Author's response to reviews

Title: High SIRT1 expression is a negative prognosticator in pancreatic ductal adenocarcinomas

Authors:

Albrecht Stenzinger (albrecht.stenzinger@med.uni-heidelberg.de)
Volker Endris (volker.endris@med.uni-heidelberg.de)
Frederick Klausch (frederick.klauschen@charite.de)
Bruno Sinn (bruno.sinn@charite.de)
Katja Lorenz (katja.lorenz@med.uni-heidelberg.de)
Arne Warth (arne.warth@med.uni-heidelberg.de)
Benjamin Goeppert (benjamin.goeppert@med.uni-heidelberg.de)
Volker Ehemann (volker.ehemann@med.uni-heidelberg.de)
Alexander Muckenhuber (alexander.muckenhuber@med.uni-heidelberg.de)
Carsten Kamphues (carsten.kamphues@charite.de)
Marcus Bahra (marcus.bahra@charite.de)
Peter Neuhaus (peter.neuhaus@charite.de)
Wilko Weichert (wilko.weichert@med.uni-heidelberg.de)

Version: 2 Date: 26 July 2013

Author's response to reviews: see over
Point-by-point reply to the reviewers’ comments

Ms. No. 4523884429784685 (first submission)

Remarks to the comments of the reviewers
We thank the reviewers for their helpful comments, and we have taken their suggestions into account in our revision of the manuscript. We have reproduced each comment along with our responses and referenced the changes in the manuscript with the corresponding page of the revised manuscript. We highlighted all changes in the revised version of our manuscript in yellow colour.

Comments to the reviewers’ points

Reviewer Ilse Rooman

**The reviewer wrote:**
In this manuscript, Sirt1 expression is studied in a cohort of 129 patients with resectable PDAC. Sirt1 was found to be an independent prognostic factor with high Sirt1 expression in patients with lower survival. Functional evidence from cell lines showed that Sirt1 overexpression in pancreatic tumour cells provided higher cell replication and lower apoptosis. A Sirt1 inhibitory drug (Cambinol) alone was effective in inhibiting tumour cells, and was more potent in combination with an EGFR inhibitor (Gefitinib).

**Major compulsory revisions**
1) The major criticism to this study is the lack of novelty. Sirt1 expression analysis in PDAC sample cohorts have been reported.

**Our reply**
As indicated by the title of the manuscript, our study mainly focuses on the expression levels of Sirt1 in 129 PDAC samples with respect to pathological and clinical parameters, and specifically with respect to patient outcome. While we agree with the reviewer that there is a single study, by Zhao et al. who reported on the association of Sirt1 expression levels in 49 PDAC specimen with different pathological variables (cited and discussed in our manuscript), we would like to emphasize that to our knowledge there are no reports that address the role of Sirt1 as a prognosticator and in this regard the tissue part of our study in our view clearly incorporates novel data with clinical implications, which have not been published before and which support mechanistic work of other groups working in this field.
Furthermore our cohort is more than twice the size of the cohort reported by Zhao and colleagues.

In the other studies by Zhao et al and Wauters et al. that the authors here refer to, the proportion of tumour samples expressing high Sirt1 is much higher. This may be explained by usage of different antibodies. Therefore, it is crucial to validate the antibody used here and possibly to compare with the antibodies used in the other studies. Are more samples positive when the Epitomics antibody is more concentrated? Please include validation by Western Blot analysis and in Sirt1 overexpression tissues/cells and Sirt1 KO's. Other studies (eg Wauters et al) have also reported on cytoplasmic Sirt1 in pancreatic tumours – is this never seen here with the Epitomics antibody?

Our reply and changes:
We thank the reviewer for this valuable advice. We determined the specificity of our antibody by siRNA-mediated knock down of the Sirt1 encoding mRNA including mock controls in cell lines. We added this information in the Material and Methods (lines 181-187) and Results section (lines 271-274) of the manuscript and included a Figure (new figure 2) to corroborate these data. The concentration of the antibody used for immunohistochemistry in our cohort has been determined prior to the study. Higher concentrations of the antibody led to unspecific background staining which we did not observe with the concentration applied in our setting.

There is a study by Tanno et al. (2007) that reports on the principal capability of Sirt1 to shuttle between the nuclear and cytoplasmic compartment in mouse cells and another study by Hisahara et al. (2008) who observed this phenomenon in mouse neural precursor cells. Wauters et al. (2013) recently provided evidence that there is nuclear to cytoplasmic shuttling of Sirt1 in rat and mouse acinar cells with potential tumorigenic implications in the acinar to ductal metaplasia carcinogenesis model of PDAC. They also reported on cytoplasmic localization of Sirt1 in human exocrine cells. However, investigating human tissue samples of fully developed pancreatic ductal adenocarcinoma, we only detected nuclear Sirt1 with the Epitomics antibody. Since i) many target proteins of Sirt1 are localized within the nuclear compartment of the cell and ii) the specific role of Sirt1 within the cytoplasm is not comprehensively explored yet and iii) there are other models of PDAC carcinogenesis, which may contribute to PDAC origin and development, we believe that our observations with an antibody, whose specificity has been corroborated by siRNA-mediated knock-down, is of interest and contributes further to the understanding of Sirt1 in PDAC. It may also be that cytoplasmic Sirt1 levels in comparison to nuclear expression levels were too low to be detected by our antibody. Another explanation might be that cytoplasmic Sirt1 plays a major role in the development of carcinogenic precursors and nuclear Sirt1 has its place in the fully developed cancer. This is being discussed in the revised version of our manuscript (lines 418-430)

2) To give an idea on the manipulation of Sirt1 expression in MiaPaCa-2 cells and Panc-1 cells, it is crucial to see the endogenous and overexpression levels of Sirt1 quantified.

Our reply and changes:
We thank the reviewer for this valuable advice and performed quantification of endogenous and overexpressed Sirt1 in both cell lines. We added this information accordingly in the Material and Methods (lines 181-187) as well as in the Results section (new figure 3, lines 302-306).

3) Effects of inhibiting Sirt1 by RNAi or drugs have been reported before and similar observations were recorded with more detailed analysis than what is provided in this new manuscript. The new observations in the present study come from the combination therapy with the EGFR inhibitor, but observations are limited and there is no mechanistic exploration as to how this synergy may take place.

**Our reply and changes:**
We agree with the reviewer that a more in depth analysis of the functional mechanisms underlying the effects of combinatory treatment reported by us is desirable. However, our study, as we already pointed out, is designed as a translational study with an emphasis on the tissue part. According to the demands of both reviewers' we already extended the part dealing with cell culture experiments significantly in the revised version of our manuscript (see other comments and replies), however, a meaningful in depth mechanistic exploration of the reasons for the effectiveness of combination therapy by far exceeds the initial scope of our study and is unfortunately not doable in the time frame given for this revision. This should be done in follow up studies with a functional but not a translational focus.

**Minor essential revisions**
Legend to Y-axis in graphs in Figure 2 is likely wrong. It reads that 0.3-0.5% of EGFP cells are viable.

**Our reply and changes:**
We thank the reviewer for this helpful advice and corrected our mistake accordingly.

**Discretionary revisions**
The data also suggest that Sirt1 protein expression is inhibited by Cambinol exposure, an interesting observation to further explore and to provide novelty to this manuscript.

**Our reply and changes:**
We thank the reviewer for this comment. Indeed, we are working on the mechanism and impact of Cambinol-mediated changes in Sirt1 expression. From our current experiments we can derive evidence that this effect is not mediated on a transcriptional level but occurs via protein degradation. However, since the biochemical work on this topic is enormous and very preliminary and the focus of our present study is on the role of Sirt1 as prognosticator, we aim at publishing these data in a second paper.
Reviewer Younh-Hwa Chung

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related death in the United States. Since the overall 5-year survival rate is approximately 5%, a search for prognosticator of PDAC is required for extending a patient's life. In this study, the authors paid an attention to SIRT1, a class III histone deacetylase.

**Major comments**

1. Based on the clinical results on PDAC samples, they concluded that strong SIRT1 expression is a significant predictor of poor survival but did not see a positive correlation of SIRT1 expression with tumor progression. It is hard to understand their result and conclusion. SIRT1 is an independent prognosticator in PDACs and plays an important role in pancreatic cancer cell growth. Please discuss this matter; Role of SIRT1 between clinical PDAC samples and pancreatic cancer cell lines.

Our reply and changes:

We thank the reviewer for the valuable comment. Our data indeed indicate, that Sirt1 expression is prognostically deleterious regardless of the initial tumor stage of the patient, this is also shown by its prognostic independency in multivariate survival analysis. That means, that a Sirt1 positive tumor regardless of the initial size and status of metastasis at the time of diagnosis shows a much higher rate of recurrence postoperatively and carries a much higher rate of patient death, presumably because these tumors have already seeded multiple clinically at the time of resection undetectable micrometastases. Such a theory would also be in line with our findings of an association of Sirt1 expression and tumor dedifferentiation (indicated by a higher tumor grade), since it is well known that dedifferentiation and the associated phenomenon of epithelial-to-mesenchymal-transition play an essential role in the development of early local and distant tumor spread. This is discussed in the revised version of our manuscript (lines 393-402).

2. They showed that over-expression of SIRT1 enhances proliferation of Panc1 or Miapaca cells. When they would like to emphasize a positive role of SIRT1 in pancreatic cancer cell growth, they should show experiments that suppression of SIRT1 expression with siRNA or inhibition of SIRT1 with Nicotinamide can decrease pancreatic cancer cell growth.

Our reply and changes:

We thank the reviewer for this helpful comment and performed experiments with Nicotinamide in cells with endogenous and overexpressed Sirt1 protein levels for both cell lines. We could show that increasing concentrations of Nicotinamide, in analogy to Cambinol, suppresses cell growth in both pancreatic cancer cell lines. In addition, again in analogy to Cambinol, we observed synergistic effects of Nicotinamide with Gefitinib. Furthermore, treatment with Nicotinamide was able to revert the increase in proliferative activity induced by forced Sirt1 overexpression. This set of new data has been included in the Results section (figures 5 and 6, lines 311-325).

3. Although they showed cellular effects of cambinol, gemcitabine and genfitinib on Panc1 or Miapaca cells, growth inhibition of Panc1 or Miapaca cells by treatment with
those drugs should be connected to a low expression of SIRT1 protein in order to support their hypothesis. They should show a relationship of growth inhibition with SIRT1 levels at different concentrations of each drug. Although they showed that treatment with cambinol at high conc. of 100 or 200 mM reduces SIRT1 protein levels in supplementary fig.3, they should show this result in a regular figure.

We thank the reviewer for this comment. We changed the supplementary figure 3 to a regular figure (now figure 9) and changed the consecutive numbering of the figures accordingly. Indeed, we believe that presence and activity of Sirt1 is connected with the activity of the respective drugs/drug combinations in pancreatic cancer. This hypothesis is underlined by the fact that Cambinol, which not only inhibits Sirt1 but also induces degradation of this enzyme (see figure 9) acts synergistically with Gefitinib. In the revised version of our manuscript we could also show that the pro-proliferative effects induced by forced overexpression of Sirt1 could be blocked by treatment with the Sirt1 inhibitor Nicotinamide, which also corroborates this assumption. Since both cell lines used in our study (as well as a variety of other pancreatic cancer cell lines tested by us, data not shown) expressed Sirt1 at moderate or high levels, we were not able to compare the effectivity of our drug combinations in cell lines with differences in endogenous Sirt1 levels.

Minor comments
1. In figure legend (Table 1,2,3 and Figure 1), we found a different letter style compared to other figure legends (Figure 2, 3, 4).

Our reply and changes:
We thank the reviewer for this advice and changed the letter style of the figure legends accordingly.

2. Please correct figure 1 labeling as a uniform style.

Our reply and changes:
We followed the reviewer’s suggestion and changed the letter style of figure 1 accordingly.

3. In 18 line 10 and line 14, they should put two sentences apart.

Our reply and changes:
We changed the structure of the sentences accordingly.

Quality of written English: Needs some language corrections before being published.

Our reply and changes:
The manuscript has been reviewed by a native speaker and errors have been corrected accordingly.