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Expression of Human Telomerase Subunits and Correlation with Telomerase Activity in Cervical Cancer

Masahiro Takakura, Satoru Kyo,[1] Taro Kanaya, Masaaki Tanaka, and Masaki Inoue

Department of Obstetrics and Gynecology, School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920, Japan

ABSTRACT

Activation of telomerase and stabilization of telomeres are thought to be required for both cellular immortality and oncogenesis. Three major components of human telomerase, human telomerase RNA (hTR), telomerase-associated protein (TP1/TLP1), and human telomerase catalytic subunit (hTRT/hEST2), have been identified recently. However, it remains unclear what roles these subunits play in the regulation of telomerase activity. In the present study, a total of 25 cervical cancers and 14 normal cervices as well as various cell lines derived from cervical cancer were examined for the expression of hTR, TP1 mRNA, and hTRT mRNA, and the correlations between expression of these and telomerase activity were evaluated in 23 cancers and 14 normal cervices. Reverse transcription-PCR analysis revealed that hTR and TP1 mRNA were commonly expressed in cancers and noncancerous tissues. However, hTRT mRNA was observed only in cervical cancers and cell lines, and more than 80% of cervical cancers expressed it, whereas neither normal cervical tissues nor normal primary fibroblast cells did. There was a strong correlation of telomerase activity with hTRT mRNA expression but not with TP1 or hTR expression. Cervical exfoliated cells were subjected to reverse transcription-PCR analysis for detection of hTRT mRNA, and approximately 70% of cervical cancers were positive for such expression. These findings provide strong evidence that expression of hTRT is a rate-limiting determinant of the enzymatic activity of human telomerase and that upregulation of hTRT expression may play a critical role in human carcinogenesis. Our findings also indicate that detection of hTRT mRNA is useful for cytological screening for cervical cancer.

INTRODUCTION

Telomeres are the distal ends of human chromosomes composed of tandem repeats of the sequence TTAGGG (1). Possible functions of telomeres include prevention of chromosome degradation, end-to-end fusions, rearrangements, and chromosome loss. Human telomeres in somatic cells undergo progressive shortening with cell division through replication-dependent sequence loss at DNA termini (2). The shortening of telomeres results in chromosomal instability, leading to cellular senescence. A possible cause of shortening of human telomeres is repression of telomerase, a specialized ribonucleoprotein polymerase containing an integral RNA with a short template element that directs the synthesis of telomeric repeats at chromosome ends (3, 4). Telomerase reactivation is thus thought to be essential for stabilizing telomere length to attain cellular immortality (5, 6).

A number of studies using PCR-based telomeric repeat amplification assay have demonstrated that telomerase is activated in a variety of malignant tumors (7–10). In contrast, telomerase activity is usually repressed in normal somatic tissues, except in some self-renewing tissues with high regenerative potential such as uterine endometrium (11). These findings suggest that telomerase activation is a major step in human carcinogenesis. However, little is known concerning the molecular mechanisms through which human telomerase is activated in tumors, because the components of human telomerase complex have not been well characterized. hTR,[2] the first such component identified, functions as a template for telomere elongation by telomerase (12). Disruption the function of telomerase RNA in *Tetrahymena* through overexpression of an inactive form of telomerase RNA has been shown to result in progressive shortening of telomeres (4). Tumor cells transfected with antisense hTR lost telomere sequence, resulting in cellular senescence (12). These findings suggest that hTR plays an essential role in the maintenance of telomeres. The level of hTR has also been shown to increase with tumor progression (13). Three proteins in different species associated with telomerase activity have also been identified. p80 and p95 were purified from ciliate *Tetrahymena* (14), and the gene encoding a mammalian homologue of p80, *TP1/TLP1*, has also been cloned (15, 16). However, recent studies demonstrated that expression of hTR and these telomerase-associated proteins is not correlated with the level of telomerase activity, leaving the role played by these proteins in the regulation of telomerase activity unclear (15–17). Recently, two related proteins, Est2p and p123, were identified as catalytic subunits of telomerase in yeast *Saccharomyces cerevisiae* and ciliate *Euplotes aediculatus*, respectively, both of which contain characteristic sequences in genes that are well conserved in catalytic regions of reverse transcriptases (18, 19). The human homologue of Est2p and p123, hEST2/hTRT, has most recently been cloned. High levels of expression of hTRT are observed in some types of tumors and telomerase-positive cell lines but not in telomerase-negative cell lines, suggesting that hTRT is a putative catalytic subunit homologue protein (20, 21). However, the expression of hTRT and its correlation with telomerase activity have not been determined clearly for a large number of clinical samples. In the present study, the expression of each telomerase subunit was determined in cervical cancers and normal cervical tissues, and the correlations between these expressions and telomerase activity were evaluated. Our findings suggest that hTRT is expressed in cervical cancers but not in normal cervical tissues, whereas hTR and TP1/TLP1 are broadly expressed in both cancers and noncancerous tissues. There was a significant correlation between telomerase activity and expression of hTRT, providing strong evidence that hTRT is a rate-limiting determinant of the enzymatic activity of human telomerase.

MATERIALS AND METHODS

Tissue Samples. Thirty cervical cancers and 14 normal cervical tissue samples were obtained at surgery in the Departments of Obstetrics and Gynecology of Kanazawa University Hospital, Toyama Prefectural Central Hospital, Toyama City Hospital, and Koseiren Takaoka Hospital. Histological diagnosis was performed with sections from the same specimens used for telomerase assay and RNA assay. The criteria used for tumor staging were those recommended by the International Federation of Gynecology and Obstetrics. All of the samples were obtained within 2 h after surgical removal or punch biopsy. Cervical scraping samples were also obtained with cotton swabs from uterine ectocervices and soaked in PBS, and cell pellets were recovered and stored at −80°C until use.

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[1] To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, School of Medicine, Kanazawa University, 13-1, Takaramachi, Kanazawa, Ishikawa 920, Japan. Phone: (81-0) 76-262-6500; Fax: (81-0) 76-234-4266; E-mail: satoruky@med.kanazawa-u.ac.jp.

[2] The abbreviations used are: hTR, human telomerase RNA component; TP1/TLP1, telomerase-associated protein; hTRT, human telomerase reverse transcriptase; TRAP, telomeric repeat amplification protocol; RT, reverse transcription.

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Cell Lines. C33A, C4-I, C4-II, CaSki, HeLa, SiHa, HT-3, and MS751 cells, all of which were derived from cervical cancers, were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% fetal bovine serum in the presence of 5% CO_2 at 37°C. Human embryonal and skin fibroblast cells were purchased from Clonetics (San Diego, CA) and were grown according to the manufacturer's protocol.

TRAP Assay. Frozen samples of 50–200 mg were resuspended in ice-cold wash buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM DTT]. After washing, the pellets were homogenized in 20–100 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (Sigma), and 10% glycerol]. After 30 min of incubation on ice, the lysate was centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatant was frozen and stored at −80°C. The protein concentration in the extract was measured by Bradford assay (22). Five micrograms of protein were used for TRAP assay. Assay tubes were prepared by sequestering 0.2 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTTAA-3′) under a wax barrier (Ampliwax: Perkin-Elmer Corp., Foster City, CA). Each extract was assayed in 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 60 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM deoxynucleotide triphosphates, 0.2 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 1 μg of T4g 32 protein (Boeringer Mannheim), and 2.5 units of *Taq* DNA polymerase (Wako Pure Chemicals, Osaka, Japan). After a 30-min incubation at 23°C for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90°C for 3 min and then subjected to 31 cycles of PCR including denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 60 s. The PCR products were electrophoresed on a 12% polyacrylamide gel and visualized with SYBR Green I nucleic acid gel stain (FMC Bioproducts, Rockland, ME).

RNA PCR Analysis. Analysis of the expression of each telomerase subunit was performed by RT-PCR amplification as described previously (21). hTRT mRNA was amplified using the primer pair 5′-CGGAAGAGTGTCTGGAGCAA-3′ (LT5) and 5′-GGATGAAGCGGAGTCTGGA-3′ (LT6); TP1 mRNA was amplified using the primer pair 5′-TCAAGCCAAACCTGAATCTGAG-3′ (TP1.1) and 5′-CCCCGAGTGAATCTTTCTACGC-3′ (TP1.2); and hTR mRNA was amplified using the primer pair 5′-TCTAACCCTAACTGAGAAGGGCGTAG-3′ (F3b) and 5′-GTTTGCTCTAGAATGAACGGTGGAAG-3′ (R3c). Total RNA was isolated from the tissues using Isogen (Nippon Gene) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using the RNA PCR kit version 2 (Takara Shuzo Co., Ltd., Kyoto, Japan) with random primers. To amplify the cDNA, 2-μl aliquots of the reverse-transcribed cDNA were subjected to 28 cycles of PCR in 50 μl of 1× buffer [10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 50 mM KCl] containing 1 mM dATP, dCTP, dGTP, and dTTP, 2.5 units of *Taq* DNA polymerase (Takara Shuzo), and 0.2 μM concentrations of specific primers. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s. PCR products were electrophoresed in a 7% polyacrylamide gel and stained with SYBR green (FMC Bioproducts). The efficiency of cDNA synthesis from each sample was estimated by PCR with glyceraldehyde-3-phosphate dehydrogenase-specific primers as described previously (21).

Statistical Analysis. The χ^2 test was used to evaluate the significance of differences. Findings of $P < 0.05$ were considered statistically significant.

RESULTS

A total of 25 cervical cancers and 14 normal cervical specimens were examined for the expression of hTR, TP1 mRNA, and hTRT mRNA. RT-PCR analysis revealed tha hTR was expressed in all of the tissues examined, both cancerous and noncancerous (Table 1). TP1 was expressed in 21 of 25 cervical cancers (84%) as well as in 10 of 14 normal cervical specimens (71%). These findings suggest that hTR and TP1 are widely expressed in both cancerous and noncancerous cervical tissues. In contrast, hTRT mRNA expression was observed in 20 of 25 cervical cancers (80%) but in none of the normal cervical specimens. There was no significant correlation between expression of hTRT mRNA and clinicopathological features of cervical cancer, such as age, clinical stage, and histological type. We also examined eight cell lines (C33A, C4-I, C4-II, CaSki, HeLa, SiHa, HT-3, and MS 751) derived from cervical cancer as well as two primary normal fibroblasts from human skin and kidney. All of the cancer cell lines expressed each of the telomerase subunits, whereas the two normal fibroblast cell lines expressed TP1 and hTR but not hTRT (Fig. 1).

Telomerase activity in each sample was examined using the TRAP assay, and the correlation between telomerase activity and expression of each telomerase subunit was evaluated. Twenty-eight cervical cancers were examined for telomerase activity, and 23 (82%) were found to be telomerase-positive, whereas none of the normal cervical specimens were (Table 1). Of 23 cervical cancers examined for both telomerase activity and hTRT mRNA, 18 (78%) were concordant for positivity or negativity (16 were both positive and 2 were both negative), whereas 5 were discordant (Table 2). Three cervical cancers (cases 3, 11, and 14) that expressed hTRT mRNA lacked telomerase activity, whereas two (cases 4 and 5) lacking hTRT mRNA expressed telomerase activity. Normal cervical samples, all of which lacked hTRT expression, were telomerase-negative without exception. When cancerous and normal samples were combined, 32 of 37 cases (86%) examined for both telomerase activity and hTRT expression were concordant for positivity or negativity ($P < 0.001$). The cervical cancer cell lines examined were all telomerase-positive and expressed hTRT mRNA. However, the two primary normal fiboblasts were both telomerase-negative and expressed no hTRT mRNA. In contrast to hTRT, there was no significant correlation between telomerase activity and hTR or TP1 mRNA expression for either cervical tissues or cell lines.

We next examined whether hTRT mRNA is also detectable in cervical exfoliated cells from patients with cervical lesions. Approximately 70% (11 of 17) of cervical cancers examined expressed hTRT mRNA in exfoliated cells, whereas none of 14 from normal cervix did (Table 1). These findings suggest that expression of hTRT mRNA is useful for cytological examination of cervical lesions.

DISCUSSION

In the present study, hTRT was expressed in most of the cervical cancers but in none of the normal cervical samples. There was a strong association between telomerase activity and hTRT mRNA expression. These findings suggest that expression of hTRT is a rate-limiting determinant of the enzymatic activity of human telomerase and are thus consistent with those of recent studies, suggesting that hTRT is a putative catalytic subunit of telomerase (20, 21). Thus, up-regulation of hTRT mRNA may play a critical role in activation of telomerase. However, we found some cases of discordancy, in which telomerase activation was not associated with hTRT mRNA expression, or expression of hTRT mRNA did not lead to telomerase activation in cervical cancers. The former type of discordance suggests the possibility that other factors may substitute for the hTRT subunit in conferring full enzymatic activity. The latter suggests several possibilities. First, the levels of expression of each subunit (hTR, TP1, and hTRT) and the balance of the levels of expression of these subunits might play critical roles in determining enzymatic activity, although in the present study we did not determine the levels of expression of these subunits. Second, it is also possible that posttranscriptional modification of the subunits plays a role in regulation of enzymatic activity. Third, unknown telomerase inhibitor(s) may exist in cell extracts in such cases and may decrease telomerase activity.

In contrast, hTR and TP1 were broadly expressed not only in cancers but also in normal cervices. There was no significant correlation between telomerase activity and expression of hTR or TP1 mRNA expression. Thus, expression of neither TP1 nor hTR appears

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Table 1 *Telomerase activity and expression of telomerase subunits in cervical cancer*

Case	Diagnosis	Stage	Histology	TRAP	hTRT	TP1	hTR	hTRT(ex)[a]
1	Cervical cancer	Ib	SCC[b]	+	+	+	+	+
2	Cervical cancer	IIb	SCC	+	+	+	+	+
3	Cervical cancer	Ib	SCC	−	+	+	+	+
4	Cervical cancer	IVa	Adeno[c]	+	−	−	+	ND
5	Cervical cancer	IIb	SCC	+	−	−	+	−
6	Cervical cancer	IVb	SCC	−	−	−	+	ND
7	Cervical cancer	Ib	SCC	+	+	+	+	+
8	Cervical cancer	Ib	SCC	+	+	+	+	+
9	Cervical cancer	Ia	Adeno	+	ND[d]	ND	ND	ND
10	Cervical cancer	IIb	Adeno	−	−	+	+	ND
11	Cervical cancer	IIIb	Verrucous	−	+	+	+	−
12	Cervical cancer	IVa	SCC	+	+	+	+	ND
13	Cervical cancer	IIb	SCC	+	+	+	+	ND
14	Cervical cancer	Ia	SCC	−	+	+	+	ND
15	Cervical cancer	Ib	SCC	+	ND	ND	ND	ND
16	Cervical cancer	IIIb	SCC	+	ND	ND	ND	ND
17	Cervical cancer	IIIb	SCC	+	ND	ND	ND	ND
18	Cervical cancer	IVb	SCC	+	ND	ND	ND	ND
19	Cervical cancer	IIb	SCC	+	+	+	+	+
20	Cervical cancer	IIb	SCC	+	+	+	+	+
21	Cervical cancer	Ib	SCC	+	+	−	+	ND
22	Cervical cancer	Ib	SCC	ND	−	+	+	ND
23	Cervical cancer	IIa	SCC	+	+	+	+	−
24	Cervical cancer	IIIb	SCC	+	+	+	+	+
25	Cervical cancer	Ib	Small cell	+	+	+	+	+
26	Cervical cancer	IIb	SCC	ND	+	+	+	−
27	Cervical cancer	Ib	SCC	+	+	+	+	−
28	Cervical cancer	Ib	SCC	+	+	+	+	+
29	Cervical cancer	IIb	Adeno	+	+	+	+	+
30	Cervical cancer	Ib	SCC	+	+	+	+	−
31	Normal			−	−	−	+	−
32	Normal			−	−	+	+	−
33	Normal			−	−	+	+	−
34	Normal			−	−	+	+	−
35	Normal			−	−	−	+	−
36	Normal			−	−	+	+	−
37	Normal			−	−	+	+	−
38	Normal			−	−	+	+	−
39	Normal			−	−	+	+	−
40	Normal			−	−	+	+	−
41	Normal			−	−	+	+	−
42	Normal			−	−	−	+	−
43	Normal			−	−	−	+	−
44	Normal			−	−	+	+	−

[a] Exfoliated cells from uterine cervix.
[b] Squamous cell carcinoma.
[c] Adenocarcinoma.
[d] Not determined.

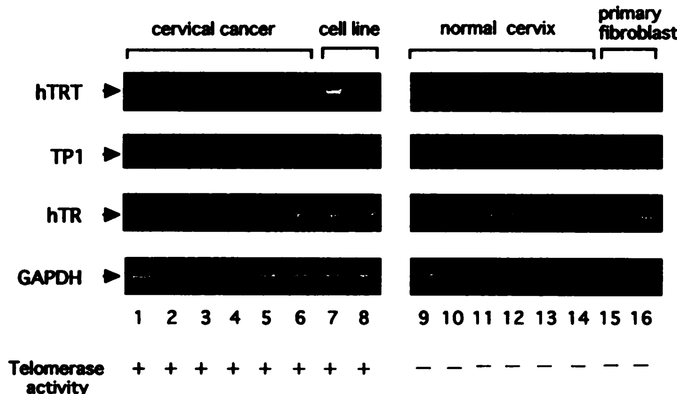

Fig. 1. Representative results of RT-PCR analysis for the expression of each telomerase subunit in cervical tissues and cell lines. Expression of hTR, TP1 mRNA, and hTRT mRNA was examined by RT-PCR. *Lanes 1–6*, cervical cancer cases; *Lane 7*, C33A cells; *Lane 8*, HeLa cells; *Lanes 9–14*, normal cervix; *Lane 15*, primary human embryonal fibroblast cells; *Lane 16*, primary human skin fibroblast cells. Results of the TRAP assay are shown below each lane.

to parallel telomerase activity. Disruption of the function of telomerase RNA in *Tetrahymena* through overexpression of an inactive form of telomerase RNA has been shown to result in progressive shortening of telomeres (4). Tumor cells transfected with antisense hTR lost telomeric DNA, resulting in cellular senescence (12). Most recently, targeting the telomerase RNA gene in mice has been shown to lead to progressive shortening of telomeres (23). These findings suggest that function of hTR is absolutely required for telomerase activity. Our findings, however, showed that expression of hTR is not sufficient by itself to ensure enzymatic activity.

TP1 has been identified as a human homologue of *Tetrahymena* p80 and has been shown to associate with telomerase *in vivo* (15, 16). The function of TP1 is yet to be determined, but the WD repeat

Table 2 *Correlation between telomerase activity and hTRT mRNA expression in cervical cancer and normal cervix*

Correlation between telomerase activity and hTRT expression is statistically significant ($P < 0.001$).

Telomerase activity	*n*	hTRT mRNA expression +	hTRT mRNA expression −
Cervical cancer			
+	18	16	2
−	5	2	3
Normal cervix			
+	0	0	0
−	14	0	14

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sequences in its gene suggest that it may play a role as an interface between telomerase and the protein member of the telosome. A recent study also suggested the possibility that posttranscriptional modification of TP1 may play a role in regulation of telomerase activity (16). Although the present study failed to find an association between TP1 mRNA expression and telomerase activity, TP1 may function as a regulatory component for telomerase activity through the mechanisms described above.

The findings of the present study suggest that hTRT mRNA is useful for the cytological screening for cervical cancers. Although hTRT mRNA was detected frequently in smear samples from cervical cancer, it was observed in none of those from normal cervix, indicating that it is very specific to cancer lesions. We previously reported the usefulness of the TRAP assay for screening for cervical lesions (24). Because hTRT mRNA was detected even in some of the TRAP-negative cancer cases, the combination of the TRAP assay and hTRT mRNA detection will be a powerful method of screening for cervical lesions.

Although our findings indicated that hTRT expression is strongly associated with cervical cancer, it remains unclear whether this is also the case for premalignant, benign lesions. In particular, it will be interesting to determine whether hTRT is expressed in normal somatic tissues with telomerase activity. Because hTRT expression was found to be a rate-limiting determinant of telomerase activity, it is most important to determine when hTRT is expressed during carcinogenesis. Understanding the molecular mechanisms through which hTRT is expressed and telomerase is activated will provide critical insights into the molecular basis of cellular immortality and human carcinogenesis.

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