Author's response to reviews

Title: Y-box protein-1/p18 fragment identifies malignancies in patients with chronic liver disease

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Author's response to reviews: see over
Dear Editor,

thank you very much for considering our manuscript (“Y-box protein-1/p18 fragment identifies malignancies in patients with chronic liver disease”) for publication in *BMC Cancer*. We greatly appreciate the insightful comments by the expert referees that indeed helped us to improve the manuscript. In the revised version, we addressed all issues raised by the reviewers.

We hope you find our manuscript now suitable for publication in *BMC Cancer*. Please find our point-to-point responses to the referees’ comments below and the revised manuscript (changes are underlined).

Kindest regards,

Frank Tacke
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**Point-to-point responses to the reviewers**

Thank you very much for the thorough and fair review of our manuscript.

**Reviewer: Hiromitsu Nagata**

*In this manuscript, authors reported the availability of YB-1/p18 measurement as a tumor marker in human plasma of patients with advanced cancerous diseases, using a novel immunoblot assay. They described that plasma YB-1/p18 could identify patients with malignancies, independent of liver dysfunction, acute inflammation or renal impairment. This is an interesting work with novel information that may be relevance in clinical medicine. However, I have some issues that need to be addressed by the authors:*

1. **As described by the authors, nuclear YB-1 overexpression is a common finding in a variety of solid tumors and has linked to tumor growth and survival of cancer patients. Furthermore, high YB-1 expression is an independent negative prognostic marker for many solid tumors. Do the authors have any information about the relationship between plasma YB-1/p18 and nuclear YB-1?**

   **Response:**
   As proposed by the referee we have collected tumor tissue specimens (n=20) and determined the subcellular YB-1 localization with monoclonal antibody by immunohistochemistry. At the same time we have determined the presence or absence of YB-1/p18 in corresponding plasma samples. As a result, we were not able to find a positive correlation (unpublished observations). Given that these tissue samples were from other origins than HCC, the low number of tissues analyzed and the high percentage of positive YB-1/p18 cancer patients, these data have to be regarded as preliminary. We thus have opted not to include the data in the revised manuscript. As suggested by the referee we have included a paragraph in the revised manuscript that addresses these important questions.

2. **In this paper, the authors mention that YB-1/p18 was the best single marker to identify patients with other malignancies among our study population and proved superiority compared with AFP, CA19-9 or CEA. Is it statically significant? I guess that the c-statistic value above 0.8 is generally significant.**

   **Response:**
Due to the low frequency of observed “events” (= other malignancies than HCC), the c-statistic values obtained by our analysis need to be viewed with caution. As stated by our consultant statistician, it is, due to the rare occurrence of other malignancies, not possible to formally compare the c-statistics derived by the different tumor markers. However, it is appropriate to plot the ROC curves (as we did in figure 4C), demonstrating the best c-statistics for YB-1/p18 in comparison to the other markers investigated. We have now included this important consideration into the revised manuscript.

Minor revisions:
1. In Figure 3 I feel there is macroscopically a gap in the relative optical density (OD). For example, Pat#57’s OD in Figure 3B is equal to Pat#11’s one in Figure 3D?

Response:
We apologize for causing confusion about the ODs given in Figure 3. Figure 3 summarizes many blots, and each blot was loaded with the same positive control. The optical densities were calculated as the relative OD in comparison to the positive control from the same blot. This allowed normalizing for possible Western Blot assay variability (e.g., exposure time). For clarity reasons, we have combined many blots into this one figure and show only one representative positive control. However, the positive (and negative) control was kept on each single blot. This is now clarified in the revised manuscript (Materials & Methods, Figure legend).

2. In the Table 2, it seems that the authors have misprinted Pat#59’s CRP level.

Response:
We apologize for this error. The CRP level was correct, but we had not highlighted it. This has now been modified in the revised manuscript.

3. Page 10, line 27: the authors refer to “(Fig.3D)”, in my opinion it should be “(Fig.3C)”.

Response:
We apologize for this error. This has now been modified in the revised manuscript.

Reviewer: Andreas Teufel

The manuscript submitted by Tacke et al. entitled “Y-box protein-1/p18 fragment identifies malignancies in patients with chronic liver disease” deals with the clinical and diagnostic value of YB-1 protein fragment p18 (YB-1/p18), a cold shock protein, in serum based screening for in patients with chronic liver diseases. The manuscript is well written. The experiments were carried out straight forward and in logical order. Generally, this is an interesting piece of work warranting publication. However, I would suggest the authors change a few issues in the manuscript.

1) The section on malignancies other than HCC is not of major interest given the low number on patients investigated. Since the manuscript investigates several diverse subgroups which is close to confusing the reader it would benefit from dropping this part. The authors should investigate this issue in subsequent studies with a larger number of patients.

Response:
As suggested by the referee we have clearly separated data on YB-1/p18 in other cancer diseases from the findings reported for HCC. However, given that the YB-1/p18 marker is novel and along the line to proof the concept of a qualitative determination we wish to opt for adherence to this section, because it strengthens the point of YB-1/p18 as a novel qualitative tumor marker. Certainly, we strongly agree with the reviewer that YB-1/p18 needs to be tested on larger cohorts of patients with different malignant diseases. In fact, based on the results presented in the current manuscript, we have already started investigating these patients. At the discretion of the reviewer, the preliminary interim analysis of this follow-up study is shown below (see Figure for the reviewer). Data on the other cohort are summarized at the discretion of the referee in the attached figure (see below). A statement was included in the revised manuscript that a larger cohort of characterized patients should be tested for the sensitivity and specificity of the YB-1/p18 plasma marker.

![Figure for review](https://example.com/image.png)

**Figure for review:** Preliminary analysis of patients with cancers of various origins. The absolute numbers of patients analysed and tested positive for YB-1/p18 in plasma are given.

2) I would further appreciate having absolute numbers of patients AND percentages of the total cohort for all subgroups.

**Response:**
We have revised table 1 and the text of the results’ section accordingly and have added absolute numbers and percentages for all subgroups.

3) Since the benefit in HCC screening seems to come from combination of several markers including AFP and YB1 the authors should discuss the available literature on those combinatory screening trials with AFP and markers other than YB1.

**Response:**
We thank the reviewer for this important remark and have included a short discussion on other markers (AFP-L3, CA242, DCP/PIVKAII or AAG) that had been suggested for HCC screening in our revised manuscript.

4) How many patients were AFP AND YB1 positive? And does this number have an influence on the statistics provided in this manuscript, especially with respect to superiority of markers.

**Response:**
Only 1 out of 15 patients prospectively analyzed for HCC tested positive for AFP AND YB-1/p18. Thus, it is clear that the combination of these two markers would not be superior to testing each marker separately. However, one might speculate that both markers may
identify different types of HCC and that YB-1/p18 may help to diagnose AFP-negative HCCs. Larger studies are needed to investigate this matter. In the revised manuscript, we have now added that the combination of both markers is not likely to increase their diagnostic accuracy.

Reviewer: Joerg Schrader

Tacke and Kanig et al. describe a novel fragment of the cold-shock protein YB-1 in the plasma of patients with malignancies. The identified a 18kDa fragment of YB-1 by Western Blot in the plasma of patients with various solid organ cancers, which is not present in healthy controls. Acute inflammation or renal impairment does not seem to have an effect on the levels of this fragment in patients, but was detected in 5 out of 60 patients in this group without a known underlying malignant disease. In two different cancer cohorts compromising 25 HCC patients and 20 metastatic cancer patients of various primary origin the specific 18kDa fragment could be detected in 44% and 80% respectively. They went on to evaluate this fragment as a screening tumour marker for malignant disease in 111 patients undergoing extensive search for non-detected tumour diseases in the process of evaluation for liver transplantation. Here 6 patients with detected cancer were positive for the YB-1/p18 fragment, whereas the same number of detected cancers were positive for the established HCC tumour marker AFP. Overall this study is interesting and novel and the identified protein fragment has the potential to become a new tumour marker. Nevertheless, a few points remain unclear and need attention.

Major Compulsory Revisions
1. Although the authors evaluated the presence of the p18 fragment of YB-1 protein with different antibodies a formal proof of identity would be helpful. If any of the antibodies would work on immunoprecipitation a mass spectometry analysis would be the method of choice – otherwise a preabsorption of the antibody with recombinant protein or corresponding peptides would be an alternative. In due course it seems puzzling, that the preparation of recombinant protein contains the p18 fragment as well – it would be interesting to speculate whether this is a transcriptional variant or a proteolytic fragment?

Response:
As suggested by the referee we have performed ample immunoprecipitation experiments with monoclonal as well as polyclonal antibodies raised against YB-1 epitopes. None of these experiments were successful in elucidating the exact fragment composition. Therefore we have designed the experiment that tests the specificity of the different antibodies and approximated the relative composition of the fragment as encompassing the cold shock domain (compare Figure 1D and the whole Figure 2).

Serum protein precipitation with ammonium sulphate and enrichment of YB-1/p18 by liquid chromatography were also performed and failed. In these experiments multimerization of the enriched protein fragment was apparent. The results of all these additional experiments are now reported in the revised manuscript (see revised Results’ section).

As suggested by the referee we have added our speculation on the nature of the fragment in the revised manuscript. Given the use of an expression plasmid with non-existing alternative splice sites and the expression in bacteria we consider the generation of an (auto-) proteolytic fragment as more likely.

2. Could the level of the p18 fragment be correlated to the total (full length) YB-1 protein in the same plasma sample?

Response:
We thank the reviewer for this comment. Indeed, the Western Blots always detected abundantly present full-length YB-1 protein as well. However, even with very short exposure time, we were not able to detect any meaningful regulation between patients and controls or...
between samples from different cancers. We therefore concluded that the detection of full-length YB-1 is not specifically regulated in serum. This is in sharp contrast to the YB-1/p18 fragment, which showed features of a specific tumor biomarker. We have clarified this in the revised results’ section of the manuscript (please see also Figure 1).

3. The authors extensively evaluate the prognostic value of YB-1/p18 in the cohort of 111 patients undergoing evaluation for liver transplantation. Although these data are interesting, the low number of positive correlations (only 6 patients YB-1/p18 positive out of 20 patients with detected malignancies) and the high number of negative correlations (14 patients YB-1/p18 positive out of 91 without detected malignancies) warrant a more cautious discussion of these findings. It would be interesting to know how many “newly” diagnosed tumours diseases would have been detected by the use of YB-1/p18 once all patients with a known tumour disease (HCC n=4, liver metastasis n=3) as a reason for liver transplantation were excluded.

Response:
The reviewer raises a very important point, but it is hard to address by our current study. Indeed, our study population with the liver disease patients was followed prospectively. Thus, it should be possible to address how many of the YB-1/p18 positive patients developed cancer upon follow-up (“detection of newly diagnosed tumors”). In fact, outlined in the last paragraph of the Results’ section, one of the YB-1/p18 positive patients developed HCC three months later, and three more patients had PSC as underlying disease which can be regarded as a precancerous condition (for cholangiocarcinoma). However, a comprehensive analysis of the predictive value of YB-1/p18 is hampered by (a) relatively small study cohort (n=111) with a rare incidence of new malignancies, and (b) short follow-up of the very high-risk patients due to the fact that these patients received liver transplantation on average within 6-12 months after evaluation. These limitations and the need for further prospective studies are now discussed more extensively in the revised manuscript. We have also rephrased the conclusions in a more cautious manner.

Minor Essential Revisions
1. The species source for the recombinant YB-1 gene sequence should be stated.

Response:
The recombinant YB-1 was synthesized with the pRSET prokaryotic coding sequence of rat YB-1 (also denoted EFIA). Rat and human YB-1 are more than 98% homologous at the amino acid level. This is now clarified in the revised Materials & Methods’ section. Figure 2 has been modified accordingly.

2. An earlier study has evaluated the prognostic significant of YB-1 positivity in HCC tissue (Yasen et al 2005) – this should be mentioned and included in the references.

Response:
We truly apologize that we had not included this very important paper into our initial submission. This is now mentioned and quoted in the revised manuscript.

3. The second last sentence on page 9 starting “The fastest running band.....” needs to be revised.

Response:
The sentence has been revised as suggested by the reviewer.
4. The statement on page 10, line 4 is difficult to understand, as it currently reads as if the absence of cirrhosis is associated with higher rates of malignancies, which is not true for the frequency of HCC in patients with liver disease.

Response:
We understand that this sentence might be misleading and have removed it from the revised manuscript.

Discretionary Revisions
1. It would be interesting to speculate on the origin of the p18 fragment in the tumour patients. Does it come from the cancer cells themselves or from infiltrating stromal cells? Is it intra- or extracellularly processed?

Response:
We have included a short paragraph in which we speculate about the origin of the YB-1/p18 fragment. Further experiments from our group indicate that YB-1 processing may occur intracellularly within the vesicles that release YB-1 from the cells (Frye at al., 2009). However, such studies are difficult to quantify, and it is unknown whether this is a phenomenon of cancer and transformed cells only. Further studies are on their way to answer these questions.

2. Is the YB-1/p18 fragment a specific (qualitative) tumour phenomenon, or is it related to the total amount of YB-1 protein produced by the tumour, hence a mere quantitative phenomenon?

Response:
Thank you for this comment. As outlined above (major comment #2) and in response to reviewer Nagata (question #1), we failed to quantitatively relate YB-1/p18 in plasma to either total full-length YB-1 in plasma or to nuclear YB-1 expression in the tumor. Therefore, we can at present only regard the 18-kD fragment as a “qualitative” tumor phenomenon. However, we are trying to develop a specific ELISA system for YB-1/p18. This may in the future allow to more quantitatively measure YB-1/p18 levels. With such a more accurate quantification tool, new quantitative associations (e.g., between full-length YB-1 in plasma or nuclear YB-1 expression levels) may be explored. This is now added to the revised discussion section.