Author's response to reviews

Title: Quality assessment of DNA derived from up to 30 years old formalin fixed paraffin embedded tissue (FFPE) for PCR-based methylation analysis using SMART-MSP and MS-HRM

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Version: 2 Date: 1 October 2009

Author's response to reviews: see over
Reviewer's report
Title: Quality assessment of DNA derived from up to 20 years old formalin fixed paraffin embedded tissue (FFPE) for PCR-based methylation analysis using SMART-MSP and MS-HRM
Version: 1 Date: 7 July 2009
Reviewer: Catherine Gratas
Reviewer's report:
Major Compulsory revisions

1) No data in fresh or frozen NSCLC tissues are presented to validate their methods in this type of tissue. This prevents comparison of their results with any other published data and of the efficiency of the techniques in paraffin versus frozen.


Furthermore, we do not have frozen material from these patients, as the samples are dates up to 30 years back in time. It is therefore not possible to present data on this type of tissue for this sample cohort.

2) It is not precised if the histology and quality of the tissue (necrosis, % of tumoral tissue) was checked before extraction of DNA.

This has been performed by the pathologist and a discussion of these results relative to the methylation content of the samples can be found in the revised version of the manuscript.

3) For a study in paraffin-embedded samples usually easily available, the initial number of samples is not sufficient: as all samples are not methylated, the number of tumors by groups to assess the reproducibility is then too small. Moreover many tumors have very low percentage of methylation.

We have now included additional 16 samples in the study. Given the low p-values we obtained when comparing the quantitative results of the two methods in the original manuscript, we did not find this strictly necessary. However, we believe that including more samples of even older dates has reinforced our conclusions, so that we now go 30 years back in time as opposed to 20 years in the original manuscript.

4) For DNA study, DNA quality from paraffin-embedded is always a big issue. However in immunohistochemistry it is well known that the quality of the samples is good enough. A correlation between % of methylation and immunostaining of the protein in the same samples would reinforce the conclusion.
We have performed immunohistochemical staining for *CDKN2A (p16)* for all samples including the 16 additional samples included in the revised version of the study, and correlated these results with the level of methylation. We found a strong correlation between methylation and negativity by immunohistochemistry, and thus we agree with the reviewer that these additional experiments have reinforced the conclusion of the article.

5) It is unclear to me if the triplicates are done at the level of the PCR or at the bisulfitation level. The reproducibility level should be studied on both levels.

This has now been made clear in the manuscript. The reproducibility in between bisulfite modifications have been studied for many PCR based methods (including MS-HRM) in the literature and does not give rise to much variation. We have bisulfite modified a subset of the samples a second time, and obtained results that matched the results derived from the first bisulfite modification.

6) The method of calculation of 2##CT is not well described. The formulae should be clearly presented.

The formulae are now clearly presented.

7) The authors do not comment on the clinical relevance of very low percentage of methylation. Is it clinically significatif? They do not comment on samples with 0% methylation. Was the quality of DNA correct?

The samples with 0% methylation is amplified when using MS-HRM and melts together with the 0% control. Samples that did not amplify using MS-HRM are scored as “failed amplification”. In SMART-MSP, the ALU control measures the quality of DNA after bisulfite modification. Samples that are scored as 0% when using SMART-MSP did all amplify in the control assay. This has now been made clearer in the text. Whether or not low level methylation is clinically significant is beyond the scope of this publication.

Minor revisions
1) The methylation *CDKN2A (p16)* and *RARB* genes is not well presented in the context of NSCLC. We have to wait until the end of the discussion to get one reference to this subject.

We appreciate this comment, and have now moved the discussion on the methylation of the *CDKN2A (p16)* and *RARB* genes in the context of NSCLC to the beginning of the discussion.

2) Some references are not well introduced in material and methods

We agree, and this has now been corrected.

3) Fig4: the non template controls do not appear clearly

The non-template controls were not amplified in any of the assays and thus does not have a melting curve.
4) For the SMART-PCR the authors do not explain why they used Alu primers instead of Col2A1 primers, used in the paper they refer to.

This has now been explained in the revised version of the manuscript.

5) The e-mails of the authors are missing
E-mails of the authors are now found on the title page.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**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.

**Reviewer’s report**

**Title:** Quality assessment of DNA derived from up to 20 years old formalin fixed paraffin embedded tissue (FFPE) for PCR-based methylation analysis using SMART-MSP and MS-HRM

**Version:** 1  **Date:** 7 July 2009

**Reviewer:** Hongdo Do

**Reviewer’s report:**
In the present study, the authors used two HRM-based technologies (MS-HRM and SMART-MSP) to assess the utility of DNA extracted from FFPE tissues for methylation studies. The methylation status of two commonly methylated genes, CDKN2A and RARB, was determined in non-small cell lung cancers by the two techniques. This study is novel in that the HRM-based techniques are directly compared to assess the DNA quality of FFPE tissues. It was shown that both MS-HRM and SMART-MSP can be used for quantitative methylation studies of DNA samples extracted from formalin-fixed paraffin-embedded tissues up to 20 years old. The following issues should be further addressed by the authors.

**Major Compulsory Revisions**

1. The authors tested the reproducibility of SMART-MSP for the CDKN2A and RARB genes and concluded that the reproducibility of the technique did not decrease with the age of samples. However, the RARB results in Figure 5 show very high intra-sample differences as seen in samples 4, 6, and 30. There are about 10 samples (10/23) having 5-fold or higher difference when the reproducibility is considered within the individual samples. As the same sample and conditions were used in the triplicate testings, it is very unlikely to have 5-fold or higher difference if the assays are reproducible. If the authors fail to verify the reproducibility of a given assay within a sample, how can the reproducibility of samples from different storage time periods be reliably compared? Does high variation in the estimate of methylation indicate that SMART-MSP is not reproducible in this context?
The high intra-sample differences seen in samples 4, 6, and 30 are due to the fact that relative differences have been plotted. The absolute differences for these samples are actually quite small (2.3%, 3.6% and 2.7% respectively). On the other hand, if absolute differences are plotted, samples showing high methylation levels seem to show high intra-sample differences in spite of showing very low differences when relative differences are plotted. For this reason, it makes more sense to plot absolute differences relative to the average methylation estimate. This has been done in the revised manuscript.

2. Figure 5 should be plotted relative to the absolute values of methylation.

As explained above, it makes more sense to plot absolute differences relative to the average methylation estimate as samples with high levels of methylation otherwise would be interpreted as not being reproducible compared to samples of low methylation level even when this is not the case.

3. In Table 2, estimates of methylation for RARB are up to 186.6%. This should be addressed.

This has been addressed in the revised version of the manuscript.

4. The authors’ statement that they developed the methodologies is both true but also misleading. Two of the authors were involved in the development of MS-HRM and SMART-MSP while they were employed at the Peter MacCallum Cancer Centre. It might be inferred that the development was done at the University of Aarhus. For this reason, it is preferable for this statement to be omitted.

The sentence is changed.

Minor Essential Revisions

5. Standard dilution series were prepared by mixing of methylated and unmethylated controls. The unmethylated controls were prepared by two rounds of whole genome amplification of DNA extracted from peripheral blood. The authors should give more details to describe how they prepared the series of dilutions.

This has been done in the revised version of the manuscript.

6. In this study, FFPE DNA was extracted from whole sections without microdissection. As DNA from non-tumour cells is present and thus each sample is likely to be contaminated by normal DNA, the levels of methylation in this study do not represent the methylation levels present in the tumour cells. Information on tumour percentage in each sample should be provided in order to fully interpret the results. It is preferable to test high purity tumour samples for methylation as methylation of both CDKN2A and RARB genes in normal lung tissues has been reported in individuals with NSCLC (Feng, 2008, Cancer Epidemiol Biomarkers Prev). The occurrence of methylation in normal tissue adds an additional degree of complexity in determining methylation levels.

The amount of tumor tissue and necrosis in the sections were determined in order to evaluate if low level of methylation were due to low amount of vital tumor or influenced by
the amount of necrosis. However, we found no difference in area of necrosis or vital tumor between the tumors with low, medium or high level of methylation. This may be due to the fact that methylation of both \textit{CDKN2A} and \textit{RARB} in adjacent normal lung tissue has been reported as pointed out by the reviewer.

7. The authors are advised to omit Figure 3. It shows very little information except the melting temperature information which is described in the text.

Figure 3 has now been omitted.

8. The name of the gene studied in Figure 4 should be included in the figure legend.

This has now been done.

\textbf{Level of interest}: An article whose findings are important to those with closely related research interests

\textbf{Quality of written English}: Needs some language corrections before being published

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

\textbf{Declaration of competing interests}:

I declare that I have no competing interests.