Methylation of the Human Telomerase Gene CpG Island

Scott K. Dessain, Hua-yin Yu, Roger R. Reddel, Roderick L. Beijersbergen, and Robert A. Weinberg

Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142 [S. K. D., H-y. Y., R. A. W.]; Department of Hematology/Oncology, Massachusetts General Hospital, Boston, Massachusetts 02114 [S. K. D.]; Harvard Medical School, Boston, Massachusetts 02115 [S. K. D.]; Children’s Medical Research Institute, Westmead, N.S.W. 2145, Australia [R. R. R.]; and Netherlands Cancer Institute, 1066 CX Amsterdam, the Netherlands [R. L. B.]

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 nonmethylated 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.

Introduction

Cancer cells possess two fundamental traits, uncontrolled proliferation and immortality. Mutations in oncogenes and tumor suppressor genes provide cancer cells with the impetus for uncontrolled cell division, but a growing body of evidence suggests that replicative immortality through the maintenance of telomere length is also required for human cells to form tumors in vivo (1, 2). Between 85% and 90% of cancer cells maintain their telomere length through the expression of the telomerase holoenzyme, in contrast to most normal tissues that do not express the enzyme, and are, therefore, mortal (3).

The activity of the telomerase holoenzyme is largely governed by the intracellular level of its catalytic subunit hTERT (4, 5). Little is known about the mechanisms regulating the expression of hTERT in normal and malignant cells. The hTERT promoter contains a high density of CpG dinucleotides that in aggregate define a typical CpG island—a DNA sequence that is a potential target for repression through DNA methylation (6–8). Most CpG island sequences are unmethylated in normal cells, and protection from DNA methylation is necessary but not sufficient for gene transcription (9–11).

The de novo methylation of CpG islands in postembryonic cells is associated with aging, the establishment of cells in culture, and tumorigenesis. In the colon, progressive DNA methylation that correlates with aging has been observed at the CpG islands of a number of genes, including the estrogen receptor gene (12, 13). Widespread CpG island methylation accompanies the establishment of nontransformed cells in culture (14). In tumors, de novo CpG island methylation has been shown to repress the expression of the von Hippel-Lindau, retinoblastoma, and p16 tumor suppressor genes, among others (9–11). A phenotype of extensive de novo CpG island methylation has been identified in colon cancers that appear to cause, among other things, the repression of the hMLH1 gene, which in turn may contribute to microsatellite instability and tumor progression (13, 15).

Because the expression of telomerase catalytic activity can be an important contributor to malignant transformation in human cells (1, 2), we reasoned that mechanisms leading to the repression of hTERT could have a potential tumor suppressor function. Consequently, we sought to determine whether the CpG island of the telomerase gene is a potential target for repression by DNA methylation.

Materials and Methods

Bisulfite Treatment of DNA (16). Approximately 1 μg of genomic DNA was digested with PstI in 10 ml 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 μM 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 Qiagen 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′-GTGGGTATAGATGTTAGGATGTT-3′; reverse primer: 5′-CCACATACACAAACCAACAC-3′; 5′ primer set, specific for methylated DNA: forward primer: 5′-GGGTATAGACGTTAGGATGTT-3′; reverse primer: 5′-CGTACGGCAACACACACAC-3′; 3′ primer set, specific for unmethylated DNA: forward primer: 5′-AGTTTTGGTTTTGGTTATTTTTGT-3′; reverse primer: 5′-AAACACTAAACCAACCAACAC-3′; 3′ primer set, specific for methylated DNA: forward primer: 5′-GTCTACGCAACACACACAC-3′; reverse primer: 5′-CAACACACACACCAACACAC-3′.

Methylation-specific PCR. PCR reactions were performed in 25 μl using published conditions (16) and 1.25 units of Taq Polymerase (Perkin-Elmer Corp.) complexed 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 mM. PCR reactions were run in standard conditions with 2.5 units of Taq Polymerase (Perkin-Elmer Corp.) complexed with TaqStart antibody (Clontech) and 32P-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

Received 9/7/99; accepted 12/1/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a Merck/MIT collaboration agreement (to R. A. W.); NIH Grant T32 CA71382, the Lauri Strauss Leukemia Foundation (to S. K. D.), and a grant from the National Health and Medical Research Council of Australia (to R. R. R.).

2 To whom requests for reprints should be addressed, at The Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142. Phone: (617) 258-5213; E-mail: hickey@wi.mit.edu.

3 The abbreviations used are: hTERT, human telomerase; MSP, methylation-specific PCR; RT-PCR, reverse transcriptase-PCR; 5-aza-C, 5-azacytidine; 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: LT5 forward (GGGAGAGGTGTCTGGGACAA) and LT6 reverse (LT6GGATGAAACGGAGTCTGGA; Ref. 17). GAPDH primers were: GAPDH1 forward (GAACCCCTTACGGCTCAAC) and GAPDH2 reverse primer (CTTCTCCATGGTTGGAAGA).

**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 MEMα 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 RNAzol (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). 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 pairs 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.

As shown in Fig. 2a, the hTERT CpG island is unmethylated in the WI38 cell strain (primary fibroblasts), the HA-1 precrisis cell strain (mortal, SV40 large T-transformed embryonic kidney cells), and the JFCEF-6/5K precrisis cell strain (mortal, SV40 large T-transformed fibroblasts), all of which are telomerase negative as judged by the highly sensitive TRAP assay (Ref. 20; data not shown). We observed identical results with DNA prepared from IMR90 and BJ primary fibroblast cells (data not shown), which are also telomerase negative.

In contrast, three immortalized telomerase-negative cell lines that use an alternative mechanism for lengthening telomeres (ALT) (21), showed hTERT CpG island methylation (Fig. 2a). The U2OS osteosarcoma cell line has partial methylation of its hTERT CpG island, and the immortal fibroblast lines GM847 and VA13 have complete methylation. These results indicate that hTERT CpG island is subject to DNA methylation in cultured cells, but that DNA methylation is not a requirement for repression of the telomerase gene in telomerase-negative cells. In addition, the lack of CpG island methylation in the primary cell fibroblasts suggests that hTERT is not an imprinted gene.

To determine whether the inhibition of DNA methylation could induce hTERT expression in cells with methylated hTERT loci, we treated GM847, U2OS, and VA13 cells with the DNA-methylation inhibitor 5-aza-C (22). Treatment of GM847 cells with 5-aza-C induced transcription of hTERT that was detectable with RT-PCR (Fig. 3a). Similarly, 5-aza-C treatment of U2OS cells reproducibly increased a low level of hTERT transcription by 2–3-fold. The VA13 cell line did not induce hTERT expression with 5-aza-C treatment despite having substantial CpG island methylation (data not shown).

As a negative control, we treated the HA-1 precrisis cell line with 5-aza-C, which is telomerase negative but does not have a methylated hTERT CpG island. As expected, hTERT was not induced. The levels of mRNA induced by 5-aza-C in U2OS and GM847 cells were much lower than in telomerase-positive HA-1 postcrisis cells, and telom-
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 postcancer 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 postcancer 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 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-
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.

Acknowledgments

We thank Drs. Laurie Jackson-Grusby, James Herman, Lisa Spirio, and Michael Powers for helpful discussions. We also thank Brain Elenbaas, William Hahn, Sheila Stewart, and Randolph Watnick for critical reading of the manuscript. We thank the Massachusetts General Hospital Tumor Bank, Mohammed Miri, and Dr. Francis Haluska for providing tumor samples.

References

Methylation of the Human Telomerase Gene CpG Island

Cancer Res 2000;60:537-541.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/3/537

Cited articles
This article cites 23 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/3/537.full.html#ref-list-1

Citing articles
This article has been cited by 36 HighWire-hosted articles. Access the articles at:
/content/60/3/537.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.