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Invited Review

Telomerase activity in cancer as a diagnositic and therapeutic target

S. Kyo, M. Takakura and M. Inoue
Department of Obstetrics and Gynecology, Kanazawa University, School of Medicine, Takaramachi, Kanazawa, Ishikawa, Japan

Summary. Major advances have been made in understanding the role of telomerase in cellular immortalization and carcinogenesis. Human telomeres undergo progressive shortening with cell division, and critical shortening of telomeres with cellular aging triggers a signal for cells to stop dividing and senesce. Telomerase is an enzyme that adds telomeric-repeated sequences to the ends of human chromosome DNA. Telomerase is active in the vast majority of tumors, but not in normal somatic tissues, and prevents progressive shortening of telomeres with cell division, probably giving tumor cells a growth advantage over normal cells. Highly-sensitive PCR-based TRAP (telomeric repeat amplification protocol) assay provided the means to analyze telomerase in a wide variety of tissues. Evidence has been accumulated that this assay may be useful as a potential diagnostic tool for cancer. The constituents of telomerase complex have recently been identified, and human telomerase reverse transcriptase (hTERT) has been found to be responsible for the enzymatic activity of telomerase. Detection of hTERT mRNA may therefore be useful for the screening and diagnosis of cancers. The mechanisms regulating hTERT expression have been extensively analyzed, and transcriptional regulation of hTERT has been found to be essential for hTERT expression, in which several nuclear factors including c-Myc play crucial roles. Understanding of such mechanisms might provide insight into molecular basis of human carcinogenesis and contributes to the development of novel cancer gene therapy targeting telomerase.

Key words: Telomeres, Telomerase, Cancer, Diagnosis, Gene therapy

Offprint requests to: Dr. Satoru Kyo, Department of Obstetrics and Gynecology, Kanazawa University, School of Medicine, 13-1, Takaramachi, Kanazawa, Ishikawa, 920-8641, Japan. Fax: +81-(0)76-234-4266. e-mail: satoruky@med.kanazawa-u.ac.jp

The roles of telomeres and telornerase in cellular aging

Normal cells have limited proliferative capacity. After a definite number of cell divisions, normal cells stop dividing and attain their finite life span. This process is referred to as cellular aging or senescence. In contrast, most malignant tumor cells have an infinite life span and continue to proliferate until their host dies. Normal cells are thus thought to have a so-called endogenous mitotic clock by which they recognize how many times they have divided. This clock is probably inactive in tumor cells. Recent progress in research in cellular aging has revealed that the DNA structures located at the ends of chromosomes, called telomeres, play essential roles in mechanisms of cellular aging.

Telomeres are the distal ends of eukaryotic chromosomes, and are composed of an average of 5000-15000 base pairs of (TTAGGG)n repeats and telomere binding proteins (Moyzis et al., 1988). It has been suggested that one of the major functions of telomeres is stabilization of chromosomes (Blackburn, 1991). In yeast, it has been shown that removal of the telomere of a nonessential chromosome causes dramatic loss of that chromosome (Sandell and Zakian, 1993). It remains unclear how telomeric structures contribute to chromosomal stability, but they may effectively shield chromosomal ends from exonuclease digestion and prevent aberrant recombination.

DNA polymerase is an enzyme that copies the strands of DNA prior to cell division by base polymerization in the 5' to 3' direction. However, it fails to fully replicate the ends of linear DNA templates (Watson, 1972). This failure is thought to be caused by the difference in replication mechanisms between leading and lagging strands. Fig. 1 shows the replication fork at which new strands of DNA are synthesized. As the replication fork proceeds, the lagging strands are synthesized as series of discrete fragments, termed Okazaki fragments, each requiring a new RNA primer to begin DNA polymerization. This is due to the inability of DNA polymerase to polymerize in the 3' to 5'

direction. In contrast, the leading strand DNA is continuously synthesized in the 5' to 3' direction without a new RNA primer, since upstream sequences synthesized by polymerization can function as a primer. As the replication fork proceeds to the end of chromosome DNA, the lagging strand sequences between the last RNA priming event and the end of the chromosome cannot be replicated since there is no DNA beyond the end to which the next RNA primer can anneal, and this gap cannot be filled in, resulting in shortening of the telomere. This is referred to as the end-replication problem. The shortened telomere is inherited by the daughter cells. Telomeres thus undergo progressive shortening with cell divisions. A cell loses 50-100 base pairs at the end of its telomeres each time it divides.

Evidence that telomeres are lost with cellular aging has been obtained in several studies. It has been reported that telomeres are shorter in somatic tissues of older than those of younger individuals (Hastie et al., 1990). Telomeres in normal cells from young individuals are known to progressively shorten when grown in cell culture (Harley et al., 1990), and children born with prognia, a disease which results in premature aging and shortened life span, have shorter telomeres than age-matched controls (Allsopp et al., 1992). Finally, the proliferative capacity of cultured cells was extended by experimental elongation of telomeres (Wright et al., 1996). These findings suggest that shrinkage of telomeres reflects the mitotic age of cells, and support a model in which telomere length is a mitotic clock showing how many times cells have divided.

When telomeres reach a critically short length with cellular aging, the cells exit from the cell cycle and stop dividing; the so-called replicative senescence (Allsopp and Harley, 1995). The molecular mechanism by which shortened telomeres lead to cellular senescence remains unclear, but induction of a DNA damage checkpoint pathway may be involved. In contrast, immortal cells as well as germline cells, which show indefinite growth during the lifespan of individuals, must have a mechanism to compensate for the progressive loss of telomeres. Telomerase is a ribonucleotide protein enzyme which functions as a DNA polymerase containing an integrated RNA template (Greider and Blackburn, 1989; Yu et al., 1990). Telomerase binds to the 3' ends of DNA strands and extends them by copying its own RNA template in multiples of hexamer repeat sequences (Fig. 2). Telomerase activity is usually repressed in normal somatic cells, causing continuous telomere erosion with normal cell division (Kim et al., 1994). Telomerase activation is thus thought to be essential for prevention of telomere shortening and stabilization of telomeres, which may permit cells to escape from replicative senescence, leading to cellular immortality (Counter et al., 1992).

Telomere-binding proteins function as regulators of telomerase activity

Fig. 3 shows a summary of current knowledge of the human telomere/telomerase complex. The two telomeric repeat-binding factors 1 and 2 (TRF1, TRF2) have recently been identified (Bilaud et al., 1997; Broccoli et al., 1997). The functions of these two proteins differ; TRF1 acts as an inhibitor of telomerase, while TRF2 functions to maintain telomere integrity. Overexpression of TRF1 results in telomere shortening, while inhibition of TRF1 increases telomere length (van Steensel and de Lange, 1997). In contrast, a dominant negative allele of TRF2 induced end-to-end chromosome fusions detectable in metaphase and anaphase cells (van Steensel et al., 1998). Furthermore, expression of mutant forms of TRF2 induced growth arrest with characteristics of senescence. These findings suggest that chromosome end fusions and senescence in human cells may be caused by loss of TRF2 from shortened telomeres. Most

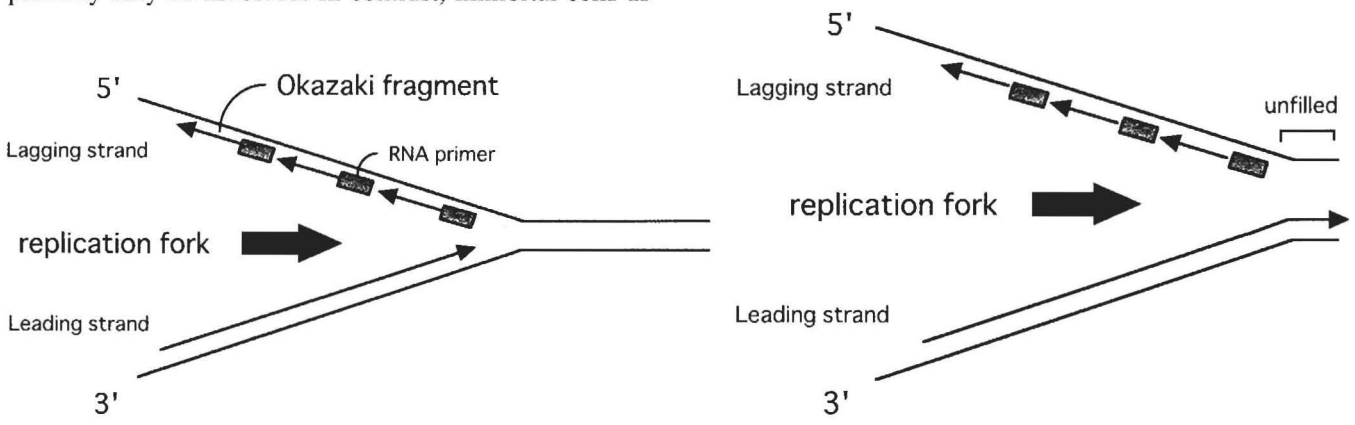

Fig. 1. End replication problem. DNA is replicated by the action of DNA polymerase, which synthesizes DNA in only the 5' to 3' direction and requires an RNA primer to initiate replication. Therefore, as the replication fork proceeds, from left to right in this figure, the leading-strand DNA is replicated continuously while the lagging-strand DNA is replicated discontinuously and backwards relative to the direction of the replication fork. Thus, lagging-strand DNA synthesis depends on the ligation of Okazaki fragments, which are primed with short RNA primers. At the end of chromosomal DNA, the lagging-strand sequences between the last RNA priming event and the end of chromosomal DNA cannot be replicated since there is no DNA beyond the end to which the next RNA primer can anneal, and this gap cannot be filled in, resulting in shortening of telomeres with cell division.

recently, a novel protein called tankyrase has been identified, which has homology with the catalytic domain of poly(adenosine diphosphate-ribose) polymerase (PARP) (Smith et al., 1998). Tankyrase binds to TRF1, and recombinant tankyrase has been found to have PARP activity in vitro. ADP-ribosylation of TRF1 diminished its ability to bind to telomeric DNA in vitro, suggesting that tankyrase functions as a PARP at telomeres and inhibits TRF-1 binding to telomeres, and thereby acts as telomerase activator. Telomere length and telomerase activity are thus modulated by various regulators interacting with telomeres.

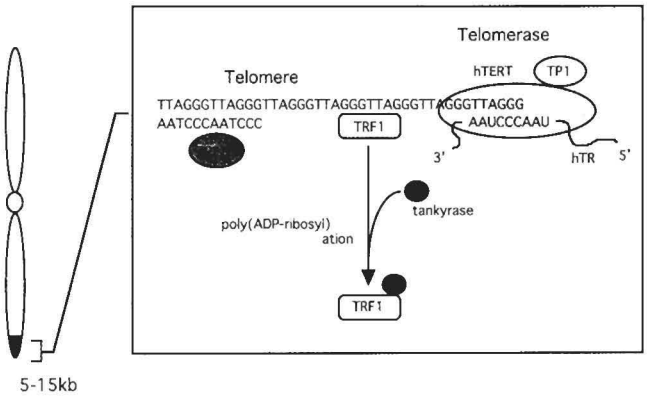

Fig. 2. Telomeres, telomerase and telomere binding proteins form complexes. The 3' overhang of single-stranded DNA at the ends of telomeres is a substrate for telomerase, which is composed of three major subunits, human telomerase RNA, telomerase-associated protein, TP1/TLP1, and telomerase catalytic component, hTERT. Two types of telomere-binding proteins have been identified, TRF-1 and TRF-2, which are involved in the regulation of telomere length and telomerase activity. Tankyrase, which has poly(adenosine diphosphate ribose) polymerase activity, adds poly(ADP) ribosyl complexes to TRF-1, resulting in the dissociation of TRF-1 from telomeres, which facilitates binding of telomerase to telomeres.

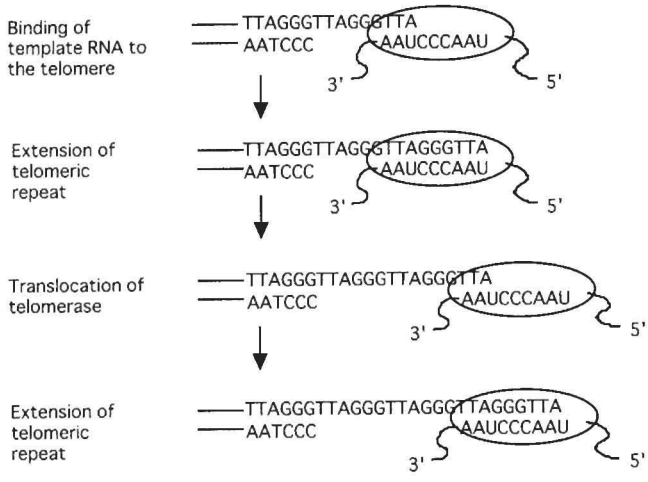

Fig. 3. Mechanism of telomere elongation by telomerase. Telomerase binds to telomeres through a part of template sequences of its RNA component (hTR). New telomere sequences are extended by DNA polymerization according to the template sequences of hTR. Telomerase then translocates and repositions at the end of telomeres and repeats the polymerization. The complementary strand is synthesized by a standard DNA polymerase.

Development of telomerase detection assay

A highly-sensitive PCR-based method to detect telomerase activity has recently been developed by Kim et al, termed TRAP (Telomeric Repeat Amplification Protocol) assay (Kim et al., 1994). The TRAP assay includes two major steps, telomerase extension reaction and amplification of extended products (Fig. 4). In the first step, cell lysates are given a telomere-specific oligonucleotide primer as a substrate. If telomerase is present in the extracts, it adds the telomeric repeat sequences to the ends of substrate oligonucleotide primers (TS primer), which allows the oligonucleotides to be extended. The second step is a PCR reaction to amplify the extended ologonucleotides with a special set of primers, TS and CX primers; the sequences of the latter are complementary to telomeric repeats. This PCR step permits measurement of telomerase activity in a small number of cells. The final PCR products are heterogeneous in their lengths, since the CX primers can anneal at random sites in TTAGGG-repeated sequences of the template oligonucleotides. As a result, the PCR products generate DNA ladders in electrophoresis, which can be visualized or measured with radioisotope or fluorescence labeling of the primers or simply by staining with special reagents (SYBR Green I) that enable detection of small amounts of DNA in gels.

Semi-quantitative telomerase detection methods have subsequently been developed (Wright et al., 1995;

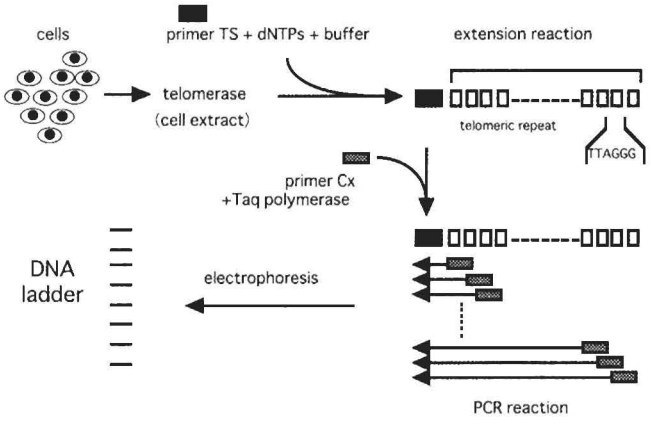

Fig. 4. Principle of the TRAP assay. Telomerase contained in the cell extracts can add telomeric-repeated sequences to the oligonucleotide primer (TS) added to the reaction. Extension products are then PCR-amplified using a set of specific primers CX and TS. The CX primer is designed to have sequences complementary to telomeric repeats. PCR products are heterogeneous in length, since Cx primer can anneal at various sites within telomeric repeats of the template DNAs produced in the PCR cycles. Electrophoresis therefore reveals PCR products as a ladder in the gel.

Tatematsu et al., 1996). In these methods, cell extracts are purified by phenol/chloroform followed by ethanol precipitation prior to the steps of PCR amplification to remove reaction inhibitors, which are included in tissue samples and therefore interfere with PCR reactions. In addition, internal control DNA fragments are included in the reactions to standardize the efficacy of PCR amplification for each sample. Determination of the ratio of telomerase products to internal standards permits semi-quantification of activity and comparison between different experiments or laboratories. Several research kits for telomerase detection are commercially available.

Telomerase activation in tumors

A number of studies have examined telomerase activity in different types of tumors using TRAP assays, and have demonstrated that more than 90% of malignant tumors, irrespective of tumor type, are telomerase-positive (Counter et al., 1994; Kim et al., 1994; Hiyama E. et al., 1995a b, 1996; Hiyama K. et al., 1995b; Tahara et al., 1995; Kyo et al., 1996, 1997a) (Fig. 5). This suggests that these malignant tumors may be composed of or include a population of immortal tumor cells and that telomerase activation is a critical step in carcinogenesis. The most interesting question is whether these tumors have longer telomeres due to the presence of telomerase activity than the corresponding normal tissues. Unexpectedly, however, telomeres in each tumor are usually shorter than those in normal tissues and maintain a constant length (Counter et al., 1994; Clark et al., 1997). This may be explained by the increased rate of cell division of tumor cells, resulting in more progressive erosion of telomeres than in normal cells. Association of telomerase activity with clinical features of tumors is another question. A number of studies have examined the correlations between telomerase activity and clinicopathological characteristics of tumors, such as histological type, pathological grade, clinical stage and prognosis. Some studies demonstrated that telomerase activity is associated with malignant features of tumors. In particular, for breast and gastric cancers, telomerase activity was significantly associated with poor survival (Hiyama E. et al., 1995a, 1996; Clark et al., 1997). These findings suggest that cells with higher telomerase activity have a selective growth advantage due to improved stability of chromosomes with restored telomeres, which may cause clonal expansion of these cells, leading to tumor agressiveness. Alternatively, higher levels of telomerase activity might reflect the presence of a larger fraction of immortalized cells in the tumor cell population, which could have accumulated all

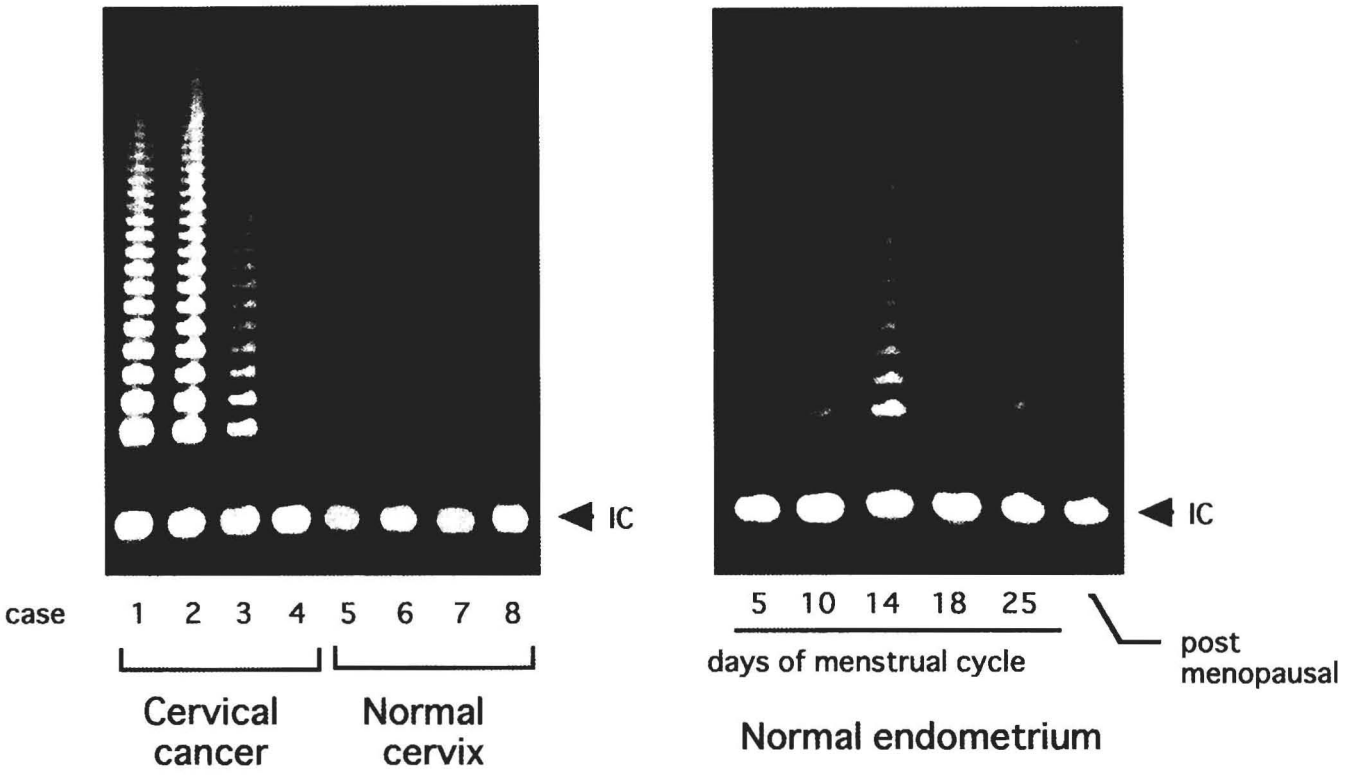

Fig. 5. Representative results of TRAP assay for samples from cervical and endometrial tissues. Cervical cancers (cases 1 to 4) exhibit significant telomerase activity while normal cervical tissues (cases 5 to 8) do not. Unlike other normal tissues, normal endometria express telomerase activity in a menstrual phase-dependent manner. The strongest activity is observed in the proliferative phase (days 1 to 14) while decreased activity is found in the secretory phase (days 15 to 28) and in postmenopausal endometria. IC: internal standard to normalize PCR efficiency.

the characteristics of the metastatic phenotype. Other striking evidence for this correlation has been shown by Hiyama et al. demonstrating that neuroblastomas in stage 4s that lack or have low levels of telomerase activity were likely to spontaneously regress, suggesting that telomerase levels insufficient to maintain telomeres may contribute to a favorable outcome (Hiyama K. et al., 1995a). Despite these findings, other studies have failed to find such a correlation (Kyo et al., 1997a; Nawaz et al., 1997; Mokbel et al., 1999). Since up to 90% of tumors have detectable telomerase activity, it is unlikely that the mere presence of telomerase has useful predictive value for clinical outcome or prognosis. Further careful examination will be needed to determine the relevance of telomerase activity in cancers with respect to clinical outcome.

Telomerase activity in normal cells

Initial studies demonstrated that telomerase activity is usually repressed in normal somatic cells. However, subsequent studies have revealed telomerase activity in some types of normal cells, such as hematopoietic progenitors, intestinal crypt cells, keratinocytes in undifferentiated layers of skin or cervical mucosa, endometrial cells and trophoblasts, all of which are highly regenerative (Broccoli et al., 1995; Counter et al., 1995; Hiyama et al., 1995c; Harle-Bachor and Boukamp, 1996; Yasumoto et al., 1996; Kyo et al., 1997c; Tanaka et al., 1998). These cells have characteristics of stem cells, which continue to divide throughout the entire life of individuals. Accumulating evidence suggests that telomerase activity is correlated with the proliferative activity of these cells. For instance, telomerase activity in hematopoietic progenitor cells is detectable at only low levels in non-stimulating conditions, but dramatically increases upon mitogenic stimulation such as that by IL-2 and PHA (Hiyama K. et al., 1995c). Similarly, telomerase activity in endometrial cells is regulated in a menstrual phase-dependent manner, with the strongest activity observed in proliferative phase, while a dramatic decrease in activity is found in the secretory phase (Kyo et al., 1997c; Tanaka et al., 1998) (Fig. 5). Immunohistochemical analysis revealed that PCNA expression was significantly correlated with telomerase activity in endometrial cells (Kyo et al., 1997c). Similarly, strong telomerase activity is observed in trophoblasts in early gestation, when they vigorously proliferate to form the placenta (Kyo et al., 1997d). In contrast, no or small amounts of telomerase are detectable in trophoblasts in the late stage of gestation when placental formation is completed with decreased proliferative activity of trophoblasts. Additional evidence of proliferation-dependent telomerase activation has been obtained using normal human urothelial cells. Biopsy samples from normal urothelial tissues were telomerase-negative, while those from bladder cancers were positive (Belair et al., 1997). However, once normal urothelial cells were cultured in vitro and allowed to proliferate, telomerase activity was detected, although it was lower than that in cultured bladder cancer cells. Taken together, these findings suggest that telomerase activity is regulated in association with cell proliferation.

The regulation of telomerase activity during the cell cycle is not fully understood. However, recent studies revealed that telomerase-positive leukemia and fibrosarcoma cells sorted by flow cytometry had telomerase activity at each stage of the cell cycle (Holt et al., 1997). In contrast, telomerase activity was repressed in quiescent cells which had exited the cell cycle. Another study demonstrated that telomerase in normal leukocytes was activated as the cell cycle progressed from G0/G1 to S phase following stimulation by PHA and IL-2 (Buchkovich and Greider, 1996). However, treatment with rapamycin, which blocks cell-cycle progression at G1 by inhibiting the phosphorylation of retinoblastoma protein (RB), resulted in failure of these mitogens to activate telomerase. In contrast, treatment with hydroxyurea, which blocks cell-cycle progression in early S phase, did not disturb activation of telomerase by PHA and IL-2. These findings suggest that telomerase is activated in G1 phase when normal cells enter the cell cycle from G0 phase, and that activity levels are preserved throughout the cell cycle.

Human telomerase reverse transcriptase (hTERT) is responsible for the enzymatic activity of telomerase

Human telomerase is composed of three main subunit components, human telomerase RNA (hTR), telomerase-associated protein (TP1/TLP1) and telomerase reverse transcriptase (hTERT). hTR provides the template sequences through which the telomerase complex recognizes telomeres at the DNA termini to initiate telomere elongation (Feng et al., 1995). Several studies have shown that disrupting the function of telomerase RNA in Tetrahymena by in vivo alteration of telomere RNA sequences leads to progressive shortening of telomeres and cellular senescence, suggesting that this component plays an essential role in telomerase function (Yu et al., 1990). Most recently, targeting the mouse telomerase RNA gene (mTR) has been shown to lead to progressive shortening of telomeres and to impair long-term viability of tissues with high rates of renewal such as testis and bone marrow (Blasco et al., 1997; Lee et al., 1998). Furthermore, a study of aging mTR-/- knockout mice revealed that age-dependent telomere loss was associated with shortened lifespan as well as reduced capacity to respond to stress (Rudolph et al., 1999). Interestingly, increased incidence of spontanous malignancies was observed in these mice. TP1 was first identified as a factor associated with telomerase activity (Harrington et al., 1997; Nakayama et al., 1997). However, the functional roles of TP1 remain unclear. Most recently, hTERT, a catalytic subunit protein of telomerase, has been cloned (Meyerson et al., 1997; Nakamura et al., 1997). hTERT protein harbors several

sequence motifs characteristic of catalytic regions of reverse transcriptase conserved among different species. Disruption of these motifs has been shown to abolish the enzymatic activity of telomerase (Meyerson et al., 1997; Nakamura et al., 1997). hTERT thus functions as a specialized reverse transcriptase. Expression of hTERT mRNA was observed at high levels in cancer tissues and cell lines, but not in normal somatic tissues or telomerase-negative cell lines (Fig. 6) (Takakura et al., 1998; Ito et al., 1998; Kanaya et al., 1998; Kyo et al., 1999a,b). There was a strong correlation between telomerase activity and expression of hTERT in a variety of tumors as well as normal cells with telomerase activity. In contrast, highly sensitive RT-PCR analyses demonstrated that hTR or TP1 was broadly expressed not only in cancers but also in normal tissues, and that their expression was not significantly correlated with telomerase activity (Ito et al., 1998; Kanaya et al., 1998; Takakura et al., 1998; Kyo et al., 1999a,b). However, some studies using in situ hybridization technique have revealed enhanced expression of hTR in some cancer tissues compared to that in normal tissues, suggesting that hTR may be up-regulated in cancers (Yashima et al., 1997; Soder et al., 1998). In vitro reconstitution assay of telomerase, in which expression vectors for hTR and hTERT were used in a transcription/translation system revealed that these two components were necessary and sufficient for the production of telomerase (Weinrich et al., 1997; Nakayama et al., 1998). Introduction of hTERT cDNA into normal cells confers telomerase activity in these cells (Weinrich et al., 1997; Nakayama et al., 1998). hTERT-expressing normal cell clones which were obtained by transfection of hTERT cDNA have an extended life span without any evidence of malignant transformation (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999). These findings strongly suggest that hTERT confers enzymatic activity of telomerase and is the best gauge of telomerase activity.

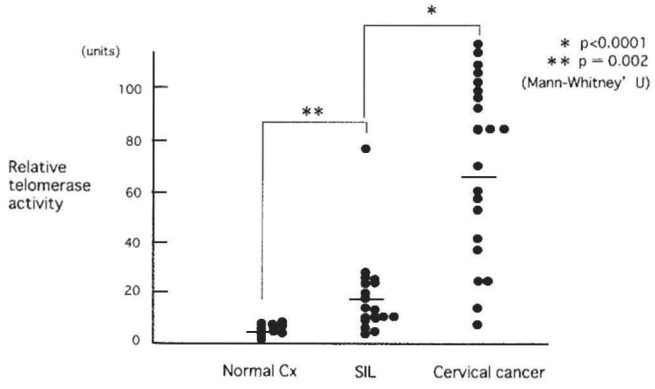

Fig. 6. Differences in amounts of telomerase activity between cancerous and non-cancerous tissues in uterine cervix. Quantitative telomerase assays reveal differences in levels of telomerase activity among cervical cancers, squamous intraepithelial lesions (SIL) and normal cervical tissues. As lesions progress, telomerase activity increases, and cervical cancers exhibit significantly higher telomerase activity than SIL. Interestingly, the telomerase activity in SIL appears to be higher than that in normal cervical tissues, suggesting that these lesions contain a population of cells with immortal characteristics or malignant potential. Telomerase activity levels are determined relative to control activity of 104 cells of the C33A cervical cancer cell line.

Telomerase activity as a tumor marker

A number of studies have examined the usefulness of telomerase activity as a tumor marker. Most studies using frozen samples from a variety of tissues found that more than 90% of malignant tumors expressed telomerase activity while few normal tissues did (Counter et al., 1994; Kim et al., 1994; Hiyama E et al., 1995a,b, 1996; Hiyama K. et al., 1995a; Tahara et al., 1995; Kyo et al., 1996, 1997a). The most serious problems in application of telomerase activity to cancer diagnosis may be false-positivity due to telomerase-positive non-cancerous tissues. Some premalignant lesions such as squamous intraepithelial lesions of uterine cervix (SILs) or ovarian low grade malignancies (LPM) express modest levels of telomerase activity (Kyo et al., 1998a,b). In addition, as noted in this review, some higly regenerative normal tissues, such as hematopoietic progenitors and endometrial cells, exhibit telomerase activity. Contamination of these cells in samples may lead to false-positive results. In particular, normal tissue samples with severe inflammation may contain increased numbers of activated lymphocytes expressing telomerase. Curettage samples from endometrial tissues to screen endometrial cancers are likely to include normal endometrial tissues, which may exhibit telomerase activity if the endometria are in the proliferative phase of the menstrual cycle. One possible

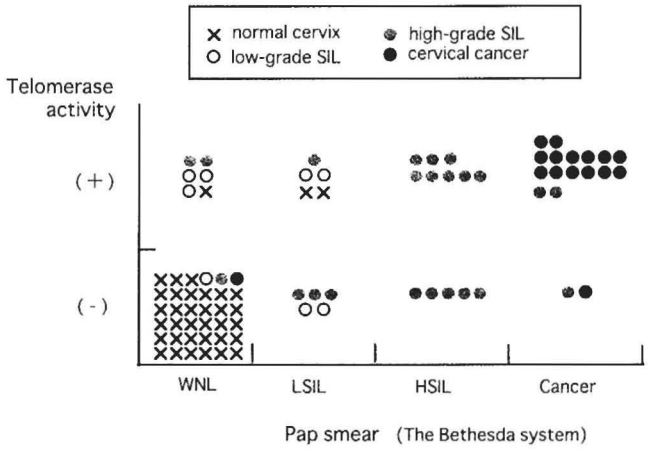

Fig. 7. Correlation between findings of smear test and telomerase activity in cervical scraping samples. Scraping samples from cervical lesions were tested by both telomerase assay and Papanicolau (Pap) smear test. About 90% of samples from cervical cancers exhibited telomerase activity, while less than 10% of those from normal cervix did. A total of 50-60% of samples from squamous intraepithelial lesions were also telomerase-positive. There was a good correlation between results of the two tests, but some discordant cases were observed. WNL: within normal limits; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade intraepithelial lesion.

way to distinguish the telomerase activity of cancerous and non-cancerous lesions may be quantification of activity levels. Quantitative telomerase assays were able to distinguish telomerase activity in cervical cancers from that in SILs since most SILs exhibited significantly lower telomerase activity than cervical cancers (Kyo et al., 1998a) (Fig. 7). However, there are differences in assay sensitivity among laboratories, and it remains unclear how to establish a cut-off value.

Samples obtained from non-invasive or minimally invasive procedures are also available for telomerase assays. Various sources of samples, such as sedimented cells in voided urine, oral rinses, colon washes, fine needle aspirates from lymph nodes, pancreatic juice, cervical scrapes, and peritoneal and pleural fluids have been used for telomerase assays, and have yielded significantly high sensitivity in cancer screening (Califano et al., 1996; Sugino et al., 1996; Kyo et al., 1997b; Suehara et al., 1997; Yoshida et al., 1997; Kavaler et al., 1998; Yang et al., 1998). We used the telomerase assay for screening of cervical lesions using cervical scraping samples (Kyo et al., 1997b). Approximately 90% of samples from cervical cancer patients exhibited telomerase activity while less than 10% of those from normal cervices did (Fig. 8). Interestingly, 50-60% of samples from squamous intraepithelial lesions also expressed modest telomerase activity. There was a strong correlation between results of telomerase assay and those of cytological examination (Papanicolau smear test). However, some discordant cases were found, such as telomerase-positive SILs with negative cytology or telomerase-negative SILs with positive cytology. The combination of both methods will improve the sensitivity and specificity of cancer screening.

Since expression of hTERT is closely correlated with telomerase activity, it may also be useful for cancer screening and diagnosis. Studies using RT-PCR assays have shown that detection of hTERT mRNA in surgical or biopsied samples as well as those obtained by non-invasive procedures is a sensitive method for cancer screening (Ito et al., 1998; Takakura et al., 1998). We used sedimented cells from voided urine of patients with or without bladder cancers. Approximately 80% of samples from patients with bladder cancers exhibited hTERT mRNA expression, while only 4% of samples from patients without bladder lesions did.

Telomerase as a target for cancer therapy

The finding that telomerase activation is present in the majority of a wide variety of tumors but not in normal cells suggests that telomerase activity may be a logical target in the development of cancer therapies. Several therapeutic approaches that target telomerase are currently being explored. The most popular strategies at present involve inhibition of telomerase activity in tumor

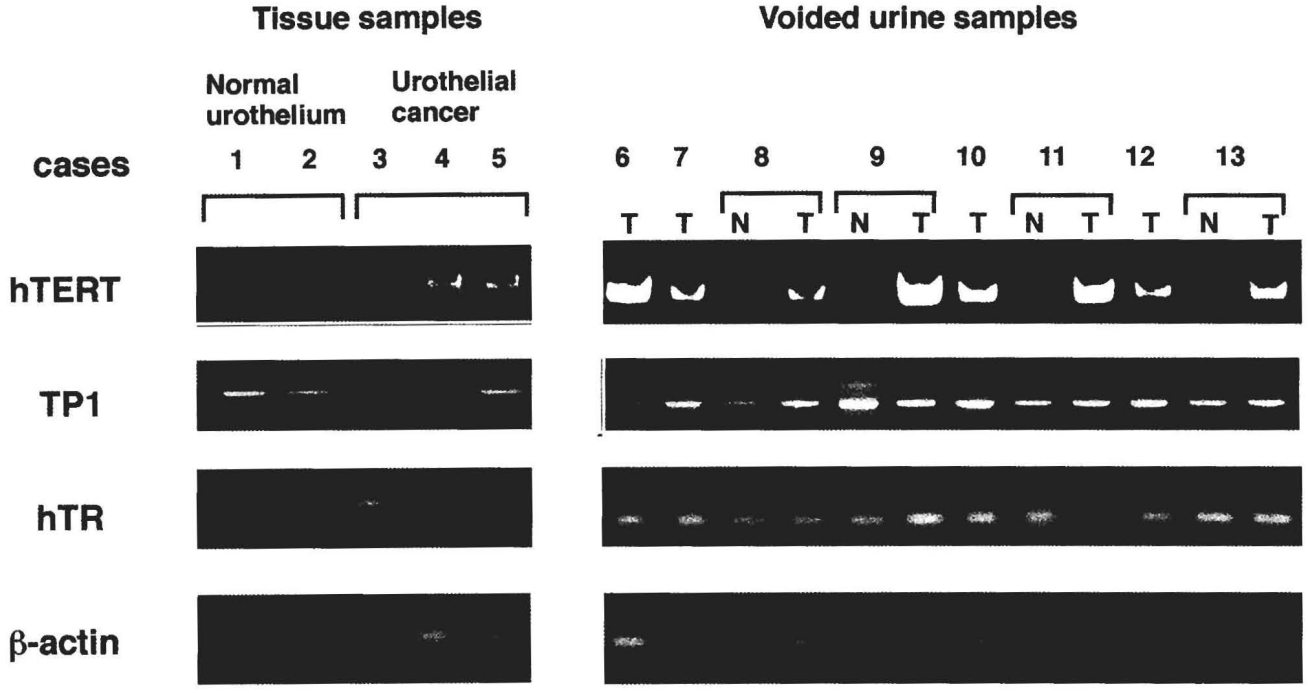

Fig. 8. Expression of telomerase subunits in urothelial tissues and voided urine. Urothelial tissues or voided urine samples from patients with or without bladder cancers were examined for telomerase subunit expression. RT-PCR analyses revealed that expression of hTERT mRNA was specific to cancer tissues (cases 3-5) while TP1 and hTR were expressed in both cancer and normal tissues (cases 1 and 2). Sedimented cells from voided urine samples were also available for the detection of subunit expression. hTERT mRNA was detected in 80% of urine samples from patients with bladder cancers (cases 6-13: T), but in less than 5% of urine samples from normal urothelia (cases 8, 9, 11, 13: N), suggesting that expression of hTERT in urine samples may be a useful diagnostic marker for bladder cancer. T: tumors; N: normal.

cells, based on the concept that telomerase inhibition leads to telomere erosion beyond threshhold levels for cellular senescence before the tumor burden kills the patient. Alternatively, it is possible that interfering with telomerase activity induces some other mechanisms of regulation of cell growth, such as apoptosis, which may prohibit extended survival of tumors. The initial trials of telomerase inhibition were performed using reverse transcriptase inhibitors such as AZT (azidothymidine). AZT treatment led to significant telomere shortening and decreased telomerase activity in some cancer cell lines (Strahl and Blackburn, 1996). A novel approach toward achieving the inhibition of telomerase is to target its substrate, the telomere. Telomere-interacting agents have been designed considering the unique DNA secondary structures associated with the telomerase extension reaction. One such structure is the guanine-rich sequences termed G-quadruplex which are formed by folding of the single-stranded G-rich overhang produced by telomerase activity. This structure is thought to play an important role in the dissociation and realignment of telomerase during the process of telomere synthesis. Several G-quadruplex interacting agents such as cationic porphyrins have been examined and found to repress telomerase activity and cell growth (Sun et al., 1997; Izbicka et al., 1999). On the other hand, since one strand of the telomere is rich in guanine nucleotides, telomeres might be sensitive to chemotherapeutic agents that target guanine. Cisplatin is a sequence-specific (GpG) intrastrand cross-linking agent, and telomeres are potential targets of it based on its affinity for G-rich regions. A study demonstrated that telomeres were markedly shortened and degraded in cancer cells treated with cisplatin (Ishibashi and Lippard, 1998). There are other points at which interventions could be devised, based on how telomerase is regulated in cells. Tankyrase, which inhibits the binding of TRF-1 to telomeres via its poly(adenosine diphosphate-ribose) polymerase (PARP) activity and facilitates binding of telomerase to telomeres, could be as effective a target for inhibitors as telomerase itself (Smith et al., 1998) (Fig. 2).

Antisense strategies for human telomerase RNA (hTR) have initially been attempted as gene therapy targeting telomerase complex proteins. Antisense expression of hTR template sequences leads to significant inhibition of proliferation of some cancer cells (Feng et al., 1995). Modified strategies have recently been developed using special antisense oligonucleotids against hTR, the 2'-*O*-Alkyl-RNAs, a second-generation class of oligonucleotids (Pitts and Corey, 1998). 2'-*O*-Alkyl-RNAs-based oligomers effectively and sequence-selectively inhibited telomerase upon transfection of human prostate cancer cells using cationic lipids. Antisense oligonucleotides linked to 2', 5'-oligoadenylate (2-5A), an activator of a single-strand- specific endonuclease, also successfully inhibited telomerase activity in human glioma cells (Kondo et al., 1998). Dramatic growth inhibition was observed for cancer cells treated with these antisense oligo-nucleotides, but not for normal cells. Surprisingly, a significant effect on cell growth appeared only 5-7 days after treatment with 2-5A antisense olgonucleotides. This is inconsistent with the developing concept that cell death is related to telomere erosion, since these cells would not have undergone a

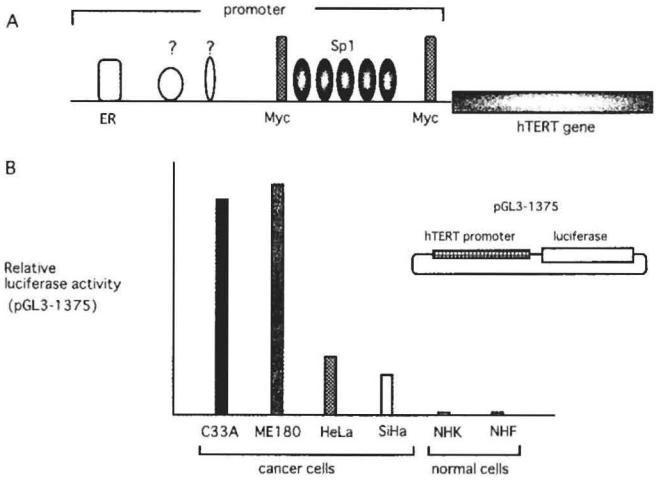

Fig. 9. Transcriptional regulation of hTERT gene. **A.** Transciption factors interacting with promoter sequences of the hTERT gene have been identified. Two c-Myc and five Sp1 sites are located around the transcription start site. These two factors were found to play critical roles in directly activating hTERT transcription. Estrogen receptor (ER) also plays a role in transactivation under stimulation by estrogen. Other regulatory factors may be identified. **B.** Transcriptional activity of hTERT promoter was evaluated in a variety of cell types by luciferase assay using hTERT promoter-luciferase reporter plasmids (pGL3-1375). Luciferase activities relative to control activity obtained with positive control reporter plasmid (pGL3-control) are shown for each cell type. hTERT promoter was active in cancer cells but not in normal cells. NHK: normal foreskin keratinocye; NHF: Normal skin fibroblasts.

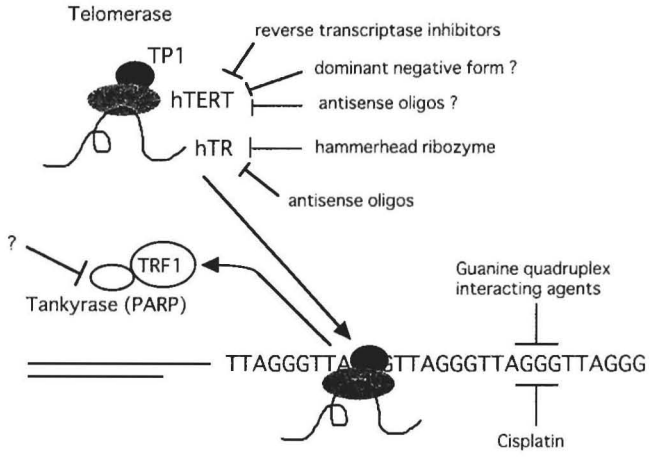

Fig. 10. Strategies for telomerase inhibition. Possible methods of telomerase inhibition based on current understanding of the structure and function of telomeres and the telomerase complex. PARP: poly(ADP-ribose)polymerase.

sufficient number of cell divisions to significantly shorten their telomeres. Thus, these findings suggest the novel concept that interfering with telomerase activity can affect other aspects of cell proliferation rather than telomere length. Hammerhead ribozyme, a catalytic RNA molecule that catalyzes and cuts specific RNA sequences, has also been used to inhibit hTR expression (Yokoyama et al., 1998). Introduction of hammerhead ribozymes designed to target the template region of hTR into cancer cell lines resulted in significant reduction of telomerase activity and hTR expression in cancer cells.Expression of a dominant-negative form of hTERT in tumor cells have also been attempted and succeeded in complete inhibition of telomerase activity, reduction in telomere length and death of tumor cells (Hahn et al., 1999).

Recently, some laboratories, including our own, have succeeded in cloning promoter sequences of the hTERT gene (Cong et al., 1999, Takakura et al., 1999). There have been extensive efforts to identify the regulatory region of the promoter. Of particular importance is identification of transcription factors interacting with such regions and regulating hTERT expression. c-Myc has been shown to be a direct transactivator of the hTERT gene (Greenberg et al., 1999; Wu et al., 1999; Kyo et al., 2000). An antisense strategy against c-Myc has been successful in inhibiting telomerase activity in leukemia cells (Fujimoto and Takahashi, 1997). Transient expression assays using hTERT-promoter reporter plasmids revealed that hTERT promoter is active in immortalized and cancer cells but not in normal cells, suggesting tumor-specific activity of hTERT promoter (Takakura et al., 1999). These findings may be applied to development of strategies for cancer gene therapy. When hTERT promoter is cloned into plasmids or virus vectors upstream of cDNA of specific genes which negatively regulate cell growth, such as suicide genes or apoptosis-inducing genes, introduction of these chimeric vectors into cells may lead to tumor-specific growth inhibition or cell death.

It might also be important to consider the side effects of telomerase-inhibition on telomerase-expressing normal cells. However, since normal cells are likely to contain longer telomeres than those of cancer cells, telomeres of cancer cells probably reach to a threshhold level for cellular senescence earlier than those of normal cells. Duration of treatment and timing of withdrawal may thus be critical for telomerase inhibition to be specifically effective in cancer cells. Genetic instability is a possible side effect of telomerase inhibitor and should be considered. Since the shortened telomeres of cancer cells are believed to result in increased genomic instability by causing end-to-end fusion or loss of heterozygosity and anueploidy (Griffith et al., 1999), treatment with telomerase inhibitors may in turn elicit clonal expansion of resistant tumor cells with such abnormalities. Fortunately, however, the effects of telomerase inhibition on cell growth appear to be quicker than expected, as shown above; we can therefore hope that telomerase inhibitors will be effective before genomic instability occurs due to eroded telomeres.

Concluding remarks

Recent experimental advances have clarified the roles of telomerase in cellular immortalization and carcinogenesis. Extensive analyses are now in progress concerning the mechanisms of regulation in telomerase activity. In particular, transcriptional regulation of hTERT has been vigorously analysed, and the molecular mechanisms by which telomerase is activated during human carcinogenesis will be resolved in the not-too-distant future. With knowledge of these mechanisms, novel approaches to inhibit telomerase activity will be developed and applied to cancer gene therapy. Future clinical trials will determine whether telomerase inhibition is a viable approach to reducing the mortality associated with advanced cancer.

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