Author’s response to reviews

Title: Elevated expression of prostate cancer-associated genes is linked to down-regulation of microRNAs

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Author’s response to reviews: see over
Dear Dr. Edwards,

Please find enclosed our revised manuscript “Elevated expression of prostate cancer-associated genes is linked to down-regulation of microRNAs” by Kati Erdmann et al. which we would like to resubmit for publication as an original research article to “BMC Cancer”. Please note that the changes in the revised manuscript are highlighted in yellow.

We would like to thank the editor as well as the reviewers for their effort and for considering our manuscript for publication in “BMC Cancer”. We are happy to answer all of the reviewers’ questions. Below you can find the reviewers’ comments with our response.

We hope we could address all issues in a sufficient manner and look forward to hearing from you at your earliest convenience.

Sincerely yours,

Kati Erdmann

*Corresponding author on behalf of all the authors*
Reviewer 1
This manuscript by Erdmann et al characterises expression of miRNAs in prostate cancer, firstly undertaking in silico analyses, and performing validation in a new cohort of patients, and making clinico-pathological correlates. Finally, some preliminary functional data is presented. The manuscript presents predominantly correlative data, but is of general interest to the prostate cancer field, specifically to those with an interest in miRNAs and gene expression profiling. The authors should be commended on producing a manuscript with exemplary presentation and technical english, which is certainly worthy of publication in this journal. I have a few comments which would strengthen the manuscript prior to publication.

Major Compulsory Revisions
1. The authors should give details of how samples were processed i.e. How were tumour rich areas were harvested for analysis? Was macrodissection or microdissection performed?

*We did not perform macrodissection or microdissection. Following removal of the prostate gland, the tissue was roughly cut into regions according to its appearance being normal or tumor suspicious. All tissues were then cryo-preserved in liquid nitrogen. For further analyses, cryosections of available tissues were prepared for RNA isolation as well as serial sections (start, middle, end). The serial sections were then used for diagnostic hematoxylin-eosin stainings to ascertain the tumor cell amount, which was estimated by an experienced pathologist. For further analyses, only tissues with a tumor cell amount of ≥50% for malignant samples or of 0% for tumor-free and BPH samples were used. Based on the critique of the reviewer we elaborate more on the tissue processing in the methods section.*

2. Authors should state why using RNU48 was used for normalisation of miRNA expression. Some snoRNAs are mis-regulated in cancer - did they consider and/or use any other "housekeeping" RNAs? Clearly, mis-regulation of RNU48 could bias results.

*The selection of RNU48 as reference RNA was based on the following literature:*
Based on studies in human breast cancer and prostate cancer tissue, RNU48 emerged as one of the three most stable control genes in both types of tissue. Due to its biological stability, RNU48 is broadly used as a reference molecule for miRNA expression studies in prostate cancer such as Mavridis et al. (reference #43 in the manuscript). We now have included an appropriate note in the methods section of the manuscript on why RNU48 was used for normalization of microRNA expression. However, we did not use other housekeeping RNAs but this is a good suggestion for future analyses and we will keep this in mind.

3. Authors need to show data demonstrating vector-based over-expression of mir26a and also baseline levels of expression for prostate cancer cell lines. This is current "data not shown", but should be shown if only in supplementary data.

As suggested by the reviewer we now have included the baseline levels of miR-26a as well as the expression levels of this miRNA following treatment with the miR-26a mimic and the control construct, respectively, in the new Table 5. However, due to the extremely low baseline expression of miR-26a (mostly below the detection threshold) in the used prostate cancer cell lines it is not appropriate to calculate fold expressions. Therefore, only the relative transcript levels of miR-26a (normalized to RNU48) are presented.

4. Error bars for luciferase assays: Is this SEM or SD? This should be clearly stated in the figure legends.
Values are depicted as averages of six independent experiments with their mean deviation. This is stated in the figure legend.

Minor Essential Revisions
1. The discussion section is rather lengthy and could be made more succinct in terms of previous data and relevance of current data to prostate cancer field.
As suggested by the reviewer, we shortened the discussion section to make it more succinct.

Discretionary Revisions
1. The authors should consider making Table 4 and 5 into graphical format. At present, the tabulated form makes data interpretation quite difficult.
As suggested by the reviewer, Table 5 is now presented in graphical format (Figures 3 & 4). However, we do feel that the data of Table 4 are better presented in tabular format and thus, we kept the table.

Reviewer 2
The authors studied the expression of miRNAs in prostate cancer, which potentially target PCa relevant genes. This is an interesting approach, especially taking into account that regulation of the target genes in PCa has not been fully elucidated in prostate cancer so far.

Major Compulsory Revisions:
1) The authors studied a comparably small set of patient specimens but were nevertheless able to show that the selected miRNAs were downregulated in prostate cancer. They did not study, whether expression of the miRNA was associated with poor survival of the patients. Especially for miR-186 this would be interesting to see and should be included in the manuscript.

We understand the objection of the reviewer. Follow up data on the prostate cancer patients are available and we did perform survival studies. The median follow up time for our patient cohort was 104 months. However, only five patients died during the follow up. Of these five patients only one patient died due to prostate cancer.
Therefore, we did not consider the survival data as presentable for this manuscript. Besides, the main focus of this study was to evaluate the expression of selected microRNAs that could be responsible for the up-regulation of prostate cancer associated genes. Nevertheless, the reviewer is right that comprehensive survival studies with regards to miR-186 would be very interesting. This could be the focus of a future study on a bigger patient cohort with the main focus on survival outcome.

2) The authors used a miRNA mimic to knockdown AMACR expression. Downregulation of AMACR in Du-145 is rather small upon miR-26a transfection. Yet, statistics are missing in table 5 and figure 3 and should be included for all transfection experiments. Statistics should also be included in figure 4 (luciferase assay).

Statistics have now been included in the respective figures.

3) The authors suggest AMACR as a novel target for miR-26a in prostate cancer. Unfortunately, they do not proof whether this regulatory mechanism is of any functional significance for prostate carcinogeneis. They should show experimentally that miR-26 overexpression functionally phenocopies the knockdown of AMACR in prostate cancer cell lines, for example in regard of AMACRs regulation of androgen-independent growth. This experiments should be included in the manuscript.

First and foremost the present study focused on the identification and expression analysis of selected microRNAs that could be responsible for the up-regulation of prostate cancer associated genes. In this regard, we could show that the down-regulation of most miRNAs inversely correlated with an up-regulation of their putative target genes. However, to prove that we identified hitherto unknown miRNA-target gene associations we selected miR-26a for further analysis. In that regard we evaluated the effect of exogenously administered miR-26a on its putative target genes EZH2 and AMACR. EZH2 has already been verified as a direct target of miR-26a as discussed in the manuscript, but AMACR has not. By using the luciferase reporter assay AMACR was validated as new target for miR-26a. Of course, the reviewer is right that the functional significance of the regulatory mechanisms has to be investigated. The necessary experiments are being conducted right now. But we do feel that the results of these experiments should be summarized in a future, more functionally oriented manuscript.
4) miR-186 is the only miRNA that has been associated with organ-confined disease and metastatic growth in this study and it shows inverse correlation with AMACR and PSMA. This makes this miRNA an interesting molecule in prostate cancer and thus it would be nice to see in this manuscript, whether this miRNA is also able to directly bind to the AMACR and PSMA 3'-UTR and thus diminish their expression.

We first selected miR-26a for further analysis because this miRNA has already been investigated by various other groups. For that matter, the prostate cancer associated gene EZH2 has already been verified as a direct target of miR-26a (references #36 & #38 in the manuscript). Thus, this miRNA was a good starting point to launch further analyses and to compare the results with previously published results. However, we do evaluate some of the differently expressed miRNAs such as miR-186 right now. Within the time frame of the revision it was not possible to complete these experiments in sufficient manner. Furthermore, comprehensive data on the regulatory mechanism of miR-186 can be - as mentioned above - subject to a subsequent, more functionally oriented manuscript.

Reviewer 3

This is a manuscript with much potential, but which could do with some restructuring, simplification and revision to make it clearer. At present, it is a bit of a 'mish-mash'.

As suggested by the reviewer, we revised and shortened the manuscript (discussion section).

My main criticisms are:

Major

The manuscript really looks like two separate bodies of work. It could be strengthened by simplification of the screening work, assembled in to one report, while the reporter assay work could be a separate manuscript, but this would need all the candidate miRNAs similarly treated. It currently adds little to the manuscript, as it appears as an incomplete add on. I have sympathy with the authors and understand
why they did this. The work is sound and rational but an incomplete fit as it presently stands.

We do understand the concerns of the reviewer. First and foremost the present study focused on the identification and expression analysis of selected microRNAs that could be responsible for the up-regulation of prostate cancer associated genes. In this regard, we could show that the down-regulation of most miRNAs inversely correlated with an up-regulation of their putative target genes. However, to prove that we identified hitherto unknown miRNA-target gene associations we selected miR-26a for continuative analysis. We thus evaluated in an initial assessment the effect of exogenously administered miR-26a on the expression of selected target genes as well as the direct regulation of a previously unknown target gene. In doing so, AMACR was validated as new target for miR-26a. With these experiments we wanted to highlight that the evaluated miRNA-target gene associations (exemplary for miR-26a) could be of functional importance in regulating genes linked to prostate cancer pathogenesis.

Of course other interesting miRNAs such as miRNA-186 will be and are investigated with regard to their functional significance of their potential regulatory mechanisms and the results will be published in a future, functionally oriented manuscript. Taking into account the opinions of all three reviewers makes it difficult to adjust the manuscript to the suggestions of all reviewers. Therefore, we decided to keep the general structure of the manuscript but we do understand the reviewer’s concerns.

**Minor**

1. The authors need to detail more specifically, what expression fold change were determined as significant, and with what confidence intervals were they used (e.g. 95 or 99%).

   As stated in the table legends we used a Mann–Whitney U test for two group comparisons. A p value <0.05 was defined to be statistically significant. We used a confidence interval of 95% - this information has now been included in the manuscript.

2. The use of 1.5 expression fold change as a cut off is low. This should be minimally x 2, like most other published work in the field and ideally at 99% CI.
As suggested by the reviewer we now report 2.0 expression fold changes and thus, adapted Table S4 and the respective results section.

3. The expression changes might be more meaningfully shown as a heat map, which is again common for this type of work, rather than tables?

As suggested by the reviewer and in addition to the tables 2 & 4 we included a heat map to further highlight the differential expression of the miRNAs and genes depending on the prostate tissue specimen (Additional File 1: Figure S1). In the heat map you can see that the miRNAs are mostly downregulated in prostate cancer tissues and higher expressed in matched non-malignant prostate tissue as well as in BPH tissue. In contrast, the prostate cancer associated genes EZH2, TRPM8, PSGR, PSMA and AMACR show an inverse expression pattern. For both genes and microRNAs the clearest expression differences occur in tumor and BPH tissue samples. However, we renounced conducting unsupervised clustering as tissue classification based on gene and microRNA expression patterns was not aim of the present work.

4. A Power calculation missing for clinco-path associations. This might help strengthen assertions made using these data.

The aim of this study was not to prove that the selected microRNAs or genes are differentially expressed in different patient groups based on the clinical-pathological parameters. Regarding the microRNAs, only miR-186 was significantly diminished in patients with non-organ confined prostate cancer and initial metastases. We discussed this result in a careful manner and did not overrate it (e.g.: miR-186 in PCa could be a factor of disease progression). To draw any fundamental conclusions, the expression of miR-186 should be analyzed in a bigger patient cohort with an appropriate power calculation before the assembly of said patient cohort. Furthermore, other studies in this field of work (e.g. reference #43 Mavridis et al.) also not showed power calculations for associations with the clinical-pathological parameters.

However, as suggested by the reviewer we performed a power calculation for the associations of the gene and microRNA expression levels with the clinical-pathological parameters which we would like to present in this cover letter. Note, that the significant associations from the manuscript are highlighted in bold.
<table>
<thead>
<tr>
<th>Gene / microRNA</th>
<th>Age</th>
<th>PSA</th>
<th>Tumor stage</th>
<th>Gleason Score</th>
<th>Initial metastases</th>
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<tr>
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<td>&lt;7 vs 7</td>
<td>&lt;7 vs &gt;7</td>
<td>7 vs &gt;7</td>
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<td>AMACR</td>
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<tr>
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</table>

5. Fig 2 plots C and D look skewed by outlier samples. This should be addressed. We calculated the Spearman correlation coefficients following the exclusion of outlier samples mentioned by the reviewer. The Spearman correlation coefficients only differed marginally compared those reported in the manuscript: $r_s$ with outliers ranging from -0.107 to -0.551 and $r_s$ without outliers ranging from -0.106 to -0.551. Furthermore, statistical associations were the same as when the outlier samples were included. Since the reported correlations between the miRNA expression and the expression of their target genes is mainly of weak to moderate nature we feel that the outliers have not a huge influence on the respective correlations. Below you can find the adapted scatter plots as well as the Spearman correlation coefficients and p values for (A) miR-26a/TRPM8 and (B) miR-26a/EZH2.
without outliers: $r_s=-0.332$, $p<0.01$
with outliers: $r_s=-0.362$, $p<0.01$

without outliers: $r_s=-0.356$, $p<0.01$
with outliers: $r_s=-0.383$, $p<0.01$