Author's response to reviews

Title: Prognostic impact of Proline-, glutamic acid- and leucine-rich protein 1 tumor expression in ovarian cancer

Authors:

stefanie aust (stefanie.aust@meduniwien.ac.at)
dietmar pils (dietmar.pils@meduniwien.ac.at)
sophie pils (sophie.pils@meduniwien.ac.at)
christoph grimm (christoph.grimm@meduniwien.ac.at)
reinhard horvat (reinhard.horvat@meduniwien.ac.at)
dan cacsire castillo tong (dan.tong@meduniwien.ac.at)
bernd schmid (bernd.schmid@wienkav.at)
paul speiser (paul.speiser@meduniwien.ac.at)
alexander reinthaller (alexander.reinthaller@meduniwien.ac.at)
stephan polterauer (stephan.polterauer@meduniwien.ac.at)

Version: 2 Date: 23 December 2012

Author's response to reviews: see over
Answers to the reviewer Ratna Vadlamudi

Reviewer's report:
The goal of this study is to evaluate the prognostic significance of PELP1 and ERbeta in epithelial ovarian cancer (EOC) patients receiving platinum/taxane-based chemotherapy using 60 patient samples. Authors detected co-expression of PELP1 and ERbeta nuclear expression in ~70% of the tumors analyzed. They concluded that ERbeta coregulator PELP1 alone and in combination with ERbeta is a positive prognostic factor for DFS and OS in EOC. Ovarian cancer is deadly cancer and prognostic markers are urgently needed. Eventhough, the presented findings are interesting, there are some aspects of this work that requires clarification, the findings as presented seems to be preliminary and additional experimental evidence needs to be provided or else the conclusion and title of the manuscript need to be modified. My suggestions / comments are given below.

1. The main conclusion of the paper as written in the title manuscript is not convincingly supported by the experimental data. Much of conclusion that PELP1 alone is a positive prognostic factor is not supported by the data and is also against the published literature that predicts PELP1 as a proto-oncogene. Further in this study, only PELP1 nuclear expression is evaluated and PELP1 cytoplasmic localization is ignored. The antibody used for IHC only recognizes nuclear PELP1.

As noticed by the reviewer, we have used an antibody only recognizing nuclear PELP1. Determining the prognostic impact of PELP1 in colorectal cancer, Grivas at all have likewise used an antibody only staining nuclear PELP1. We have added this reverence in the discussion section.

As the study population we have used for our first version of the manuscript was too small to provide profound results, we have included more EOC patients in our analysis, with a patient number of 86 EOC patients. As a histotype specific analysis was required by one of the reviewers only patients with serous histology were included in the validation set. We have rewritten the manuscript, showing a prognostic significance for the combined expression of ERbeta and PELP1 in the test set. Interestingly the combined expression of ERbeta and PELP1 expression did not significantly influence patients’ survival in this validation set of only serous histology. As little is known about the role of PELP1 expression in human EOC we believe that the data of both, the test set and the histotype specific validation set are important and should be presented. We have extended the discussion on the role of PELP1 in breast and ovarian cancer.

As recommended by the reviewer we have rewritten the conclusion and title of the manuscript. We have also rewritten the Discussion section of the revised version of the manuscript.

2. Most significant results seem to be with ERbeta and data presented for combined expression of the ERbeta+PELP1 convincing. This may fit to the emerging finding of tumor suppressor functions for ERbeta and PELP1 may
probably enhancing ER beta tumor suppressor functions as a coactivator. In that scenario, authors should consider including ERbeta-PELP1 axis as prognostic factor in the title of the paper rather than PELP1 alone.

Thank you for this comment; as we have included the new data of the validation analysis in the manuscript, we have adapted the title and the discussion section of the revised version of the manuscript. The title has been changed to: The prognostic value of estrogen receptor beta and Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) expression in ovarian cancer

3. Sample numbers are too small (n=60) considering PELP1 staining is observed only 70% tumors and the PELP1 +ve tumors belongs to four distinct ovarian types. Conclusion of prognostic significance by combining four subtypes with small number of tumors may be premature.

We thank the reviewer for this important remark that has helped us to provide a manuscript of higher quality and more profound statistic results. As mentioned above we have now included a validation analysis comprising 86 EOC patients of only serous histology to validate the survival data in a histotype specific setting.

4. Half of the samples seem to be obtained from patients treated with chemotherapy. The data should also be segregated / analyzed based on therapy and this further reduces the sample number for prognostic analysis.

Only patients receiving chemotherapy were included in the survival analysis of the study (n=50). It is true, that our inclusion criteria were a limitation of our first analysis. Finally, we provide a larger and well-described validation set of only serous EOC patients that have all been treated with chemotherapy according to therapeutic standards. In the revised version survival analysis includes only patients receiving chemotherapy in a) the test set (n=50, Table 2A) and b) the validation set (n=86, Table 2B).

5. No IHC data on ERbeta is included. ER beta antibodies are controversial and several ERbeta isoforms are identified. In the absence of evidence for the specificity of the ER beta antibody used in this study and ERbeta isoform information, the conclusions of ERbeta as a prognostic marker may be premature.

We have included the ERbeta IHC data: “ERbeta1, 1:20, clone PPG5/10, mouse IgG2a, Dako, Denmark”

6. The positive and negative controls used for PELP1 and ERbeta used should included in the methods section.

As positive controls, FFPE sections of ER positive human breast adenocarcinoma were used. Negative control mouse and rabbit isotypes were used as negative controls.
Answers to the reviewer Charlie Gourley
Reviewer's report:

Major Compulsory Revisions
1) The main issue in this study is its size (63 pts is too few with only 50 included in the univariate and multivariate analysis) and the lack of an analysis in a histotype-specific fashion. I accept that multivariate analysis has been performed but this is limited by subdividing histology only into serous and non-serous. It is clear that if studies seeking prognostic biomarkers in ovarian cancer do not adequately account for histotype then the majority of associations detected are erroneous (Kobel et al PLoS Medicine, 2008). In order to validate this data, a histotype-specific analysis in a larger tumour set is required. It is important for the reader to know whether their is an association between PELP1 and/or ERbeta expression and DFS/OS within the context of a specific ovarian cancer histotype. Analysis without such subdivision is effectively meaningless.

Thank you for this important remark. As our study population was small and included only 28 patients of serous histology, we agree that it was necessary to provide an overview over a broader spectrum of histological subtypes.

We thank the reviewer for the remark that the association between PELP1 and ERbeta expression and DFS/OS should also be investigated within the context of a specific ovarian cancer histotype. We believe that serous ovarian cancer is the most relevant subtype regarding studies seeking prognostic biomarkers in ovarian cancer. Thus we have included a validation set in the revised version of the manuscript with a total of 86 EOC patients of only serous histology.

Unfortunately we could not reproduce our findings regarding the protective effect of the coexpression of ERbeta and PELP1. Still, we believe that both, the results from the test and validation set are of relevance as little is known about the role of PELP1 in EOC. We have embedded the validation data in the manuscript and we have adapted the discussion. We believe this has enhanced the quality of the data and we hope to provide more profound data in this revised version of the manuscript.

Minor Essential Revisions
1) Describe acronyms at first mention in the text (NRs; Introduction, line 50).

In the revised version of the manuscript all acronyms have been described at first mention in the text.

2) Please clarify what 'at least 2' means in Material and Methods, lines 101 to 102.
The sentence has been clarified: “Recurrent disease was defined as an at least a twofold increase in the nadir serum CA-125 level”

3) Results, line 154: Please clarify how response was assessed. It is not clear how 73% of patients were deemed to have responded to initial chemotherapy when 30% of the patients had stage 1 disease so should not have been assessable for response.

We have rewritten the whole paragraph to present the results more clearly. Only patients receiving chemotherapy were included in the survival analysis (n=50). We have added a validation set of 86 serous EOC patients to validate the survival results, whereby only patients receiving chemotherapy according to therapeutic standards were included.

4) Please confirm that every patient who progressed also died during the follow-up period (Results, lines 155-156). This would be extremely unusual in an ovarian cancer cohort, even of this limited size, to have no survivors living with their disease. In addition, the Kaplan-Meier curves suggest this is not the case.

Unfortunately this paragraph was misleading; therefore the whole paragraph has been rewritten in the revised version. We now clarified that not all patients died during the observation period.

Discretionary Revisions
1) Add 'apart from FIGO stage' after 'had the strongest impact on survival' in line 196 and p250.

We have added the suggested comment: “Besides the clinicopathological factors FIGO stage and residual tumor load after cytoreductive surgery, this coexpression pattern (ERbeta+/PELP1+) turned out to be the most relevant prognostic factor in univariate and multivariate survival analysis, revealing a significantly longer DFS (HR 0.3 [0.1-0.7], p = 0.004) and OS (HR 0.3[0.1-0.7], p = 0.005).”
Answers to the reviewer Ansgar Brüning

Reviewer's report:
The authors have analyzed the expression of PELP1 and estrogen receptors in 63 tissue samples of ovarian cancer patients and came to the conclusion that coexpression of PELP1 and ERbeta is associated with a better prognosis for ovarian cancer patients.

Data analysis and presentation:
The authors show Kaplan-Meier curves of their “positive” results only. They should also show survival data of their other calculations and factors tested, at least for comparative reasons.

As requested by the reviewer we present the survival data of the other calculations below in the Supplementary Figure S1. The Kaplan-Meier estimates of ERalpha, ERbeta and PELP1 are presented to provide a better overview. If wished by the reviewer or the editor we can redesign figure 2 in the manuscript and include the additional Kaplan-Meier curves.

The quality and resolution of the IHC shown in Fig. 1 is impressive. Is appears to be that the authors achieved a strong nucleolar staining in Fig. 1A but homogeneous nuclear staining in Fig. 1B (do Figs 1A and 1B really represent the same magnification?).

In Fig.1 A-D we provide pictures of four different TMA cores, in 1A and 1D the nucleoli can be seen in this examples of low differentiated EOC tissues. We have provided pictures including the borders of the singles cores. This might help to compare the size of the pictures and to understand, that the same magnification has been used.

The Vadlamudi group recently described nucleolar localization of PELP1 (Gonugunta VK, Nair BC, Rajhans R, Sareddy GR, Nair SS, Vadlamudi RK. Regulation of rDNA transcription by proto-oncogene PELP1. PLoS One. 2011;6(6):e21095). Since intranuclear distribution of PELP1 has been reported to be cell cycle-dependent [Vadlamudi group], it might be worthwhile to perform a subgroup analysis of nucleoplasmic vs. nucleolar PELP1-expressing tissue samples in relation to patients’ survival, because higher proliferation rates (Ki67!) are known to be associated with poor prognosis. Did the authors observe any cytoplasmic staining that might be involved in the non-genomic interaction of PELP1 with ERs? This could lead to a new sub-group to be differentially analyzed. Breast cancer cells for example have been shown to express a high percentage of cytoplasmic PELP1 [Kumar group].

Thank you very much for this interesting comment. Vadlamudi et al. speculated that PELP1modulates rDNA transcription and accelerates cell cycle progression. Thus we decided to additionally perform immunofluorescence to analyze nucleolar expression of PELP1 in ovarian cancer tumor tissue. We observed intensive PELP1 staining within
the nucleoli of the tumor cells. We included a new figure in the revised version of the manuscript showing nuclear and especially intra-nuclear expression of PELP1. As we have used an antibody detecting only nuclear PELP1 (polyclonal rabbit, No. IHC-00013, Bethyl Laboratories, USA) we did not observe any cytoplasmic staining.

It could further be of interest to have a closer look at the intranuclear PELP distribution by performing immunofluorescence on cultured ovarian cancer cell lines. Because the authors stress on the relevance of PELP1/ERbeta co-expression, a co-IF of PELP1 and ERbeta at various steps of the cell cycle may lead to further insights.

Thank you for this inspiring comment. As described above, we have included an immunofluorescence staining in the revised version of the manuscript to determine intra-nuclear expression of PELP1. Unfortunately we are not able to perform co-expression analysis using ovarian cancer cell lines. We have included the following sentence in the discussion section: “Still, further studies are required to determine PELP1 expression during cell-cycle progression in EOC”.

What is the expression level of PELP1 in non-malignant human ovarian surface epithelial (HOSE) cells, from which most of the epithelial ovarian cancer cells arise?

Unfortunately we were not able to determine PELP1 expression in non-malignant human ovarian surface epithelial. This might be an interesting approach for further immunohistochemical and immunofluorescence analyses.

Discussion:
Expression of PELP1 in ovarian cancer has previously been studied [Dimple C, Nair SS, Rajhans R, Pitcheswara PR, Liu J, Balasenthil S, Le XF, Burow ME, Auersperg N, Tekmal RR, Broaddus RR, Vadlamudi RK. Role of PELP1/MNAR signaling in ovarian tumorigenesis. Cancer Res. 2008 Jun 15;68(12):4902-9 and Chakravarty D, Roy SS, Babu CR, Dandamudi R, Curiel TJ, Vivas-Mejia P, Lopez-Berestein G, Sood AK, Vadlamudi RK. Therapeutic targeting of PELP1 prevents ovarian cancer growth and metastasis. Clin Cancer Res. 2011 Apr 15;17(8):2250-9.]. These important contributions have been cited [ref.12 and13], but not really been discussed by the authors, in particular since they report contradictory results. PELP1 has been described as a “proto-oncogene”. The authors themselves mention this in their abstract and introduction. Accordingly, but in contrast to the data presented by the authors, PELP1 expression should be associated with poor prognosis. Therefore, there are still many open questions left, which should at least be discussed. Interestingly, Grivas et al. [Grivas PD, Tzelepi V, Sotiropoulou-Bonikou G, Kefalopoulou Z, Papavassiliou AG, Kalofonos H. Expression of ERAlpha, ERbeta and co-regulator PELP1/MNAR in colorectal cancer: prognostic significance and clinicopathologic correlations. Cell Oncol. 2009;31(3):235-47] recently published
data on PELP1 in colorectal cancer which are similar to those obtained by the authors of the present study. Therefore, based on the similarity and –in part supportsive nature of the data, this reference could be included and discussed.

We thankfully included and discussed the mentioned publication by Grivas et al. in the Discussion section of the revised manuscript. As required by the reviewers, we have validated the survival data of our patient cohort (n=63) in a validation set. The validation was performed in an independent and more homogenous set of 86 EOC patients with only serous histology. Unfortunately we could not reproduce our findings regarding the protective effect of the coexpression of ERbeta and PELP1. Still, we believe that both, the results from the test and validation set are of relevance as little is known about the role of PELP1 in EOC. Therefore we have included the data of the validation analysis in the revised version of the manuscript. We have extended the discussion on the role of PELP in breast and ovarian cancer as recommended by the reviewer.

Minor points:
Title: If possible, the authors should mention PELP1 in the title in brackets after “proline-…….”, or use PELP1 directly in its abbreviated form. Then, they will probably notice that the word “tumor” might be superfluous.
Some language mistakes should be corrected, for example:
“...accounts for more death women..”
British or American English should be used consistently and some more hyphens, if appropriate, could be used.

Thank you very much for these precise suggestions. We have changed the title to: “The prognostic value of estrogen receptor beta and Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) expression in ovarian cancer”. We have corrected the language mistakes and we have adapted some phrases and paragraphs.