Author's response to reviews

Title: Impact of RNA degradation on gene expression profiling

Authors:

Lennart Opitz (lopitz@uni-goettingen.de)
Gabriela Salinas-Riester (gsalina@uni-goettingen.de)
Marian Grade (mgrade@uni-goettingen.de)
Klaus Jung (klaus.jung@ams.med.uni-goettingen.de)
Peter Jo (jo.peter@chirurgie-goettingen.de)
Georg Emons (georg.emons@googlemail.com)
B. Michael Ghadimi (mghadim@uni-goettingen.de)
Tim Beissbarth (tim.beissbarth@ams.med.uni-goettingen.de)
Jochen Gaedcke (jochen.gaedcke@med.uni-goettingen.de)

Version: 3 Date: 22 July 2010

Author's response to reviews: see over
Dear Sirs and Madams!

Please find attached a second revised version of our manuscript "Impact of RNA degradation on gene expression profiling". We want to thank you very much for still considering our manuscript.

In summary, two of the initial reviewers were in favor of publication. The third reviewer in his first review has stated some concerns, which we have addressed, and suggested some further experiments, which we have performed. However, in his re-review, he did not regard his initial concerns or our responses or neither acknowledge the new experiments. Instead he states that he very generally dislikes the contents of the manuscript. We respect the fact, that there can be different opinions and also that there are people who dislike certain research areas, like e.g. microarray analysis, in general. However, we would like to express our concern that it is bad practice as a reviewer, to suggest specific additional experiments, and in the re-review generally reject the contents of a manuscript for completely different reasons after the researchers did spend additional time and money.

Apparently the editorial board reached a similar conclusion and asked a fourth reviewer for his opinion. The additional review was very concise and helpful. In the following we respond to this reviewers concerns and questions as suggested by the editor. We believe that we could address all the reviewers comments and improve the manuscript.

Kind regards

Tim Beissbarth

Referee 4:

- The authors should make clear that their results may not be valid for other chips (e.g. Affymetrix, short oligos).

We added this point in the Abstract:
These results are limited to the Agilent 44k microarray platform and should be carefully interpreted when transferring to other settings.

Further, we added this point as part of the Discussion:
However, these results have some limitations. The platform used within these analyses has to be taken into consideration. Apart from different methods of reverse transcription as for example the use of oligo-dT primers versus random hexamers the platform itself might play a major role. While the probes that are spotted on the Agilent 44K array used here are 60mer long those from the Affymetrix chip for example are only 25 bp long. This difference might strongly influences the binding characteristics to the microarray, especially when degraded RNA is used.

- The authors claim that they are the first to achieve RNA degradation by heat treatment ("We are the first to achieve degradation by heat-treatment of isolatedRNA..."). Heat treatment of purified RNA is used quite generally and it is certainly not a first or a new
procedure. Heat treatment is often the preferred method because one does not introduce salt that may interfere with downstream applications.

We modified this sentence to:

*We performed degradation by heat-treatment of isolated RNA to avoid transcriptional processes in the context of cell death.*

- I suggest that the authors completely remove the terms up-regulation and down-regulation. Purified RNA is partially degraded by a more or less random process. Apparently, some of the probes tend to be under-represented as compared to the average RNA population leading to a lower signal in partially degraded RNA as compared to intact RNA. Similarly, some RNA species are more stable than the rest of the RNA leading to an increased signal. Major parameters are probably the distance between the poly(A) tail at the 3´ end as cDNA synthesis is primed by oligo(dT) primers.

The terms up- and down-regulated RNAs were replaced by under-represented and more stable or over-represented RNAs. The term non-regulated was replaced by normally-represented.

- The authors show the absolute distance oligo on the chip relative to the 5´ end of the mRNA (Fig. 4B) but it would probably be more adequate to show the distance from the 3´ end where reverse transcription starts. Obviously, synthesis of a probe is more difficult if this distance is long especially when the RNA is partially degraded.

We added a supplementary Figure 1 where the distance from the 3' end to the probe position is shown and reference it in the text. The under-represented genes also tend to be further away from the 3' end.

- The authors claim that the differences shown in Fig. 4C are significant. This is not very obvious and the authors should document this with numbers.

We added the p-value to the text: *Figure 4C shows that the mRNA-lengths of down-regulated genes are significantly shorter (Bonferroni corrected p-value from two-sample Wilcoxon test was < 0.001).*

- The authors conclude that short RNAs are "lost" for detection more rapidly during heat treatment because the distance between the end of the RNA and the probe is shorter. This is highly speculative. In fact this may only be correct if degradation occurs from the end rather than randomly. Instead, the bioanalyzer data strongly suggest that long RNAs are degraded more quickly than short RNAs. This is apparent when the 18S rRNA peak and the 28S rRNA peak are compared during heat treatment. Degradation from the end would not lead to a preferential loss of the 28S rRNA peak but rather to the formation of a similar shoulder on the left side of 28S and 18S rRNA. But this is clearly not observed.

We modified our argumentation in the manuscript:

*The RNA degradation process is not entirely explained, yet. On the one hand it is postulated, that the process starts from the 5´ end, on the other hand a more random mechanism including endonucleases activity has to be expected. This becomes evident interpreting the bioanalyzer data. If the mRNA would be degraded only from one side a broader peak within the 28S and 18S peak would be observable. For both assumptions it could be hypothesized that probes which are closer to the 3’ end should be efficiently hybridized and be more stable than those located closer to the 5’ end. This is also accounted for by the fact that the Agilent*
microarray probes are predominantly located near the 3’ end of the mRNA but also due to the important step of reverse transcription. In case of random degradation incomplete cDNA synthesis becomes more and more probable by the increases of the distance between 3’ end (location of poly-A-tail that serves as starting point) and the mRNA sequence that later serves as the probe. ...

This is consistent with the comparison of the supposed mRNA lengths between the different groups of genes. Here, we also found significant differences between under-represented and normally-represented genes. Figure 4C shows that the mRNA-lengths of under-represented genes are significantly shorter (Bonferroni corrected p-value from two-sample Wilcoxon test was < 0.001). This might be due to the degradation mechanisms that result in a higher probability of loss of detection. However, it does not mean that short RNAs are degraded quicker or more prone to degradation.

- Table 3 and Fig. 5. The authors present primers for RT-QPCR for 9 genes plus three reference genes (Table 3) but in Fig. 5 they only show the results of 6 genes. What was the result with the 3 missing genes?

Thanks for this comment. We have made a mistake there. After the suggestion to perform further RT-QPCR validations, we started to check all the three different groups of genes (i.e. over-represented, under-represented and normally-represented). However, we did not plan initially RT-QPCR validation due to two reasons. 1. We usually observe a very good validation using RT-QPCR. 2. Due to the special setting here, the results are likely very platform dependent, as the reviewers noticed. Therefore, RT-QPCR validation might not actually measure the same thing but a different mode of RNA stability. Therefore, we also did not have RNA left and had to repeat the entire degradation experiment. In the results we noticed that the results for the normally-represented group were inconclusive with a slight trend of the normally-represented group showing under-representation. Since it is very difficult to quantify no-change or insignificance and since there are many variables like stability of the housekeeping genes, or slightly changed degradation, we decided that it would be besides the point of this paper to add a long discussion about this group and to only show the results for the over- and under-represented genes. Furthermore, the normally-expressed genes only appear to be not affected on the microarray due to the normalization effect that mimics no expression changes of the majority of genes. In fact there may be a slight degradation over the majority of genes. This difference may become evident in RT-QPCR, where normalization is based on housekeeping genes and may therefore detect this “expression” difference. We have now tried to explain this better in the text and have also changed Figure 5 to include the results of all three groups.

- The authors realize that the difference in sensitivity is smaller in QPCR than in chip analysis but they do not explain the reason for this: as described in Materials and Methods, cDNAs is made by random priming for QPCR studies and by oligo(dT) for DNA chip experiments. Obviously, random priming can occur at any position within the RNA and is not restricted to the 3´end. Subsequent PCR will be successful if the entire amplicon is contained within a single cDNA molecule. This is dependent on the size of the RNA fragments present during RT and the size of the amplicon during QPCR but the absolute position of the amplicon within the RNA is not critical. This is different for chip experiments where cDNA synthesis is initiated at the 3’ end of the mRNA. As a result, qPCR tends to be more robust than DNA chip analyses.

In the results part we added the following paragraph:
This finding might be explained by the different techniques of reverse transcription that is carried out by random priming for the qPCR and by oligo-dT for the chip experiments. Since random priming can occur at any position within the RNA and is not restricted to the 3' end subsequent PCR will be successful if the entire amplicon is contained within a single cDNA molecule. This is dependent on the size of the RNA fragments present during RT and the size of the amplicon during qPCR but the absolute position of the amplicon within the RNA is not critical. This is different for microarray experiments where cDNA synthesis is initiated at the 3' end of the mRNA. As a result, qPCR may tend to be more robust than DNA chip analyses.

- At least part of the chip experiments should have been carried out in replicates or triplicates to document the reproducibility of the data.

We checked the technical reproducibility of Agilent microarray results in another study using the same patient samples. Here we investigated the robustness of gene expression profiles by changing the experimenter (E) or repeating the labelling (L), hybrization (H) or washing (W) process. These types of technical replicates where highly correlated and clustered together (see the attached Supplement Figure 2). Also from other experiments we have the experience that purely technical replicates are very robust and do not offer much additional information.

- Finally, the authors compared the results of three tumors and they conclude that the difference between these tumors is much more pronounced than the difference between the time points of degradation. Although this seems to be correct for this situation it cannot be extrapolated to other tumors. In fact, other tumors may be much more homogeneous and the role of degradation may become problem.

We do not believe that our samples display considerably more variability than other tumors. However, we tried to clarify this in the text:

*Furthermore we investigated a small group of different rectal cancers. The results, that implicit a much higher difference based on biology than on degradation might only hold true as long as such heterogeneity within the investigated samples can be found.*

- The authors should discuss that heat-induced fragmentation of purified RNA may be substantially different to RNA degradation which occurs in the tissue during preparation. In fact it is not clear whether the results shown by the authors can adequately mimic the situation in the lab.

According this recommendation the text was changed as follows:

*To assess this influence, we simulated RNA degradation in vitro by using heat treatment of patient samples. We believe that this approach provides data that is more relevant to our approach of gene expression profiling. However, it remains unclear to which extent this resembles the in-vitro situation or changes during tissue preparation. Since in previous studies, tissue was treated to achieve degradation [Strand2007, Thompson2007, Jochumsen2007a]. Those studies have to deal with the problem of differences due to functional changes in living cells such as apoptotic changes for example. We therefore used heat induced degradation that has previously been used in [Ravo2008].*

- The authors might comment (in Material and Methods) on the quality of their chip results based on internal controls present on the chip

We performed a quality control check during feature extraction using Agilent's protocol GE1_107_Sep09. Here all 10 QC metrics, based on internal controls, were in a good range
for all chips:

*Intensity data were extracted using Agilent's Feature Extraction (FE) software (version 9.5.3.1) including a quality control based on internal controls using Agilent's protocol GE1_107_Sep09. All chips passed the quality control ...*

- page 13: replace "side" by "site"

Modified.