Author's response to reviews

Title: Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients

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Author's response to reviews: see over
Sao Paulo, September 30th, 2014.

Ref: Article title: Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients
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Dear Editors,

Thank you for the opportunity to address the reviewers’ comments and concerns. Following their suggestions, we performed additional experiments and provided more information, which significantly improved our manuscript. We hope that after addressing all the points raised by both reviewers, our manuscript is ready for publication on BMC Medical Genomics.

Please, let me know if any further information is needed.

Kind regards,

Raphael Parmigiani

Reviewer’s report
Title: Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients
Version: 1
Date: 14 July 2014
Reviewer: Pasqualino de Antonellis
Reviewer’s report:

Dear editor
I have read this manuscript with great interest and my first opinion is that this is a good manuscript and potentially identifies new biomarkers for predicting complete responder to nCRT although the number of patients analyzed was a limiting factor. The workplan is comprehensive, exhaustive and the methodology is based on established published work.
However, at many places authors have not provided enough justification to support their results that require to be addressed. I have made some suggestions and comments, listed below, which I feel would improve this manuscript.

Authors: The authors highly appreciated the careful reading and favorable evaluation of our work. Below, we have addressed all the points raised by the reviewer. In the revised version of the manuscript we have highlighted in blue the additional information that was included to address the referee’s comments.

Major Points:
Firstly in their study the authors in result section (page 7) claim “The number of sequences for each specific miRNA was used to estimate its expression, and low expressed miRNAs were removed in order to increase detection power of statistic tests “ this conceptually should be a correct way to obtain more robust results but the authors didn’t have any explicit control for assume low and high expression. How many miRNAs are down or up regulated in this samples compared to normal tissue? Their analyses were performed using only complete vs incomplete responder. In order to define a possible predictive use of these identified miRNA the author must evaluate the expression also in normal samples.

Authors: We understand the referee’s concern about the expression of miRNAs on normal tissue. It’s worth mentioning that the exclusion of low abundant miRNAs in differential expression analysis is very common. Indeed, other authors have been performing even more stringent analyses while dealing with miRNA-Seq data, removing from their analyses miRNAs whose expression was less than 200cpm (see: Voellenkle C, et al. RNA. 18(3):472-84, 2012; and Volinia S, et al. Proc Natl Acad Sci U S A. 109(8):3024-9, 2012). Here we have used 20cpm as a cutoff. Moreover, considering that the standard treatment for rectal cancer patients is neoadjuvant chemoradiation, if we would evaluate miR-21-5p expression in normal tissue, we only would have access to post treatment samples whose miRNA expression profile probably do not reflect a healthy tissue. Besides, the relative expression level that we are interested in is based on comparison among tumor samples only. A given miRNA can be upregulated in a given tumor sample when compared to the corresponding normal tissue, but still, when compared to other tumor samples, this miRNA might not have a high expression level. Finally, considering the clinical application of this predictive marker, it would be much more reasonable to access its expression on pretreatment tumor biopsies only (already collected for tumor diagnosis), without the need of testing the normal paired tissue.

Then the authors claim that “Using this approach, we found a strong negative correlation (r = -0.5 and p-value = 0.03) for SATB1 gene”. These claim implied that only SATB1 gene was found inversely correlated to miR-21. The author should provide data on others genes that were found inversely correlated to miR21.

Authors: We agree with the referee that the sentence should be clearer and we should provide more details. Thus, we have modified the manuscript and also
included a new table in the revised version (page 16 and Additional file 3: Table S3). Indeed, among all putative miR-21-5p conserved targets (in our whole transcriptome data we were able to detect the expression of 249 out of 307 predicted targets), only SATB1 expression was inversely correlated in our samples. We also included another table (Additional file 2: Table S4) containing the miR-21-5-p cpm data as well as the RPKM data of SATB1 and 5 of miR-21-5-p predicted targets (PTEN, MSH2, Cdc25A, SPRY2 and PDCD4), which have been claimed by other authors to be involved in treatment response in other tumors. As can be seen, indeed, only SATB1 is inversely correlated with miR-21-5p expression in our samples.

Moreover between early recurrence and incomplete response the miR 21 expression was found to be more or less the same. Authors need to discuss this issue on how early recurrence and incomplete response showed potentially the same molecular signature in their dataset. Are these entities only distinct by physician, but molecularly are the same?

Authors: Early local recurrence patients and clinical complete responders are very similar based on clinical and imaging exams. They are both easily distinguished from incomplete responders by physicians. However, as mentioned, it is most likely that early recurrence patients had clinically undetectable residual disease after nCRT. Our hypothesis is that although the physician cannot distinguish between early recurrence patients and clinical complete responders, they are molecularly different, specially regarding miR-21-5p expression. Therefore, considering that both incomplete responders and early recurrence patients still have viable tumor cells after nCRT, they might share molecular similarities such as lower miR-21-5p expression.

Finally, miR-21-5p overexpression could be considered a complete response biomarker. If used in the clinic, miR-21-5p expression should be evaluated in patients who seemed to present a complete clinical response. Hypothetically, if a given biopsy shows low levels of miR-21-5p expression, that patient should undergo surgery in spite of good clinical response after nCRT. We have included more information in the revised manuscript to make it clearer (see page 14).

Minor Points:
Page 9 the claim must be reworded “we analyzed the expression pattern of its target genes using whole transcriptome” as “we analyzed the expression pattern of its putative target genes using whole transcriptome” target scan such as other algorithms used for target prediction needs in vitro validation.
Authors: We agree with the referee and we have corrected the text in the revised manuscript (see page 15).

Discretionary Revisions
In methods section page 17 “Only miRNAs with a minimum of 20 cpm in at least seven samples were kept in the analyses.” My suggestion is corroborate this table, with min and max cpm value for each miRNA and preferably with a table supplementary table, that show cpm for each miR in each sample. This table could be helpful for the reader.
Authors: As suggested by the referee, we have provided this table with cpm values for all miRNAs that remained in our analysis after such filter. We have generated the table containing data from all samples (training and validation sets), including the average, minimum and maximum expression for each miRNA (See Additional file 1: Table S1).

Reviewer 2: Pedro Borralho
Reviewer's report:
Major Compulsory Revisions:
1. General approach by the authors is sound, and the clinical question is relevant. The identification of biomarkers to predict complete pathological response in rectal tumors, allowing the identification of patients not requiring surgery, would be extremely relevant. However, the present study presents some limitations, particularly relating to the size of sample populations used both for training and validation sets, which is quite limited. The inclusion of additional samples in both sets is highly recommended. The inclusion of non-responders in the analysis (therapy resistant) would also be interesting.

Authors: Firstly, we highly appreciated the careful reading and overall favorable evaluation of our work. We are thankful for the time and attention given by the reviewer on evaluating work. In the revised version of the manuscript we have highlighted in blue the additional information that was included to address the referee’s comments.

We agree that the number of samples is limited although we have performed differential expression analyses in two independent sets of samples (training and validation sets). Unfortunately we don’t have more samples that fulfill our study’s criteria, specially the minimum time of follow up for complete clinical responders (24 months).

It is worth mentioning that our incomplete response group is actually composed mainly by therapy resistant patients. As described on page 7 (section “Groups for Comparison”) we intentionally chose samples from patients that didn’t respond well to nCRT in order to increase the chances of finding molecular differences when compared to complete responders. We prefer to name such patients as incomplete responders because almost every patient presents some response to nCRT.

2. In addition, it would be important to validate the data by an additional independent method, for instance qRT-PCR, for hsa-miR-21 expression, to validate and develop a faster, more easily implemented, and cheaper method compared to deep-sequencing. (alternatively, in situ hybridization for mature miR-21 in FFPE sections may also be an alternative).

Authors: We totally agree with the reviewer and therefore we have performed qPCR analysis evaluating miR-21-5p in the 10 of the 11 samples that we used for validation of SATB1 expression (one of them there was no RNA available). It’s well known that normalization of miRNA qPCR data is challenging. We initially tested
commonly used small RNAs such as SNORD95 and RNU6b and observed a high variability in their expression levels among our rectal cancer samples. To overcome this problem we applied a similar strategy of others authors: we looked up in our deep-sequencing data for the most stably expressed miRNAs in our samples [Chang KH, et al. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. BMC Cancer. 2010;10:173 and Viprey VF, et al. Identification of reference microRNAs and suitability of archived hemopoietic samples for robust microRNA expression profiling. Anal Biochem. 2012; 421(2):566-72]. According to the deep-sequencing data, their expression levels measured by qPCR were considerably more stable than the other small RNAs and thus were combined for miR-21-5p normalization. Similarly to SATB1, probably due to the small number of samples, the difference between complete and incomplete responders isn’t statistically significant, although the complete responders present a higher expression. We added a new supplementary figure in the revised version of the manuscript (Additional file 4: Figure S1).

3. Regarding data from the “potential role of miR-21-5p on treatment response”, the analysis of the miR-21-5p putative target expression should be included, at least as supplementary data. Since it is widely known that miR-21 expression is increased in the vast majority of human cancers, and it is also widely associated with decreased response to chemotherapy, it is somewhat counter-intuitive that increased expression of miR-21 may be a biomarker for complete pathological response following neoadjuvant chemoreadiotherapy. Nevertheless, the suggested link between increased expression of miR-21 from incomplete responders to complete responders, associated to a concomitant decreased expression of SATB1 protein from incomplete responders to complete responders, may be relevant to provide mechanistic explanation to the data here reported by the authors. In this regard, the authors evaluated SATB1 expression by qRT-PCR in 11 samples, obtaining concordant results, although not reaching statistical significance. This data is relevant and should be complemented by evaluation of STAB1 protein expression (e.g. by western blot in total protein extracts obtained from tumor biopsies of incomplete and complete responders), and also by increasing the sample universe to use. Evaluation of protein expression is particularly relevant since miRNA target gene regulation in some instances does not lead to major changes in RNA abundance, but does in fact lead to abundant reduction of the target protein. If possible, parallel evaluation of RNA and protein from the same sample would be ideal, and currently there are simple methods allowing this simultaneous isolation and evaluation of both molecular species from the same tumor specimens.

Authors: We agree with the referee that more details regarding miR-21-5p putative targets should be provided. Thus, we have modified the manuscript and also included two tables in the revised version (page 16, Additional files 2-3: Tables S3-S4). As mentioned now in the manuscript, we were able to detect the expression of 249 out of the 307 putative miR-21-5p conserved target genes in our whole transcriptome data. Among them, only SATB1 expression was inversely correlated in our samples (p < 0.05). We included in this new table (Additional file 2: Table S4) the miR-21-5p cpm data as well as the RPKM data of SATB1 and 5 of its predicted targets (PTEN, MSH2, Cdc25A, SPRY2 and PDCD4), which have been
claimed by other authors to be involved in treatment response in other tumors. As can be observed, in our rectal cancer samples miR-21-5p expression is not inversely correlated to all of these other predicted targets.

Regarding STAB1 protein expression, we agree with the referee but, as mentioned above, we don’t have more fresh tissues to be tested by western-blot in parallel with SATB1 RNA expression and miR-21-5-p, since these samples were all biopsies and were completely used for RNA extraction. We have however, tried to evaluate SATB1 protein expression by immunohistochemistry in FFPE samples. Unfortunately we were unable to obtain reasonable staining with the antibodies tested (Abcam Anti-SATB1 antibody, # AB70004 and Santa Cruz anti-SATB1 (N14) antibody # sc-5989). Although we cannot ensure that the same results would be true at the protein level, besides all the evidences on the patients’ samples, our in vitro experiments strongly support the regulation of SATB1 gene expression by miR-21-5p. Finally, we believe that if SATB1 protein levels weren’t different among the transfected cell lines, we wouldn’t have observed a change on their sensitivity to chemoradiation.

Discretionary Revisions:
4. The authors data would be importantly complemented if they could perform experiments to demonstrate in vitro that miR-21 directly binds to SATB1 3’ UTR (e.g. luciferase reporter assays) and if possible, that increased miR-21 expression in rectal cancer cell lines does increase tumor cell response to chemoradiotherapy used in rectal cancer management. These aspects would importantly enrich the present manuscript, and increase its relevance. In addition, it would be important to include whole transcriptome data mentioned by the authors, from these samples regarding the expression of known and validated miR-21 direct targets, to better grasp all possible changes occurring in these miR-21 target genes.

Authors: We agree with the referee that the in vitro experiments are important. Therefore, we have performed in vitro manipulation of miR-21-5p expression in two different colorectal cancer cell lines. In one cell line, named SW480, which expresses low levels of miR-21-5p, we have transfected miR-21-5p mimic and observed a reduction on SATB1 mRNA levels (Figure 5B). In the other hand, the other cell line (HCT116), which expresses high levels of miR-21-5p, we performed transfection using miR-21-5p inhibitor and observed an increased expression of SATB1 mRNA (Figure 5C). These experiments were included in the revised version of the manuscript (see page 16).

More importantly, we have performed transient transfections in different colorectal cancer cell lines to manipulate miR-21-5-p expression and challenged them to chemoradiation. As can be observed in the revised version of the manuscript, increased miR-21-5p expression resulted in decreased expression of SATB1 as well as increased sensitivity to treatment (Figure 6).

As suggested by the referee, we have included additional tables (Additional file 2: Table S4) on the revised version of the manuscript in which the expression of SATB1 and other 5 miR-21-5p target genes is provided. Another table containing
the Pearson correlation for the expression of all predicted miR-21-5p targets was also provided (Additional file 3: Table S3). The data containing the complete whole transcriptome data is part of another manuscript, which has been submitted elsewhere. It will certainly be publicly available after publication.

5. The manuscript by Drebber et al, 2011 (INTERNATIONAL JOURNAL OF ONCOLOGY 39: 409-415, 2011) should be included in the discussion of the present manuscript.
Authors: As suggested, we have also discussed the above-mentioned reference in the revised manuscript (page 21).