Author's response to reviews

Title: Genome-wide analysis of three-way interplay between gene expression, cancer cell invasion and anti-cancer compound sensitivity

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Author's response to reviews: see over
Dear Dr. Claire Tree-Booker,

On behalf of my coauthors, I am submitting the revision of "Genome-wide analysis of three-way interplay between gene expression, cancer cell invasion, and anti-cancer compound sensitivity" (MS: 8072089817355886).

We have followed your suggestions to modify the manuscript. We would like to thank all three reviewers for their insightful comments which have led to a great improvement of the presentation. We have incorporated the reviewers’ suggestions and performed additional analysis. An item-to-item response to show how we answer the reviewers’ questions is attached. We also have asked a native English speaker to help edit the manuscript.

Thank you very much for kindly giving us the opportunity to revise our Manuscript. We are looking forward to hearing your favorable decision soon.

Sincerely yours,

Ker-Chau Li
Corresponding Author
Response to Reviewer 1

1. In the Background, (para 2) the authors state that they intend to characterize genes associated with invasion heterogeneity and drug sensitivity heterogeneity. Heterogeneity is an important issue in cancer biology these days. Yet I am confused by the authors’ approach to addressing heterogeneity in the cell lines they use. As seen in Figure 2, it appears that there is heterogeneity among cell lines within a tissue type. In fact, there are about 12 of the 60 cell lines that each is significantly higher in their invasiveness than the mean for their tissue type. Without labels, it’s unclear which specific lines these are, but they include 2 RE lines, 1BR, 2OV, 2LC, 1ME, 1PR, 2CO and 1CNS line. It would be interesting, if not essential to analyze expression in these specific lines to see whether the relevance of the 8 gene signature is confirmed. To me this provides a ‘truer’ signature related to the common phenotype of invasiveness in these particular cell lines. Furthermore, regarding Figure 2b, I am unclear as to the relevance of this histogram to the manuscript. What’s your point in including a histogram of this type, and what exactly do you mean by ‘within group residual’; does it refer to tissue of origin? Also regarding this figure and the manuscript in general, since you ultimately focus this paper on lung and breast cancer cohorts, analyses specifically focused on these cell lines would be appropriate.

Our response:

Thank you very much for pointing out the confusion in our original submission. To help the clarification of our approach, we have clearly stated the four aims of this study (see Background: third paragraph) in the revision. Please note that the clinical application is our last Aim (Aim 4). Although we eventually focused on lung and breast cancer cohorts, this is only because we are unable to find other cohorts suitable for Aim 4. Our Aims 1 to 3 are not restrictive to the lung and breast cancers.

It is our first aim to identify the set of invasion-associated genes whose expression levels are likely to be indicative of the invasion potential of a tumor. The purpose of showing Figure 2b (Figure S1 in the revision) is to support our viewpoint that tissue of origin might not be an essential factor in characterizing invasion heterogeneity between cancer cell lines. We agree that the term “within-group residual “ is unclear, although it is not uncommon in statistical literature of ANOVA. It does refer to how the invasion count of each individual cell line deviates from the average values of the cell lines of the same tissue origin. To clarify the confusion, we now used “ within-group residual ICC” (invasion cell counts). Because the dispersion of group means is smaller than the dispersion of the individual cell lines’ deviations from their respective group means and because the
number of cancer cell lines in each tissue group also varies greatly, we are unable to
detect statistically-significant differences between the high and the low invasion cell
lines in each tissue group separately for our 8-gene signature. But our 8-gene
signature does correlate significantly with the invasion profile when all 53 cancer cell
lines are considered.

Although one may argue that there is some visual subjectivity in detecting the gap
between high and low values of invasion counts, we agree that special attention to this
group of cell lines can help elucidation of our gene signature. From the following
table, we can see that the mean expression of each signature gene in the group of the
12 cell lines identified by the reviewer is higher than the mean expression of other cell
lines.

**Table:** The mean expression of each of the 8 signature genes in 12 cell lines of high
invasion and that of the remaining 41 cell lines.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Higher invasion group</th>
<th>Lower invasion group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGA3</td>
<td>0.244376656</td>
<td>-0.071524875</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.230951851</td>
<td>-0.067595664</td>
</tr>
<tr>
<td>RAI14</td>
<td>0.150239852</td>
<td>-0.04397264</td>
</tr>
<tr>
<td>NNMT</td>
<td>0.227677731</td>
<td>-0.066637385</td>
</tr>
<tr>
<td>MYLK</td>
<td>0.149838926</td>
<td>-0.043855295</td>
</tr>
<tr>
<td>IL32</td>
<td>0.085524973</td>
<td>-0.025031699</td>
</tr>
<tr>
<td>AHNNAK</td>
<td>0.272119342</td>
<td>-0.079644685</td>
</tr>
<tr>
<td>GLS</td>
<td>0.235319711</td>
<td>-0.068874062</td>
</tr>
</tbody>
</table>
2. Regarding Figure 3A, the authors need to show on the left axis the location of the dividing line between the probe panels for invasive ability (positive vs negative) of the NC160 lines. Also, there appear to be common responses in invasive abilities among the cell lines. For instances, the first 14 cell lines appear to share common expression patterns across the genes probes evaluated. Why is this so? Is it related to the specific cell lines (i.e. RE7860, RBT549, etc)? Furthermore, are these specific cell lines the same cell lines that show high invasive characteristics in Figure 2A?

**Our response:**
Thank you very much for the suggestion. A dividing line that separates the top probe panel with negative invasion-expression correlations from the bottom probe panel with positive ones is added. Thank you very much for pointing out the clustering of the first 14 cell-lines in gene expression. Yes, this is indeed because the cell lines are ordered according to the invasion counts, highest on the left and lowest on the right. The first 14 cell lines as detected by the reviewer’s keen eyes are indeed more invasive than others and they matched those showing the highest invasion characteristics (regardless the tissue types) in Figure 2A (Figure 2 in the revision). Because of the positive (negative, respectively) correlation with the bottom (top, respective) panel of the IA genes, the expression levels of these more invasive cell lines tend to be higher (lower, respectively) than other cell lines. This resulted in the similar expression patterns for these 14 cell lines.

3. Regarding Figure 3b: this Figure is superficial, poorly described, confusing and its purpose is unclear. A better description of the data presented is definitely required. Furthermore, the specific patterns of the eight signature genes should be enlarged and added as a Figure 3C so we can specifically see the ‘complementary’ responses that are described in the text.

**Our response:**
Thank you very much for the criticism. We have added more description about how Figure 3B (Figure 5A in the revision) is constructed and what the purpose is. Following the reviewer’s suggestion, we have added Figure 3C (Figure 5B in the revision) to highlight the pattern on the eight genes.

4. Figure 4 is a generic, commercial pathway diagram that was copied (and uncredited) from the Metacore webpage. It contains too much irrelevant information. Furthermore, the relevant red labels are difficult to identify and
locate. This figure must be redrawn and simplified to support the manuscript. Are the eight signature genes present on this Figure?

**Our response:**
We have redrawn the pathway diagram (Figure S2 in the revision) which highlighted the relevance to the eight-gene signature and two drugs we used in the study.

5. The probe selection criteria discussed (in Results: Selection of invasion and drug sensitivity associated genes) are poorly described. This section must be re-written.

**Our response:**
Thank you very much for the suggestion. We have provided detailed information to clarify how our genes are selected in the revision (see Results).

6. In the Results (Validating gene signature with independent cell lines) you refer to previous work regarding 29 lung cell lines exposed to paclitaxel and docetaxel. Did these include any of the cell lines you used on the NCI-60 panel? A similar question regards the previous study looking at breast cell lines. If so, did you look specifically at your 8-gene signature in relation to these specific cell lines?

**Our response:**
Thank you very much for the nice suggestion. As it turns out, a total of 9 cell lines out of the 78 validation cell lines are from NCI-60 cell lines. Although these validation data sources were independent from the NCI-60 data sources, certain degree of consistency was observed. See Supplementary Table S1. We conducted an additional comparison analysis by removing these cell lines. The result showed that not much was changed. Please see Supplementary Figure S5.

7. In the Results (Clinical Outcome prediction; last para), your statement that ‘patients predicted to have higher metastasis potential and to be more drug-resistant had a significantly increased risk for poor survival’ is not surprising and hardly seemed provocative as an adequate interpretation of the results.

**Our response:**
Thank you very much. This paragraph was not written well. We have modified it in the revision.

8. Overall, the Discussion does not provide a convincing description of the
methods applied or the relevance of the data generated. You state in the Discussion (para 2) that a previous study from your group (reference [40]) generated a 4-gene signature for clinical outcome prediction. How do the data generated by the present study correlate with your previous study? In the Discussion (para 3), you infer that combined use of anti-MT drugs with dasatinib/erlotinib may increase efficacy. What literature supports this conjecture? Has this been tested in clinical trials? Furthermore, much of the Discussion reads as a literature review without sufficient integration of the impact of the authors’ new data as it relates to the field.

Our response:
Thank you very much for the constructive criticism. We have revised the Discussion session completely by incorporating your suggestions. The relationship with the previous 4-gene signature in lung cancer study is discussed. We have shown 4-gene signature did not work for chemotherapy patients (Figure S7 in the revision).

The revision now focused on the relevance of our data in discussing the efficacy issue of dasatinib/erlotinib and anti-MT drugs. We provided literature showing the synergistic effect of combining anti-M and targeted therapy agents, including clinical trials.

Minor Essential Revisions
1. In the Results (Gene-Drug heat maps for compound selection) the MYB and TOB1 genes are mentioned, as are others, yet these cannot be identified or located in Figure 3. Also in this paragraph on page 14, please define or clarify what you mean by saying that the ‘efficacy of the two groups of compounds are complementary’ … perhaps you mean inversely correlated?

Our response:
Thanks for your suggestion. We have labeled MYB and TOB1 in the Figure 3 and Figure 5 in the revision.
We have rewritten the paragraph. It meant “inversely correlated” as the reviewer said.
Response to Reviewer 2

1. The major clinical implication of this work rests on their ability to predict outcome in two recent adjuvant chemotherapy cohorts including a vinorelbine containing regimen for lung cancer and a taxane-containing regimen for treating invasive breast cancer. The authors should demonstrate that the predictive capacity of their 8 genes signature is specific to the cancer treatments in those studies. A negative control is needed. How do the 8 genes perform as predictors of outcome in the many publically available gene-expression datasets of lung and breast cancer where clinical outcome data is available?

Our response:
Thank you very much for the very nice suggestion. We have conducted the additional studies as suggested. For breast cancers, we found three cohorts without adjuvant chemotherapy to use as the negative control. As anticipated, the 8 gene signature failed to predict clinical outcomes. For lung cancer, we reconsidered the GSE14814 lung cancer cohort used in our original version. This cohort has two part groups of patients: OBS (After surgery, patients under observation), and ACT (After surgery, patients treated with adjuvant chemotherapy). We had shown that our 8-gene signature worked for ACT in our original submission. Now we used OBS as negative control and found the eight-gene signature failed. A section on Control Cohort is added and the survival curves were shown in the Figure S6.

2. It is unclear what their rationale was for developing a gene-expression signature predictive of response to those specific 5 anticancer therapies. They mention that they selected the “richest” drugs from Figure 3b, but a clear rationale for the selection of these 5 drugs is needed. Additionally, why did the authors attempt to develop a single signature (composed of 8 genes) for all 5 drugs? Why not develop separate gene-expression signatures that would best predict response to each drug (i.e. 5 distinct signatures)?

Our response:
Thank you very much for these questions which help us to present a better description about how we used Figure 3b to guide the decision of drug selection. Visually, the three targeted therapy drugs formed a cluster and the two anti-MT formed another cluster. We now provided the correlation matrix in Table S4 to confirm our observation. As it turned out, paclitaxel and docetaxel have much higher correlation than others in the anti-MT group of drugs. Similarly, among the targeted therapy drugs, Everolimus, Dasatinib,
Erlotinib have the highest correlations between them. We added Table S4A and S4B to help the explanation. The section on compound selection was rewritten. Figure 3b is called Figure 5A in the revision.

In defending our decision to find one signature for five drugs, our reasoning is that while it certainly makes sense to find one gene signature for each drug separately, we think that after obtaining five separate gene signatures, an immediate question to ask would be what genes are shared by these signatures. Our procedure is one way of providing the answer. The core set of genes between targeted therapy and anti-MT can lay down the foundation for studying the potential cross talk between different pathways affecting drug sensitivities.

Of course, it would still be interesting to ask what genes are predictive of one drug but are not predictive of another drug among these selected drugs. However, our data might not be informative enough to make such subtle distinctions. For example, paclitaxel and docetaxel have very strong correlation (=0.7) in their drug response profiles.

3. The authors show that the 8 gene score differs between drug resistant and drug sensitive cell lines (figure 6). Can their 8 gene biomarker accurately classify cell lines into resistant vs. sensitive in those studies?

Our response:
No. Thank you very much for pointing out the limitation of our gene signature. There are substantial overlaps as can be seen in Figure 6. This indicates that drug response in cell lines is a very complex phenotype and is not fully characterized by our gene signature. Other types of genetic markers besides gene expression such as SNPs, DNA copy number, methylation, microRNA etc., might be helpful but we have not examined such possibilities yet. The lab environment is also a factor but it is hard to assess the effect.

4. In the discussion, the authors conclude that in a neoplasm, the subpopulation of tumor cells with higher metastasis potential, which are harder to eradicate by anti-microtubule compounds, may be more likely to succumb to the targeted therapy. Given the heterogeneity in invasive potential of subpopulations of cells within a tumor that is profiled, how would their 8-gene signature work in the clinic? Wouldn’t there be differing sensitivities to the drugs among the various subpopulation of cells whose expression profiles are averaged? More discussion on this issue is needed.

Our response:
Thank you very much for asking this important question. We have given more discussion on this issue in the completely revised Discussion.

5. What was the statistical cutoff and FDR for the 633 genes associated with invasion?

Our response:
Thank you very much. We have added a section to describe procedure for finding the 633 genes, see Supplementary Information Text I. Because there were two gene expression datasets available (U95 and U133), we applied to two stage procedure. First we used the U95 (the older chip) at screening stage and the used U133 chip (the more popular chip) in the confirmation stage. We estimated the statistical FDR in confirmation stage to be about 0.08.
Response to Reviewer 3

1. The functional enrichment analysis should follow the “identification of 633 invasion-associated genes”.

Our response:
Thank you very much for the suggestion. We have rearranged sections according to the reviewer’s suggestion.

2. The gene-drug analysis is a complicated one and the specific aim or advantage of performing the analysis in this manner is not clear. First, the aim should be clearly stated. Second, if the goal is to identify drugs that preferentially target invasive tumor cells, would not a more straightforward approach simply be to correlate invasion with drug response? Once the drugs have been identified, genes correlated with response to these drugs could be explored to identify predictive signatures for each drug.

Our response:
Thank you very much for pointing out the weakness in our presentation. We clarified the aims of our study in the revision. The reviewer kindly offered an alternative way of studying the relationship between drug response, invasion profile and gene expression. A discussion on the rationale difference between these two approaches is given in the Discussion section.

We have four aims in mind, which were stated in the Background section. The four aims are
(1) Molecular markers of tumor invasion potential: to identify the set of IA genes whose expression levels are likely to be indicative of the invasion potential of a tumor;
(2) Drug sensitivity prediction by tumor-invasion markers: to evaluate how the expression levels of IA genes in a tumor are likely to be indicative of the tumor’s resistance or responsiveness to an anti-cancer drug;
(3) Drugs discovery with predictable sensitivity: to find anti-cancer drugs whose efficacies correlate with tumor-invasion potential and can be predicted by tumor-invasion markers;
(4) Clinical validation: to demonstrate the use of IA gene signature for predicting clinical outcome.

Our approach is based on the observation that invasion ability and drug sensitivity are
both phenotypes of the cell lines available for study. Each phenotype is naturally associated with its own set of molecular determinants. We hypothesize the potential overlap between the set for invasion ability and the set for drug response. If we are able to identify these common determinants, then we may measure these shared determinants to estimate the cancer cells’ overall invasion potential in a tumor and also used it to predict the drug response at the same time. However, the tumor microenvironment in a patient is different from the growth environment of cancer cell lines monitored in the lab. The robustness of the invasion molecular markers becomes an important factor for increasing the chance of success in clinical applications.

We agree that the reviewer’s suggestion is a natural way of analyzing the data. But an important reason that we did not pursue this line of analysis much further is because the phenotype-phenotype correlation can be weaker than phenotype-genomic determinant correlation. As a matter of fact, our data showed that most drug-invasion correlations appeared weak, only four drugs showed statistical significance. Among them, the highest two drugs only had correlations of 0.39 and -0.35. Our approach overcomes the limitation of weak phenotype-phenotype correlation by looking for statistical evidence of correlations directly from the phenotype-genomic determinant correlation. This helps improve the robustness of the genetic marker thus obtained.

3. The authors initially describe 17 drugs which have the highest number of correlated probes. The statistical basis for limiting this number to 17 is not provided. The 17 drugs are then further refined to 5, although again, the rationale for this refinement is not clear.

Our response:
Thank you very much. We did not explain it well before. In the revision, We have provided more information to explain the rationale behind these decisions. Briefly, we studied the number of invasion-associated genes that are correlated with the sensitivity of each drug. We found that on the average, the number is higher for tubulin-binding agents and the targeted therapy agents. We then focused on the 17 compounds from these two group of agents. There are three reasons for the further reduction to the five compounds: (A) the number in these five are higher than others; (B) the opposing patterns of correlation observed (C) molecular similarity between the two anti-microtubule agents.

4. Among these 5 drugs, the 2 anti-tubulin and 3 targeted agents show opposing patterns of correlation for several genes in the heatmap. The authors interpret
this by stating that the “compounds tend be complementary”. It is not clear how this conclusion is made, or whether they meant “contrary” instead of “complementary”. This should be clarified.

**Our response:**
Yes. It meant “contrary”. We have made the clarification in the revision.

5. In the context of the study I think it is important to formally demonstrate that invasive cells are more sensitive to microtubule-targeting agents and more refractory to targeted therapies. This could be done using the existing data.

**Our response:**
Thank you very much for the suggestion. In Table S5, we showed the negative correlation between invasion profiles and the drug sensitivity profile of each of the anti-MT agents and the positive correlation with the target therapy drugs.

6. The authors settle on a list of 8 genes following application of various cutoff criteria. The average expression of these 8 genes was applied to microarray data from cell line panels treated with paclitaxel, docetaxel, dasatinib and erlotinib. In each case, the 8 gene signature separated the sensitive and resistant groups. While it does appear to be the case, can the authors clarify whether the orientation is as expected? (higher expression of the signature in paclitaxel/docetaxel resistant cells, but lower expression in dasatinib/erlotinib resistant cells)?

**Our response:**
The orientation is consistent with what the signature predicted because in the NCI-60 data, all of the eight genes have positive correlation with the sensitivity of dasatinib and erlotinib and have negative correlation with the sensitivity of with paclitaxel, docetaxel. At the molecular level, the exact mechanism involved is still unclear to explain the directions of correlation. However, EGFR (one in our 8 gene signature ) is the key target of erlotinib. See also Figure S2 for how erlotinib and dasatinib interact with EGFR and MYLK.

7. Were any cell line or patient cohorts tested that did not yield a statistically significant result?

**Our response:**
Per suggestion by another reviewer, we made the additional tests on four control cohorts in which the patients were systemically untreated. We found our eight gene signature
failed the test. There additional results were shown in Figure S6.

Minor revisions

1. Authors should state how many times the invasion assay was performed for each cell line and what the reproducibility is of the assay.

Our response:
The experiment was repeated three times (n=3). We used the average ICC in Figure 2.