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Usefulness of immunological detection of the human telomerase reverse transcriptase [1]

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Abstract. *Objective*: Recent years have seen a considerable wealth of studies conducted on the potential usefulness of telomerase determination in diagnosis, prognosis and targeted cancer therapy. The frequently used Telomeric Repeat Amplification Protocol assay suffers from some drawbacks, the most important being the rate of false positives. *In situ* analysis using well characterised antibodies directed against the human telomerase reverse transcriptase (hTERT) would therefore appear to be important to morphologically identify the nature of telomerase positive cells. *Methods*: We performed immunostaining in a series of cultured cells and in normal, preneoplastic and tumour tissues from different organs using a monoclonal antibody directed against the catalytic subunit of telomerase. *Results*: Immunoreactivity was not observed in perennial cells of terminally differentiated cardiac and skeletal muscular tissues or in small pyramidal cells of the cerebral cortex. Conversely, it was found in other normal somatic tissues as well as in precancerous lesions and in all tumour histotypes. *Conclusions*: Immunohistochemistry with a well characterised hTERT-specific monoclonal antibody permitted the identification of hTERT immunopositive cells in normal somatic tissues. Whether hTERT protein detected by immunostaining with hTERT-specific Tel 3 36-10 antibody is actually the degraded form of the protein that retains hTERT antigenicity but not enzymatic function, or whether it represents the real, potentially functional catalytic subunit of the enzyme, immunohistochemistry would not seem to represent a useful tool to investigate the role of telomerase and the mechanisms involved in its regulation.

Keywords: hTERT immunohistochemical expression, normal and tumour tissue, telomerase

1. Introduction

One mechanism leading to the natural death of a cell is the erosion of structures that cap the ends of chromosomes, known as telomeres, which shorten with each round of DNA replication. At the end of the cell lifespan this causes chromosomes to become "sticky" and unstable and the cells, no longer capable of dividing, undergo apoptosis. However, in cells such as stem cells or tissue regeneration-committed cells in which this mechanism would be harmful, nature has provided a reparative tool to overcome telomere erosion, the telomerase enzyme, which contains the template for telomere reconstitution. It is thought that most somatic cells switch off telomerase activity [6,21], but it has been shown that the enzyme is reactivated in cancer cells [14]. This reactivation has prompted a considerable wealth of studies aimed at evaluating the potential usefulness of telomerase determination in diagnosis, prognosis and targeted cancer therapy.

In some instances, telomerase activity appears to be enhanced not only in invasive tumour cells but also at the pre-malignant stage or in *in situ* tumours [23], thus potentially enabling the early detection of cells undergoing neoplastic transformation.

The most frequently used method for detecting telomerase is the Telomeric Repeat Amplification Protocol (TRAP) assay, which has proven to have high

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sensitivity and permits quantitative evaluations to be made. TRAP assay, however, suffers from some drawbacks, the most important being the rate of false positive results when the analysis includes contaminating telomerase-positive nontumour cells [7,9,31].

Consequently, an *in situ* analysis would seem to be important to morphologically identify the nature of telomerase positive cells. As surrogate markers of telomerase activity at cellular level in paraffin-embedded tissues, the evaluation by *in situ* hybridisation of the intrinsic RNA component (hTR) of the enzyme and the immunohistochemical determination of the expression of its catalytic subunit, human telomerase reverse transcriptase (hTERT), have both been applied.

hTR evaluation by *in situ* hybridization makes quantitative analysis difficult and cannot be carried out in all pathology laboratories. Conversely, the evaluation of the expression of hTERT protein by immunohistochemical staining using well characterised antibodies [24] has opened up the possibility for studying large series of normal and neoplastic tissues. In fact, the relatively recent cloning of hTERT has permitted its immunohistochemical evaluation in different tissues, bringing to light some unexpected findings such as the expression of the protein in normal somatic cells [8,19, 25] or in telomerase RNA-negative cancer cells, and not only in the nucleus, but also in cell cytoplasm [16].

In the present paper, we report our experience of immunostaining in a series of cultured cells and in normal, pre-malignant and tumour tissue samples from different organs using a recently characterised monoclonal antibody directed against the catalytic subunit of telomerase.

2. Materials and methods

Different established cell lines and fresh human cells obtained specifically for the present study, together with several human tissues were tested with a rat anti hTERT monoclonal antibody (Tel 3 36-10, DIESSE Diagnostica Senese, Siena, Italy, commercialised by Alexis Corporation, Lausanne, Switzerland). Telomerase expression was determined using the above-mentioned monoclonal antibody at a final concentration of 40 μg/ml diluted in background reducing components (Dako Corporation, Carpinteria, CA) for 1 h at room temperature.

2.1. Cultured cells

All the established cell lines came from American Type Culture Collection (ATCC-LGC Promochem, Middlesex, UK) with the exception of the LRWZ colon cancer and MCR 110 bladder cancer cell lines, which was isolated and characterised in our laboratory [20]. Fibroblasts were obtained from primary cultures of human skin, and lymphocytes derived from metastatic lymph nodes of a melanoma patient were isolated and expanded in IL-2-containing medium.

All cells, apart from tumour infiltrating lymphocytes (TIL), were cultured and propagated in our laboratory with MEM/F12 medium (1 : 1) supplemented with 2 mM glutamine and 10% foetal bovine serum (FBS).

Cells were used in the exponential growth phase for the preparation of histological blocks. Briefly, cells were centrifuged at 1200 rpm for 10 min. The medium was removed and the cell pellet was suspended in human plasma. After the addition of thromboplastin (bioMERIEUX, Marcy l'Etoile, France), test tubes were agitated at 37°C until an agglomerate was obtained. Following fixation in 10% neutral buffered formalin for 6 h, samples were paraffin-embedded and the blocks obtained handled as routine tissue blocks.

2.2. Tissues

Freshly excised human tissues from different organs were fixed in 10% neutral buffered formalin for 6–24 h and embedded in paraffin. Whilst most of the specimens were clearly neoplastic, noncancerous biopsy samples of the skin, breast, uterine cervix, bladder, pancreas, prostate and skeletal muscle were also examined. Normal cerebral cortex, cardiac and skeletal muscle tissue was collected during autopsy.

2.3. Immunohistochemistry

The anti hTERT monoclonal antibody Tel 3 36-10 was obtained by rat immunisation with a fragment of cDNA directed against the hTERT polypeptide sequence of the telomerase enzyme. The clone was purified by chromatography on Sepharose-Protein G [24].

Four-micrometer thick paraffin-embedded sections were mounted on positive-charged slides (Bio Optica, Milan, Italy) deparaffinised in xylene, rehydrated twice in 100% alcohol for 2 min, 90% alcohol for 1 min, and finally rinsed in 70% alcohol for 1 min. Endogenous peroxidase was quenched in 3% H_2O_2 for 10 min. Antigen retrieval was carried out by incubating the sec-

tions in citrate buffer (pH 6.0) at 98.5°C for 45 min. After washing for 5 min in PBS (Phosphate Buffer Saline), aspecific sites were blocked with 1% BSA (Bovine Serum Albumin) in PBS for 20 min.

The primary antibody was diluted with background reducing components (Dako Corporation) and placed in contact with the sections for 1 h at room temperature. The samples were washed twice in PBS–Tween (0.05%), incubated for 30 min with anti-rat biotinylated secondary antibody (Dako Corporation) diluted 1 : 300, washed again in PBS–Tween (0.05%), incubated in streptavidin–peroxidase conjugate (LSAB + kit Dako Corporation) for 15 min, and washed twice in PBS–Tween for 5 min. The final enzymatic reaction was developed to a brown stain with diaminobenzidine/hydrogen peroxidase chromogen solution (DAB + liquid substrate-chromogen solution, Dako Corporation) for 5 min.

Sections were rinsed in deionised water, cell nuclei were counterstained blue by haematoxylin and the slides were mounted in Eukitt (Bio Optica). Immunostaining localisation within cells (nuclear, cytoplasmatic or both), types of immunoreactive cell elements in the different tissues, and staining intensity, when assessable, were recorded.

Table 1
Telomerase immunoreactivity in cultured cells

Cultured cells		Immunoreactivity signal
		Tel 3 36-10
UV irradiated human fibroblast		N,C*
Tumour infiltrating lymphocytes		N
Breast cancer	MCF7	N
	SKBR3	N
	BRC 230	N
Colon cancer	WIDR	N,C
	LRWZ	N
Bladder cancer	MCR 110	N
Lymphatic malignancy	Molt-4	N
	HL60	N
	Daudi	N
	Karpas 422	N
	K562	N
	Jurkat	C
Uterine cervical cancer	Hela	N
Kidney	Cos	N
	Cos T	N,C**

N, nucleus; C, cytoplasm.
* ⩽10% immunopositive cells.
**Strong positivity in all of the cells.

3. Results

All the cultured cells analysed (Table 1) showed a nuclear immunopositivity, with the exception of WIDR cell line and UV irradiated fibroblasts, which showed both a nuclear and cytoplasmatic immunoreactivity, and acute leukaemia Jurkat T cells, which presented only cytoplasmatic immunoreactivity.

Higher staining intensity was observed in monkey kidney Cos cell lines transfected with hTERT-containing plasmid (Cos T) than in nontransfected Cos cells. Moreover, the localisation was both nuclear and cytoplasmatic for the former and exclusively nuclear for the latter. A total lack of immunoreactivity was observed in skeletal and cardiac normal muscle tissue (Table 2, Fig. 1A) and in small pyramidal cells of normal cerebral cortex samples, whereas large pyramidal cells showed both nuclear and cytoplasmatic staining (Fig. 1B).

In all of the other organs, immunoreactivity was always observed in the nucleus and frequently in the cytoplasm, regardless of their histology (normal, dysplastic or neoplastic), and there were no differences in terms of staining intensity between normal and pathological tissues. Inflammatory endothelial cells and adipocytes were always immunostained.

Skin tissue displayed immunoreactivity to the hTERT antibody in regenerative basal layer cells and in several cells throughout the whole thickness of the epithelium up to the more superficial layers (Fig. 1C). Normal bladder epithelium also showed diffusely immunostained cells (Fig. 1D).

Similarly, hTERT immunoreactivity was expressed in the epithelial basal layers of both normal cervical tissue and dysplastic lesions, and immunostained dysplastic cells were observed throughout the entire thickness of intraepithelial lesions.

Normal pancreatic and prostatic tissue cells presented diffuse immunoreactivity. Mastopathic, hyperplastic and metaplastic areas in breast specimens, as well as fibroadenoma biopsies, showed a large proportion of immunostained cells. Neoplastic tissue generally exhibited a very high proportion of immunoreactive cancer cells (Fig. 1E–F).

Table 2
Telomerase immunoreactivity in human tissue

Organ	Histology	Immunoreactivity signal
		Tel 3 36-10
Skeletal muscle tissue		–
Cardiac muscle tissue		–
Cerebral cortex		–*
		N,C**
Skin		N,C
Prostate	Normal	N,C
Breast	Hyperplasia	N,C
	Metaplasia	N,C
	Mastopathy	N,C
	Fibroadenoma	N,C
	Carcinoma *in situ*	N,C
	Infiltrating carcinoma	N,C
Uterine cervix	Normal	N,C
	Dysplastic	N,C
	Neoplastic	N,C
Bladder	Normal	N,C
	Neoplastic	N,C
Kidney	Neoplastic	N,C
Pancreas	Normal	N,C
	Neoplastic	N,C
Colon	Neoplastic	N,C

N, nucleus; C, cytoplasm.
*Small pyramidal cells.
**Large pyramidal cells.

4. Discussion

Few studies have focused on the *in situ* immunohistochemical detection of telomerase catalytic reverse transcriptase subunit, hTERT, in normal and cancer cells. In the present study, a series of cell cultures and tissues with different proliferative status were immunostained with a rat monoclonal antibody raised against hTERT [24]. Immunoreactivity was not observed in perennial cells of terminally differentiated cardiac and skeletal muscle tissues or in small pyramidal cells of the cerebral cortex. Conversely, it was seen in other normal somatic tissues such as skin, mammary gland, uterine cervix, bladder, pancreas and prostate, as well as in a generally very high percentage of cells in precancerous lesions and in all tumour histotypes. The results obtained in normal tissues were confirmed by the superimposible immunostaining results obtained on the same specimens with the commercially available Novocastra monoclonal antibody (data not shown). The detection of hTERT expression in normal tissue other than regenerative epithelial compartments is not new, and has also been reported by other authors [12,16,25].

The detection of immunopositive large pyramidal cells in the cerebral cortex and of endothelial cells in cardiac muscle confirmed the reliability of the autopsy tissue, also used by other authors [8].

Some studies would seem to indicate that hTERT mRNA expression is strongly associated with telomerase activity and represents the rate-limiting step in enzymatic activity [3,5,15,26]. However, it has recently been shown that some tissues may result positive for hTERT mRNA but negative for telomerase activity [28] or vice versa [10,22,25]. Moreover, it was recently reported that hTERT protein may be present in lymphocytes independently of their telomerase activity, thus allowing for the hypothesis that the presence of hTERT protein is not sufficient to determine telomerase activity [17].

All these observations, together with the results from the present study, which highlighted a very frequent immunoreactivity for telomerase catalytic subunit in cultured cells and in normal, preneoplastic and neoplastic tissues, open the way for speculative considerations. Our findings are somewhat surprising as it would be logical to expect the expression of the telomerase catalytic subunit to be found only in cells in which enzymatic function is necessary or reactivated.

In normal, preneoplastic or cancerous tissues, hTERT immunoreactivity was detectable in both the nucleus and cytoplasm of cells. The detection of hTERT in subcellular compartments other than nuclei, already reported [2,4,10,16,30], has been tentatively attributed to the disruption of the normal hTERT nuclear translocation process during malignant transformation. However, our observations on normal tissues do not permit the same conclusions to be drawn.

Different hypotheses can be advanced to explain the presence of hTERT protein in cells without telomerase reactivation, the most plausible concerning the existence of post-transcriptional/post-translational modes of telomerase regulation consisting in qualitative modifications, such as reversible hTERT protein phosphorylation [2,17]. This regulates the enzyme structure and configuration, localisation/translocation and activity. However, other post-translational mechanisms may

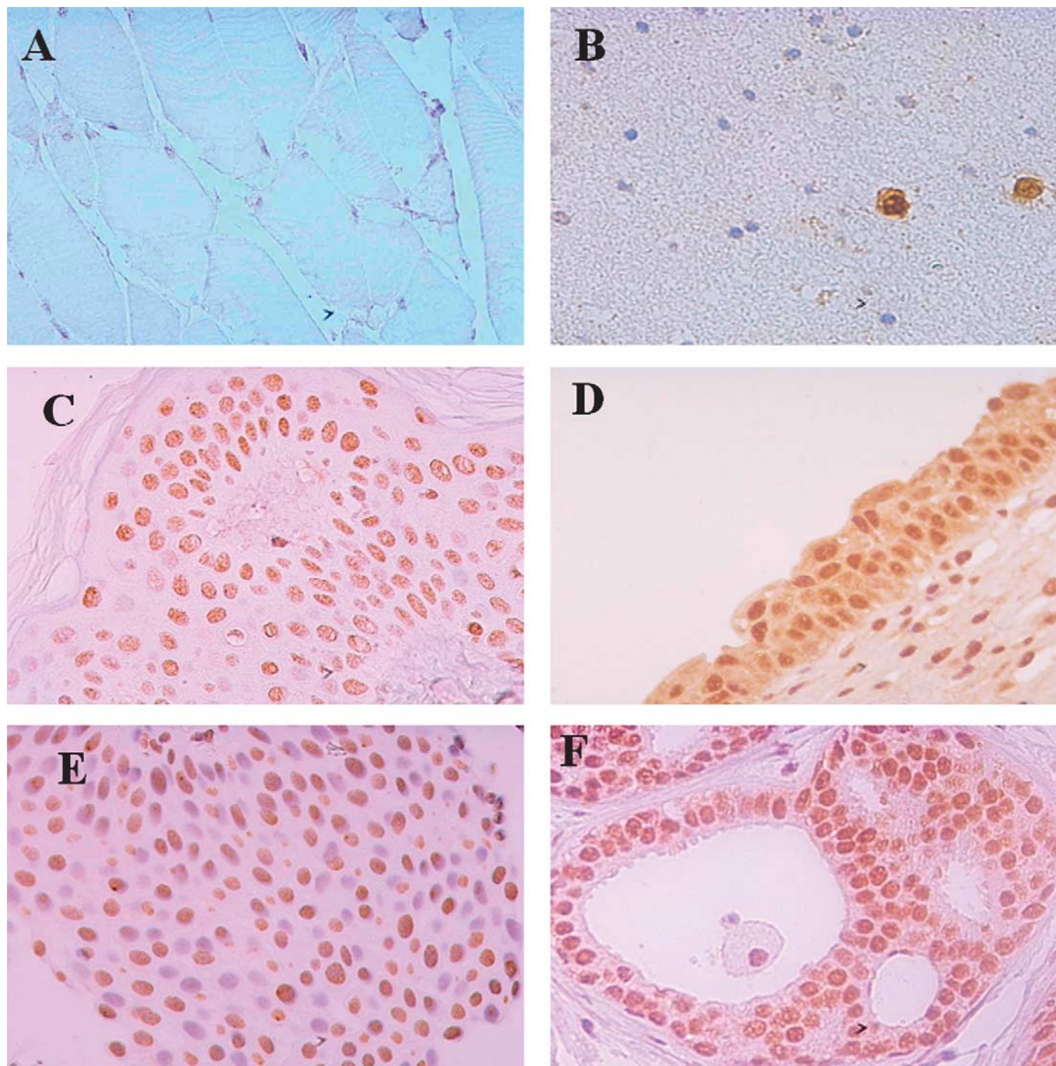

Fig. 1. hTERT immunostaining in perennial cells of terminally differentiated somatic human tissues (magnification 400×): (A) skeletal muscle; (B) cerebral cortex, in which it can be seen that small pyramidal cells do not react with the antibody, whereas large pyramidal cells exhibit immunoreactivity. hTERT immunostaining in somatic human tissues (magnification 400×): (C) normal skin; (D) normal bladder epithelium. Almost all of the cells show nuclear and cytoplasmatic immunoreactivity. hTERT immunostaining in neoplastic tissues (magnification 400×): (E) bladder cancer; (F) *in situ* breast carcinoma.

account for the presence of hTERT when no telomerase activity is detectable, such as the influence of inhibitory factors that can block hTERT protein functions or the existence of inactive splice variants [13, 18,27]. Moreover, various purification protocols have revealed that the telomerase enzyme exists as a large complex which acts as a dimer or multimer [1,29], rendering necessary the correct assembly of the different components for catalytic activity [11].

Overall, these aspects of telomerase regulation would seem to support new evidence that the presence of the enzyme catalytic subunit is not necessarily associated with telomerase activity and is therefore not a surrogate marker for telomerase reactivation.

Whether hTERT protein detected by immunostaining is actually the degraded form of the protein that retains hTERT antigenicity but not enzymatic function, or whether it represents the real, potentially functional catalytic subunit of the enzyme whose ectopic expression can immortalise different normal human cell lines and is required for the long-term proliferation of cancer cells, immortalisation and oncogenesis immunohistochemistry would not seem to represent a useful tool to

investigate the role of telomerase and the mechanisms involved in its regulation.

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