Reviewer's report

Title: hTERT promoter activity and CpG methylation in HPV-induced carcinogenesis

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Reviewer: Elizabeth R Unger

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As hTERT expression is important in cervical carcinogenesis, the authors explore the activity of hTERT regulatory sequences in HPV transformed cells. They determine the methylation status of hTERT regulatory sequences in primary keratinocytes, HPV immortalized cells, cervical cancer cell lines and cervical samples from the spectrum of cervical neoplastic progression to evaluate whether methylation could play a role in hTERT regulation. The study is well designed and of significant interest to those addressing mechanisms of hTERT regulation and cervical carcinogenesis. The authors present a lot of information and several areas require clarification.

Major Compulsory Revisions

1. The prior studies of hTERT promoter methylation are summarized in the introduction, but without sufficient detail to understand the specific regions and CgG sequences analyzed in the prior and current studies. Additional diagrams of the regulatory regions for which methylation was reported to repress hTERT transcription (ref. 15 and 17), show no correlation with transcription (ref. 12, 13) and increase transcription (ref. 11, 14) are needed, along with those targeted in current study, in order to evaluate how consistent or inconsistent the findings are. Presumably the 200 base pair core promoter region described in introduction p. 4 and the core promoter region -150 to +150 described on p. 5 are the same, but the flow of information makes it unclear.

2. The authors use luciferase reporter constructs of potential regulatory regions of hTERT to directly demonstrate the regions that repress expression. They then use bisulfite sequencing and quantitative methylation specific PCR to determine if methylation of sequences associated with hHERT repression correlate with the grade of cervical neoplasia (normal through dysplasia and cancer). Testing samples from patients does determine whether methylation occurs during cervical neoplasia, but only indirectly indicates the role of this methylation. Direct modification of the targeted Cpg sequences in the luciferase reporter constructs would provide direct evidence that modification would release the inhibition. Conclusion states “CpG methylation of transcriptionally repressive sequences in the hTERT promoter and proximal exonic sequences is associated with deregulated hTERT transcription”, however this has not been fully demonstrated. There is a correlation of methylation with disease progression which in other studies has been correlated with hTERT expression. In the absence of direct
demonstration of CpG methylation with transcription this conclusion should be tempered.

3. The DNA isolation and bisulfite treatment methods are cited, but it would help the reader to provide a brief summary of how the different types of samples were handled. In particular, for the formalin fixed paraffin embedded (FFPE) material: How many sections of what thickness were extracted? Was H&E verification of the lesion in sections before and after those used for extraction done? What proportion of the tissue was lesion versus non-lesion? For cell scrapes, what was the method of preservation or fixation and what volume of cells were extracted?

4. More details are needed to understand the qMSP method. Reference 40 is cited (citation is incomplete in references), but that reference in turn refers to other papers for details. The authors need to clarify which CpG group or groups are targeted by the primer pairs and whether the probe also targets CpG groups. This information is needed to know how thoroughly the CpG groups in M1 and M2 are targeted by the assay. In addition, the method used to quantify the product needs clarification. What is the quantity ratio? How many assays were “discrepant” requiring 4 replicates?

5. The authors compare methylation of 4 regions in keratinocytes, HPV immortalized cells and cervical cancer lines with clinical samples representing varying degrees of neoplasia. They conclude that methylation is less frequent in the clinical samples, but fail to consider that the cervical scrapes, even from the cancer, are a mixture of cells with only a minority actually representing the lesion. In addition it is hard to be certain that the few cervical scrapes examined can be representative. It is clear that these assays were used to guide the regions examined by qMSP in a larger sample set, but the limitation to the observation about methylation in clinical samples should be noted.

Minor Essential Revisions

1. In methods, it would help to specifically state which samples are used for RNA extraction and quantitative RT-PCR (presumably only cell lines). Why was PBGD used as normalizing gene?

2. Methods indicate that 21 SCCs were studied, but Figure 4 presents results for only 20.

3. Figure legend 5, the description of the x and y axes are switched. X = samples grouped for disease; y = levels of methylation. Presumably all qualitative data from Figure 4 is represented quantitatively in Figure 5, but the number of samples do not match for the 22 normal samples (in Figure 4, 2 normal samples are M1 positive and 6 are M2 positive, but Figure 5 graphs 3 samples for M1 and 5 for M2) and are hard to determine for the other disease groups. It would be helpful to include the number of samples graphed in Figure 5 for each disease category and region.

4. Abbreviations need to be defined in text (Pap 1, BMD, Pap 3a2).
Level of interest: An article of outstanding merit and interest in its field

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 not competing interests.