Author's response to reviews

Title: Concerted down-regulation of immune-system related genes predicts metastasis in colorectal carcinoma

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Author's response to reviews: see over
Dear editorial board of BMC Cancer,

Please find enclosed a resubmission of the manuscript “Concerted down-regulation of immune-system related genes predicts metastasis in colorectal carcinoma” by Marion Fehlker, Matthew Huska, Thomas Jöns, Miguel Andrade, and Wolfgang Kemmner after careful modification according to the reviewers’ comments.

The manuscript has been modified for resubmission as stated below in a point-to-point fashion. Significant changes within the manuscript are red marked.

Reviewer’s report (Feng Feng):

Major:
1) The generation of classifier might have an issue. It could be that the description is a little ambiguous. If I understand it correctly, the classifier depends upon large number of samples. It has to compare the specific sample signature gene with the median of many other sample signature genes. This limits the application of this method. In order to use this method, large number of microarray samples must be available so as to come up with an accurate median value for the gene. Or the authors have to propose a standard “median” value for each signature genes.

Reply of the authors: We totally agree that classifiers perform better if the number of examples available is large, but this depends also on the quality of the data to be classified. Hopefully, in our reply to minor point #3 from reviewer #1, the question of the quality of data becomes clearer. In any case, in our opinion the sample size that we use in our manuscript is comparable to what is usually available in similar studies presented in BMC Cancer.

2) There is no validation of the method/classifier. It might be better to apply the proposed method on a new set of samples to validate the method. Or if there is no other samples available, authors could consider using the bootstrapping/resampling approach.

Reply of the authors: Our method is justified by the result that we found a signature based on a small number of genes, which were then associated to a function that made sense from the biological point of view. We nevertheless validated the R-SVM method using cross validation as suggested by the referee: we did 5 runs of 5-fold cross validation of the method and the mean accuracy was 67.6%, mean AUC-ROC (area under the receiver operating characteristic) was 0.61 and mean AUC-PR (area under the precision-recall curve) was 0.79. ROC and PR curves were added as a supplementary figure and these performance values have been included in the figure legend.

Minor:
1) It is better to present the qPCR results and also compare them with the microarray data. For example, are qPCR results showing the comparable difference/decrease between recurrent and non-recurrent samples as seen in the microarray data?

Reply of the authors: According to the suggestions of both reviewers, the qPCR-data were included. Please see Materials &Methods section 2.5, and Results section 3.3. Both chapters were modified and supplemented with regard to the calibrator, and the statistical analysis. Moreover, a table (Table 4) describing the sample characteristics and a figure (Figure 4) showing the results of the qPCR of the five genes were included. The results of the qPCR
analysis show that the expression of these genes is lower in cases with later metastasis for all of the examined genes, thereby confirming the microarray results.

2) What does the result look like for the principal component analysis on the identified signature genes between recurrent and non-recurrent samples? Can the PCA clearly separate the samples from different patients?

Reply of the authors: The PCA of the hybridization values of the probe sets of the signature genes indicates a trend but does not separate recurrent from non-recurrent samples. We include the graph as supplementary material.

3) In figure 2B, simply summing the normalized values of 14 genes doesn’t seem meaningful. The authors should consider removing this or find meaningful explanation.

Reply of the authors: Figure 2b was removed.

4) The discussion is a little too long. Be concise and make it shorter.

Reply of the authors: The discussion section has been condensed strongly. The inherent problem of such microarray studies is that there are always a large number of key genes which need to be discussed at least briefly.

Reviewer's report (Andreas Scorilas):
Major Compulsory Revisions

1. Which calibrator did the authors use in qPCR? This should be stated in the Materials and Methods section.

Reply of the authors: According to the suggestions of both reviewers, the qPCR-data were included. Please see Materials &Methods section 2.5, and Results section 3.3. Both chapters were modified and supplemented with regard to the calibrator, and the statistical analysis. Moreover, a new table (Table 4) which describes the sample characteristics and a new figure (Figure 4) which depicts the results of the qPCR of the five genes were included. The results of the qPCR analysis show that the expression of these genes is lower in cases with later metastasis for all of the examined genes, thereby confirming the microarray results.

2. In the Materials and Methods section, the authors mention: “Correlations between clinical parameters and gene expression as measured by qPCR were analyzed by Mann-Whitney test.” This is false: Mann-Whitney U test cannot used for testing the existence of correlations. Mann-Whitney U test is used for the comparison of the distribution of a variable between two cohorts of samples. The existence of correlations is tested using Spearman’s analysis. Therefore, this should be corrected appropriately.

Reply of the authors: The reviewer is right. This has been corrected and modified accordingly in Materials and Methods section, 2.5 on page 5.

3. Using qPCR, the authors validated their microarray results regarding only 5 genes of the gene signature that they propose. They should also verify the rest of the genes of this signature in the same number of samples.

Reply of the authors: In our view, the fact that the qPCR of 5 genes was in complete agreement with the microarray data shows another time the high quality of the microarray data. Thus, we don’t think we need more assays to confirm the microarray results. An examination of all 14
genes of the 55 patients by qPCR would need to do about 630 assays in triplicate and with a replicate at least. Another problem is that for most of the cases we don’t have any more samples. Since the Tumorbank of the former Robert Roessle Hospital is no more collecting samples, unfortunately, we are not able to get more/new samples for such a study.

4. Figure 2B does not make sense. In more detail: The “sum of normalized expression values of the 14 immune response genes in non-recurrent (n = 33) and metastatic (n = 12) cases; p = 1.69×10^-4 (Mann-Whitney Test)” does not have any biological sense, as expression levels of different genes cannot be added to give a sum. This should be completely changed or removed.

Reply of the authors: Figure 2b was removed.

Minor Essential Revisions

5. The order of figures is currently wrong. Figure 3 appears first (not third) in the text. Moreover, Figure 3 bears the legend “Figure 3. ROC curves”. Nevertheless, Figure 3B is NOT a ROC curve, but a Kaplan-Meier Curve. Thus, it would be better to rename Figure 3B into Figure 4, write the appropriate legend, and state it separately in the text.

Reply of the authors: The text (page 5) has been changed according to the reviewer’s comments, so that Figure 3 appears in the right order. Indeed, because Figure 3B is not a ROC curve the title was changed, which is now “Predictive value of the immune response signature”. Because Fig. 3B depends on Fig. 3A we suggest keeping both sub-figures together in one figure.

6. The whole manuscript should be carefully checked for English language (grammatical and syntax) errors and these should be corrected.

Reply of the authors: The manuscript has been checked by a native speaker.

Thus, we hope that we were able to address your concerns in a sufficient way. Thank you for giving us the opportunity to resubmit our paper for publication in BMC Cancer. We believe that this study is another step in our efforts for early detection of those patients who later will suffer from metastasis. We hope that this will be of interest for the readership of BMC Cancer.

Thank you for your consideration.

Sincerely yours,

Wolfgang Kemmner, PhD

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