Author's response to reviews

Title: Circulating miR-200c and miR-141 and outcomes in patients with breast cancer

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Author's response to reviews: see over
We kindly appreciate the constructive and relevant comments of reviewers. We have incorporated all the recommendations into a revised version of the manuscript.

We include a point-by-point reply to the reviewers’ describing how their comments have been addressed.

Reply to: ATHINA MARKOU

Major points

1. The author refer that they used 5S and RNAU6 as internal controls. Which are the external controls that they used?

ANSWER: The use of external controls, such as exogenous spike-in (mi)RNAs, is specially recommended for studies in which samples with low yields of (mi)RNAs (e.g. serum and plasma) are utilized. In this kind of samples, the low levels of targets and the consequent elevated Cq for endogenous controls and samples can lead to important experimental and random variability which can difficult the generation of valid expression data. However, this is not the case of blood samples in which we obtain stable and consistent Cq values, both for endogenous references and target genes. Moreover spiked-in exogenous miRNAs method presumes that by adding a known quantity of spiked in miRNA to an equal volume of serum/plasma, a stable quantity of reference gene is obtained. However, this technique leaves room for technical and human error.

In each RTqPCR experiment we have included external positive controls as breast cancer tissues or cell lines.

2. It is not clear how the authors define the cut off value. This point is too important to characterized that specific miRNA is over- or down-expressed?

ANSWER: The best method for cutoff determination may depend on the biomarker, the assay and the clinical application under investigation. Currently, there is no standard method for biomarker cutoff determination. As stated in the section about “Study design and statistical analyses”, the Cutoff Finder software was used. We clarified these aspects in the text. In Lines 237 to 249, we described the methods for cut off determination, as follows:

“The Cutoff Finder software [31] was used for receiver operating characteristic (ROC) curves analysis and miRNAs expression cutoff determination. The ROC curves were constructed by plotting sensitivity (Y-axis) vs 1-specificity (X-axis) and the areas under the curve (AUC) were calculated. The method used was based on the maximization of Youden's J statistics. In this first step, the cutoff is optimized for discriminating controls and BC patients based on miRNAs expression. In the second step, the Cutoff Finder tool fits Cox proportional hazard models to the dichotomized miRNA expression in the BC cohort and the survival variables (OS and PFS). These prognostic cutoffs are defined as the points with the most significant (log-rank test) split. Hazard ratios (HRs) including 95% confidence intervals are calculated to assess the stability and significance of the dichotomization.”

3. The reviewer believe that the number of samples and the events are not enough for Kaplan Meier analysis

ANSWER: For the effect of a tumour marker on a time-to-event outcome, a Kaplan-Meier plot is recommended (Altman et al. BMC Medicine 2012 10:51 doi: 10.1186/1741-7015-10-51). We give rationale for sample size in the section about “Study design and statistical analyses” from “Methods”:

“The statistical power of the study was estimated post-hoc, taking into account a probability of survival at the end of the study of 0.75 in the low-risk miRNA signature group and 0.35 in the poor-prognostic subgroup. The poor-prognostic subgroup was defined by an increased expression of miR-200c and/or down-regulation of miR-141 in the patient’s bloods. With the
sample size of 57 patients, the study was able to demonstrate by two-sided log-rank test, a significant difference in OS, with an alpha-error of 0.05 and a statistical power higher than 80%.

However we recognized in the “Discussion” section that our promising results must be confirmed and validates in additional studies:

Lines 617-619: “Although our preliminary results are promising, several limitations in this study are addressed: (i) as the sample size is still small, further validations in large cohorts and in different ethnic groups are recommended”

Lines 640-642: “These results will have to be further verified in large study cohorts that include the different stages and molecular subtypes of BC with adequate follow-up.

We discuss this aspect also in reply to nº 1 major point of the second reviewer

4. The reviewer wonder if the author had studied the influence of RNAlater in PB and furthermore in the expression levels of miRNAs?

ANSWER: RNAlater has been widely used to stabilize blood RNA with good results in previous studies, including ours. In this work, we have used the RiboPure-Blood Kit (Cat. No. AM1928, Ambion Life Technologies, http://www.lifetechnologies.com/order/catalog/product/AM1928) to perform the isolation of small RNA-enriched total RNA from blood samples, and this kit includes the option of RNAlater utilization for blood stabilization and storage. Moreover, Weber et al. (Biomark Insights. 2010 Sep 22;5:95-102) showed that RNAlater is not only suitable for the preservation of mRNA and miRNA specimens in blood but also RNAlater demonstrated to be superior to PAXgene Blood RNA tubes in the yield and integrity of RNA obtained.

Minor Points

1. Page 20. It is a spelling mistake in the sentence 19.

ANSWER: Corrected. We have deleted “agnostic”.

2. In Table 1. The number of patients is not correct in some like Hormonal Receptors, Her2, MIB1 etc

ANSWER: The number of patients in several sections of Tables 1 and 2 does not match with the overall number of patients included in the study because there are some missing data. To address this issue, we have added an explanation at the end of Table 1 and 2. We thank the reviewer for noticing this mistake.
3. The data are not entirely sound and do not perfectly fit into the recent data by other groups. Different studies were not able to find an association of miR-200c or miR-141 with breast cancer. Moreover, the association of miR-200c and miR-141 expression with survival was not confirmed by different GEO data sets.

ANSWER: We think that our and other previous studies pursue different objectives but obtain closely related results. First, most of studies are focused on demonstrating the association of miRNAs with breast cancer in order to develop diagnostic biomarkers but they do not analyse the miRNAs expression as prognostic factors. Second, it is also important to keep in mind that different studies utilize different sample sources (tissues, serum, plasma, whole blood) so data and conclusions drawn from these studies may not be comparable.

Thus, in most of the previous studies, the analysis of miRNA expression as potential prognostic biomarkers was made in tumour tissue from biopsies and not in blood.

The use of whole blood as sample source for biomarker determination is, in our opinion, a strength of our study, since opens the possibility to develop minimally invasive assays. We could only find two studies that analysed the prognostic value of serum or plasma miRNAs in breast cancer patients. In the work by Madhavan et al. (Clin Cancer Res. 2012 Nov 1; 18(21): 5972-82), miR-200c and miR-141 levels were found as part of a complex signature with prognostic capacity. However, this study is focused on metastatic breast cancer patients and it is intended for the consecution of a subrogate miRNA signature for circulating tumour cell count.

In addition, in the work by Roth et al. (Breast Cancer Res. 2010; 12(6): R90), miR-141 serum levels were unable to discriminate breast cancer patients from healthy women but their association with the clinical outcome reached borderline significance. Thus our results are clearly in line with these previous results.

On the other hand, in the GEO dataset number GSE37405 (PLoS One. 2012;7 (5):e36170), miR-141 and miR-200c expression was associated with survival, although using FFPE tumour tissue from high-risk ER+ patients as sample source. In addition, data from Korpal et al. (Nature Medicine Volume: 17, Pages: 1101–1108 (2011) DOI: 10.1038/nm.2401) showed that deregulated expression of miR-200 family of microRNA in breast tumours was associated with disease free survival in patients. Clearly our data are in line also with these reports.

4. The manuscript adheres to the relevant standards for reporting but I found no reference that the presented data was made public on e.g. GEO?

ANSWER: We did not deposit data in public repositories. This is only mandatory for high throughput array or RT-qPCR studies.

5. The discussion and conclusions are well balanced but not completely supported by the data.

ANSWER: We have made some modifications in discussion.

6. Most of the limitations of the study are clearly stated, but one major problem of RT-PCR-analyses is not discussed: sample normalization. The authors used U6 snRNA and 5S rRNA to control input variability and sample normalization. This approach is criticized by many experts.

---ANSWER: Data normalization is an essential part of a meaningful RT-qPCR assay and requires standardization. Unfortunately, normalization is a rather problematic area and, as yet, there is no universally accepted method for data normalization that accounts for all variables.
encountered during the course of a RT-qPCR experiment. All proposed methods represent a compromise and the selection depends upon the study aims and acceptable tolerance (Taken from: Bustin, et al. Nat Protoc. 2006; 1 (3):1559-82). To date, there is no consistent internal control microRNA available for normalizing circulating microRNA expression (Shen J, Hu Q, Schrauder M, et al. Oncotarget. 2014 Jul 30; 5 (14): 5284-94).

Thus, the selection of references to normalize miRNA levels is still rather empirical (Taken from Vladimir Benes, Mirco Castoldi. Methods 50 (2010) 244–249). We now know that serum and plasma samples contain extremely low levels of larger RNA species, such as U6 and 5S, but these genes can be used for normalization in other samples, such as blood. In absence of standard reference genes for miRNA analysis in blood samples, we use the combination of U6 and 5S in this and in our previous study (J Transl Med. 2012 Sep 6; 10:186), as the combination of two commonly utilized reference genes. A combination of miRNAs for normalization augments the reliability of the data produced, and has been advocated by different studies. In line with this, recent evidences support the use of U6 in combination with other reference gene for the analysis of blood samples in breast cancer (PLoS One. 2013 Dec 31;8(12):e83718). For example, the pair U6/miR-16 was identified by NormFinder as one of the best combinations of reference genes for the normalization of this kind of samples (PLoS One. 2013 Dec 31;8(12):e83718).

We discuss this issue in lines 596-601.

Major Compulsory Revisions

1. The first shortcoming of the work is the small number of patients (and especially controls) analyzed.

ANSWER: The study has been designed as an exploratory analysis for establishing the reliability of blood miR-200c and miR-141 expression as biomarkers for breast cancer detection and prognosis (Phase II-III, according to Pepe MS, Etzioni R, Feng Z, et al. J Natl Cancer Inst 2001; 93: 1054 – 61). We give rationale for sample size in the section about “Study design and statistical analyses” from “Methods”. We discuss this aspect also in reply to nº 3 point of the first rewiever

2. The second major point is the one step approach: no “screening step” (e.g. array or NGS) was conducted to identify candidate miRNAs, which could then be confirmed by RT-PCR. The candidate miRNAs were taken from previous studies in patients with gastric cancer.

ANSWER: High-troughput methods (as NGS and arrays) are popular models for the development of Phase I studies, i.e. the discovery of potential biomarkers, specially for the early detection of cancer (Pepe MS, Etzioni R, Feng Z, et al. J Natl Cancer Inst 2001;93:1054 – 61). In stead of NGS or arrays, we first performed a Phase I preclinical study by means of computational tools for miRNAs profiling. Selected miRNAs were evaluated by means of qRT-PCR in breast cancer and hematopoietic cell lines, control bloods, and blood from metastatic breast cancer patients. Data from this preliminary study had been presented elsewhere (Medina Villaamil V, Blanco Calvo M, Díaz Prado SM, Antolín Novoa S, Calvo Martínez L, Santamarina I , Haz Conde M, Antón Aparicio LM, Valladares-Ayerbes M. MicroRNA for circulating tumor cells detection in breast cancer: In silico and in vitro analysis. J Clin Oncol 27, 2009 (suppl; abstr e22027). MiR-141 and mir-200c were chosen for further analysis in BC patients. We introduce these data in the text (lines 125-129) and a new reference (23).

MiR-200s, including miR-200c and miR-14, exhibit biological functions mechanistically linked to breast tumour progression, one of the hallmarks to be considered as reliable biomarkers. It has been increasingly recognized that miR-200 family of microRNAs plays an important role in the
proliferation, invasiveness and migratory properties of breast cancer (BC) cells in cell lines and experimental models. This rational is included in the discussion section (lines 618-620)

3. The blood collection of 49% of the patients was done “after R0 surgery” of the tumor. This is in sharp contrast to the published work by Heneghan et al. (cit.: ‘levels of miR-195 and let-7a decreased in cancer patients postoperatively, to levels comparable with control subjects’)


ANSWER: From a clinical perspective, assessment of miRNAs in the whole blood obtained after surgery, as definitive loco-regional treatment, reflects the “minimal residual disease” status that might better predict the clinical behaviour and/or therapeutic response. Postoperative sampling time combines, in theory, the baseline level of circulating miRNAs, the potential release of miRNAs due to the surgical manipulation and the rapid death of in transit cells within the bloodstream but with reduced survival ability. Moreover, the behaviour of miRNAs analysed in our study may differ from the behaviour of other miRNAs, such as those cited by the reviewer. In addition, when we perform a comparative analysis of miR-141 and miR-200c expression levels in blood of stage I to III patients, we did not find significant differences between patients who underwent surgery and those who receive neoadjuvant therapy. These data are incorporated in the results section (lines 334-339) and table 2.

4. The authors used a completely heterogeneous cohort of patients including patients with metastasis, with positive and negative hormonal receptors, HER2 positive and negative, . Taken into account the major differences of distinct breast cancer subtypes and the small number of patients in this study this heterogeneity makes it very hard to interpret the data.

ANSWER

Breast cancer is a clinical, genetically and phenotypically complex disease. Although the inclusion of BC patients with different clinical characteristics, stages and molecular subtypes could be considered a limitation of this study, we suggest that this pragmatic design accurately reflects the patients treated everyday in the oncology clinic. Thus, the prognostic value of blood miRNA quantification has been estimated in a cohort of patients truly representative of those found in the clinical setting. In our BC cohort, neither miR-200c nor miR-141 circulating levels were significantly associated with age, menopausal status, histological subtype, tumour grade, hormonal receptors or IHC-based subtypes (lines 529-531). Moreover, as we stated in Discussion “[…] we used previously reported data on miRNAs profiling studies in BC. Similar to our findings, miR-200c and miR-141 were not associated with molecular subtypes or clinic and pathologic factors analysed [9, 10].”

Furthermore, prognostic impact of miRNA quantification remains significant in multivariate model adjusting for age, tumour stage and hormonal receptors status.

These findings suggest that deregulated blood miR-200c and miR-141 levels can be detected throughout the wide spectrum of breast carcinoma and therefore underscore its potential role as a clinical biomarker.

Minor Essential Revisions

Space character is missing on page 7 line 17 ‘141expression levels’

ANSWER: the mistake has been corrected.