Histochem Cell Biol (2004) 121:391–397
DOI 10.1007/s00418-004-0645-5

ORIGINAL PAPER

Pu Yan · Jean Benhattar · Walter Seelentag · Jean-Christophe Stehle · Fred T. Bosman

Immunohistochemical localization of hTERT protein in human tissues

Accepted: 16 March 2004 / Published online: 7 May 2004

Abstract Telomerase is a ribonucleoprotein complex mainly composed of a reverse transcriptase catalytic subunit (telomerase reverse transcriptase gene, hTERT) that copies a template region of its RNA subunit to the end of the telomere. For detecting telomerase activity in a tissue specimen the TRAP assay is a relatively sensitive and specific method, but it can be used only on fresh tissue extracts and offers no information at the single cell level. Immunohistochemistry (IHC) allows to detect hTERT protein expression at an individual cell level in human tissues. We have tested commercially available anti-hTERT antibodies in formalin-fixed and paraffin-embedded human tissues by IHC. Only one monoclonal antibody (NCL-hTERT; Novacastra) was sufficiently specific and this was applied to human tissues in which telomerase activity had been shown by TRAP assay and hTERT mRNA expression by RT-PCR. hTERT protein localized diffusely in the nucleoplasm and more intensely in the nucleoli of cancer cells and proliferating normal cells. Mitotic cells showed diffuse staining of the entire cell. Granular cytoplasmic staining was occasionally found in some tumor cells. In telomerase-positive tumors not all the tumor cells showed hTERT immunoreactivity. A significantly heterogeneous hTERT protein expression was observed in human tumor tissues. The hTERT immunostaining in fixed tissues was concordant with telomerase activity and hTERT mRNA expression in corresponding non-fixed samples. Quantitative RT-PCR of microdissected sections showed that hTERT mRNA expression was higher in cells with nuclear expression than in those with cytoplasmic expression. Double staining with the M30 antibody showed that a subpopulation of hTERT-negative cells is apoptotic. We conclude that: (1) hTERT protein can be detected by IHC in fixed human tissues, but the choice of the antibody, tissue processing, and reaction conditions are critical, (2) hTERT protein localizes in the nucleoplasm, more strongly in the nucleolus, and occasionally in the cytoplasm, (3) telomerase-positive tumors show significant heterogeneity of hTERT protein expression, and (4) a subpopulation of hTERT protein negative tumor cells is identified as apoptotic cells.

Keywords hTERT · Immunohistochemistry · Telomerase · TRAP assay · RT-PCR · Apoptotic cells

P. Yan (✉) · J. Benhattar · W. Seelentag · J.-C. Stehle · F. T. Bosman
Institut de Pathologie,
Centre Hospitalier Universitaire Vaudois,
Bugnon 25, 1011 Lausanne, Switzerland
e-mail: Pu.Yan@chuv.hospvd.ch
Fax: +41-21-3147115

Introduction

Telomerase is a large complex which consists of many components, among which two are essential for the enzyme activity in vivo and in vitro: hTERT protein with reverse transcriptase activity and the RNA template for elongating telomeres. Telomerase activity has been found in almost all human cancer tissues and cancer cell lines examined to date (Shay and Bacchetti 1997; Hiyama and Hiyama 2002). However the majority of somatic cells does not express the enzyme activity, except for stem cells and proliferative cells in renewable tissues (Broccoli et al. 1995; Effros and Globerson 2002). The detection of telomerase activity is customarily performed by TRAP (Telomeric Repeat Amplification Protocol) assay. This assay is relatively sensitive and specific, but it is based on detecting the enzyme activity in extracts of fresh cells or tissues. As a consequence, the question as to whether this activity originates from all tumor cells in a cancer or only from a subpopulation or whether in positive normal tissues this activity originates from stem cells or from proliferative cells or, alternatively, from activated lymphocytes, can not be answered. Expression of hTERT mRNA is very closely associated with telomerase activity in human tumors and can be detected by RT-PCR. However, this approach again does not offer any information at the level of the individual cell. Therefore in situ hybridization of hTERT mRNA and hTERC RNA and immunohisto-

chemistry (IHC) of hTERT protein have been exploited in order to localize telomerase expression in human tissues. Competitive RT-PCR data have indicated that most tumor cells contain 15,000–60,000 molecules of hTERC and only 1–30 copies of hTERT mRNA (Yi et al. 2001). The number of hTERC molecules allows to detect hTERC by in situ hybridization, but it would be extremely difficult for in situ detection of hTERT mRNA in human tissues without a PCR amplification step. Given the availability of a sufficient number of protein molecules, IHC might be used for the detection of hTERT protein at an individual cell level in fresh or processed tissue sections. IHC of hTERT has been attempted in a variety of tumors and nuclear staining of cancer cells was found in most of the studies. However, unexpected hTERT-positive staining in normal cells and in cytoplasm of tumor cells has been observed, depending on the antibodies used (Tahara et al. 1999; Hiyama et al. 2001; Wei and Younes 2002; Kyo et al. 2003). Accurate localization of hTERT protein in human tissues is needed to resolve these issues and to this end well-characterized antibodies and validated tissue processing and IHC reaction conditions are essential.

The aim of this study was to test the specificity of commercial anti-hTERT antibodies. With one particular monoclonal antibody (NCL-hTERT; Novocastra) reproducible and reliable immunostaining was obtained under very strict conditions. Immunostaining results were validated using quantitative RT-PCR (in microdissected tissue sections) and TRAP assay (in corresponding fresh tissue) as corroborative evidence. IHC was then performed on a variety of human tissue samples in order to define localization of hTERT protein in human tumor and normal tissues.

Materials and methods

Human tissues

Two groups of formalin-fixed and paraffin-embedded human tissues have been used for localization of hTERT protein. The first group consisted of various human tissues, including normal tissues (two samples of normal bladder, two of normal kidney, two of normal muscle, one of a placenta, and three of normal testis) and tumor tissues (three samples of bladder cancer, three of breast cancer, one of a kidney cancer, two of lung cancer, and two of soft tissue sarcoma). In this group of tissues, telomerase activity and hTERT mRNA expression had been detected in frozen samples by TRAP assay and RT-PCR in a previous study (Guilleret et al. 2002). hTERT protein expression was examined by IHC in formalin-fixed adjacent tissue samples. The second group consisted of 34 cases of colorectal adenocarcinoma, including 6 well-differentiated, 20 moderately differentiated, and 8 poorly differentiated carcinomas.

Antibodies

The three polyclonal antibodies against human hTERT protein (PC563, Oncogene, San Diego; ab177, abcam, Cambridge; Ab-2, Calbiochem, San Diego) and a monoclonal one (NCL-hTERT, IgG 2a, Novocastra, UK) were of commercial origin. Mouse monoclonal antibody M30 CytoDEATH was purchased from Roche (Mannheim, Germany).

Immunohistochemistry of hTERT

The immunohistochemical reaction with the commercial antibodies (PC563, Ab177, Ab2, and NCL-hTERT) was first tested on two telomerase-positive (one sample of a colorectal carcinoma and one of a normal testis) and two telomerase-negative tissue samples (one sample of a normal muscle and one of a normal kidney) according to the manufacturer's instructions and then under different antigen retrieval protocols (Table 1). A sensitive Dako EnVision (rabbit or mouse) kit was used as detection system. In extensive preliminary experiments no specific and reproducible immunostaining was obtained with PC563, Ab177, and Ab2. On the basis of these preliminary results the monoclonal antibody NCL-hTERT was selected for localization of hTERT using the sensitive Dako EnVision+System (mouse) detection system. Deparaffinized sections were treated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature (RT) to block endogenous peroxidase activity. Then the sections were incubated in preheated 0.01 M, pH 6.0 sodium citrate buffer for 40 min at 90°C and cooled immediately with cold running water for 10 min. After blocking with 10% normal goat serum for 1 h at RT, the slides were subsequently incubated overnight with NCL-hTERT diluted in Dako Antibody Diluent with Background Reducing Components (Dako, CA) containing 0.5 M NaCl at a dilution of 1:100. After extensive washing with PBS, the slides were incubated with Dako EnVision+System (mouse) for 30 min and developed with DAB solution (Dako). The sections were then counterstained with hematoxylin and mounted for microscopic examination. Negative controls included: (1) a blank control: omission of the primary antibody; (2) a subclass-matched monoclonal immunoglobulin control: substitution of anti-hTERT with a monoclonal antibody of identical isotype (anti-neurofilament 160 kDa; NCL-NF160, IgG2a; Novocastra); (3) negative tissue controls: seven normal (two bladder, two kidney, two muscle, and one placenta) and one tumor tissue (a sarcoma) in which telomerase activity and hTERT mRNA expression had been proven absent by TRAP assay and RT-PCR.

Table 1 Antigen retrieval

	Power or temperature	Buffer 1	Buffer 2	Time (min)
Microwave	750 W	Sodium citrate, 0.1 M, pH 6.0	EDTA, 1 mM, pH 7.5	5, 10, 15
Pressure cooker	115°C	Sodium citrate, 0.1 M, pH 6.0	EDTA, 1 mM, pH 7.5	1, 2, 5, 10
Autoclave	121°C	Sodium citrate, 0.1 M, pH 6.0	EDTA, 1 mM, pH 7.5	10
0.05% Pronase	Room temperature	–	–	2, 5, 10, 15
Proteinase K	Room temperature	–	–	2, 5, 10, 15
Incubator	90°C	Sodium citrate, 0.1 M	–	30, 40, 60

Double staining of hTERT and M30

The tissue slides were first stained with anti-hTERT (NCL-hTERT) as described above with a slight modification in which the incubation of anti-hTERT was carried out for only 2 h at RT. The slides were incubated with EnVision+System (mouse) for 30 min and developed with DAB solution. After incubating overnight with M30 diluted at 1:100 in PBS containing 1% BSA and 0.2% Tween 20, the immunoreaction was performed by the APPAP method. An APPAP detection kit (Dako) with fast red as substrate was used to visualize the localization of M30 according to the manufacturer's instructions.

DNA-free RNA preparation from paraffin-embedded tissues

Total RNA was extracted from fixed tissues as described previously (Guillou et al. 2001). Total RNA, 1–5 μg, was exposed to RNase-free DNase I (3.75 U; Pharmacia Biotech) at 37°C for 30 min in 40 μl of a solution containing 40 mM TRIS-HCL (pH 7.9), 6 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM NaCl, 10 mM DTT, and 4 U of RNasine (Promega, Madison, WI). The digestion was stopped by the addition of 5 μl of a stop solution (50 mM EDTA and 1.5 M sodium acetate). The DNA-free RNA solution was extracted with phenol–chloroform and precipitated in ethanol in the presence of 10 μg glycogen.

RT-PCR

DNA-free total RNA, 100 ng, was separately amplified for hTERT mRNA and β-actin mRNA in a total volume of 15 μl with the Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen, Life Technologies, Carlsbad, CA). The primers for hTERT mRNA and β-actin mRNA amplification were: (1) hTERT: hTERT-L5:5′-CGG AAG AGT GTC TGG AGC AA-3′; L6:5′-GGA TGA AGC GGA GTC TGG A-3′ and (2) β-actin: β-actin-A: 5′-AGG CCA ACC GCG AGA AGA TGA-3′; β-actin-B: GGC CGT GGT GGT GAA GCT GTA G-3′. Reverse transcription was done with pNd6 random primer under the conditions of 65°C 5 min, 55°C 25 min, and 95°C 5 min, and 40 cycles were then performed at 95°C 30 s, 62°C 45 s, and 72°C 45 s.

Quantitative RT-PCR

Three colorectal adenocarcinomas with cytoplasmic and significant heterogeneous nuclear expression of hTERT by IHC were chosen for quantitative RT-PCR analysis. According to the localization of hTERT protein detected by IHC, tissue regions with cytoplasmic and with nuclear staining were isolated by manual microdissection from sections consecutive to those used for hTERT immunostaining. Total RNA was then extracted after microdissection and quantitative RT-PCR was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Bio-systems, Foster City, CA). Human TATA-box binding protein (TBP) was used as an endogenous control. The primers and probes for hTERT mRNA and human TBP mRNA were purchased from Applied Biosystems. The probe for hTERT was labeled with 6FAM dye-MGB and human TBP with VIC dye-MGB. DNA-free RNA, 100 ng, was amplified for hTERT mRNA and human TBP mRNA in separate tubes with TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems). The PCR amplification was performed in a 384-well plate using the following cycling conditions: 30 min at 48°C; 10 min at 95°C, followed by 50 cycles for 15 s at 95°C and for 1 min at 60°C. The results were expressed as relative levels of hTERT mRNA referring to a calibrator which was arbitrarily chosen from the studied tissues because of its lowest target expression level. The amount of target, normalized to the endogenous reference (human TBP) and relative to the calibrator is determined by the $2^{-\Delta\Delta CT}$, where

$$\Delta\Delta CT=[C_T(hTERT_{unknown})-C_T(TBP_{unknown})]-[C_T(hTERT_{calibrator}) - C_T(TBP_{calibrator})],$$

as described in Sequence Detector User Bulletin 2 (Applied Biosystems).

Results

Reproducible immunohistochemical staining results were obtained with only one of four anti-hTERT antibodies (NCL-hTERT)

For optimizing the conditions of hTERT immunostaining, the four commercial anti-hTERT antibodies (PC563, Ab117, Ab2, and NCL-hTERT) were tested on four paraffin-embedded tissue samples with confirmed telomerase activity by TRAP assay (one sample of a colorectal adenocarcinoma, one of a normal testis, one of a normal muscle, and one of a normal kidney) under different conditions of antigen retrieval (Table 1). With PC563 antibody there was almost no staining in any tissue section, regardless of the TRAP assay results and after application of a range of antigen retrieval conditions. The Ab177 antibody produced exclusively cytoplasmic staining as well in telomerase-positive as in telomerase-negative tissues, which was interpreted as non-specific staining. With Ab2 antibody nuclear and cytoplasmic staining was found in both telomerase-positive and telomerase-negative tissues, but more strongly in the nuclei of telomerase-positive tumor cells. The non-specific staining in normal smooth muscle cells and epithelial cells of kidney was not significantly reduced by modification of the antigen retrieval method nor by varying the incubation conditions. In contrast, the monoclonal antibody NCL-hTERT labeled mainly the nucleoplasm and more intensely the nucleoli of tumor cells as well as the nucleoli of spermatogonia. However, under non-optimized conditions, non-specific cytoplasmic staining was observed in both tumor and normal cells, especially in normal smooth muscle cells. When antigen retrieval was performed as indicated in Materials and methods at 90°C for 40 min, nuclear staining was more intense and cytoplasmic staining was significantly reduced, but background staining of smooth muscle cells remained. To reduce this background, different concentrations of NaCl (from 0.1 to 1 M) were tested in the incubation buffer starting at a concentration of 0.4 M. This background disappeared entirely with increasing NaCl concentrations, but at a concentration over 0.7 M nuclear staining was also lost. We therefore chose a concentration of 0.5 M NaCl in the incubation buffer as a standard condition. Under these conditions there was no staining of the negative tissue control sections. Substitution of NCL-hTERT with an isotype-matched monoclonal antibody (NCL-NF160) showed no background staining.

Table 2 Expression of telomerase activity, hTERT mRNA, and hTERT protein in human tissues

Case	Samples	Frozen tissue		Formalin-fixed tissue
		Telomerase activity[a]	hTERT mRNA[b]	hTERT labeling (%)[c]
	Normal tissues			
1	Bladder	–	–	A few cells at basic layer
2	Bladder	–	–	A few cells at basic layer
3	Kidney	–	–	–
4	Kidney	–	–	–
5	Muscle	–	–	–
6	Muscle	–	–	–
7	Placenta	–	–	–
8	Testis	++	+	Spermatogonia
9	Testis	+++	+	Spermatogonia
10	Testis	++	+	Spermatogonia
	Tumor tissues			
11	Bladder	+	–	95
12	Bladder	+++	+	80
13	Bladder	+	+	80 (70–90)
14	Breast	+++	+	85
15	Breast	+++	+	80 (50–90)
16	Breast	++	+	80 (60–95)
17	Kidney	+	+	40 (10–50)
18	Lung	++	+	50 (50–80)
19	Lung	+++	+	50 (10–80)
20	Sarcoma	+	+	80 (50–90)
21	Sarcoma	–	–	–

[a] Telomerase activity by TRAP assay: +++ marked activity, ++ moderate activity, + low activity, – no activity
[b] hTERT mRNA by RT-PCR: + positive, – negative
[c] Numbers indicate percentage of positive cells: outside brackets, average positivity; inside brackets, regions with lowest positivity and regions with highest positivity. In case of homogeneous staining only one value is shown

hTERT protein is heterogeneously expressed in nucleoli of some normal and most cancer tissues

hTERT staining in various human tissues is summarized in Table 2. With the exception of the spermatogonia in testis, a few basal cells in bladder epithelium, and the basal part of colon crypts, all examined normal tissues were negative. hTERT nuclear staining was observed in tumor cells in all ten telomerase-positive tumors, but not in the telomerase-negative sarcoma. hTERT immunostaining in fixed tissues was therefore concordant with the telomerase activity and hTERT mRNA expression determined by TRAP assay and RT-PCR in corresponding fresh tissues (Table 2). hTERT immunostaining localized diffusely in the nucleoplasm and more strongly in the nucleolus (Fig. 1A–C). Diffuse cytoplasmic staining was found in all mitotic cells (Fig. 1D) and granular cytoplasmic staining occasionally in subpopulations of tumor cells (Fig. 1E). In telomerase-positive tumors, considerable heterogeneity of hTERT protein expression was found, as shown in Table 2. Lymphocytes were most prominently stained in germinal centers (Fig. 1F).

Thirty-four colon adenocarcinomas showed expression of hTERT protein. Of 34 cases, 27 (79.4%) showed significantly heterogeneous hTERT protein expression in the nuclei of colorectal cancer cells (Fig. 2A–C). In general, more than 50% of tumor cells expressed hTERT, but the percentage of positive cells varied in an individual case between less than 5% to 95%. Mitotic cells consistently showed diffuse staining. Granular cytoplasmic staining was occasionally found in 7 cases, confined to small tumor cell subpopulations (Fig. 2D). In remnants of adenomas adjacent to adenocarcinoma hTERT labeling was much weaker than in the carcinoma (Fig. 2E). However, there was no significant difference between carcinoma and adenoma in the percentage of labeled cells. Staining intensity tended to increase in high grade adenoma, but remained weaker than in tumor cells. In the adjacent normal colon crypts, hTERT expression was generally confined to the lower half of the crypts (Fig. 2F).

hTERT immunostaining concurs with hTERT mRNA expression

To verify the specificity of hTERT immunostaining in human tissues, hTERT mRNA expression was examined by RT-PCR in two hTERT immunopositive (a kidney cancer and a sarcoma) and two immunonegative tissues (a placenta and a sarcoma). The DNA-free RNA was extracted from serial sections following those used for hTERT immunostaining. RT-PCR detected hTERT mRNA in the hTERT immunopositive, but not in the immunonegative tissues.

In order to further confirm the specificity of hTERT immunostaining, quantitative RT-PCR was performed to

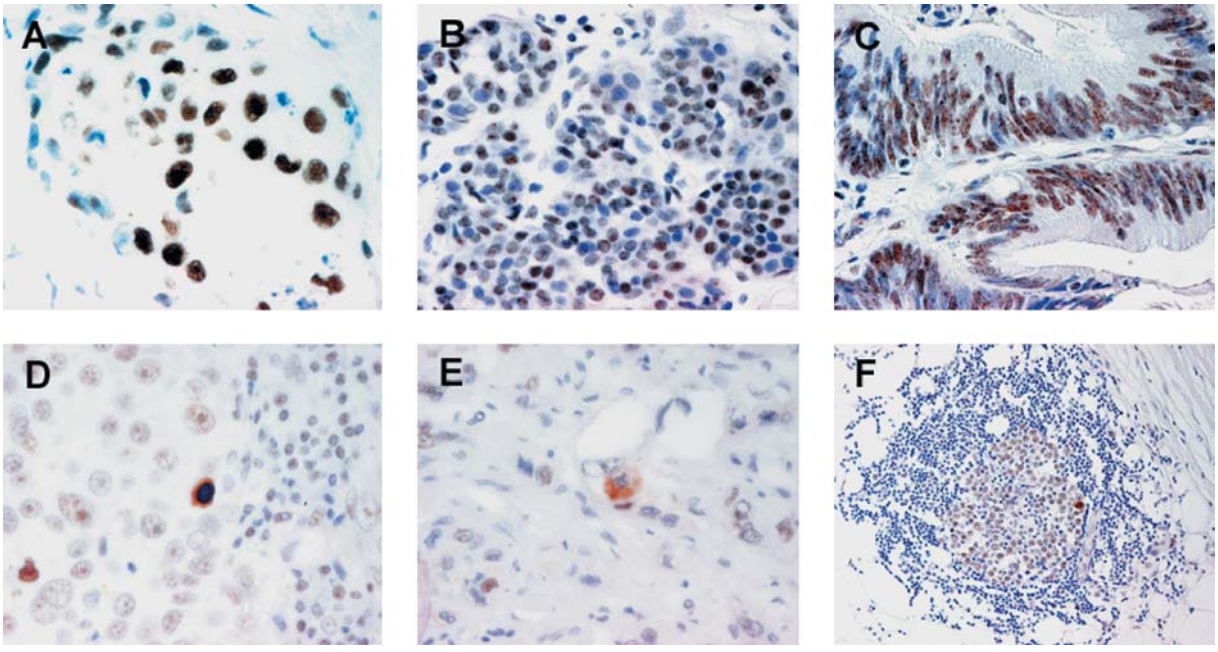

Fig. 1A–F The localization of hTERT protein in human tumor tissues. hTERT immunostaining localized diffusely in the nucleoplasm and more strongly in the nucleolus of tumor cells: bladder cancer (**A**), breast cancer (**B**), and lung cancer (**C**). Cytoplasmic staining was found in mitotic cells (**D**) and some population of tumor cells (**E**). Lymphocytes were most prominently stained in germinal centers (**F**)

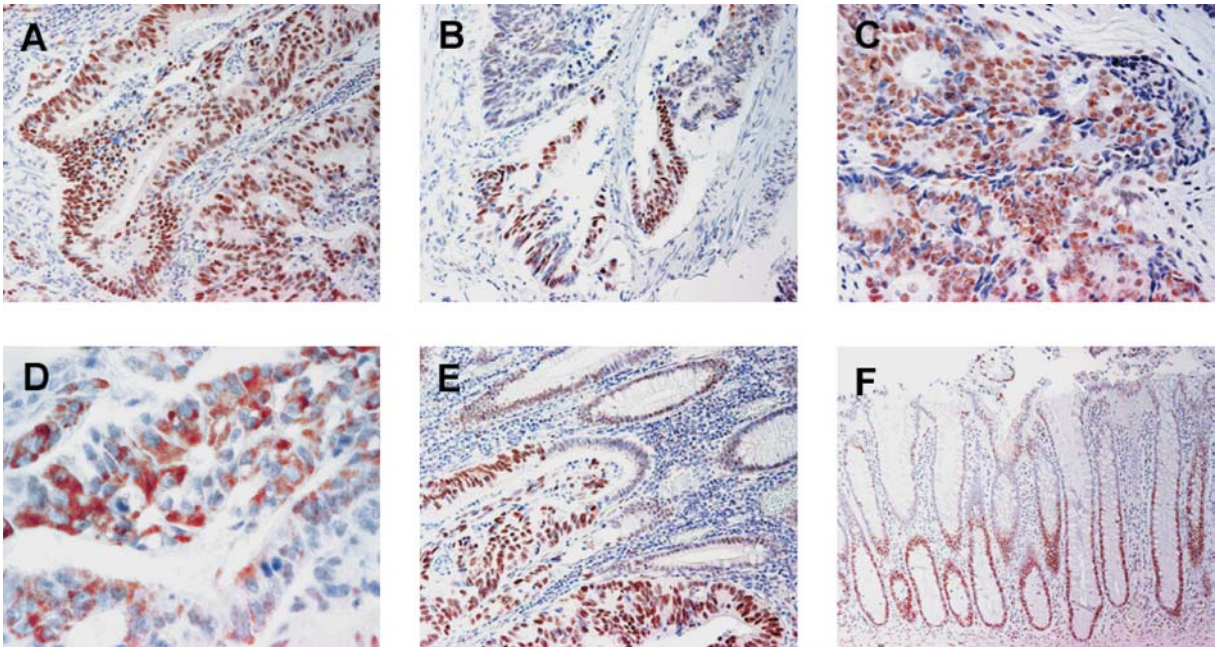

Fig. 2A–F Expression of hTERT protein in colorectal adenocarcinoma. Homogeneous and heterogeneous hTERT protein expression in the nuclei of colorectal cancer cells (**A–C**). Granular cytoplasmic staining was confined to small tumor cell subpopulations (**D**). In remnants of adenomas adjacent to adenocarcinoma hTERT labeling was much weaker than in the carcinoma (**E**). In the adjacent normal colon crypts, hTERT expression was generally confined to the lower half of the crypts (**F**)

detect hTERT mRNA expression level in three colorectal adenocarcinomas with significantly heterogeneous hTERT immunostaining. RNA was extracted by microdissection from two or three regions, selecting for: (1) strong nuclear staining; (2) weak nuclear staining; and (3) cytoplasmic staining. Quantitative RT-PCR revealed that the hTERT mRNA level was two to three times higher in regions with strong nuclear staining than in regions with weak nuclear staining or cytoplasmic staining.

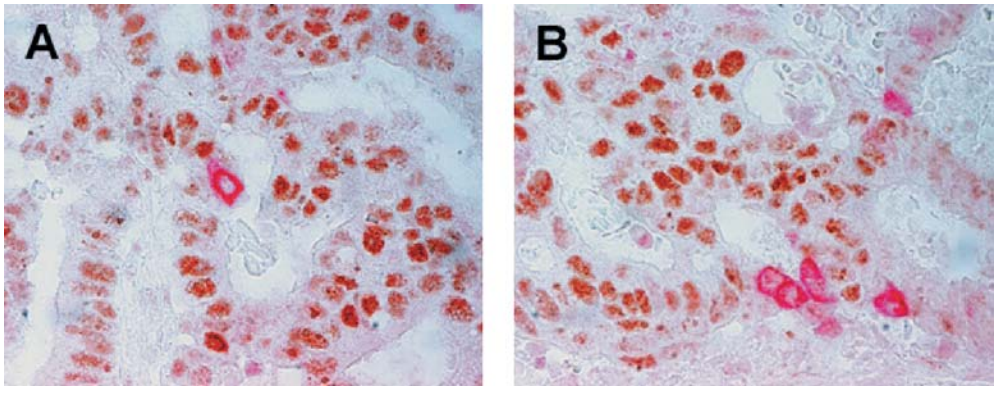

Fig. 3A, B hTERT-negative cells are partly apoptotic. Double immunostaining of hTERT and M30 showed that almost all M30-immunoreactive cells were hTERT negative and hTERT-positive cells were normally M30 negative (**A**, **B**)

hTERT-negative cells are partly apoptotic

To explore the mechanisms of hTERT protein negative tumor cells in telomerase-positive tumors, an anti-cleaved cytokeratin antibody (M30) was applied to detect apoptotic cells. M30 immunoreactivity localized in the cytoplasm of some tumor cells, scattered or clustered in distinct regions. To localize hTERT and M30 protein in the same tissue section, double immunostaining was performed. Almost all M30-immunoreactive cells were hTERT negative. hTERT-positive cells were characteristically M30 negative (Fig. 3A, B).

Discussion

For the detection of telomerase activity and the expression of the associated genes in tissues, TRAP assay and RT-PCR are customarily used. However, these methods do not provide any information about telomerase activation at an individual cell level. Regarding immunolocalization of hTERT, conflicting results were reported, at least partly depending on the antibodies used and the tissue processing conditions (Tahara et al. 1999; Dhaene et al. 2000; Hiyama et al. 2001; Yi et al. 2001; Wei and Younes 2002; Kyo et al. 2003). In the present study we compared four commercial anti-hTERT antibodies (PC563, ab177, Ab2, and NCL-hTERT) in telomerase-positive and telomerase-negative tissues by IHC. On the basis of the results of hTERT immunostaining, the NCL-hTERT monoclonal antibody was chosen for investigating the localization of hTERT protein in formalin-fixed human tissues. In order to optimize conditions for IHC of hTERT, the NCL-hTERT antibody was first tested in telomerase-positive and telomerase-negative tissue samples, following different antigen retrieval procedures. The best result was obtained after incubation of the slides in preheated sodium citrate buffer (0.01 M, pH 6.0) at 90°C for 40 min and with NCL-hTERT antibody diluted in Dako Antibody Diluent in the presence of 0.5 M NaCl.

The localization and expression of hTERT protein was then investigated on formalin-fixed human tissue samples using the NCL-hTERT monoclonal antibody. hTERT immunoreactivity predominantly localized in the nucleoplasm, more strongly in the nucleolus, of tumor cells in various tumors. The dominant nucleolar hTERT staining is in agreement with earlier observations (Wong et al. 2002). In this report strong nucleolar fluorescence was observed in GFP-hTERT transfected cells in G1 phase and a more diffuse intranuclear distribution in cells that had progressed to late S/G2. In mitotic cells we constantly found diffuse staining of the cell. Theoretically, nuclear localization of hTERT is required to promote elongation of telomere sequences. This corresponds to what Akiyama and Wong (Wong et al. 2002; Akiyama et al. 2003) observed, in that their GFP-hTERT signal became diffuse throughout the whole cell after breakdown of the nuclear envelope in mitosis. We found granular cytoplasmic staining in subpopulations of tumor cells, generally confined to small regions. We hypothesize that this might be due to alternative splicing of hTERT, with failure of translocation of a variant hTERT protein from the cytoplasm to the nucleus. Akiyama (Akiyama et al. 2003) observed translocation of hTERT protein from cytoplasm to nucleus and direct interaction with NF-κB p65 in multiple myeloma cells (MM.1 s) modulated by TNFα. NF-κB-specific inhibitors blocked TNFα-induced hTERT nuclear translocation. We hypothesize that cytoplasmic staining of tumor cells might be due to variant hTERT proteins which fail to contact with NF-κB p65. We are presently exploring this explanation by RT-PCR, focusing on the splice variants that have been reported earlier (Ulaner et al. 2001; Yi et al. 2001).

In normal tissues hTERT protein was only found in spermatogonia of testis, proliferative cells in colon crypts, and some basal cells in transitional epithelium, but not in telomerase-negative tissues such as kidney, muscle, or placenta. hTERT protein immunoreactivity was in agreement with telomerase activity detected by TRAP assay and hTERT mRNA expression detected by RT-PCR. These findings support the specificity of the immunoreactivity obtained with NCL-hTERT antibody and validate the conditions we established for reliable IHC of hTERT protein.

In tumor tissues usually more than 50% of the cells were hTERT protein positive, but this percentage varied between less than 5% and 95%. In the majority of tumors, hTERT protein expression was rather heterogeneous.

Quantitative RT-PCR showed that hTERT mRNA expression level was higher in regions with strong nuclear staining than regions with weak nuclear staining or cytoplasmic staining.

We hypothesize that at least part of the hTERT protein negative tumor cells do not cycle because they might have entered into apoptosis. The antibody M30 CytoDEATH, which stains a caspase-cleaved cytokeratin 18 epitope, was used to identify apoptotic cells in colorectal cancer tissue sections by double staining. M30-positive cells were characteristically hTERT negative, where hTERT-positive cells were rarely M30 positive. This observation confirms that part of the observed heterogeneity is due to hTERT-negative apoptotic cells. This, however, does not offer a full explanation for hTERT heterogeneous immunoreactivity. Other mechanisms might be involved.

In conclusion, hTERT protein can reliably be detected by IHC in fixed human tissues, but the choice of the antibody and tissue processing conditions are crucial. The sensitivity and specificity of hTERT immunoreactivity was confirmed by concurrent RT-PCR and TRAP assay. hTERT protein localized in the nuclei, notably nucleoli and occasionally in the cytoplasm of tumor cells. Telomerase-positive tumors showed significant heterogeneity of hTERT protein expression, part of hTERT protein negative tumor cells being in apoptosis. Additional mechanisms responsible for heterogeneous hTERT expression in telomerase-positive tumors need to be elucidated.

Acknowledgements The authors thank B. Jaspers for helpful discussions. This work was supported by a contract with Aventis Pharma Recherche-Developpement, France and by the Swiss National Science Foundation (grant number: 3100AO-101732).

References

Akiyama M, Hideshima T, Hayashi T, Tai YT, Mitsiades CS, MItsiades N, Chauhan D, Richardson P, Munshi NC, Anderson KC (2003) Nuclear factor-kappaB p65 mediates tumor necrosis factor alpha-induced nuclear translocation of telomerase reverse transcriptase protein. Cancer Res 63:18–21

Broccoli D, Young JW, de Lange T (1995) Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci U S A 92:9082–9086

Dhaene K, Wauters J, Weyn B, Timmermans JP, Van Marck E (2000) Expression profile of telomerase subunits in human pleural mesothelioma. J Pathol 190:80–85

Effros RB, Globerson A (2002) Hematopoietic cells and replicative senescence. Exp Gerontol 37:191–196

Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT, Benhattar J (2002) Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. Int J Cancer 101:335–341

Guillou L, Coindre J, Gallagher G, Terrier P, Gebhard S, Saint Aubain SN, Michels J, Jundt G, Vince DR, Collin F, Trassard M, Le DV, Benhattar J (2001) Detection of the synovial sarcoma translocation t(X;18) (SYT;SSX) in paraffin-embedded tissues using reverse transcriptase-polymerase chain reaction: a reliable and powerful diagnostic tool for pathologists. A molecular analysis of 221 mesenchymal tumors fixed in different fixatives. Hum Pathol 32:105–112

Hiyama E, Hiyama K (2002) Clinical utility of telomerase in cancer. Oncogene 21:643–649

Hiyama E, Hiyama K, Yokoyama T, Shay JW (2001) Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. Neoplasia 3:17–26

Kyo S, Masutomi K, Maida Y, Kanaya T, Yatabe N, Nakamura M, Tanaka M, Takarada M, Sugawara I, Murakami S, Taira T, Inoue M (2003) Significance of immunological detection of human telomerase reverse transcriptase: re-evaluation of expression and localization of human telomerase reverse transcriptase. Am J Pathol 163:859–867

Shay JW, Bacchetti S (1997) A survey of telomerase activity in human cancer. Eur J Cancer 33:787–791

Tahara H, Yasui W, Tahara E, Fujimoto J, Ito K, Tamai K, Nakayama J, Ishikawa F, Tahara E, Ide T (1999) Immunohistochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. Oncogene 18:1561–1567

Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR (2001) Tissue-specific alternate splicing of human telomerase reverse transcriptase (hTERT) influences telomere lengths during human development. Int J Cancer 91:644–649

Wei R, Younes M (2002) Immunohistochemical detection of telomerase reverse transcriptase in colorectal adenocarcinoma and benign colonic mucosa. Hum Pathol 33:693–696

Wong JM, Kusdra L, Collins K (2002) Subnuclear shuttling of human telomerase induced by transformation and DNA damage. Nat Cell Biol 4:731–736

Yi X, Shay JW, Wright WE (2001) Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. Nucleic Acids Res 29:4818–4825