Author's response to reviews

Title: MicroRNA signature associated with outcome of gastric cancer patients following chemotherapy

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Author's response to reviews: see over
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Editor
BMC Medical Genomics

Dear Editor,

Thank you for your recent letter indicating that our manuscript would be of interest to the readers of BMC Medical Genomics, but that it required extensive revisions. We appreciate the many excellent criticisms made by the reviewers and have revised our manuscript to address their points as described below.

**Reviewer #1**
Major Compulsory Revisions

1. The GC signature
   *Most of the differentially expressed miRs in the Gastric Cancer (GC) signature were previously reported*. This GC signature does not provide more much more information than what has already been published so far. The authors do not seem to make good use of GC signature data and should clearly state why this signature is important and how it is connected to the “chemoresistance” signature.

   We agree with the reviewer that we did not stress the novel findings of our manuscript. While we did expect to find many previously reported miRNAs in our gastric cancer vs. normal stomach tissue signature, we performed a revised analysis using new cutoff of \( P<0.05 \) and have identified novel gastric cancer-specific microRNAs that have not been previously reported as gastric cancer-specific (Table 2). However, the fact that our signature does overlap with what has been previously reported serves as validation of our results and the miRNA platform that we used. The novelty of this manuscript is the development of a predictor of response to CF therapy and its initial validation using a highly unusual and extremely difficult to obtain set of 8 pre- and post-treatment samples from patients who initially responded but later became resistant to CF therapy. The ability to predict who will or will not respond to standard therapy is an important clinical advance since predicted non-responders can immediately be placed on a different therapeutic regimen. This signature, of course, needs to be validated in larger, future studies.

2. The chemoresistance signature
   *The authors have to describe more clearly their findings, including the presence of several star (*) and clustered microRNAs (on chromosome 19) in their “chemoresistance” signature (Table 2).*
We agree with the reviewer that this important observation was not discussed in the earlier version of the manuscript. We have addressed this finding by pointing out the observations in the results and adding the following to the discussion:

Interestingly, we identified six *miRNAs that were associated with chemoresistance, including miR-518f*, miR-520a, miR-520d*, miR-519e*, miR-363*, and miR-517*, whereas no miRNAs were associated with chemosensitivity. Only one miR, miR-302*, was identified in the gastric cancer miR signature. miR*s are considered to be passenger strands that are thought to normally be degraded from the pre-miR which results in the mature 22 nt strand that enters the RISC complex. The functions of *miRNAs remain unclear, although it is possible that they result from impaired processing of pre-miRNAs (Tchernitsa et al J of Pathology, 2010) or may play a role in targeting mRNA translation (Gu and Lu, Plos One, 2010).

The proof-of-principle test set and Table3 should be part of the results.
We agree with the reviewer’s criticism and have revised the manuscript accordingly.

The authors have to provide quantitative PCR data for miR-122a and -195, which are the miRs with the most significant p value for disease progression prediction.

We agree with the reviewer that this would be a valuable validation of our results. However, the amount of RNA that we were able to obtain from these biopsies was extremely limited and we unfortunately do not have any remaining from the 8 sample pairs to perform these analyses.

3. Assays validation
The authors have to indicate how their assays were validated in the Materials and Methods or Results sections. Required information, which can be supported by the manufacturer’s data includes: 1. Sensitivity of the detection of known over-expressed miRs (e.g. miR-372 in AGS cells), 2. Specificity of the detection of miRs from the same family (e.g. let-7, miR-146), 3. Quantitative PCR validation of their microarray findings (e.g. miR-195, -122a, and -486).

We agree with the review that the important information about assay validation was not included in the original manuscript. We have added a new section to the Methods section to describe how this platform was validated and compared to other commercial miRNA microarray platforms. We have also included an Additional table and two Additional figures to provide data related to these comparisons.

4. miR-486 over-expression
It is required that the authors check the miR-486 expression in their sample sets (as in Figure 2) with the TaqMan MicroRNA Assay kit for miR-486 quantitation (preferred), as reported in Oh et al. Clin Cancer Res (2011). It is also required that the authors comment on their finding as the loss of miR-486 expression in the Oh et al. paper was supported by CGH data.

We have performed this assay as requested and have confirmed our results. We can not explain why we see upregulation of this miRNA in gastric cancer compared to normal stomach epithelium, whereas Oh et al. found that expression of this miR was commonly lost in their sample set. It is possible that the difference arises from different patient populations between the two studies or that Oh et al. compared gastric cancer with adjacent “normal tissue,” whereas we compared cancer patients with normal stomach tissue from healthy volunteers. Our microarray data was confirmed by RT-PCR experiments on 14 samples. We have added this point to the discussion.
I have noticed that several miRs in Table 2 have been renamed since the release 9 of miRbase (2006). miR-526c does not exist anymore, and seems to be miR-519c-5p (see MI0003148 in miRbase). Similarly, miR-524* has been renamed (miR-524-3p?). miR-486 has 2 arms but there is no indication in this table. miR-146 appears in the “underexpressed in cancer” category while miR-146a and -b are listed in the “chemoresistance”-related miRs. I strongly recommend the authors to contact the vendor(s) to get an updated version of the miR annotation.

We agree with the reviewer and have updated the annotations of the miRNAs in the text and tables.

6. Discordance regarding miR-25 between figure 2 and Table 2
The authors have to comment on the fact that miR-25 appears in their “overexpressed” list while miR-25 amount does not significantly differ between normal and tumors in Table 2.

We agree with the reviewer that this apparent discrepancy should be commented on. We believe this discrepancy is due to the small number of tumor samples (4) we could use for Q-PCR measurements compared to the large number of samples (90) we were able to analyze by array. One of the four tumor samples gave a higher value than the other three which resulted in a lack of significance. We have added the following to the results:

Although miR-25 was significantly up-regulated by array analysis in the tumors, this did not reach statistical significance by Q-PCR, likely due to the limited number of samples that were available for that assay.

7. miR star sequences = deficient pri-miR processing?
One important finding in Table 2 is the presence of several star (*) miRs:
“Chemoresistance” list: 8/28 – “Chemosensitive” list: 0/30. A possible explanation is that the processing of the primary miR transcript is impaired (See Tchernitsa et al., J Pathol, 2010). The authors have to discuss this result.

We agree with the reviewer that this observation deserves further discussion. We have pointed out in the results the observation that several *miRNAs were found in the chemoresistance list compared to miRNAs associated with chemosensitivity. We have added the following to the discussion:

Interestingly, we identified six *miRNAs that were associated with chemoresistance, including miR-518f*, miR-520a, miR-520d*, miR-519e*, miR-363*, and miR-517*, whereas no miRNAs were associated with chemosensitivity. Only one miR, miR-302*, was identified in the gastric cancer miR signature. miR*s are considered to be passenger strands that are thought to normally be degraded from the pre-miR which results in the mature 22 nt strand that enters the RISC complex. The functions of *miRNAs remain unclear, although it is possible that they result from impaired processing of pre-miRNAs (Tchernitsa et al J of Pathology, 2010) or may play a role in targeting mRNA translation (Gu and Lu, Plos One, 2010).

Minor revision #2
With regards to the question if less than 58 microRNAs can be used for chemoresponse prediction,
We agree with the reviewer that a robust predictor using fewer than 58 miRNAs would be advantageous. A permutation significance level for the log-rank statistic of leave-one-out cross-validated Kaplan-Meier curves was 0.021, suggesting that the association of the miRNA expression data to TTP is statistically significant. We also performed the same cross-validation analysis using smaller numbers of miRNAs, but using a smaller number of features could not adequately stratify patients according to their TTP. Hence, 2-5 miRNAs cannot replace the 58-miRNA predictor proposed in this paper, perhaps due to tumor heterogeneity.

Minor revision #4

With regards to the definition of prediction accuracy;
Prediction accuracy is defined as the percentage of samples whose predicted class label matches the actual class label.

Reviewer #2

Major revision

Referee #2

The methods seem to have a large arbitrary element, giving the impression of data fitting. Different p-values are used to define significance for miRNA differentially expressed between 90 tumours and 34 normal tissue samples (p<0.001), best performing predictors in the training set (p<0.005), and predictors of chemoresistance (p<0.05). miRNA validated by qPCR, and highlighted for discussion, are not the top ranked miRNA. In fact it is unclear which miRNA are the ‘best performing predictors’, as all that is reported are miRNA significantly differentially expressed between 90 tumours and 34 normal samples. It is difficult to appreciate an index if details on the components, their selection and their relative weightings are not disclosed. The thresholds for defining the number and proportions of cases in chemoresistance risk groups seem quite arbitrary as well. The selection of 8 cases for validation of the chemoresistance index does not comply with norms for dividing series into training and validation sets. The study needs to have more recognized, objective and consistent approaches to threshold setting and sample grouping, or improved justification of choices to improve confidence in the reliability of findings.

We thank the reviewer for pointing out important statistical issues to improve the paper. Regarding the use of different p value cutoffs, we agree that this could appear arbitrary and therefore, have revised the manuscript using the fixed cutoff of p<0.05 for all analyses. Hence, the controversial concept of “best performing predictor” was not used in the revised manuscript. We have added the following to the Results on p.5:

“To estimate the predictive power of cancer-specific miRNA profiles, class prediction analyses were also performed by randomly dividing the entire group of samples into two (training and test) subsets at a 1-to-1 ratio. Randomization was performed using nQuery Advisor software (version 7.0, Statistical Solutions, Saugus, MA). Then, the class label of each sample in the test set was predicted for each of 100 random training to test partitions according to a compound covariate predictor (CCP), diagonal linear discriminant analysis (LDA), 1- and 3-nearest neighbors (NN), nearest centroid (NC), and support vector machine (SVM). At a feature selection P<0.05, the median prediction accuracy in test sets was >90% in all classifiers (91.9%, 90.3%, 90.3%, 93.5%, 93.5%, and 91.9%, for CCP, LDA, 1-NN, 3-NN, NC, and SVM, respectively), in 100 random training-to-test partitions.

Table 2 lists discriminatory miRNAs differentially expressed between 90 tumors and 34 normal samples at the feature selection P<0.05. Many overexpressed miRNAs belong to
the miR-17-92 and 106b-25 clusters, as previously reported\textsuperscript{13,14}. In previous miRNA microarray studies in gastric cancer, adjacent non-tumor tissue was used as the control as opposed to tissue from healthy normal volunteers\textsuperscript{13,14}. Since molecular abnormalities are often found in histologically normal-appearing tissue adjacent to tumor tissue, true normal endoscopic biopsy samples obtained from cancer-free volunteers, as used in this study, are better suited for such comparative studies. Most of differential miRNAs of previous microarray studies\textsuperscript{13,14} were also identified as members of a cancer signature by the current analyses. In addition, tumor suppressor miRNAs which were not identified by these previous microarray studies (at $P<0.05$\textsuperscript{13} and $P<0.01$\textsuperscript{14}), such as miR-1\textsuperscript{15} and let-7\textsuperscript{16}, were included among our underexpressed miRNAs (at $P<0.001$) (Table 2). Quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR) analyses confirmed differential expression of these miRNAs (Fig 1B).

With regards to the training-to-test set allocation, conventional training and test allocation (2:1 or 1:1) was not performed, since 91 patient samples were estimated to be required for the training set only. Hence, we planned to use as many samples as possible for the training set to build a robust predictive model and test it using 8 \textit{pairs} of proof-of-principle test set samples (pre- and post-treatment). These 8 pairs of samples were collected from 8 clinical responders who underwent second endoscopic biopsies at the time of disease progression, thus serving as a means of validation for the chemoresistance predictor. Given the difficulty in obtaining such matched biopsy samples, we were only able to collect these samples from 8 patients who underwent second biopsies at the time of disease progression.

With regards to the cutoff for the predictive index percentile, time to progression is a continuous variable, and there is no good landmark time to define “rapid” vs. “delayed” time to progression. Among those patients with measurable disease, 63.1\% of patients had a clinical benefit from therapy (partial response and stable disease according to a radiographic response) and 36.9\% of patients experienced no clinical benefit (progressive disease). A predictive profile will be most useful if it can predict those patients who will not benefit from chemotherapy. Hence, two risk groups were delineated using a predictive index percentile of 67\% (1/3 of patients considered high-risk and 2/3 of patients considered low-risk). Please note that 6 out of the 8 pairs in the test set showing progressive disease following treatment were correctly identified as having chemoresistant tumors by the predictor (\textit{i.e.}, posttreatment samples were assigned a higher predictive index than pretreatment samples, and therefore, predicted to be more resistant), \textit{irrespective of} cutoff values dividing the risk groups.

As mentioned in the Discussion, let-7g and miR-181b microRNAs were found to be associated with chemosensitivity and are positively correlated with clinical responsiveness of colon cancer to S-1, an oral fluorouracil (Nakajima G, Hayashi K, Xi Y, et al. Non-coding MicroRNAs hsa-let-7g and hsa-miR-181b are associated with chemoresponse to S-1 in colon cancer. \textit{Cancer Genomics Proteomics} 2006;3(5):317-24). However, the molecular mechanisms responsible for tumor resistance to fluorouracil and cisplatin are not well understood. Even ERCC1 or thymidylate synthase, which are recognized markers for cisplatin/5-FU resistance, have not been consistently validated as predictive markers for chemotherapy resistance. Therefore, we avoided discussing potential microRNA-protein correlations since this seems premature at this time.

\textbf{Minor essential revision}

\textit{Minor essential revisions}
\textit{The decision to divide samples series based on patient enrollment used to derive signature (1) should be revisited. Time-related effects including sample stability differences, disease and patient management trends are possible over a 5-year span.}
We understand the reviewer’s concern. In the revised manuscript, we performed repeated analyses through randomization of the datasets. This was performed 100 times using randomized training-to-test allocations of the datasets. We then reported the median classification accuracies among the random datasets, in the revised manuscript.

The source of tissue samples (eg. department, hospital) should be disclosed.
The tissue samples were collected at the hospital of Korean National Cancer Center. This has been added to the manuscript.

The source of normal stomach mucosa from ‘healthy individuals’ should be disclosed as well, as states such as gastritis or inflammation could influence miRNA levels.
This has been added to the manuscript.

It is relevant to include confidence intervals and/or p-values in Table 2 to allow appreciation of the rankings.
This has been added to Table 2. We include 125 microRNAs that were significant at P<0.05

Clarification of quantification procedures for real-time PCR analysis is required.
The relationship between Ct differences and fold-change is logarithmic. The scales in Figure 2 and methods text make it unclear if this is appreciated.
We have added this clarification to the Methods.

It is unclear what the acronym “LMT” stands for. Evidence to support the specifications described for the custom array platform is required.
LMT stands for Laboratory of Molecular Technology. We have added this to the manuscript and added additional information to describe the custom array platform.

Discretionary revisions
The statement on sample size estimation needs to be clarified. It is unclear why the sample size estimation was considered valid, as it appears based on mRNA and not miRNA data.
We appreciate the reviewer’s comment. We have deleted the original statement about size estimation from the manuscript.

Commentary on the trends in Table 1 should be provided. There appears to be some significant differences in the frequencies of some clinicopathological features between training and test sets. What does relative dose intensity relate to? Why is there no median follow-up time for survivors disclosed for the test set?
Why is distant metastasis % displayed when the frequency is 100% in both the training and test sets. The formatting of Table 1 is irregular, making for difficult comprehension.

All of the test set cases demonstrated a clinical response, while part of the training set patients did. This analysis was not designed to have an equal distribution of clinicopathological features between training and test sets.

We added the following legend for Table 1;
-Relative dose intensity*
  *Mean of relative dose intensities of cisplatin and fluorouracil. Dose intensity is defined as the amount of drug administered per unit of time, expressed as milligrams per square meter per week. Relative dose intensity is defined as the actual dose intensity relative to the planned dose intensity of each drug.
There were no survivors among 8 patients in the test set, at the time of writing. We have removed the \% metastases from the table since all patients in this study had metastatic disease as is mentioned in the text. We have corrected the formatting of the table.

[Page 4, line 5] “proof of concept” changed to “proof of principle” to be more consistent.
We have changed all “proof of concept to “proof of principle” throughout the manuscript.

Throughout the manuscript, the text and are interchangeably used. The authors should be consistent in their annotation.
We have changed all “microRNAs” to “miRNAs.”

[Page 6, line 1] Reference not updated as miR-1 has been reported to be downregulated by Liu et al, Eur J cancer, 2011.
We have added this reference to the discussion where this section had been moved.

Figure 1. “Does prognostic index increased after chemotherapy” could be changed to “Does predictive index increased after chemotherapy” to be more consistent.
We have corrected the wording as requested by the reviewer.

We trust that we have satisfactorily addressed the many excellent points raised by the reviewers that have greatly improved the quality of the manuscript and that the manuscript is now acceptable for publication.

We look forward to your response.

Sincerely,

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