Histol Histopathol (2006) 21: 1249-1261

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

## Review

# Telomeres and telomerase. A survey about methods and recent advances in cancer diagnostic and therapy

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**Summary.** Since the discovery that telomerase is repressed in most normal human somatic cells but strongly expressed in most human tumours, telomerase emerged as an attractive target for diagnostic, prognostic and therapeutic purposes to combat human cancer. In this review, a synopsis of methods detecting telomerase is presented evaluating their potential for diagnostic and prognostic use. Also, the most promising telomerase therapeutics are discussed in the light of recent advances in the field.

**Key words:** Telomeres, Telomerase, Tumorigenesis, Telomerase inhibitors

## Introduction

### *Telomeres and cellular senescence*

Telomeres are complex structures composed of the telomeric DNA and telomere binding proteins and may form a loop structure to seal chromosome ends. Telomeric DNA consists of a tandem array of GT-rich repeats (e.g. TTGGGG in Tetrahymena and TTAGGG in humans and other vertebrates). The number of the repeats and consequently the length of telomeric DNA varies among species ranging from 36 nucleotides present at the ends of macronuclear chromosomes of ciliated protozoans (Klobutcher et al., 1981), ~300 bp in *S. cerevisiae* (Zakian, 1989) to ~150000 bp in mice (Kipling and Cooke, 1990). In human somatic cells telomeres consist of 7000-10000 bp telomeric DNA and of about 20000 bp in germ cells (Moyzis et al., 1988; Allshire et al., 1989). During replication, DNA polymerase cannot completely replicate the ends of chromosomes, due to the incompetence of the conventional DNA-dependent DNA polymerases to initiate DNA synthesis. In the absence of a specific telomere replication mechanism telomeres shorten with each replication cycle due to the end-replication problem (Watson, 1972). Olovnikov (Olovnikov, 1971, 1973) hypothesized that telomere shortening owing to the end-replication problem could explain early observations by Hayflick and Moorhead that primary fibroblasts possess a finite number of cell divisions and eventually enter a state which they called ‘senescence at the cellular level’ due to ‘intrinsic factors’ (Hayflick and Moorhead, 1961). Olovnikov’s hypothesis was proven in the early 90’s by showing that the amount and length of telomeric DNA in human fibroblasts do in fact decrease as a function of serial passage of cells *in vitro* (Harley et al., 1990) and during ageing in individuals *in vivo* (Lindsey et al., 1991; Allsopp et al., 1992). On the other hand, telomere length from sperm DNA did not decrease but increased as a function of donor age (Allsopp et al., 1992). The authors suggested that a telomere maintaining mechanism may exist in germ-line tissue and this may be the expression of telomerase, an enzymatic activity responsible for complete telomere-DNA replication.

### *Telomerase, cellular senescence and cancer*

Telomerase, a ribonucleo-protein complex with reverse transcriptase activity, was first described in the ciliated protozoan *tetrahymena* by Greider and Blackburn who demonstrated that telomerase enzymatic activity is capable of catalyzing the synthesis of telomeric DNA *de novo* (Greider and Blackburn, 1987). Subsequently, active telomerase complex was purified from HeLa cells, a human tumour cell line, indicating evolutionary conservation of this enzyme activity (Morin, 1989). Importantly, telomerase activity could be detected in a variety of tumour cell lines and transformed cells in culture but not in normal fibroblasts or embryonic kidney cells, indicative of a causal link between telomerase activity and immortality (Counter et al., 1992).

First experimental evidence for a role of telomerase and telomere length maintenance came from the studies of Counter et al. (1992) who showed that human cultured embryonic kidney cells expressing SV40 Large-

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T Ag and adenovirus 5 oncogenes exhibited extended life-span. Interestingly, telomeres continued to shorten until a 'crisis' point where most cells died. They observed rare clones that survived crisis and could show that these clones possessed telomerase activity. Based on these observations and their results that telomere length shortens as a function of age in primary fibroblasts but not in germ cells, Allsopp et al. proposed a model for 'cell ageing and immortalization' (Allsopp et al., 1992). Since their proposal, the telomere hypothesis of 'cell ageing and immortalization' has been modified. The identification of telomerase components and the information on their regulation have led to the following model (Fig. 1).

In the following years, telomerase activity has been observed in the vast majority of human tumour cell lines and human tumours of different origin (Kim et al., 1994). These observations together with the findings that telomerase activity can be detected in early human development but is down-regulated in most adult tissues (Wright et al., 1996; Ulaner and Giudice, 1997) have led to the hypothesis that the down-regulation of telomerase activity in somatic cells may be a tumour-protective mechanism (Counter, 1996). According to this point of view, during early development, telomerase activity is required for massive cell proliferation but continuous expression of telomerase would be a bad risk to develop cancer. The demonstration that telomerase activity is required for tumourigenic conversion of human cells provided experimental evidence for this hypothesis (Hahn et al., 1999). Thus, down-regulation of telomerase would reduce the risk for tumour formation in adult cells. Although telomerase activity is undetectable in most human tissues some cell types maintain weak but detectable telomerase activity or telomerase activity may be induced upon stimulation. These include fetal tissue, normal bone marrow stem cells, testes, peripheral blood lymphocytes, skin epidermis and intestinal crypt cells (Table 1). All of these cells have high turnover rates or are in a continuously replicating pool of differentiating cells. It is important to note that the level of telomerase activity found in these normal cell populations is significantly less per cell than that found in cancer cell populations.

Despite considerable variation in telomere length and telomere-sequence, telomerase activity is the major telomere maintenance mechanism among the eukaryotes, with only few exceptions (e.g. *D. melanogaster*). It should be noted that no telomerase activity could be detected in some human tumours. Tumours that lack telomerase activity maintain their telomere length via a recombination based mechanism (ALT for Alternative Lengthening of Telomeres) (Bryan et al., 1997). Understanding the mechanism underlying ALT is an area of great interest and molecular details are emerging piece by piece, as reviewed recently (Muntoni and

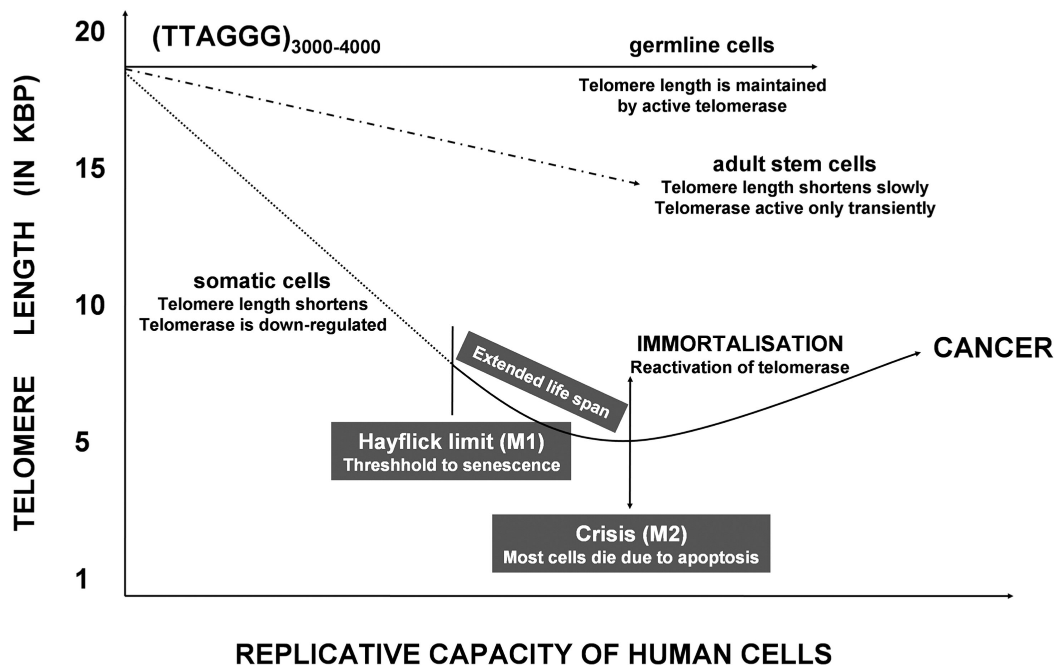


**Fig. 1.** Telomere hypothesis of senescence and cancer. The length of human telomeres is about 15-20 kbp at birth. Length of telomeres shortens during differentiation due to down-regulation of telomerase activity, except in germline cells. As a result, proliferating cells lose about 50-200 bp of telomeric DNA with each replication. This telomere shortening sets a molecular clock for the proliferation capacity of normal cells. In culture, telomerase negative normal human cells enter a telomere length dependent growth arrest, defined as the replicative senescence (Hayflick Limit, also called mortality stage 1: M1). This mechanism is thought to function as an additional barrier against an accumulation of DNA damage and malignant transformation of cells. Loss of tumour suppressor mechanisms (e.g. mutations in p53 or pRb) may overcome this barrier and result in extended life span. In the absence of telomerase however, shortening of telomeres initiates detrimental chromosomal rearrangements in such proliferating cells which end up in crisis (mortality stage 2: M2) characterized by apoptotic cell death. Activation of telomerase is one the of key events to overcome crisis in vitro and is thought to occur during tumourigenesis in vivo to stabilize telomere length. (Modified from Allsopp et al., 1992).

Reddel, 2005). Thus, telomerase or another mechanism for telomere maintenance is required for continuous tumour cell proliferation.

*Telomerase components*

The human telomerase enzyme is composed of two essential components, the RNA component (hTERC: human Telomerase RNA component) which acts as a template for reverse transcription (Blasco et al., 1995); and the catalytic subunit hTERT (human Telomerase reverse transcriptase) with the reverse transcriptase activity (Meyerson et al., 1997; Nakamura et al., 1997) (Fig. 2). Using an *in vitro* transcription/translation system, both the RNA component (TERC) and the reverse transcriptase protein subunit (TERT) can be expressed *in vitro* and telomerase activity can be reconstituted with properties similar to those of native telomerase (Weinrich et al., 1997; Beattie et al., 1998). Transfection of telomerase-negative cells with a hTERT cDNA has demonstrated that hTERT expression is rate-limiting for telomerase activity (Weinrich et al., 1997; Nakayama et al., 1998). These experiments also provided causal evidence for the telomere based replicative senescence, since ectopic expression of hTERT in primary human cells prevented telomere erosion and immortalized human cells (Bodnar et al., 1998; Morales et al., 1999).

In human somatic cells and tumour cell lines the RNA component hTERC is constitutively expressed independently of telomerase activity (Avilion et al., 1996). In contrast, the expression of the human catalytic subunit hTERT correlates very well with telomerase activity: *hTERT* gene expression is generally repressed in normal human cells and up-regulated in tumour cells (Meyerson et al., 1997; Kolquist et al., 1998; Nakayama et al., 1998; Ritz et al., 2005).

## Detecting telomerase activity

*Detecting enzymatic activity of telomerase: Telomeric Repeat Amplification Protocol (TRAP Assay)*

The demonstration that telomerase is absent in somatic cells but readily detectable in tumour cell lines has opened up the potential for telomerase to be used in cancer diagnosis, prognosis and therapy.

Originally, determination of telomerase activity relied on a primer extension assay where elongation of a telomere template primer by telomerase was measured by incorporation of radioactive nucleotides. The elongation products were resolved on a denaturing PAA-Gel for subsequent autoradiography. Although this method was convenient for experimental use (Greider

**Table 1.** Telomerase activity in normal human tissues/cells.

| ADULT HUMAN TISSUES / CELLS | SELECTED REFERENCES |
|---|---|
| Germline cells | high telomerase activity in testis; (Wright et al., 1996) |
| Hematopoietic cells | telomerase activity in bone marrow and peripheral blood leukocytes; (Broccoli et al., 1995; Counter et al., 1995) |
| Gastrointestinal tract epithelium | telomerase activity in intestinal mucosa with putative stem cells in the crypts; (Hiyama et al., 2001) |
| Fibroblasts | telomerase activity in S phase; (Masutomi et al., 2003) |
| Germinal centre cells | (Norrback et al., 1996) |
| Hair follicle bulbs | (Ramirez et al., 1997) |
| Endometrium | telomerase activity in the late proliferative phase; (Kyo et al., 1997) |
| Basal cells of skin | telomerase activity in regenerative basal layer of the epidermis; (Harle-Bachor and Boukamp, 1996) |

**Fig. 2:** Telomerase activity is necessary for complete replication of chromosomal ends. Essential components and mechanism of telomerase activity. The RNA component of telomerase (TERC) recognizes telomeric-DNA and serves as an inherent primer (underlined italic) for the catalytic subunit of telomerase, namely the TERT component.
Telomerase acts on the lagging strand (upper) and elongates telomeric DNA by the repeated addition of the GGTTAG hexa-nucleotide. The synthesis of the complementary leading strand is accomplished by the normal DNA replication machinery. The number of newly added repeats (underlined black) depends on many factors such as amount of functional telomerase complex. Several other factors (e.g. Tankyrases, TRF1, Pot1) influence the activity of telomerase *in vivo* (not shown here).

and Blackburn, 1985, 1987; Morin, 1989) it was not easily applicable for most of the clinical samples. Not only because it was a laborious method, it also required large amounts of telomerase positive cells and provided weak signal intensity. The development of a telomeric repeat amplification protocol (TRAP Assay) (Kim et al., 1994) led to a rapid analysis of a large number of samples in a relatively simple assay system which helped to further investigate the role of telomerase activity in normal and malignant human tissues.

Variations of the original TRAP assay exist by now which all are based on the elongation of a template sequence by a telomerase positive cell extract in a first step. Subsequently, the elongation products are amplified by PCR to increase the signal intensity. The TRAP-products are separated by a non-denaturing PAA-Gel (Fig. 3). Usually the assay is done with radioactive end-labelling of the telomere template oligonucleotide (in the first step) or by the incorporation of radioactively labelled dCTP during the PCR reaction (second step), but also visualization of the reaction products in a non-radioactive manner is possible by post-PCR staining with ethidium bromide or SYBR Green/SYBR Gold. The use of flourescently labelled primers has been described as an alternative detection method. We have the experience that the radioactive detection method gives the most reproducible and reliable results. The use of SYBR Gold is a true alternative but does not reach the salient results obtained by the radioactive detection method.

In our experience, the most critical parameter is extract preparation, especially tissue homogenization if tissue samples are used. A commercially available telomerase activity detection kit ($TRAP_{EZE}^{®}$) exists containing several quality and quantification controls and prevailed over most other methods. Alternatively, quantification can be performed with an ELISA TRAP, developed by Roche Diagnostics. However, PCR-derived artefacts, which can be sorted out by analysing the gel cannot be recognized in an ELISA assay. Therefore, a careful interpretation of the results is mandatory. This is also true for recently described modified forms of the conventional TRAP assay such as the real-time (Hou et al., 2001; Wege et al., 2003) or the *in situ* (Ohyashiki et al., 1997; Feng et al., 1999) TRAP assay (Table 2). On the other hand, if carefully performed, these alternative methods are useful for high-throughput screening of many samples simultaneously. More recent publications indicate that the in situ TRAP assay could be of great use to detect telomerase activity in individual cells (Tanemura et al., 2005).

### *Detecting telomerase components: hTERT and hTERC levels*

Despite the many advantages of this simple assay system, one drawback of determining telomerase activity by the TRAP assay is that the tissue has to be fresh or snap-frozen, which is rarely the case in routine surgery. As an alternative, hTERT mRNA levels may be measured by quantitative RT-PCR analysis. Regulation of telomerase activity primarily occurs at the level of transcriptional initiation of the *TERT* gene (Gunes et al., 2000; Ducrest et al., 2001). Accordingly, hTERT expression is limiting for telomerase activity and hTERT expression correlates well with telomerase activity in human tumours and tumour cell lines (Meyerson et al., 1997; Weinrich et al., 1997).

Different approaches correlate telomerase activity, as well as hTERT and/or hTERC expression levels in cancer with diagnosis (Table 3) and prognosis. Whereas hTERC is expressed in most human somatic tissues (Avilion et al., 1996), with some exceptions (Weikert et al., 2005), expression of hTERT as well as telomerase activity is down-regulated during differentiation (Gunes et al., 2000). On the other hand, hTERT expression is detectable in about 90% of human tumours and highly correlates with telomerase activity (Meyerson et al.,

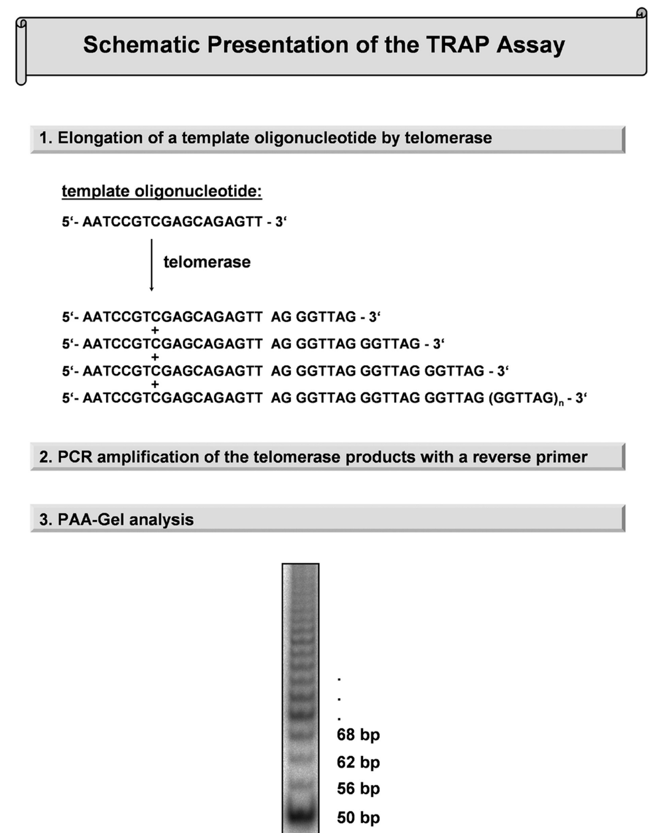


**Fig. 3.** Scheme of the telomeric repeat amplification protocol (TRAP) assay (modified from $TRAP_{EZE}^{®}$ Telomerase Detection Systems, CHEMICON International, Serologicals Corporation). The TRAP assay was developed in 1994 (Kim et al., 1994). In a first reaction telomerase elongates a substrate oligonucleotide by adding telomeric repeats (TTAGGG). The products differ in their length depending on the processivity of the enzyme. In a second step the elongated oligonucleotides are amplified in a PCR reaction and separated by non-denaturing PAA gel electrophoresis resulting in a characteristic 6 bp ladder in the gel.

1997; Kolquist et al., 1998; Nakayama et al., 1998). Thus, evaluation of hTERT level may be more suitable for estimating telomerase activity than investigating hTERC expression. RNA isolation and RT-PCR for hTERT can be easily done from fresh, frozen or even from paraffin embedded tissue of archival material (Poremba et al., 2000; Krams et al., 2003). The levels of hTERT and hTERC expression are usually determined by RT-PCR. For quantification, radioactive RT-PCR is more recommendable as this method is most sensitive and allows to sort out false positive signals (Gunes et al., 2000; Ritz et al., 2005). Real-time RT-PCR is also commonly used and can be performed with the LightCycler hTERT and hTERC Quantification Kit (Roche Diagnostics).

Additional information on telomerase activity in individual cells may be derived by detection of hTERT mRNA by *in situ* hybridization (ISH) or hTERT protein by immunohistochemistry (IHC). With telomerase as a potential tumour target in mind, this information is not inconsequential since not all cells in a tumour may be telomerase positive (Yan et al., 2004). In contrast, quantification of telomerase activity by *in situ* hybridization/*in situ* TRAP and immunohistochemistry is quite laborious and partly unreproducible. To date, a series of reports have been published applying these methods with conflicting results. These discrepancies may in part be due to the antibodies and the tissue processing conditions used in different studies (Yan et al., 2004). Another important issue is that telomerase complex and the TERT component are present at a low level in human cells, aggravating their detection. It should also be noted that several hTERT alternative splice variants exist in normal and tumour cells, although protein products remain to be shown as yet (Ulaner et al., 2001). It is unclear to what extent they do contribute to background staining by histological detection methods. This information is important supposing that TERT/telomerase activity may be a potential prognostic or diagnostic tool in human cancers.

### Use of telomerase activity and its components in diagnosis and prognosis

To evaluate if telomerase and its components may serve as tumour markers and prognostic factors, telomerase activity was monitored in human tumour samples and correlated with the clinical course of the disease. In the following table some of the multiple examples for these studies are listed.

One promising approach for diagnostic and prognostic purposes is the detection of telomerase activity or telomerase components in serum or body fluids. In one such attempt, Miura et al. showed elevated hTERT mRNA in serum of patients with hepatocellular carcinoma (HCC) diagnosis via quantitative real-time

**Table 2.** Methods for detecting enzymatic activity of telomerase.

| METHOD | SELECTED REFERENCES |
|---|---|
| Standard TRAP Assay | (Kim et al., 1994) |
| (TRAP-kit: TRAPEZE®) | (Serologicals) |
| Stretch PCR Assay | (Tatematsu et al., 1996) |
| Flourescent TRAP Assay | (Aldous and Grabill, 1997) |
| Real-time TRAP Assay | (Hou et al., 2001) |
| Telomerase PCR ELISA | Roche Diagnostics |
| *in situ* TRAP Assay | (Ohyashiki et al., 1997; Tanemura et al., 2005) |

**Table 3.** Telomerase activity/hTERT applied for diagnostic values in human tumours.

| METHOD | CANCER TYPE | SELECTED REFERENCES |
|---|---|---|
| TRAP Assay | neuroblastoma | (Poremba et al., 2000) |
| Real-time TRAP | body fluids of cancer patients | (Shim et al., 2005) |
| ELISA TRAP | bladder cancer | (Longchampt et al., 2003) |
| | gastric adenocarcinoma | (Yoo et al., 2003) |
| | renal cell carcinoma | (Fan et al., 2005) |
| | colon cancer | (Sanz-Casla et al., 2005) |
| in situ TRAP | lung cancer | (Yahata et al., 1998) |
| RT-PCR | neuroblastoma | (Poremba et al., 2000; Krams et al., 2003) |
| | bladder cancer | (Longchampt et al., 2003; Weikert et al., 2005) |
| | hepatocellular carcinoma | (Miura et al., 2005) |
| | acute myelogenous leukemia | (Huh et al., 2005) |
| | renal cell carcinoma | (Fan et al., 2005) |
| in situ Hybridization (hTERT mRNA) | gastric adenocarcinoma | (Yoo et al., 2003) |
| | DCIS of the breast | (Liu et al., 2004) |
| Immunohistochemistry | neuroblastoma | (Poremba et al., 2000) |
| | breast cancer | (Poremba et al., 2002) |
| | gastric adenocarcinoma | (Yoo et al., 2003) |
| | hepatic colorectal metastases | (Domont et al., 2005) |
| | lung cancer | (Miyazu et al., 2005) |

RT-PCR which is associated with hTERT expression in HCC tissue (Miura et al., 2005). The specificity of hTERT mRNA in HCC diagnosis was superior to conventional tumour markers. Evaluation of hTERT and in particular of hTERC mRNA in urine using real-time RT-PCR may serve as a better biomarker for non-invasive detection of bladder cancer than cytology (Weikert et al., 2005). With a quite novel technique Shim et al performed real-time TRAP assay in body fluids of cancer patients (Shim et al., 2005). The real-time TRAP assay displays an improved sensitivity for quantitative detection of telomerase activity compared to the conventional TRAP assay and cytology. Cancer patients that had telomerase negative body fluids showed a longer progression-free duration.

Certainly, immunostaining of hTERT protein has the greatest potential for routine applications for diagnostic or prognostic values. In many studies, using hTERT antibodies, a inverse correlation has been described between survival and hTERT positive signal intensity: hTERT positive immunostaining is associated with worse survival in specimen after resection of hepatic colorectal metastases (CRM) (Domont et al., 2005). Similarly, high hTERT (and telomerase activity) signals served as an independent prognostic factor and correlated with poor prognosis in neuroblastoma (Poremba et al., 2000) and breast cancer (Poremba et al., 2002).

If the hTERT component is used for detection, either at the RNA or the protein level, care must be taken in determining splice variants as several hTERT splice variants have been described (Krams et al., 2001, 2003; Ulaner et al., 2001; Fujiwara et al., 2004). The diagnostic and prognostic relevance of full length versus truncated splice variants of *hTERT* has been addressed in two studies. Krams et al. distinguished between full-length and truncated *hTERT* transcripts and showed that full-length *hTERT* expression is an independent prognostic factor in neuroblastoma (Krams et al., 2003). For this purpose they isolated RNA from paraffin-embedded tumour tissue and performed RT-PCR. About 24% of tumours contained full-length hTERT mRNA and expression of full-length hTERT was highly correlated with poor outcome. Similarly, Fan et al. compared *hTERT* expression in normal and malignant renal tissues via RT-PCR and revealed that full-length *hTERT* expression is restricted to renal cell carcinoma, whereas normal renal cells express splice variants of *hTERT* (Fan et al., 2005). In conclusion, only the cancerous renal cells show telomerase activity which was confirmed by ELISA TRAP.

One recent important contribution on this issue shows that activation of *hTERT* expression is an early event in tumour development and could be a marker for premalignant lesions (Miyazu et al., 2005). The authors evaluated hTERT protein by immunohistochemistry in noncancerous epithelia and correlated its presence with the risk for lung cancer. Telomerase activity in normal bronchial epithelia in particular of smokers indicates a higher susceptibility for lung cancer development.

Despite the overwhelming evidence of enhanced telomerase activity in tumour versus normal tissue one report critically evaluated *hTERT* mRNA expression in normal breast tissues versus ductal carcinoma in situ (DCIS) by *in situ* hybridization (Liu et al., 2004). Surprisingly, the authors showed that hTERT expression was significantly higher in normal breast cells compared with adjacent DCIS. This finding is contradictory to the conventional understanding that hTERT is repressed in most somatic tissues but up-regulated in malignant cells. They hypothesized that prior studies in DCIS have been small and are founded on PCR-based methods like RT-PCR and TRAP assay using heterogenous tissue extracts. By *in situ* hybridization they could show the topographic and cellular distribution of *hTERT* mRNA on samples that had normal areas and DCIS lesions on the same slide. It remains to be determined whether they detected the full-length hTERT mRNA or the splice variants as described by Fan et al. for normal versus malignant renal tissues (Fan et al., 2005). Nevertheless, most data implicate that evaluation of telomerase activity and/or hTERT expression status may provide a suitable marker for tumour diagnostics and may discriminate between prognostically different subsets of tumours. Taken together, it is obvious that up-regulation of telomerase and its components is a general marker for tumour tissues and often is accompanied by a bad prognosis.

## Targeting telomerase and telomeres for cancer therapeutics

As telomerase activity is usually repressed in human somatic tissues and highly active in most tumours, telomerase can be considered as an ideal target for cancer therapy. Thus, the remaining healthy tissues would be largely unaffected when the organism is treated with telomerase inhibitors. On the other hand, one could assume that the telomeres itself could be a target for tumour therapy for two reasons. Firstly, telomere length in tumours is usually shorter than in the corresponding normal tissue and secondly, functional telomeres have to be rebuilt more often in highly proliferating cells, such as tumour cells than in normal cells. Therefore, interfering with telomeric structure should be more detrimental for tumour cells than for normal cells although the same factors are thought to contribute to telomere function both in normal and malignant cells.

Different approaches have been made to turn telomerase off: 1) directly targeting telomerase; 2) telomere targeting agents (TTAs); 3) targeted gene therapy; 4) telomerase immunotherapy of cancer.

Both telomerase and the telomeres were targeted *via* drugs or immunotherapy leading to continuous shortening of telomeric DNA, activation of DNA repair mechanisms or an immune response against hTERT expressing cells (Table 4). In conclusion the affected cells exhibit a limited life span, enter senescence or

apoptosis.

A recent review by Kelland gives an update overview over most telomere and telomerase based therapies (Kelland, 2005). Therefore, here, we will focus on most recent publications and discuss their implications.

### *Directly targeting telomerase*

There are several promising agents for cancer therapeutics which target telomerase directly. BIBR1532 (2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid), is a synthetic, non-nucleosidic telomerase inhibitor (Damm et al., 2001; Pascolo et al., 2002). BIBR1532 inhibits enzymatic activity of native and recombinant telomerase. It is thought to block telomerase activity selectively in a non-competitive manner. Treatment of tumour cells with BIBR1532 leads to progressive telomere shortening followed by senescence resulting in delayed growth of tumour cells. In a mouse xenograft model drug-treated tumour cells exhibited a reduced tumourigenic potential (Damm et al., 2001). Human telomerase in the injected cells was selectively repressed by the drug and no or only small tumours developed. BIBR1532 was well tolerated by the mice. In a current report, BIBR1532 was tested in combination with chemotherapeutic drugs (Ward and Autexier, 2005). In this study, BIBR1532 helped to sensitize leukemia and breast cancer cells to chemotherapeutic treatment opening up the potential for telomerase inhibitors for combination therapies or for treatment of drug resistant tumours.

Somewhat puzzling are the observations by El-Daly et al. who investigated the activity of BIBR1532 in normal and malignant hematopoietic cells from acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) patients (El-Daly et al., 2005). They described that in addition to its known role in telomerase inhibition, high-dose BIBR1532 (30-80 $\mu$m which is about 50-fold higher than the previously published 100nM $IC_{50}$ value by (Damm et al., 2001) has a direct cytotoxic effect in leukemia cells but not in normal hematopoietic stem cells. This effect is independent of initial telomere length or the telomerase activity since telomerase positive and telomerase negative leukemia cells responded similarly. However, analysis of individual telomere length revealed progressive loss of individual telomeres and end-to-end fusions indicating that treatment of these cells with high dose of BIBR1532 provokes telomere dysfunction. Interestingly, the authors found loss of the telomeric repeat binding factor 2 (TRF2) and increased phosphorylation of the tumour suppressor protein p53. It is unclear how BIBR1532 treatment results in loss of TRF2 leading to telomeric uncapping which could also explain phosphorylation of p53. p53 in turn may induce apoptotic cell death. Whether the cytotoxic effect was due to apoptosis or not is not addressed by the authors. It would be of importance to see the cytotoxic effects of BIBR1532 in p53 negative tumour cells. Under the conditions used in this study, BIBR1532 rather seems to function as a telomere targeting agent (TTA) interfering with telomere structure.

GRN163L is another promising agent which seems to hold a great potential for telomerase based therapies. GRN163L, developed by Geron Corporation, is a highly potent and specific inhibitor of telomerase (Herbert et al., 2005). The agent, a lipid-conjugated thio-phosphoramidate (N3'-P5') 13-mer oligonucleotide, tightly binds (*in vitro* $IC_{50}$ value ~ 7.8 nM) to the complementary target strand of telomerase RNA subunit, hTERC. The binding is not antisense-based as activation

**Table 4.** Strategies to target telomerase and telomeres and their potential

| | Selected Examples | Mode of Action | Advantages | Disadvantages |
|---|---|---|---|---|
| Directly Targeting Telomerase | • BIBR 1532<br>• GRN163L | • inhibition of telomerase components hTERT / hTERC<br>• telomere shortening at each cell division | • only cells with telomerase activity, such as tumour cells, are affected | • time delay before telomeres become critically short<br>• small subset of normal human cells that express hTERT are also affected |
| Targeting Telomeres (TTA) | • BRACO-19<br>• Telomestatin | • disruption of telomeric cap<br>• activation of DNA repair mechanisms<br>• accessibility of telomeres for telomerase is disturbed | • fast cell response upon treatment<br>• telomere maintenance is interrupted independently on the telomere lengthening mechanism via telomerase or ALT | • telomeres of all cells are affected |
| Targeted Gene Therapy | • hTERT-promoter driven suicide genes | • targeting tumour cells via hTERT promoter, that drives expression of a pro-apoptotic gene or of adenoviral vectors | • high specificity for hTERT expressing cells, such as tumour cells<br>• fast cell response upon treatment | • small subset of normal human cells that express hTERT are also affected |
| Immunotherapy | • vaccination with hTERT peptides | • generation of hTERT-specific CTLs | • high specificity for hTERT expressing cells, such as tumour cells<br>• fast cell response upon treatment<br>• option for preventive immunotherapy | • small subset of normal human cells that express hTERT are also affected |

of RNaseH based degradation of hTERC mRNA is not initiated (Asai et al., 2003). Rather, telomerase activity is inhibited by blocking substrate (telomeric DNA) recognition. As expected, treatment of cells with GRN163L results in progressive shortening of telomeric DNA and growth inhibition of a variety of tumour cells including lung, breast, prostate, liver and prostate (Herbert et al., 2005). GRN163L was also assayed in xenograft animal models and shows inhibition of telomerase activity and tumour growth (Dikmen et al., 2005; Djojosubroto et al., 2005). Dikmen et al. demonstrated that GRN163L reduced colony formation in vitro. Moreover, using an elegant xenograft metastasis model they showed that treatment of mice with GRN163L prevented formation of lung tumours. Similarly, Djojosubroto et al. revealed growth inhibitory effects of GRN163L on two human hepatoma cell lines (Hep3B and Huh7), both *in vitro* and *in vivo*. Remarkably, initial telomere length in these cell lines is above 10 kbp and thus considerably longer than in most human liver carcinomas (Kitada et al., 1995; Miura et al., 1997; Aikata et al., 2000). Another interesting aspect was that despite significant difference in the mean telomere length between the two hepatoma cell lines, growth inhibition followed similar kinetics (K-L. Rudolph, personal communication). A similar observation was also reported by Gellert et al. (2006). This group used two different breast carcinoma cell lines (MDA-MB-231 and MDA-MB-435) with similar telomere length and comparable telomerase activity. Although treatment of these cells inhibited telomerase activity with similar kinetics, and resulted in significant reduction of telomere length, MDA-MB-231 cells continued to grow in the presence of GRN163L. Interestingly, however, the ability of both cell lines to invade through Matrigel™ was completely inhibited (Gellert et al., 2006). These studies hint either at a novel, telomere length independent function of telomerase or, alternatively, some telomeres may be critically short in some cell lines and further shortening may be more detrimental to them. Further, the agent may interfere with other cellular functions.

### Telomere targeting agents (TTAs)

The single-stranded 3’-telomeric overhang is capable of forming four-stranded DNA G-quadruplex structures. Although not demonstrated in human cells yet, there is good evidence that the G-quadruplex can form at telomeres *in vivo* (Schaffitzel et al., 2001; Paeschke et al., 2005). Most recently, Granotier et al. demonstrated that a G-quadruplex binding ligand preferentially binds to human chromosome ends supporting this idea (Granotier et al., 2005).

Sun et al. were the first who demonstrated a non-nucleoside agent (2,6 diamidoanthraquinone) that inhibits telomerase by targeting G-quadruplex structures (Sun et al., 1997). Since this discovery several G-quadruplex ligands were examined to induce the 3’-single stranded telomeric overhang to fold into a quadruplex intramolecular structure. Some of these molecules have been shown to induce telomere shortening in cell lines probably resulting in telomere uncapping and leading to senescence/apoptosis (Riou et al., 2002; Burger et al., 2005). In vitro, TTAs have been shown to inhibit elongation of substrate telomeric DNA. The ligands do so by stabilizing the G-quadruplex structure thereby preventing the access of the telomerase complex to the telomere template DNA. However, the precise mechanism of action of these molecules still remains unclear. Since at least some G-quadruplex ligands were effective on telomerase-positive cell lines with short telomeres and also on telomerase negative ALT cell lines it remains to be clarified whether inhibition of telomerase enzymatic activity by these ligands in telomerase positive cell lines does play a role at all for their therapeutic potential. For telomestatin, a promising candidate TTA, it was demonstrated recently that this agent probably induces telomeric dysfunction independent of telomerase inhibition (Tahara et al., 2006). Telomestatin, isolated from *Streptomyces anulatus*, is a naturally occurring molecule which is known to stabilize G-quadruplex structures at 3' single-stranded telomeric overhangs (G-tails) and to selectively induce prompt cell death in cancer cells (Tahara et al., 2006). The authors show that telomestatin treatment leads to dissociation of the telomere binding protein TRF2 from telomeres and that the observed effects resemble those of dominant negative TRF2 expression in tumour cells indicating that telomestatin exerts its anticancer effect not only through inhibiting telomere elongation, but also by disrupting the capping function at the very ends of telomeres.

Another candidate, BRACO-19, which holds potential as a TTA in cancer therapy will be mentioned here. BRACO-19, a synthetic molecule, is a 3,6,9-trisubstituted acridine (Read et al., 2001) which shows a selective binding to G-quadruplex DNA and a potent inhibition of telomerase *in vitro* and *in vivo* (Burger et al., 2005). The *in vivo* activity of BRACO-19 was proven in human tumour xenografts. Interestingly, in xenograft experiments treatment with BRACO-19 led to loss of nuclear hTERT localisation and colocalisation of hTERT with ubiquitin in the cytoplasm. The relevance of hTERT degradation upon treatment is not fully clear yet. It may be helpful to test telomerase positive and ALT cell lines under similar conditions to understand the role of telomerase enzyme in this context.

Direct targeting of the telomeres instead of the enzyme telomerase has a broad-spectrum of application in cancer therapy and is independent of the initial telomere length in the treated cells. The G-quadruplex ligands interfere with telomere maintenance independently on the telomere lengthening mechanism via telomerase or ALT. The onset of antitumour effects is quite fast (within days) compared to the direct inhibition of telomerase, which exhibits a lag period between enzyme inhibition and tumour regression.

*Targeted gene therapy*

Telomerase activity is primarily regulated at the level of *hTERT* gene transcription (Gunes et al., 2000; Ducrest et al., 2001). *hTERT* promoter activity is repressed in most human somatic cells and up-regulated in tumour cells. Thus, *hTERT* promoter may be suitable to drive the expression of cytotoxic or pro-apoptotic genes specifically in tumour cells. Several genes like Bax (Gu et al., 2000), caspase 8 (Koga et al., 2000), rev-caspase 6 (Komata et al., 2001), TRAIL (Lin et al., 2002), FADD (Koga et al., 2001) or tBid (Kazhdan et al., 2006) have been analysed to induce specific death of the tumour cells

One strategy to deliver these "suicide genes" is by injection of the plasmid or a replication-defective, E1-deleted adenoviral vector with the *hTERT* promoter driven pro-apoptotic gene directly into the tumour tissue. This has the advantage that side effects on normal cells with hTERT expression will be low due to the limited ability of these vectors to go to distant sites. On the other hand, probably only a subset of tumour cells in the vicinity of the injection will be targeted and eliminated. For an effective infection of the target tumour tissue an increase of viral spread is required. An auspicious improvement on this issue is targeting cancer cells by tumour-specific replication-competent adenoviruses (TRAD) which has been used for virotherapy using *hTERT* promoter driven expression of adenoviral genes E1A and E1B (Kawashima et al., 2004). To this end, conditionally replicating adenoviruses (CRAs) are promising tools for telomerase dependent gene therapy because they are specifically targeted to the tumour cells. Normal somatic cells that are infected by the virus are not affected due to the lack of active telomerase (Wirth et al., 2003).

Basically, all of these gene therapy experiments were performed using approximately 200-400 bp of human *TERT* promoter. Although several factors responsible for human *TERT* gene expression have been located to this 'core promoter' these results are restricted to *in vitro* studies and may not fully reflect human TERT gene regulation *in vivo*. We recently demonstrated that lacZ reporter gene expression under the control of an 8.0 kbp fragment of human TERT gene promoter recapitulates human TERT gene expression in transgenic mice and is reactivated in spontaneous mouse mammary tumours (Ritz et al., 2005) making this promoter suitable for targeted gene therapy.

*Telomerase immunotherapy of cancer*

Therapeutic cancer vaccines aspire to provoke an immune response to cancer cells by immunogenic hTERT epitopes. Several T cell epitopes from hTERT have been described that can be recognized by cytotoxic T lymphocytes (CTLs) which kill the hTERT expressing cancer cells in an antigen-specific MHC response (Vonderheide et al., 1999, 2001; Minev et al., 2000). Following this line, a clinical phase I study was performed with patients with progressive metastatic breast cancer or progressive hormone-independent prostate cancer (Vonderheide et al., 2004). Vaccination was performed with dendritic cells (DCs) exposing the hTERT peptide1540 (ILAKFLHWL). This peptide strongly binds to human leukocyte antigen (HLA) HLA-A2, the most frequently expressed HLA allele. The hTERT 1540 peptide presented on the DCs induced functional anti-tumour T-cells in humans. Immunity was achieved in the absence of significant toxicity. In a current report, artificial antigen-presenting cells (AAPC) were generated by transducing murine fibroblasts with retroviral vectors encoding hTERT epitopes or the full-length hTERT cDNA (Dupont et al., 2005). The authors showed efficient tumouricidal activity of hTERT-specific CTLs against human tumour cell lines, proportional to telomerase activity in the analyzed cell lines. This approach demonstrates that hTERT-specific CTLs can be produced in clinically relevant numbers, one of the major limitations achieving CTL responses to tumour antigens.

## Conclusions

Telomerase activity is essential for unlimited growth potential of human cells. This is reflected by the fact that telomerase is up-regulated in about 90% of human tumours (Kim et al., 1994; Meyerson et al., 1997; Nakayama et al., 1998).

Since the description of a PCR-based telomerase activity detection method a huge number of reports have evaluated telomerase activity in almost all cancer types. To date, TRAP assay is the most powerful and reliable molecular tool to detect telomerase activity. This assay is so far the only method which really detects the enzyme activity. RT-PCR detection of *hTERT* component is usually done in combination with TRAP assay and is helpful to better quantify telomerase. For an accurate quantification of hTERT mRNA expression levels RT-PCR has to be standardized to graduate between low, intermediate and high expression. On the other hand, correlation of *hTERT* expression level and telomerase activity can be misleading when splice variants instead of the full-length *hTERT* transcript are present that lead to truncated proteins which are not able to form a functional telomerase complex. For this reason, discrimination between full-length and truncated *hTERT* transcripts is essential for diagnosis and prognosis. It is also worth noting that the same discrepancy may occur between telomerase activity and detection of hTERT mRNA or protein. This may especially be the case for non-malignant tissues with proliferative potential depending on the cellular proliferation status as it was described for normal human endometrium where telomerase activity is altered during the menstrual cycle (Kyo et al., 1997). Additional techniques (immunohistochemistry, *in situ* hybridization) need to be optimized for routine use but may provide very useful

tools for diagnostic and prognostic purposes. In fact, although a wealth of reports correlate telomerase activity or detection of telomerase components to tumour progression and/or prognosis there is no standard protocol that can be applied to clinical samples for routine use. Evaluation of telomerase activation in animal tumourigenesis models could provide a basis for clinical applications.

Obviously, telomere and telomerase based cancer therapeutics promise admirable potential in overcoming the immortality of tumour cells. The above described findings that anti-telomerase therapy could sensitize tumour cells to conventional chemotherapeutic drugs indicate that these strategies could complement each other very well. On the other hand, activation of telomerase independent ALT pathway is one of the major concerns when telomerase activity is abrogated in cancer cells. There is at least some experimental evidence cautioning to be alert for problems (Bechter et al., 2004). Switching of tumour cells to ALT could weaken the effectiveness of telomerase inhibitors in cancer therapy. Nevertheless, G-quadruplex ligands were shown to work on telomerase-positive glioma cell lines with short telomeres as well as on ALT cell lines (Pennarun et al., 2005). Finally, a more detailed understanding of the ALT mechanism is essential to potentially combine anti-telomerase and anti-ALT therapies. Another issue to be considered is the presence of telomerase activity in normal human cells like stem cells, hematopoetic progenitor cells and cells of the renewal tissues (Wright et al., 1996; Forsyth et al., 2002). Inhibition of telomerase activity could provoke detrimental consequences in these cells. However, these consequences may not be more detrimental than treatment with the conventional drugs which target fast proliferating cells. Taking into account that stem cells usually are in a quiescent state and have quite long telomeres compared to tumour cells, anti-telomerase based therapies may even prove better. In fact, clinical trials using hTERT vaccination did not impair normal human cells. It may well be that telomerase activity is modulated differentially in normal and tumour cells (e.g. by TRFs, Tanykrases and Pot1). Research on telomere and telomerase has opened up great opportunities in the struggle against cancer. Yet, many questions wait to be answered.

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Accepted May 24, 2006