirrhotic patients and not healthy subjects as controls.

   **Answer:**
   We have tried ROC analysis as suggested, but AFP is still sensitive for HCC with LC. The tendency is not so different from Fig 2. Also, hTERTmRNA expression is specific HCC oncogenesis, suggesting that the measurement is not influenced by the background lesion. Whatever the background is, hTERT upregulation is induced by tumor proliferation accompanied with dedifferentiation. Therefore, I do not add another ROC curve on the basis of liver background in this report.

2. The clinical significance of monitoring the effect of therapies using hTERTmRNA has been reported only in two representative cases. It is unclear whether the concordance with clinical outcome is a common behavior in all patients or whether this has not been evaluated in all treated patients. At list 12 months follow-up after treatment should be required to draw any conclusion on the prognostic value of the behavior of this marker.

   **Answer:** hTERTmRNA quantification is followed by the sensitivity and specificity. We have a lot of recurrence cases received therapeutic modalities. It is naturally difficult for us to predict the outcome. However, hTERTmRNA tend to upregulate at the early phase of post therapy in recurrence case. We can not analyze all the cases of multi-facilities. We will report the outcome soon.

3. The authors describe that the level of hTERT mRNA correlates with tumor differentiation. However, in Fig. 6 (panel D) the higher levels were shown in poorly differentiated cases, while in the discussion (pag.16) they report that “hTERT mRNA expression was closely associated with well to moderate differentiation degree.” This point should be clarified.

   **Answer:** I am terribly sorry, but this is due to limited English. I meant that hTERT
suddenly upregulation during well to moderate and the upregulation enhances after the rapid proliferation and dedifferentiation accompanied with heterogeneous genetic alteration. We added this explanation to Discussion section you pointed out as “and was enhanced with the proliferation”

Minor comments:
- In the Patients and Methods section the list of all the clinical parameters, also reported in Fig 2 (and not in Figure 1) is redundant.
  **Answer:** I thoroughly agree with your opinion but and we changed this order rightly. Also, I replaced this Fig 1 with 2.

- The authors should specify how the diagnosis of HCC was achieved in tumors of size <10mm.
  **Answer:** We added the explanation as “the differentiation of HCC was diagnosed by liver biopsy” to Patients and Methods section (p.6, L9).

- English should be substantially improved
  **Answer:** We corrected the abstract, introduction, and discussion, except for the indispensable content, resulting that several overlapping expressions and words were corrected and manuscript was shortened. Afterward, we corrected this MS by requesting another company the correction. We erased 21 and 72 words from background sand Introduction.

**Reviewer:** Dr. Michael Grusch

**Reviewer's report:**

The manuscript “A novel biomarker TERTmRNA is applicable for an early detection of hepatoma” by Miura et al describes that in a study including 638 subjects (303 with HCC) analysis of telomerase mRNA in serum by real-time RT-PCR was a superior biomarker for compared to AFP, AFP-L3 and DCP in diagnosis and detection of recurrence of HCC.

There is indeed an urgent need - and ongoing search - for improved HCC biomarkers and the data reported here for hTERT mRNA, if confirmed, seem promising. Important clarifications/improvements, however, have to be made by the authors before this study can be published.

Compulsory revisions:
1) Regarding their method of TERT mRNA detection, the authors refer to an earlier
publication, but say that they used more efficient primers than in the earlier report. Primer sequences should be given and it should be explained how RNA controls for quantification were performed and copy numbers per ml were derived. Are logarithmic values log2 or log10? How was exclusion of false negatives performed? No reference at all is made to the detection of the other markers.

**Answer:** We used the original primer set only because PCR is primer-sensitive and, if we change the primer set in this HCC project, this assay will be changed, resulting in the different outcome. The primer sequences are in 2003 Oncology. Using this original primer, this study was performed. In different large-scale study of HCC in Japan, we are using modified primer set. Also, logarithmic value is 10. Furthermore, we never exclude false negative case and we treated statistically and added OR to the table 1-(b) as the evidence. Regretfully, we could not measure other markers the volunteer. In this regard, we think that this report has been rejected in other journals as you pointed out. It would be more expensive, though we have all the serum still now.

2) Language editing is essential, as multiple statements are hard to interpret in their present form.

**Answer:** We corrected the abstract, introduction, and discussion, except for the indispensable content, resulting that several overlapping expressions and words were corrected and manuscript was shortened. We erased 21 and 72 words from *background sand Introduction*. Afterward, I corrected this MS by requesting another company the correction.

3) Although a different approach was used, the conflict with recent data of Kong et al (J Cancer Res Clin Oncol) should be discussed.

**Answer:** I do not know the reason but our methods using SYBR Green I and excellent primer is superior to detect circulating hTERT mRNA. I think their AFP mRNA quantification is less sensitive and specific than outcomes by our methods. As they are performing different approach, we can not compare with ours because we started this study to avoid Taqman method for less sensitivity. Also, they used $2000 \times g$ during RNA purification, inducing the pick-up the hTERT derived from broken lymphocyte.

4) Given the assumed specificity of hTERT for immortalized cancer cells, how do the authors interpret the apparently increased level in LC and CH compare to healthy controls and what were the statistics of this effect?

**Answer:** As we described in previous report, hTERT in hepatocytes infected by virus is
upregulated. We think that hTERTmRNA were released from liver through the viral damage (inflammation, necrosis, and regeneration). Immunoreaction in circulating situation causes it partly or majorly. We do not know the details yet because we would like to know them. However, we think hTERTmRNA expression cancer cells have is essentially different from that in infected hepatocytes.

Reviewer: Dr. Hans Spangenberg

Reviewer's report:
The paper entitled „A novel biomarker TERTmRNA is applicable for an early detection of hepatoma” by Miura. describes the detection of TERTmRNA in the serum of patients with HCC. The paper is well written, the methods used are state of the art. Even though the results of the study are quite intriguing, some points should be addressed prior to publication.

1. The study population is not well described. A table with the characteristics should be added. (Figure 1 with should include the characteristics) could not be found.

Answer: To save the number of figures, we were obliged to do this style as we needed to combine characteristics with the Results. We replaced Fig 1 with Fig 2.

2. The control population is not well chosen, since most HCC patients are male, however in this paper the majority of control patients is female.

Answer: I agree with your opinion but we could not single out gender of volunteer when we obtain the control samples. It is very difficult to get more. We think this mismatch caused the rejection by other journals.

3. For better statistical comparison more patients with liver cirrhosis with different child classification should be evaluated.

Answer: Child classification did not have significant correlation with hTERT. Regarding LC, measurement of markers was not statistically significant in comparison with CH from the result of ROC curve analysis. We added the explanation as “hTERT and other markers in LC was not statistically significantly different in comparison with that in CH.” to Result section (p.10, L5-6).

4. Longitudinal analysis of TERTmRNA should be done to analyse the predictive value of this marker to detect HCCs.

Answer: We described the predictive value in Table 1-(b).
5. Was the diagnosis of HCC according to AASLD guidelines, this should be stated in M and M.

**Answer:** I appreciate this suggestion very much. We added this phrase in P and M (p.6, L8-9), as “HCC was diagnosed according to AASLD guidelines.”

6. The recurrence of HCC after TACE, was there any correlation of TERT with imaging (Ultrasound, CT, MR)?

**Answer:** In all the cases, any other imaging modalities than hTERT could not detect the recurrence at the earlier phase, but when we could find HCC in image such as US, CT, or MR, other markers began to rise up. We added this phrase in Result section (p.11, L4-6) as “In all the cases that hTERT detected recurrence at the earlier stage, any other imaging modalities than hTERT could not detect it at the same time, but when we could find HCC in image such as US, CT, or MR, other markers began to arise.”

I believe the manuscript has been improved satisfactorily and hope it will be accepted for publication in BMC Gastroenterology.

Best regards,

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