Methylation of the Human Telomerase Gene CpG Island

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Abstract

The acquisition of expression of hTERT, the catalytic subunit of the telomerase enzyme, seems to be an essential step in the development of a majority of human tumors. However, little is known about the mechanisms preventing telomerase gene expression in normal and transformed cells that do not express hTERT. Using a methylation-specific PCR-based assay, we have found that the CpG island associated with the hTERT gene is unmethylated in telomerase-negative primary tissues and nonimmortalized cultured cells, indicating that mechanisms independent of DNA methylation are sufficient to prevent hTERT expression. The hTERT CpG island is methylated in many telomerase-negative and telomerase-positive cultured cells and tumors, but the extent of methylation did not correlate with expression of hTERT. Demethylation of DNA with 5-aza-cytidine in two cell lines induced expression of hTERT, suggesting that DNA methylation can contribute to hTERT repression in some cells. Together, these data show that the hTERT CpG island can undergo cytosine methylation in cultured cells and tumors and that DNA methylation may contribute to the regulation of the hTERT gene, but that CpG island methylation is not responsible for repressing hTERT expression in most telomerase-negative cells.

Materials and Methods

Bisulfite Treatment of DNA (16). Approximately 1 µg of genomic DNA was digested with PstI in 10 µl and then denatured in 0.3 M NaOH at 37°C for 10 min. Six microliters of 10 mM hydroquinone (Sigma Chemical Co.) and 104 µl of 3 M sodium bisulfite (Sigma Chemical Co.; both freshly mixed) were added. The samples were treated with 20 cycles of 95°C for 30 s, 50°C for 15 min, followed by 10 h at 50°C. The DNA was purified using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer’s protocol. The eluted samples were incubated in 0.3 M NaOH for 5 min at 20°C; ethanol precipitated, washed in 70% ethanol, and resuspended in 10 µl of H2O. DNA (1.5 µl) was used in each PCR reaction. Methylation-specific primers were: 5’ primer set, specific for unmethylated DNA: forward primer: 5’-GGGTTATGATGGTTAGGATGGT-3’; reverse primer: 5’-CATATACACAAACAACACACACCA-3’. 5’ primer set, specific for methylated DNA: forward primer: 5’-GGGTATAGACGTTAGGATCGC-3’; reverse primer: 5’-CGTACGACAAACCGAAGC3’. 3’ primer set, specific for unmethylated DNA: forward primer: 5’-AGTTTTGTGTTGTTATTTTTGTT-3; reverse primer: 3’-AAAAACATAAAACCAACAAACACCA-3’. 3’ primer set, specific for methylated DNA: forward primer: 5’-GGGTATAGACGTTAGGATCGC-3’. 5’-CGTACGACAAACCGAAGC3’. 3’ primer set, specific for unmethylated DNA: forward primer: 5’-AGTTTTGTGTTGTTATTTTTGTT-3; reverse primer: 3’-AAAAACATAAAACCAACAAACACCA-3’. 3’ primer set, specific for methylated DNA: forward primer: 5’-GGGTATAGACGTTAGGATCGC-3’. 5’-CGTACGACAAACCGAAGC3’. 3’ primer set, specific for unmethylated DNA: forward primer: 5’-AGTTTTGTGTTGTTATTTTTGTT-3; reverse primer: 3’-AAAAACATAAAACCAACAAACACCA-3’. 3’ primer set, specific for methylated DNA: forward primer: 5’-GGGTATAGACGTTAGGATCGC-3’. 5’-CGTACGACAAACCGAAGC3’.

Methylation-specific PCR. PCR reactions were performed in 25 µl, using published conditions (16) and 1.25 units of Taq Polymerase (Perkin-Elmer Corp.) complicated with TaqStart antibody (Clontech). Reactions were assembled on ice and hot-started at 95°C for 5 min, followed by PCR: 10 cycles of 95°C for 30 s; 70°C for 30 s (decreasing by 1°C with each cycle); and 72°C for 30 s. This was followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and concluded at 72°C for 600 s. Reactions were analyzed on ethidium bromide-stained 2.5% agarose gels in 1× Tris-borate EDTA buffer.

RT-PCR for hTERT mRNA. Total RNA (5 µg) was used in a cDNA synthesis reaction using the First-strand cDNA synthesis kit (Pharmacia), with the reverse hTERT and GAPDH primers, each at 2 nm. PCR reactions were run in standard conditions with 2.5 units of Taq Polymerase (Perkin-Elmer Corp.) complicated with Taq Start antibody (Clontech) and 5P-labeled forward primers. cDNA (5 µl) was used in the hTERT reactions; one microliter of a 1:400 dilution of the cDNA was used in the GAPDH reactions. PCR reactions

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3 The abbreviations used are: hTERT, human telomerase; MSP, methylation-specific PCR; RT-PCR: reverse transcriptase-PCR; 5-aza-C, 5-aza-cytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

4 P. Steiner, personal communication.
were cycled 25 times: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. Ten microliters of each reaction were analyzed with 8% PAGE, 1% Tris-borate EDTA. hTERT primers were: LTS forward (CGGAAGAGTGTCTGGGACAA) and LT6 reverse (LT6GGATGAAGCGGAGTCTGGA; Ref. 17). GAPDH primers were: GAPDHI forward (GACCCCTCTTGGACTCAAC) and GAPDH2 reverse primer (CTTCTCCATGGTGTAAGA).

**Sequencing of MSP Products.** MSP products were subcloned into plasmid vectors using the Topo TA cloning kit (Invitrogen), following the manufacturer’s instructions. Plasmid DNAs were purified using the Wizard Plus miniprep kit (Promega), and then sequenced by Research Genetics (Huntsville, AL).

**Cell Culture.** Cultured cells were maintained in standard conditions in DME with 10% heat-inactivated fetal bovine serum. For 5-aza-C treatment, cells were incubated in MEM with 10 μM 5-aza-C for 24 h on day 1 and day 4, and then harvested on day 7 for analysis (15). Total cell RNA was prepared using RNeasy (Tel-Test).

**Results**

The telomerase gene has a typical CpG island domain overlying putative transcription start site and a number of potential transcriptional regulatory sequences (6–8). On the basis of the quantitative criteria proposed by Antequera and Bird (18), this CpG island is from 654 bp upstream of the putative transcription start site (6) to 510 bp downstream of the transcription start site, ending 56 bp after the start of the first intron (6–8, 18). Within this region, the DNA has a GC content of 74% and a CG:GC ratio of 0.87.

The MSP technique we have used to determine the methylation state of the hTERT CpG island is based on the observation that sodium bisulfite treatment of DNA converts cytosines into uridines and that this reaction is strongly inhibited when cytosines are methylated (19). DNA that has been treated with sodium bisulfite, therefore, retains only methylated cytosines, and can subsequently be tested with PCR primers that amplify specifically either modified or unmodified DNA (16). We generated PCR primers that distinguish between bisulfite-modified and -unmodified cytosines in the hTERT CpG island. For this study, we chose primer sets to assay the methylation state of the transcription start site, as well as regions 3’ to the translation start site that are near the first intron and which may contribute to hTERT activation (Refs. 6–8; Fig. 1a).5 Primer sets generated to test sequences near the 5’ boundary of the hTERT CpG island revealed complete DNA methylation in most of the cell lines and tumors examined, independent of the state of expression of the hTERT gene, suggesting that methylation at the extreme 5’ end of the CpG island does not play a significant role in hTERT regulation (data not shown). Additional primer sets at ~300 and 140 bp upstream of the transcription start site yielded results that correlated closely with the results described below for the 5’ primer pairs (data not shown) and, therefore, will not be discussed further.

To establish the specificity of our PCR primers, we used hTERT promoter plasmid DNA before and after treatment with Sss I DNA methylase as controls. This enzyme methylates all CpG cytosines. As shown in Fig. 1b, each primer set generated a PCR product specifically with either methylated or unmethylated control DNA. None of the sets generated a product with plasmid DNA that had not been treated with bisulfite (data not shown), indicating that DNA that has been incompletely treated with sodium bisulfite was unlikely to be amplified by primer sets specific for methylated sequences and, therefore, inadvertently interpreted as being methylated.

We sought to establish whether telomerase-negative cells have hTERT CpG island methylation that might underlie the absence of hTERT expression in these cells. To do so, we tested DNA prepared from a series of primary, mortal cell strains and immortal cell lines.

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5 P. Steiner and R. A. Weinberg, unpublished data.
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Fig. 2. MSP of the human hTERT CpG island in cultured cells. Genomic DNA was tested with 5’ and 3’ primer sets specific for either unmethylated (U) or methylated (M) hTERT CpG island DNA. a, telomerase-negative cells: WI38, primary fibroblasts; HA-1 pre, mortal SV40 large T transformed primary embryonic kidney cells; JFCF-6T/5K, mortal SV40 large T-transformed primary fibroblasts; U2OS, immortal osteosarcoma cells; GM847 and VA13, immortal SV40 large T-transformed human fibroblasts. b, telomerase-positive cell lines. HA-1 post, immortalized postcrisis HA-1 cells (SV40 large T transformed primary embryonic kidney cells); JFCF 6T/1J.6B, immortalized postcrisis SV40 large T-transformed fibroblast cells; 293, immortalized adenosine-transfected human embryonic kidney cells; HT29 and SW480, colon carcinoma cells; HeLa, cervical carcinoma cells.

Methylation of the hTERT CpG island

The catalytic activity could not be detected in the 5-aza-C-treated GM847 or U2OS cells using the TRAP assay (Ref. 20; data not shown). Taken together, these data indicate that DNA methylation of the hTERT CpG island can contribute to repression the hTERT gene, but that additional regulatory mechanisms operate to limit hTERT expression in U2OS and GM847 cells, and to prevent hTERT expression in VA13 and HA-1 cells.

The MSP technique used here only allowed us to assess the methylation state of the hTERT genomic sequences represented in the primers themselves. We wished additionally to determine the state of methylation of the 42 CpG cytosines that lie between our MSP primer pairs. To do so, we cloned and sequenced MSP products from bisulfite-treated GM847 DNA (Fig. 3). Because we observed only methylation-specific products with the GM847 cell DNA, we concluded that use of these methylation-specific primers to amplify GM847 DNA would enable us to survey all hTERT gene copies carried by these cells. For comparison, and as a positive control for the completeness of the bisulfite modification, we analyzed human placenta DNA, which has an unmethylated hTERT CpG island (data not shown). This sequencing revealed that most of the 42 CpG cytosines in the hTERT CpG island of GM847 cells are methylated, with the exception of a cluster of 5 CpGs that were largely unmethylated and lie between +71 and +152 bp downstream of the transcription start site. Analysis of the placenta control DNA revealed a virtually complete conversion of cytosines to uridines, indicating effective bisulfite treatment and a lack of cytosine methylation. The extent of hTERT CpG island methylation in GM847 cells is similar to the extent of methylation described by others as being responsible for the repression of the gene encoding tissue inhibitor of metalloproteinase-3 (TIMP-3) (23). Hence, this level of methylation may account, on its own, for the absence of hTERT expression in these cells, explaining thereby the inducibility of hTERT expression upon 5-aza-C treatment.

In general, CpG island methylation correlates inversely with gene expression (9–11). We, therefore, expected that the hTERT CpG islands of telomerase-positive cell lines would not be methylated. The telomerase-positive postcrisis HA-1 embryonic kidney and the JFCF-6T/1J.6B fibroblast cell lines both revealed unmethylated hTERT loci (Fig. 2b). However, we observed partial methylation of the hTERT CpG island in the 293 human embryonic kidney cell line, the HeLa cervical carcinoma cell line, and the SW480 and HT29 colon carcinoma cell lines, all of which are strongly telomerase positive (Fig. 2b).

Specifically, all four lines revealed DNA methylation with the 5’ primer set (specific for the transcription start site), with the HeLa and SW480 cells also exhibiting partial methylation with the 3’ primer set (downstream of the translation start site). Partial methylation did not correlate with the level of hTERT mRNA expression because the 293 cells have steady-state hTERT mRNA levels 8-fold greater than HA-1 postcrisis cells (data not shown). These results indicate that partial hTERT CpG island methylation can exist in telomerase-positive cells and is not inhibitory of telomerase gene expression.

The de novo methylation of CpG islands is a frequent accompaniment to malignant transformation (9–11). We, therefore, sought to evaluate whether hTERT CpG island methylation occurs during tumorigenesis and is a feature of telomerase-negative tumors. We used MSP to test for a correlation between hTERT CpG island methylation and telomerase expression in a series of primary tumor and normal control tissues (Fig. 4). A telomerase-negative adrenal carcinoma (Adrenal ALT), that has the telomere length pattern characteristic of all telomerase-negative, immortalized human cells studied to date (21), exhibited no hTERT CpG island methylation, as did the telomerase-positive CT1485 colon carcinoma (Fig. 4). A telomerase-negative breast carcinoma (Breast ALT) (21) and a telomerase-negative colon carcinoma (Col1310) both revealed partial CpG island methylation. However, the extent of methylation, as indicated by the pattern of primer pairs that amplified the bisulfite-treated DNA, was identical to that seen in the telomerase-positive Br1958 and Col1229 carcinomas, respectively. In accord with the observations made with telomerase-negative cell lines, these results do not reveal a correlation between telomerase-expression and hTERT CpG island methylation in tumors. Furthermore, they reveal that methylation of the hTERT CpG island is not a prerequisite for the telomerase-negative phenotype in tumors.

For three of the tumor DNAs that we examined we obtained normal control tissues taken from the surgical margins of the tumors. All three were telomerase-negative and showed no hTERT CpG island methylation. This indicates that methylation of the hTERT CpG island is not a feature of the telomerase-negative state in normal colon tissues and suggests that the partial methylation we observed in the tumor DNAs occurred de novo during tumorigenesis.

Discussion

Using the MSP technique, we have found that the hTERT CpG island is unmethylated in normal tissues and in nonimmortalized mortal, telomerase-negative cell strains. This indicates that hTERT is not an imprinted gene and that mechanisms operating independently of DNA methylation are sufficient to prevent hTERT expression in most telomerase-negative cells. Such mechanisms could include the absence of positively acting transcription factors or the presence of active repressors of hTERT transcription.

In contrast to normal and primary tissues, we found that the hTERT
CpG island methylation is methylated in a variety of primary tumor tissues and cultured cells. However, both telomerase-positive and telomerase-negative cell lines and tumors exhibited partial methylation of their hTERT CpG islands, and we observed no correlation between the degree of methylation and telomerase activity. Therefore, hTERT CpG island methylation is unlikely to play a substantial role in the regulation of hTERT in vivo, and the CpG island methylation we observed likely reflects the de novo CpG island methylation that is a frequent accompaniment of tumorigenesis and the establishment of cells in culture (10, 11, 14).

Nonetheless, indirect evidence suggests that CpG island methylation is able to contribute to the repression of hTERT in certain cultured cell lines. The level of cytosine methylation of individual hTERT alleles in GM847 cells is consistent with the level of methylation implicated in the repression of the TIMP-3 gene (23). Furthermore, the methylation-inhibitor 5-aza-C was able to induce expression of hTERT in GM847 cells and in U2OS cells that have partial methylation of the hTERT CpG island. 5-aza-C treatment did not induce hTERT expression in the HA-1 precrisis cell strain, which has an unmethylated hTERT CpG island. This argues against the possibility that the activity of 5-aza-C on DNA methylation at sites other than the hTERT CpG island was responsible for the activation of hTERT in GM847 and U2OS cells. Although our data suggest that DNA methylation can repress hTERT, the low level of 5-aza-C-induced expression in GM847 and U2OS cell lines indicates that additional transcriptional regulatory mechanisms in those cells prevent hTERT from attaining expression levels typical of telomerase-positive cell lines.

It is plausible that in cells with a high de novo DNA methylase activity, the hTERT CpG island is under continuous pressure to become increasingly methylated. Such a mechanism could explain the partial methylation we observed in some telomerase-positive cell lines and tumors. One possible consequence of such pressure would be the emergence of cell clones that have repressed the telomerase gene. This may explain the observation that subclones derived from the telomerase-positive 293 and HeLa cell lines were occasionally telomerase negative (24). In cultured cells and tumor cells that depend on telomerase activity for continued proliferation, repression of the telomerase gene would lead to the loss of telomere maintenance and subsequent cell death. Such an effect could potentially slow the growth of tumor cell clones that express a high level of methylase activity, counteracting, in part, the detrimental effects of methylation of tumor sup-

Fig. 3. Induction of hTERT gene expression by 5-aza-C and extent of methylation of the GM847 CpG island. a, cells were treated with 10 μM 5-aza-C on days 1 and 4 and analyzed on day 7 for hTERT gene expression using RT-PCR. The autoradiographs are overexposed to show the induced hTERT expression; the RT-PCR assay was designed to be linear for hTERT and GAPDH mRNA concentrations close to those in the telomerase-positive HA-1 postcrisis cell line (data not shown). No RT, a control reaction without reverse transcriptase; BJ, a telomerase-negative primary fibroblast cell strain; H2O, a water-only control; GM847, untreated; G+aza, 5-aza-C-treated GM847 cells; HA-1, untreated; H+aza, 5-aza-C-treated HA-1 cells; U2OS, untreated; U+aza, 5-aza-C-treated U2OS cells. b, GM847 MSP products were subcloned into plasmid vectors, and individual subclones were sequenced. The locations of the CpG sites are given in reference to the transcription start site. ●, methylation; ○, no methylation. Each row represents one sequenced allele. Placenta DNA (Plac) was sequenced as a control for the bisulfite reaction. Coordinates are given relative to the putative transcription start site (Ref. 6). The black bar denotes a cluster of CpG cytosines that are hypomethylated in GM847 cells.

Fig. 4. MSP of primary human tissues, depicted as in Fig. 2. The telomerase expression of each of the samples is indicated by a + or - suffix. In the left column are data from telomerase-negative tumors from the adrenal gland (Adrenal ALT) and breast (Breast ALT) that both have the characteristic phenotype of cells that use the alternate mechanism of telomere maintenance (Ref. 21), and a telomerase-positive breast carcinoma (BT1958). The middle and right columns depict data from paired normal and tumor DNAs taken from the same surgical specimens. CN1483 and CN1231 are normal, telomerase-negative colon tissues that correspond to the telomerase-positive colon tumors positive CT1485 and CT1229, respectively. CN1312 is a telomerase-negative normal colon sample that corresponds to the telomerase-negative colon carcinoma CT1310.
pressor gene CpG islands. We suggest that the analysis of the methylation patterns of hTERT CpG island alleles in telomerase-positive cell lines may help to delineate regulatory elements that are essential for the regulation of the telomerase gene.

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