Author's response to reviews

Title: MicroRNA expression signature in human abdominal aortic aneurysms

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Editor-In-Chief

BMC Medical Genomics

Greetings!

I am submitting a revised version of a manuscript entitled “MicroRNA expression signature in human abdominal aortic aneurysms” by Matthew C. Pahl, Kimberly Derr, Gabor Gäbel, Irene Hinterseher, James R. Elmore, Charles M. Schworer, Thomas C Peeler, David P. Franklin, John L. Gray, David J. Carey, Gerard Tromp, and myself, and would like to ask you to kindly re-consider it for publication in BMC Medical Genomics.

We are grateful for the insightful comments by the two expert reviewers. Their thorough review has improved our manuscript. Below we provide point-by-point responses to the comments and have revised the manuscript accordingly.

The manuscript has not been published and is not being considered for publication elsewhere in whole or part in any language except as an abstract. None of the authors have any conflicts of interest.
Sincerely,

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Comments by Reviewer Koichi Yoshimura

This study by Pahl et al. provides important information to understand the molecular mechanisms of AAA. All the experiments were designed and performed well. The manuscript was written very well, and the data were presented clearly. Overall, this is a very interesting manuscript. I have the following concerns and suggestions.

RESPONSE: We thank Dr. Yoshimura for his time, his careful review and his valuable comments for improving our manuscript.

Minor Essential Revisions:

1) Line 2 of the second paragraph in the Results and Discussion. The phrase “twelve AAA” may be wrong and should be replaced with “36 AAA.”

RESPONSE: It appears we did not describe our study design with sufficient clarity. We performed the study in 3 stages: a) discovery of differentially expressed miRNAs using a gene chip; b) replication and estimation of biological variability of expression of miRNAs identified in the discovery stage using qRT-PCR and a new set of twelve AAA samples and seven controls; and c) more extensive validation of differential miRNA expression for those five miRNAs whose expression and variance were consistent in replication. We have revised the Materials and Methods (see page 6 of the revised manuscript), and Results and Discussion (see page 8 of the revised manuscript) to reflect the design more clearly. We also present the qRT-PCR results from stage 2 (part b above; the first phase of the validation) as a new figure (Figure 2A in the revised manuscript). The results from stage 3 (c above; the second validation phase) are now shown in Figure 2B. While generating the new figure 2A, we detected an...
error in processing the data to generate the combined data set. Figure 2B therefore has a slightly different appearance and the significance of a few individual comparisons changed slightly (e.g., miR-30c2* is no longer significantly different between unruptured and ruptured AAA). The overall results and conclusions have not changed.

2) The third paragraph in the Results and Discussion. It is not possible to discuss AAA and thoracic aortic dissection in the same framework. The description of thoracic aortic dissection can be deleted.

RESPONSE: The intended purpose of the third paragraph in the Results and Discussion is to emphasize the distinction between the two diseases. We have addressed this point by modifying the paragraph to clarify this intention (see page 9 of the revised manuscript).

3) Line 1 of the tenth paragraph in the Results and Discussion. There is the phrase “Response to organic stimulus”, while the phrase “Response to organic substance” is used in Figure 4. Is this okay?

RESPONSE: We thank the Dr. Yoshimura for detecting our error in the text. The phrase “Response to Organic Stimulus” has been changed to “Response to Organic Substance” in the text on page 12.

4) Figure 1. There is the name of miRNA “miR-181#”, while the name “miR-181a#” is used in Line 6 of the first paragraph in the Results and Discussion. Is this okay?

RESPONSE: Again, we are grateful for Dr. Yoshimura in pointing out this inconsistency. We have modified Figure 1 by changing miR-181* to miR-181a*.

Discretionary Revisions:

1) The seventh paragraph in the Results and Discussion. The description “we are looking at later stages of the human aneurysmal disease” is very significant. Unfortunately, this study lacks the data supporting this explanation. It is already known that histopathology of human AAA walls can be categorized in three pathological stages: inflammatory, active and amorphous (J Clin Invest 1998; 102: 1900-10, Atherosclerosis 2011; 218: 285-6). If the authors could provide information regarding which of these stages was used in this study, the results of this study would be much more informative.

RESPONSE: We thank Dr. Yoshimura for his insightful comment. Although we included no histological information about the samples we used in this manuscript, a detailed histological analysis of our samples was included in Hinterseher et al. (2011; ref # 8) and Hinterseher et al. (in press; ref # 9). We
have added this information to the text on pages 13-14 and included the papers mentioned by Dr. Yoshimura (ref. #s 55 and 56) and our two previous studies (ref. #s 8 and 9) as references. The phrase “we are looking at later stages of the human aneurysmal disease” has been modified to “we are looking at late stages of the human aneurysmal disease requiring surgical intervention” on page 11 to better reflect the intended meaning of the sentence.

Comments by Reviewer Jan Lindeman

Thank you for giving me the opportunity to review this well-written and timely paper. The authors rightly point out that the mechanisms of AAA formation; progression and rupture are unknown as such a study as this is relevant. Yet, an inherent and unavoidable limitation of this study is the fact that all AAA samples were from patients with advanced disease. As such data from the study can not be extrapolated to understanding of processes underlying AAA formation. A second, and again unavoidable limitation of the study is that the findings can not differentiate between cause and consequence. I.e. do the findings from the study reflect involvement and activation of for example apoptotic pathways or do they merely reflect a clear differences in cellular content (i.e. increased cytotoxic T-cells and NK cell content in AAA). These issues should be brought up in the discussion.

RESPONSE: We thank the Dr. Lindeman for his time and valuable insight. We agree that our observational study is unable to differentiate between cause and effect, and have added a paragraph that describes the limitations of our study to the Results and Discussion (see pages 13-14 of the revised manuscript).

Another point is whether correction for multiple testing is indicated, I am surprised by the extremely low number of differentially expressed miRNAs in the AAA and control samples. Although the authors already performed the mildest correction for multiple testing it presumably introduces a major type I statistical error. Given the fact that the authors performed independent validation of their results a much more liberal correction appears to be more appropriate (although I realize that this would not allow for a detailed bio-informatics approach as performed in the paper). Do the differentially expressed (before correction) fit in distinct pathways??

RESPONSE: Dr. Lindeman raises an important subject that has been debated for many decades, namely “what is the appropriate statistical threshold for a given question?” The choice of threshold is a balance between false positive (type 1) and false negative (type 2) errors. Multiple testing using the same (non-independent) samples compounds the problem. Due to the genome-wide nature of our study (nearly a thousand tests performed), some correction for multiple testing is required to control the type 1 error. We chose to control the false-discovery rate, i.e., control type 1 error, at the cost of the type 2 error. The low number of miRNAs (approximately 1% of the 847 miRNAs test on the
microarray) that are significant after Benjamini-Hochberg correction suggests that there was a high degree of variance in the miRNA expression within classes. Although we have performed independent validation, use of a more liberal threshold, either of the FDR or of nominal P values, would require independent validation of all miRNAs identified by a specific threshold, a choice that would quickly exhaust the available specimens. To allow readers to set their own threshold we have provided a list of all miRNAs that were nominally significant (i.e., without correction) in the supplemental material (Additional files 2 and 3). The lists contain 139 miRNAs and 78 other small non-coding RNAs, representing 16.4% of the 847 miRNAs probes and 8.5% of the 922 snoRNA probes on the array.

In response to Dr. Linderman's question, we performed an exploratory bioinformatic analysis with the expanded set of miRNAs to investigate the use of alternative thresholds. We analyzed the functions of predicted gene targets of miRNAs, and used significance cutoff points of \( P < 0.01 \) (nominal) and \( P < 0.02 \). We predicted 667 unique targets of 21 of the 31 downregulated miRNA and 713 unique targets of 13 of the 20 upregulated miRNA that were significant at \( P < 0.01 \). At the \( P < 0.02 \) cutoff, we predicted 790 unique targets of 33 of the 46 downregulated miRNA and 1,017 unique targets of 22 of the 30 upregulated miRNA.

The GO annotations of both expanded downregulated miRNA target sets are consistent with our original predictions. The significant (adjusted \( P < 0.001 \)) GO annotations for biological processes of the gene set targeted by upregulated miRNAs include “muscle tissue development”, “nervous system development”, “embryonic development”, “tube development”, “muscle cell differentiation”, “cell junction assembly”. In the expanded analysis (unadjusted \( P < 0.02 \)) of the GO annotations, “negative regulation of transcription”, “insulin-like growth factor receptor signaling pathway”, “cell morphogenesis”, and “cell migration” were added to the significant (\( P < 0.001 \)) GO annotations for biological processes, while “tube development” was lost.

In addition to the predicted gene targets, we used the validated set of gene targets for each miRNA in our expanded sets from Ingenuity Pathway Analysis (IPA). There were 183 genes that were validated targets of 15 of the 31 downregulated miRNAs (\( P < 0.01 \)) and 132 genes that were validated targets of 9 of the 20 upregulated miRNAs, which increased to 409 unique genes targets of 22 of the 46 down regulated miRNAs (\( P < 0.02 \)) and 148 genes of 14 of the 30 upregulated miRNAs.

The significant GO annotations of the biological processes at the down regulated miRNAs at \( P < 0.01 \) included, “cell-matrix adhesion”, “negative regulation of apoptosis”, “regulation of proliferation”, “cell development”, and “positive regulation of biosynthetic synthesis”, “pattern specification process”, “angiogenesis”, “skeletal system morphogenesis”, “response to nutrient levels”, and “response to external stimulus”. By increasing the significance cutoff to \( P <
0.02, there was no change in the significant GO terms but a general trend of slightly decreased significance. The significant GO annotations of the validated targets of the upregulated miRNAs included “I-kappaB kinase/NF-kappaB cascade”, “regulation of cell proliferation”, “regulation of immune system process”, “innate immune response”, “defense response to bacterium”, “regulation of interleukin-12 production”, and “organ morphogenesis”. Expanding the analysis to P < 0.02 resulted in losing significance of the annotations for “I-kappaB kinase/NF-kappaB cascade” and interleukin-12 production.”

The significant GO terms differ substantially depending whether one uses the predicted target or validated target dataset. The down regulated targets differ in the positive and negative regulation of apoptosis. The analyses of predicted targets of the upregulated miRNAs are suggestive of involvement in smooth muscle cells while the validated set seems related to immune function, which suggest miRNAs play roles in multiple diverse pathways in AAA. The predicted target dataset was much larger than the experimentally validated set. Both datasets have their own limitations. The predicted target data lack experimental evidence for the miRNA-mRNA interactions, but the experimentally validated target set is limited to only what is already known, which may not be a complete picture. It is interesting that apoptosis is important to both up and down regulated miRNAs.

In conclusion, the results of the exploratory analysis presented above demonstrate that genome-wide analyses require correction for multiple testing. Incompleteness of available data sources (e.g. validated and predicted targets of miRNAs) complicates the interpretation of the results. Re-analysis of the results can be accomplished in the future.

Minor points:

1) I am surprised by the fact that PCR failed to confirm the results for the up regulated miRNAs, may this relate to a problem with the use of U6 for normalization?

RESPONSE: The choice of internal standard is difficult. We chose to use the U6 small noncoding RNA since it is a ubiquitous RNA of similar size to miRNA. We verified that the U6 RNA expression was not different between our control and AAA group (P = 0.67). We see no obvious problem with using U6 as an internal standard for these experiments.

2) In the discussion the authors point to an apparent paradox between positive regulation of apoptosis and SMC proliferation. Isn't this a logical consequence which indicates accelerated SMC turn-over?

RESPONSE: We have taken Dr. Lindeman's comment into consideration. In the discovery analysis of the validated targets of the downregulated miRNAs (unadjusted P <0.05), we found that the validated targets were significantly
annotated as being involved in the negative regulation of apoptosis. Concerning the previously published studies on apoptosis, we have added a sentence “Another possible explanation is that our results indicate high turnover of vascular smooth muscle cells.” on page 11.

3) Many theoretical pathways have been labeled on basis of the context of their initial clustering. Could the authors relate response to organic stimulus etc. to mechanistic pathways involved in AAA?

RESPONSE: We have expanded the paragraph about to response to organic substance on page 12. About half the genes in the “response to organic substance” GO category were also in other enriched categories. The organic substance in the definition includes organic signaling molecules such as endocrine and paracrine activators, and cytokines relevant to many of the other enriched categories. Since none of the child categories of “response to organic substance”, which include “response to cytokine stimulus”, “response to growth factor stimulus”, and “response to hormone stimulus”, are enriched, the category was not sufficiently specific to provide more insight into pathobiology or mechanism.

Other Changes

1) A paragraph (see page 9) has been added to the Results and Discussion on two recent studies on the roles of miRNAs in aortic aneurysms, which were published while the current study was under review.

2) A spelling mistake has been corrected in Figure 4.

3) Several literature citations on AAA pathobiology have been added.

4) Author affiliations and e-mail addresses have been update