Author's response to reviews

Title: Primary microRNA 221/222 is strongly overexpressed in the majority of patients with acute myeloid leukemia

Authors:

Anna Rommer (anna.rommer@meduniwien.ac.at)  
Katarina Steinleitner (katarina.steinleitner@meduniwien.ac.at)  
Hubert Hackl (hubert.hackl@i-med.ac.at)  
Christine Schneckenleithner (christine.schneckenleithner@vetmeduni.ac.at)  
Maria Engelmann (maria.engelmann@googlemail.com)  
Marcel Scheideler (marcel.scheideler@tugraz.at)  
Irena Vlatkovic (irena.vlatkovic@brain.mpg.de)  
Robert Kralovics (robert.kralovics@cemm.oeaw.ac.at)  
Sabine Cerny-Reiterer (sabine.cerny-reiterer@meduniwien.ac.at)  
Peter Valent (peter.valent@meduniwien.ac.at)  
Heinz Sill (heinz.sill@medunigraz.at)  
Rotraud Wieser (rotraud.wieser@meduniwien.ac.at)

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Author's response to reviews:

Reviewer: Amanda Dixon-McIver

A very comprehensive piece of work and one that was a pleasure to review.

The following comments are discretionary revisions only:

1. The description of AML as an "aggressive" haematopoietic malignancy with a "dismal" outcome.

   This statement depends on the type of AML, the age at presentation and the molecular profile of your disease amongst other prognostic predictors. Whilst there are patients who definitely have an aggressive disease and therefore dismal outcome, those with the favourable and/or intermediate prognosis may not fear as bad. Consider the removal of aggressive and dismal.

   Reply: We have adjusted our wording by removing the word 'aggressive', and adding 'in the majority of cases' to the statement about the dismal outcome in the abstract. Since AML is mostly a disease of the elderly, and age is a strong prognostic factor, we believe that this modified statement is appropriate.

2. Leukaemia-associated misexpression of miRNAs has been studied but no coherent picture has emerged yet.

   This statement contradicts the paragraph later in the paper where you describe miRNA signatures predictive of survival and specific patterns of expression that
are associated with recurrent genetic abnormalities in AML.

Reply: While it is true that a number of relevant and important papers have reported miRNA expression patterns in AML (vs healthy controls, in relation to cytogenetic and molecular genetic aberrations, and/or with respect to outcome), the results (i.e., the actual miRNA lists) of these studies agree only to a very limited extent as pointed out in the same paragraph of the introduction (and described in detail in the referenced review). We therefore believe that the statement that no coherent picture has emerged yet is appropriate.

3. Little is known about the regulation of miRNA expression.
I am not sure that this is entirely correct given that there is mounting evidence of the regulation of miRNA expression by promoter transcription, methylation, miRNA processing, RNA editing, miRNA-target interactions etc.

Reply: We agree that a lot of progress has been made in this field in the recent past. We therefore removed this sentence from the abstract.

4. pri-miR-221/222 was overexpressed to a substantially higher degree than its mature derivatives in AML versus healthy controls.
Primary miRNA may encode for a number of mature miRNA, so could this perhaps provide an explanation (not discussed) for the difference?

Reply: This seems unlikely because according to hg19 no additional miRNAs are present in the relevant genomic region. However, even if this was the case, our data would still show that the maturation of miR-221 and miR-222 is compromised in AML. Therefore, even though this point is well made, we believe that discussing it would probably create more confusion than clarification, and refrained from doing so.

5. "Parsimonious" explanation
A delightful word but not the right use of this word in this context.
Reply: Replaced by 'plausible'.

Reviewer: Spencer B Gibson
Rommer et al has submitted a manuscript entitled “Primary microRNA 221/222 is strongly overexpressed in the majority of patients with acute myeloid leukemia” for consideration for publication. The authors show that there is a higher ratio of pri-miRNA 221/222 compared to miRNA 221 in primary AML cells compared to normal controls. The author conclude that there is a processing rate limiting step in AML cells to allow for this accumulation. The high pri-miRNA 221/222 could represent a novel biomarker for this disease. Even though the data is interesting and could reflect the biology of AML, there are significant issues with interpreting the data and the functional consequence of these miRNAs. Specific comments
are listed below.

Major Compulsory Revisions

1. The high level of pri-miRNA 221/222 is likely due to a limitation in processing but it was unclear whether this was due to an increased over expression of miRNAs or a defect in miRNA processing.

   Reply: This point has already been addressed in the discussion (second paragraph, starting on page 9): in a number of studies, defective Drosha processing did not lead to an accumulation of pri-miRNAs, making it unlikely that pri-miR-221/222 overexpression in AML would be due to defective processing in the absence of an elevated rate of transcription. In addition, even if defective Drosha processing could by itself cause an accumulation of pri-miRNAs in certain cases, if AML cells acquired a (general or pri-miR-221/222 specific) pri-miRNA processing defect it would be difficult to explain why the levels of mature miR-221 and miR-222 are nevertheless elevated in AML, as has been observed by us and others. We therefore believe our data are best explained by assuming that myeloid cells have an intrinsically limited capacity to process pri-miR-221/222, which becomes saturated when pri-miR-221/222 levels are transcriptionally upregulated in AML.

2. miRNA 221/222 are increased in AML cells but this increase is somewhat limited by processing of the miRNAs. What is the functional outcome of this? The level of pri-miRNA might not be important unless there were changes in processing. This was not directly addressed by the authors.

   Reply: Even though at present the miRNA literature focusses very much on the functions of mature miRNAs, there is some reports (see our discussion) that pri-miRNAs, too, have biological and biochemical functions. Since defective Drosha processing did not lead to primiR accumulation in a number of systems, cells are apparently able to get rid of unneeded pri-miRNAs. If they do not, as is the case with pri-miR-221/222 in AML, this may indicate that such pri-miRNAs do indeed serve some function.

   We are of course aware of the fact that we have not experimentally demonstrated any such function for pri-miR-221/222. However, it is less than trivial to overexpress a >23kb nucleic acid, or, RNAi being a cytoplasmic mechanism, to knock down a nuclear RNA. Moreover, to discriminate pri-miR from mature miR function, any manipulation of pri-miR levels would require simultaneous manipulations to keep mature miRNA levels constant, which would make such experiments even more challenging. We therefore hope that it can be accepted that such experiments would be beyond the scope of the work presented in this manuscript.

3. In figure 4, there was a huge range of expression of pri-microRNAs in the AML samples. It will be hard to interpret the results unless larger number of AML samples was used. Overall, it seems that most pri-miRNAs were increased in
some AML cells. This also causes concern over the importance of pri-miR-221/222 in AML.

Reply: We agree that our data do not rule out the possibility that other pri-miRNAs may play a role in AML as well. However, comparing the scales of the y-axes in Fig. 4, it is quite evident that, of the five pri-miRNAs investigated, pri-miR-221/222 overexpression was by far the most pronounced. In addition, it was significant even in this small number of samples, which was not the case for 3/4 of the other pri-miRNAs. We would like to point out that all pri-miRNAs were measured on the same day and from the same cDNA dilution.

While more general investigations into the expression levels of pri-miRNAs in AML would certainly be highly interesting, to comprehensively address this point one should study all known pri-miRNAs in a large, homogenously treated patient cohort using custom made arrays or next generation sequencing. We hope that it can be accepted that such experiments are beyond the scope of this study.

4. In Figure 5 G, why was MCF-7 cells used. This is a breast cancer line and not close to an AML cells. It is hard to interpret these results.

Reply: Human hematopoietic cells are notoriously difficult to transfect, often yielding transfection efficiencies in the range of 1% or less. In addition, we have observed that some of these cell lines are not readily lysed by the luciferase assay lysis buffer, so that the little luciferase that is produced after inefficient transfection cannot be recovered. Since this is a mostly confirmatory experiment, designed to investigate whether the region near the predicted pri-miR-221/222 TSS as derived from GRO-seq, RNA-seq, and qRT-PCR data has basal promoter activity in human cells, we decided to do the luciferase assays in the way most researchers do this kind of experiment, i.e. in an easily transfectable adherent cell line. MCF7 are no less appropriate then the more often used 293T cells in that respect, and in fact, as shown in Fig. S2, 293T cells yielded a similar result. To make this point better understandable to readers from outside the AML field, we have included corresponding statements in this paragraph.

5. In Figure 6, the lower levels of pri-miR221/222 in remission patients is not surprising since the high levels of pri-miR221/222 in AML cells. There was also no major difference in pri-miR221/222 in relapsed patients.

Reply: The purpose of this experiment was to confirm the association between pri-miR-221/222 overexpression and AML. Based on the previous data it indeed had an expected result (i.e., pri-miR-221/222 levels returning to basal in remission and rising again at relapse). That reality matches this expectation precisely makes the whole point of this experiment.

The value of pri-miRNA as a biomarker is hard to determine. It would be good to correlated pri-miR221/222 with overall survival to evaluate it as a biomarker. One troubling issue is patients with low pri-miRNA levels still relapsed.
Reply: In our understanding a biomarker for a disease is a marker that discriminates healthy from diseased cells (and therefore, e.g., is of potential use for disease monitoring).

If we had shown that pri-miR-221/222 levels correlated with survival (be it OS or DFS) - which we have not claimed at any point in this manuscript - we would refer to it as a prognostic marker, and in this case Dr Gibson's comment of course would be entirely valid.

If our use of the word 'biomarker' is not deemed appropriate by the reviewer or the editor, we will be happy to change it for whatever they suggest to be more correct.

6. The major question not addressed by author is why pri-miRNA is generally elevated in AML. Is this due to changes in DNA methylation or chromatin changes. This is currently unclear and lowers the significance of this work.

Reply: We fully agree that it would be interesting to study the mechanism of regulation of pri-miR-221/222. However, according to the UCSC genome browser no CpG island is located anywhere near the pri-miR-221/222 transcriptional start site. Also, we would not expect pri-miR-221/222 to be repressed by methylation in healthy hematopoietic cells based on the fact that methylation usually leads to a complete shutdown in gene expression, which is not observed in this case. Since this makes regulation by methylation, a very straightforward and general mechanism of gene regulation, very unlikely for pri-miR-221/222, the question as to which mechanisms or transcription factors are involved in its upregulation in AML is a very open one, with no straightforward assumptions or educated guesses being possible. Of course one could study histone modifications and show that indeed the pri-miR-221/222 gene is in an open chromatin conformation when being actively transcribed, but this would not teach us a lot about the specific regulation of this gene. So for as interesting as the question how pri-miR-221/222 overexpression is brought about in AML is, in our opinion addressing it is far beyond the scope of this paper.