Hypermethylation of the human telomerase catalytic subunit (TERT) gene correlates with telomerase activity

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · OCTOBER 2002

Impact Factor: 5.09 · DOI: 10.1002/ijc.10593 · Source: PubMed

6 AUTHORS, INCLUDING:

Fred T Bosman
University Hospital of Lausanne
295 PUBLICATIONS **7,900** CITATIONS
SEE PROFILE

Jean Benhattar
Aurigen
120 PUBLICATIONS **5,046** CITATIONS
SEE PROFILE

Int. J. Cancer: **101,** 335–341 (2002)

Publication of the International Union Against Cancer

HYPERMETHYLATION OF THE HUMAN TELOMERASE CATALYTIC SUBUNIT (*hTERT*) GENE CORRELATES WITH TELOMERASE ACTIVITY

Isabelle GUILLERET, Pu YAN, Fabienne GRANGE, Richard BRAUNSCHWEIG, Fred T. BOSMAN and Jean BENHATTAR*

Institut de Pathologie and Institut de Médecine Légale, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

DNA methylation is an epigenetic process involved in embryonic development, differentiation and aging. It is 1 of the mechanisms resulting in gene silencing in carcinogenesis, especially in tumor suppressor genes (e.g., *p16*, *Rb*). Telomerase, the DNA polymerase adding TTAGGG repeats to the chromosome end, is involved in the regulation of the replicative life span by maintaining telomere length. This enzyme is activated in germ and stem cells, repressed in normal somatic cells and reactivated in a large majority of tumor cells. The promoter region of the *hTERT* gene, encoding for the catalytic subunit of human telomerase, has been located in a CpG island and may therefore be regulated at least in part by DNA methylation. We analyzed the methylation status of 27 CpG sites within the *hTERT* promoter core region by methylation-sensitive single-strand conformation analysis (MS-SSCA) and direct sequencing using bisulfite-modified DNA in 56 human tumor cell lines, as well as tumor and normal tissues from different organs. A positive correlation was observed among hypermethylation of the *hTERT* promoter, *hTERT* mRNA expression and telomerase activity ($p < 0.00001$). Furthermore, this correlation was confirmed in normal tissues where hypermethylation of the *hTERT* promoter was found exclusively in *hTERT*-expressing telomerase-positive samples and was absent in telomerase-negative samples ($p < 0.00002$). Since tumor tissues contain also non-neoplastic stromal elements, we performed microdissection to allow confirmation that the *hTERT* promoter methylation truly occurred in tumor cells. Our results suggest that methylation may be involved in the regulation of *hTERT* gene expression. To our knowledge, this is the first gene in which methylation of its promoter sequence has been found to be positively correlated with gene expression.

Key words: *telomerase;* hTERT*; DNA methylation; microdissection; TRAP assay*

Telomerase, the DNA polymerase that adds TTAGGG repeats to the end of chromosomes, is involved in the regulation of the replicative life span by maintaining telomere length. In humans, telomerase consists of a complex of proteins, including a reverse transcriptase subunit (encoded by *hTERT*), an RNA template (encoded by *hTR*) and a telomerase-associated protein (encoded by *hTEP1*), as well as many other proteins.[1] Telomerase was found to be activated in germ and stem cells, repressed in normal somatic cells and reactivated in a large majority of tumor cells.[2] An *in vitro* reconstitution experiment showed that the expression of hTERT and hTR was sufficient to obtain telomerase activity.[3] It is therefore of interest to understand how the expression of the genes encoding different subunits of human telomerase is upregulated when a cell becomes immortal.

Recently, the promoter region of the *hTERT* gene has been characterized.[4–6] This region harbors a CpG island and may therefore be under the regulation of DNA methylation.[7,8] CpG methylation is an epigenetic process involved in tumorigenesis, differentiation, X chromosome inactivation and imprinting.[9,10] Promoter region hypermethylation associated with transcriptional loss is an alternative mode of gene inactivation during cancer development—as demonstrated for several tumor suppressor genes.[11,12]

hTERT methylation has been recently assessed in a variety of cell lines, including normal, immortalized and cancer cell lines, as well as in several tumor tissues.[7,8] The promoter of the *hTERT* gene was found to be hypermethylated in many telomerase-negative and telomerase-positive tumors and cell lines. In contrast, hypomethylation was detected in 3 normal tissues[8] and in most of the analyzed normal somatic cell lines[7] without telomerase expression. In general, CpG island methylation correlates inversely with gene expression.[13] In these studies, *hTERT* promoter methylation was also observed in telomerase-positive cells. Although these results are complex, they indicate that the level of methylation of the *hTERT* promoter is variable and thus led to the possibility that methylation plays a role in *hTERT* gene regulation.

De novo CpG island methylation is a frequent accompaniment of the establishment of cells in culture.[14] In the case of *hTERT*, the question was whether the methylation observed was due to this phenomenon or whether this methylation was specifically implied in the regulation of this gene.

To elucidate the relationship between methylation and *hTERT* gene expression, we have focused the present study on a variety of human normal and tumor tissues, as well as cell lines established from tumors. *hTERT* gene expression and telomerase activity were determined for each sample by RT-PCR and TRAP assay, respectively. After sodium bisulfite modification of genomic DNA and PCR amplification using primers without CpG repeats, methylation status of the *hTERT* promoter was determined using 2 complementary techniques: direct sequencing, which indicates whether each CpG site is methylated, and methylation-sensitive single-strand conformation analysis (MS-SSCA), which allows determination of the clonal nature of the methylation pattern.[15] Although methylation-specific PCR (MSP) is the most current approach used for the analysis of DNA methylation,[16] it was not applied in our study. Indeed, this method of analysis is sometimes too sensitive[17] and not quantitative. Moreover, MSP does not give any information about the clonality of the analyzed DNA region. Finally, microdissection was achieved to determine specifically the *hTERT* methylation status of tumor cells isolated from tumor tissues.

We found that the *hTERT* promoter is frequently hypermethylated when *hTERT* mRNA is expressed, which suggests that methylation of the *hTERT* promoter may be implicated in the regulation of *hTERT*, but in a different way from other genes regulated by promoter methylation.

Grant sponsor: Ligue Vaudoise contre le Cancer (Lausanne, Switzerland); Grant sponsor: Swiss National Science Foundation; Grant number: 3200-061624.00.

*Correspondence to: Institut de Pathologie, CHUV, Bugnon 25, CH-1011 Lausanne, Switzerland. Fax: +41 21-314 7115;
E-mail: Jean.Benhattar@chuv.hospvd.ch

Received 25 October 2001; Revised 5 February, 19 April 2002; Accepted 19 June 2002

DOI 10.1002/ijc.10593

MATERIAL AND METHODS

Tissue samples

Normal and tumor tissues were obtained from the Tissue Bank of the Institute of Pathology of Lausanne. Twenty-four human normal tissues or blood samples (bladder, brain, leukapheresis from healthy patients, colon, heart, kidney, muscle, placenta, non-stimulated skin and testis from fertile men) and 19 tumor tissue samples (bladder, brain, breast, colon, kidney, lung and soft parts) were analyzed in our study. The nature of all tissue samples was histologically checked by experienced pathologists.

Tumor cell lines

Thirteen human malignant tumor cell lines (bladder: J82, T24; breast: MCF-7; cervix: A431, HeLa; colon: Co115, HT29, SW480; lung: H520, SW2; prostate: PC-3; soft tissue: Saos-2, U2-OS) were used in our study. With few exceptions, these cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in DMEM with glutamax-1 supplemented with 10% FBS (5% for Saos-2 and U2-OS), or Leibovitz medium (L15) with 5% FBS and 0.2% $NaHCO_3$ for SW480 (all products from GIBCO BRL, Paisley, Scotland).

Plasmids

Control plasmid was generated by subcloning 1.1 kb, bases -700 to +405 bp of the *hTERT* promoter region,[5] in the pGEM-T vector (Promega, Madison, WI). The plasmid was divided into 2 parts: 1 was left completely unmethylated and the other was fully methylated at all CpG sites using *Sss*I methylase (Biolabs, Hitchin, England) according to the manufacturer's protocol. Unmethylated and methylated plasmids were mixed at different ratios. The bisulfite modification was performed on fully methylated and unmethylated plasmids as well as on different mixes.

DNA and RNA extraction, RT-PCR and TRAP assay

DNA, RNA and proteins were extracted from whole consecutive tissue sections. To establish the methylation status of the *hTERT* gene promoter, genomic DNA was isolated using the DNeasy tissue kit (Qiagen, Hilden, Germany). Total RNA was extracted from frozen tissue sections or cells using Trizol (Life Technologies, Rockville, MD). RT-PCR on *hTERT* RNA was done as described previously.[18] The TRAP assay was performed according to a modified protocol described in Yan *et al.*[19]

Sodium bisulfite modification

Genomic DNA was modified using a protocol adapted from Raizis *et al.*[20]and Bian *et al.*[15] After 3 μg of DNA heating in 60 μl of water for 10 min at 100°C, DNA was denaturated for 10 min at 37°C by addition of 4.5 μl of 3 M NaOH. Sodium bisulfite (750 μl) and hydroxyquinone (42 μl) were then added to a concentration of 40.5% and 10 mM respectively. The reaction was performed at 55°C for 5 hr. After addition of 160 μl of pure ethanol, DNA was purified using the DNeasy tissue kit columns (Qiagen). After washing, desulfonation was performed on the column by addition of 500 μl of 0.3 M NaOH/80% EtOH. Incubation was performed at RT for 20 min in the dark. After washing, the modified DNA was eluted from the column with 50 μl of 10 mM Tris-HCl (pH 8.0).

PCR, direct sequencing and MS-SSCA analysis

PCR on *hTERT* promoter was performed using the primers 5′-GGGTTATTTTATAGTTTAGGT-3′ and 5′-AATCCCCAA-TCCCTC-3′, in a final concentration of 5% DMSO, under the following PCR cycling conditions: 35 cycles of 94°C for 30 sec, 53°C for 45 sec and 72°C for 45 sec, followed by 10 min at 72°C. Direct sequencing of all PCR products was done on a ABI prism 310 sequencer (Perkin-Elmer, Oak Brook, IL). Each PCR product was analyzed by MS-SSCA.[15] First, PCR products (5 μl) were denatured for 10 min at 50°C by addition of 1 μl of 0.5 M NaOH/10 mM EDTA. After addition of 2 μl of formamide dye (95% formamide, 10 mM EDTA pH 8.0, 0.05% bromophenol, 0.025% xylene cyanol), the samples were immediately loaded on a 40% MDE gel (FMC BioProducts, Rockland, ME) and electrophoresed overnight in 0.5× TBE buffer at 20°C and 220 V (14.5 V/cm). After electrophoresis, gels were released from plates, stained for 20 min in the dark with SYBR Gold gel stain (Molecular Probes, Eugene, OR) and visualized under U.V. light using a CCD camera.

Microdissection

Microdissection and DNA extraction were performed as described previously.[21] Briefly, paraffin-embedded tissue was cut (7 μm thickness) and the sections were mounted on glass slides. Before the slides were stained with toluidin blue, the sections were deparaffinized in xylol and rinsed with methanol. Then normal cells were removed by scratching. Verification that the remaining cells were indeed tumor cells was performed by an experienced pathologist (R.B.) before they were harvested. After extraction using the phenol/chloroform method, DNA was modified by sodium bisulfite without the preliminary heating step and amplified as above.

RESULTS

Calibration of the methylation analysis by direct sequencing and MS-SSCA

Genomic DNA was modified using the sodium bisulfite method, and DNA fragments were amplified by PCR using primers without CpG repeats and complementary to the deaminated DNA strand. No PCR amplification was obtained with these primers on non-modified genomic DNA. Methylation patterns of the *hTERT* promoter were determined by direct sequencing and MS-SSCA.[15] To check the reliability of the PCR reaction, we subcloned the promoter of *hTERT*[4–6] in a pGEM-T vector. After modification by sodium bisulfite, PCR reactions were done by using the completely unmethylated plasmid, fully methylated plasmid and a mixture of unmethylated and methylated plasmid. Direct sequencing of these PCR products revealed that all the CpG sites were TpG, CpG and T/CpG, respectively. Moreover, all cytosine from non-CpG sites was transformed into uracile after bisulfite treatment. Band intensity ratios obtained by MS-SSCA were similar to the methylated/unmethylated plasmid ratios before bisulfite treatment (Fig. 1, lanes 1–3).

hTERT *promoter methylation positively correlates with* hTERT *MRNA expression and telomerase activity*

According to MS-SSCA and direct sequencing, the *hTERT* promoter region was found to be hypomethylated in all the 15 normal telomerase-negative tissue samples analyzed. In contrast, promoter hypermethylation was observed in all 11 telomerase-positive cell lines (Figs. 1, 2). All the 27 CpG sites of the 224 bp sequence (−441 to −218 from the ATG translational start site) of the *hTERT* promoter region were methylated, with the exception of 2 bladder cancer cell lines, which exhibited a different pattern. In the latter cell lines, direct sequencing revealed a mixture of T and C at all CpG sites, and MS-SSCA showed equidense bands at the fully methylated and the completely unmethylated positions. As cell lines are clonal, this observation is compatible with the hypothesis that 1 allele of the *hTERT* promoter was methylated at all CpG sites, and the other allele was totally unmethylated (Fig. 1, lane 12). RT-PCR revealed that *hTERT* was expressed in all telomerase-positive cell lines but not in telomerase-negative normal tissues.

Tumor tissues with strong or weak telomerase activity were analyzed for methylation of the *hTERT* promoter (Table I). In 11 tumor tissues that all expressed *hTERT* mRNA and had strong telomerase activity (the 6 pb ladder extending all the way to the top of the gel), we found by sequencing analysis a combination of T and C at all CpG sites. A mixture of fully methylated and unmethylated DNA was observed by MS-SSCA (Fig. 1, lanes 15–17; Fig. 2, tumor tissues with strong *hTERT* mRNA expression). We also

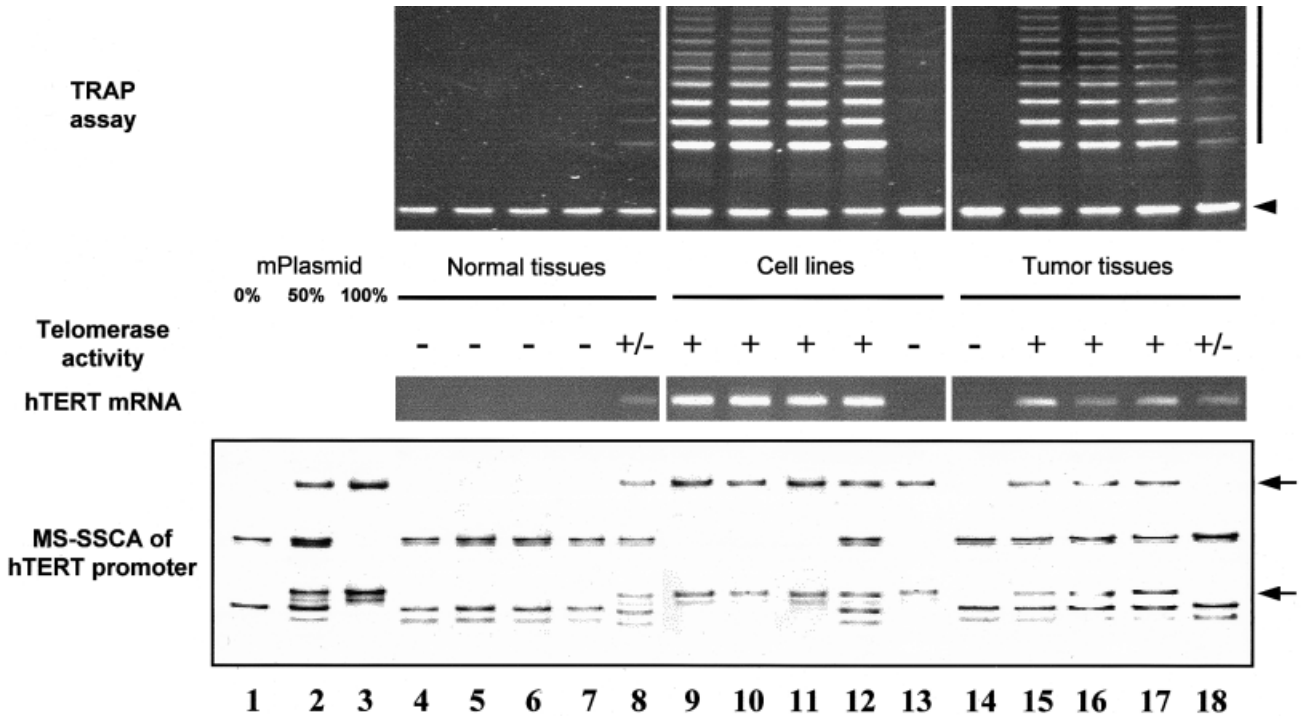

FIGURE 1 – Telomerase activity, *hTERT* expression and methylation patterns of *hTERT* gene promoter in human tissues and tumor cell lines. Lanes 1–3, MS-SSCA controls obtained from plasmids containing *hTERT* sequences; lane 1, MS-SSCA from unmethylated plasmid; lane 2, MS-SSCA from a mixture of fully methylated and unmethylated plasmids (50% of the population was methylated at all CpG sites); lane 3, MS-SSCA from a fully methylated plasmid; lanes 4–7, normal tissues without telomerase activity from respectively bladder, kidney, muscle and skin; lane 8, normal colon with weak telomerase activity; lanes 9–12, telomerase-positive cell lines (SW480, PC-3, SW2, J82); lane 13, Saos-2 (osteosarcoma cell line without telomerase activity); lane 14, sarcoma without telomerase activity; lanes 15–17, tumors with strong telomerase activity (brain, breast, lung); lane 18, bladder tumor with weak telomerase activity. In the TRAP assay, a 36 bp internal positive control band was seen in every lane (indicated by an arrow), and it was used to identify non-informative specimens due to inhibitors of *Taq* DNA polymerase. *hTERT* mRNA expression was detected by RT-PCR. Arrows in the MS-SSCA of the *hTERT* promoter indicate the fully methylated bands.

studied 4 tumors with weak telomerase activity (Table I). In 3 of them, we detected intratumor variations in telomerase activity.

This observation was not surprising, as intratumoral heterogeneity for telomerase activity has already been described by us and others.[18,22,23] Interestingly, intratumor heterogeneity was not detected in the 11 tumors with strong telomerase activity. Direct sequencing revealed that several CpG sites of the *hTERT* promoter were partially methylated in the 4 tumors with relatively weak telomerase activity (Table I, Fig. 2). A mixture of fully methylated and unmethylated DNA was observed by MS-SSCA in only 1 case, a breast tumor, whereas the other cases were found to be unmethylated (Fig. 1, lane 18). This apparent inconsistency between the results from direct sequencing and MS-SSCA can be explained on the assumption that the methylated CpG sites observed in sequencing come from partially methylated subclones smaller than 5%, which is the lower limit of the MS-SSCA detection for each clone.[24]

In summary, hypermethylation of the *hTERT* promoter was observed in all tumor tissues and tumor cell lines that expressed *hTERT* mRNA and had telomerase activity (Table II). In contrast, hypomethylation was detected in telomerase-negative normal tissues. Thus, a positive correlation could exist among hypermethylation of the *hTERT* promoter, *hTERT* mRNA expression and telomerase activity.

hTERT *promoter methylation occurs in tumor cells from telomerase-positive tumor tissues*

As tissues contain different cell types, the observed methylation and telomerase activity could have been derived from different cell types (tumor cells or stromal cells). We therefore isolated tumor cells by microdissection from paraffin-embedded fixed tissue sections. To perform this analysis, we selected tumor sections from 4 cases corresponding to the frozen tumor tissues studied above (Table I, bladder B, colon B, kidney and soft tissue E). The *hTERT* core promoter was methylated at all CpG sites in telomerase-positive and *hTERT*-expressing carcinomas from kidney and colon, as observed in colon and kidney tumor cell lines (Fig. 3). A telomerase-positive bladder transitional carcinoma exhibited a mixture of fully methylated and unmethylated patterns. The mixture observed might be due to the presence of 1 allele methylated at all CpG sites and the other allele totally unmethylated. Interestingly, this mixture was also seen in 2 bladder cancer cell lines that also came from transitional carcinomas (Fig. 1, lane 12). As a negative control, we used a telomerase-negative and *hTERT*-non-expressing sarcoma (Table I, liposarcoma) in which microdissected tumor cells were unmethylated. These results indicate that, in tumor cells with high telomerase activity, the *hTERT* promoter is hypermethylated.

hTERT *promoter methylation occurs in some telomerase-negative tumors*

We analyzed 4 telomerase-negative tumor samples (Table I) and 2 telomerase-negative tumor cell lines. In 3 of these 4 tumors (1 brain and 2 soft tissue), many CpG sites were found to be partially methylated by sequence analysis (Fig 2, tumor tissues without *hTERT* mRNA expression). However, no fully or partially methylated clone was detected by MS-SSCA (Fig. 1, lane 14). In contrast, the 2 telomerase-negative cell lines (Saos-2 and U2-OS) exhibited complete methylation of the *hTERT* core promoter by direct sequencing (Fig. 2) as well as by MS-SSCA (Fig. 1, lane 13).

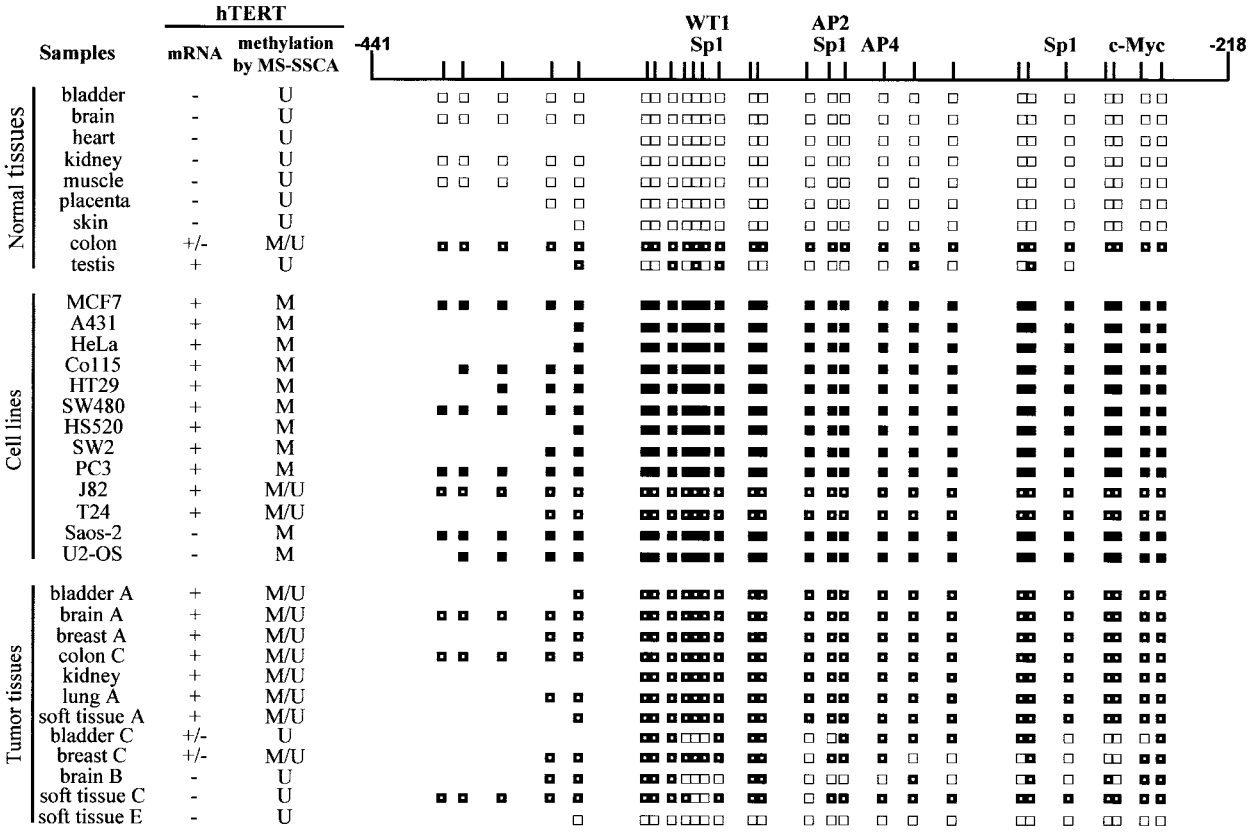

FIGURE 2 – Genomic bisulfite sequencing of *hTERT* promoter from −441 to −218 nucleotide bases upstream of the ATG translational start site. Each line on the scale represents 1 CpG in the sequence studied. Methylation status at each CpG site is indicated as follows: black boxes, complete methylation; black boxes with open center, partial methylation; open boxes, no methylation; blank, not assessed. Results from RT-PCR and MS-SSCA are summarized on the left: +, strong *hTERT* mRNA expression; +/−, relatively weak *hTERT* mRNA expression; −, no *hTERT* mRNA expression; U, unmethylated pattern only; M, methylated pattern only; M/U, mix of unmethylated and methylated patterns.

Normal tissues containing stem or germ cells and with telomerase activity may also show hTERT *promoter methylation*

We analyzed normal tissues including colon, testis and white blood cells collected by leukapheresis. *hTERT* expression was observed by RT-PCR in all 9 samples analyzed. Normal colon mucosa exhibited a weak telomerase activity by TRAP assay and a mixture of unmethylated and totally methylated *hTERT* promoter by direct sequencing and MS-SSCA (Fig. 1, lane 8; Fig 2). In all testis and in 1 of the 2 leukapheresis samples, sequencing revealed that several CpG sites were partially methylated (Fig. 2, testis), whereas no clonal methylation of the *hTERT* promoter was observed by MS-SSCA.

It is interesting to note that no telomerase activity, or *hTERT* expression and promoter methylation, was detectable in the majority of the analyzed normal tissues containing *a priori* only nonstimulated stem cells, skin, for example. These findings were in good agreement with the data from the literature, which indicated that telomerase activity was not detectable in most nonstimulated normal adult somatic tissues.[25,26]

In summary, in normal samples, *hTERT* promoter hypermethylation was observed in 8 of the 9 telomerase-positive cases, whereas all the 15 telomerase-negative tissues were found to be hypomethylated. Therefore, a strong correlation was shown between methylation of the *hTERT* promoter and telomerase activity in normal human samples ($p < 0.00002$).

DISCUSSION

Since the 5′-region of the *hTERT* gene has been found to harbor a CpG island, it was of interest to look at the methylation status of the promoter of this gene in various normal and tumor tissue samples as well as in tumor cell lines. In our study, we show that hypermethylation of the *hTERT* promoter gene is positively correlated with its activation and telomerase activity ($p < 0.00001$, Table II). Indeed, by direct sequencing of sodium bisulfite-modified genomic DNAs, we found that all but 1 of the 35 samples examined (tumor tissues, normal tissues containing germ or stem cells and tumor cell lines), which expressed *hTERT* gene and with detectable telomerase activity, contained a methylated *hTERT* promoter. In contrast, 76% (16/21) of samples lacking telomerase activity harbored a hypomethylated *hTERT* promoter (results summarized in Table II). Interestingly, all 5 telomerase-negative samples with a hypermethylated *hTERT* promoter had a tumor origin. Hence, the *hTERT* promoter hypermethylation seems to occur during almost all tumor cell transformations regardless of *hTERT* gene regulation. However, when this is applied to normal tissues only, the correlation between *hTERT* methylation and telomerase activity remained highly significant ($p < 0.00002$).

MS-SSCA, another approach to methylation analysis, was performed in our study. This technique allows a clonal analysis of DNA population mixtures,[15] in which any clone larger than 5% can be easily detected.[24] In contrast, direct sequencing only gives data about the level of methylation of each single CpG site, without considering the potential presence of distinct cell populations presenting different levels of methylation. Therefore, MS-SSCA can be used to show the clonal nature of the methylation detected. When applied to our samples, MS-SSCA revealed a mixture of fully methylated and fully unmethylated DNA in the 11 highly telomerase-positive tumors. Manual microdissection to exclude normal cells allowed us to study the tumor cells specifi-

TABLE I – TELOMERASE ACTIVITY AND *hTERT* PROMOTER METHYLATION OF MALIGNANT TUMORS

Tissue of origin	Histology[1]	Grade[2]	Telomerase activity[3]	*hTERT* methylation	
				MS-SSCA[4]	Sequencing[5]
Bladder A	Transitional carcinoma	3	+++	M/U	+
Bladder B	Transitional carcinoma	2	+++	M/U	+
Brain A	Glioblastoma	4	+++	M/U	+
Breast A	Ductal carcinoma	2	+++	M/U	+
Breast B	Ductal carcinoma	2	+++	M/U	+
Colon A	Adenocarcinoma	3	+++	M/U	+
Colon B	Adenocarcinoma	2	+++	M/U	+
Colon C	Adenocarcinoma	2	+++	M/U	+
Kidney	Renal carcinoma	4	+++	M/U	+
Lung A	NSCLC	2	+++	M/U	+
Soft tissue A	MFH, giant cell type	2	+++	M/U	+
Bladder C	Transitional carcinoma	3	+	U	+/−
Breast C	Lobular carcinoma	2	+	M/U	+/−
Lung B	NSCLC	3	+	U	+/−
Soft tissue B	MPNST	3	+	U	+/−
Brain B	Glioblastoma	4	−	U	+/−
Soft tissue C	Leiomyosarcoma	3	−	U	+/−
Soft tissue D	MFH, storiform-pleom.	2	−	U	+/−
Soft tissue E	Liposarcoma	3	−	U	−

[1]NSCLC, nonsmall cell lung carcinoma; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor.–[2]2, moderately differentiated (colon, TNM 1997, 5th ed.), grade II (bladder and lung, TNM 1997, 5th edition), grade II (breast, Elston CW, 1987), grade II (soft tissue sarcoma, FNCLCC classification); 3, poorly differentiated (colon, TNM 1997, 5th ed.), grade III (bladder and lung, TNM 1997, 5th ed.), grade III (soft tissue sarcoma, FNCLCC classification); 4, grade IV (kidney, TNM 1997, 5th ed.), grade IV (brain, WHO classification).–[3]Telomerase activity by TRAP assay: +++, marked activity; +, low activity; −, no activity.–[4]M, methylated pattern only; U, unmethylated pattern only; M/U, mix of methylated and unmethylated patterns.–[5]+, partial methylation observed at all CpG sites; −, no methylation at all CpG sites; +/−, some CpG sites were partially methylated.

TABLE II – *hTERT* PROMOTER METHYLATION, AS DETERMINED BY MS-SSCA AND SEQUENCING ANALYSIS, IN HUMAN TISSUES AND CELL LINES AND CORRELATION WITH TELOMERASE ACTIVITY

	Telomerase activity[1]								
	Cell lines		Tumors			Normal tissues[2]		All samples[3]	
	+++	−	+++	+	−	+	−	Yes	No
Hypermethylation	11	2[4]	11	4[5]	3[6]	8[7]	0	34	5
Hypomethylation	0	0	0	0	1	1[8]	15	1	16
Number of cases	11	2	11	4	4	9	15	35	21

[1]Telomerase activity by TRAP assay: +++, marked activity; +, low activity; −, no activity.–[2]$P < 0.00002$ (Fisher's exact test).–[3]$P < 0.00001$ (Fisher's exact test).–[4] Cell lines without telomerase activity: Saos-2 and U2-OS.–[5] Weak telomerase-positive tumors: 1 tumor presented a methylated clone by MS-SSCA, but direct sequencing allowed to find at least some CpG sites, partially methylated, for all these 4 tumors.–[6]Telomerase-negative tumors that harbored several methylated CpG sites but only by sequencing.–[7]Normal tissues (colon, leukapheresis and testis) containing stem or germ cells.–[8]Leukapheresis from a healthy donor in whom no methylation at CpG sites was identified by sequencing.

cally.[21] Performed on telomerase-positive tissues, it revealed that tumor cells possess a hypermethylated *hTERT* promoter. Thus, *hTERT* promoter methylation correlates with *hTERT* transcription and telomerase activity in telomerase-positive tumors.

Independently of telomerase activity, the *hTERT* promoter was found to be hypermethylated by sequencing in 18 of the 19 analyzed tumors (Table I). Clonal methylation, as demonstrated by MS-SSCA, was observed in all the tumors with strong telomerase activity. In contrast, the presence of methylated CpG sites within the *hTERT* promoter but in the absence of a clonal situation was observed in most tumors without or with only weak telomerase activity (6 of 7 cases). These data indicate that, in this kind of tumor, several coexisting subclones would present different partial methylation patterns, which, sequenced together, would lead to a partially methylated sequence. As the lower limit of MS-SSCA sensitivity is around 5%, each subclone could represent less than 5% of the analyzed sample. All but 1 of the tumors with weak or without telomerase activity have exhibited a nonclonal methylation, at least by MS-SSCA analysis. In contrast, the methylated clonal pattern present in the 2 telomerase-negative cell lines could be explained by selection during the establishment of the cell line.[14]

Analysis of DNA from leukapheresis and testis revealed a nonmonoclonal *hTERT* core promoter methylation. The percentage of stem cells was estimated to be around 1% in leukapheresis samples.[27] The number of germ cells relative to the total cell number in testis has not yet been assessed; nevertheless, *hTR* expression was only observed by *in situ* hybridization in spermatocytes but not in differentiated spermatozoa.[28] Spermatocytes represent a small proportion of the total cell sample, and MS-SSCA might not have been sensitive enough to detect them. In contrast, in normal colon samples, an *hTERT* clonal fully methylated DNA population was observed. Our results are in good agreement with those obtained by immunohistochemistry, in which the colonic crypt epithelial cells were stained with anti-hTERT antibodies except those at the tip.[29] Furthermore, recent data indicate that normal human colonic crypt could contain a niche in which multiple stem cells are present and are replaced through periodic symmetric divisions.[30]

For tissues containing a small percentage of stem or germ cells, as in leukapheresis and testis, it is reasonable to propose that the *hTERT* promoter becomes demethylated during cell differentiation. During this process, the level of *hTERT* methylation can fluctuate from cell to cell. A similar process could also occur in tumors with weak or no telomerase activity. These tumors could maintain their telomeres in the absence of telomerase by a mechanism referred to as alternative lengthening of telomeres (ALT).[31] In this situation, the *hTERT* promoter could occasionally become

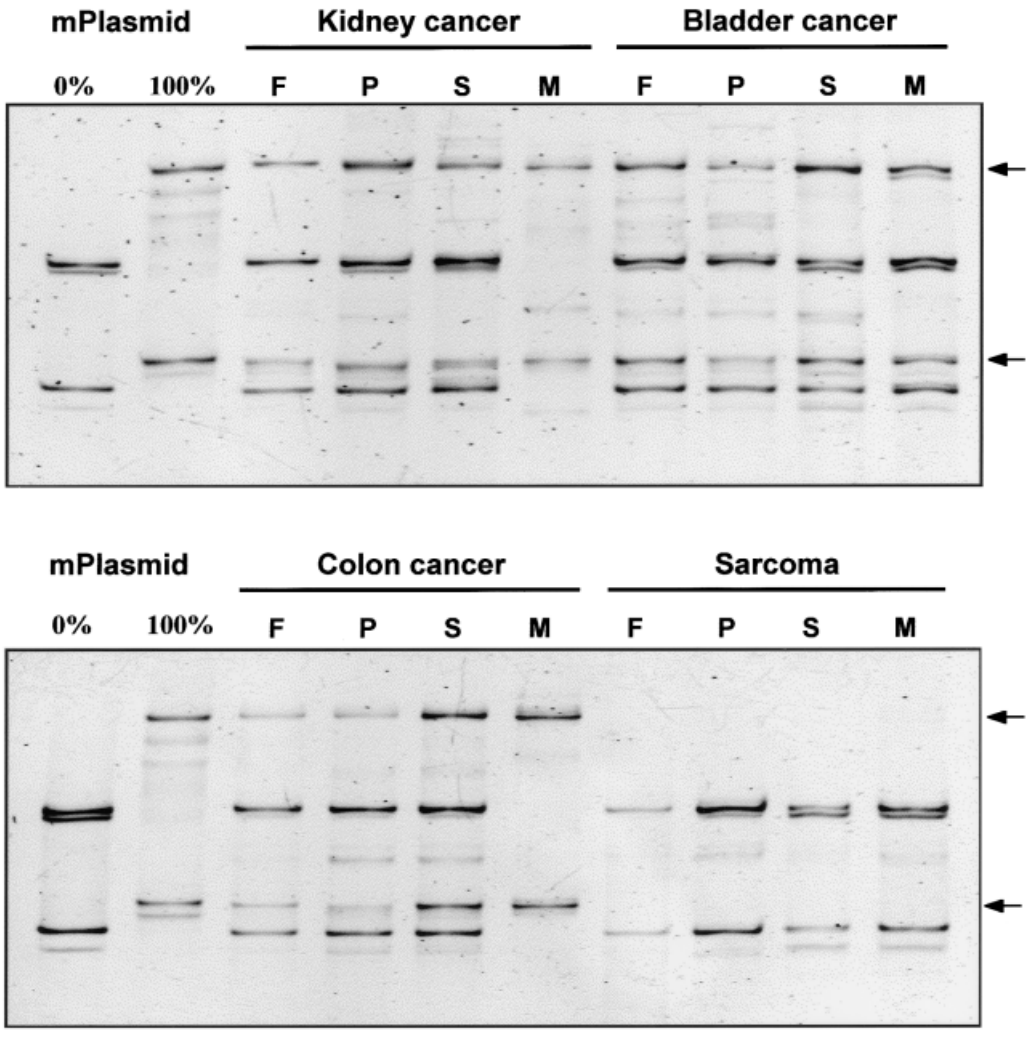

FIGURE 3 – Complete methylation of *hTERT* promoter in isolated tumor cells from telomerase-positive tissues. PCR products obtained after bisulfite modification were purified on acrylamide gel, reamplified (12 cycles) and analyzed by MS-SSCA. F, fresh tissue containing all kind of cells; P, tissues embedded in paraffin from which slides were obtained; S, area on slide containing all kind of cells and identical to the zone microdissected; M, microdissected area containing at least 95% of tumor cells. Bladder, colon and kidney tumors were telomerase-positive samples whereas sarcoma was a telomerase-negative case. Arrows indicate the fully methylated bands.

hypermethylated in few tumor cells. Its demethylation would occur during subsequent cellular divisions and thus, in a certain number of tumor cells, variable levels of methylation would be observed. A more complete study will be necessary to confirm this hypothesis.

All the results of our study are concordant with those obtained by Dessain *et al.*[7] and Devereux *et al.*[8] However, it was not possible for those authors to determine whether a correlation exists between the methylation level of the *hTERT* promoter and expression of the gene. In our study, the nature of the cases (tumor and normal tissues as well as tumor cell lines) and the number of samples examined (56) allow us to claim that the methylation of *hTERT* we observed could not be a random phenomenon. Consequently, our main conclusion is in stark contrast to the general model of gene regulation by promoter methylation.[32,33] In this model, methylation of DNA helps to stabilize chromatin in an inactive configuration and inhibits gene expression.

According to our results, 2 hypotheses have to be evaluated. First, it should be considered that the region that has been described as the *hTERT* core promoter is not the main regulatory sequence. Other DNA regions might act on the regulation of *hTERT* gene expression. In this view, the methylation we observed would be a side effect of this regulation. Numerous articles have characterized the *hTERT* promoter region,[34]and thus it seems difficult to believe that this region is not the main regulatory region of *hTERT*.

Second, considering the strong correlation observed in our study between *hTERT* promoter methylation and *hTERT* expression ($p < 0.00001$), methylation might play a role in *hTERT* regulation. DNA methylation is an epigenetic process known as a general inhibiting mechanism. It has been shown to interfere directly with the binding of activating transcription factors, such as for AP-2 and NF-κ_B.[35,36] On the other hand, transcriptional repressors, such as MeCP1 and MeCP2, can bind specifically to methylated DNA and mediate transcriptional repression.[37–39] For the *hTERT* promoter, such inhibition might occur on inhibitor(s). When *hTERT* is hypomethylated, the inhibitor(s) would bind to the promoter and block the transcriptional machinery. Hypermethylation of *hTERT* would prevent this binding and therefore would allow the promoter to be activated by appropriate transcriptional factors such as Sp1 or estrogens.[34,40] c-Myc seems to be a major activator of the *hTERT* promoter.[41,42] Its binding to DNA was demonstrated to be affected

by methylation of the internal CpG site.[43] According to our results and those of Devereux *et al.*,[7] the internal cytosine of the distal and proximal E-boxes was found to be hypermethylated in several telomerase-positive cell lines. These results may suggest that c-Myc binds, even weakly, to the hypermethylated *hTERT* promoter.

In conclusion, a strong correlation among *hTERT* promoter methylation, *hTERT* expression and telomerase activity was observed. Thus, methylation of the *hTERT* promoter may be 1 of the mechanisms regulating *hTERT* gene expression. To our knowledge, it is the first time that methylation in promoter sequences has been correlated with gene activity.

ACKNOWLEDGEMENTS

We thank L. Guillou and E. Strom for reviewing some hematoxylin and eosin sections, R. Janzer for providing brain samples, J.-C. Pages for skin samples, L. Juillerat and P. Beard for cell lines, and C. Brandt and N. Dimo for the access to the sequencer. We also thank M. Cusin for her help with microdissection.

REFERENCES

1. Krupp G, Klapper W, Parwaresh R. Cell proliferation, carcinogenesis and diverse mechanisms of telomerase regulation. Cell Mol Life Sci 2000;57:464–86.
2. Liu JP. Studies of the molecular mechanisms in the regulation of telomerase activity. FASEB J 2000;13:2091–104.
3. Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VW, Holt SE, Bodnar AG, Lichsteiner S, Kim NW, Trager JB, Taylor RD, Carlos R, et al. Reconstitution of human telomerase with the template RNA component hTR and catalytic protein subunit hTRT. Nat Genet 1997; 17:498–502.
4. Cong YS, Wen J, Bacchetti S. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum Mol Genet 1999;8:137–42.
5. Horikawa I, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res 1999;59:826–30.
6. Takakura M, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, Inoue M. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res 1999;59:551–7.
7. Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. Cancer Res 1999;59: 6087–90.
8. Dessain S, Yu HY, Reddel R, Baijersbergen RL, Weinberg RA. Methylation of the human telomerase gene island. Cancer Res 2000; 60:537–41.
9. Battistuzzi G, D'Urso M, Toniolo D, Persico GM, Luzzatto L. Tissue-specific levels of human glucose-6-phosphate dehydrogenase correlate with methylation of specific sites at the 3′ end of the gene. Proc Natl Acad Sci USA 1985;82:1465–69.
10. Razin A, Kafri T. DNA methylation from embryo to adult. Prog Nucleic Acid Res Mol Biol 1994;48:53–81.
11. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–96.
12. Herman JG. Hypermethylation of tumor suppressor genes in cancer. Semin Cancer Biol 1999;9:359–67.
13. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis 1997;18:869–82.
14. Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 1990;62: 503–14.
15. Bian YS, Yan P, Osterheld MC, Fontolliet C, Benhattar J. Promoter methylation analysis on microdissected paraffin-embedded tissues using bisulfite treatment and PCR-SSCP. Biotechniques 2001;30:66–74.
16. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93:9821–6.
17. Oakeley EJ. DNA methylation analysis: a review of current methodologies. Pharmacol Ther 1999;84:389–400.
18. Yan P, Coindre JM, Benhattar J, Bosman FT, Guillou L. Telomerase activity and human telomerase reverse transcriptase mRNA expression in soft tissue tumors: correlation with grade, histology, and proliferative activity. Cancer Res 1999;59:3166–70.
19. Yan P, Bosman FT, Benhattar J. Tissue quality is an important determinant of telomerase activity as measured by the TRAP assay. Biotechniques 1998;25:660–2.
20. Raizis AM, Schmitt F, Jost JP. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. Anal Biochem 1995; 226:161–6.
21. Baisse B, Bian YS, Benhattar J. Microdissection by exclusion and DNA extraction for multiple PCR analyses from archival tissue section. Biotechniques 2000;28:856–62.
22. Harley CB, Villeponteau B. Telomeres and telomerase in aging and cancer. Curr Opin Genet Dev 1995;5:249–55.
23. Wullich B, Rohde V, Oehlenschlager B, Bonkhoff H, Ketter R, Zwergel T, Sattler HP. Focal intratumoral heterogeneity for telomerase activity in human prostate cancer. J Urol 1999;161:1997–2001.
24. Chaubert P, Bautista D, Benhattar J. An improved method for rapid screening of DNA mutations by nonradioactive single-strand conformation polymorphism procedure. Biotechniques 1993;15:586.
25. Forsyth NR, Wright WE, Shay JW. Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. Differentiation 2002;69:188–97
26. Wu A, Ichihashi M, Ueda M. Correlation of the expression of human telomerase subunits with telomerase activity in normal skin and skin tumors. Cancer 1999;86:2038–44
27. Fritsch G, Stimpfl M, Kurz M, Printz D, Buchinger P, Fischmeister G, Hoecker P, Gadner H. The composition of CD34 subpopulations differs between bone marrow, blood and cord blood. Bone Marrow Transplant 1996;17:169–78.
28. Delgado R, Rathi A, Albores-Saavedra J, Gazdar AF. Expression of the RNA component of human telomerase in adult testicular germ cell neoplasia. Cancer 1999;86:1802–11.
29. Tahara H, Yasui W, Tahara E, Fujimoto J, Ito K, Tamai K, Nakayama J, Ishikawa F, Tahara E, Ide T. Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. Oncogene 1999;18: 1561–7.
30. Yatabe Y, Tavare S, Shibata D. Investigating stem cells in human colon by using methylation patterns. Proc Natl Acad Sci USA 2001; 98:10839–44.
31. Dunham M, Neumann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. Nat Genet 2000;26: 447–50.
32. Bird A. The essentials of DNA methylation. Cell 1992;70:5–8.
33. Bird A, Tate P, Nan X, Campoy J, Meehan R, Cross S, Tweedie S, Charlton J, Macleod D. Studies of DNA methylation in animals. J Cell Sci 1995;19:37–9.
34. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene 2001;269:1–12.
35. Bednarik DP, Duckett C, Kim SU, Perez VL, Griffis K, Guenthner PC, Folks TM. DNA CpG methylation inhibits binding of NF-kappa B proteins to the HIV-1 long terminal repeat cognate DNA motifs. New Biol 1991;3:969–76.
36. Hermann R, Doerfler W. Interference with protein binding at AP2 sites by sequence-specific methylation in the late E2A promoter of adenovirus type 2 DNA. FEBS Lett 1991;281:191–5.
37. Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell 1991;64:1123–34.
38. Nan X, Campoy FJ, Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell 1997;88:471–81.
39. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 1998;19:187–91.
40. Kyo S, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, Orimo A, Inoue M. Estrogen activates telomerase. Cancer Res 2000; 59:5917–21.
41. Oh S, Song YH, Kim UJ, Yim J, Kim TK. In vivo and in vitro analyses of Myc for differential promoter activities of the human telomerase (hTERT) gene in normal and tumor cells. Biochem Biophys Res Commun 1999;263:361–5.
42. Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, Dalla-Favera R. Direct activation of TERT transcription by c-MYC. Nat Genet 2000;21:220–4.
43. Prendergast GC, Ziff EB. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science 1991;251:186–9.