Author's response to reviews

Title: Y-box protein-1/p18 fragment in plasma is a sensitive and specific novel disease marker in patients with malignancies of different origin

Authors:

Frank Tacke (frank.tacke@gmx.net)
Oliver Galm (ogalm@ukaachen.de)
Nicolas Kanig (Kanig@gmx.net)
Eray Yagmur (eyagmur@labor-stein.de)
Sabine Brandt (Sabine.Brandt@med.ovgu.de)
Jonathan A Lindquist (Jon.Lindquist@med.ovgu.de)
Christiane S Eberhardt (Christiane.Eberhardt@gmail.com)
Ute Raffetseder (uraffetseder@ukaachen.de)
Peter R Mertens (peter.mertens@med.ovgu.de)

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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 in plasma is a sensitive and specific novel disease marker in patients with malignancies of different origin”) 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  
University Hospital Aachen, Germany

Peter Mertens  
University Hospital Madgeburg, Germany

Point-to-point responses to the reviewers

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

Reviewer: Reinhold Schäfer

The authors have determined serum levels of the YB-1 p18 polypeptide in cancer patients suffering from different carcinomas and demonstrated a high prevalence. The paper extends a previous publication from the same group (Tacke et al., BMC Cancer 11:185, 2011), showing that YB-1 p18 is present in serum of hepatocellular carcinoma patients but not detectable in sera of non-diseased individuals or patients with inflammatory disease. Hence, the claim suggesting specificity for malignant diseases is further strengthened. The authors have compared the sensitivity of p18 detection with that of 13 other tumor markers present in serum (e.g. CEA) and report overall superior performance of their marker. However, YB-1 p18 does not have any prognostic value. In my view, this limits the chances of translating YB-1 p18 detection into a clinical or diagnostic setting. I wonder, if the authors have considered to apply YB-1 p18 serum detection in a prospective setting or as an early marker for cancerogenesis. The paper would gain much more value as a tumor marker provided that the authors could correlate p18 detection with adenomas or any other benign precursor lesions.

The major drawback of work on p18 is the lack of functional data that would describe any specific function, particularly in view of the multifaceted roles of YB-1 in multi-drug resistance, control of transcription and translation etc. (although this was not the focus of their paper).

Specific points:
1. The authors have analyzed a broad range of tumors which limits their conclusions because the individual tumor cohorts are relatively small (Table 1-3).

Response:
We agree with the reviewer and fully understand his/her concern. Nevertheless, our study was intended to provide a proof-of-concept for this yet unrecognized circulating YB-1/p18 fragment. After having conducted a rather narrow analysis with a defined subset of patients (liver cirrhosis and liver cancer) previously (Tacke F, et al. BMC Cancer 2011; 11:185), this
current study now intended to screen a large number of patients with very different tumor entities and tumor stages, aiming at identifying whether YB-1/p18 is a rather general characteristic in malignancies. However, we agree with the reviewer that we cannot draw specific conclusions for very distinct subgroups of malignancies. We have now clearly stated this potential limitation of our study in the revised manuscript.

2. **Indicating a percentage for tumor groups in which the total number equals 1, 2 or 3 does not make sense (table 3).**

**Response:**
We agree with the referee and have modified the table 3 accordingly. We now present the absolute numbers of patients in all subgroups with positivity for YB-1/p18.

3. **Although immunoblotting is a semi-quantitative method at best, it would be interesting to determine the limit of detection using other markers for normalization (if possible) (Fig. 1). In hematological malignancies, one could try to correlate p18 concentration with the number of blasts present in the circulation.**

**Response:**
We fully agree with the reviewer that a true quantitative method is required before YB-1/p18 could be useful in clinical scenarios. The quantitation of the p18 band was performed in order to avoid biased interpretations of the bands by the researchers (although he was blinded to the sample when analysing the results). We are currently working on establishing a ELISA method, which would then allow to accurately also determine the lowest limit of detection. At the moment, however, these results are too preliminary to include them into the manuscript, because we are afraid that the antibody employed in our ELISA approach has considerable cross-reactivity with (yet unknown) other protein fragments (of different size). This is now incorporated into the revised discussion part of the manuscript.

With respect to the other suggestion of the reviewer, we have tried to correlate the p18 concentration with the presence and number of blasts. However, this analysis was inconclusive due to the relatively small subgroups. For instance, in the AML subgroup, we had two patients with extremely high numbers of circulating blasts (total WBC of 155 and 75 G/L). Both were positive for YB-1/p18, but the intensity of the p18 band did not remarkably differ from the positive cases with lower leukocyte or blast counts. We believe that such analyses will be way more conclusive once a quantitative ELISA method is available, and should then be repeated in a larger cohort of patients with haematological malignancies. This consideration of the reviewer has now been also incorporated into the revised manuscript (discussion section).

**Reviewer: Kimitoshi Kohno**

This is an interesting investigation of Y-box protein-1/p18 fragment in plasma in patients with malignancies. The manuscript is easy to understand for reader. It is good point. However, I believe the manuscript could be improved by addressing several issues described below:

**Major comments**

1. It did not provide a story or strong rationale for the current study. I think more effort needs to be made to provide a rationale about the importance and novelty of this study in introduction.

**Response:**
We truly appreciate this helpful comment by the reviewer. The main hypothesis when starting our study was that YB-1/p18 detection might represent novel, yet unrecognized characteristic
of patients with malignancies. We have now included the hypothesis as well as the rationale of the study into the revised introduction.

2. Part of introduction is too long and has no mention of the main issue.

Response:
We have significantly shortened the introduction part, especially the parts which were maybe not closely linked to the main issue of the manuscript. For instance, we now erased the detailed descriptions on associations between YB-1 expression and outcome in different cancers and now only summarize main findings from these prior studies.

3. What is YB-1/p18 fragment? Which part in the full length of YB-1?

Response:
The reviewer addresses a very important point, which we have been working on for a long time. In order to investigate the identity of the 18-kD-fragment detectable in human plasma, we have performed ample immunoprecipitation experiments with the monoclonal as well as the polyclonal antibodies raised against YB-1 epitopes. None of these experiments was successful in elucidating the exact fragment composition. Moreover, serum protein precipitation with ammonium sulphate and enrichment of YB-1/p18 by liquid chromatography also failed, most likely due to multimerization of the enriched protein fragment.

We thus used a different approach to further elucidate this unexpected banding pattern of YB-1. Recombinant affinity purified full-length YB-1 protein was subjected to similar gel electrophoresis and coomassie blue staining. Without further manipulation and under neutral pH (7.4), there were two dominant bands at about 50 and 25 kDa, alongside several other bands including one at 66 kDa and the p18 band. To ascertain that these bands of interest matching the signals from human plasma are all derivatives of the His-tagged recombinant protein, MS/MS analysis was performed on excised bands. Interestingly, also the band running at 66 kDa only contains YB-1 protein, thereby likely reflecting dimers of different YB-1 fragments. The band at 18 kDa was identified as truncated cold-shock domain with peptides corresponding to aa81-137. This is in full agreement with the findings obtained from plasma samples using the polyclonal and monoclonal YB-1 antibodies. Given the use of an expression plasmid with non-existing alternative splice sites and the expression in bacteria, it appears likely that YB-1/p18 is generated as an (auto-)proteolytic fragment.

The detailed results of these experiments and the experimental details have been previously described by our group (Tacke F, et al. BMC Cancer 2011). However, we now included the essential information in the revised manuscript (introduction section).

4. Which part of epitope for their newly established monoclonal YB-1 antibody?

Response:
Our laboratory is currently conducting a larger series of experiments to fully understand the epitope of the newly established monoclonal YB-1 antibody, but also to test and develop new antibodies for the YB-1/p18 fragment, which might be suitable for ELISA techniques as well. To this end, the antibody detected different fragments tested by binding assays, which narrow down the epitope to aa 21-262. We will further continue to elaborate on this question, but we feel that this would be beyond the scope of our manuscript and merit a separate, more methodological publication.

5. What is the function for YB-1/p18 fragment?

Response:
We truly appreciate this very important comment by the reviewer. In fact, it is currently unclear, whether circulating YB-1/p18 fragments are functionally active in patients with malignancies. Inside tumor cells, YB-1 has been shown to fulfill critical cellular functions, such as the transcriptional upregulation of proliferation-associated and downregulation of apoptosis-related genes or induction of drug-transporter genes (like MDR-1) involved in chemoresistance. Data from our own laboratories indicated that also extracellular YB-1 may be involved in tumor progression, since adding recombinant YB-1 protein to cancer cell-lines in vitro revealed profound pro-mitogenic effects suggesting that secreted YB-1 or its fragments could act as a tumor growth-promoting factor (Frye BC, et al. EMBO Rep 2009). These considerations have now been included in the revised manuscript.

6. Where is the localization of YB-1/p18 fragment?

Response: In this study, we have analysed YB-1/p18 fragment in plasma samples, thus we cannot provide information of intracellular localisations of this fragment. However, it is tempting to speculate that the localisation of the p18 fragment outside the cell might be important for its function. For instance, if released primarily from malignant cells, secreted p18 fragment could indeed be localized in peritumoral stroma or at higher concentrations in the extracellular matrix of the tumor. Further analyses are needed to define such possible additional localizations of the YB-1/p18 fragment. In fact, we have detected YB-1/p18 fragments in cell culture medium of transformed cells. This is now included and discussed in the revised manuscript.

7. Aim of authors is clinical use. If so, they should discuss the action assignment and interpretation such as the difference of local existence of YB-1/p18 fragment and measurement means with citation use (Yoshimatsu, et al, Anticancer Res. 2005, Shibahara et al, Clin Cancer Res. 2001, Hyogotani et al, Clin Lung Cancer. 2012)

Response: We sincerely thank the reviewer for this expert comment. We now included that nuclear expression of YB-1 in tumor samples has been associated with disease progression and prognosis in patients with non-small cell lung cancer and have included the suggested three important references into our revised manuscript.

8. How authors get the obtaining written informed consent from healthy blood donors?

Response: We apologize if this was unclear to the reviewer. In our institution, healthy persons that voluntarily donate blood at our transfusion medicine department sign a written informed consent, which allows using their serum samples for further research analyses.

9. What would the investigators propose as the next step in evaluating this marker for clinical usefulness? Please discuss the clinical impact.

Response: As outlined in the responses to the other reviewer, we fully agree with you that further studies and methodological advances are needed before this new marker can be implemented into clinical algorithms. One important prerequisite, to our opinion, is the development of a specific and quantitative ELISA method. This is now incorporated into the revised discussion section.
Minor comments

1. How authors select randomly the patients in this study? Consecutive patients?
   
   Response: Thank you for this remark. Indeed, the patients were consecutively recruited from the outpatient cancer clinic at our institution. This is now clarified in the revised manuscript.

2. Below sentence is too difficult to understand for readers. (For instance, in patients with lung cancers b2-microglobulin ……depending on the underlying histology (Fig.4).) To begin with, b2-microglobulin was not the tumor markers tested most frequently.
   
   Response: We thank the reviewer for this remark. We have shortened the sentence and deleted the statement about b2-microglobulin. We hope that this part of the results is now easier to understand for the readers.

3. Author should change the Figure 5 from Figure to graph including the CT graphical content to understand for readers.
   
   Response: We understand that the referee feels a presentation of the YB-1/p18 band intensity as a graph would fit better to the presentation of the other tumor markers. However, the other referee had legitimate concerns about the semi-quantitative nature of the immunoblotting method. Thus, we feel that it might be misleading to present the OD values as “absolute” numbers, as it might be misinterpreted as a fully quantitative assessment of YB-1/p18 fragments. We therefore prefer to show the original data (original band from immunoblotting) in order to avoid misinterpretations at this point.

4. Author should discuss the prognostic impact with the object of organ specific.
   
   Response: We thank the reviewer for this suggestion, which is similar to comment #3 from the other referee. As outlined above, we have attempted to address the prognostic impact of YB-1/p18 fragments in the different subgroups (e.g., AML, see above). However, this analysis is limited by the rather small patient numbers in the subgroups / organ-specific cancer groups as well as by the semi-quantitative nature of the (technically demanding) immunoblotting methodology. We believe that such subgroup analyses will be way more successful once a quantitative and specific ELISA method (which we are working on at the moment) is available. We have incorporated the reviewer’s comment and our response into the revised manuscript (discussion section).