Author's response to reviews

Title: Decreased expression of key tumour suppressor microRNAs is associated with lymph node metastases in triple negative breast cancer.

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Author's response to reviews: see over
Dear Dr Khalil Helou,

Re: MS: 1669647981047013 “Decreased expression of key tumour suppressor microRNAs is associated with lymph node metastases in triple negative breast cancer.”

Thank you for your email on the 5/11/13 regarding MS: 1669647981047013 “Decreased expression of key tumour suppressor microRNAs is associated with lymph node metastases in triple negative breast cancer”, and for giving us the opportunity to resubmit this manuscript. Please find uploaded our revised manuscript by Avery-Kiejda et al that we would like to be reconsidered for publication in BMC Cancer. We would like to thank the reviewer’s for their valuable feedback on this manuscript. The manuscript has been altered to address the reviewers’ concerns. The specific alterations are described below in point form where an underline indicates addition of a word and a strikethrough indicates removal of a word. These changes have been highlighted throughout the manuscript.

- An additional author has been added, Page 1 and 21:
  Andrea Mathe (Andrea.Mathe@uon.edu.au)
  AM: analysis and interpretation of data.

Reviewer 1: John Martens

1. Major compulsory revision:

   I have one major concern with the paper. In my view if healthy tissue between patients with and without lymph node metastasis and their primary tumors are identical, I find it surprising the authors find significant differences for patients with and without lymph nodes when their healthy tissue is compared with the corresponding primary tumors. I believe this could be a matter of sampling error. Have the authors tried to switch the labels of the samples (random assignment label lymph node involvement yes/no) to show that the effect is specific and not due to sampling. The differences between primary tumors and healthy tissue are large thus the change of finding differences by chance is significant. Alternatively, conformation in an independent cohort would be required (possibly exploiting public data, if available).

Although there are a number of miRNA expression data sets that are publicly available (Buffa et al, 2011; Farazi et al, 2011; Gravgaard et al, Breast Cancer Res Treat 2012 Jul;134(1):207-17; Romero-Cordoba et al, PLoS One 2012;7(3):e31904), none of these have used TNBCs with matched normal tissue and matched lymph node metastases, which is required to truly validate the findings. This is one of the unique features of our study. The only exception is the recent study conducted by Cascione et al, 2013. We have confirmed that the results of the normal versus tumour comparison are 70% concordant with Cascione et al, however, it is
not possible to validate our findings of our 27 miRNAs identified in LN+ patients since they did not perform this comparison and this information is not available in GEO (miRNA data from lymph node metastases and normal tissue is missing in Accession: GSE41970). Thus, it is not possible to validate these findings on publicly available datasets at present. In addition, TNBCs account for only a small percentage of all breast cancers diagnosed (10-24%) - the cohort used in the current study was collected over a five year period (2004-2009). We are currently collecting a larger TNBC cohort with follow-up data to validate the role of these miRNAs in metastasis but this is a time-consuming activity and can therefore not be included in the submitted manuscript.

We have therefore, randomised our tumour and normal cases into lymph node positive and negative cases to determine if the results are due to chance as Reviewer 1 has suggested. We randomised all tumour and normal cases into the sub-groups using Research Randomiser v4.0 (http://www.randomizer.org/form.htm). We performed 3 separate randomisations and we then compared the miRNAs differentially regulated in these comparisons with our true results (see Tables A and B below).

In our first comparison, we determined what the effect would be of simply switching the normal cases i.e. if we compared all lymph node positive IDC with lymph node negative NAT and vice versa. Overall, we found a high concordance (76.6% and 79.2%) in the particular miRNAs that were regulated in these comparisons (highlighted in yellow - Table A).

One of the major findings of our study was that the majority of miRs that were identified in lymph node negative IDCs in this study were up-regulated (91.9%) while those in lymph node positive IDCs were down-regulated (76.09%) when compared to their matched normal tissue. However, when we looked at the effect of simply switching the NAT in this comparison (highlighted in blue - Table B) we see a very different proportion of miRNAs being up- or down regulated. Similar results were achieved in 3 separate randomisation experiments. Again, while there was high concordance in the particular miRNAs that were regulated (Table A), the proportion of up- and down-regulated miRNAs in each of the comparisons (Table B) was very different to that of the true result further confirming that the effects observed in our study are true and not simply due to chance.

Table A: Proportion of miRNAs in common when lymph node positive and negative cases are randomised. Numbers in brackets represent the number of miRs regulated in total.

<table>
<thead>
<tr>
<th></th>
<th>Proportion of miRNAs in list A that are in list B</th>
<th>Proportion of miRNAs in list B that are in list A</th>
</tr>
</thead>
<tbody>
<tr>
<td>A→B→</td>
<td>IDC- v NAT- (37)</td>
<td>IDC+ v NAT+ (46)</td>
</tr>
<tr>
<td>IDC- v NAT-</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>IDC+ v NAT+</td>
<td>100.0</td>
<td>76.6</td>
</tr>
<tr>
<td>Random 1</td>
<td>IDC- v NAT- (50)</td>
<td>IDC+ v NAT+ (59)</td>
</tr>
<tr>
<td>IDC- v NAT-</td>
<td>64.0</td>
<td>78.0</td>
</tr>
<tr>
<td>IDC+ v NAT+</td>
<td>60.4</td>
<td>22.6</td>
</tr>
<tr>
<td>Random 2</td>
<td>IDC- v NAT- (23)</td>
<td>IDC+ v NAT+ (87)</td>
</tr>
<tr>
<td>IDC- v NAT-</td>
<td>56.3</td>
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</tr>
<tr>
<td>IDC+ v NAT+</td>
<td>62.8</td>
<td></td>
</tr>
<tr>
<td>Random 3</td>
<td>IDC- v NAT- (64)</td>
<td>IDC+ v NAT+ (58)</td>
</tr>
<tr>
<td>IDC- v NAT-</td>
<td>79.3</td>
<td>59.0</td>
</tr>
<tr>
<td>IDC+ v NAT+</td>
<td>64.2</td>
<td>59.0</td>
</tr>
</tbody>
</table>
Reviewer 2: Stefano Caramuta

1. The authors use two different reference genes (RNU44 and RNU49), however only one was shown in the results. Did the authors get same results (in term of miRNA relative expression) with the two reference genes? Did the authors try to use both genes as normalizer (e.g. geometric mean)?

Yes, the same results were achieved using both reference genes. No, we did not use the geometric mean of both genes to normalise the samples. We have now included a Figure (Supplementary Figure 3) showing examples of the results achieved with both normalisers and depicting the relative expression of RNU44 and RNU49 demonstrating that there was no significant difference in the expression of these normalisers between sub-groups.

- The following sentence has now been added to the methods Page 9, Paragraph 1, Line 1:

   .....(data not shown). The same relative expression patterns for the miRs analysed was observed when normalised with RNU49 (examples shown in Supplementary Figure S3). The relative expression RNU44 and RNU49 was not significantly different between the subgroups analysed and therefore served as an appropriate normaliser for this analysis (Supplementary Figure S3). All pre-amplified multiplex miRNA assays were validated against uniplex miRNA assays to verify that the multiplex reaction did not affect miRNA quantitation (Supplementary Methods, Supplementary Table S1 and Supplementary Figure S4). hsa-miR-126* and hsa-miR-205 were outside our range of acceptable PCR efficiencies and were not used for further validations.

- The following Figure and Figure legend have been added to the Supplementary Material.

**Supplementary Figure S3: Validation of RNU44 and RNU49 as normalisers.** Relative quantification of miR-210 and miR-100 by real-time RT-PCR in normal (n=24) and tumour samples (n=35). Results are shown as a scatter plot of the relative normalised expression of the target miRNA to A) RNU 44 and B) RNU49 (2-ΔCt). Values represent the median ± interquartile range. *p<0.0006, **p=0.0016, ***p<0.0001. C) The relative expression of RNU44 (white bars) and RNU 49 (grey bars) in NAT and IDC from lymph node negative tissues (-) and lymph node positive tissues (+). Values represent the mean ± SD.
Supplementary Figure S3 has now been changed to Supplementary Figure S4 throughout the manuscript to accommodate this extra Figure.

2. To verify microarray results from the first comparison (IDC vs NAT), the authors chose 6 miRNAs: how were these miRNAs selected among the 71 deregulated miRNAs? Moreover, data for miR-205 are not reported in Fig.1B.

These miRs were chosen because they were also differentially regulated in our list of 27 miRs, meaning that we could validate their expression in all tumour versus normal cases and then validate the differences that we were seeing when we compared the lymph node positive and negative cases separately.

miR-205 is not shown in Figure 1B as “All pre-amplified multiplex miRNA assays were validated against uniplex miRNA assays to verify that the multiplex reaction did not affect miRNA quantitation (Supplementary Methods, Supplementary Table S1 and Supplementary Figure S3). hsa-miR-126* and hsa-miR-205 were outside our range of acceptable PCR efficiencies and were not used for further validations.” (Page 9, Paragraph 1, Line 4).

References to miR-205 validation have now been removed. The results section on Page 10, Paragraph 1, Line 6 now reads:

......(miR-210, miR-100, miR-130a, miR-205, let-7b, let-7c) were verified by real-time PCR and were shown to be significantly different in expression between IDC and NAT in all cases (Figure 1B). In addition, the fold change in expression (IDC versus NAT) of the six miRNAs

Figure 1, Line 14 now reads:

.....100, miR-130a, miR-205, let-7b and let-7c between normal and tumour samples.....

3. Among the 71 deregulated miRNAs, the authors claim that 5 miRNAs (miR-130a, miR-1280, miR-590-5p, miR-1308, miR-17®) were not previously reported to be implicated in breast cancer and might be associated with TNBC phenotype. It would be worth to investigate a bit more on these miRNAs and maybe include them for PCR validation. Did author check possible pathways or target genes for these miRNAs? Did the authors try to perform a clustering analysis to check if these 5 miRNAs could be sufficient to distinguish IDC from NAT?

We agree that this was an interesting finding and miR-130a is one of the five miRs that was validated by real-time PCR in this study (Figure 1B). These five miRs alone were not sufficient to distinguish IDC (red branches-top of dendrogram) from NAT (blue branches- top of dendrogram) (see figure next page), nor would we expect them to be. This analysis was not included in the manuscript as it draws attention away from our major findings which are the differences in miR expression when lymph node positive and negative breast cancers are compared.
We have added a more detailed discussion regarding these miRs and validated targets in the following way:

- The following text has been added to the results section, Page 10, Paragraph 2, Line 16:

> ......identified 5 miRNAs: miR-130a, miR-1280, miR-590-5p, miR-1308, miR-17*, which to the best of our knowledge, have not previously been implicated in breast cancer (Table 2). These may represent novel miRNAs associated with the TNBC phenotype. Of note, miR-130a has been shown to be associated with chemotherapy response in ovarian cancer and lung cancer cell lines, while miR-1280 has recently been demonstrated to inhibit invasion and metastasis by targeting ROCK1 when over-expressed in bladder cancer [22-24]. miR-590-5p has been reported to enhance (via the tumour suppressor PBRM1) or inhibit (via S100A10) cell growth and invasion depending on the cellular context, however, the function of miR-1308 and miR-17* has not been extensively studied [25, 26].

- Three additional references have been added:
We agree that further characterization of the genes and pathways targeted by these five miRNAs would allow us to truly identify the role that they play in the TNBC subtype. We have performed gene expression microarray analysis on the samples used in the current study, to determine the exact target genes that are altered by the miRNAs and we are experimentally validating these miRNAs and target genes in a panel of breast cancer cell lines. We wish to publish the genes regulated by all miRNAs identified in this study (not just these five novel miRs) as a subsequent manuscript.

4. When the authors compare LN- vs NAT and LN+ vs NAT they find different miRNA profiling but also a set of 10 miRNAs which were commonly deregulated in both groups. However, none of the 5 miRNAs (which authors claim to be potentially associated with TNBC: miR-130a, miR-1280, miR-590-5p, miR-1308, miR-17*) were deregulated in both LN- and LN+ tumors. If they were associated with TNBC phenotype they should be commonly deregulated in LN- and LN+ tumors. It would be worth to discuss this issue.

It is true that although these miRs were differentially regulated when all tumours were compared to all normal tissues, they were not commonly regulated when lymph node positive and negative cases were compared separately to matched normal tissue.

- The following text has been added to the results section, Page 12, Paragraph 2:
  Of note, the five unique miRNAs that were differentially regulated when all tumours were compared to all normal tissues (Table 2), were not commonly regulated when lymph node positive and negative cases were compared separately to matched normal tissue. Instead, the miRs that were positively regulated (miR-17*- 3.03, miR-590-5p- 2.15, miR-1280- 2.10) when all tumours were analysed became more highly up-regulated (miR-17*-4.66, miR-590-5p- 3.13, miR-1280- 2.40) in the comparison of lymph node negative tumours compared to matched normal tissue, but were not regulated in lymph node positive tumours. In contrast, while miR-130a was down-regulated in the comparison of all tumour tissues (-2.21), it became more strongly down-regulated in lymph node positive tumours (-3.32) when they were compared to matched normal tissues, and was not regulated in lymph node negative cases. This supports our data, that there are intrinsic differences between lymph node positive and negative TNBCs: miRs are mainly down-regulated in patients with lymph node metastases, and up-regulated in patients who do not have lymph node metastases and suggests that it is these patients (i.e. lymph node positive or negative tumours) that are driving the differential regulation (negative and positive respectively) when all patients are combined.

5. How did the authors choose the 9 miRNAs (among the 27) for PCR validation?

These miRNAs were chosen as they showed the highest variability (>2-fold) between LN+ and LN- tumour samples. For example, in the comparison of IDC v NAT for let-7a, there was a fold change of -2.36 in LN+ cases and 1.05 in LN- cases. This is a -2.25 fold change between these two groups. While miR-210 did not show as high variability between LN+ and LN- cases, it was chosen because it is commonly known to be deregulated in breast cancer.
6. Since the author mentioned the possible involvement of Dicer in the overall down-regulation of miRNAs observed in LN+ tumors, it would be interesting to check at least the mRNA expression levels of Dicer in the tumors used in this study.

We have examined the expression of both Dicer and Drosha in these samples. This manuscript entitled “The expression of Dicer or Drosha is not associated with lymph node metastases in triple negative breast cancer” is currently under review by BMC Cancer (MS ID: 9268457611046810) and was submitted concurrently with this manuscript.

We trust that these changes to the manuscript will make it acceptable for publication in BMC Cancer and we look forward to a favourable response.

Yours Sincerely,

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