Author's response to reviews

Title: Microarray analysis of RNA extracted from formalin-fixed, paraffin-embedded and matched fresh-frozen ovarian adenocarcinomas

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Author's response to reviews: see over
Dear Editor,

Thank you for reviewing our manuscript entitled ‘Microarray analysis of RNA extracted from formalin-fixed, paraffin-embedded and matched fresh-frozen ovarian adenocarcinomas’. We are pleased to hear that you are willing to consider the manuscript for a publication in *BMC Medical Genomics*. We found the reviewers’ comments to be relevant and helpful; we have addressed them in a detailed list below. We hope you find our response to the comments satisfactory and decide to accept this improved manuscript for publication.

Please do not hesitate to contact us if you have any questions or require additional clarification.

Sincerely,

Zora Modrusan
Reviewer: Giorgio Stanta

1. We discussed that most FFPE samples used in this study were fixed at 4°C and thus the resulting RNA may be of better quality than RNA obtained from routinely fixed FFPE samples (see Discussion, page 13, line 3).

2. RIN numbers have been included in Figure 1A and in the Results (see page 9, line 6).

3. A comment indicating that slightly more CLDN3 product was made when using Ambion extracted RNA was added in the Results (see page 10, line 3).

4. FFPE sample 390 was removed from Figure 2 because it was not feasible to generate amplification products for the FFPE-Ambion method.

5. The publication by Lassmann et al. on microarray analysis of microdissected FFPE and fresh colorectal tumor tissue has been included in the Discussion (see page 14, line 16) and in the References (see #20).

Reviewer: Zhiyuan Hu

1. The reviewer’s main concern is that the amplification protocol used in this study is not suitable for amplifying RNA from FFPE samples. Although true that the protocol relies on oligo (dT) priming and thus is 3’ end biased, it is widely accepted and routinely used for processing samples on microarrays. While some other methods may be more suitable for amplifying RNA from FFPE samples, our rationale was to perform direct comparison between FF and FFPE samples by using one standard method.

2. We believe that Bioanalyzer trace data for FFPE samples (see Figure 1A) was somewhat misinterpreted. While FFPE RNA shows the highest peak at ~200 nt, there is a significant population of high molecular weight RNA in all samples, except for 390, which extends to and beyond 4000 nt.
3. Similar to the previous point, Bioanalyzer profiles of labeled cRNA (see Figure 1B) show the highest peak at 200 nt and extend further into >500 nt size range. Thus, labeled cRNA can hybridize to probes designed within the last ~500 nt of each transcript; this corresponds well with the array design that was used in this study.

Additional comments:

We added a new paragraph that discusses potential issues with the amplification method used in this study (see last paragraph of the Discussion) and furthermore, illustrates some alternatives such as NuGEN’s whole transcriptome labeling (see page 15, line 22).

Regarding the verification of our results with IHC, we have addressed it by including some references that describe an increased level of CLDN3 protein in ovarian adenocarcinoma (see #2 and #5; page 3, line 22).

Although Pearson correlation of 0.95 is often considered as a threshold, we believe that the correlations observed here are in agreement with those reported previously (see #17 and #23, page 14, line 19-22).

Reviewer: Hermann-Josef Grone

1. We agree that normal ovarian counterparts would have added value to this study. However, it is very difficult to obtain such samples and in our case they were not available. We have recognized and addressed this concern by including gene expression profiles of five normal ovary samples from other patients.

2. Several publications that deal with microarray expression profiling of FFPE samples were reviewed in the Discussion (see pages 14 and 15) and were added to the References (see #20, #21, #22, #23, #24, #36, #37 and #39).
3. A set of 56 genes identified as >2 fold expressed in ovarian serous adenocarcinoma compared to normal ovary (Table 1) was obtained using very stringent cut-off criteria that required their presence in all replicates of the four patient samples and in all three sample processing methods (i.e. FF-Qiagen, FFPE-Agencourt, FFPE-Ambion, see page 11, line 24). Relaxing the cut-off criteria would have increased the number of overexpressed gene signatures.

4. One way of having a standard set of genes for comparison of FF and FFPE samples is to introduce external spike-in controls. Starting in 2005, the External RNA Controls Consortium (ERCC) has been leading the effort towards identifying “gold standard” reagents (i.e. RNA spikes) for assessing microarray data quality between different samples and array platforms. Although progress was made (see publications by Tong et al., Nature Biotechnology, 2006 and Kerr et al., BMC Bioinformatics, 2007), a standard set of controls have not yet been identified. However, further effort of the ERCC will likely result in a set of control genes that will prove valuable when comparing the performance of FF and FFPE samples.

Reviewer: Falko Fend

Regarding the comparison of our methods and findings with those from other groups, we have included some additional references, for example, on FFPE sample handling (see #33 and #34, page 13, line 7), on microarray profiling of FFPE samples (see #20, #21, #22, #23, #24 and #39, pages 14 and 15) and on identification of ovarian cancer signatures (see #36 and #37, page 15, line 8-9).