Reviewer's report

Title: Why are results on the prognostic value of the methylation status in colon cancers conflicting? The role of the preservation method

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This manuscript presents a comparison of the performance of bisulfite conversion and pyrosequencing methylation between 40 matched colorectal FFPE and cryopreserved tumors. The methods describe a number of quality control measurements and comprehensive methods for the analysis of methylation of LINE-1, MLH-1 and MGMT. Reproducibility was assessed using measurements on serial bisulfite conversions from the same sample and comparisons between sample types. The authors conclude that the variation in measurement on FFPE samples could explain the conflicting results regarding CIMP and colorectal cancer, and should not be used for methylation analyses. The authors took great care to quantify reasonable expected experimental error with serial measurements on cryo-preserved DNA – a strength of the analyses. However, there are several mitigating concerns regarding the analyses and conclusions that weaken the significance of the paper considerably.

Major Compulsory Revisions

1. While there was considerable care taken with quality control of the samples, the authors only required 40% tumor cellularity for analyses. However, most studies require >70% cellularity for methylation analyses. It is possible that variation in tumor cellularity between FFPE and frozen samples can account for the variation in methylation measurements.

2. DNA from FFPE tumors display greater degradation and fragmentation. Thus, many experimental modifications can be made for methylation analyses of these samples compared to DNA from frozen tissue. These include a greater input of DNA for bisulfite conversion, decreased annealing temperature in the amplification step and modified primers for the PCR and sequencing steps. This is why many laboratories that conduct pyrosequencing for methylation analyses do not rely on commercially available assays, particularly for FFPE DNA.

3. It would be useful to report the number of samples that failed to amplify based on gel electrophoresis of the PCR reaction vs. failed pyrosequencing.

4. The proportion of failed bisulfite conversions is troubling, particularly with respect to the frozen samples – do the authors have any explanation? It appears that the conversions failed for some assays but not others in the same sample; could this be due to experimental error in pyrosequencing or flawed assays?

5. The difference in methylation values between FFPE and frozen tumors was often (but not always) a large difference (> the arbitrary 6%) but the absolute
difference does not correspond to a biologically meaningful difference. For example, for MGMT, tumor 3 had values of 57.5% and 71.9% for cryo-preserved and FFPE tissue, respectively. This difference could be due to a difference in tumor cellularity. This indicates that, more likely, one should be cautious in interpreting the absolute levels of methylation in both FFPE and cryo-preserved samples as, barring analysis of laser microdissected pure tumor cells, these analyses measure a mixed cell population.

Level of interest: An article of limited interest

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests'