**Referee: 1 Comments to the author(s)**

The manuscript details determination of the MGMT promoter methylation status in glioma using nanopore sequencing. Multiple datasets are analyzed and compared to the results of the original MGMTp methylation classification by distinct methods commonly used. The overall conclusion is that nanopore sequencing is a good and rapid method to determine the MGMTp methylation status for diagnostic use.

The methodology is well described, and the source code for data analyses is made available. The limitations of the study are discussed.

This is an interesting manuscript, and the analyses are well performed and described.

Specific comments:

1. How do the authors envision the classification for individual patient samples using the nanopore sequencing. - Amend manuscript text “Every institution has it’s own standard operating procedures and cut-off points as to how patient samples are classified as MGMT methylated or unmethylated. Here we provide a framework for how nanopore sequencing can be used to evaluate MGMT promoter methylation in the context of established methods (the MGMT-Pyro kit and STP-27 classifier) or based on full sequencing of the MGMT promoter CpG -island (for example by hierarchical clustering of samples). We also provide MGMT promoter methylation values for 160 samples. Institutions and research groups are encouraged to download our data and compare our results to their own samples. Although we believe the method holds great promise, further evaluation is necessary to decide the optimal way to classify patients according to nanopore sequencing.”
2. The issue of the grey zone samples is not addressed, it would require large datasets to have the statistical power. The grey zone certainly plays a role when decisions of omitting TMZ are addressed e.g. in clinical trials. - An interesting yet difficult comment. “ Grey-zone samples continue to be an active area of research and debate. The MGMT methylation grey-zone is poorly defined as it is influenced by the method of detection. As the reviewer points out, our data lack the necessary statistical power to provide a definitive answer as to how grey-zone samples should be treated. ...”

- Can we annotate which of our samples are defined as grey-zone?

- if so, do the grey-zone samples cluster together?

- The additional information provided via nanopore sequencing may provide data that can improve our understanding of grey-zone samples.

- Nanopore sequencing data is comparible between labs and platforms, pooling data from multiple studies will with time provide a solid foundation for future studies.

Additional comment

The reference list is messed up (likely because the first 2 references have been fused and all references thereafter seem to be wrongly annotated in the text). Hence the rational for parts of the text cannot be followed, as the proper references cannot be linked to get respective background information. The origin and quality of cutoffs chosen in this work from the literature cannot be looked at. Since this is a crucial part for the study, this needs to be corrected for proper evaluation of this work. - We apologize for this embarrassing mistake, which is a product of converting the manuscript text between different formats. The reference list has been fixed in the reviewed version.

The tables at the end of the document cannot be evaluated due to the unfortunate formatting that cuts the tables into multiple pieces. - We apologize again for the unfortunate formatting of the tables. This error is a product of the Wiley online submission platform that does not provide the submitter with a final version to review before final submission. We have uploaded a finalized .pdf version of the manuscript that we hope will find it’s way to the reviewer.

**Referee: 2 Comments to the author(s)**

MGMT promoter methylation status remains the most important predictive biomarker for treatment of IDH-wildtype glioblastoma. The authors performed comparative MGMT promoter methylation analysis using nanopore sequencing, methylation microarray and pyrosequencing. The study provides important performance metrics for nanopore sequencing and is of strong interest to neuropathologists. The study is well written and well illustrated.

Specific aspects:

1. - An important factor to guide implementation of nanopore sequencing for MGMT promoter analysis would be the minimally required read depth for reliable quantitative estimation of promoter methylation. A dedicated analysis to derive a cut-off using e.g. random downsampling would be very valuable. -This is an valid point. We have now added a paragraph and supplementary figure on read depth analysis.

Examples of overall smoothed methylation patterns from 100 reads to 10, 5 or 2 reads can be seen in (Supplementary figure 1A). To estimate the effects of decreasing read depth in the MGMT CpG-island on sample classification, we performed random downsampling of all samples with over 20 reads 10 or 5 reads per sample and re-classified the samples via the MGMT-Pyro logistic regression model described above. Of the 29 samples classified as “unmethylated” based on the full sequence depth, 27 (93%) maintained this classification through downsampling to 10 and 5 reads. Of the 13 samples classified as “Methylated” based on full sequence depth, 11 (85%) were stable through downsampling to 5 reads.

1. - For logistic models, it is not clear how training and test cohorts were chosen (retrospective nCATS vs other, Rapid-CNS vs other). Would not random splits of the entire cohort be more appropriate? - Good observation, our approach needs more clarity. The objective of the logistic regression models (figure 2c and 3b) was to show that extracting the relevant CpG sites for either the MGMT-pyro kit or STP27 can be used to accurately predict the classification as long as the “known status” was established using the same sites. However, the regression models perform poorly when used to predict the outcome of different classifiers. The text and figures have been amended accordingly.
2. Should maybe non-glioblastoma cases be excluded from regression model fitting/validation because they appear to show different methylation patterns across the entire island (see point below) and predictive value is only relevant for IDH-wildtype glioblastoma? - This is a valid point. Ideally, all our samples would have been IDHwt-GBMs. This is unfortunately not the case as only 103 of our 165 samples (62%) are in fact IDHwt-GBMs. We were therefor faced with the choice of excluding a substantial part of our samples because they may not be clinically relevant (as we did in figure 5) even though the have relevant data for the comparisons or including them under the assumption that they provide relevant information for MGMT promoter methylation classification. In this study we chose the latter although arguments can certainly be made for both routes.
3. - Hierarchical clustering (Fig 4a) suggests that the majority of IDH-mutant gliomas have a distinct methylation profile compared to MGMT-methylated IDH-wildtype glioblastoma (showing similar methylation approximately along CpG sites 1-14 and 65-98, but distinct methylation along CpG sites 20-54 approximately). This is an interesting finding that might warrant further analysis and discussion (e.g. hierarchical clustering of CpG sites of the CpG island). - This is an interesting observation by the reviewer and worth a closer look. To this extent we have examined clustering of IDH-glioma samples in more detail, for example by clustering of CpG sites as suggested. It is true that methylation of IDH-glioma and GBM samples appears to differ in the central region of the CpG island but there is also in general somewhat lower methylation in the cluster containing most of the IDH-glioma samples. We also performed the clustering with only methylated IDH-glioma and IDHwt-GBM samples and observe two main clusters; one, characterized by lower methylation. included 17 of 25 IDH-glioma samples (68%) and 16 of 39 IDHmut GBM samples (41%) while the other, characterized by higher overall methylation, contained 8 of 25 IDH-glioma samples (32%) and 23 of 39 IDHmut GBM samples (59%).

We also analysed the data via principal component analysis (PCA). As expected, known MGMT methylation status was the main separator of samples. No separation of IDH-glioma samples and IDHwt-GBM was observed within the first 3 principal components. Finally, we performed supervised clustering via partial least squares discriminatory analysis (PLS-DA) to specifically search for patterns that might separate methylated IDHglioma samples from methylated GBM samples. Some level of separation is apparent but there is still considerable overlap between IDHglioma samples and GBM samples. To conclude; while the observation is interesting, the MGMT methylation pattern separating GBM and IDHgliomna samples is subtle. We believe it does not warrant further discussion in the manuscript at this point. Our primary focus is to show that data generated via nanopore sequencing covering the MGMT promoter is as good, if not better in separating methylated samples from unmethylated samples.

1. - Figure 3: In analogy to Figure 2a, it would be interesting to see the correlation between nanopore methylated allele frequency and microarray beta value for the two CpG sites of the MGMT-STP27 model. -We completely agree, a more through comparison of nanopore sequencing data and methylation bead array data is warranted. Unfortunately, the array beta values are not available to us.
2. - Line 110f: The authors claim that coverage differed between methylated and unmethylated cases in the adaptive sampling cohort. Have MGMT copy number and genome-wide mean depth been accounted for? (There likely is a strong correlation between MGMT/chr10 copy number, read depth and IDH-mutant glioma, which usually are MGMT methylated.) - An excellent observation. We assume the reviewer is referring to the gain of chromosome 7 and loss of chromosome 10 typically observed in IDHwt-GBM and if this may influence MGMT promoter read depth in methylated versus unmethylated samples. Of the samples analyzed via adaptive sampling, 49 of 67 were IDHmut GBM samples (40 of which were classified as unmethylated) while 13 of 67 were IDHmut glioma samples (12 of which were methylated). This does in fact create a bias where unmethylated samples are far more likely to have loss of heterozygosity on chromosome 10 (and by extension the MGMT gene) while methylated samples are likely to retain both copies. We have added this into the discussion.

Minor aspects:

1. - Abstract: “95%” is not an appropriate metric for correlation. Please consider rephrasing as agreement or concordance or provide a correlation coefficient. - This has been corrected in the abstract.
2. Figure 3a: the axis range should be 0-100. - This has been corrected.
3. - STP27 probe and pyrosequencing CpG locations could maybe be indicated in Figures 4a, 4b, 4c and 5a in consistent fashion (annotation track) as in Figure 1a for easier recognition. - This has been implemented, the STP-27 and MGMT-Pyro sites have been annotated in figure 4a in the same way as in figure 4b. A column annotation track has been added to the heatmaps (figures 4c and 5a)
4. - In heatmaps (Fig. 4a and 5a), it might be interesting to indicate MGMT status separately for pyrosequencing vs. STP27 and also include their quantitative readouts. - A row annotation track has been added to figure 4c that indicates how the samples were originally classified (MGMT-Pyro or STP-27). We do not feel that including this annotation track to figure 5a (heatmap of only GBM samples) adds value. Regarding the quantitative readout of these assays, we agree with the reviewer that this could have been a valuable addition. However, as stated in the comment above, we do not have access to the beta values for the samples classified via the STP-27 classifier, these samples (67 of 165) would therefor have missing values in the annotation track.







