U. ESKİOCAK

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INVESTIGATION OF TELOMERASE ACTIVITY IN DIAGNOSIS OF ENDOMETRIAL AND CERVICAL CANCER

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INVESTIGATION OF TELOMERASE ACTIVITY IN DIAGNOSIS OF ENDOMETRIAL AND CERVICAL CANCER

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UĞUR ESKİOCAK

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

INVESTIGATION OF TELOMERASE ACTIVITY IN DIAGNOSIS OF ENDOMETRIAL AND CERVICAL CANCER

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Human telomerase is a ribonucleoprotein complex that adds hexameric TTAGGG repeats to the ends of chromosomes in order to prevent their shortening. Telomerase activity has been evaluated for its diagnostic and prognostic value in cancer since it is observed in most malignancies but not in most normal somatic tissues.

In this study telomerase activity was examined in tumor specimens obtained from cervix, endometrium and their non-cancerous regions by an improved telomeric repeat amplification protocol (TRAP) – silver staining assay. Appearance of characteristic TRAP leader with 6 base pair increments indicate a positive result and was observed in all cancerous and some of the non-cancerous tissues. Telomerase activities of carcinoma tissues and normal counterparts were compared by densitometric analysis after PCR. Significantly higher telomerase activity was observed in cervical carcinoma samples compared to normal adjacent tissue. No significant difference was observed between endometrium carcinomas and normal endometrial tissue in terms of telomerase activity.

High telomerase activity in normal endometrium restricts the use of assay for detection of carcinogenesis. However, in cervical tissues an accurate quantification of telomerase activity by TRAP – silver stain assay may be valuable as a confirmatory assay.

Keywords: Telomerase, cervical cancer, endometrial cancer, TRAP assay

ÖZ

ENDOMETRİYUM VE SERVİKS KANSERLERİNİN TANISINDA TELOMERAZ AKTİVİTESİNİN İNCELENMESİ

Eskiocak, Uğur
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İnsan telomeraz enzimi, kromozomların kısalmasını engellemek için telomerlere TTAGGG ardışık tekrarlarını ekleyen ve yapısında RNA bulunduran bir enzimdir. Normal dokuların bir çoğunda telomeraz aktivitesi gözlenmezken, bir çok tümör dokusunda aktivite gözlenmesi, telomerazın kanser tanı ve prognozunda bir gösterge olarak kullanılabilmesine olanak sağlar.

Bu çalışmada tümörlü serviks, endometriyum ve çevrelerindeki normal doku örneklerinde, geliştirilmiş TRAP (telomeric repeat amplification protocol) – gümüş boyama yöntemiyle telomeraz aktivitesi belirlenmiştir. İzlenen protokolde telomeraz aktivitesinin göstergesi olan karakteristik 6 bç'lik aralıklarla artan bantlar, kanserli dokuların hemen hepsinde, normal dokuların bazılarında gözlenmiştir. Kanserli dokularla çevresindeki normal dokuların telomeraz aktiviteleri densitometrik yöntemle karşılaştırılmıştır. Servikal karsinom örneklerinde, çevresindeki normal dokularla karşılaştırıldığında, istatistiksel olarak daha yüksek telomeraz aktivitesi gözlenmiştir.

Normal ve kanserli endometriyum dokularında telomeraz aktivite düzeyleri karşılaştırıdığında istatistiksel olarak anlamlı bir fark bulunamamıştır. Normal endometriyumda gözlenen telomeraz aktivite düzeyleri bu yöntemin endometriyum kanserinde kullanımı kısıtılamaktadır. Ancak, serviks kanserinde TRAP – gümüş boyama yöntemiyle telomeraz aktivite düzeylerinin belirlenmesi bu kanserin tanısında yardımcı bir yöntem olarak kullanılabileceğine işaret etmektedir.

Keywords: Telomeraz, serviks kanseri, endometrium kanseri, TRAP yöntemi

To My Family

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ALT	Alternative Lengthening of Telomeres
BSA	Bovine Serum Albumin
bp	base pair
DEPC	Diethylpyrocarbonate
dNTP	Deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
FIGO	Federation of Gynecologists and Obstetricians
IC	Internal Control
kb	kilobase
M1	Mortality Stage 1
M2	Mortality Stage 2
NHEJ	Non-Homologous End-Joining
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
POT1	Protection of Telomeres 1
RTA	Relative Telomerase Activity
RNP	Ribonucleoprotein
Rpm	Rotation per minute
TBE	Template Boundary Element
TEMED	N, N, N, N'-Tetramethylethylene diamine
TER	Telomerase RNA
TERT	Telomerase Reverse Transcriptase
TIN2	TRF1 interacting protein 2
TP	Telomerase Products
TRAP	Telomeric Repeat Amplification Protocol
TRD	Telomere Rapid Deletion
TRF	Tlomere Repeat Binding Factor
UV	Ultra Violet

CHAPTER I

INTRODUCTION

1.1. Telomeres

1.1.1. Structure of Telomeres

Telomeres are specialized structures protecting the ends of linear chromosomes. They were first observed in *Drosophila* in a genetic analysis of chromosome terminal deletions and inversions (Muller, 1938). Telomeres are composed of double-stranded short repeats and/or single-stranded G-rich tails that also contain telomere-binding proteins (Haussman *et al.*, 2003).

Telomeres in eukaryotes consist of 6-8 bp tandemly repeating DNA sequences (Blackburn, 1991). The first identified telomeric repeat sequence (TTGGGG) belongs to *Tetrahymena* telomeres (Blackburn *et al.*, 1978). The sequence of the repeat for humans and other vertebrates is TTAGGG (Moyzis *et al.*, 1988; Morin *et al.*, 1989). These G-rich telomeric sequences makes about one part in 3000 by weight of human genome (Blackburn, 1990).

Southern blot analysis of the chromosome terminal restriction fragments (TRFs) revealed that the number of repeats per telomere varies among the different chromosomes of the same cell, among the same chromosomes in different cells, and among organisms (de Lange *et al.*, 1990; Starling *et al.*, 1990; Murnane *et al.*, 1994). In human telomeres TTAGGG repeats extends up to 10 to 15 kb (Greider, 1991; Blackburn, 1991).

Telomeric sequences are not packed into nucleosomes because they associate with non-histone structural proteins (Blackburn, 1990). Telomere binding proteins can be categorized in to two groups: those that bind to double stranded telomeres, e.g. Rap1p and Taz1p in yeast (Shore *et al.*, 1987; Cooper *et al.*, 1997), TRF1 and TRF2 (telomere repeat binding factor) in humans (Chong *et al.*, 1995; Brocolli *et al.*, 1997), and those that bind to single stranded overhang cdc 13p in yeast (Nugent *et al.*, 1996), POT1 (protection of telomeres) in humans (Baumann *et al.*, 2001).

Human telomeres (Figure 1.1.A) developed strategies to protect its G rich 3' overhang from nuclease degradation. One such strategy is the formation of T-loop/D-loop structure (telomere loop and displacement loop, respectively; Griffith *et al.*, 1999) with the involvement of telomere binding proteins and proteins that bridge between them (Figure 1.1.B). In this model free G strand overhangs are tucked back inside the double-stranded DNA several kilobases away from the end to form the big T-loop and the single-strand tails overrun the double-stranded region to form the smaller D-loop. The T-loop structure is dynamic and reforms after each round of DNA synthesis (Griffith *et al.*, 1999). It has been shown that TRF2 alone has the ability to form loop structures when telomeric substrates are given *in vitro* suggesting that it is the main player of the protein complex that mediates T-loop formation *in vivo* (Griffith *et al.*, 1999; Stansel *et al.*, 2001). Two proteins, PTOP and Tin2 help to stabilize T-loop by connecting TRF1 and TRF2 with POT1 (Colgin *et al.*, 2004). The growing list of proteins that are directly or indirectly associated with telomere binding proteins indicates the importance of these players for telomere maintenance.

Another approach to protect G-strand overhang involves folding of the overhang in to a four-stranded G-quadruplex structure (Parkinson *et al.*, 2002; Figure 1.1.C). The formation of thermodynamically stable G-quadruplex conformation has been well documented *in vitro* (Yu *et al.*, 2006) and *in vivo* (Chang *et al.*, 2006). During the course of the cell cyle progression telomere caps take different forms (discussed above) to perform different functions; however the timing of the formation of these protective structures remains unclear.

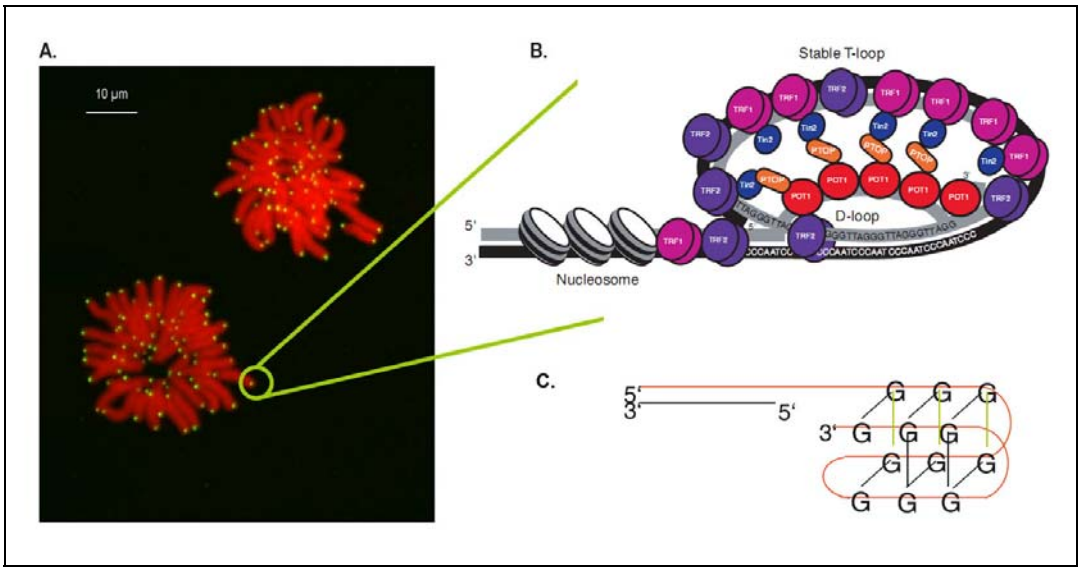

Figure 1.1. The end of the human chromosome. A) Extended focus (confocal) image of an embryonic mitotic murine cell (8-cellstage) after insitu hybridization with FITC-labeled telomere-specific PNA probes (green) and DNA counterstaining with propidiumiodide(red; Bekaert *et al.*, 2004). B) T-loop/D-loop structure (Colgin *et al.*, 2004). C) Schematic view of the G-quadruplex structure of the single-strand overhang (Boukamp *et al.*, 2007).

1.1.2. Functions of Telomeres

The first insights on the function of telomeres came 40 years after its discovery when scientists showed that telomeres protect the genetic material during replication (Blackburn and Gall, 1978). Later it was shown that protection of the genetic material by telomeres depends both on its specific DNA sequence (van Steensel *et al.*, 1998) and telomere binding proteins (Griffith *et al.*, 1999). For instance, inhibition of TRF2 results in the activation of the ATM/p53-dependent DNA damage check point pathway, and leads to cell cycle arrest and apoptosis (Karlseder *et al.*, 1999). Formation of T-loop/D-loop structure not only prevents recognition of chromosomal ends as damaged DNA but also provides a protection against nonhomologous end-joining (NHEJ), the direct rejoining of broken ends.

When telomeres are too short to form stable T-loop/D-loop structures NHEJ fuses two telomeres together, the result is a dicentric chromosome that is inherently unstable (McEachern *et al.*, 2000).

Telomeres also seem to play important roles in meiotic chromosome pairing, meiotic and mitotic chromosome segregation as well as in nuclear organization (Pandita *et al.*, 2007). Telomeres also repress the expression of genes placed near them (Aparicio *et al.*, 1991). However, the molecular mechanism of transcriptional silencing by telomeres is not clear.

1.1.3. Telomere Dynamics

Another unique property that distinguishes telomeres from the rest of the chromosomal DNA is the constant loss and replacement of telomeric DNA sequences in a cell cycle dependent manner. One of the reasons for telomere shortening is related with the DNA "end replication problem" (Olovnikov, 1996). Conventional DNA polymerases can only replicate DNA in the 5' to 3' direction, and they require free 3' hydroxyl group of an RNA primer to be able to synthesize DNA. The RNA primer is later removed and replaced by the DNA polymerase that synthesizes the upstream okazaki fragment. However the gap generated after the removal of the RNA primer at the very end of the lagging strand can not be filled because of the absence of a 3' hydroxyl group. Therefore some of the telomeric DNA will be lost after each replication, as observed with progressive cell division *in vitro* and ageing cells *in vivo* (de Lange *et al.*, 1990; Harley *et al.*, 1990; Hastie *et al.*, 1990; Lindsey *et al.*, 1991). This progressive shortening of telomeres acts as a molecular clock that monitors the replicative history of the cells (Figure 1.2; Wright and Shay, 1992).

The maximum number of times that a normal mammalian somatic cell can divide is known as the Hayflick limit (Hayflick, 1965) at which one or more critically shortened telomeres induce cell cycle exit, a stage referred as mortality stage 1 (M1) (Wright *et al.*, 1989; Pandita, 2002). M1 cell cycle check point can be

bypassed by inactivation of critical tumor suppressor genes such as p53 in which case the cells continue to divide and loose telomerase until mortality stage 2 (M2) (Counter *et al.*, 1992; Shay *et al.*, 1993) characterized by a crisis in which most of the cells die due to critically short telomeres. Rarely normal cells can escape from M2 by stabilizing their telomeres, and this leads to cellular immortalization.

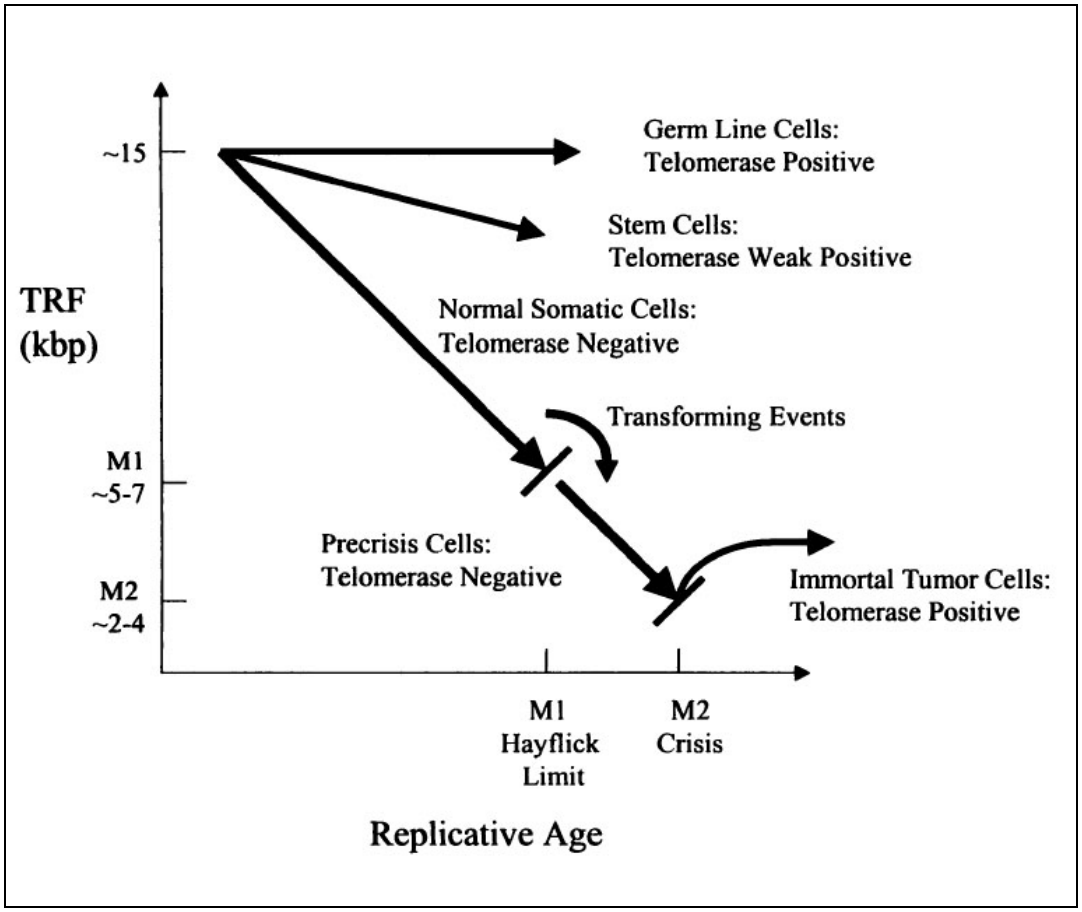

Figure 1.2. Two step hypothesis of cellular senescence and immortalization.

The end replication problem alone can not account for the total loss of telomeric DNA for two reasons. First, end replication problem suggests that only half of the chromosomes will have a 3' overhang since the DNA replication in leading strand will be complete. However, it has been shown that all of the chromosome ends have long 3' overhangs (Wellinger *et al.*, 1996; Makarov *et al.*, 1997). Second, the

DNA loss predicted by DNA end replication problem is the length of the RNA primer which is 8-12 bp, whereas the actual loss was shown to be longer, i.e. 77 bp per telomere per cycle in normal human fibroblasts (Makarov *et al.*, 1997). The differences between observed and the expected telomere loss can be explained by other mechanisms which can also shorten telomeric DNA. These mechanisms generally take place at a time different from DNA replication and are not affected by DNA polymerase inhibitors (Vermeesch *et al.*, 1993). In yeast the flap endonuclease Fen-1/Rad27p has been proposed to process telomeres in late S-phase to generate long 3' overhangs (Wellinger *et al.*, 1996). There is yet another mechanism that shortens telomeres through recombination events: telomere rapid deletion (TRD) which reduces over-elongated telomeres to wild-type size (Lustig, 2003).

There are two mechanisms that can replenish the telomeric loss: 1) active telomerase, a reverse transcriptase that synthesizes de novo telomeric repeats using an RNA template (Greider and Blackburn, 1989); 2) alternative lengthening of telomeres (ALT) pathway which is caused by homologous recombination events between telomeric repeats that are still unclear (Reddel *et al.*, 2001; Bailey *et al.*, 2004).

In human germ-line cells and most of the tumor cells telomere shortening and elongation mechanisms are well balanced that there is no net loss of telomeric DNA. However, telomere shortening mechanisms dominates in most of the normal somatic cells (Counter *et al.*, 1992; Kim *et al.*, 1994). Telomere dynamics are regulated at many levels. First, telomere binding proteins TRF1 and TRF2 are important determinants of telomere length. For instance, over expression of TRF1 or TRF2 results with shortening of telomeres even in telomerase positive human cells (van Stansel and de Lange, 1997; Smogorzewska *et al.*, 2000; Ancelin *et al.*, 2002). It has been shown that the amount of TRF1 at telomeres is proportional to its length (Loayza and de Lange, 2003) and the amount of TRF1 controls the action of telomerase in each telomere (van Stansel and de Lange, 1997). TRF2 on the other hand stimulates the forming of T-loops and thereby restricts access of

telomerase to telomeres (Stansel *et al.*, 2001). Similarly, POT1 masks the 3' overhang and makes them inaccessible to telomerase and thus probably inhibits telomere elongation (Lei *et al.*, 2004). Recently it has been shown that POT1-TPP1 complex can both positively and negatively regulate telomerase access to telomeres (Xin *et al.*, 2007) more over the same complex increases the activity and processivity of the human telomerase core enzyme (Wang *et al.*, 2007).

Second, proteins that interact with telomere binding proteins are also involved in regulating telomere lengths. For instance, tankyrase is a TRF1 binding protein that ADP-ribosylate TRF1 and thereby diminish its telomere binding ability (Smith *et al.*, 1998). There is yet another protein, TIN2 (TRF1 interacting protein 2), that binds to TRF1 and protects it from tankyrase action and there by regulates the access of telomerase to telomeres (Kim *et al.*, 1999). PINX1 is another TRF1 binding protein that is shown to bind hTERT and directly inhibit its activity *in vitro* (Zhou and Lu, 2001). Third, the possible G-quadruplex formation at the chromosome ends acts as a negative regulator by blocking telomerase access to 3' overhang (Zahler *et al.*, 1991). Fourth, telomerase itself is tightly regulated (discussed below). Finally, centromere position effect and repetitive subtelomeric DNA sequences can affect telomere lengths (Slijepcevic, 1998).

1.2. Telomerase

Telomerase is an enzyme that maintains the telomeric repeats at the ends of chromosomes. It was first discovered in *Tetrahymena* (Greider and Blackburn, 1985), and later in several other eukaryotes including Oxytricha, Euplotes, yeast, mice, human, etc. (Greider, 1996; Morin 1989; Cohn and Blackburn 1995).

1.2.1. Structure of Telomerase

Telomerase is a ribonucleoprotein (RNP) complex whose activity depends both on its RNA and protein components, which was confirmed by the loss of its activity either by RNase or heat pre-treatment (Greider and Blackburn, 1987; Morin, 1989).

The structure of the human telomerase was elicited very recently and is composed of two molecules each of human Telomerase Reverse Transcriptase (hTERT), Telomerase RNA (hTER) and dyskerin (Cohen *et al.*, 2007).

The telomerase RNA (TER) component was first identified in *Tetrahymena* (Greider and Blackburn, 1987). Presence of a complementary region on TR to telomeric repeats suggested that the RNA might serve as a template for telomeric DNA synthesis (Greider and Blackburn, 1987) which was later confirmed by the mutational studies on the RNA template and observation of the corresponding mutant telomeric DNA (Feng *et al.*, 1995). Although they share an essential and conserved function, TERs vary greatly between species in terms of size and DNA sequence (Greider, 1996; Table 1.1. Five bases at the 3' end of the template region of TER in humans (hTER) binds to 3' ends of the telomeric ends where as the other 6 bases located at 5' of hTER is the templating domain which specifies the telomeric sequence (Gilley and Blackburn, 1996).

Despite of the divergent sequence composition and length of the TER among species, the secondary structure is quite conserved (Figure 1.3; Chen and Greider, 2004). In addition to the template, all TERs contain a 5' template boundary element (TBE) and a large loop that includes the template, a potential pseudoknot and a loop-closing helical region (Theimer and Feigon, 2006). hTER has also H/ACA domain that is missing in lower eukaryotes but shown to be essential for hTER accumulation, hTER 3' processing and telomerase activity in cells (Dez *et al.*, 2001; Mitchell and Collins 2000; Dragon *et al.*, 2000). hTER is constitutively expressed in all tissues regardless of telomerase activity (Avilion *et al.*, 1996), with cancer cells generally having several folds higher expression than normal cells (Yi *et al.*, 1999).

Table 1.1. Telomerase RNA Component

Organism	Telomere Sequence	RNA template Sequence	RNA length
Tetrahymena	TTGGGG	CAACCCCAA	160
Euplotes	TTTTGGGG	CAAAACCCCAAAACC	190
Oxytricha	TTTTGGGG	CAAAACCCCAAAACC	190
Human	TTAGGG	CUAACCCUAAC	450
Mouse	TTAGGG	CCUAACCCU	450
S. cerevisiae	TG(1-3)	CACCACACCCACACAC	1300
K. lactis	TTTGATTAGGTATGT-GGTGTCGGA	UCAAAUUUCCGUACACC-AAUACCUAAUCAAA	1300

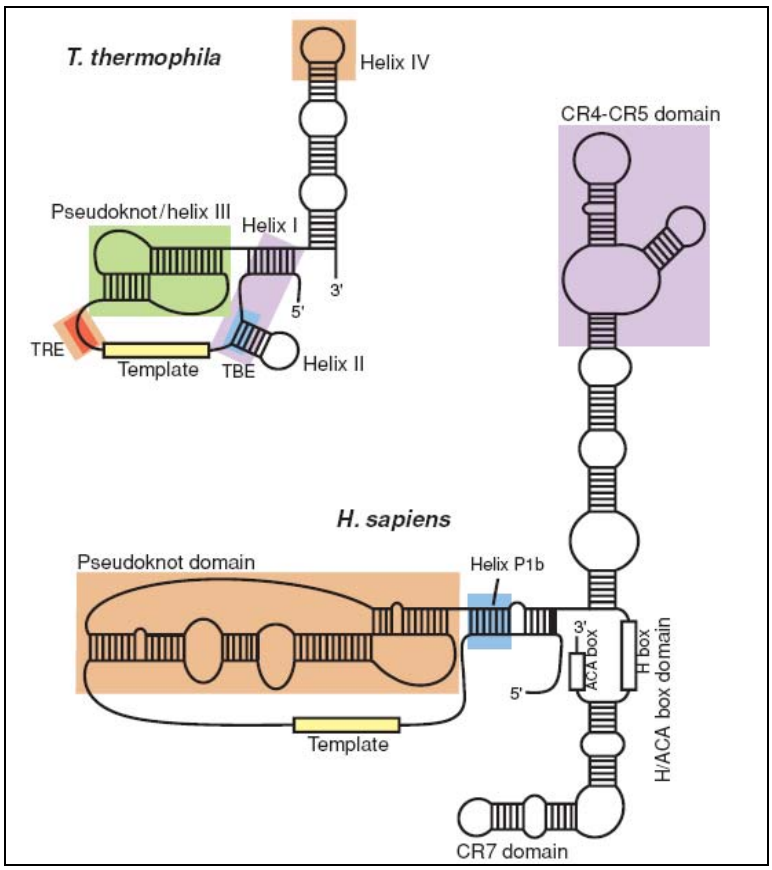

Figure 1.3. Structures of telomerase RNAs. Secondary structures of *T. thermophila* and *H. sapiens* telomerase RNAs are illustrated. Template regions (yellow), main TERT-binding regions (highlighted in purple boxes), and template boundary regulating elements (boxed in blue) are indicated.

The catalytic subunit of telomerase was first purified biochemically from *Saccharomyces cerevisiae* (Lendvay *et al.*, 1996) and subsequently in *Euplotes aediculatus* (Lingner *et al.*, 1997). Based on the conserved sequence information with in the reverse transcriptase motifs the human homologue of the catalytic subunit of telomerase (hTERT) was identified (Harrington *et al.*, 1997; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). Structure and organization of TERTs from different organisms are strikingly similar (Figure 1.4). They all contain 7 universally conserved reverse transcriptase motives in the central region of the protein. In addition to RT domain, they also have telomerase-specific T motif, a long N terminal extension and a short C-terminal extension.

Although hTER and hTERT is shown to be sufficient to reconstitute catalytic activity *in vitro*, size measurements of human telomerase have indicated a complex larger than expected for a composition of one hTERT (127 kD) and one hTR (153 kD) (Schnapp *et al.*, 1998; Wenz *et al.*, 2001). Recently catalytically active form of human telomerase has been successfully isolated from various cell lines (cell lines (MCF-7, A2182, HCT-116, TE-85, HT- 1080, and HEK-293, derived from cancers of the breast, lung, colon, bone, and connective tissue, and from embryonic kidney cells, respectively) and out of at least 32 distinct telomerase associated proteins dysekrin is the only one that is found to be present in the core cataytic complex with hTERT and hTER (Cohen *et al.*, 2007). Previously it has been reported that mutations in dyskerin resulted in poor telomere maintenance and lower telomerase activity, and the data supported an association of dyskerin with telomerase (Mitchell *et al.*, 1999). Dyskerin is a putative pseudouridine synthase within the class of H/ACA box ribonucleoproteins (Heiss *et al.*, 1998). Other proteins that are shown to associate with telomerase may be involved in its biogenesis, trafficking, recruitment to the telomere, and degradation (Cohen *et al.*, 2007).

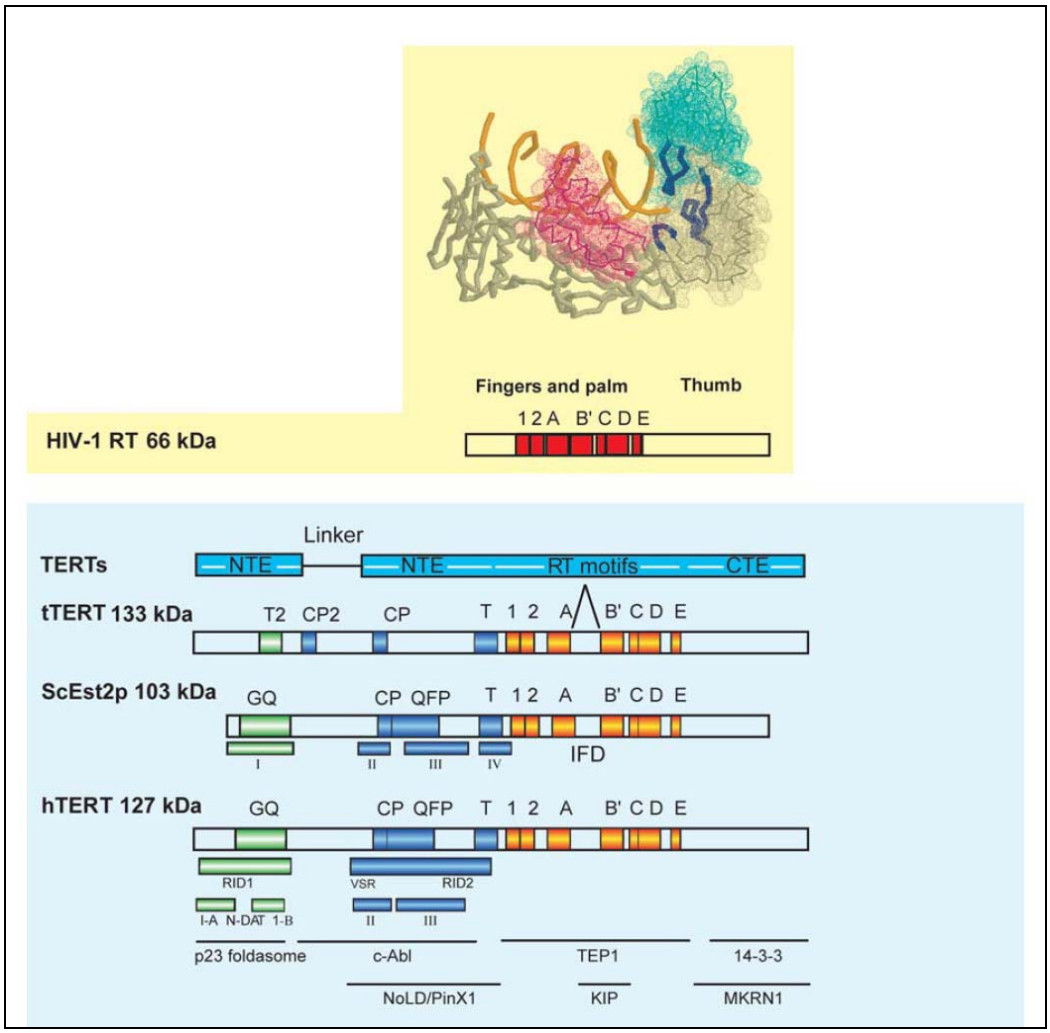

Figure 1.4. Structure and organization of TERTs. The organization of TERT is illustrated for the *Tetrahymena thermophila* (tTERT), *Saccharomyces cerevisiae* (ScEst2p), *Homo sapiens* (hTERT), *Plasmodium falciparum* (PfTERT), and *Caenorhabditis elegans* (CeTERT) proteins in comparison with HIV-1 RT. Features of TERTs include the RT motifs (1, 2, A, B', C, D, and E) and the telomerase-specific T motif and N- and C-terminal extensions (NTE, CTE). Regions of hTERT implicated in binding to other proteins are also illustrated. (Autexier and Lue, 2007).

1.2.2. Functions of Telomerase

Telomerase can be classified as a reverse transcriptase, because its mechanism of action involves the copying of an RNA template into DNA. However it is an unusual reverse transcriptase, because it contains its own RNA template as an integral part of the enzyme (Blackburn, 1992) and it has repeat addition processivity which is the ability of the enzyme to reverse transcribe a small segment of the template repetitively (Figure 1.5). The main function of telomerase, to extend 3' end of telomeres by de novo synthesis of the G-rich telomeric repeats, is catalyzed by a process involving binding of the telomeric DNA to the RNA template and to other domains of the RNP (i.e. anchor sites; Figure 1.5.a); successive addition of nucleotides to the 3' end of the DNA (nucleotide addition processivity; Figure 1.5.b); translocation and repositioning of the 3' end of the DNA upon reaching the 5' template boundary (Figure 1.5.c); and subsequent rounds of elongation (Figure 1.5.d).

In addition to reverse transcription activity, substantial evidence suggests that telomerase also have intrinsic nuclease activity that removes nucleotides from the 3' terminus of the chromosomal strand prior to telomere synthesis (Collins and Greider, 1993; Cohn and Blackburn, 1995; Greene *et al.*, 1998; Huard and Autexier, 2004). It has been proposed that nuclease activity enhances the fidelity of telomerase (Huard and Autexier, 2004). However, preferential removal of mismatched nucleotides by nuclease activity of telomerase has not been experimentally proved. Another proposed role of nucleolytic cleavage is the rescue of the stalled or arrested complexes by generating a substrate that can be processively elongated (Huard and Autexier, 2004).

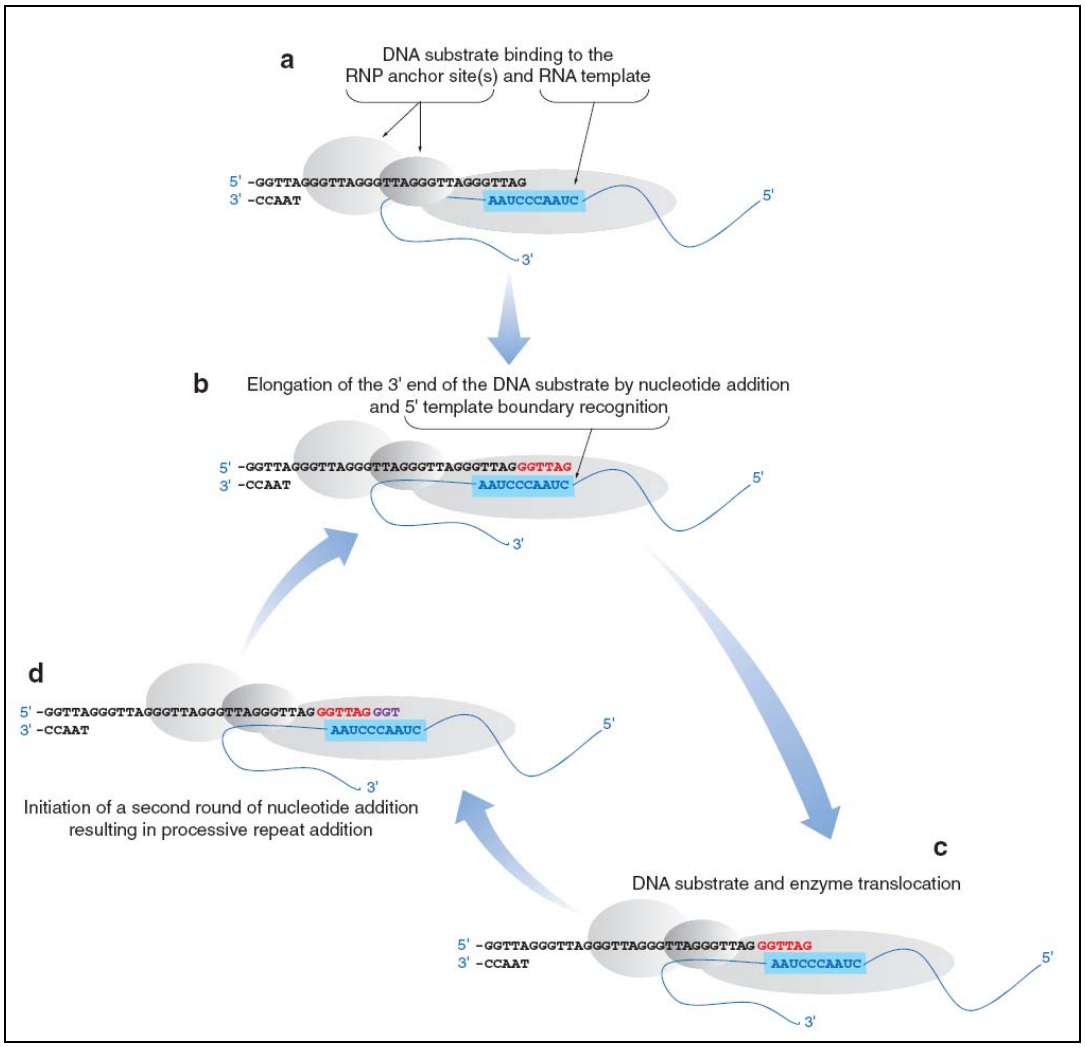

Figure 1.5. Model for processive elongation by telomerase (Autexier and Lue, 2006).

1.2.3. Regulation of Telomerase

Telomerase activity is regulated in many levels including transcription, mRNA splicing, maturation and modifications of hTER and hTERT, transport and subcellular localization of each component, and accessibility of telomerase to telomeres (discussed above). The rate limiting factor for telomerase activity, hTERT, is primarily under transcriptional regulation (Meyerson *et al.*, 1997;

Nakamura *et al.*, 1997). The hTERT gene, located on human chromosome 5p (Meyerson *et al.*, 1997), has been found to be differentially spliced (Kilian *et al.*, 1997). Alternatively spliced forms include the full-length transcript; the α spliced transcript, which lacks 36 nucleotides from the 5' end of exon 6; the β spliced transcript, which lacks exon 7 and exon 8; both α and β spliced transcripts; and insertional alternative transcripts, including transcripts with a 159-nucleotide insertion of intron 14, a 38-nucleotide insertion of intron 4, a partial insertion of intron 14, and the replacement of exon 15 and part of exon 16 with the first 600 nucleotides of intron 14. Although all of the transcripts are observed in a development and tissue dependent manner, it should be noted that only the full length transcript is associated with telomerase activity (Ulaner *et al.*, 1998; Ulaner *et al.*, 2000).

Presence of many transcription factor binding sites at hTERT promoter suggests that the regulation of hTERT expression is subject to multiple levels of control by different controls in different cellular contexts (Cong *et al.*, 1999). For example, the hTERT promoter contains the Myc/Mad binding site (E-box) and is a direct transcriptional target of c-Myc (Wang *et al.*, 1998; Wu *et al.*, 1999). Consistently overexpression of Mad 1, a c-Myc antagonist, suppresses hTERT expression (Oh *et al.*, 2000). Another inducer of telomerase activity primary human keratinocytes and mammary epithelial cells is human papillomavirus 16 E6 protein (Klingelhutz *et al.*, 1996). Steroid sex hormones also regulate telomerase. For instance, estrogen activates telomerase in mammary epithelial cells that express the estrogen receptor by both direct and indirect effects on the hTERT promoter (Kyo *et al.*, 1999), and androgen signaling appears to upregulate hTERT expression (Guo *et al.*, 2003).

It has been proposed that lack of telomerase activity in most somatic human cells is due to transcriptional repression of the hTERT gene. Accordingly, transfer of some chromosomes from normal somatic cells to telomerase positive cells resulted with the repression of telomerase activity (Oshimura and Barrett, 1997). Tumor suppressor pathways other than Mad1 have also been identified as negative regulators of hTERT transcription: p53, acting through Sp1; TGFB, acting through

SIP1; Menin, binding directly to the hTERT promoter (Lin and Elledge, 2003). Wilms' tumor 1, pRB and E2F also negatively regulate hTERT transcription (Cong *et al.*, 2002). It is also reported that BCL2 modulates telomerase activity (Mandal and Kumar, 1997) with an unknown mechanism.

The observation that normal ovarian and tissues and uterine uterine leiomyoma cells has no detectable telomerase activity despite of expression of both hTER and hTERT (Ulaner *et al.*, 2000) suggests the involvement of post-translational modifications as an additional layer of control of telomerase activity. Evidence suggests that phosphorylation hTERT by PKCa or AKT/PKB activates whereas phosphorylation by c-Abl inhibits human telomerase activity (Cong *et al.*, 2002). Induction of telomerase activity by kinases is believed to be a result of phosphorylation-dependent translocation of hTERT from cytoplasm to its functional nuclear compartment (Minamino *et al.*, 2001).

1.2.4. Telomerase and Cancer

Immortality, an important characteristic of cancer cells, is achieved by activation of telomerase in over %85 of human tumors with various origins (Shay and Wright, 1996). During tumorigenesis activation of telomerase in a clone of the cells, give the cells the proliferative capacity to accumulate necessary mutations to become malignant (Holt *et al.*, 1996). It should be noted that the cells containing telomerase activity is potentially immortal but not necessarily malignant. However, some tumors maintain telomere lengths by ALT mechanism are also immortal (Bryan *et al.*, 1997).

Growing number of studies suggest that telomerase contributes to cancer development beyond its effects on immortalization and telomere length. For example, the expression of an oncogenic RAS allele in an ALT cell line that expresses the SV40 ER but lacks telomerase activity failed to permit tumor formation when injected into sublethally irradiated, immunodeficient nude mice (Stewart *et al.*, 2002). However, co-expressing functional hTERT in this, the non-

tumorigenic RAS-expressing ALT cell line converted such cells into cells capable for growing as tumors in mice. Similarly, murine tumors derived from mTerc null mice that exhibit an ALT phenotype were markedly deficient in their ability to metastasize after tail vein injection when compared to tumors in which mTerc was reintroduced (Chang *et al.*, 2003). Taken together, these findings suggest that telomerase contributes to tumorigenicity independent of its effects on telomere length.

1.2.5. Clinical Significance of Telomerase in Cancer

The development of highly sensitive PCR based telomerase assay, Telomeric Repeat Amplification Protocol (TRAP), enabled detection of telomerase activity from low amount of human cells (Kim *et al.*, 1994). Telomerase is shown to be reactivated in over 85% of human cancers (Kim *et al.*, 1994; Shay and Wright, 1996; Shay and Bacchetti, 1997). If this activation occurs early during tumorigenesis, then it may be useful for early detection and diagnosis. However, if it is a late event, then it may be useful for staging and prognosis.

Cancers in which there is evidence that activation occurs early include bladder (Yoshida *et al.*, 1997), liver (Tahara *et al.*, 1995; Lee *et al.*, 2004), head and neck (Mutirangura *et al.*, 1996; Mao *et al.*, 1996; Zhang *et al.*, 2001), lung (Yashima et al, 1997; Shibuya *et al.*, 2001), kidney (Mehle *et al.*, 1996; Segawa *et al.*, 1993), prostate (Sommerfeld *et al.*, 1996; Botchkina *et al.*, 2005), and thyroid (Umbricht *et al.*, 1997). However, in these tumors both telomerase positivity and the levels of telomerase activity did not correlate with markers known to have prognostic value.

Cancers in which there seems to be correlations between telomerase activity and tumor stage or prognostic factors include meningiomas (Demasters *et al.*, 1997; Simon *et al.*, 2000), neuroblastomas (Hiyama *et al.*, 1995; Poremba *et al.*, 1999), non-Hodgkins lymphoma (Norrback *et al.*, 1996), and leukemia (Ohyashiki *et al.*, 1997; Verstovsek., 2003). Conflicting results were reported for gastric and breast cancers. In gastric cancers Hiyama *et al.* reported that telomerase activity

correlated with tumor size, presence of metastasis to lymph nodes, and survival (Hiyama *et al.*, 1995) which was not observed in another study (Tahara *et al.*, 1995). In breast cancer, activity has been found in pre-malignant lesions and most studies has failed to find any correlation between telomerase activity and prognostic factors (Sugino *et al.*, 1996; Bednarek *et al.*, 1997; Nawaz *et al.*, 1997) where as Hiyama *et al.* observed significant correlation between lack of telomerase activity and early tumor stage (Hiyama *et al.*, 1996).

Peripheral blood leukocytes and bone marrow cells were the first normal tissues found to possess telomerase activity (Broccoli *et al.*, 1995; Counter *et al.*, 1995). In addition, blood telomerase has been detected in several other normal tissues. Typically, it is the mitotically active stem cells in a given tissue that express telomerase activity. For instance the proliferative basal layer of the skin was found to be telomerase positive, where as the quiescent dermis was not (Harle-Bachor and Boukamp, 1996). Consistent with these findings normal epithelial cells of various tissues also bears telomerase activity (Kunimura *et al.*, 1998; Colitz *et al.*, 1999). Other normal tissues that possess telomerase activity includes hair follicles (Taylor *et al.*, 1996; Ramirez *et al.*, 1997), vascular endothelial cells (Hisiao *et al.*, 1997), low portions of the crypt within the colonic mucosa (Hiyama *et al.*, 1996) as well as the endometrium of the uterus (Brien *et al.*, 1997; Kyo *et al.*, 1997; Saito *et al.*, 1997; Yokoyoma *et al.*, 1998; Bonatz *et al.*, 2001; Maida *et al.*, 2002; Nemos *et al.*, 2003).

1.3. Endometrium and Endometrial Cancer

1.3.1. Normal Anatomy and Function of the Endometirum

The uterus is divided into body (upper two-thirds) and cervix (Figure 1.6). The walls of the uterus are composed of a mucosal layer, the endometrium, and a fibromuscular layer, the myometrium. The endometrium consists of a simple columnar epithelium (ciliated cells and secretory cells) and an underlying thick connective tissue stroma. The mucosa is invaginated to form many simple tubular

uterine glands. The glands extend through the entire thickness of the stroma. The stromal cells of the endometrium are embedded in a network of reticular fibres. The endometrium is subject to cyclic changes that result in menstruation. Only the mucosa of the body of the uterus takes part in the menstrual cycle.

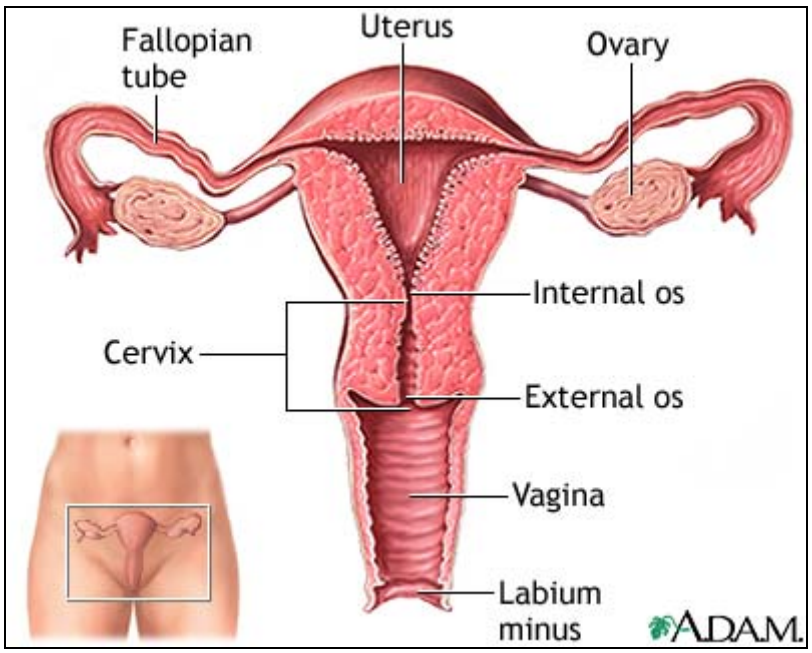

Figure 1.6. Female reproductive anatomy (Web site of MedlinePlus Medical Encylopedia).

Hormone changes during a woman's menstrual cycle cause the endometrium to change. During the menstrual cycle, the endometrium grows to a thick, blood vessel rich, glandular tissue layer. This represents an optimal environment for the implantation of a blastocyst upon its arrival in the uterus. During pregnancy, the glands and blood vessels in the endometrium further increase in size and number. Vascular spaces fuse and become interconnected, forming the placenta, which supplies oxygen and nutrition to the embryo and fetus.

1.3.2. Endometrial Cancer

Endometrial cancer accounted for 199 000 new cases (3.9% of cancers in women) and 50 000 deaths (1.7% of all cancer deaths in women) worldwide in 2002 (Sankaranarayanan and Ferlay, 2006). Endometrial cancer has a low incidence in the third world, as most other hormone-dependent tumors (Tkeshelashvili *et al.*, 1993). International variation in endometrial cancer rates may reflect the differences in distribution of known risk factors, like familiarity, obesity, diabetes, ovarian dysfunction, infertility, nulliparity, and tamoxifen use (Basile *et al.*, 2006).

Most endometrial cancers are adenocarcinomas, meaning that they originate from the single layer of epithelial cells which line the endometrium and form the endometrial glands. There are many microscopic subtypes of endometrial carcinoma, including the common endometrioid type, in which the cancer cells grow in patterns resembling normal endometrium, and the far more aggressive papillary serous and clear cell endometrial carcinomas.

Endometrial carcinomas can be classified into two pathogenetic groups (Bokhman, 1983). Type I cancers occur most commonly in pre- and peri-menopausal women and associates with increased exposure to estrogen. They are often minimally invasive into the underlying uterine wall, are of the low-grade endometrioid type, and carry a good prognosis. Type II cancers occur in older, post-menopausal women and are not associated with increased exposure to estrogen. They are typically of the high-grade endometrioid, papillary serous or clear cell types, and carry a generally poor prognosis.

In contrast to endometrial carcinomas, the uncommon endometrial stromal sarcomas are cancers which originate in the non-glandular connective tissue of the endometrium. Malignant mixed müllerian tumor is a rare endometrial cancer which contains cancerous cells of both glandular and connective tissue (Takano *et al.*, 2003).

1.3.3. Staging of Endometrial Cancer

The staging of endometrial carcinoma is done according to the Federation of Gynecologists and Obstetricians (FIGO) staging system, which is based on clinical examination. However, clinical staging of uterine MMMTs is unreliable; tumors are often upstaged after thorough surgical staging (Callister *et al.*, 2004). The staging procedure, although not defined by FIGO, typically includes hysterectomy, bilateral salpingo-oophorectomy, washings for cytologic evaluation, pelvic nodal sampling, para-aortic nodal sampling, and biopsy of any suspicious areas. Staging criteria is listed in Table 1.2.

Table 1.2. Staging of Endometrial Cancer (Benedet *et al.*, 2000)

Stage	
I	
IA	Tumor limited to endometrium
IB	Invasion to < 1/2 the myometrium
IC	Invasion to > 1/2 the myometrium
II	
IIA	Endocervical glandular involvement
IIB	Cervical stromal invasion
III	
IIIA	Tumor invades serosa and/or adnexa and/or positive peritoneal cytology
IIIB	Vaginal involvement
IIIC	Metastases to pelvic and/or para-aortic lymph nodes
IV	
IVA	Tumor invasion of bladder and/or bowel mucosa
IVB	Distant metastases including intra-abdominal and/or inguinal lymph nodes

1.4. Cervix and Cervical Cancer

1.4.1. Normal Anatomy and Function of the Cervix

The cervix (from Latin "neck") is the lower and narrow portion of the uterus that extends into the upper vagina (Fereneczy and Wright, 1994). It is cylindrical or conical in shape and protrudes through the upper anterior vaginal wall.

The part next to the vagina is the ectocervix. On average, the ectocervix is 3 cm long and 2.5 cm wide. The opening of the ectocervix is called the external os. The size and shape of the external os and the ectocervix varies widely with age, hormonal state, and whether the woman has had a vaginal birth. In women who have had a vaginal birth, the ectocervix appears bulkier and the external os appears wider. The part of the cervix closest to the body of the uterus is called the endocervix. The passageway between the external os and the uterine cavity is referred to as the endocervical canal which measures 7 to 8 mm at its widest in reproductive-aged women and terminates with internal os (Figure 1.6).

The main function of the cervix is to allow flow of menstrual blood from the uterus into the vagina and direct the sperms into the uterus during intercourse. The opening of the cervical canal is normally very narrow. However under the influence of the body hormones and the pressure from the fetal head, this opening widens to about 10 cm during labor, to allow the birth of a baby. If the opening is loose, as observed in some women, it can lead to miscarriages during pregnancy.

1.4.2. Cervical Cancer

Cervical cancer is the second most common cancer in women worldwide (Parkin *et al.*, 2001) and ranks third in Europe (Bray *et al.*, 2002). The incidence of invasive cervical cancer is decreasing worldwide because of the early detection of the premalignant lesions by cytological screening known as papnicolaou (PAP) smear and early treatment of the disease. It has been observed that screening women ages 35 to 64 for cervical cancer precursors by conventional cytology every 3 to 5 years

within high-quality programs reduces incidence of invasive cervical cancer by at least 80% among those screened (IARC, 2005).

Cervical cancer is one of the most preventable types of cancer. By reducing risk factors such as smoking, practicing unsafe sex, and using birth control pills for prolonged periods of time, women can reduce their chances of developing cervical cancer. As in most forms of cancer, cervical cancer will also metastasize to distal sites if it is left untreated. Typical sites of metastasis for cervical cancer are the aortic and mediastinal lymph nodes, the lungs, and the skeleton (Parkin *et al.*, 2002).

There are two main types of cervical cancers: squamous cell carcinomas arise from the squamous epithelium of the exocervix and adenocarcinoma which arise from the glandular lining of the endocervical canal. Approximately 80% to 90% of cervical cancers are squamous cell carcinomas and the remaining 10% to 20% of cervical cancers are adenocarcinomas. Rarely cervical cancers have features of both squamous cell carcinomas and adenocarcinomas in which case they are called adenosquamous carcinomas or mixed carcinomas.

1.4.3. Staging of Cervical Cancer

Cervical cancer is staged according to the FIGO staging system based on clinical examination. The stage is determined by a series of diagnostic tests including palpation, inspection, colposcopy, endocervical curettage, hysteroscopy, cystoscopy, proctoscopy, intravenous urography, and X-ray examination of the lungs and skeleton, and cervical conization. Staging criteria is listed in Table 1.3.

Table 1.3. Staging of Cervical Cancer (Benedet *et al.*, 2000)

Stage	
0	Carcinoma in situ
I	Cervical carcinoma confined to uterus (extension to corpus should be disregarded)
IA	Invasive carcinoma, diagnosed only by microscopy. All macroscopically visible lesions—even with superficial invasion—are stage IB/T1b. Stromal invasion with a maximum depth of 5 mm measured from the base of the epithelium and horizontal spread of 7 mm or less. Vascular space involvement, venous or lymphatic, does not affect classification.
IA1	Measured stromal invasion 3 mm or less and 7 mm or less in horizontal spread
IA2	Measured stromal invasion more than 3 mm and not more than 5 mm with a horizontal spread of 7 mm or less
IB	Clearly visible lesion confined to the cervix or microscopic lesion greater than T1a2/IA2
IB1	Clearly visible lesion 4 cm or less in greatest dimension
IB2	Clearly visible lesion more than 4 cm in greatest dimension
II	Cervical carcinoma invades beyond uterus but not to pelvic wall or to the lower third of vagina
IIA	Tumor without parametrial invasion
IIB	Tumor with parametrial invasion
III	Cervical carcinoma extends to the pelvic wall and/or involves lower third of vagina or causes hydronephrosis or nonfunctioning kidney
IIIA	Tumor involves lower third of the vagina, no extension to pelvic wall
IIIB	Tumor extends to pelvic wall or causes hydronephrosis or nonfunctioning kidney
IVA	Tumor invades mucosa of bladder or rectum and/or extends beyond true pelvis
IVB	Distant metastasis

1.5. Aim of the Study

In this study the aim is:

I) Optimization of a quantitative telomeric repeat amplification protocol (TRAP) – silver staining assay.

II) To detect telomerase activity from surgical specimens of endometrial and cervical caner and their adjacent non-neoplastic tissues.

III) To determine the clinical significance of telomerase activity in endometrial and cervical cancer.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

All Chemicals used were molecular biology grade. RNAlater®, bovine serum albumin (BSA), Tris-HCl, Tris-base, ethylenediaminetetraacetic acid (EDTA), ammonium persulfate (APS), silver nitrate and N,N′-(1,2-Dihydroxyethylene) bisacrylamide were from Sigma-Aldrich. Diethylpyrocarbonate (DEPC), Coomassie® Brilliant Blue G-250, acrylamide, glycerol and N,N,N′,N′-Tetramethylethylenediamine (TEMED) were purchased from Applichem. Sodium hydroxide and ethanol was from Riedel-de Haën; formaldehyde, phosphoric acid, boric acid and acetic acid from Merck, bromophenol blue from Bio-Rad and xylene cyanol from JT-Baker. Taq DNA polymerase, ribonuclease inhibitor and DNA ladder (GeneRuler® 100 bp DNA Ladder Plus) were from Fermentas. Deoxynucleoside triphosphate (dNTPs) was purchased from Promega. TRAPeze® telomerase detection kit was from Chemicon.

2.1.2. Patients

Fifteen tissue samples of endometrial cancer, 15 adjacent non-neoplastic endometrium, 17 tissue samples of cervical cancer and 10 adjacent non-neoplastic cervical tissue samples were obtained from 32 women who underwent hysterectomy or biopsy at Balcalı Hospital, Çukurova University Medical Center, Adana. All samples were collected in RNAlater storage solution, frozen in liquid nitrogen with in two hours after surgical removal and stored in liquid nitrogen until

protein extraction. The histological diagnosis was performed from the same specimens that were used for TRAP assay.

2.2. Methods

2.2.1. Staging and Classification of Cancer Specimens

The histopathology of endometrial and cervical cancer specimens were examined by a pathologist (Dr. H. Zeren, Department of Pathology, Çukurova University Medical Center, Adana) according to World Health Organization (WHO) specifications. The clinical staging of samples was according to the International Federation of Gynecology and Obsterics (FIGO) staging system.

2.2.2 Protein Extraction

The frozen tissue sample was sliced with a disposable surgical blade on sterile mortar. The sliced tissue (40-50 mg) was immediately frozen with liquid nitrogen and grinded to a fine powder with a matching pestle. The frozen powder was transferred to a 1.5 mL microcentrifuge tube containing 200µL ice-cold CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-Mercaptoethanol, 0.5% CHAPS, 10% Glycerol) supplied with the TRAPeze® telomerase detection kit and 40 unit of ribonuclease inhibitor. The suspension was then incubated on ice for 30 minutes and spun in a benchtop microcentrifuge at 12,000 X g for 20 minutes at 4°C. Aliquots of supernatant fluids were carefully collected and immediately stored at -80°C.

2.2.3. Determination of Protein Concentration

The serial dilutions of BSA standards were prepared in triplicate according to following table (Table 2.1)

Table 2.1. Preparation of protein standards.

Cuvette No	*1X Coomassie Solution (Appendix)*	*dH_2O*	*BSA (1 mg/mL)*
1 (blank)	5 mL	500 µL	0 µL
2 – 2` – 2``	5 mL	495 µL	5 µL
3 – 3` – 3``	5 mL	490 µL	10 µL
4 – 4` – 4``	5 mL	480 µL	20 µL
5 – 5` – 5``	5 mL	470 µL	30 µL
6 – 6` – 6``	5 mL	450 µL	50 µL
7 – 7` – 7``	5 mL	420 µL	80 µL

The tubes were vortexed and incubated at room temperature for 10 minutes. Then the absorbances were measured against the blank and a standard curve was obtained by plotting absorbance at 595 nm versus protein concentration.

2.2.4. Determination of Protein Concentration in Extracts

Sample tubes contained 10 µL of protein extract diluted in 490 µL dH_2O and mixed with 1X coomassie brilant blue solution (Appendix). After 10 min incubation at room temperature, absorbance at 595 nm was measured against blank. Trend line equation of the standard curve (y = mx + b, where y = absorbance at 595 nm and x = protein concentration) was used to calculate protein concentration according to following formula:

Protein concentration of extracts (µg/mL) = [(y-b)/m] X dilution factor

2.2.5. Telomerase Assay

Telomerase is an RNA dependent DNA polymerase therefore precautions were taken to eliminate contaminating ribonucleases which can cause false-negative

results. Pipette tips, microcentrifuge and PCR tubes, mortars and pestles were washed with active DEPC treated ddH_2O and kept under hood overnight for DEPC volatilization. All DEPC treated equipments were autoclaved and dried at 80°C before use.

The principle of the TRAP assay is illustrated in Figure 2-1. In the first step of the reaction telomerase adds hexameric GGTTAG repeats to the 3' end of the substrate oligonucleotide (TS). In the second step, elongated products are amplified with reverse primer (RP) to generate an incremental ladder of 6 bp starting form 50 bp. Furthermore, a primer (K1) and a template (TSK1) are added to each reaction to generate a 36 bp internal control (IC) which eliminates false-negatives and allows for more accurate quantitation.

Telomerase reaction and PCR were performed according to TRAPeze® telomerase detection kit manual. All reagents were thawed and kept on ice. A "master mix" was prepared by combining the following reagents in a 1.5 mL microcentrifuge tube: 5 µL of 10X TRAP reaction buffer (200 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA), 1 µL of 50 X dNTP mix (2.5 mM each of dATP, dTTP, dGTP, dCTP), 1 µL TS primer, 1 µL TRAP primer mix (RP primer, K1 primer, TSK1 template), 0.4 µl of Taq polymerase (5 units/µL) and 39.6 µL of PCR grade water. After the "master mix" was vortexed and spun briefly in a microcentrifuge, 48 µL of aliquots were dispensed into 0.5 mL PCR tubes. Two µL of one of the following was added to reaction mixture: protein extract (diluted to 0.2 µg/µL with CHAPS), heat-inactivated protein extract (negative control), CHAPS lysis buffer (negative control) or telomerase-positive cell extract (provided with the kit). The tubes were then incubated in thermocyler at 30°C for 30 min for the elongation of TS primer by telomerase and followed by 33 cycles of PCR amplification (94°C for 30 seconds, 59°C for 30 seconds and 72°C for 60 seconds).

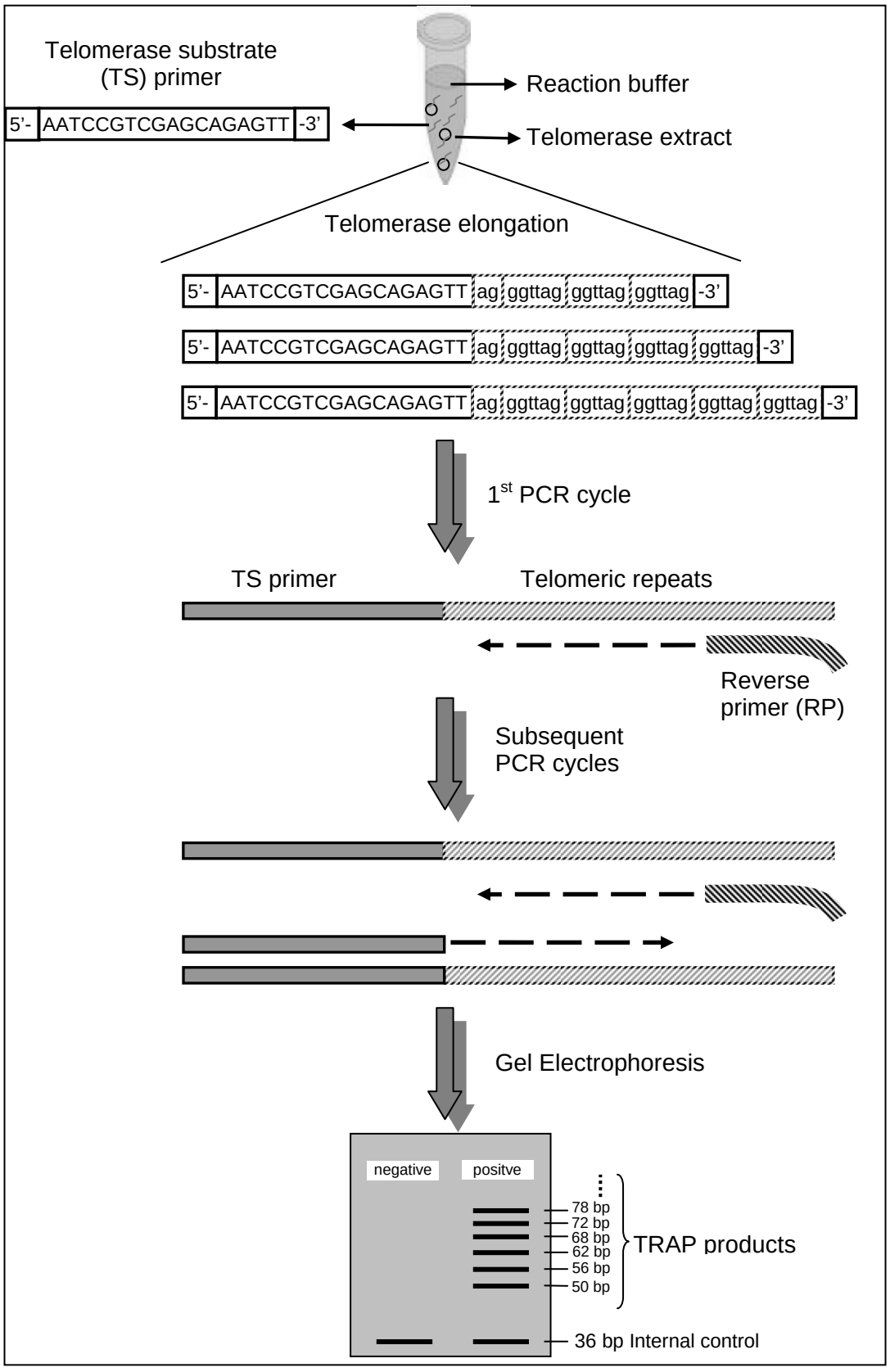

Figure 2.1. Schematic illustration or TRAP assay.

2.2.6. Polyacrylamide Gel Electrophoresis (PAGE)

TRAP reaction products were analyzed on a 1 mm 10 % non-denaturating PAGE containing 0.5X TBE. First, the glasses were set up using 1 mm thick spacers, comb and paper clips to hold them together. 50 mL gel solution was prepared by mixing 49.5 mL 10 % Polacrylamide stock in 0.5 X TBE (Appendix) and 0.5 mL 10 % APS. 5 mL of this solution was mixed with 15 µL TEMED and poured between glasses to form a layer at the bottom of the glasses that will block leakage. Remaining 45 mL gel solution was mixed with 45 µL TEMED and poured between glasses. The comb was placed and the gel was left for polymerization. Following polymerization, the comb and the lower spacer was removed. The gel was mounted into electrophoresis unit (Sigma-Techware) and the upper and lower reservoirs were filled with 0.5 X TBE buffer. Before loading the samples, the gel was pre-electrophoresed at 300 V for 10 minutes. 5 µL of loading dye (Appendix) was added to each TRAP reaction. The tubes were than vortexed and spun in a microcentrifuge. Twenty five µL of each reaction was loaded into each lane and the gel was electrophoresed at 300 V until the xylene cyanol ran 8-9 cm from the top of the gel.

2.2.7. Silver Staining of Polyacrylamide Gels

The glass plates were separated and the gel was soaked in to 300 mL fixing solution containing 10 % ethanol and 0.5 % acetic acid. Following 3-4 minutes incubation on a shaker the solution was poured and this step is repeated with fresh fixing solution. After pouring fixing solution, the gel was stained in 500 mL 0.1 % (w/v) silver nitrate for 15 min on the shaker. Then the silver nitrate solution was removed and the gel was incubated in 300 mL containing 1.5 % (w/v) sodium hydroxide and 0.15 % formaldehyde until all the bands are clear (~10 minutes).

2.2.8. Densitometric Analysis of Polyacrylamide Gels

A sample was considered positive for TA when characteristic ladder of TRAP products (TP) with 6 bp increments and a 36-bp internal control (IC) were observed and negative when only IC was present. The gel photographs were processed with ImageJ software (National Institutes of Health, Maryland, USA). Relative telomerase activity (RTA) was calculated by the following formula:

$$RTA = \frac{(X_{TP}/X_{IC})}{(Tel_{TP}/Tel_{IC})}$$

where densitometric band intensities were designated by X and Tel for the samples and telomerase positive control, respectively. Values for TP were obtained from the area containing all the bands of the 6 bp increment ladder starting from 50 bp. Whereas values for IC was obtained only from the densitometric signal of 36 bp band.

2.2.9. Statistical Analysis

The results were subject to statistical tests by using SPSS Software (SPSS Inc., Chicago, Illinois, USA) to determine significant difference between means of groups ($\alpha = 0.05$) .

CHAPTER III

RESULTS AND DISCUSSION

In this study, each sample was assigned a three character code starting with “C” for cervical or “E” for endometrial tissue, followed by a patient number in order of surgery and terminates with “T” for tumors or “N” for normal tissues.

3.1. Determination of Protein Concentrations in Tissue Extracts

Extracts were successfully prepared from each tissue and protein concentrations of samples were calculated according to procedure presented in section 2.2.2.3. As it can be seen in Table 3.1 each extract yielded sufficient amount of protein to make necessary dilutions.

3.2. TRAP-Silver Staining Assay

In telomerase positive samples both the characteristic TRAP ladder with six base pair increments starting from 50 bp and the 36 bp IC bands were clearly visible after staining with the modified technique, whereas only the IC was observed in telomerase negative samples as seen in Figure 3.1. The presence of an extra band between 36 bp internal control band and the TRAP ladder bands starting from 50 bp indicates that telomerase activity in the extracts are too high. The extra band does not affect overall detection of telomerase activity and it can be eliminated by diluting the extracts. Amplification of 36 bp internal control and TRAP products are competitive. Hence the lanes with strong telomerase ladder bands have weak internal control bands, whereas negative control lanes have strong internal control bands (Figure 3.1).

Table 3.1. Protein concentrations of tissue extracts

Code	Concentration (µg/mL)	Code	Concentration (µg/mL)	Code	Concentration (µg/mL)
E1T	3265	E10N	449	C6N	8547
E1N	4465	E11T	5946	C7T	1623
E2T	8261	E11N	3046	C7N	6805
E2N	4155	E12T	2250	C8T	5542
E3T	2661	E12N	1587	C8N	6339
E3N	3552	E13T	6952	C9T	2500
E4T	8120	E13N	4433	C10T	3230
E4N	4521	E14T	5071	C11T	3158
E5T	8260	E14N	4039	C11N	1219
E5N	3576	E15T	6735	C12T	4152
E6T	7771	E15N	3120	C12N	4044
E6N	6522	C1T	5402	C13T	1914
E7T	5617	C2T	4610	C13N	8139
E7N	6157	C2N	6531	C14T	8564
E8T	4372	C3T	5226	C15T	4170
E8N	4923	C4T	435	C15N	5085
E9T	4164	C5T	3818	C16T	6866
E9N	6614	C5N	350	C16T	7393
E10T	1479	C6T	8351	C17N	6005

Heat sensitivity of telomerase is used to demonstrate the specificity of the assay. Activity of telomerase was lost by heat treatment prior to assay (Figure 3.1).

Processivity of telomerase in the sample extracts can be estimated due to special design of reverse primer (RP) which is complementary to three telomeric repeats and contains a 3' cap to prevent telomerase elongation during PCR (Kim NW *et al*, 1997). Therefore, lengths of the products that we see on the gel are representatives of the length of the starting template and presence of x number of ladder bands indicate that the longest telomerase extended products have x+3 telomeric repeats. Three repeats are added to the observed number of products because the smallest product that can be amplified by RP should contain 3 telomeric repeats.

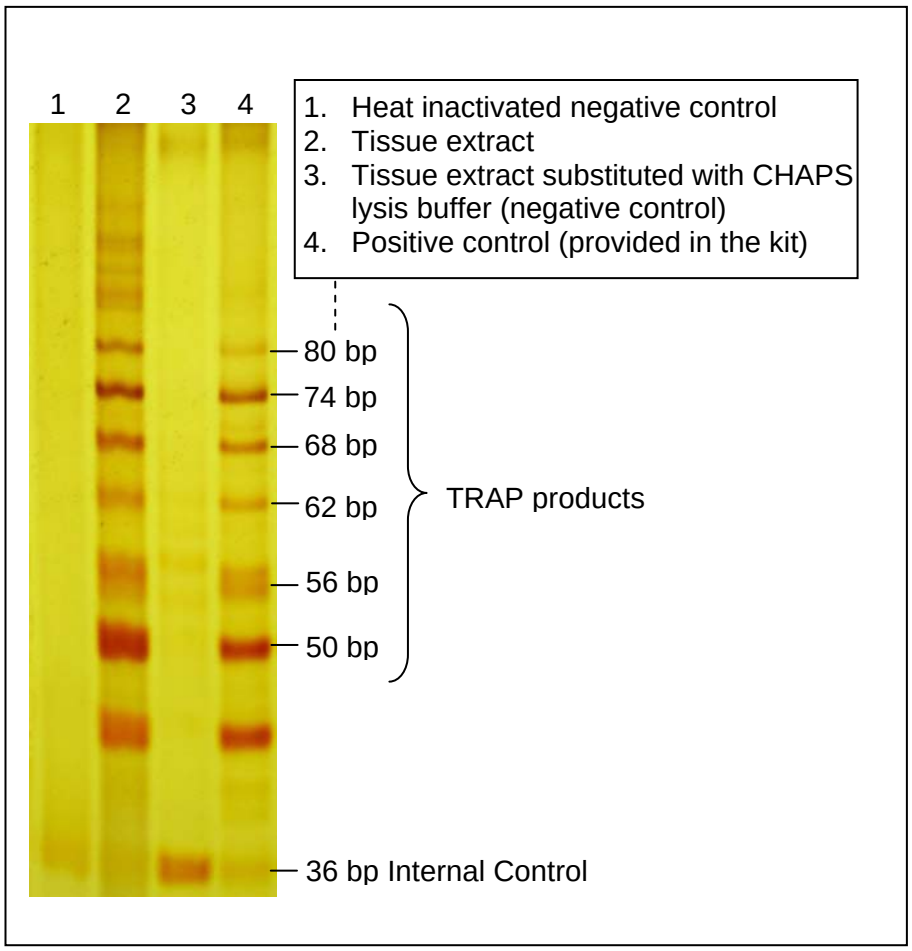

Figure 3.1. Test of positive and negative controls of the TRAP assay.

3.2.1. Optimization of TRAP-Silver Staining Assay

In order to have a reliable quantitative assay number of PCR cycles and protein content in the assay were optimized. Various staining conditions were also tried for the finest staining. Finally staining procedure was modified so that it required minumum time and chemicals compared to assay originally reported by Bassam *et al.* (1991). The modified TRAP – silver staining assay was a rapid, non-radioactive and low cost procedure developed to quantify telomerase activity from tissue samples.

3.2.1.1. Optimization of PCR Cycle Number

In a typical PCR reaction there are three stages: exponential, linear and plateau. For a PCR reaction to represent starting amount of target, measurements should be taken in exponential or linear phase. An extract which is shown to be positive (Figure 3.1, lane 2) was used in all tubes. RTA values were calculated as described in section 2.2.8. using densitometric analysis of the polyacrylamide gel shown in Figure 3.2.A. From the graph (Figure 3.2.B) 33 cycles were found to be appropriate for quantitative analysis.

3.2.1.2. Optimization of the Protein Content

Several dilutions of the positive extract (Figure 3.1, lane 2) were used to determine the optimum protein content for the assay. Telomerase activity was observable in extracts containing as low as 200 ng of protein (Figure 3.3.A, lane 3). Extract containing 20 ng protein (Figure 3.3.A, lane 2) and heat inactivated control (Figure 3.3.A, lane 8) yielded patterns of bands resembling PCR artifacts (as it is explained in the manufacturer's instructions). These were not regular TRAP ladder bands and thus they were considered as telomerase negative. Although occurring at low frequency, these artifacts are unavoidable even when the optimal assay conditions are employed and they usually occur in negative controls. Highest telomerase activity was not correlated with extract containing the highest amount of protein (4000 ng – Figure 3.2.A, lane 7). This can be explained by the presence of Taq DNA polymerase inhibitors in tumor samples (Kim NW *et al*, 1997). Increase in protein content results in an increase in Taq DNA Polymerase inhibitors which in turn decreases the efficiency of PCR. Even in such a case existence of the internal control accounts for an accurate quantification. Presence or absence of PCR inhibitors does not change their relative ratios since they are co-amplified in the same tube. These features of the assay are very useful for determining telomerase activity levels of clinical samples that may contain different amount of PCR inhibitors. Therefore dividend of the RTA formula (densitometric intensity ratio of TRAP products to internal control in samples) achieves normalization of each tube in the assay; where as divider (densitometric intensity ratio of TRAP products to

internal control in telomerase positive controls) provides normalization between assays.

There was a significant linear correlation between RTA and protein content (r = 0.984) within 200 – 800ng range (Figure 3.3.B). 400 ng was selected for further studies. All extracts were diluted to 200 ng/µL to obtain 400 ng protein in each assay.

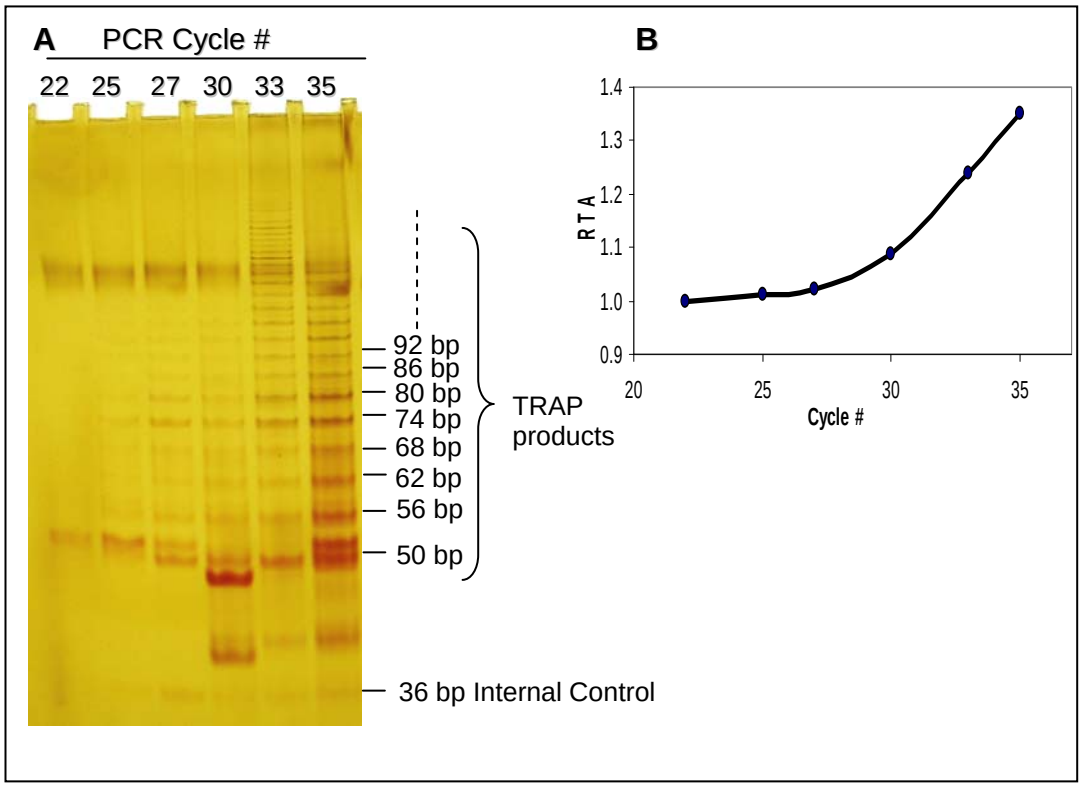

Figure 3.2. Optimization of cycle number for TRAP. A) TRAP products on 10% polyacrylamide gel with varying cell numbers. Number of cycles is shown at the top of the lanes. B) Change of RTA with varying number of PCR cycles. RTA values were calculated as described in section 2.2.8.

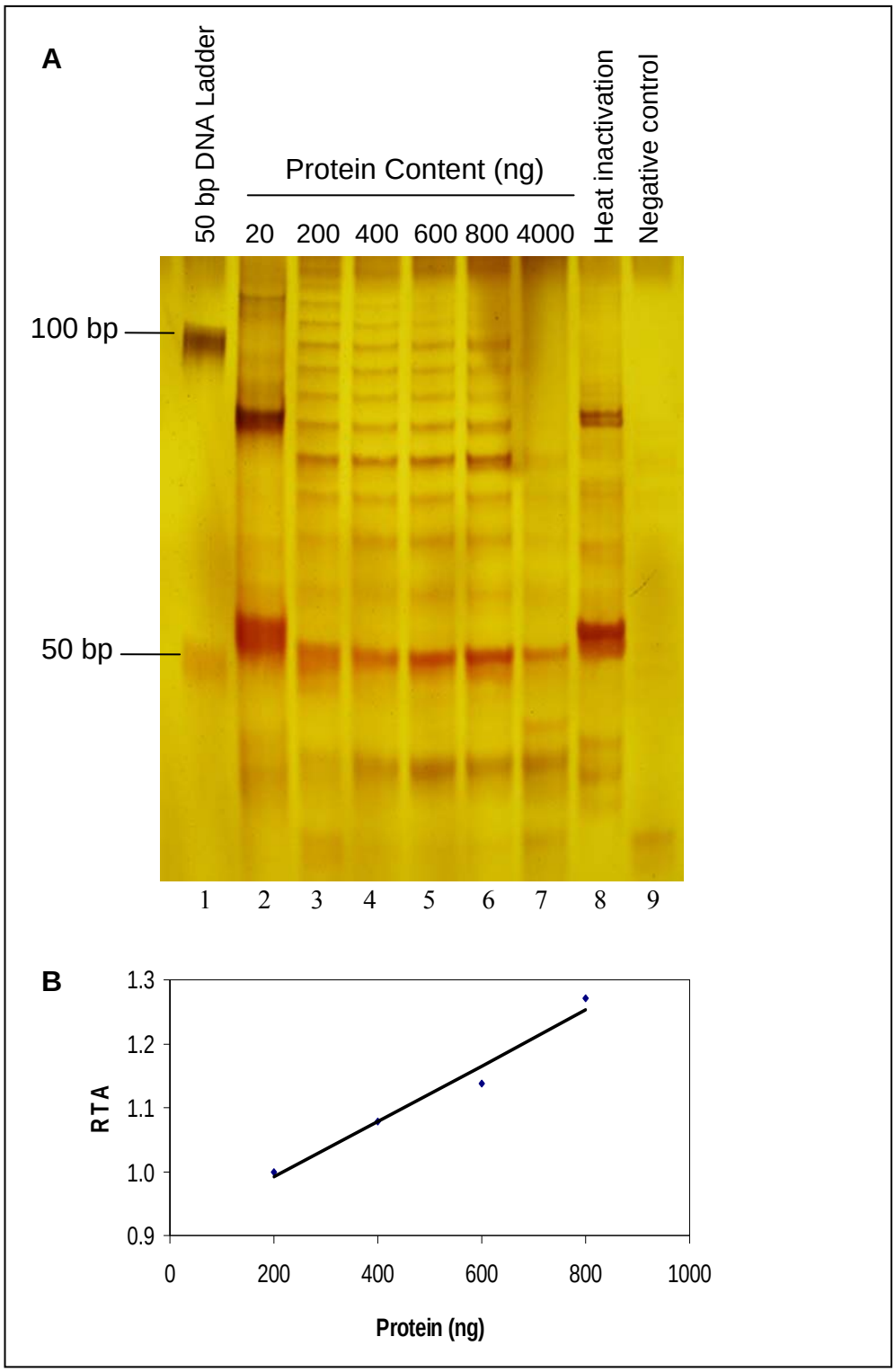

Figure 3.3. Optimization of protein content for TRAP. A) TRAP products on 10% polyacrylamide gel with varying protein contents in the assay indicated at the top of the

lanes. B) Change of RTA with varying protein content. RTA values were calculated as described in section 2.2.8.

3.3. Telomerase Activity in Tissue Samples

Optimized TRAP – silver staining assay was used for quantitative determination of telomerase activity in total of 57 tissue samples from 32 endometrial and cervical cancer patients. Some features of patients were summarized in Table 3.2.

Table 3.2. Some features of cancer patients

		Number	Percentage
Clinical Diagnosis			
	Endometrial Cancer	15	46.9%
	Cervical Cancer	17	53.1%
	Total	32	
Stage (FIGO)			
	I	1	3.3%
	II	21	70.0%
	III	8	26.7%
	Total	30	
Age			
	<40	6	18.8%
	40-60	17	53.1%
	>60	9	28.1%
	Total	32	

3.3.1. Telomerase Activity in Endometrial Cancer

A total of 15 cases of endometrial cancers were examined for telomerase activity. Two samples, one from carcinoma and the other from adjacent normal tissue was taken from each patient. The clinicopathological characteristics of these patients

were summarized in Table 3.3. In this study, endometrioid adenocarcinoma constituted the major histological subtype of endometrial cancer (Table 3.3), which is also the common histological subtype of endometrial cancer worldwide (Sankaranarayanan and Ferlay, 2006). Serous papillary adenocarcinoma, mucinous adenocarcinoma and clear cell carcinoma were other observed histological subtypes. Despite the clinical diagnosis, pathology was not observed in one of the patients. About 80% of all endometrium cancers used in this study was stage II and half of them were in 40-60 age group.

Clinicopathological data, presence or absence of telomerase activity and its quantitation is given in Table 3.4. Sample E2T was not considered for further analysis because it did not show pathology however it was positive for telomerase activity.

The first indication that telomerase activity was possessed by normal human tissues came from findings that blood leukocytes and bone marrow cells from normal donors had detectable levels of telomerase activity (Brocoli *et al.*, 1995; Counter *et al.*, 1995; Hiyama *et al.*, 1995). Telomerase activity in hematopoietic system was not restricted to the stem cells, but was also found in mature T-cells, B-cells, macrophages and granulocytes (Brocoli *et al.*, 1995; Hiyama *et al.*, 1995). Later on many other normal tissues including skin (Taylor *et al.*, 1996; Yasumoto *et al.*, 1996), hair follicles (Taylor *et al.*, 1996; Ramirez *et al.*, 1997), vascular endothelial cells (Hisiao *et al.*, 1997), low portions of the crypt within the colonic mucosa (Hiyama *et al.*, 1996) as well as the endometrium of the uterus (Brien *et al.*, 1997; Kyo *et al.*, 1997; Saito *et al.*, 1997; Yokoyoma *et al.*, 1998; Bonatz *et al.*, 2001; Maida *et al.*, 2002; Nemos *et al.*, 2003) were found to be telomerase positive.

Table 3.3. Summary of clinicopathological features of endometrial cancer

		Number	Percentage
Histological Subtype			
	NP	1	6.7%
	EA	10	66.7%
	SPA + CCC	1	6.7%
	EA + MA	2	13.3%
	EA + CCC	1	6.7%
	Total	15	
Stage (FIGO)			
	I	1	7.1%
	II	11	78.6%
	III	2	14.3%
	Total	14	
Age			
	<40	1	6.7%
	40-60	8	53.3%
	>60	6	40.0%
	Total	15	

NP: no pathology detected, EA: endometrioid adenocarcinoma, SPA: serous papillary adenocarcinoma, MA: mucinous adenocarcinoma, CCC: clear cell carcinoma.

Positive telomerase activity was detected in all cases of endometrial cancer and in 60% of normal adjacent tissue (Table 3.4 and 3.5). Positivity of telomerase in endometrial cancer found in this study (100%) is similar to others which detected telomerase activity in 19 out of 20 (95%; Brien *et al.*, 1997), 18 out of 22 (87%; Nagai *et al.*, 1998), 28 out of 28 (100%; Yokoyama *et al.*, 1998), 50 out of 53 (94%; Bonatz *et al.*, 2001), 12 out of 15 (80%; Maida *et al.*, 2002) and 11 out of 11 (100%; Nemos *et al.*, 2003) cases.

Table 3.4. Clinicopathological data in endometrial carcinomas

Code	Age	Histology	Myometrial invasion (%)	Stage	TA	RTA
E1T	63	EA	70	III	+	1.296346
E1N					-	-
E2T	58	NP			+	0.728674
E2N					+	0.692288
E3T	51	EA	40	II	+	0.896518
E3N					+	0.914736
E4T	67	EA	35	II	+	0.894065
E4N					-	-
E5T	55	EA	50	II	+	0.944752
E5N					+	0.860378
E6T	70	EA	68	III	+	1.929361
E6N					-	-
E7T	75	SPA + CCC	53	II	+	0.951731
E7N					-	-
E8T	58	EA + MA	18	II	+	1.121236
E8N					+	1.10322
E9T	57	EA + MA	75	II	+	1.092044
E9N					+	0.7888
E10T	57	EA	29	II	+	0.897437
E10N					+	0.876428
E11T	39	EA	50	II	+	1.827119
E11N					+	1.821613
E12T	60	EA	22	II	+	1.652132
E12N					+	1.587531
E13T	65	EA	30	I	+	1.948597
E13N					-	-
E14T	55	EA + CCC	45	II	+	2.58649
E14N					+	0.898107
E15T	75	EA	95	II	+	1.093981
E15N					-	-

TA: Telomerase activity, RTA: relative telomerase activity, NP: no pathology detected, EA: endometrioid adenocarcinoma, SPA: serous papillary adenocarcinoma, MA: mucinous adenocarcinoma, CCC: clear cell carcinoma.

Table 3.5. Positivity of telomerase activity in endometrium

Tissue	Telomerase activity		
	Negative	Positive	Total
Endometrial carcinoma	0	14 (100%)	14
Normal adjacent endometrium	6 (40%)	9 (60%)	15
Total			29

Telomerase activity has been reported in normal endometrium (Brien *et al.*, 1997; Kyo *et al.*, 1997; Saito *et al.*, 1997; Yokoyoma *et al.*, 1998; Bonatz *et al.*, 2001; Maida *et al.*, 2002; Nemos *et al.*, 2003) and was explained by the highly regenerative nature of the tissue. Telomerase was detected in 33 out of 49 (67%; Kyo *et al.*, 1997), 39 out of 44 (89%; Yokoyoma *et al.*, 1998) and 17 out of 71 (24% Maida *et al.*, 2002). The variation in these results could be explained by the pre- and post-menopausal frequencies of the sample population.

It has been shown that telomerase activity in premenopausal normal endometrium can easily be detected and its levels are comparable to that of endometrial cancers while postmenopausal normal endometrium has low and often undetectable activity (Kyo *et al.*, 1997). Our results were in accord with previous findings in which telomerase was detected in 20 out of 21 (95%; Kyo *et al.*,1997) and 10 out of 15 (67%; Maida *et al.*,2002) cases of proliferating endometrium. In this study all cases below the age of 60 showed telomerase activity whereas only one (%14) case above the age of 60 was positive for telomerase activity in normal endometrium (Table 3.6). Age and telomerase positivity seemed to be significantly related ($p=0.01$, Fisher's exact test) in normal endometrium. This may be the reason why telomerase activity was observed in the sample coded E2T (age: 58) although it didn't show pathology. However it should be noted that in this study telomerase activity in normal endometrium tissue adjacent to carcinoma was analyzed. The possibility of contamination of those tissues with cancer cells can not be excluded.

Table 3.6. Age of patients and telomerase activity in normal endometrium

Age Group	**Telomerase activity**		
	Negative	Positive	Total
<60	0	8 (100%)	8
>60	6 (86%)	1 (14%)	7
Total			15

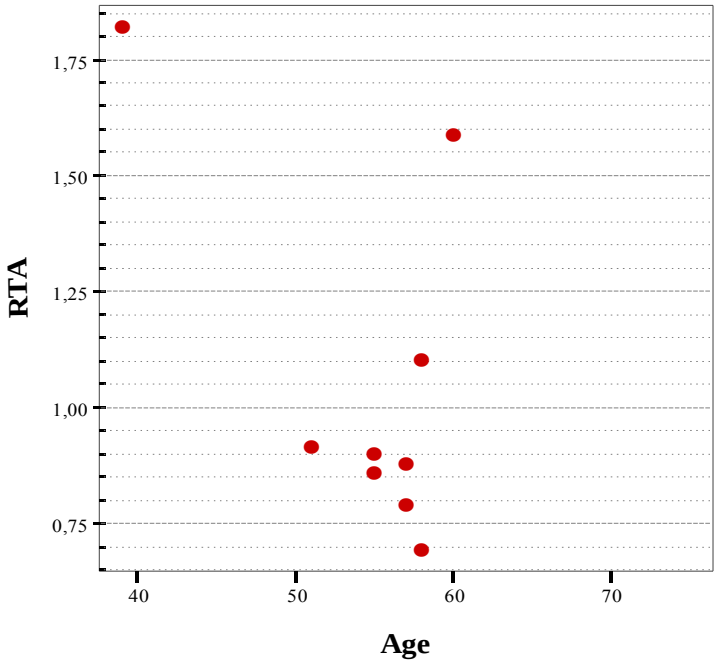

Figure 3.4. Relation of age and telomerase activity in normal endometrium.

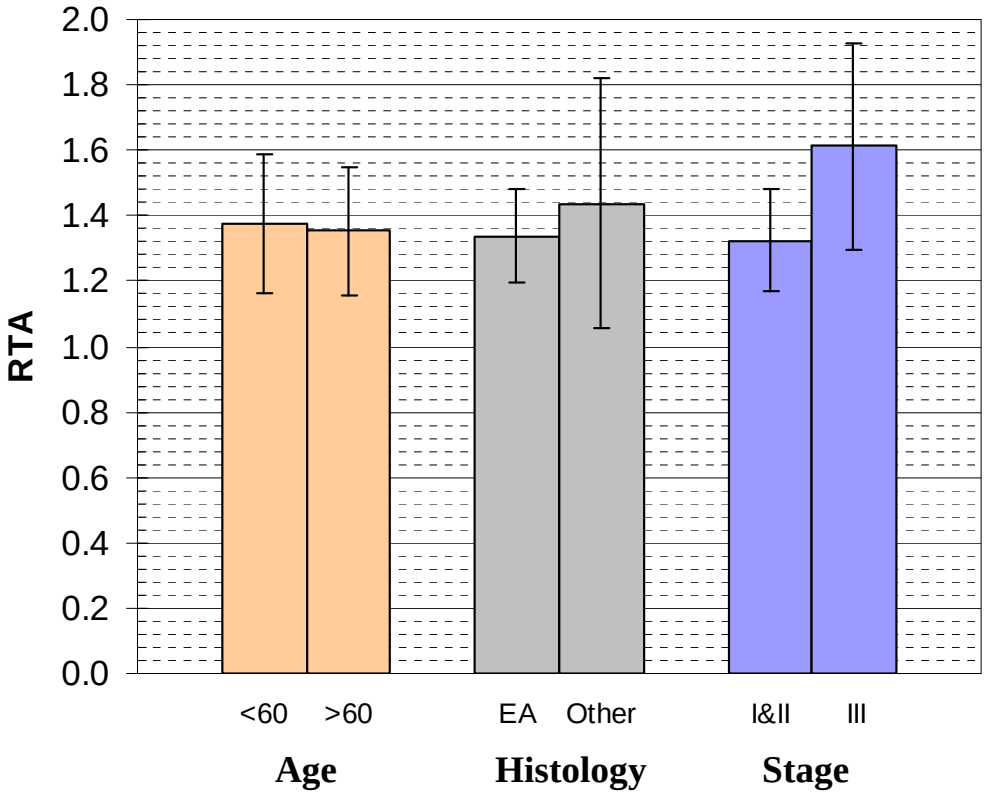

Figure 3.5. Quantitation of telomerase activity in different age, histology and stage groups of endometrial cancer. RTA values represented as means ± SEM.

Although high telomerase positivity was observed in cases younger than 60, telomerase activities were not higher (P=0.92). There was no correlation between RTA levels in normal endometrium and age of the patient (Figure 3.4, r=0.556). Concordantly, there was no significant difference in RTA levels of age groups for endometrial cancer tissues (Figure 3.5; p = 0.94, 2-tailed Student's *t*-test).

Telomerase activity was found in all histological subtypes of endometrial cancer. There was no significant difference in RTA levels between the major histological subtype (endometriod adenocarcinoma) and other subtypes (Figure 3.5; p=0.764, 2-tailed Student's *t*-test).

Telomerase activity could be detected in all stages of endometrial cancer suggesting that telomerase activation is an early event in carcinogenesis. Telomerase activation was also found to be an early event in other cancers: bladder (Yoshida *et al.*, 1997; Kyo *et al.*, 1997), liver (Tahara *et al.*, 1995; Lee *et al.*, 2004), head and neck (Mutirangura *et al.*, 1996; Mao *et al.*, 1996; Zhang *et al.*, 2001), lung (Yashima et al, 1997; Shibuya *et al.*, 2001), kidney (Mehle *et al.*, 1996; Segawa *et al.*, 1993), prostate (Sommerfeld *et al.*, 1996; Botchkina *et al.*, 2005), and thyroid (Umbricht *et al.*, 1997). However, the difference in RTA levels of samples from stage III and stage I-II patients was not significant (Figure 3.5; p=0.501, 2-tailed Student's *t*-test). There was only one case of stage I. Thus it was grouped with stage II to be able to perform statistical analysis.

Telomerase activity was observed in all cases showing myometrial invasion from 18% to 95%. However there was no correlation between RTA levels and degree of myometrial invasion (Figure 3.6, r=0.179).

There was no significant relationship between telomerase activity and clinopathological characteristics of endometrial cancer examined which supports previous findings (Nagai *et al.*, 1998 and Bonatz *et al.*, 2001). However the sample size may be too small for these observations to be conclusive.

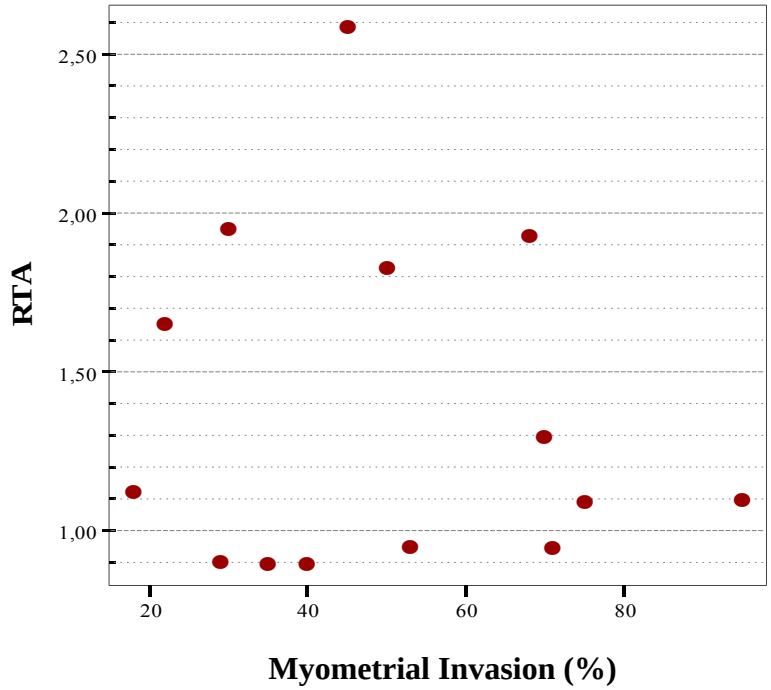

Figure 3.6. Myometrial invasion and telomerase activity in endometrial carcinoma.

Presence of telomerase activity in normal endometrium eliminates the qualitative use of telomerase as a diagnostic/prognostic marker for endometrium cancer. Quantitative analysis was carried on to see if cancerous versus normal differentiation was possible by comparing telomerase activity levels. RTA was 1.1 ± 0.13 (mean ± SEM) for adjacent normal endometrium and 1.4 ± 0.14 for endometrial carcinoma (Figure 3.7). However, the difference was not statistically significant (P = 0.15, 2-tailed Student's *t*-test). Previous studies resulted with contradicting findings regarding the difference between normal and cancerous tissues in activity levels of telomerase. The difference reported by Yokoyoma *et al.* (1998) was not significant where as Lehner *et al.* (2002) observed significantly higher levels of telomerase activity in carcinoma. However those studies gathered normal and cancer tissue from different patients where as in this study both normal and cancer tissue was collected from same patient.

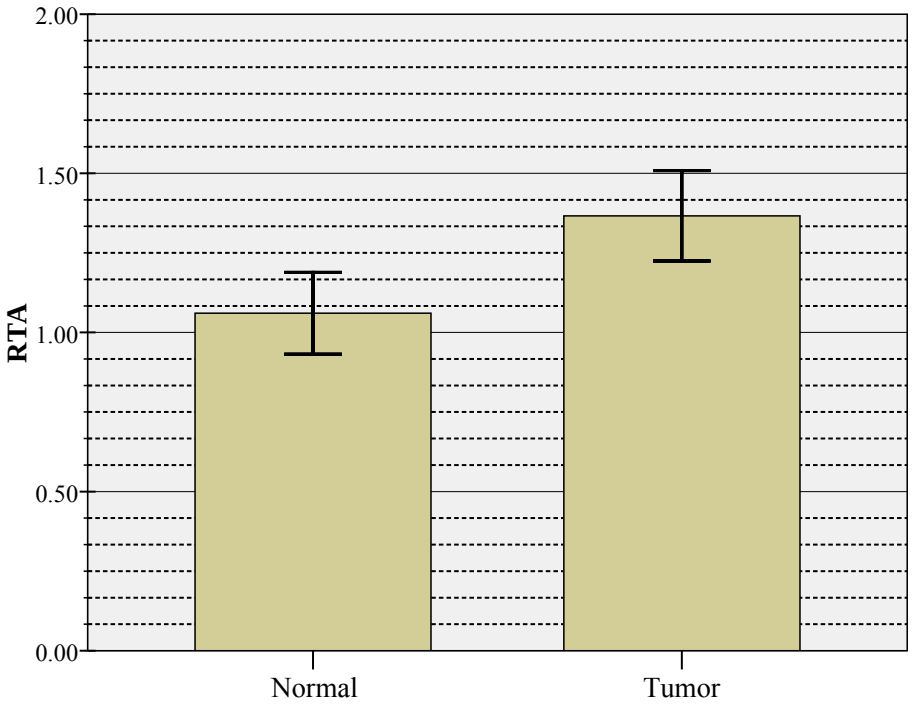

Figure 3.7. Quantitation of telomerase activity in endometrium. RTA values represented as means ± SEM.

3.3.2. Telomerase Activity in Cervical Cancers

A total of 17 cases of cervical cancers were examined for telomerase activity. Two samples, one from carcinoma and the other from adjacent normal tissue were collected from 10 patients whereas only cancerous tissue samples were collected from 7 patients. The characteristics of these patients were summarized in Table 3.7. Large cell non-keratinizing epidermoid carcinoma (LNKEC) constituted the major histological group of cervical cancer in this study (Table 3.7), which is also the common histological subtype worldwide. Large cell keratinizing epidermoid carcinoma, adenosquamus carcinoma and clear cell carcinoma were other observed histological subtypes. Despite the clinical diagnosis of cervical cancer, pathology

was not observed in one of the cases. The majority of the cases were stage II (62.5%) and the remaining cases were stage III. Half of the patients were in 40-60 age group.

Table 3.7. Clinicopathological features of cervical cancer patients

		Number	Percentage
Histological Subtype			
	NP	1	5.9%
	LNKEC	11	64.7%
	LKEC	3	17.6%
	AC	1	5.9%
	CCC	1	5.9%
	Total	17	
Stage (FIGO)			
	I	0	0%
	II	10	62.5%
	III	6	37.5%
	Total	16	
Age			
	<40	5	29.4%
	40-60	9	52.9%
	>60	3	17.6%
	Total	17	

NP: no pathology detected, LKEC: large cell keratinizing epidermoid carcinoma, LNKEC: large cell non-keratinizing epidermoid carcinoma, AC: adenosquamus carcinoma, CCC: clear cell carcinoma.

Clinopathological findings, presence or absence of telomerase activity and its quantitation is given in Table 3.8. Sample, C15T, was telomerase positive although no pathology was observed. This sample might be a normal tissue with activated telomerase or a tissue in process of carcinogenesis. In any case, this sample was excluded in further statistical calculations because it lacked cancer cells.

Telomerase activity was found to be a common event in cervical cancer. Positive telomerase activity could be detected in 16 out of 17 cases (94%) of cervical cancer examined (Table 3.9). Although shown to be cancerous, telomerase activity was not detected in C13T. There are several possible explanations for this observation. Firstly, the enzyme may have been inactivated due to improper storage of samples. Secondly, the tissue may be truly telomerase negative and maintaining their telomeres by a different mechanism as reported by others (Bryan *et al.*, 1995; Willinger *et al.*, 1996). It is also possible that activation of telomerase may not be a prerequisite for carcinogenesis in all cancers.

Telomerase activity in cervical cancers was also reported in several independent studies (Kyo *et al.*, 1996; Anderson *et al.*, 1997; Zheng *et al.*, 1997; Pao *et al.*, 1997; Zhang *et al.*, 1999). The positivity of telomerase activity detected in this study (94%) is comparable to these other reported studies which detected telomerase activity in 10 out of 10 cases (100%; Anderson *et al.*, 1997), 20 cases out of 20 (100%; Zheng *et al.*, 1997), 22 cases out of 24 (91%; Pao *et al.*, 1997) and 66 out of 71 cases (93%) of cervical cancer (Zhang *et al.*, 1999), respectively.

Table 3.8. Clinicopathological data in cervical carcinomas

Code	Age	Histology	Stage	TA	RTA
C1T	64	LNKEC	III	+	1.15288
C2T	44	LKEC	III	+	1.39471
C2N				+	0.7831
C3T	34	LKEC	II	+	1.29548
C4T	54	LNKEC	II	+	1.14901
C5T	56	LNKEC	II	+	1.34805
C5N				+	0.80001
C6T	55	LNKEC	III	+	1.29529
C6N				+	0.88552
C7T	59	LNKEC	II	+	1.27693
C7N				-	-
C8T	50	LNKEC	III	+	1.2872
C8N				-	-
C9T	36	LNKEC	II	+	1.29104
C10T	47	LKEC	II	+	0.82973
C11T	45	LNKEC	II	+	1.15288
C11N				-	-
C12T	29	LNKEC	II	+	2.17274
C12N				+	0.71707
C13T	38	LNKEC	II	-	-
C14T	65	LNKEC	III	+	2.30343
C14N				+	1.31244
C15T	47	NP		+	0.81502
C16T	39	AC	III	+	1.28454
C16N				-	-
C17T	68	CCC	II	+	1.28416
C17N				-	-

TA, Telomerase activity; RTA, relative telomerase activity; NP, no pathology detected; LKEC, large cell keratinizing epidermoid carcinoma; LNKEC, large cell non-keratinizing epidermoid carcinoma; AC, adenosquamus carcinoma; CCC, clear cell carcinoma.

Table 3.9. Positivity of telomerase activity in cervix

Tissue	Telomerase activity		
	Negative	Positive	Total
Cervical carcinoma	1 (6%)	16(94%)	17
Normal adjacent cervix	5 (50%)	5 (50 %)	10
Total			27

Telomerase activity was also found in 50% of the normal cervical tissues adjacent to tumor. Positivity of telomerase activity in normal cervical tissues from normal women reported to be lower in other studies (7% Kyo *et al.*, 1996; 0% Poa *et al.*, 1997; 7% Zhang *et al.*, 1999). However, 50% telomerase positivity was detected for tumor adjacent normal tissue (Zhang *et al.*, 1999). The higher positivity observed in normal tissue adjacent to cancerous lesions may indicate the spread of cancer cells to those tissues. Telomerase activation is shown to be an early event in carcinogenesis. Therefore it is possible that those tissues showing normal clinical and pathological characteristics might contain some cancerous cells.

Presence of telomerase activity in normal cervix rules out the qualitative use of telomerase as a diagnostic/prognostic marker for endometrium cancer. Quantitative analysis was carried out to see if cancerous versus normal differentiation was possible by comparing telomerase activity levels. The mean values of RTA was 0.90 ± 0.11 for adjacent normal cervix and 1.33 ± 0.10 for cervical carcinoma (Figure 3.8). There was a significant difference in RTA levels of normal and cancerous cervix (P = 0.03, 2-tailed Student's *t*-test), suggesting that telomerase activity levels could be a diagnostic value for cervical cancers.

The relationship between telomerase activity and clinicopathological features of cervical cancer samples were analyzed. Telomerase activity was found in all histological subtypes of cervical cancer. There was no significant difference in RTA levels between the major histological subtype (LNKEC) and other subtypes (Figure 3.9; p=0.293, 2-tailed Student's *t*-test).

There was also no significant relationship of telomerase activity with stage (Figure 3.9; p=0.497, 2-tailed Student's *t*-test) and age (Figure 3.10; p=0.827). These findings support the findings of Zhang *et al.* (1999). However, they have also reported a significant correlation of telomerase activity and stage of the tumor in cervical carcinoma.

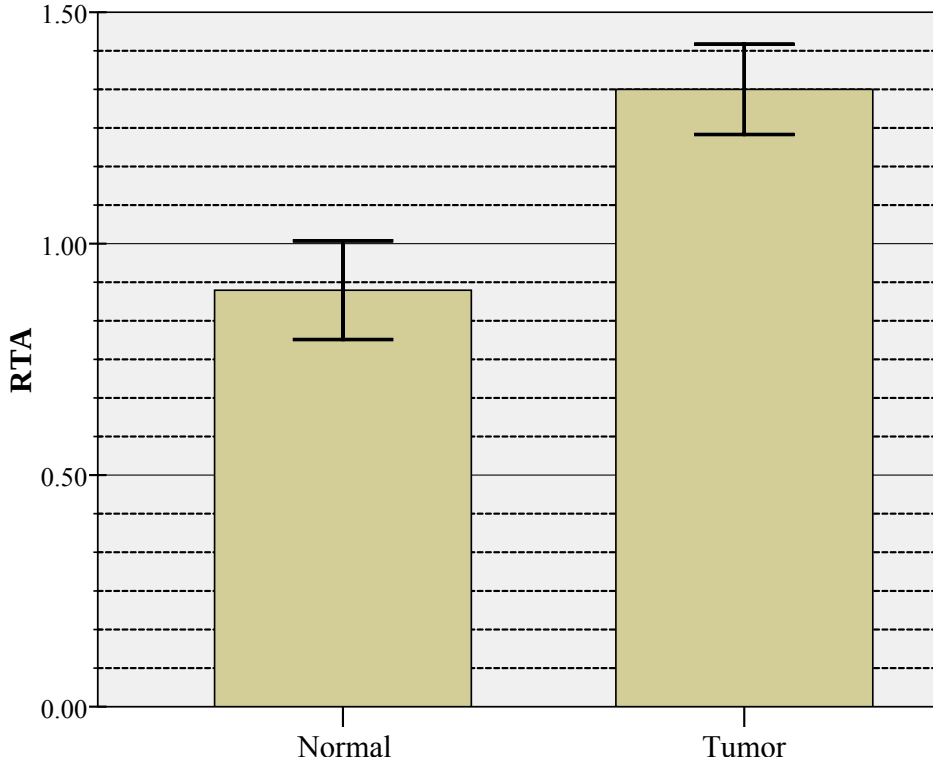

Figure 3.8. Quantitation of telomerase activity in cervix. RTA values represented as means ± SEM.

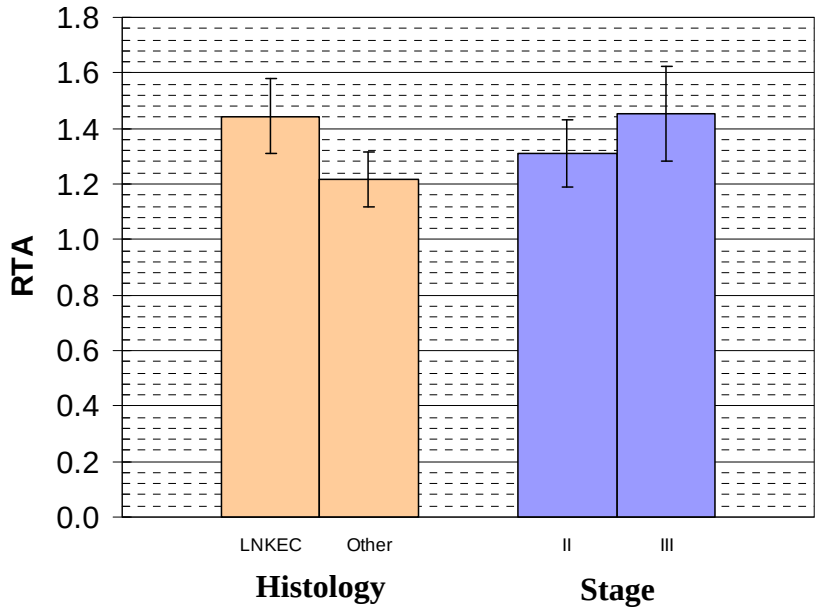

Figure 3.9. Quantitation of telomerase activity in different histology and stage groups of endometrial cancer. RTA values represented as means ± SEM.

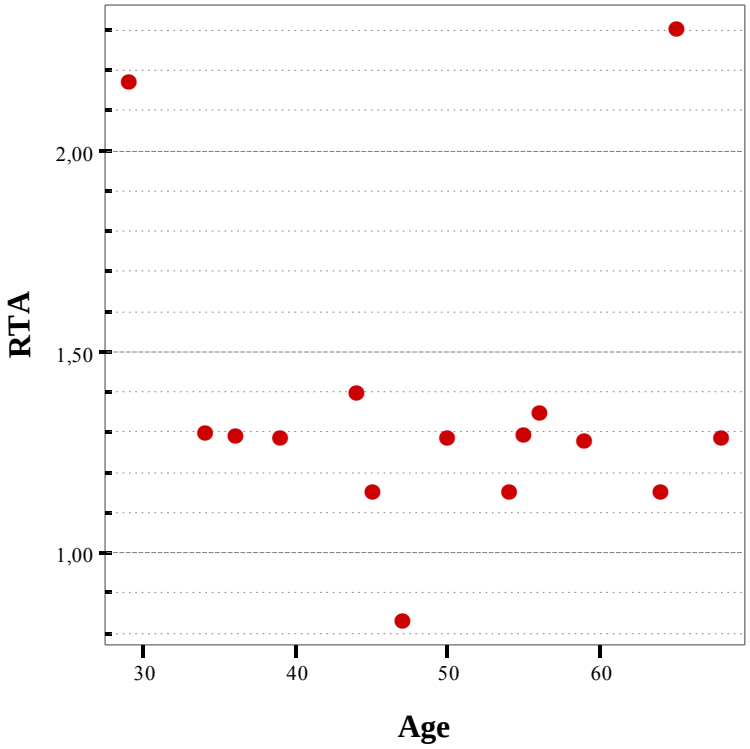

Figure 3.10. Relation of age and telomerase activity in cervical carcinoma.

CHAPTER IV

CONCLUSION

In order to sustain the continuous proliferation ability, the telomeric DNA of cancer cells has to be maintained at a steady length. Telomerase activation appears to be the main mechanism used by 85% of all cancers (Kim *et al.*, 1994) to maintain telomere lengths. The fact that telomerase is frequently activated in the majority of tumor types suggests that telomerase activity may have applications in diagnosis and prognosis of cancer.

This study has indicated that qualitative analysis of telomerase activity can not be used for diagnostic/prognostic purposes in pre-menopausal women but may be feasible in post-menopausal women. Quantitative analysis of telomerase activity can not be employed in diagnosis/prognosis of endometrial cancer because telomerase activity level in normal endometrium was not found significantly lower than that of cancerous endometrium.

Telomerase activity was generally observed in normal cervix adjacent to tumor tissues. In cervical cancer, contrary to monoclonal cancers, separate transformation events by HPV infection could contribute to cancer. Thus, this result may indicate that those tissues are in the processes of carcinogenesis but further studies are needed to confirm this. However if it is confirmed, telomerase may become a potential molecular marker to determine the surgical boundaries of the cancerous tissue in order to decrease the relapse. Positivity of telomerase in normal cervix eliminates its qualitative use as a diagnostic/prognostic marker. However, significantly higher telomerase activity levels were found in cervical cancers suggesting that telomerase may be employed for cervical cancer screening with accurate quantitation.

In both endometrial and cervical cancer, telomerase activity levels were not significantly associated with other clinicopathological features such as age, histological subtype, stage and myometiral invasion. The number of samples examined should be increased in future studies in order to assure these conclusions.

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APPENDIX

COMPOSITIONS OF BUFFERS AND SOLUTIONS

5X Coomassie brilliant blue solution

- 60 mM Coomassie Brilliant Blue G-250
- 25 % Ethanol
- 42.5 % Phosphoric acid

Filter through Whatman no. 1 filter paper. Store at 4 °C.

5X Tris-Borate-EDTA (TBE) Buffer

- 0.45 M Tris-base
- 0.45 M Boric acid
- 10 mM EDTA, pH 8.0

Adjust pH: 8.1-8.5

Acrylamide/ Bis, 19:1, 40% Solution

- 38 % Acrylamide
- 2 % N,N′-(1,2-Dihydroxyethylene)bisacrylamide

Filter through Whatman no. 1 filter paper. Store protected from light at 4 °C.

10 % Polacrylamide stock in 0.5X TBE

- 250 mL Acrylamide/ Bis, 19:1, 40% Solution
- 160 mL 5X TBE
- 650 mL Deionized water

Loading Dye Solution

- 0.25 % Bromophenol blue
- 0.25 % Xylene cyanol
- 50 % Glycerol
- 1 mM EDTA