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The human telomerase RNA gene (*hTERC*) is regulated during carcinogenesis but is not dependent on DNA methylation

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Telomerase, the ribonucleoprotein complex involved in telomere maintenance, is composed of two main components: hTERT and hTERC. hTERT seems to be the rate-limiting factor for telomerase activity, although *hTERC* expression was also shown to correlate to a certain extent with telomerase reactivation. To determine whether the absence of *hTERC* expression could be the consequence of DNA methylation, we quantified *hTERC* RNA in 60 human samples (19 telomerase-negative normal tissues, nine telomerase-positive and 22 telomerase-negative tumor tissues, eight telomerase-positive and two telomerase-negative cell lines) using a quantitative dot blot on RT–PCR products. Most of the normal tissues did not express *hTERC* whereas, in telomerase-positive cell lines and in telomerase-positive tumor tissues, a strong up-regulation was observed, suggesting that *hTERC* transcription is up-regulated during tumorigenesis. The two telomerase-negative cell lines did not express *hTERC*. In a series of 22 telomerase-negative soft tissue sarcomas (STS), half did not express *hTERC* at all, or only weakly, whereas a wide range of expression was observed in the other half. As methylation might be involved in *hTERC* silencing, we examined the methylation pattern in all samples by direct sequencing and methylation-specific single stand conformation analysis after bisulfite modification. *hTERC* methylation was never observed, neither in normal nor in tumor tissues. Furthermore, there was no correlation between *hTERC* expression and proliferation, telomere length or *hTERT* expression in telomerase-negative STS. In contrast, three of eight telomerase-positive cell lines and the two telomerase-negative cell lines were found to be hypermethylated, suggesting that the methylation observed may occur during cell line establishment. In conclusion, this study shows that *hTERC* expression is indeed regulated during carcinogenesis, but this regulation is unlikely to depend on *hTERC* methylation, cell proliferation rate, telomere length or *hTERT* expression.

Introduction

Human telomeres are nucleoprotein complexes located at the extremity of linear chromosomes (1). They are considered to play a key role in controlling the mitotic clock, via their length (2). In normal human somatic cells, they shorten at each cell division. In contrast, their length is maintained in most tumor cells by the action of a ribonucleoproteic enzyme called telomerase (3). The telomerase large complex consists of many components, among which two seem to be essential for the activity *in vivo* and *in vitro*: the first, hTERT protein, shows reverse transcriptase activity (4); the second, hTERC, is the RNA matrix used to elongate telomeres. Because of their potential role in tumorigenesis, these components have been extensively studied. *In vitro*, hTERT and hTERC have been shown to form the minimal complex required for telomerase activity (5). More recently, *hTERT* gene expression was shown to correlate closely with telomerase activity *in vitro* and *in vivo*, and thus was thought to be a crucial determinant for telomerase activity (6,7).

The *hTERC* gene was identified a few years ago (8). Since most RT–PCR-based experiments showed that this RNA was widely expressed in both tumoral and non-tumoral tissues (9,10), it was concluded that *hTERC* was not essential to telomerase reactivation. However, the examination of a series of tumors using *in situ* hybridization revealed an up-regulation of *hTERC* expression in tumor tissues (11,12). Weak expression was also occasionally detected in some normal tissues, namely gastric, esophageal and prostate epithelial basal layers, and activated lymphocytes (12–14). In a previous study, we demonstrated that *hTERC* expression was closely linked to telomerase activity in colorectal carcinogenesis (15) suggesting that this gene could play a role during the process of telomerase reactivation. Based on these observations, we thought it would be interesting to see if *hTERC* regulation could be a tumor-specific phenomenon. Characterization of the human TR gene revealed that several sites might be involved in its regulation (8). In a recent report, Zhao *et al*. (16) showed that the *hTERC* gene may be activated by the transcription complex NF-Y, also by transcription factors such as Sp1 and pRB (Retinoblastoma protein), and may be repressed by Sp3. Furthermore, the presence of a large CpG island within the *hTERC* gene suggests that methylation could be implicated in *hTERC* regulation as well. Recently, a strong correlation between *hTERC* promoter methylation and lack of *hTERC* expression was observed exclusively in telomerase-negative cell lines (17). Therefore, it is possible that *hTERC* methylation results in *hTERC* silencing in at least a subset of telomerase-negative tumors.

Immortalized telomerase-negative tumors are malignant cells, which use a telomerase-independent mechanism (18). A subset of this tumor category, using the ALT mechanism (alternative lengthening of telomeres) (19) has been shown to exhibit ultra-long, heterogeneously sized telomeres and characteristic multiprotein structures (20). Recent studies suggested that the length of telomeres in ALT cells might be obtained by homologous recombination and copies switching between telomeric tracts (21,22). Soft tissue sarcomas (STS) constitute a large and heterogeneous group of malignant mesenchymal tumors. About half of them do not express

Abbreviations: ALT, alternative lengthening of telomeres; MS-SSCA, methylation-specific single stand conformation analysis; STS, soft tissue sarcomas.

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telomerase (23,24). These telomerase-negative STS might constitute an appropriate material for studying the regulation of *hTERC* expression.

In the present study, we examined *hTERC* expression and *hTERC* methylation in a series of human normal tissues, non-soft tissue tumors, and tumor cell lines, as well as in a series of telomerase-negative STS. We observed strong variations of *hTERC* expression according to the cell type studied. In addition, the analysis of methylation patterns showed that the *hTERC* gene is unlikely to be regulated by methylation-based mechanisms in telomerase-negative normal tissues and in tumor tissues. The few partial methylation patterns observed in telomerase-positive cell lines could represent a side effect of cell culture.

Materials and methods

Tissue samples

Normal and tumor tissues were obtained from the Frozen Tissue Bank of the University Institute of Pathology of Lausanne. Nineteen human normal tissue samples (bladder, brain, breast, colon, heart, liver, muscle, placenta and prostate) and nine telomerase-positive human tumor tissues (bladder/invasive transitional carcinoma G3, non-invasive transitional carcinoma G2, invasive transitional carcinoma G3; breast/invasive ductal carcinoma G2; colon/ invasive adenocarcinoma moderately differentiated G2, invasive adenocarcinoma poorly differentiated G3, invasive adenocarcinoma moderately differentiated G2; kidney/non papillomary conventional clear cell sarcomatoid invasive carcinoma G4; and lung/invasive adenocarcinoma moderately differentiated G2) were examined in this study. Twenty-two telomerase-negative STS examined previously by TRAP assay, *hTERT* expression, Mib-1 expression, and telomere lengths (25) were also analyzed. All samples were carefully checked by experienced pathologists (L.Guillou and R.Braunschweig) and contained at least 70% of tumor cells.

Tumor cell lines

Ten human tumor cell lines (breast, MCF-7; cervix, A431, HeLa; colon, Co115, SW480; lung, H520, SW2; prostate, PC-3; osteosarcoma, Saos-2, U-2 os) were also studied. With few exceptions (SW480, SW2 and Co115, from the Swiss Institute for Cancer Research, ISREC, Lausanne, Switzerland), these cells were obtained from the American Type Culture Collection. Cells were routinely cultured in Dulbecco's modified medium with glutamax-1 supplemented with 10% fetal bovine serum (5% for Saos-2 and U-2 os), or Leibovitz medium (L15) with 5% FBS and 0.2% $NaHCO_3$ for SW480 (all products from Gibco BRL, Paisley, UK). All cells lines were tested and found to be negative for *Mycoplasma* contamination.

DNA and RNA extraction and TRAP assay

DNA, RNA and proteins were extracted from consecutive tissue sections. To establish the methylation status of *hTERC* promoter and exon, genomic DNA was isolated using the DNeasy tissue kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA was extracted from frozen tissue sections or cells using Trizol (Life Technologies, Rockville, MD). RT–PCR on *hTERT* RNA was performed as described previously (23). TRAP assay was performed according to the modified protocol described by Yan *et al*. (23).

Analysis of hTERC expression by RT–PCR and quantitative dot blot

Total RNA (2.5 µg) was first digested by DNase I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. RNA was purified by phenol–chloroform extraction followed by ethanol precipitation. cDNA was obtained using pd(N)6 random primer (Amersham, Freiburg, Germany) and Expand Reverse Transcriptase (Roche Diagnostics) as per manufacturer's protocol. PCR on *hTERC* cDNA was performed by using the primer set 5′-CGCCGTGCTTTTGCTCC-3′ and 5′-ACTCGCTCCGTTCCT-CTTCC-3′, in a final 5% DMSO, and the following PCR conditions: 23 cycles of 94°C for 30 s, 62°C for 45 s and 72°C for 45 s, followed by 10 min at 72°C on a Primus (MWG-Biotech) apparatus. cDNA quality was checked by PCR amplification of *p53* and *GAPDH* cDNA (23). Calibration scales were realized by mixing total RNA from an *hTERC* positive cell line (HeLa) with total RNA from a negative sample (U-2 os) in 100 ng final. Percentages used were: 100, 50, 25, 10, 2.5, 1, 0.25 and 0%. Then, RT–PCR was realized in the conditions described above. All tissue samples and calibration scales were amplified together in the same PCR reactions. A DIG-labeled probe was produced by re-amplification of an hTERC positive RT–PCR, in a PCR including dUTP-DIG.

For quantitative dot blot, RT–PCR products were denatured 10 min at 100°C and put immediately on ice. Two microliters of each product were loaded on Eletran® N+ nylon membrane (BDH Laboratory Supplies, Poole, UK) and fixed under UV. The membrane was pre-hybridized in a 5× SSC/2× blocking/0.02% SDS/0.1% *N*-lauroyl/50% formamide for 1 h at 42°C. Hybridization with the DIG-labeled probe (100 ng in 3 ml) was done for 2 h at 42°C. The membrane was then washed twice in 2× SSC/0.1% SDS for 5 min at room temperature, and twice in 0.2× SSC/0.1% SDS for 15 min at 68°C. After 2 min incubation in maleate buffer pH 7.5/Tween® 20, and 30 min in maleate buffer pH 7.5/1× blocking, the antibody anti-DIG (Roche Diagnostics) was added. The chemiluminescence reaction was performed after three washes with maleate buffer pH 7.5/Tween® 20, and one wash in 100 mM Tris pH 9.5/100 mM NaCl. Detection was realized with CDP-Star+ ready-to-use (Roche Diagnostics) according to the manufacturer's protocol. Signals were analyzed from X-omat film (Eastman Kodak Company, Rochester, NY) after different times of exposure, by comparing intensities with the internal calibration scale.

hTERC methylation analysis by MS-SSCA and sequencing after bisulfite modification

In order to differentiate methylated from unmethylated cytosine, genomic DNA was modified by sodium bisulfite using a protocol adapted from Raizis *et al*. (26) and Bian *et al.* (27). Two microliters of DNA in 36 µl of water were cleaved by 4 µl of 1 N HCl for 2 min exactly at room temperature. Then, 4.5 µl of 3 M NaOH was added and DNA denaturation was performed for 20 min at 37°C. Sodium bisulfite (500 µl) and hydroxyquinone (28 µl) were then added to a final concentration of 40.5% and 10 mM, respectively. The reaction was performed overnight at 55°C. After addition of 80 µl of water and 365 µ1 of pure ethanol, DNA was purified using the DNeasy tissue kit columns (Qiagen). Following washing with the kit wash buffer, desulfonation was performed on the column by addition of 500 µl of 0.15 M NaOH I 90% EtOH. Incubation was performed for 10 min at room temperature and in the dark. After washing, the modified DNA was eluted from the column with 50 µl of 10 mM of Tris–HCl pH 8.0.

Two sets of primers were used for PCR on *hTERC*, one for the promoter region: 5′-GGAAATGGAATTTTAATTTTT-3′ and 5′-AACCAACAACTAA-CATTTTTT-3′, and one for the exon region: 5′-TAAATAAAAAATGTTAGT-TGT-3′ and 5′-ACCTAAAAAACCTAAACC-3′. PCR conditions used to amplify *hTERC* promoter were 40 cycles of 94°C for 45 s, 51°C for 45 s and 72°C for 75 s, followed by 15 min at 72°C. The same PCR cycling conditions in a final concentration of 5% DMSO and an annealing temperature of 48°C were used for *hTERC* exon. Sequencing of all PCR products was done on an ABI prism 310 sequencer (Perkin-Elmer, Branchburg, NJ). Each PCR product was analyzed by methylation-specific single stand conformation analysis (MS-SSCA) as described previously (27).

Control plasmid was generated by subcloning 672 bp, bases –212 to +459 bp of the *hTERC* gene (GenBank accession no. U86046), in the pGEM-T vector (Promega, Madison, WI). The plasmid was divided in two parts: one was left unmethylated and the other was fully methylated at all CpG sites using *SssI* methylase (New England Biolabs, Hertfordshire, UK) 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.

5-aza-dC treatment

Cells were immediately treated after seeding in standard conditions, with 3 µM of 5-aza-dC every 48 h for 1 week. The cells were then collected for DNA and RNA extraction.

Results

hTERC expression and methylation in tumor cell lines and in normal and human tumor tissues

Telomerase-positive cell lines expressed *hTERC* RNA at a very high level, with marked variations from one cell line to another (from 50 to 385%, Table I), as compared with our reference set (HeLa cells, 100%). In contrast, no *hTERC* RNA could be detected in the telomerase-negative cell lines, U-2 os and Saos-2. Tumors from various organs showed a wide range of *hTERC* expression (from 10 to 69%, Table I). Finally, *hTERC* RNA expression could not be detected in 68% (13/19) of normal tissues with our RT–PCR and dot blotting assay. In contrast, five normal samples showed a low level of transcription and one sample presented a 6.5% relative *hTERC*

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Table I. *hTERC* expression and methylation in tumor cell lines, normal tissue and tumor samples

Samples	Telomerase activity[a]	*hTERC* expression[b] (%)	*hTERC* methylation[c]
Tumor cell lines			
SW2	+++	50	–
A431	+++	65	+/–
SW480	+++	87	–
HeLa[d]	+++	100	+/–
PC-3	+++	115	–
MCF-7	+++	125	+/–
Co115	+++	350	–
H520	+++	385	–
Saos-2	–	0	+/–
U-2 os	–	0	+
Normal tissues			
Bladder	–	0	–
Bladder	–	0	–
Bladder	–	0	–
Brain	–	0	–
Colon	–	0	–
Heart	–	0	–
Heart	–	0	–
Kidney	–	0	–
Kidney	–	0	–
Kidney	–	0	–
Kidney	–	0	–
Kidney	–	1	–
Liver	–	0.25	–
Muscle	–	0	–
Muscle	–	1	–
Muscle	–	1.25	–
Placenta	–	0	–
Placenta	–	2.5	–
Tumor tissues			
Kidney	+++	10	–
Bladder	+++	11	–
Breast	+++	11	–
Colon	+++	12	–
Colon	+++	13	–
Bladder	+	15	–
Bladder	+	25	–
Lung	+++	25	–
Colon	+++	69	–

[a]Telomerase activity by TRAP assay: +++, marked activity; +, low activity; –, no activity.
[b]As determined by quantitative dot blot of RT–PCR products. 100% control determined in HeLa cells.
[c]As determined by direct sequencing and MS-SSCA. +, methylated pattern ; –, unmethylated pattern ; +/–, mixture of methylated and unmethylated patterns.
[d]100% control of *hTERC* expression.

expression (Table I). When comparing telomerase-negative normal tissues to telomerase-positive tumor tissues, *hTERC* expression ratios were markedly different.

In order to determine the putative origin of these variations in *hTERC* expression, we examined the methylation status of the *hTERC* promoter and exon. Bisulfite modification of DNA changes all unmethylated cytosines to uracils while leaving methylated cytosines intact. The modified DNA was then amplified with primers without CpG repeats and directly sequenced. To determine the clonal nature of the methylated pattern, each PCR product was also analyzed by MS-SSCA. This approach can identify patterns of band mobility corresponding to the presence or absence of methylated CpG sites. All tumors and normal tissues were found to be hypomethylated by sequencing and MS-SSCA in their promoter and exon

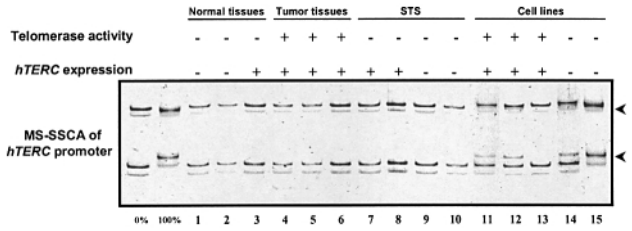

Fig. 1. Telomerase activity, *hTERC* expression and methylation patterns of *hTERC* promoter in human tissues and tumor cell lines. Lanes 0 and 100%, MS-SSCA controls obtained from plasmids containing *hTERC* sequences; lane 0%, MS-SSCA from unmethylated plasmid; lane 100%, MS-SSCA from a fully methylated plasmid; lanes 1–3, normal tissues without telomerase activity from bladder, muscle and prostate respectively; lanes 4–6, telomerase-positive tumor tissues (colon, kidney and lung); lanes 7–10, telomerase-negative STS; lanes 11–13, telomerase-positive cell lines (A431, HeLa and SW2); lanes 14 and 15, osteosarcoma cell line without telomerase activity (Saos-2 and U-2 os, respectively). Telomerase activity, as obtained by the TRAP assay, was reported in the top of the gel: +, marked activity and –, no activity. *hTERC* expression was summarized by expression (+) or with no expression (–). See Table I for values. Arrows in the MS-SSCA of the *hTERC* promoter indicate the fully methylated bands. Identical patterns were obtained in *hTERC* exon.

regions (Figure 1, lanes 1–6). Using MS-SSCA only, three of the eight telomerase-positive tumors (MCF-7, HeLa and A431) presented a mixed pattern of fully methylated and fully unmethylated promoter and exon regions (Figure 1, lanes 11 and 12), but expressed *hTERC* RNA at a high level (Table I). The two telomerase-negative and *hTERC*-negative cell lines displayed a partially methylated (Saos-2, Figure 1, lane 14) or a fully methylated pattern (U-2 os, Figure 1, lane 15) for *hTERC* promoter as well as for *hTERC* exon.

As these preliminary results suggest that methylation might be involved in *hTERC* repression in telomerase-negative cell lines, we treated U-2 os with the demethylating reagent 5-aza-dC. The MS-SSCA analysis revealed a clonal fully demethylated DNA population of ~10%. However, no *hTERC* re-expression could be detected by dot blot analysis after RT–PCR amplification.

hTERC expression and methylation in telomerase-negative soft tissue sarcomas

Twenty-two telomerase-negative STS were analyzed in this study. *hTERC* expression showed wide variations (range from 0 to 186%) (Table II). Interestingly, half of the tumors (11/22) expressed *hTERC* at a level similar to that of normal tissues (between 0 and 6%) (Figure 2), including four of the five liposarcomas and three of the five leiomyosarcomas examined. In 41% (9/22) of the telomerase-negative STS, the *hTERC* expression level was similar to that observed in the telomerase-positive tumors (10–70%) (Table II). Only two samples showed an expression as strong as that of the cell lines (⩾70%). Methylation status was analyzed by MS-SSCA and by sequencing after bisulfite modification in all these telomerase-negative STS (Figure 1, lanes 7–10). No methylation could be detected in any of them.

Correlation between hTERC expression and proliferation, telomere length and hTERT expression, in telomerase-negative soft tissue sarcomas

Since *hTERC* regulation might be related to proliferation rate (14), we tried to correlate its expression with Mib-1 expression in 20 telomerase-negative STS. This nuclear protein distinguishes between proliferative and non-proliferative cells. Mib-1 expression of <5% was considered as weakly proliferative,

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Table II. Expression and methylation of *hTERC*, TRF, Mib-1 and *hTERT* expression in telomerase-negative STS

Case	Histology	Grade[a]	*hTERC* expression[b] (%)	*hTERC* methylation[c]	TRF[d]	Mib-1[e]	*hTERT* expression[f]
1	MPNST (M)	2	0	–	M	1	–
2	Liposarcoma (Pleomorphic)	3	0	–	L[g]	5	–
3	Liposarcoma (Pleomorphic)	3	0	–	L	40	–
4	Rhabdomyosarcoma (alveolar)	3	0.25	–	S	NA[h]	–
5	Leiomyosarcoma (M)	3	0.25	–	M	15	–
6	MFH, storiform-pleophic type (M)	3	0.25	–	L[g]	10	–
7	Leiomyosarcoma	3	0.9	–	L	NA[h]	–
8	Liposarcoma (myxoid) (R)	1	1	–	S	<1	+
9	Leiomyosarcoma	3	1.5	–	L[g]	15	–
10	Liposarcoma (differentiated)	2	1.9	–	M	<1	+
11	Unclassified spindle cell STS/FS	2	2.5	-	M	10	–
12	MFH, storiform-pleophic type	2	11	–	S	<1	–
13	Unclassified spindle cell STS/FS	1	12	-	S	<1	–
14	MFH, storiform-pleophic type (M)	3	12.5	-	M	10	–
15	Chondrosarcoma (myxoid)	2	20	–	M	<1	–
16	Liposarcoma (Myxoid/round cell)	2	21	-	S	2	+
17	MPNST	3	22.5	–	M	70	–
18	Leiomyosarcoma	3	27	–	S	<1	–
19	MFH, myxoid type	3	62	–	L	15	–
20	MFH, storiform-pleophic type (R)	2	67	-	S	1	–
21	Leiomyosarcoma	3	81	–	L[g]	<5	–
22	MFH, storiform-pleophic	2	150	–	M	5	–

[a]According to the FNCLCC grading system.
[b]As determined by quantitative dot blot of RT-PCR products. 100% control determined in HeLa cells.
[c]As determined by direct sequencing and MS-SSCA.
[d]As determined by Southern blot. L, long; M, medium; and S, small.
[e]Mib-1 staining has been assessed semi-quantitatively as a proportion of positively stained nuclei for a total of 200–300 nuclei assessed per case.
[f]As determined by RT-PCR. +, Expression; –, no expression.
[g]Exhibiting the elongated and heterogeneous phenotype characteristic of ALT cells.
[h]NA, not available.

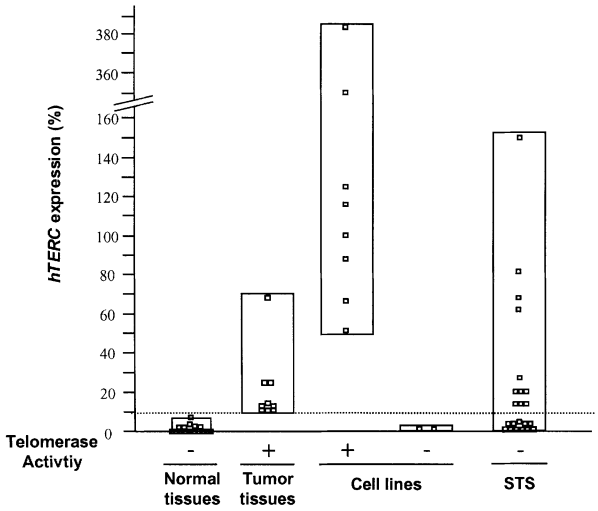

Fig. 2. *hTERC* expression varies according to the cell type. Relative expression levels of *hTERC* in various tumor and normal tissues as well as in tumor cell lines were reported according to the cell type. A clear relationship between *hTERC* expression and the tumor histology can be defined in epithelial telomerase-positive tumors. In contrast, two subgroups could be seen in telomerase-negative STS. The dotted line indicates the 10% of *hTERC* expression chosen as cut off.

and ⩾5%, moderately or highly proliferative (23). The mean of *hTERC* expression was found similar in proliferative (10/20) and non-proliferative (10/20) telomerase-negative STS, with a relative *hTERC* expression of 25 and 29%, respectively. Wide ranges were observed in both categories (Table II).

A previous study revealed that telomere length was heterogeneous and did not correlate with telomerase activity (25). In the present report, we tried to correlate *hTERC* expression with telomere length in the 22 telomerase-negative STS. TRF fragments were assessed as short (S), medium (M) or long (L) when the medium size was situated under 9.6 kb (20% shorter than mean normal skeletal muscle TRF length), between 9.6 and 14.4 kb, and over 14.4 kb, respectively (25). In our series, samples were evenly distributed: seven small, eight medium and seven long. Means of *hTERC* expressions were 20, 26 and 21% (relative to the 100% in HeLa cells) for short, medium and long telomeres, respectively.

Table III. Expression of *hTERC* and *hTERT* RNA in telomerase-negative STS

hTERC expression	*hTERT* expression	
	hTERT +	hTERT –
hTERC +	3	16
hTERC –	0	3
Total	3	19

Finally, we tried to correlate *hTERC* and *hTERT* expressions. As shown in Table III, most telomerase-negative tumors expressed *hTERC* but not *hTERT* (16/22, 73%). Surprisingly, three cases expressed both genes, without any detectable telomerase activity. Finally, only three cases (one MPNST and two pleomorphic liposarcomas) were found that presented no *hTERC* nor *hTERT* expression (Table II).

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Discussion

Telomerase activity has been shown to depend on at least two components: the reverse transcriptase, hTERT protein and the RNA matrix, hTERC. Recent studies identified hTERT as the rate-limiting factor for telomerase activity. In contrast, little is known about *hTERC* regulation and its role in the telomerase re-activation mechanism is the subject of conflicting results. These discrepancies raised the following questions: is *hTERC* expression up-regulated during tumorigenesis, and which mechanisms could control this phenomenon?

In order to answer these questions, *hTERC* expression was quantified by dot blot after RT–PCR in a series of 60 samples (Tables I and II). As *hTERC* gene does not contain any intron, DNase I digestion was an absolute requirement to avoid a possible contamination of the results with genomic DNA. This quantitative PCR and dot blot showed that most normal human tissues did not express *hTERC* RNA. Although a few normal cases revealed weak transcription, the mean of *hTERC* expression was at least 30 times less in normal tissues than in telomerase-positive tumor tissues and 250 times less than in telomerase-positive tumor cell lines. This strongly suggests that *hTERC* gene is strongly up-regulated during tumorigenesis. A similar ratio in *hTERT* re-expression (about 24 times) has been observed between normal renal tissue and sporadic renal cell carcinoma (28). Thus, it appears that both hTERT and hTERC seem to be induced or strongly up-regulated during carcinogenesis. Therefore, as *hTERT*, *hTERC* expression can be considered as a marker of cell transformation. Few studies have tried to quantify and compare *hTERC* and *hTERT* RNAs in the same tumor tissue sample. In three types of human cancer (gliomas, hepatocarcinomas and breast carcinomas), a linear relationship between both RNA types was observed when *hTERT* expression reached a certain level of expression. In *in situ* carcinoma of the uterine cervix, a linear relationship between *hTERC* RNA and *hTERT* mRNA could be defined whereas such a correlation was not observed in precancerous dysplasia of the cervix (29). Therefore, transcriptional levels of both genes might be cross-regulated when the cancer becomes established.

hTERC expression was also investigated in telomerase-negative tumor cell lines and in tumor tissues. hTERC RNA was not detected in the tumor cell lines, Saos-2 and U-2 os. In contrast, marked variations (0–150%) of *hTERC* expression were observed in the group composed of 22 telomerase-negative STS (Table II). In comparison with the *hTERC* levels observed in telomerase-negative normal tissues and in telomerase-positive tumor tissues, STS samples could be divided in to two groups: the first group, comprising half the samples, showed a level of *hTERC* expression similar to normal tissue, whereas the other half showed levels similar to the telomerase-positive tumors (Figure 2). Therefore, *hTERC* expression could not be used as a tumor marker in soft tissue sarcoma.

The presence of a CpG-rich region in the promoter and in the exon of *hTERC* led us to hypothesize that it could be one of the putative *hTERC* regulatory elements. In a recent study, Hoare *et al*. (17) examined the role of methylation in *hTERC* transcription. Their results indicated that methylation might be implicated in telomerase-negative cell lines, but not in telomerase-negative normal tissues nor in telomerase-positive tumor tissues. In the present study, using direct sequencing and MS-SSCA after bisulfite modification of genomic DNA, identical results were obtained in normal tissue and tumor samples of various histology and location. MS-SSCA allows a clonal analysis of DNA population mixture where any clone >5–10% can be easily detected (27). As all tumor samples contained at least 70% of tumor cells, it is probable that *hTERC* methylation is not implicated in *hTERC* transcription fluctuations. Interestingly, three of the eight telomerase-positive cell lines (HeLa, MCF-7 and A431) showed a hypermethylated pattern by sequencing. Similar results were obtained for HeLa and MCF-7 (17). In the present study, the use of MS-SSCA allowed us to demonstrate that one allele was fully methylated whereas the other one was fully unmethylated, indicating that one allele is sufficient to induce *hTERC* gene transcription (Figure 1).

We next examined *hTERC* methylation patterns in 22 telomerase-negative STS. In contrast to the two telomerase-negative cell lines which were found to be hypermethylated, *hTERC* was not methylated in this series of 22 tumors, suggesting that methylation is not important in the *hTERC* regulation mechanism within this interesting group of telomerase-negative tumors.

How can we explain the occurrence of *hTERC* methylation in some of the analyzed tumor cell lines? Aberrant methylation has already been reported in cultured normal fibroblasts (30). In this particular experiment, growth constraints altered CpG island methylation, leading to alterations in epigenetic stability. Furthermore, we observed that treatment with 5-aza-dC of the telomerase-negative cell line U-2 os did not lead to the re-expression of the *hTERC* gene. Thus, the *hTERC* methylation detected in the two telomerase-negative cell lines as well as in the three telomerase-positive cell lines might merely correspond to a non-specific side effect of cell culture.

Recently, *in situ* hybridization performed on STS suggested that telomerase RNA expression may be up-regulated in tumor cells and may precede morphological transduction (31). In the present study, a huge range of *hTERC* expressions were obtained (from 0 to 186%), indicating that hTERC RNA is not an appropriate tumoral marker for STS, whereas its use might be relevant for epithelial tumors. Further studies will be necessary to investigate this hypothesis. In telomerase-negative STS, we tried to correlate *hTERC* expression with several other parameters, such as the cell proliferation rate as assessed by Mib-1 staining, telomere length and *hTERT* expression in the group of telomerase-negative STS. Our results indicate that *hTERC* expression does not correlate with Mib-1 staining or telomere length. Therefore, other parameters, which could interfere with *hTERC* transcription have to be identified and studied. In a recent report, Zhao *et al*. (16) showed that *hTERC* might be activated by the transcription complex NF-Y, by the transcription factors Sp1 and pRB (retinoblastoma) and could be repressed by Sp3. Such transcription factors might play a role in the variations observed in *hTERC* transcription. On the other hand, we found that most telomerase-negative STS did not express *hTERT*, suggesting that the lack of telomerase activity probably came from the lack of *hTERT* transcription. Along the same lines, we failed to find a tumor in our series, which expressed *hTERT* but not *hTERC*. Interestingly, three samples lacked expression of both genes, whereas three others did not show any telomerase activity in spite of expressing both genes. This latter finding lends weight to the notion that not only telomerase genes expression but also post-transcriptional modifications are required to obtain telomerase activity (32).

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In summary, the present study shows that the levels of *hTERC* expression may vary according to the cell type examined (normal tissue versus telomerase-positive tumor versus telomerase-positive tumor cell lines) and are up-regulated during tumorigenesis. With the exception of telomerase-negative cell lines, variations in hTERC RNA expression are not related to a change in the methylation status of the *hTERC* gene. In addition, variations in *hTERC* expression in telomerase-negative STS were not linked to the methylation status, proliferation rate, telomere length, or *hTERT* expression.

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