Author's response to reviews

Title: Prognostic prediction of glioblastoma by quantitative assessment of the methylation status of the entire MGMT promoter region

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Version: 3 Date: 2 August 2014

Author's response to reviews: see over
Dear Editor,

We are submitting a revision of our manuscript entitled “Prognostic prediction of glioblastoma by quantitative assessment of the methylation status of the entire MGMT promoter region“. We added Katsumi Nakanishi as an author, because he performed experiments to answer the query from the second reviewer. Following the suggestion from the reviewers, the manuscript was subjected to a language-editing service (Nature Publishing Group Language Editing Service). Response to reviewers’ comments is as follows. Changed parts are marked with red characters in the marked up manuscript.

The first reviewer:
In this manuscript, authors describe a classifier method based on MGMT methylation to classify glioblastoma patients into good or poor prognosis categories. They performed Sanger sequencing and MiSeq sequencing to generate MGMT methylation data and devised a methylation score called M-score, which is weighted sum of methylation proportion of certain CpG sites where weights are coefficients of univariate regression analysis of methylation against overall survival or progression-free survival.

The results of the manuscript show that their scoring metric could classify the groups pretty well. However, there are some additional works needs to be done before I could determine whether the work is publishable or not.

Below are my detailed comments.

**Minor essential revisions**

pg 14 line 4: "A total of 96 colonies of each sample" whereas in pg 16, line "up to 96 molecules of the MGMT promoter region from each sample". Please be clear about number of colonies per sample.
We revised the corresponding parts according to this comment. The number of colonies that yielded sequence data was shown in the second line from the bottom of page 16.

pg 17, line 1: hierarchical clustering method is not described in detail. Any standardization on the data before clustering? What kind of linkage method was used?

We added short description in page 17.

In Figure 1A, the column bars below the clustering indicate the MSP results for 53 samples? I don't see a definition of how MSP is computed.

MSP results were judged by visual inspection. We did not apply digital or image analysis for MSP results interpretation. We added comment in page 12.

Major compulsory revisions

pg 20, line 4: The authors use the methylation proportion of CpG51 - CpG74 sites to build their diagnostic model. However, they do not explain why only these sites are selected. All the results are based on this decision so it must be clearly justified.

This is due to restriction of read length of Illumina sequencers. We added explanation in page 20.

pg 20, line 16: The choice of M-score threshold is not clearly described. The authors describe that the threshold that minimizes the log-rank p-value is chosen however there could be more than one threshold value for the minimum log-rank p-value. How do they choose the final threshold value for each iteration of LOOCV.
We added the rule for the same p-value in page 21.

pg 22, line 9, multivariate cox regression analysis. In this analysis, age, gender and several other covariates are included and only M-score and extent of surgical resection were significant predictors. However, age is known to be an independent significant prognostic factor of survival in glioblastoma. The authors should address why they do not find age as a significant factor.

We added discussion on this subject. Page 25, first paragraph.

The second reviewer:

Major Compulsory Revisions
The biggest flaw in this MS is that while the authors had the fortune to work with frozen tissues, the majority of diagnostics is done on FFPE DNA. Thus in the nested PCR used for the next generation protocol, the outer primers amplify a PCR product size of 289 base pairs which is not optimal for FFPE DNA which is often highly fragmented. A recommendation of a region of 150bp or shorter thus should be made.

We designed a new set of primers, and confirmed the validity of primers using DNA extracted form FFPE tissue. The method and result are shown in Additional File 4. In the text, we described in the second paragraph of page 23.

Minor Essential Revisions
MGMT should be italicised when referring to the gene.

Fixed as suggested.
The sentence “Only cluster 1 and cluster 4 (p=0.00491) and clusters 2 and 4 (p=0.0204) had statistically significant associations with PFS (Figure 1C).” should be rewritten as it is misleading as it stands.

We rewrote the sentence in page 18.

Discretionary Revisions

In the statement “we built a classifier to predict the malignancy of GB”, outcome might be preferable to malignancy.

It would have been useful to mention the MGMT SNP promoter SNP in the context of false positive MSP results. E.g. see McDonald, et al. The T genotype of the MGMT C>T (rs16906252) enhancer single-nucleotide polymorphism (SNP) is associated with promoter methylation and longer survival in glioblastoma patients Eur J Cancer (2013) 49 2 360-8

We added this reference in page 26.

Although the authors state “It would be preferable to utilize information from all methylation sites.” their results show that specific regions are the best predictors of OS.

We rewrote the sentence in page 9.