DNA Methylation Analysis of the Promoter Region of the Human Telomerase Reverse Transcriptase (hTERT) Gene

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Abstract

The promoter of the hTERT gene encoding the catalytic subunit of telomerase was recently cloned and has a dense CG-rich CpG island, suggesting a role for methylation in regulation of hTERT expression. In this study, we have initiated the analysis of the regulation of hTERT expression by examining the methylation status of up to 72 CpG sites extending from 500 bases upstream of the transcriptional start site of the hTERT gene into the first exon in 37 cell lines. These cell lines represent a variety of cell and tissue types, including normal, immortalized, and cancer cell lines from lung, breast, and other tissues. Using bisulfite genomic sequencing, we did not find a generalized pattern of site-specific or region-specific methylation that correlated with expression of the hTERT gene: most of the hTERT-negative normal cells and about one-third of the hTERT-expressing cell lines had the unmethylated/hypomethylated promoter, whereas the other hTERT-expressing cell lines showed partial or total methylation of the promoter. The promoter of one hTERT-negative fibroblast cell line, SUSM-1, was methylated at all sites examined. Treatment of SUSM-1 cells with the demethylating agent 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor trichostatin A induced the cells to express hTERT, suggesting a potential role for DNA methylation and/or histone deacetylation in negative regulation of hTERT. This study indicates that there are multiple levels of regulation of hTERT expression in CpG island methylation-dependent and -independent manners.

Introduction

Normal human somatic cells exhibit little or no telomerase activity. Consequently, telomeres shorten as these cells divide, and this may be responsible for cellular senescence (1, 2). In contrast, telomerase activity often becomes activated in cancer cells to elongate or maintain telomere length indefinitely during cancer progression (3). Thus, the up-regulation of telomerase is a critical step in the carcinogenic process in humans.

The telomerase enzyme consists of at least three components, including an RNA component (hTERC), a telomerase-associated protein (TEP1), and a telomerase catalytic subunit (hTERT) with sequence similarity to reverse transcriptase enzymes. The majority of cells that are telomerase negative express the hTERC and TEP1 components but lack hTERT expression; there is a strong correlation between hTERT expression and telomerase activity. Recently, the hTERT gene promoter has been cloned in our laboratory (4) and in other laboratories (5, 6), and several putative regulatory motifs have been identified. The presence of a large CpG island with a dense CG-rich content suggests that DNA methylation and chromatin structure may play a role in the regulation of hTERT expression.

Different patterns of methylation in promoters of certain genes are important for the regulation of gene expression during normal development and in some cancers. For example, accumulation of random CpG-site methylation in the promoter of p16INK4a is responsible for loss of expression of this gene in some human cancers (7, 8). Region-specific methylation of the hMLH1 promoter appears to correlate with the loss of its expression in colorectal carcinoma cell lines (9). Instances of site-specific methylation and methylation-sensitive transcription factors have also been identified that regulate the expression of certain genes (10).

Our present study focuses on assessing the methylation status of the promoter of the hTERT gene in normal, immortal, and cancer cell lines to begin to evaluate a possible role for DNA methylation in the regulation of this gene. We use the bisulfite genomic sequencing method to determine whether site-specific or region-specific methylation correlates with hTERT expression.

Materials and Methods

Cell Lines. The human cell lines used in this study were NHF (normal human fibroblasts), MRC-5 (fetal lung fibroblasts), CMV-Mj-HEL-1 (CMV-immortal fibroblasts), SUSM-1 (immortal fibroblasts), SiHa (cervical cancer), RCCC23 (kidney cancer), Calu1 (lung cancer), Calu3 (lung adenocarcinoma), Calu6 (lung cancer), HTB182 (lung squamous cell cancer), HTB57 (lung adenocarcinoma), HTB178 (lung adenosquamous cancer), A549 (lung cancer), HTB183 (lung cancer metastatic cell line), HCT116 (colon cancer), SW480 (colon cancer), 184FB (breast fibroblasts), 184 (breast epithelial cell strain), 90P (breast epithelial cell strain), 90P-TERT (immortalized with hTERT), 90P-E6E7 (immortalized with human papillomavirus-16 viral E6/E7 genes), and other breast cancer cell lines [A1, B5, BBL100 (SV40-transformed cell line), BT20, HS578T, HCC1937, MDA-MB 231, MDA-MB435, MDA-MB-436, MDA-MB453, MCF-7, BT483, BT549, T47D, ZR751, and SKBR3]. With a few exceptions, these cells were obtained from the American Type Culture Collection. NHF normal human fibroblast cells were generously provided by M. Cordeiro-Stone (University of North Carolina, Chapel Hill, NC). Breast epithelial cell strains 184 and 90P, breast fibroblast cell strain 184FB, and the chemically immortalized cell line 184-A1 were kindly provided by Dr. Martha Stumper (Lawrence Berkeley Laboratory, University of California, Berkeley, CA). 90P cells were immortalized after infection with either a retrovirus containing the human papillomavirus 16 viral E6/E7 genes, and other breast cancer cell lines [A1, B5, BBL100 (SV40-transformed cell line), BT20, HS578T, HCC1937, MDA-MB 231, MDA-MB435, MDA-MB-436, MDA-MB453, MCF-7, BT483, BT549, T47D, ZR751, and SKBR3]. With a few exceptions, these cells were obtained from the American Type Culture Collection. NHF normal human fibroblast cells were generously provided by M. Cordeiro-Stone (University of North Carolina, Chapel Hill, NC). Breast epithelial cell strains 184 and 90P, breast fibroblast cell strain 184FB, and the chemically immortalized cell line 184-A1 were kindly provided by Dr. Martha Stumper (Lawrence Berkeley Laboratory, University of California, Berkeley, CA). 90P cells were immortalized after infection with either a retrovirus containing the human papillomavirus 16 viral E6/E7 genes, and other breast cancer cell lines [A1, B5, BBL100 (SV40-transformed cell line), BT20, HS578T, HCC1937, MDA-MB 231, MDA-MB435, MDA-MB-436, MDA-MB453, MCF-7, BT483, BT549, T47D, ZR751, and SKBR3]. With a few exceptions, these cells were obtained from the American Type Culture Collection. NHF normal human fibroblast cells were generously provided by M. Cordeiro-Stone (University of North Carolina, Chapel Hill, NC). Breast epithelial cell strains 184 and 90P, breast fibroblast cell strain 184FB, and the chemically immortalized cell line 184-A1 were kindly provided by Dr. Martha Stumper (Lawrence Berkeley Laboratory, University of California, Berkeley, CA). 90P cells were immortalized after infection with either a retrovirus containing the human papillomavirus 16 viral E6/E7 genes, and other breast cancer cell lines [A1, B5, BBL100 (SV40-transformed cell line), BT20, HS578T, HCC1937, MDA-MB 231, MDA-MB435, MDA-MB-436, MDA-MB453, MCF-7, BT483, BT549, T47D, ZR751, and SKBR3]. With a few exceptions, these cells were obtained from the American Type Culture Collection. NHF normal human fibroblast cells were generously provided by M. Cordeiro-Stone (University of North Carolina, Chapel Hill, NC). Breast epithelial cell strains 184 and 90P, breast fibroblast cell strain 184FB, and the chemically immortalized cell line 184-A1 were kindly provided by Dr. Martha Stumper (Lawrence Berkeley Laboratory, University of California, Berkeley, CA). Cell lines and other breast cancer cell lines [A1, B5, BBL100 (SV40-transformed cell line), BT20, HS578T, HCC1937, MDA-MB 231, MDA-MB435, MDA-MB-436, MDA-MB453, MCF-7, BT483, BT549, T47D, ZR751, and SKBR3]. With a few exceptions, these cells were obtained from the American Type Culture Collection.

Expression of hTERT. Reverse transcription-PCR was used to evaluate the expression of hTERT mRNA. RNA (2 μg) was reverse-transcribed with the oligo(dT)20 primer using the SuperScript preamplification kit (Invitrogen, Inc., Carlsbad, CA) and the RNeasy Mini kit (Qiagen, Inc., Valencia, CA). PCR was performed using the following primer sets: (core promoter) 5'-CTGAGCTTATGCCTTCCTGGCTC-3' and 5'-GCTCTCGTGAGTGCAACGG-3', or (gene region) 5'-CTGAGCTTATGCCTTCCTGGCTC-3' and 5'-GCTCTCGTGAGTGCAACGG-3'. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. A part (1 μl) of the reaction was used as template for 10 μl of PCR.

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2 The abbreviations used are: CMV, cytomegalovirus; 5′-AZC, 5′-aza-2′-deoxycytidine; TSA, trichostatin A.


Additional content from the original text has been removed for brevity and relevance to the DNA methylation analysis of the hTERT gene.
amplification using the Advantage cDNA PCR kit (Clontech Labs, Inc., Palo Alto, CA). The PCR conditions were as described previously (11). The following two independent primer pairs were used for the detection of hTERT mRNA: (a) 5'-TGAAAGCCAAGAACGCAGGGA-3' and 5'-GGGAAGTGGAAGACGGCAGGT-3' (11); and (b) 5'-CGGAAGAGTGTCTGGAGCAAT-3' and 5'-GGATGAAGCGGAGTCTGGA-3' (12). The control amplification of glyceraldehyde-3-phosphate dehydrogenase mRNA was performed as described previously (11).

Methylation Analysis. To assess methylation of the hTERT promoter, bisulfite genomic sequencing was used (13, 14). Before bisulfite modification, 1–3 μg of DNA were digested with EcoRI at 37°C overnight in a volume of 35 μl. The DNA was then denatured by the addition of 5 μl of 3 N NaOH, heated at 75°C for 15 min, and placed on ice. A total of 300 μl of freshly made 4.4 μM sodium bisulfite (pH 5.0) and 17 μl of 10 mM hydroquinone were added to the DNA, covered with mineral oil, and incubated at 55°C for 5 h. The DNA was then washed on a Centricon 30 column (Amicon, Inc., Beverly, MA), desulfonated with 5 N NaOH (10 μl/150 μl sample), and incubated at 37°C for 15 min. The DNA was neutralized with 75 μl of 5 M ammonium acetate and stored at −20°C. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 μl of dH2O for PCR.

Seventy-two CpG sites of the hTERT promoter, extending from 500 bases upstream of the transcriptional start site to 50 bases downstream of the translational start site, were examined for methylation. The primers used for amplification of this region were as follows: 3F (bases −522 to −501), 5'-GGTATTTGTGGTGTTCGGAGTT-3' (unmethylated/methylated); 4R (bases 115–135), 5'-AACATACCAAACAAACACCTT-3' (unmethylated/methylated); 5R (same as 4R, but methylated), 5'-AACGTAACCAAACACCTT-3'; 7R (bases −176 to −155), 5'-AATACCCCAAGTTCCCAATCC-3' (methylated); 8F (bases −431 to −410), 5'-GTACGTATTTGTTTTAGGGTT-3' (methylated); 9F (bases −223 to −203), 5'-GGATTCGGGGTGATAGACGTT-3' (methylated); and 13F (same as 9F, but unmethylated), 5'-GGATTTGTGTTATAGACGTT-3'.

After bisulfite modification, nested PCR was performed with primers designed to amplify the methylated or unmethylated sequences in the hTERT promoter. Samples were amplified first with outer primers 3F 3-5R (modified methylated) and 3F 3-4R (modified unmethylated). Inner primer pairs used were 3F 3-7R, 8F 3-7R, 9F 3-5R (methylated), or 13F 3-4R (unmethylated). Because of the density of the CpG sites in this region, all primers included some CpG sites. Even with primers containing the most Cs in potentially methylated CpG sites, PCR products from some cells had many partially methylated or unmethylated sites, suggesting that these primers are not just amplifying a minority of cells that are methylated.

Fig. 1. Bisulfite genomic sequencing of hTERT promoter from CpG sites 41–47 in MRC-5, MDA-MB435, and SiHa cells. Methylated CpG sites show bands in the C lane, whereas unmethylated Cs are changed to Ts by this method.

Fig. 2. Methylation of the hTERT promoter region from 500 nucleotide bases upstream of transcriptional start site extending into exon 1 in normal and cancer cell lines. CpG sites are numbered from 1–72, with density shown below (4). CpG sites are indicated as follows: complete methylation (■); partial methylation (○); unmethylated (●); and not assessed (□).
After PCR the products were electrophoresed on 1.5% low-melting-point agarose gels, the bands were excised and purified on QiaGen columns (Qiagen Inc., Santa Clarita, CA). The products were then cycle sequenced with a United States Biochemical/Amersham 33P Thermo-sequenase kit (United States Biochemical, Cleveland, OH). Amplification primers were used as sequencing primers.

Cell Treatments to Study the Effect of Demethylation and Chromatin Structure on hTERT Expression. SUSM-1 cells were treated with 5-AZC at a final concentration of 3 μM or the same volume of PBS for 96 h. After this initial incubation, TSA at a final concentration of 500 nM or the same volume of ethanol was added to the media. After an additional 24-h incubation, the cells were collected for isolation of DNA and RNA. These procedures made a set of four differentially treated cells: (a) untreated cells; (b) cells treated with TSA alone; (c) cells treated with 5-AZC alone; and (d) cells treated with both 5-AZC and TSA. Expression of hTERT and methylation of the hTERT promoter were then measured in these cells as described above.

Results

Using bisulfite genomic sequencing (Fig. 1), we first assessed the methylation status of the CpG island in the hTERT promoter in a diverse set of normal, immortalized, and cancer cell lines. The region of the hTERT promoter examined includes 72 CpG sites as well as several potential transcriptional regulatory motifs (4). Bisulfite modification of DNA changes all unmethylated deoxycytidines to uracils (read as thymidines), while leaving methylated deoxycytidines intact. This method allowed us to examine all CpG sites at once for site-specific and region-specific methylation. Results for eight cell lines are shown in Fig. 2. Methylation of either specific CpG sites or groups of CpG sites did not necessarily correlate with hTERT expression in all of these cell lines. For example, the hTERT 5′-region from NHF cells and MRC-5 fetal lung fibroblasts, which lack hTERT expression and telomerase activity, had almost no methylated CpG sites. On the other hand, the CMV immortal fibroblast cell line that expresses hTERT and the SUSM-1 fibroblasts that do not express hTERT both showed complete methylation at all CpG sites examined. In some of the hTERT-expressing cancer cell lines, most of the CpG sites were partially or totally methylated.

In case there might be common methylation patterns among different cell lines within tissue types that might suggest a mechanism of hTERT regulation in certain cell types, we examined additional cell lines. We assessed the methylation status of the hTERT promoter from CpG site 35 (−196 nucleotide relative to the transcriptional start site) to site 72 (+112 nucleotide) in eight lung cancer and two colon cancer cell lines. Similar to the results shown in Fig. 2, we saw no common methylation pattern that correlated with hTERT expression among these cancer cell lines (Fig. 3A). Among this group of hTERT-expressing cancer cell lines, the HTB 182, HTB 183, A549, and HCT 116 cell lines exhibited mostly unmethylated CpG sites in this region, whereas the other six cell lines showed partial or complete methylation at most of the CpG sites examined. We also tested 21 breast normal, immortal, and cancer cell lines for CpG methylation in the region of a c-MYC binding motif and the translational start site (Fig. 3B). About half of the hTERT-expressing breast cancer cell lines exhibited partial or complete methylation at the sites examined, whereas the other half appeared almost totally unmethylated.

Although the hTERT expression from the methylated promoter suggests that CpG island methylation is not a general mechanism of
down-regulation for this gene, it may play a role in the regulation of hTERT expression in specific cell types and/or at some stage of development or carcinogenesis. To provide evidence for a possible role for DNA methylation in the regulation of hTERT expression, we grew SUSM-1 cells, which lacked hTERT expression and exhibited complete methylation of the hTERT promoter, in the presence or absence of the demethylating agent 5-AZC and the chromatin/histone deacetylase inhibitor TSA (Fig. 4). Treatment with 5-AZC induced expression of hTERT and reduced methylation of promoter CpG sites. Two experiments with different bisulfite treatment times (2.5 and 5 h) for DNA from cells treated with TSA or TSA + 5-AZC showed incomplete modification of the DNA in some regions, although no reduction in methylation was evident at some CpG sites nearby non-CpG Cs that were totally converted to Ts. However, TSA cooperated with 5-AZC to markedly enhance the hTERT expression, although no effect was observed with TSA alone (Fig. 4). In normal human fibroblasts in which the promoter is hypomethylated, the same treatments with 5-AZC and/or TSA did not induce hTERT expression (data not shown). These results suggest that DNA methylation and associated histone deacetylation are at least partially responsible for the repression of hTERT in the SUSM-1 cells.

Discussion

Regulation and maintenance of telomere length is complex and appears to involve multiple mechanisms. A role for regulation of hTERT expression and telomerase activity by methylation is suggested by the presence of a dense CpG-rich region in the promoter of hTERT (4). To begin to evaluate these regulatory mechanisms, we assessed the methylation status of the hTERT CpG island in a variety of human normal, immortal, and cancer cell lines. We found that the hTERT promoter was completely or partially methylated in many cell lines. Moreover, treatment of hTERT-negative SUSM-1 cells with 5-AZC and TSA induced hTERT expression. These results indicate that hTERT promoter methylation is involved in the regulation of hTERT expression and telomerase activity, at least in some cells.

No specific methylation patterns at certain CpG sites or regions of the hTERT promoter emerged that correlated with expression in all of the diverse cell lines examined. Numerous transcriptional binding motifs have also been identified in the hTERT promoter, indicating that the regulation of hTERT expression is complicated. Several recent reports have identified c-MYC binding sites (E-boxes) in the hTERT promoter (4, 6) and found that c-MYC positively regulates hTERT expression (4, 6). Our results suggest that a generalized mechanism of CpG methylation within or adjacent to these E-boxes does not affect the transcriptional regulation of hTERT in the cells studied.

Some of our results were surprising, but they provide some new insights into hTERT regulation. Most of the normal human somatic cells that do not express the hTERT gene (e.g., NHF, MRC-5, 184FB, and P90 cells) had an unmethylated/hypomethylated promoter. This suggests that a DNA methylation-mediated mechanism is not primarily responsible for the stringent repression of hTERT expression in these cells. This could also mean that the hTERT-expressing immortalized cells with the unmethylated/hypomethylated promoter underwent a mutation(s) that permitted the up-regulation, or derepression, of hTERT transcription from such a promoter. On the other hand, some cell lines (e.g., CMV and SiHa) with a completely hypermethylated promoter in the region examined expressed hTERT mRNA. This indicates that hypermethylation itself is not necessarily associated with transcriptional repression of hTERT. However, treatment with 5-AZC and TSA induced hTERT expression in an immortalized fibroblast cell line SUSM-1 that is otherwise hTERT negative and has the completely methylated promoter. This suggests the presence of a DNA methylation/chromatin structure-mediated mechanism for repression of the hTERT gene transcription, at least in this cell line. It is known that some cells maintain their telomeres by telomerase-independent mechanisms (3); thus, CpG methylation may be involved in the regulation of hTERT expression and telomerase activity in some cells or tissue types, but not others.

CpG islands within gene promoters generally become methylated during human carcinogenesis, which can result in methylation-mediated loss of expression of tumor suppressor genes (e.g., p16INK4a, hMLH1, and E-cadherin). Our data suggest that the promoter of the hTERT gene also becomes methylated during the development of some but not all tumors. However, in contrast to the tumor suppressor genes, promoter hypermethylation of tumor-promoting genes such as hTERT does not contribute to tumor progression. Instead, it could function as a fail-safe mechanism against carcinogenesis. If that is the case, it is possible that the hTERT-expressing cells with the hypermethylated promoter have gained an additional mutation(s) to overcome the DNA methylation/chromatin structure-mediated repression of the hTERT gene.

It is possible that methylation of another region upstream or downstream functions in the regulation of hTERT expression. However, based on the CpG density within the promoter region cloned to date, we tested the major region of the promoter CpG island without finding a good correlation with expression. The additional cloned region upstream of the area we examined has only a sparse representation of CpG sites. Our findings suggest that control of hTERT expression is complex and appears to involve both methylation-dependent and methylation-independent mechanisms.

References


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