Author's response to reviews

Title: Cell cycle and aging, morphogenesis, and response to stimuli genes are individualized biomarkers of glioblastoma progression and survival

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Author's response to reviews: see over
Dear Editor

We wish to express our appreciation to the reviewer Dr. Kun Yu and the editor Dr. Jen-Tsan Chi for their thoughtful comments and suggestions. We have addressed all the recommendations and modified the manuscript accordingly. Below is a point-by-point reply to each recommendation.
Do not hesitate to contact us if further information is needed. We appreciate your support.

Sincerely
The authors

MS: 1171187099493423
Cell cycle and aging, morphogenesis, and response to stimuli genes are individualized biomarkers of glioblastoma progression and survival
Serao et al.

Essential Editorial Requests:

Copyedit
- Please get your manuscript copy edited. Perhaps a colleague, preferably a native English speaker, could look it over for you. Alternatively, we can supply the contact details of a professional copy editing service.

REPLY. Agreed. The revised manuscript was reviewed in three independent occasions by experts from the University of Illinois, The Center for Writing Studies Writers workshop (http://www.cws.illinois.edu/workshop/) that provided writing assistance. Our manuscript was reviewed by consultants who are experienced writers and teachers of writing. In addition, three co-authors of this manuscript are native English speakers (Kristin Delfino, Bruce Southey and Jonathan Beever) and also proof-read the manuscript.

Section Editor comments:

The reviewer has stated that the work is interesting and potentially worthy of publication. However there is a concern regarding the purported superiority of the five step approach described in this study compared to other more conventional analysis methods. In their revised manuscript, the authors are strongly encouraged to address this specific point in their revision.

REPLY. Done. The data from the three survival indicators were reanalyzed using the alternative approach proposed by the reviewer and the results were compared to the original five-step approach. The five-step approach was consistently superior to the simpler approach (one-step Cox analysis with P-value < 0.001) on all three survival indicators based on the overlap of results between approaches and the confirmation of results in independent studies. For the three variables studied; overall and post-
diagnostic survival and post-diagnostic progression (renamed following recommendations of the reviewer to lifetime, overall and progression-free survival, respectively), the simpler approach identified 60, 71 and 67 probes of which 19, 17, and 23 respectively overlapped with the corresponding 61, 47, and 60 probes identified in the five-step analyses of the three survival indicators. Of the 139 probes identified by the simpler approach and not identified by our approach, the vast majority (123 probes across all three variables) have not been associated with glioblastoma.

The results from the additional analysis and comparison to the five-step approach were included in the Results section (prior to Functional and Gene Network Analyses).

Referee 1
Reviewer's report
Title: Cell cycle and aging, morphogenesis, and response to stimuli genes are individualized biomarkers of glioblastoma progression and survival
Version: 2 Date: 26 February 2011
Reviewer: Kun Yu
Reviewer's report:
Comments on Serao et al: “Cell cycle and aging, morphogenesis, and response to stimuli genes are individualized biomarkers of glioblastoma progression and survival”
Major comments:
1) The key component of this paper is to apply five-step approach to “reduce the dimensionality of the data set caused by the large number of probes and few records of the individuals in this experiment”. Later (page 8) the authors concluded that “this approach would have the same effect than reducing the threshold in the first step with the added benefit of minimizing the loss of true positive”.
To support their statement, I suggest that authors should add one section in the Result or Discussion to demonstrate the superior of five-step approach over one-step Cox with stringent cut-off. For instance, the authors may conduct one-step Cox separately; compare the output between two approaches; and comment on whether the difference reflects the increase of true positives.

REPLY. Done. The data from the three survival indicators were reanalyzed using the alternative approach proposed by the reviewer and the results were compared to the original five-step approach. The five-step approach was consistently superior to the simpler approach (one-step Cox analysis with P-value < 0.001) on all three survival indicators based on the overlap of results between approaches and the confirmation of results in independent studies. For the three variables studied; overall and post-diagnostic survival and post-diagnostic progression (renamed following recommendations of the reviewer to lifetime, overall and progression-free survival, respectively), the simpler approach identified 60, 71 and 67 probes of which 19, 17, and 23 respectively overlapped with the corresponding 61, 47, and 60 probes identified in the five-step analyses of the three survival indicators. Of the 139 probes identified by the simpler approach and not identified by our approach, the vast majority (123 probes across all three variables) have not been associated with glioblastoma.
The results from the additional analysis and comparison to the five-step approach were included in the Results section (prior to Functional and Gene Network Analyses).

2) It’s not clear to me why independent validation cannot be performed because of the different cohort structure (page 10). These reported prognostic markers (particular cohort-independent ones) should hold true in other GBM patients. There are a few studies (such as REMBRANDT (Madhavan et al, 2009) and Phillips et al (2006)) on the patient population similar to TCGA.

REPLY. Done. We appreciate the helpful references to the REMBRANDT database and the manuscript by Phillips et al. (2006). Following the suggestion of the reviewer we used the gene expression information in REMBRANDT to confirm our findings. Although the glioblastoma data was not available for download from REMBRANDT (https://caintegrator.nci.nih.gov/rembrandt/download.do?method=caarray), we queried the database for associations between survival and the 10 genes with the most extreme hazard ratio estimate for each of the three survival variables studied that did not interacted with other cohort variables. The query was performed using the Kaplan-Meier survival plot for Gene Expression Data. Of these, 8 have the same significant trend observed in our study, 4 did not have information on the REMBRANDT database, one gene had a trend opposite to the one observed in this and in previous independent studies (Fstl1), and the rest and the rest were not significant although most had the same trend observed in our analysis. The latter results are consistent with the simpler analytical approach based on Kaplan-Meier curves available in REMBRANDT compared to the more flexible Cox survival analysis used in our study. The Kaplan-Meier approach relies on non-parametric rank-based test to compare the survival between individuals with high and low gene expression. These groups are obtained by setting up an arbitrary expression threshold. Non-parametric rank-based approaches tend to have lower power to detect significant variation than semi- and parametric approaches such as the Cox survival analysis. In addition, the Kaplan-Meier analysis only allows the consideration of one explanatory variable at a time and this variable has to be discrete (thus the reason for comparing high and low expression groups in REMBRANDT). This approach does not allow the simultaneous consideration of multiple continuous covariates (i.e. gene expression) and factors (e.g. race, gender, therapy, progression) or interactions. The Cox-survival analysis implemented in our study allows the consideration of multiple factors, covariates and interactions simultaneously and it does not require the discretization of the gene expression values that could result in potential loss of information. Thus, the Cox approach used in our study is able to capture the association between continuous gene expression values and survival conditional on all other model terms and is able to detect associations that are likely not to reach statistical significance using the Kaplan-Meier comparison of survival between high and low gene expression groups. The absence of information on race, therapy or progression severely hampered the use of the Phillips’ data for confirmation. Specially, the lack of information on race hindered the testing of the association between genes and survival adjusting for potential population stratification by race.
The comparison of the association between genes and glioblastoma survival detected in this study and available in REMBRANDT was presented in the last paragraph of the Materials and Methods and the results were included on the final paragraph of the Discussion section of the manuscript (page 20, cross-validation paragraph).

With respect to the consideration of independent validation, validation of the predictive hazard equation and risk factors detected in a training data set on an independent test data set is desirable, followed by X-fold cross-validation. Our predictive hazard functions include the cohort information such as race and therapy (in addition to the interactions between genes and cohort levels) and the estimates are adjusted by this information. The representation of all races, genders, therapies and recurrences on training and test sets was necessary since the hazard prediction function included adjustments for all the cohort variables to ensure that the detected biomarkers were not a spurious artifact of ignored cohort effects. Consideration of race is particular critical for validation of the results in this study because lack of adjustment for this cohort factor could result in the identification of associations that are due to genetic background and not the particular gene expression profile. Accurate validation of the biomarkers would require the consideration of an independent test data including similar information to that in the training hazard predictive equation and these data sets are not available. Likewise, the size of our data set forbids a X-fold cross-validation because there are insufficient individuals across cohort factors to partition the data into training and test sets with adequate representation of factors. Leave-one-out-crossvalidation (LOOCV) is specially recommended in data sets of limited size, providing an almost unbiased estimator and identifying the same best classifiers as other X-fold training-test data partitions (Chuang et al., 2009 OMICS, Petrausch et al., 2008). Thus, we opted to use LOOCV to confirm the results of our study. LOOCV allows the consideration of the same cohort information used to obtain parameter estimates and train the hazard predictive equations.

We modified the Cross-validation paragraph in the Materials and Methods section to clarify the strengths of the cross-validation approach for the particular data set analyzed.

3) “overall death” defined by authors here is the period from birth to death. This is quite different from standard definition of “overall survival”, which is same as the ‘post-diagnosis survival” defined by authors. One question is whether “overall death” simply reflects aging. In fact, authors found that top GO for genes associated with “overall death” is aging (Table 9). I wonder whether these markers are generally associated with aging, rather than GBM patients. Authors may further check the association of these markers with ‘overall death” in other diseases or healthy population.

REPLY. Done. First, the term “overall death” was replaced with “lifetime” across the manuscript to remove all possible confusion, and “post-diagnosis survival” was replaced by “overall survival”. Second, there is evidence that the gene profiles associated with lifetime survival, which resulted in the enriched functional category of aging, are not simply reflection of the natural aging process. Two results confirm that our results are
not mere confounding with aging. First, the genes in the GO terms "aging (GO:0007568)" and "cell aging (GO:0007569)" Pdcd4, Cdkn2a, and Tp53 have all been associated with GBM in previous independent studies. Please refer to Table 3 that lists independent studies supporting each gene identified in this study. In addition, Tp53 was associated with progression-free survival (Table 7). Second, the biological processes of cell death and cell cycle were enriched for both lifetime and progression-free survival.

The previous discussion of the evidence supporting association between genes on the aging biological process category and lifetime survival was added to the manuscript on the Discussion section (page 22).

4) this is a suggestion: authors may want to explore the association between the reported biomarkers and molecular subtype of GBM, which has been reported to associate with prognosis of GBM (TCGA paper, REMBRANDT paper and Phillips)

REPLY. We agree with the reviewer that further studies that would allow the identification of gene profiles associated with survival corresponding to specific glioblastoma types will shed additional insights into the genomic regulation of this disease. We have addressed this on the Conclusions section and included the three references on relevant studies to the manuscript.

Minor comments:
1) leave-one-out cross-validation may overestimate the performance since tumor population is heterogeneous. Could authors try 3-fold or 10-fold cross-validation to better reassemble the reality?

REPLY. Please refer to the reply to 2). Briefly, X-fold validation approach, the specification of suitable training and testing data sets would have required at least 200 patients in each data set (5 patients x 2 races x 2 genders x 5 therapies x 2 recurrence groups) and only 287 patients were available. The representation of all races, genders, therapies and recurrences was necessary since the hazard prediction function included adjustments for all the cohort variables so as to ensure that the gene associations detected were not a spurious artifact of ignored cohort effects. Thus, the LOOCV approach was implemented. LOOCV is recommended specially in data sets of limited size, providing an almost unbiased estimator and identifying the same best classifiers as other X-fold training-test data partitions (Chuang et al., 2009 OMICS, Petrausch et al., 2008).

We added to the manuscript the rationale for using LOOCV to address the reviewer’s suggestion.

2) for the reported genes (Table 2-8), could authors explicitly state which has been reported to be linked to GBM?

REPLY. Done. Tables 3-8 include for each gene identified to be associated with survival, an indicator of a previous study reporting a similar association in glioblastoma (superscript G) or to other cancer (O) and the associated reference to the study (number between square brackets).
A description of the literature review information available in each table was added to the footnote of each table.

**Level of interest:** An article of limited interest  
**Quality of written English:** Acceptable  
**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.  
**Declaration of competing interests:**  
I declare that I have no competing interests