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Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections

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Human telomerase is expressed in germ tissues and in the majority of primary tumors. Cell renewal tissues and some pre-cancerous tissues also have weak telomerase activity. Yet, neither the exact location and frequency of telomerase-positive cells nor the changes in telomerase expression during differentiation or carcinogenesis of individual cells are known. This paper reports on the expression of hTERT (telomerase reverse transcriptase) protein in tumor and non-tumor colorectal tissues by Western blotting and tissue sections by immuno-histochemistry using antibodies raised against partial peptides of hTERT. Though telomerase activity and hTERT expression at both mRNA and protein levels were generally higher in tumor part than in non-tumor part, these two were not always correlated: expression of hTERT did not always give rise to high telomerase activity. Colonic carcinoma cell nuclei were stained with anti-hTERT antibodies but not with antigen-preabsorbed antibodies. In normal mucosa, hTERT protein was expressed, though weaker than in carcinoma, in all colonic crypt epithelial cells except those at the tip; the expressing-cell distribution was much wider than that of Ki-67 positive cells which were located at the bottom of the crypt. Isolated crypt contained a significant level of hTERT protein revealed by Western blotting, while having very weak telomerase activity. Telomerase activity was detected in epithelial cells only at the bottom half of the crypt. Specific hTERT-staining was positive in tissue lymphocytes but negative in almost all other stromal cells. It is of interest to see whether a significant level of hTERT expression with low telomerase activity is characteristic of physiologically regenerating tissues containing stem cells. *In situ* detection of the hTERT protein will permit further analysis of cancer diagnosis and stem cell differentiation.

Keywords: telomerase; *in situ* detection; hTERT; immuno-histochemistry; colorectal carcinoma

Introduction

Telomerase is a reverse transcriptase that synthesizes telomere DNA thereby compensating for telomere loss that occurs with each replication cycle, and limits proliferation of cells. Telomerase is expressed in human germ tissues and in the majority of primary human tumors (Kim *et al*., 1994). Physiologically renewing tissues such as bone marrow and gastrointestinal tissues have weak but significant telomerase activity, which is assumed to be derived from proliferating stem cells (Harle Bachor and Boukamp, 1996; Hiyama *et al*., 1995, 1996). We do not know, however, the exact location and frequency of telomerase-positive cells within tissues nor the changes in telomerase expression of individual cells during differentiation or under different proliferative conditions. Some pre-cancerous lesions, such as adenomas or intestinal metaplasia, show higher levels of telomerase activity compared to surrounding normal tissues (Shay and Bacchetti, 1997; Tahara *et al*., 1995), but we do not know whether this activity is due to a small number of carcinoma cells, proliferation-stimulated stem cells, or activated lymphocytes migrating into the lesions. *In situ* detection of telomerase activity at the individual cell level should resolve these ambiguities, but prior attempts in tissue sections have not been successful (Ohyashiki *et al*., 1997). Telomerase components such as hTERC (human telomerase RNA component also known as hTR (Avilion *et al*., 1996; Feng *et al*., 1995)) and hTEP1 (telomerase protein 1 also known as TLP1/TP1 (Harrington *et al*., 1997a; Nakayama *et al*., 1997)) are expressed constitutively in both normal (telomerase-negative) and tumor (telomerase-positive) tissues and expression levels of these components do not correlate with telomerase activity, although some differences in expression level of hTERC was reported between normal and tumor tissues (Kuniyasu *et al*., 1997). Recently, the catalytic subunit of human telomerase, hTERT (human telomerase reverse transcriptase also known as hTRT/hEST2/TP2 (Harrington *et al*., 1997b; Meyerson *et al*., 1997; Nakamura *et al*., 1997; Nakayama *et al*., 1998), was cloned, whose expression, detected by RT–PCR, closely correlates with telomerase activity in cells *in vitro* and in tissues *in vivo* (Nakayama *et al*., 1998). Expression of hTERT mRNA by *in situ* hybridization was reported in early premalignant lesions and in normal tissues (Kolquist *et al*., 1998). We report here our success in *in situ* immuno-histochemical detection of anti-hTERT antibody-reactive proteins in colorectal tissue sections.

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Results

Telomerase activity by TRAP and expressions of hTERT, hTERC and hTEP1 by RT–PCR in human colorectal tissues

To characterize tissue samples used for immunodetection of hTERT protein, telomerase activity and expression levels of hTEP1, hTERC and hTERT were assayed by TRAP and RT–PCR assays in the colorectal tissue samples. We confirmed microscopically that each tumor tissue sample (T) consisted mainly of carcinoma tissue and non-neoplastic (normal) mucosal sample (N) neither contained tumor component including adenoma nor showed significant inflammatory involvement. Telomerase activity was higher in T than in N of paired samples from the same patients (Figure 1), while its level in T was highly variable among patients as previously reported (Tahara *et al*., 1995). Expression levels of hTERC and hTERT were also generally higher in T than in N between the paired samples from the same patients (Figure 1). hTEP1 was expressed independent of telomerase activity level. However, there were some discrepancies between telomerase activity and expression of hTERT. Such discrepancies were most obvious in cases (No. 5, 6), (No. 33, 34) and (No. 37, 38), where significant levels of telomerase activity were observed in T but not in N whereas the hTERT expression levels were almost equal between T and N. In cases of No. 5 and 6, however, levels of hTERT protein were significantly higher in No. 6 (T) than in No. 5 (N) (Figure 1, middle panel). In other words, the N parts of these samples have negligible telomerase activity with significant levels of hTERT expression at mRNA level. The results were reproducible and therefore cannot be attributed to experimental error. The same powdered samples (see Materials and methods) were used for both the RT–PCR and TRAP methods. The RT–PCR reaction was confirmed (Nakayama *et al*., 1998) to be linear range when greater than 0.01 μg of RNA from a telomerase-positive control cell line, HSC39, was used. And finally the signal intensity of the internal telomerase assay standard (ITAS) band was almost identical among samples, especially in those that were telomerase negative/weak. Nevertheless we cannot rule out the possible differential effect on enzyme activity and mRNA integrity of the conditions under which the tissue samples were subjected to following surgical resection prior to cryopreservation (even when as short as within 30 min). These results suggest that while expression of hTERT and hTERC is essential for telomerase activity, other factors are involved in the regulation of telomerase activity especially in non-neoplastic colorectal mucosa.

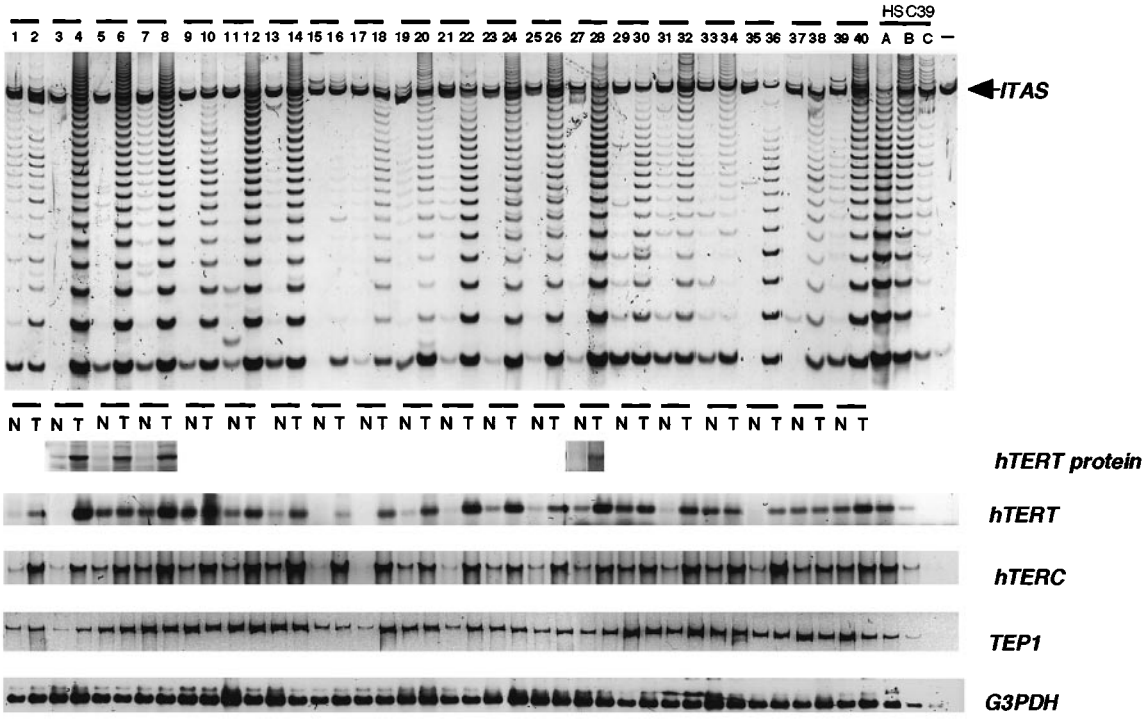

Figure 1 Telomerase activity and hTERT, hTERC and hTEP1 expression in human colorectal tissues. Paired tumor (T) and non-tumor (N) parts from 20 colorectal cancer patients were assayed for telomerase activity by TRAP method (upper panel), for the expression of hTERT protein by Western blotting (middle panel) and for the mRNA expression of hTERT, hTERC, hTEP1 and G3PDH (as a control) by RT–PCR (lower panel). Tumor part consisted mainly of carcinoma and non-tumor (normal) mucosal part neither contained such tumor component as adenoma nor showed significant inflammatory involvement. The numbers at the top of the upper panel represent the sample number. Samples containing 0.1 μg protein, 50 μg protein and 0.1 μg RNA were used for each TRAP, Western blotting and RT–PCR assay, respectively. Anti-hTERT1.0 antibody was used for Western blotting. HSC39 is a human gastric tumor cell line used as a positive control; A, 1000 cells for TRAP and 0.1 μg RNA for RT–PCR; B, 100 cells for TRAP and 0.01 μg RNA for RT–PCR; and C, 10 cells for TRAP and 0.001 μg RNA for RT–PCR. (–), buffer containing no sample extract assayed as a negative control. ITAS, internal telomerase assay standard

Detection of hTERT protein by Western blotting

By Western blot using two anti-hTERT rabbit polyclonal antibodies, a protein band (hTERT arrow in Figure 2), with an estimated molecular mass of 120 kD, close to that predicted from the amino acid sequence (Nakayama *et al*., 1998), was detected in extracts from telomerase-positive cultured cells (YT-3) and from a colorectal tumor sample (No. 4) but not in extracts from a telomerase-negative normal fibroblast strain (TIG-3) (Figure 2). Both antibodies, however, reacted with several other bands in addition to p120, with considerable variation among samples and between antibodies. Harrington *et al*. also reported that anti-hTERT antibody detected a couple of bands besides p120 (Harrington *et al*., 1997b). Though we did not further characterize these extra bands, these bands disappeared when the antibody was preabsorbed with the corresponding antigen (Figure 2) or when pre-immune serum was used (data not shown). Some of these might be unrelated proteins, degradation products of hTERT proteins or products from alternatively spliced transcripts. Alternatively spliced hTERT transcripts were reported in several normal tissues (colonic crypt and testis) and various tumors with considerable differences in splicing patterns (Kilian *et al*., 1997). We next surveyed the expression of hTERT by Western blot in some of the colorectal tissues (Figure 1, middle panel), in which the paired non-tumor part (N) from the same patient gave a lower intensity p120 band than tumor part (T).

Immuno-histochemical detection of hTERT protein in colorectal tissue sections

hTERT protein was detected in colorectal tissue sections by immuno-histochemical method. Colonic carcinoma cells (No. 4) were strongly positive to the specific antibody (Figure 3a) whereas not to preabsorbed antibody (Figure 3b). Nuclear localization of the signal was observed in most carcinoma cells at higher magnification (Figure 3c). Carcinoma cells in 10 tumor samples out of 12 examined were also stained by specific antibodies but not by preabsorbed ones. Epithelial cell nuclei of normal colonic crypt were stained with the specific antibody with weaker intensity than tumor cell nuclei (Figure 3d) except for those near the tip of the crypt (Figure 3f), while the preabsorbed antibody gave negative staining (Figure 3e). Expression of hTERT at mRNA level was also reported in normal epithelial cells that retain long proliferative potential by *in situ* detection of hTERT mRNA (Kolquist *et al*., 1998). Because proliferating stem cells are considered to be located exclusively near the bottom of the colonic crypt, as shown in Ki-67 positive cells in Figure 3h, it was unexpected that hTERT-stained cells were distributed from the bottom to nearly the tip of the crypt (Figure 3d,f,g) where the cells are differentiation-committed and non-proliferating. These differentiated cells might contain degraded proteins that retain antigenicity of hTERT although enzymatic function is lost. Such an explanation would be consistent with the finding that significant telomerase activity was present only in the bottom half of the isolated crypt and not in the upper half (Figure 4c,d). However, isolated whole crypt which had weak but significant telomerase activity (Figure 4b), contained p120 hTERT protein as a major band as revealed by Western blot (Figure 4a) suggesting that the majority of immuno-stained material was not degraded peptides. Although Western blotting is not sufficiently quantitative to make definitive conclusion, it appeared that isolated crypts contained higher amounts of p120 (Figure 4a) than expected based on telomerase activity (Figure 4b) as compared with cases of tumor tissues presented in Figure 1. It was confirmed, using HSC39 cells, that the process of crypt isolation did not reduce telomerase activity. The amount of material extracted from manually-separated halves of the crypts was too small to detect hTERT protein by Western blotting. It is of interest to see whether these differentiation-committed cells contain dormant hTERT protein and retain proliferative potential as reserver cells when proliferating stem cells are lost. Phosphorylation of hTERT protein was suggested as a requirement for its enzymatic activity (Li *et al*., 1997), but preincubation

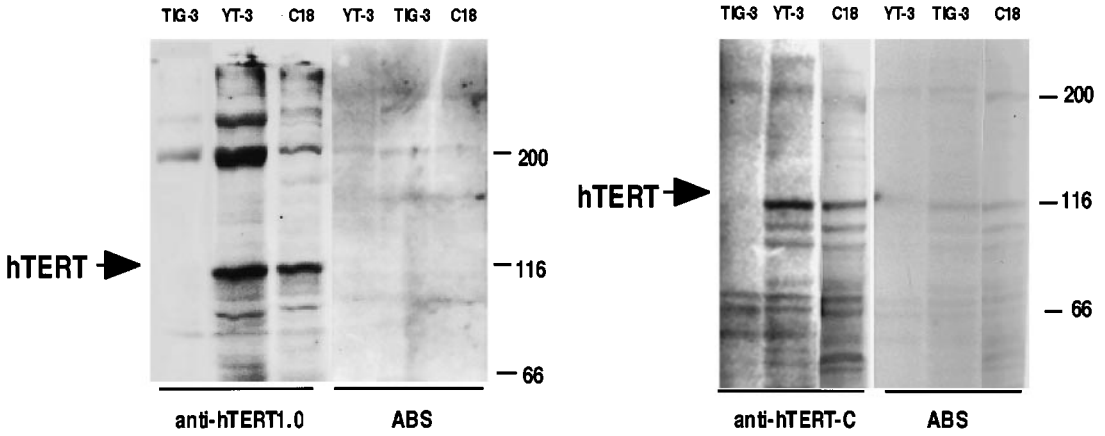

Figure 2 Western blot of hTERT protein in human cultured cells and colorectal carcinoma tissue. Cell extracts (50 μg protein) from TIG-3 (telomerase-negative normal fibroblast strain), YT-3 (a cell clone which became telomerase-positive after transfection of TIG-3 with hTERT cDNA) and C18 (colorectal carcinoma, tissue No. 4) were examined for hTERT protein by Western blotting. Anti-hTERT1.0 and anti-hTERT-C antibodies were used as designated below. ABS indicates that the antibodies were pre-absorbed for 2 h with a tenfold excess of the corresponding antigens prior to immunostaining. Arrow indicates the presumptive hTERT protein

of extracts from isolated crypts and from tissue samples 5, 33 and 37 with ATP did not enhance telomerase activity (data not shown). Lymphocytes in colorectal tissues were positive for hTERT staining (Figure 3d). Stromal cells such as fibroblasts, smooth muscle cells and vascular endothelial cells were not positive for hTERT staining, though the possible presence of small amounts of contaminating immunologically positive materials was not completely ruled out.

Discussion

The present paper reports our success in *in situ* immuno-histochemical detection of anti-hTERT antibody-reactive proteins in colorectal tissue sections. Colonic carcinoma cell nuclei were strongly positive to the specific antibody (Figure 3a,c) whereas not to preabsorbed antibody (Figure 3b). Epithelial cell nuclei of normal colonic crypt were also stained with the

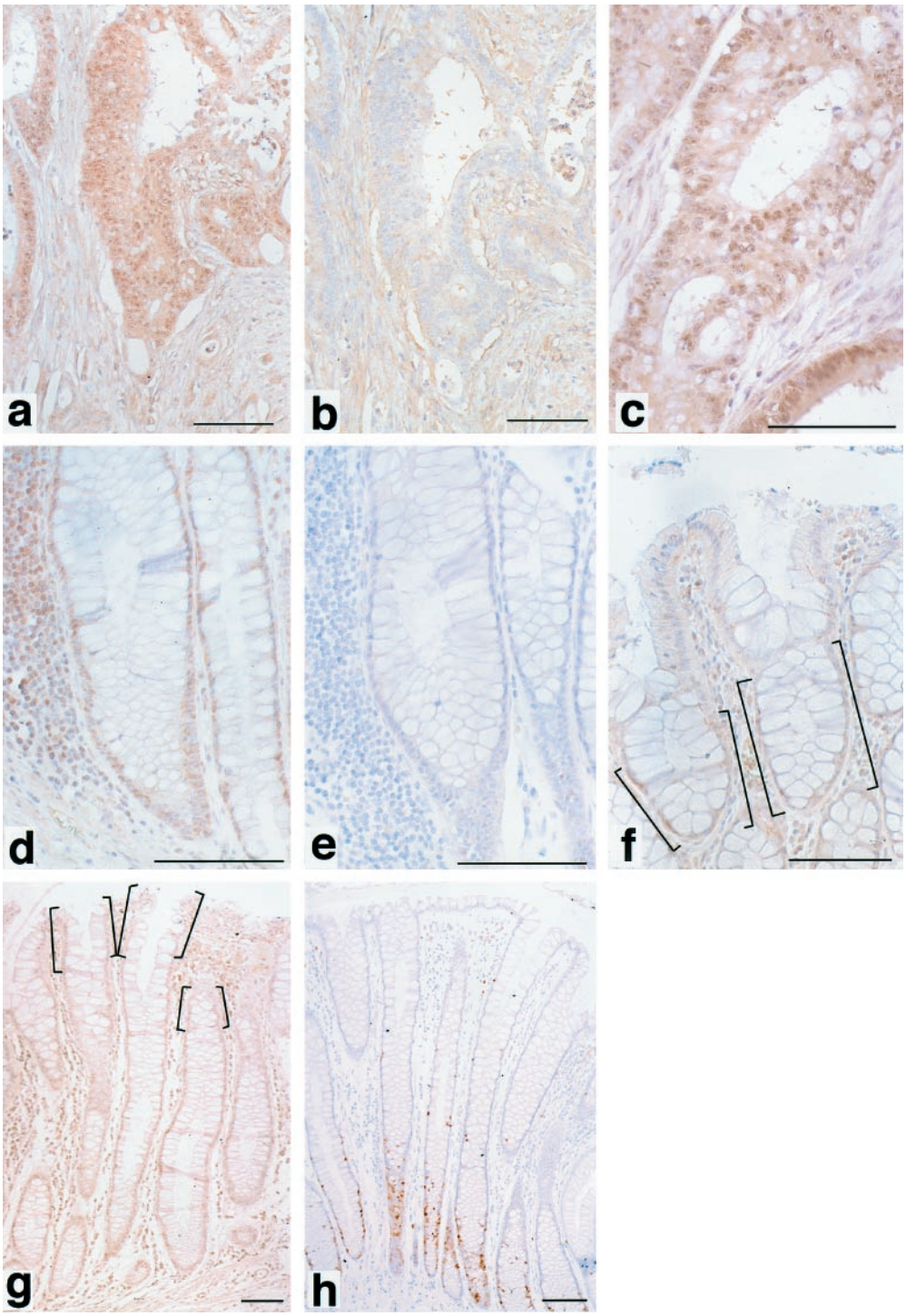

Figure 3 Immuno-staining of human colorectal tissues by anti-hTERT antibody. A tissue section containing human colorectal carcinoma was stained with anti-hTERT1.0 antibody (**a**,**c**) or with the antibody preabsorbed with the corresponding antigen peptide (**b**). Normal colorectal tissue sections were stained with anti-hTERT1.0 antibody (**d**,**f**,**g**), with the antibody preabsorbed with the antigen (**e**) or with anti-Ki-67 antibody (**h**). Brackets in **f** and **g** indicate crypt regions with positively- and negatively-stained epithelial cells, respectively. Bar indicates 100 μm

specific antibody with weaker intensity than tumor cell nuclei (Figure 3d) except for those near the tip of the crypt (Figure 3f), while the preabsorbed antibody gave negative staining (Figure 3e). Specific antibodies also stained tissue lymphocytes but not stroma cells. While the antibodies used here detected a couple of protein bands in addition to p120, a positive signal by immuno-histochemical detection is considered a specific signal of hTERT protein, because (1) it was positive in tumor cells of telomerase-positive colorectal carcinomas, (2) it was positive in presumptive stem cells and in tissue lymphocytes, (3) it was negative in epithelial cells near the surface of colonic crypt and stromal cells in normal colorectal tissues, and (4) signals disappeared by preabsorption of antibody with hTERT peptide used as immunogen, although final conclusion should await until a specific antibody is available which reacts only with p120 protein. *In situ* detection of hTERT protein permits further analysis of carcinogenesis and cancer diagnosis.

While telomerase activity by TRAP and hTERT expression level by RT–PCR were generally higher in T than in N of paired samples from the same patients, there were some discrepancies between telomerase activity and expression of hTERT as the most obviously observed in cases (No. 5, 6), (No. 33, 34) and (No. 37, 38), where significant levels of telomerase activity were observed in T but not in N whereas the hTERT mRNA expression levels were almost equal between T and N (Figure 1). In hepatic tissue samples, including tumor and non-tumor tissues, the levels of telomerase activity and hTERT mRNA expression showed good correlation and there was no non-tumor liver tissue that expressed hTERT (Nakayama *et al*.,

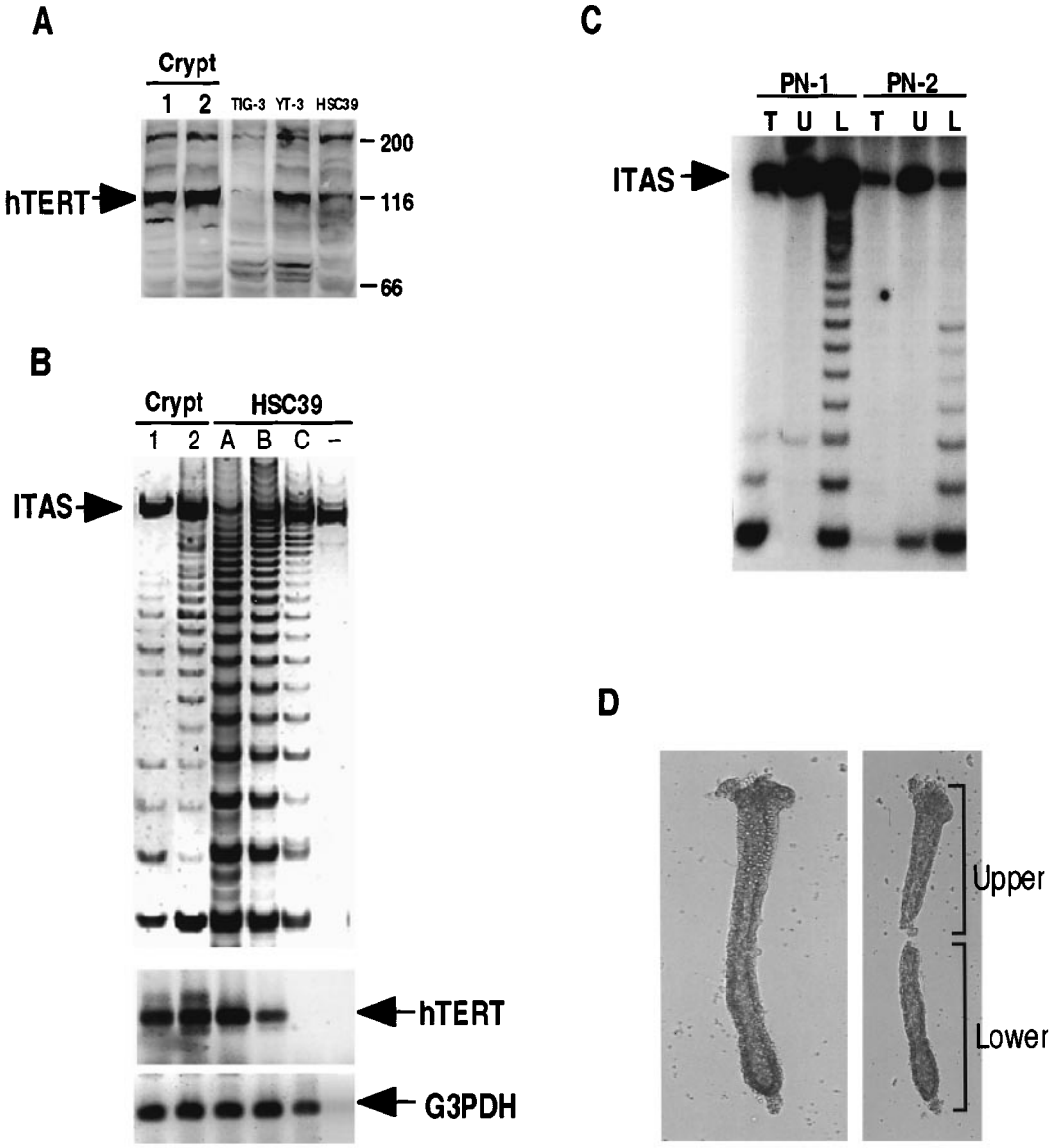

Figure 4 Western blot of hTERT protein and telomerase activity in human colonic crypt. (**a**) Extracts (50 μg protein) from two independently isolated colonic crypt preparations were examined for hTERT protein by Western blotting using the anti-hTERT-C antibody. Arrow indicates the presumptive hTERT protein. (**b**) Telomerase activity in isolated colonic crypt samples. HSC39 is the same as in Figure 1. Expression levels of hTERT and G3PDH (as control) by RT–PCR are also presented. (**c**) Isolated crypts were cut and separated into upper and bottom halves (as shown in (**d**) under microscope and extracts were assayed for telomerase activity (0.1 μg protein per assay). PN–1 and 2 were from patient 1 and 2 (different patients from those in Figure 1), respectively; T, whole tissue of colonic mucosa; U, upper half of the crypt; L, bottom half of the crypt

1998). Discrepancy between protein and mRNA levels of hTERT was observed in that the level of hTERT protein was significantly higher in No. 6 (T) than in No. 5 (N) (Figure 1, middle panel) whereas the level of mRNA was almost equal. Moreover, discrepancy was also observed that all epithelial cell nuclei of normal colonic crypt were stained with the specific antibody (Figure 3d) except for those near the tip of the crypt (Figure 3f), whereas significant telomerase activity was present only in the bottom half of the isolated crypt (Figure 4c,d) where proliferating stem cells were located (Figure 3d,f,g). Isolated whole crypt contained p120 hTERT protein as a major band as revealed by Western blot (Figure 4a). Lymphocytes in colorectal tissues were strongly positive for hTERT staining (Figure 3d), whereas stromal cells were not. Though we could not measure telomerase activity in tissue lymphocytes, peripheral blood lymphocytes in adults had very weak telomerase activity but they showed strong activity after growth stimulation (Hiyama *et al*., 1995). These results suggested that, in colorectal tissues, expression of hTERT at mRNA or protein level did not always result in significant expression of telomerase activity. Low level expression of hTERT mRNA has been reported by *in situ* hybridization in normal colonic epithelial cells which located within the proliferative zone of the crypts (Kolquist *et al*., 1998). hTERT related protein detected by immuno-staining in the upper part of the proliferative zone of the crypts might be a survivor synthesized within the proliferative zone, though other possibilities were not excluded such that hTERT mRNA was also present in the upper part of the crypts but the level was undetectably low by *in situ* hybridization. It is of interest to see whether a significant level of hTERT expression with low telomerase activity, a possible presence of post-transcriptional and/or post-translational regulation for telomerase activity, is characteristic of physiologically regenerating tissues containing stem cells and lymphocytes. This will shed a new light in the study of kinetics and differentiation of somatic and germinal stem cells, and in the study of cellular senescence of stem cells.

Materials and methods

Telomerase activity and expression of hTERC, hTEP1 and hTERT mRNA

Colorectal tissue samples were collected at surgery and stored as described (Tahara *et al*., 1995). Frozen tissue (approximately 20 mg) was powdered in liquid nitrogen and divided into two samples, one for the telomerase assay and the other for RT–PCR, allowing the telomerase assay and RT–PCR assay with the same samples. Preparation of extracts and assay of telomerase activity by TRAP (telomeric repeat amplification protocol) was done as described (Kim *et al*., 1994; Nakayama *et al*., 1998). RNA extraction and RT–PCR were also performed as described (Nakayama *et al*., 1998).

Crypt isolation

Colonic crypts were isolated as follows. The resected colon tissue piece (about 500 mg) was rinsed briefly with cold Ca,Mg-free phosphate buffered saline (PBS(−)) once and with cold 30 mM EDTA in PBS(−) three to four times until major debris could not be seen in the supernatant. The crypts were isolated from washed tissues by adding 20 ml of 30 mM EDTA in PBS(−) and vortexing for 1 min at maximum speed. The crypts recovered in the supernatant were collected. This step was repeated three to four times. Collected crypts were washed three times with cold PBS(−) and pelleted by centrifugation at 2000 r.p.m. for 5 min. It was confirmed, using a cell pellet of cultured tumor cell line, HSC39, that this process did not affect telomerase activity. Isolated crypts were spread onto a microscope slide and each crypt was manually divided under microscope using a scalpel into upper and lower halves and collected by micropipette into 1.5 ml tubes for TRAP assay.

Antibodies

A part of hTERT cDNA, corresponding to the nucleotide 1687–2042 of the GenBank accession number AF015950 clone, was fused with the GST (glutathione S-transferase) gene, and the fused recombinant protein was produced in *E. coli*. An oligopeptide, corresponding to the carboxyl-terminal 13 amino acids [NPALPSDFKTILD] deduced from the same GenBank hTERT cDNA clone, was chemically synthesized. The hTERT recombinant protein and oligopeptide were used as antigens to immunize rabbits separately. Thus prepared anti-recombinant protein antibody (anti-hTERT1.0) and anti-oligopeptide antibody (anti-hTERT-C) were purified by affinity chromatography and used in this study.

Western blot of hTERT protein

Cell or tissue extracts for TRAP assay containing 50 μg protein were fractionated in 6% SDS–PAGE and electroblotted onto membrane. The membrane was treated with anti-hTERT antibody, followed by an anti-rabbit IgG horseradish peroxidase (Amersham). The ECL Plus kit (Amersham) was used to detect antigen/antibody complexes and chemiluminescent signals were detected using X-ray film.

Immuno-histochemical detection of hTERT protein

Colorectal tissues obtained by surgery were fixed in 10% buffered-formalin and embedded in paraffin. Deparaffinized sections were immersed in methanol containing 0.03% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. Microwave pretreatment in citrate buffer was performed for 10 min three times to retrieve the antigenicity, and the sections were incubated with blocking solution for 10 min. The sections were treated at room temperature with anti-hTERT antibody (3–5 μg/ml) or Ki-67 antibody (MIB-1; Medical and Biological Laboratories, Nagoya, Japan; diluted 1 : 100) for 90 min. The immuno-complex was visualized by a modification of the immunoglobulin enzyme bridge technique (ABC method) using the SensiTek HRP kit (ScyTek Laboratories, Logan, UT, USA) (Yasui *et al*., 1996). Since 3,3′-diaminobenzidine tetrahydrochloride was used as substrate, positive reaction was detected as a brown color. The sections were weakly counterstained with 0.1% hematoxylin. The specificity of hTERT-immunostaining was determined by preabsorption of the antibody with an excess of specific antigen.

Abbreviations

RT–PCR, reverse transcribed-polymerase chain reaction; TRAP, telomeric repeat amplification protocol; hTERT, human telomerase reverse transcriptase; hTERC, human telomerase RNA component; hTEP1, human telomerase protein 1.

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