Author's response to reviews

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

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Version: 3 Date: 23 March 2010

Author's response to reviews: see over
Comments to the reviewers

Reviewer 1

1. As suggested by the reviewer we have rewritten the results shown in Figure 2B and C to better clarify the results shown.

Page 15, second paragraph:
“The repression of core promoter activity upon inclusion of both 5’ sequences is shown in Figure 2B, in which the luciferase activity of both constructs relative to the hTERT-297/-23 core promoter construct is depicted”.

Page 16, first paragraph:
“The repression of core promoter activity upon inclusion of both 3’ sequences is shown in Figure 2C by plotting luciferase activity of both constructs relative to the hTERT-297/-23 core promoter construct.”

2. We have corrected our mistake on page 15 (1st version) with respect to region S2.

Page 16 2nd paragraph:
“Using overlapping PCR products the following 4 regions were analyzed: -442 to -219 bp (region S1), -208 to +104 bp (region S2), +88 to +348 bp (region S3), and +319 to +566 bp (region S4), all relative to the ATG site (Figure 3A).”
Reviewer 2

Major compulsory revisions

1. We can understand the reviewer getting confused by the summary of literature. We tried to be more explicit on previously published data and have rewritten this in the Introduction:

Page 4-5: “In embryonic stem cells and normal lymphocytes, which express hTERT, almost all CpGs around the transcription start site of the hTERT gene are unmethylated [17]. However, hTERT expression is not always associated with hypomethylation. In many cancer cell lines hTERT expression is often correlated with hypermethylation [11,14], although the expression level in these cells is usually much lower than in ES cells [17]. Other studies did not find a clear correlation between hTERT methylation and hTERT expression [12,13]. Zinn et al. [17] showed that in most cancer cell lines the hTERT promoter is densely methylated, but that CpGs around the transcription start site of a substantial number of hTERT alleles is unmethylated. This suggests that methylation of hTERT promoters is heterogeneous and that only unmethylated alleles are expressed. Indeed, histones at a hypomethylated core-promoter carry transcription active marks (H3K9Ac and H3K4me2) whereas a methylated core-promoter carries inactive marks (H3K9me3 and H3K27me3) [17]. The importance of this core promoter region is also illustrated by the observation that sequences surrounding the hypomethylated and active core promoter may be hypermethylated [18]. In addition to the importance of the methylation status of the core-promoter region other sequences in hTERT CpG island may also be important. Choi et al. [11] observed in colorectal cancer hypermethylation of a specific CpG outside known transcription factor binding sites, that together with hypermethylation of a number of CpGs closer to the transcription start site was associated with increased hTERT expression. It is unknown how methylation of these CpGs induce hTERT expression. The CpG rich region encompassing the 5’ proximal region of the gene has also been shown to affect hTERT expression [18].”

We would like to add that although the suggestion made by the reviewer to make a diagram may indeed be informative, this is not possible as data presented in previous papers are not always defined to specific CpGs. The 200 bp core fragment mentioned earlier on page 4 is indeed within the -150 to +150 bp region around the transcription start site as described in the previous version. To avoid confusion we left the latter out.

2. We agree that we have not formerly proven a direct association between methylation and deregulated gene transcription, which may be suggested from our conclusion.

We have now adjusted our conclusions both in the Abstract (Page 2) and Conclusions (page 26) to:

“Methylation of transcriptionally repressive sequences in the hTERT promoter and proximal exonic sequences is correlated to deregulated hTERT transcription in HPV-immortalized cells and cervical cancer cells”
3. The reviewer asks for more detailed information on DNA isolation and bisulfite treatment methods.

We have now included a detailed description of the methods used, see page 11-12:

**DNA isolation and bisulfite modification**

Genomic DNA from paraffin-embedded tissue specimens was isolated as described previously [38]. Ten paraffin-embedded tissue sections of 5µm were digested in 450µL proteinase K buffer [100 mmol/L Tris-HCl (pH 9.0), 10 mmol/L NaCl, 1% SDS, and 5 mmol/L EDTA] with 50 µL Proteinase K (1 mg/mL; Boehringer-Ingelheim, Alkmaar, The Netherlands) for 24 hours at 52°C with shaking. Thereafter, DNA was isolated by phenol/chloroform/isoamylalcohol (25:24:1) extraction, followed by ethanol precipitation. DNA was dissolved in H₂O. The histology of sections used for DNA isolation was checked according to the sandwich method in which the first and last section were stained by H&E and checked by a pathologist for presence of the lesions. Tumor sections contained in general 50-80% of tumor cells, whereas section of CIN lesions contained 10-50% of dysplastic cells. Genomic DNA of cervical scrapings was isolated using the high pure PCR template preparation kit (HPPTP, Roche, Mannheim, Germany). After a classic cervical smear was made on a slide, cervical scrapes were collected by placing the brush in 5 ml sterile phosphate-buffered saline (PBS, 0.82% (w/v); NaCl, 0.19% (w/v); Na₂HPO₄·2H₂O, 0.03% (w/v); NaH₂PO₄·2H₂O, adjusted to pH 7.4 with HCl) 0.005% merthiolate. Upon arrival in the laboratory, cells were pelleted at 300g for 10 min and resuspended in 1 ml 10 mM Tris-HCl (pH 7.4). A 100-µl Tris-HCl suspensions was used for DNA isolation by the HPPTP kit (Roche) according to the recommendations of the manufacturer, except that samples were eluted with 100 µl of elution buffer. Ultimately, 10 µl of eluate was used for HPV detection and 500 ng of DNA for bisulfite modification.

Sodium bisulfite modification, which induces chemical conversion of unmethylated cytosines into uracils, whereas methylated cytosines are protected from this conversion, was performed with the EZ DNA Methylation Kit, according to the manufacturer’s guidelines (Zymo Research, Orange, CA).

4. As requested by the reviewer we have also included more details on the MSP method used (and also completed citation 41 in the reference list), see page 13.

**Quantitative methylation-specific PCR (qMSP)**

qMSP was performed using two primer and Taqman probe combinations, one of which was located in the promoter (region 1) and the other in the first intron and exon 2 (region 2) (Table 1). As indicated in bold in Table 1 each primer and both Taqman probes contained two to four CpG dinucleotides, to ensure specific detection of methylated DNA. A standard curve of bisulfite-treated DNA of SiHa was included in each qMSP. As negative controls, H₂O, unmodified genomic DNA obtained from SiHa cells and unmethylated DNA obtained from primary keratinocytes were included. The house keeping gene β-actin was included as a quality control (Table 1) [40, 41]. qMSP reactions were carried out in a 12 µl reaction volume containing 50 ng of bisulfite-treated DNA, 417 nM of each primer, 208 nM probe and 1x QuantiTect Probe PCR Kit master mix (Qiagen, Westburg, Leusden, The Netherlands) using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Nieuwerkerk a/d IJssel,
The Netherlands). For each qMSP assay the threshold was fixed at 0.01. All samples analysed in the study showed an ACTB CT value that was equal to or below 31 (CT ≤ 31). qMSP values of target genes were adjusted for DNA input by expressing the results as ratios between the absolute target measurement and the ACTB measurement (mean quantity of methylated hTERT DNA/mean DNA quantity for β-actin * 1000).

All samples were tested in duplicate and in case of discrepancy (in < 10% of cases) in quadruplicate. Samples scored positive if at least two test results (quantity ratios) were above zero.

5. We agree with the reviewer that clinical samples tested are a mixture of normal and abnormal cells, which indeed explains the lower detection rates of hTERT methylation. Moreover, it would be interesting to test more clinical samples by bisulfite sequencing, but due to the labor intensity of the sequencing procedure and limitation of the clinical materials this was unfortunately not possible.

We have now included the following sentence in the Results, page 18: “Methylation levels in clinical specimens were generally lower than in cell cultures, most likely resulting from the admixture of normal cells in these samples.”

Minor essential revisions

1. The reviewer asks for some more information on the RNA extraction and RT-PCR procedures, which has now been included on page 9 of the Methods section:

RNA isolation and quantitative RT-PCR

Total RNA was isolated from cell lines using RNA Bee (Tel-test, Friendswood, TX) following the manufacturer’s instructions. hTERT mRNA was quantified by real time RT-PCR using the TeloTAGGG hTERT kit (Roche, Woerden, The Netherlands) according to the manufacturer’s manual. Relative hTERT levels were determined by dividing hTERT expression levels by expression levels of the housekeeping gene Porphobilinogen Deaminase (PBGD), which was also included in the TeloTAGGG hTERT kit (Roche).

2. The reviewer asks why 21 SCC are described in the Methods, whereas 20 SCC are shown in Figure 4.

Next to the 20 SCC used for MSP we also analysed another SCC by bisulfite sequencing, adding up to 21 SCC.

3. We very much appreciate the careful review of the Figures included in the paper, and we would like to express our apologies for the inaccuracies. We have adjusted the description of the y- and x-axes of Figure 5 and also made a correction to Figure 4, in which 1 positive sample for the M1 region was incorrectly shown as negative. The final remark that for normal samples 5 dots are shown for the M2 region in Figure 5, whereas 6 positives are shown in Figure 2 results from the fact that 2 samples had nearly equal quantities (0.29 and 0.30) due to which the dots are overlapping in Figure 5.
We have also included the number of samples graphed in Figure 5 as suggested by the reviewer.

4. We have now replaced the abbreviations Pap and BMD in the text by their full descriptions, see page 8.
   We included three scrapings of women without CIN disease (classified as normal cytology n=2, and borderline mild dyskaryosis n=1) and one scrape (classified as moderate dyskaryosis) of a woman with CIN disease.