Author's response to reviews

Title: MicroRNA expression as risk biomarker of breast cancer metastasis: a pilot retrospective case-cohort study.

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

We are resubmitting the revised form of the manuscript entitled “MicroRNA expression as risk biomarker of breast cancer metastasis: a pilot retrospective case-cohort study”, containing the major and minor concerns of the Referees.

Below you will find the discriminated answers of every question with the page reference to final form of the manuscript.

Looking forward to hearing from you in the near future,

Best Regards,

Marcia M.C. Marques

Referee 1 (Athina Markou) major compulsory concerns:

Question 1. The main limitation of this study is the small number of clinical samples. It is too risky to evaluate miRNA as biomarker and to perform statistical analysis in this limited group of patients. Many other studies in the literature have already studied miRNAs as biomarkers in a larger group of breast cancer patients.

Answer: Despite the present study contains a small number of clinical samples, the major characteristic of this study is the well defined case-cohort study with 10 years
of follow-up which is rare, increases the reliability of the results and is different of the previous publication. According to our knowledge, this is the first study stratifying patients according to clinical staging (CS), specially including CSI, which is an extremely rare population to metastasize. Considering a starting search of thousands patients in our hospital and approximately 300.000 samples in the local biobank, it is possible to find only four metastatic cases of CSI population. This information was included in methods at page 7 and discussion at page 15 in the manuscript.

**Question 2.** Secondly, although the authors found **7 miRNAs** that were differentially expressed between metastasis and non-metastasis patients stratified according to clinical stage, 2 of them were finally selected for further evaluation by RT-qPCR. You should state the reason why these miRNAs were selected, additionally why you didn’t study all these miRNAs and why you preferred to study miR-183 which not included in this group of seven.

**Answer:** A better explanation of the selection of the miRNAs for real-time PCR is now in methods section, page 11 and discussion section at page 18. In addition, and a new Venn diagram was included in figure 2 to better explain this issue. In summary, it was selected 2 miRNAs between these 7 shared by all CS (hsa-miR-494 and hsa-miR-21), and 1 miRNA specific to each stage, after ROC curve analysis (hsa-miR-21 in CSI, hsa-miR-183 in CSII and hsa-miR-140 in CSIII). Curiously, hsa-miR-21 was found as differentially expressed in all CS but only presented significative sentitivity and specificity in CSI. We tested these possibilities to discover the best form to select miRNAs for confirmation and in the first version we did not mentioned the miR-140 because it was not confirmed by real-time PCR.

**Question 3.** It is necessary the authors inform us about the concordance of microarray data and RT-qPCR analysis.

**Answer:** About the concordance of microarray data and RT-qPCR analysis we found medium correlation (about 60%) and in our knowledge the reason of this result can be explained by differences in normalization methods. The microarrays analysis is
based on global normalization that adjust the probability distributions while RT-qPCR use an endogenous probe as \( (2^{-\Delta\Delta CT}) \) a normalization method. Therefore all microRNAs were found induced in both methods except to miR-140-3p that was not shown in our results.

**Question 4.** The three selected miRNAs shared PTEN as target, the reviewer wonder if you have studied the expression of this protein in this group of patients.

**Answer:** A tissue microarray (TMA) of these patients have been constructed to evaluate the protein expression of selected targets, including PTEN and it is now included in methods at page 12 and as Figure 4, and discussion at page 19.

**Referee 2 (Jeremy Squire) major compulsory concerns:**

**Question 1.** The three miRs were also selected because of their functional role in addition to their expression differences shown in the arrays. For example the rationale for choosing miR183 for further study has not been presented. The reason miR21 and 494 were selected from the overlapping Venn circles and the other 5 excluded also needs to be explained more clearly. The overall rationale for the study as presented in the abstract and the background is not really described in an accurate way. The selection of the three miRs was based not only on their differential expression but also on the likely function for their target pathways. This part of the study rationale was not apparent until page 13.

**Answer:** As previously explained in Question 2 of the Referee 1, a new figure and better explanations about the selection of miR-183 was included in the final version of the manuscript. It was now also included in the abstract and in the background, page 6.
2. Please provide more information on the extraction of RNA from FFPE and details on the quality metrics of the templates obtained. Formalin fixation is notorious for RNA degradation, so it would be reassuring to see that the approach taken in this paper is robust. Without this information there is the concern that the results are related to differential RNA stability from fixation damage and/or the extraction procedure.

Answer: Previous studies in literature have shown the stability of miRNAs in FFPE, with a high correlation with frozen tissues. This information is important to exclude some bias related to RNA degradation (Reference 8). This topic is better discussed at page 16. In addition, in page 9 of methods, a quality control was included, and corresponds to a supplementary figure of a scatterplot correlating frozen with FFPE for comparison.

Question 3. Figure 1 does not contribute to gene selection. It should be moved to supplementary but it would be helpful to arrow the three selected miRs (183, 494 and 21) so that readers can easily see how well expression levels are associated with the phenotypes.

Answer: Figure 1 is now called Supplementary Figure 1, and miRNAs selected were featured in this final version.

Question 4. The discussion is too general in many places. Please focus on the findings from the study and the potential future directions.

Answer: Some new topics were included in discussion, as suggested. Including the overall relevance of the present study (page 16), a better explanation about the FFPE quality (page 16) and the selection of the miRNAs (page 18),

Referee 2 (Jeremy Squire) minor compulsory concerns:

Question 1. The probes table 2 can be moved to supplementary.
**Answer:** Table 2 is now called Supplementary Table 1.

**Question 2.** Please briefly explain use of ROC curves for more general readership.

**Answer:** A better explanation about ROC curve principles was included in methods’ section, page 10.