INVESTIGATION OF TELOMERASE ACTIVITY AND GENE EXPRESSION IN COLORECTAL CANCER

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

AHU İZGİ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

JUNE 2012

Approval of the thesis:

INVESTIGATION OF TELOMERASE ACTIVITY AND GENE EXPRESSION IN COLORECTAL CANCER

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ABSTRACT

INVESTIGATION OF TELOMERASE ACTIVITY AND GENE EXPRESSION IN COLORECTAL CANCER

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June 2012, 89 pages

Human telomerase is a reverse transcriptase which synthesizes telomeric repeat sequences at the ends of chromosomes. The telomerase enzyme has two essential subunits to be functional which are called telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR). Telomerase uses its RNA subunit as a template for the addition of hexameric repeats at the ends of chromosomes. The activity of telomerase has been detected in immortal cells but not in most normal somatic cells. Therefore, its activity could serve as diagnostic or prognostic marker in malignancies.

Telomeres are heterochromatic DNA sequences bound by a number of telomere binding proteins in order to maintain the stability of chromosomes. Protection of telomere 1(POT1) is a single stranded telomere binding protein which is thought to have significant role in the recruitment of telomerase to telomeres.

The objective of the current study to investigate telomerase activity and gene expression of hTERT and hPOT1 in human colorectal cancer tissues. The activity of telomerase was examined in colorectal tumors and normal adjacent specimens by and improved telomeric repeat amplification protocol (TRAP)-Silver Staining Assay. The expression levels of hTERT and hPOT1 genes was analysed by qPCR.

The results showed that colorectal cancer tumors showed significantly high telomerase activity whereas normal adjacent tissues were found to be telomerase negative. Among clinicopathological parameters; the stage, histological type, distant metastasis and lymph node metastasis status of tumors were found to show significant differences in terms of telomerase activity. Moreover, the expression of human telomerase reverse transcriptase (hTERT) was found to be overexpressed in tumor tissues compared to normal adjacent tissues. Likewise, colorectal tumors expressed high level of hPOT1 compared to normal tissues. Both the expression of hTERT and hPOT1 correlated with telomerase activity.

It can be concluded from the results of the current study that high telomerase activity and overexpression of hTERT and hPOT1, may indicate that they could serve as diagnostic or prognostic indicators in colorectal cancer.

Key words: Telomerase, colorectal cancer, hTERT, hPOT1.

ÖZ

KOLON KANSERİNDE TELOMERAZ AKTİVİTESİ VE GEN İFADESİNİN İNCELENMESİ

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Tez Yöneticisi: Prof.Dr.Ufuk Gündüz

Haziran 2012, 89 sayfa

İnsan telomeraz enzimi kromozomların ucuna telomerik tekrar dizilerini sentezleyen bir enzimdir. Telomeraz enzimi fonksiyonel olabilmesi için, insan telomeraz RNA'sı ve insan telomeraz ters transkriptazı olarak adlandırılan iki önemli alt birime sahiptir. Telomeraz enzimi, hekzamerik tekrarların telomere uçlarına eklenmesi için kendi RNA'sını kalıp olarak kullanır. Telomeraz aktivitesi ölümsüz hücrelerde saptanmıştır fakat normal vücut hücrelerinde saptanmamıştır. Bu nedenle, telomeraz aktivitesi kötü huylu kanserlerde tanısal ve prognostik belirteç olarak hizmet edebilir.

Telomerler, kromozomların kararlılığını sağlamak için çok sayıda telomere özgü proteinlerle bağlanmış heterokromatik DNA dizileridir. Telomer korunması 1 (POT1) proteini, telomerin tek zincirine bağlanan ve telomerazın telomerlere taşınmasında önemli rol alan bir proteindir.

Bu çalışmanın amacı, insan kolon kanseri dokularında telomeraz aktivitesi, hTERT ve hPOT1 genlerinin ifade düzeylerinin incelenmesidir. Telomeraz aktivitesi kolon tümör örneklerinde ve normal doku örneklerinde geliştirilmiş telomerik tekrar çoğaltma-gümüş boyama yöntemiyle incelenmiştir. hTERT ve hPOT1 genlerinin ifade düzeyleri kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile analiz edilmiştir. Sonuçlar, kolon tümörlerinin önemli ölçüde yüksek telomeraz aktivitesinin gösterdiğini, buna karşın normal dokuların telomeraz aktivitesi yönünden negatif bulunduklarını göstermiştir. Klinik ve patolojik parametreler arasında; tumorun evresi, histolojik türü, lenf nodu metastaz durumu ve organ metastaz durumu, telomeraz aktivitesi açısından önemli ölçüde farklılık göstermiştir. Ayrıca, telomerazın katalitik alt biriminin tumorlu dokularda normal dokulara göre aşırı düzeyde ifade edildiği bulunmuştur. Benzer şekilde, kolon tümörleri, hPOT1 genini, normal dokulara göre daha fazla ifade etmişlerdir. hTERT ve hPOT1 genlerinin her ikiside telomeraz aktivitesi ile ilişkilidir.

Bu çalışmanın sonuçlarından, yüksek telomeraz aktivitesinin, hTERT ve hPOT1 genlerinin çok fazla ifade edilmesi kolon kanserinde tanısal veya prognostik belirteçler olarak hizmet edebilecekleri çıkarılabilir.

Anahtar kelimeler: Telomeraz, kolon kanseri, hTERT, hPOT1.

To My Family

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude towards my supervisor, Prof.Dr.Ufuk Gündüz for her leadership, support, encouragement, guidance, insight at every stage in this study.

I greatefully acknowledge Assist.Prof.Dr.Armağan Günal for his advise, supervision and crucial contributions.

I also feel great appreciation to Examining Committee members for their contributions.

Completing a M.Sc is truly a marathon event, and I would not have been able to complete this journey without the aid and support of countless people over the past three years. I would like to thank Yaprak Dönmez for being the first person who helped me for every aspects of my study. I also want to thank my dearest friends, Esra Kaplan, Zelha Nil, Tuğba Keskin and Gülistan Tansık for their friendship, contributions and support throughout this thesis. I would like to acknowledge the youngest but the most clever lab member, Çağrı Urfalı, for her support, trust and help in this thesis. I would like to thank Gülşah Pekgöz, Aktan Alpsoy, Neşe Çakmak for their supports.

My parents deserve special mention for their inseparable support and prayers. My mother Münire İzgi, in the first place is the person who put the fundament for my learning character, showing me the joy of intellectual pursuit ever since I was a child. My father, Ali İzgi is the one who sincerely raised me with his caring and gently love, and my sister Banu İzgi for her sincere support in every ways during my thesis.

Words fail me to express my appreciation to my friend, Kadir Arıoğlu, whose dedication and persistent confidence in me, has taken the load off my shoulders most of the times.

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BSA	Bovine Serum Albumin
DEPC	Diethylpyrocarbonate
D-Loop	Displacement loop
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
POT1	Protection of Telomeres 1
RTA	Relative Telomerase Activity
RNP	Ribonucleoprotein
TEMED	N, N, N, N'-Tetramethylethylene diamine
TR	Telomerase RNA
TERT	Telomerase Reverse Transcriptase
T- Loop	Telomere loop
TIN2	TRF1 interacting protein 2
TP	Telomerase Products
TRAP	Telomeric repeat amplification protocol
TRF	Telomere Repeat Binding Factor

CHAPTER 1

INTRODUCTION

1.1 Biology of Cancer

Cancer is one of the most serious health problem for centuries and leading cause of death. About 1,638,910 new cancer cases are expected to be diagnosed in 2012 and also about 577,190 Americans are expected to die of cancer (American Cancer Society, 2012).

Cancer can be defined as a genetic disease that leads uncontrolled cell growth and proliferation resulting in tumor formation in any part of the body through series of acquired or/and inherited mutations (Rieger, 2004). Factors that cause cancer can be classified as external factors and internal factors. External factors are tobacco, infectious diseases, radiation, chemical irritants, whereas immune conditions, mutations, hormones are internal factors. Both external and internal factors, also called carcinogens, lead to initiate carcinogenesis which is a process composed of initiation, promotion and progression steps required in development of cancer (Martin-Preston *et. al*, 1990).

There are two type of tumors; benign and malignant. The former is noninvasive, localized, therefore non-cancerous. However, the latter is invasive, metastatic, therefore cancerous (Hanahan & Weinberg, 2011). Malignant tumors have more aggressive properties during their development (Duesberg and Rasnick, 2000).

The genes responsible for cancer development are classified as proto-oncogenes and tumor suppressor genes (Futreal, Kasprzyk *et. al*, 2001). Cancer causing mutations occur generally on proto-oncogenes or/and tumor suppressor genes (Ambler K., 2006). During cancer development, abnormal cells acquire and accumulate stepweise many genetic changes such as gain of function of proto-oncogenes, loss of function of tumor suppressor genes, chromosomal rearrangements, aneuploidy, gene amplifications, translocations. Cancer cells have in common with a set of acquired properties: self sufficiency in growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, insentivity to anti-growth signals, sustained angiogenesis, tissue invasion and metastasis (Weinberg, & Francisco, 2000).

The types of cancer can be classified in five main groups in terms of the origin of the tumor formation: carcinoma (in skin or in epithelial tissue that line or cover internal organs), sarcoma (in bone, cartilage, fat, muscle, blood vessels, connective or supportive tissue), leukemia (bone marrow), lymphoma and myeloma (immune system), central nervous system (brain and spinal cord) (National Cancer Institute, 2011).

1.2 Colorectal Cancer

Colorectal cancer is the third most common and also second leading cause of cancer death worldwide among malignancies both in men and women. About 103,170 cases of colon and 40,290 cases of rectal cancer are expected to occur in 2012, in addition, about 51,690 deaths from colorectal cancer are expected to occur in 2012 in USA (American Cancer Society, 2011). Turkey is ranked fourth on the incidence of colorectal cancer in males and fifth in females and it is estimated that around 6,000 new cases are seen each year (Ministery of Health of Turkey, 2010).

Colorectal cancer shows no symptoms in earlier stages, however, there are a number of symptoms observed in moderate or late stages: bleeding from the rectum, blood in the stool, dark- or black-colored stools, a change in the shape of the stool, cramping pain in the lower stomach, unintentional weight loss, new onset of constipation or diarrhea that lasts for more than a few days (American Cancer Society, 2010).

1.2.1 Treatment of Colorectal Cancer

The treatment of colorectal cancer involves mainly 4 types of treatment. These are surgery, radiation therapy, chemotherapy and targeted therapy. Based on the stage of colorectal cancer, patients get neoadjuvant (pre-operative) and adjuvant therapies (post-operative). Neoadjuvant therapy (radiotherapy, chemotherapy, chemoradiotherapy) is used to decrease tumor size before surgery. In earlier stages, mainly removal of tumors carried out by surgery which is the oldest treatment type of cancer. Depending on the morphological appearance of tumor, if cancer is suspected to come back, adjuvant therapies such as radiation therapy and/or chemotherapy could be recommended.

Radiation therapy is used after surgery to kill any remnant cancerous cells generally if tumor is grown into adjacent tissues (American Cancer Society, 2012).

Chemotherapy treatment which is systemic treatment involving the usage of chemotherapeutic drugs such as fluorouracil (5-FU), bevacizumab, cetuximab, panitumumab, leucovorin, irinotecan, and oxaliplatin (Wolpin *et. al*, 2007).

In rectal cancers, neoadjuvant chemoradiotherapy is most widely used and found to be most effective compared to adjuvant therapy. Adjuvant therapy can also be used after surgical resection to prevent metastatis and kill the cancer cells (Sauer *et. al*, 2004).

1.2.2 Anatomy and Function of Colon and Rectum

Colorectal cancer occurs in colon and rectum which are the parts of gastrointestinal system (G.I), also called as digestive system. The colon is divided into 4 sections; ascending colon, transverse colon, descending colon and sigmoid colon (American Cancer Society, 2012). The colon is the first and longest part of the large intestine, a muscular tube about 5 feet long, whose final six inches is the rectum. Both the colon and rectum are encircled by following layers from inner to outer: the mucosa (epithelial inner surface), the submucosa (fibrous tissue), the muscularis propria (a thick layer of muscle), the subserosa (fat and peritoneal layer), the serosa (a thin outer layer of peritoneal) (Figure 1.1) (Cancer Journal for Clinicians, 2004). The function of colon and rectum is the absorbtion of water and nutrients from food. The rest is discarded as waste from anus (National Cancer Institute, 2006).

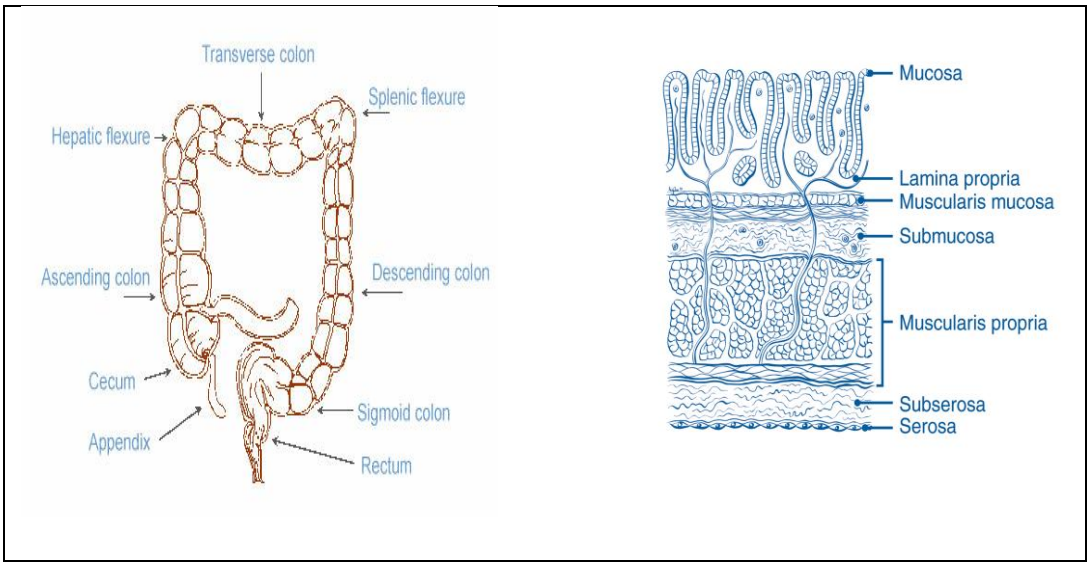


Figure 1.1 Anatomy of colon and rectum in humans (American Cancer Society, 2009)

1.2.3 Staging of Colorectal Cancer

American Joint Committee on Cancer (AJCC) defined tumor-node-metastasis (TNM) system which is used for staging of colorectal cancer. TNM classification is based on the depth of tumor invasion into the wall of the intestine, lymph node metastasis and presence or absence of distant meastatis to other organs. Pathological staging provides significant informations for the prognosis of patients (Wolpin *et. al*, 2007). Table 1.1 represents TNM classification.

Table 1.1 Pathological Classification (ACJJ, 2009)

Primary tumor (T)

- T_x Primary tumor cannot be assessed
- T_{is} Carcinoma in situ
- T_1 Tumor invades submucosa
- T_2 Tumor invades muscularis propia
- T_3 Tumor invades through the muscularis propria into the subserosa
- T_4 Tumor directly invades other organs or structures, or perforates visceral peritoneum

Regional lymph nodes (N)

- N_x Regional lymph nodes cannot be assessed
- N_0 No regional lymph node metastases
- N_1 Metastases in one to three regional lymph nodes
- N_2 Metastases in four or more regional lymph nodes

Distant metastases (M)

- M_x Presence or absence of distant metastases cannot be determined
- M_0 No distant metastases detected
- M_1 Distant metastases detected

Stage	Grouping	Five-year survival
I	T_{1-2}, N_0, M_0	>90%
IIA	T_3, N_0, M_0	60–85%
IIB	T_4, N_0, M_0	60–85%
IIIA	T_{1-2}, N_1, M_0	55–60%
IIIB	T_{3-4}, N_1, M_0	35–42%
IIIC	T_{1-4}, N_2, M_0	25–27%
IV	T_{1-4}, N_{0-2}, M_1	5–7%

1.2.4 Molecular Mechanisms Underlying Colorectal Cancer

Colorectal cancer is suggested to arise from adenomas by stepwise alterations in size and morphology of clonal structure (Sugarbaker *et. al*, 1985). A genetic model for colorectal cancer has been developed by demonstrating stepweise accumulation of gene defects (Fearon and Vogelstein, 1990). Therefore, colorectal cancer has been known to be a perfect genetic model for multistep tumorigenesis.

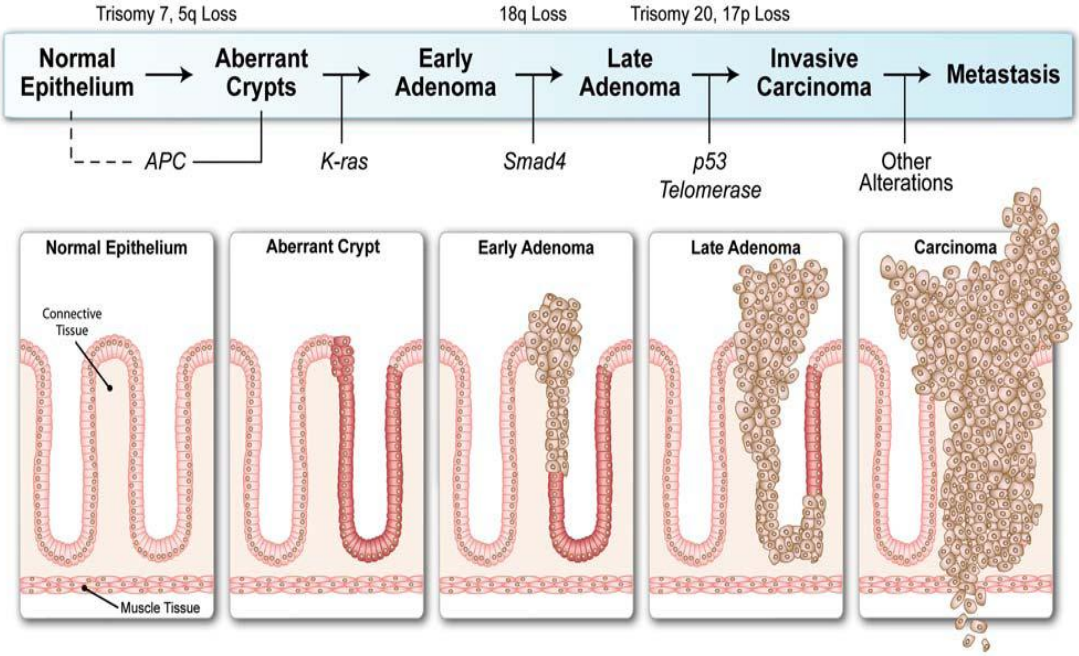


Figure 1.2 Multistep model of human colon cancer progression (Roig *et. al*, 2009)

APC (adenomatous polyposis coli) gene located on chromosome 5q is mutated nearly 85% of all colorectal cancer cells which is an early and also key event for development of colorectal tumorigenesis. APC gene has been reported to play critical roles in cell cycle control, apoptosis, migration and adhesion (Morin *et. al*, 1997).

APC gene especially in colon cancer is thought to behave as an initiator, whose inactivation certainly causes tumor formation by increasing cell proliferation in colon cancer (Huang *et. al*, 2004).

Transition from normal epithelium to adenoma is triggered most frequently by K-Ras mutations which is an oncogene (Kinzler and Vogelstein, 1996). Loss of heterozygosity at chromosome 18q has been found in about 50% of colorectal cancers in association with a poor prognosis which is probably owing to loss of the putative tumor supressor gene deleted in colorectal cancer (Shibata *et. al*,1996). As described in the figure above, inactivation of other tumor supressor genes such as p53, SMAD 4 genes and activation of telomerase gene lead to invasive carcinoma successively. Not only the accumulation of mutations but also the order of mutations have been suggested to be determinant for tumorigenesis in the colon (Knudson, 1993).

1.3 Telomere

Telomeres are special DNA sequences at the ends of linear chromosomes (Blackburn, 1991). Telomeres contain tandemly repeated sequences rich in guanosine (Blackburn,1990). The first discovered telomeric sequence $(TTGGGG)_n$ belongs to *Tetrahymena thermophilia* (Blackburn, 1978). In vertebtares, $(TTAGGG)_n$ hexameric repeat was found (Moyzis *et. al*, 1988).

Telomere length is different from species to species, among the chromosomes of the same cell and among the same chromosome in different cell types (de Lange *et. a*.,1990; Murnane *et. al*, 1994; Blackburn, 2001). The telomeres of mammalian systems are approximately 10 kb length (Chai *et. al*, 2006; Wright *et. al*, 1999).

Table 1.2 Telomerase sequence and lenghts among telomerase dependent organisms (Moon and Jarstfer, 2007)

Group	Organism	Telomere length, dsDNA	Telomere Length, G-overhang	Telomere sequence (3' strand, orientated 5'-3')
Vertebrate	Human	5 - 15 kb	60-600 nt	TTAGGG
Vertebrate	Lab mouse	~30 to 120 kb	150-200 nt	TTAGGG
Filamentous fungi	*Neurospora crassa*	~150 bp	ND	TTAGGG
Filamentous fungi	*Didymium*	100-400 bp	ND	TTAGGG
Kinetoplastid protozoa	*Trypanosoma brucei*	3 - 20 kb	< 30 nt	TTAGGG
Ciliate	*Tetrahymena*	250-400 bp	14-15 or 20-21 nt	TTGGGG
Ciliate	*Euplotes*	exactly 28 bp	exactly 14 nt	TTTTGGGG
Higher plant	*Arabidopsis thaliana*	2.5 - 5 kb	>20-30 nt	TTAGGG(T/C)
Green algae	*Chlamydomonas*	300-350 bp	ND	TTTAGGG
Insect	*Bombyx mori*	6 - 8 kb	ND	TTAGG
Roundworm	*C. elegans*	2-4 kb	ND	TTAC(A)(C)G(1-8)
Budding yeast	*Saccharomyces cerevisiae*	~300 ± 75 bp	12-16 nt (50 - 100 nt at the end of S phase)	G(2-3)(TG)(1-6)T
Budding yeast	Candida albicans	0.5 - 2.5 kb	ND	GGTGTACGGATGTCTAACTTCTT

Most repeated DNA sequences vary fairly among species, however, similar telomeric sequences are present in most other organisms. This similarity in telomere sequences indicates that they are conserved sequences and they own conserved functions among organisms (Blackburn, 1990).

1.3.1 Structure of Telomere

Telomeric DNA is composed of both double-stranded tandemly repeated sequences and single-stranded G-rich overhang together with specialized proteins (Henderson and Blackburn, 1989). Single-stranded G-rich overhang has the ability to fold back onto itself to form T-Loop (telomere loop) and invade the double- stranded telomeric DNA to form D-Loop (displacement loop; Stansel *et. al*, 2001).

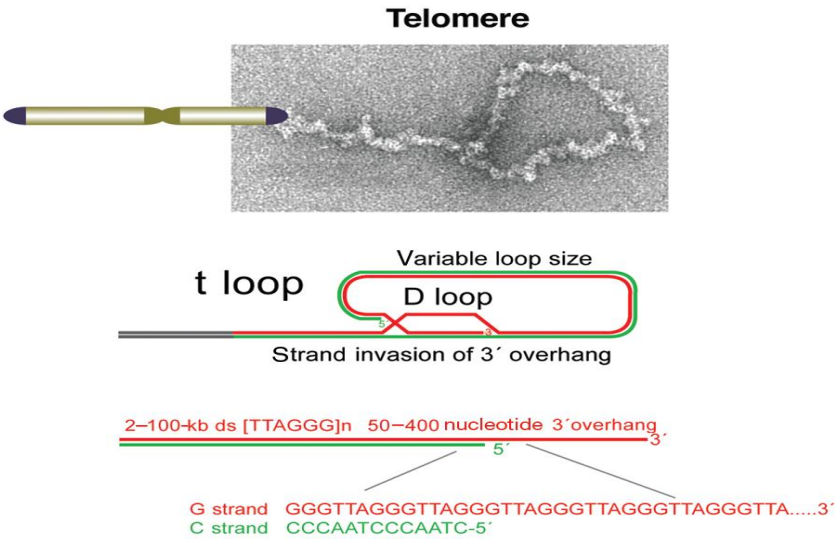


Figure 1.3 Structure of mammalian telomeres (Nikitina and Woodcock, 2004)

Telomeres are protected and shaped by six main proteins which form altogether ‘shelterin complex’. These proteins can be categorized into two groups: those that bind to specifically double stranded telomeric DNA and those that bind to single stranded G-overhang (Cooper *et. al*, 1997. ; Zhong *et. al*, 1992).

POT1 (in human), cdc 13 (in budding yeast), TEBP (telomere end binding protein in ciliate) bind to specifically to single stranded G-overhang of telomere whereas TRF1, TRF2, TIN2, RAP 1 bind to double stranded telomeric DNA (Martinez and Blasco, 2011).

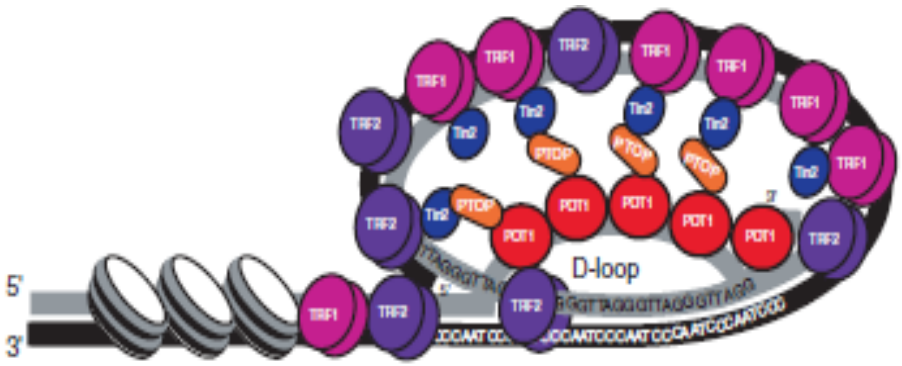


Figure 1.4 Model of stable T-Loop structure of telomeres (Colgin *et. al*, 2004)

TRF1 and TRF2 bind to double-stranded telomeric DNA independently. Both TRF1 and TRF2 are bound by TIN 2 through different domains (Martinez and Blasco, 2011). TPP 1 recruitment to the protein complex is achieved by TIN2. POT 1 (in human) which is the unique protein, binding to single stranded G-overhang (Lei *et. al*,2003), is recruited by TPP1 which serves as a bridge to accumulate all six functinal proteins to telomeres (Ye and de Lange, 2004). TRF2 is capable of forming telomere loop when other telomere substrates are supplied in vitro, therefore this indicates that TRF2 is a vital component of shelterin complex which mediates T-loop formation in vivo through its DNA binding domain (Griffith *et. al*,1999; de Lange, 2011).

To stabilize T and D-loop structures, additinal proteins are required such as PTOP, PIP1, Tankyrase1 which help to mediate the interaction between POT1 and TIN2 (Colgin *et. al*, 2004). The components of shelterin complex and other telomere binding proteins have different functions as shown in Table 1.3 (Greenberg and Rudolph, 2005). Maintenance and stabilization of chromosomal ends require specific interaction between telomeric proteins and telomeric DNA. T and D-Loops together with specific telomere binding proteins provide stability to chromosomes and form a protective cap to telomeres against degredation, end to end fusion and aberrant recombination (Edsö *et. al*, 2011).

Table 1.3 Human telomere binding proteins (Greenberg and Rudolpf, 2005)

A) single strand telomere binding proteins		
POT1	Similar to cdc13 in yeast; protects the G-strand from degradation by exonuclease 1, recruits telomerase to the telomere, inhibits telomere elongation of long telomeres	Colgin *et al.*, 2003; Ye *et al.*, 2004
Telomerase: TERC TERT	Necessary for telomere elongation, telomere capping function, not necessary for cell for organsimal viability over several generations.	Bryan *et al.*, 1998; Collins and Gandhi, 1998; Greenberg *et al.*, 1998; Lingner *et al.*, 1997
EST1a-c	Three homologs of yeast EST1. EST1a is necessary for unfolding of telomeres and telomere elongation by telomerase	Reichenbach *et al.*, 2003
B) double strand telomere binding proteins		
PIP1	Mediates binding of POT1 to the TRF1/TIN2 complex, negative regulator of telomere length	Ye *et al.*, 2004
TRF1	Negative regulator of telomere length, telomere independent function necessary for organismal viability	Karlseder *et al.*, 2002; Karlseder *et al.*, 2003
Tankyrase1, Tankyrase2	poly (ADP-ribose) polymerases that inhibit TRF1 binding to the telomere, positive regulators of telomere length, involved in sister chromatid separation during anaphase	Smith and de Lange, 2000; Smith *et al.*, 1998; Dyneck *et al.*, 2004
TIN2	Protects TRF1 from Tankyrase action, negative regulator of telomere length	Smogorzewska and De Lange, 2004
PinX2	Negative regulator of telomerase	Zhou and Lu, 2001
TRF2	Stabilises the T-Loop, necessary for telomere function, inhibits activation of ATM	Karlseder *et al.*, 2004
RAP1	Binds to TRF2, negative regulator of telomere length	Li and de Lange, 2003; Li *et al.*, 2000
RIF1	Binds to dysfunctional telomeres and activates ATM and p53bp	Silverman *et al.*, 2004

Chromosomal ends are bound by non-shelterin proteins, namely telomeric binding associated proteins, as well (Table 1.4). The significant difference between shelterin proteins and non shelterin proteins is that shelterin proteins are present at telomeres along with cell cycle and they are telomere specific. Additionally, the functions of telomere binding proteins are rectricted to telomeres (de Lange, 2011).

Table 1.4 Examples of non-shelterin proteins at human telomeres (de Lange,2005)

Protein complex	Nontelomeric function	Effects at telomeres	Interactions
Mre11/Rad50/Nbs1	recombinational repair DNA damage sensor	t-loop formation/resolution? required for t-loop HR	associated with shelterin
ERCC1/XPF	NER, crosslink repair 3′ flap endonuclease	deficiency leads to formation of TDMs; implicated in overhang processing after TRF2 loss	associated with shelterin
WRN helicase	branch migration G4 DNA resolution	deficiency results in loss of lagging-strand telomeres	TRF2
BLM helicases	branch migration crossover repression	t-loop formation/resolution?	TRF2
DNA-PK	NHEJ	deficiency leads to mild fusion phenotype	associated with shelterin
PARP-2	BER	not known	TRF2
Tankyrases	role in mitosis (tankyrase1)	positive regulator of telomere length through inhibition of TRF1	TRF1
Rad51D	unknown (HR?)	deficiency leads to mild fusion phenotype	unknown

According to genetic and biochemical studies conducted with yeast and ciliate, cells lacking telomere end binding proteins showed telomeric loss which is followed chromosome end fusions and segregation defects. These results implied that proteins bound to specifically G-rich overhang are responsible for protection of chromosomal ends and genomic stability (Baumann and Cech, 2002). However, studies with vertebrates demonstrated that knockdown of POT1 caused predominantly telomere elongation (Hockemeyer *et. al*, 2004).

1.3.2 Human Protection of Telomere 1 (hPOT1) Protein

Protection of telomere 1 protein (POT1) protein is a single stranded telomeric DNA binding protein and binds to G-overhang of telomeres through the NH_2 terminal oligonucleotide binding folds (Lei *et. al*, 2004). In human, only one POT1 gene is found whereas mice have two POT1 genes which encodes two different proteins (POT1a and POT1b) (Hockemeyer *et. al*, 2006). POT1 has been reported to have a full lenght form (variant 1) and four truncated different isoforms (variant 2, 3, 4, 5) due to alternative splicing. However, the functions of these four isofoms are still unclear (Baumann *et al*.,2002).

Studies revealed that hPOT1 has distinct functions effecting telomere lenght and the activity of telomerase. The functions of POT1 are thought to prevent telomeric DNA loss by nucleases which provides the stability of cap structure of telomeres and to recruit the telomerase to the ends of chromosomes (Colgin *et. al*, 2003 ; Baumann *et al.*, 2001).

POT1 recruitment to chromosomal ends is mediated by TPP1 protein. These complex has been shown in a study to enhance the processivity of telomerase enzyme in vitro (Wang *et al.*, 2007). In addition, in vivo studies showed the involvement of human POT1 in the regulation of telomerase function positively and negatively (Yang *et. al*, 2007).

1.3.3 Telomere Dynamics

DNA polymerase is able to synthesize DNA in 5’ to 3’ direction during replication. Leading strand is synthesized continously, while lagging strand is synthesized as Okazaki fragments which require RNA primer to be extended by DNA polymerase. After removal of RNA primer at lagging strand, the gap can not be completed by DNA polymerase. Therefore, telomeres are shortened gradually after each cell division due to the inability of DNA polymerase to fully replicate the lagging strand which is called as ‘ end replication problem’ (Watson, 1972).

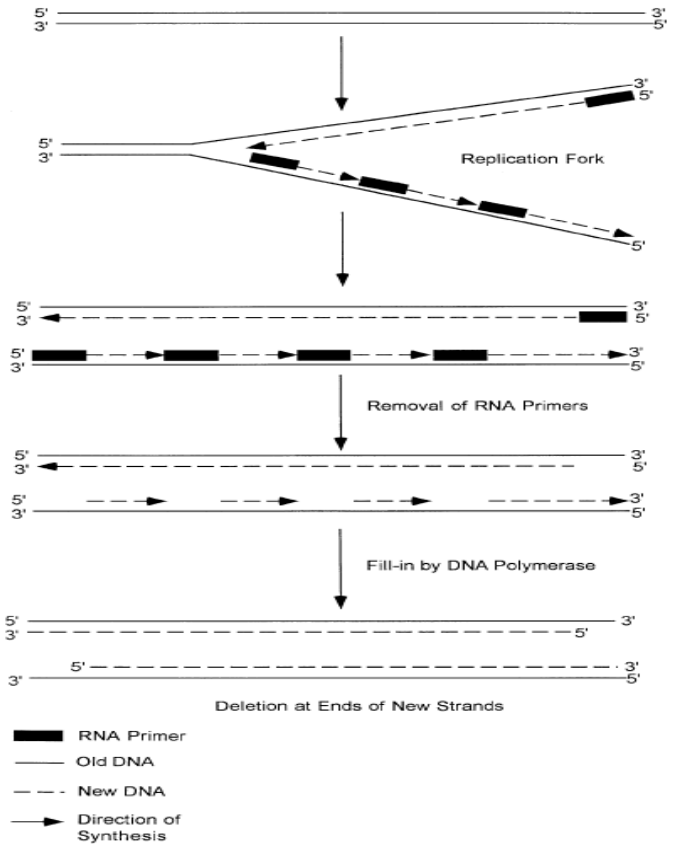


Figure 1.5 Mechanism of DNA loss at chromosome termini (Klingelhutz,1997)

1.4 Senescence and Immortalization

According to the study conducted by Hayflick, normal fibroblast cells in culture stopped dividing approximately after 50 cell cycles. Hayflick proposed that an intrinsic counter in somatic cells forced them to enter into cellular senescence (Hayflick *et. al*, 1965).

Normal somatic cells have been shown to have limited replicative ability and this point indicates that limited proliferation of normal somatic cells could be related with shortened telomeres. When telomeres become critically shortened, it can not cap and protect terminal ends anymore and thus, this causes a DNA damage response followed by growth arrest (Vallejo J, 2008).

It was proposed that there are 2 significant barriers that prevent normal cells to become cancerous cells: M1 (mortality stage 1) and M2 (mortalitiy stage 2) (Wright and Shay, 1992).

M1 stage is characterized by growth arrest which may lead soon senescence permanently. Most of normal cells are arrested at M1 and become senescence, namely, they do not divide anymore. Although some cells have shortened telomeres, they are capable of escaping from M1 through inhibited p53 and RB pathways and thus, they can continue to divide. Since the telomeres become more and more short and cells enter into M2 stage, also called crisis. The second barrier is the last station for some cells to go into senescence, however, few cells can bypass M2 to become immortal through activating their specific mechanisms for which their telomeres can be stabilized (Wright *et. al*, 1989).

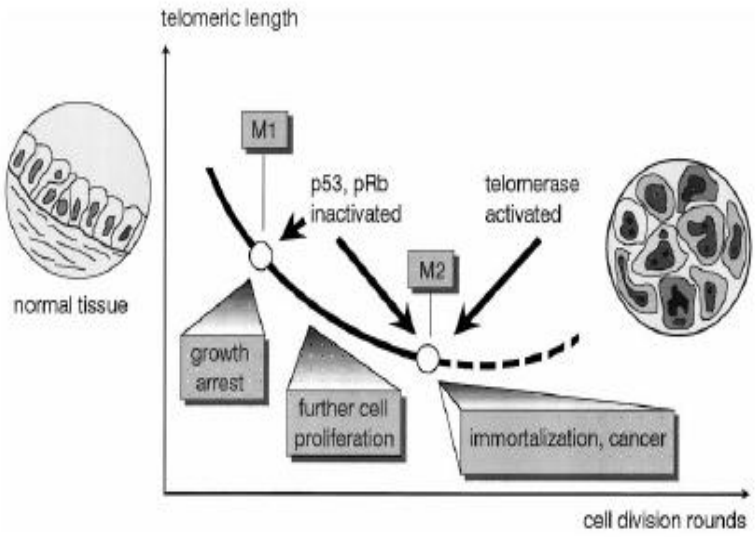


Figure 1.6 Hypothetical model of cellular immortalization (Fiedler and Ernst, 1997)

Stem cells, germline cells, hematopoietic cells, immortal cells and cancer cells are able to proliferate indefinetely thanks to the ability of stabilizing their telomere length (Wright and Shay, 2001). As described in Figure 1.6, most of cancer cells are able to prevent telomere attrition through activation of telomerase, whereas a minority of cancer cells can maintain telomeres without telomerase activity through Alternative Lengthening of Telomere pathway which is based on homologous recombination or non-homologous end joining (Cesare and Reddel, 2010).

Telomeres can show its effect in different ways in terms of pro- and anti-tumorigenesis. In cells with functional signaling and checkpoint pathways, short terminal ends of chromosomes lead to senescence or apoptosis. This consequently restricts cell division. Because genomic instability is triggered through increased end to end fusions and aberrant recombinations in cells with dysfunctional checkpoint and signaling pathways (Counter C.M *et. al*, 1992).

1.5 Telomerase

Telomerase is a cellular reverse transcriptase which synthesizes telomeric repeats onto the chromosomes. Telomerase has two essential subunits: TERT (telomerase reverse transcriptase) and TR (telomerase RNA) (Theimer and Feigon, 2006). hTERT gene is located on human chromosome 5p and has several splicing variants. hTR is located on human chromosome 3q (Kilian *et. al*, 1997). Telomerase enzyme activity has been found to increase the cell proliferation. About % 85 cancer types have shown telomerase activity (Shay and Bacchetti, 1997).

1.5.1 Structure of Telomerase

Special association of TERT and TR subunits provide the catalytic activity of telomerase. Telomerase utilizes hTR subunit as a template since hTR contains complemantary sequence to telomeric DNA sequences.

Mature h TR (human telomerase RNA) molecule is distinguished from other transcripts due to lacking of poly A tail of a mRNA and having a 5' tri-methyl rather than mono-methyl guanosine cap (Feng *et. al*, 1995). Telomerase template RNA has functional domains which are necessary for telomerase activity. Biogenesis box (BIO box) is a motif required for in vivo accumulation of hTR (Fu and Collins, 2003). In addition to, stem-Hinge-stem ACA (H/ACA) motif is an important and conserved motif responsible for determination of the site specific conversion to pseudouridine, processing and stability of telomerase RNA. The pseudoknot domain is responsible for the dimerization of TR with TERT and regulation of telomerase activity (Carrol and Ly, 2009).

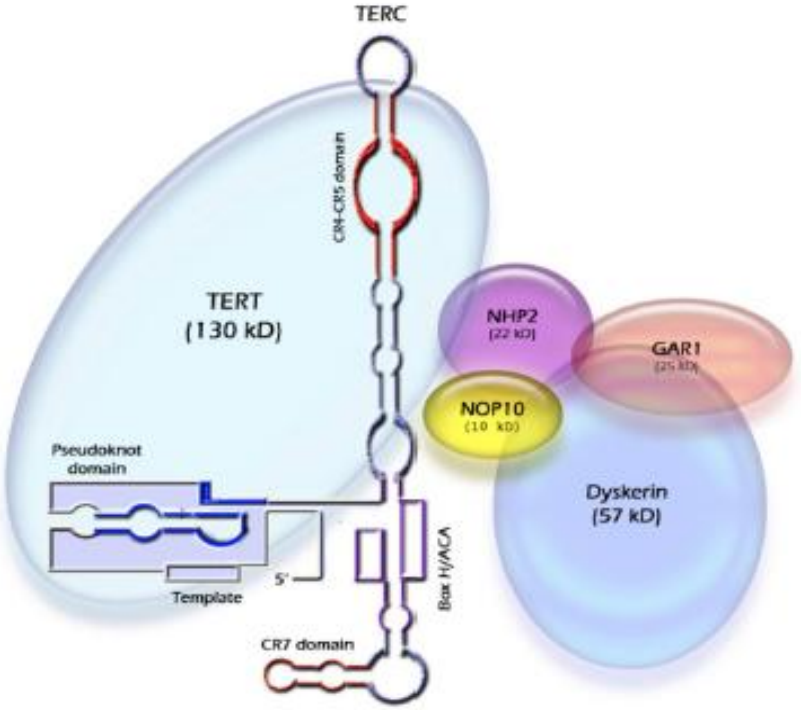


Figure 1.7 Telomerase holoenzyme (Carrol and Ly, 2009)

The N terminus of catalytic subunit of telomerase was found to have a number of conserved domains among species (Chen and Greider, 2004). TERT subunit represents DNA and RNA binding sites together with polymerase active site (Collins, 2008). N and C terminals contain TERT -specific domains which have been shown to play significant role in the interaction between telomerase RNA and single stranded telomeric DNA. Additionally, catalytic domain of TERT subunit is required to process nucleotide joining (Autexier and Lue, 2006). Studies revealed the association of telomerase complex with a number of proteins that have vital roles in ribonucleprotein complex assembly and proper enzyme activity (Forsythe *et. al*, 2001, Le *et. al*, 2000). The telomerase associated proteins in human can be divided in two groups: hTR associated proteins and hTERT associated proteins (Table 1.5) (Cong *et. al*, 2002).

Table 1.5 Human telomerase associated proteins and functions (Cong *et. al*, 2002)

Protein	Interacting region	Function
hTERT associated		
TEP1	aa 1–350, 601–927	Unknown
P23/p90	aa 1–195	Assembly/conformation
14-3-3	aa 1004–1132	Nuclear localization
hTR associated		
TEP1	nt 1–871	Unknown
hGAR1	hTR H/ACA domain	Stability, maturation, localization
Dyskerin/NAP57	hTR H/ACA domain	Stability, maturation, localization
hNOP10	hTR H/ACA domain	Unknown
hNHP2	hTR H/ACA domain	Stability, maturation, localization
C1/C2	nt 33–147	Stability, maturation, localization
La	nt 1–205, 250–451	Accessibility to telomeres?
A1/UP1	nt 1–208	Unknown
hStau	nt 64–222	Accessibility to telomeres?
L22	nt 64–222	hTR processing, localization?

[a] aa, amino acids; nt, nucleotides.

1.5.2 Action of Telomerase

Telomerase differs from other reverse transcriptases because of having own RNA template. During cell cycle, telomeric ends are extended by telomerase through the addition of telomeric repeats onto the chromosomes (Blackburn, 1992). Telomere extention process by telomerase consists three steps: recognition of telomeric ends, elongation and translocation (Harrington, 2002). G-rich hexameric repeats are added by telomerase complex until the end of template region of TR subunit. Translocation of telomerase complex along the newly synthesized strand and elongation of 3' end of telomeric DNA are continued in 5' to 3' direction (Urguidi *et al*., 2000). Since telomeric 3' single stranded overhang which forms T-loop structure is not an open and accessible structure, telomerase can not extent in vivo the ends of chromosomes all the time (Munoz-Jordan *et. al*, 2001).

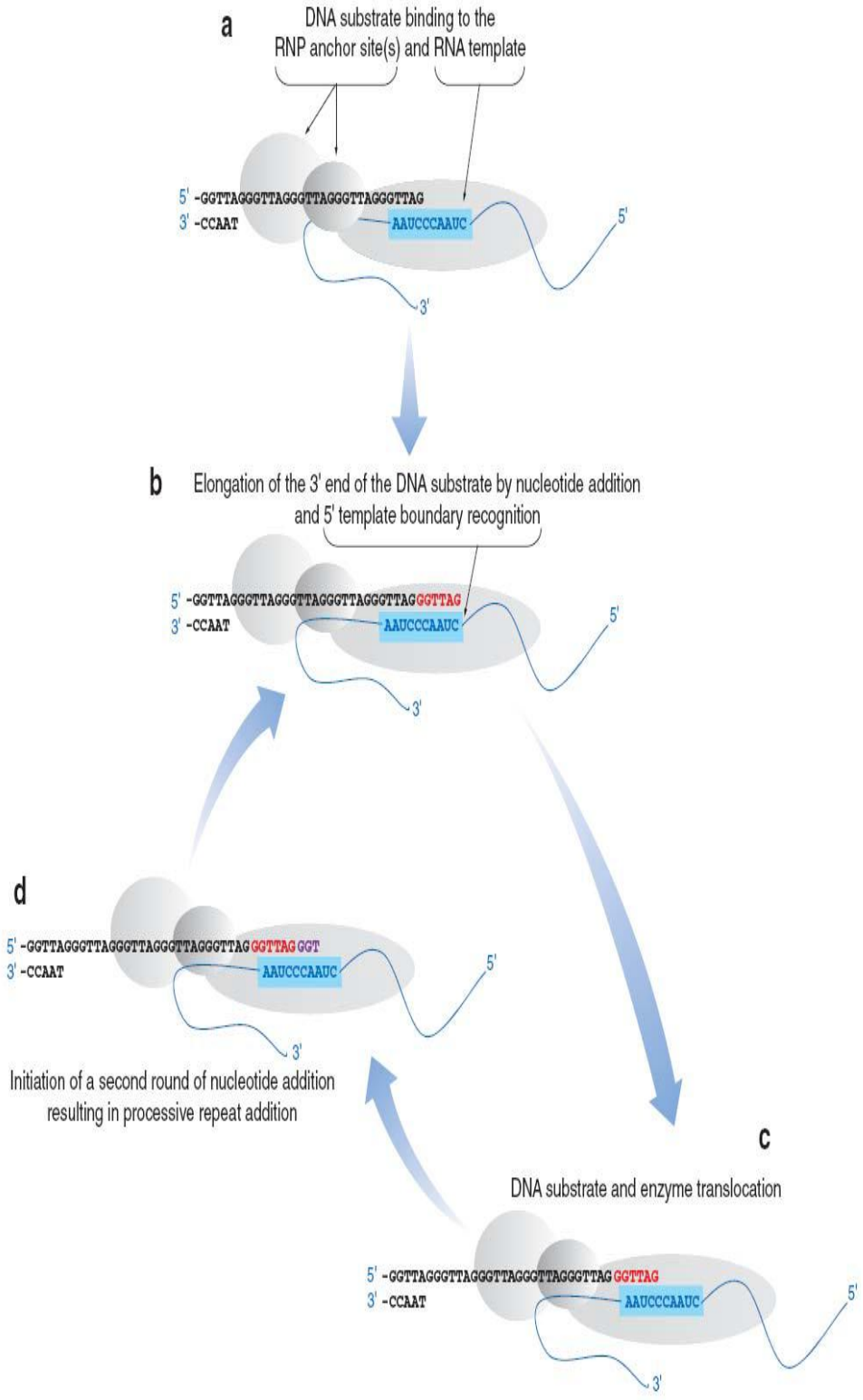


Figure 1.8 Model for processive elongation by telomerase (Autexier and Lue, 2006)

1.5.3 Regulation of Telomerase

The telomerase holoenzyme has a complex structure with two functional components which effect on its regulation. The expression properties of TERT and TR are different in vertebrates during development. The telomerase RNA in vertebrates has been found to be expressed in a variety of tissues regardless of telomerase activity (Blasco, M.A, *et. al*, 1995). In contrast to TR, the expression of telomerase protein subunit has been shown to be repressed transcriptionally in normal somatic cells. Therefore, telomerase catalytic subunit, TERT, may be considered as a limiting factor for telomerase activity since the expression has been detected in immortal cells (Cong *et. al*, 2002).

Telomerase activity is regulated through a number of level including transcriptional and postranscriptional regulation of TERT, phosphorylation of TERT protein, telomeric binding proteins, cellular transport and association of each components required for the holoenzyme assembly (Hiyama *et. al*, 1995).

The promoter sequence of hTERT gene is rich in guanosine and cytosine nucleotides. TATA box and CAAT boxes are lacking in the promoter region of hTERT gene, unlike other promoters (Wick *et. al*, 1999). However, in the promoter region, (CCTCTCC) sequence is located as an recognition site for RNA polymerase II to start the transcription (Depcrynski *et. al*, 1999). The hTERT promoter includes several binding sites for transcription factors. Transcriptional activators of hTERT gene are c-myc, Sp1, human papilloma virus 16 (E6), steroid hormones. Transcription of hTERT is repressed through the actions of Mad1, p53, RB/E2F, WT1, MZF-2 (Poole et. al, 2001).

Besides transcriptional regulation of hTERT promoter, posttranscriptional regulations through phosphorylation of hTERT protein by specific kinases play crucial role in activity of telomerase (Aisner *et. al*, 2002).

Phosphorylation of TERT by c-Abl at specific tyrosine residue has been shown to decrease telomerase activity (Sawyers *et. al*, 1994). Protein phosphatase 2A (PP2A) is another negative regulator of telomerase activity. PP2A shows its effect on telomerase activity through dephosphorylation of TERT protein on Ser and/or Thr residue (Haendeler *et. al*, 2003). In contrast, telomerase activity is shown to be increased by phosphorylation by protein kinase B, also known as Akt kinase, and protein kinase C (Kang *et. al*, 1999; Li *et. al*, 1998). Furthermore, POT 1 which is a single stranded telomere binding protein, has been shown to act on telomere extention positively and negatively by telomerase depending on its position at telomeres. It has been suggested that telomerase activity is positively regulated when POT1 protein binds to more internally on the telomeric DNA, thereby leaving more open and accessible 3' terminal (Lei *et. al*, 2005). On the other hand, the binding of POT1 near the 3' terminal leads to unaccessible structure for telomerase to bind to the ends of chromosomes (Kelleher *et. al*, 2005).

1.5.4 Significance of Telomerase in Cancer

Activation of telomerase gene has been found in about %85 cancer types with the help of a sensitive PCR-based assay. Its activation leads to immortalization of cells through increasing proliferation capacity (Kim *et. al*.,1994).

Telomerase activity is shown to be associated with cancer progression (Klingelhutz *et. al*, 1997). Its clinical importance varies among different type of cancers. Since there was no significant difference in telomerase activity between normal adjacent and tumor tissues, telomerase activity was found not to be a diagnostic or prognostic marker for some type of cancers. Whereas telomerase activity was found to be correlated with poor prognosis in certain type of cancers such as neuroblastoma, lung cancer, gastric cancer (Hiyama *et. al*, 1996; Tahara *et. al*, 1999).

Several studies have been conducted also for expression analysis of telomerase subunits to display whether there is a relation among its activity and gene expression profile. Gene expression level of hTERT, catalytic subunit of telomerase, has been shown to be correlated with telomerase activity in neuroblastoma (Hiyama *et. al*, 1995). Another study demonstrated that hTERT m RNA levels were associated with poor prognosis in breast cancer and suggested to be involved as a prognostic marker for further analysis (Elkak *et. al*, 2006).

1.6 Objectives of the study

The objectives of this study are:

- Investigation of clinical significance of telomerase activity in colorectal cancer
- Optimization of a quantitative telomeric repeat amplification protocol (TRAP)-silver staining assay
- Determination of telomerase activity in surgical specimens of colorectal cancer and their matched non-cancerous normal tissues
- Evaluation of clinicopathological features of patients with respect to telomerase activity
- Determination of expression levels of hTERT and hPOT1 genes
- Investigation of the association between telomerase activity and the expressions of hTERT and hPOT1 genes

CHAPTER 2

MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

RNAlater®, bovine serum albumin (BSA), ammonium persulfate (APS), silver nitrate, Tris-HCl, sodium acetate, ethylenediaminetetraacetic acid (EDTA), N,N′-(1,2-Dihydroxyethylene) bisacrylamide, ethidium bromide, glycerol, phosphate buffered saline tablets (PBS), Coomassie® Brilliant Blue G-250, TRI Reagent were purchased from Sigma-Aldrich. Diethylpyrocarbonate (DEPC), isopropanol, agarose, N,N,N′,N′-Tetramethylethylenediamine (TEMED) were purchased from Applichem, Germany. Formaldehyde, phosphoric acid, boric acid and acetic acid were purchased from Merck, Germany. High range RNA ladder, Taq DNA polymerase, 6X loading buffer, RNAse Inhibitor, dNTP mix, Moloney-Murine Leukemia virus reverse transcriptase were obtained from Fermantas, Lithuania.

TRAPeze telomerase detection kit was purchased from Millipore, Germany. QuantiTect Rev. Transcription Kit was obtained from Qiagen, Germany. Light-Cycler-Fast Start DNA master SYBR Green I kit was purchased from Roche Diagnostics, Switzerland.

2.1.2 Patients

23 tissue specimens of colorectal cancer and 23 adjacent non-neoplastic colorectal tissue samples were obtained at the time of surgery from Gülhane Millitary Medical Academy (GATA).

Ethical approval was obtained from the committee for the collection of tissues samples and further studies. Patient data were obtained from the Department of Pathology, Colorectal Cancer Database at GATA.

All tissue specimens were collected freshly and stored in RNAlater solution after surgical removal. Following overnight incubation at 4 °C, tissues were stored at -80 °C.

2.1.2.1 Clinicopathological Features of Patients

For the current study, 23 matched tumor (T) and normal (N) adjacent tissues were processed for the determination of telomerase activity in colorectal cancer. However, 3 out of 23 tissue specimens were found to be degraded. Therefore, they were not included for further analysis. 20 tissue specimens were used for detection of telomerase activity and gene expression studies. The same amount of tissue were used for telomerase gene expression analysis and determination of enzymatic activity.

Patients under 40 age were counted as young in the current study; in contrast, above 40 age was counted as old.

The association between telomerase activity and clinicopathological features (such as age, gender, histological type, stage of disease, histological grade, tumor site, and size of tumors) was evaluated for the study. Besides these criteria, depth of tumor, lymph node status and distant metastasis were also analysed.

According to histopathological assesment, the major histological type was found to be adenocarcinoma which is considered as usual type or classical type and 2 out of 20 were evaluated as unusual types, mucinous adenocarcinomas. In addition, most of the tumors were poorly differentiated (Grade III).

Tumors were localized mostly (13/20) in left colon and %65 of tumors in left colon were found in rectum and remaining were found in sigmoid colon. Tumors in right colon (7/20) localized in hepatic flexure, ascending colon, cecum. Tumor size ranged from 1.8 cm to 10 cm.

According to TNM classification, 14 out of 20 patients were evaluated as late stage (stage III and IV) whereas 6 out of 20 patients were at early stage (stage I and stage II). One of the patient was diagnosed at early onset with Stage I which is a rare event in turkish population. Table 2.1 summarizes all the clinopathological features of patients involved in the study.

Tumors which invades mucosa and muscle layer assessed as intramural involving pT1 and pT2. 1 out of 20 cases were found to be pT2 whereas 19 out of 20 tumors invaded through muscle layer into pericolorectal tissues assesses as pT3 and pT4 (extramural).

About %65 of patients showed lymph node metastasis. The number lymph of node metastasis was variable, however metastasis in 4 or more regional lymph node was common among patients.

Distant metastasis (M) status was also evaluated and 2 out of 20 cases showed metastasis to liver and peritoneum. Table 2.2 represents the TNM staiging results of patients according to ACJJ Version 7. Patients with rectal tumors took chemoradiotheraphy as neoadjuvant therapy, however, other patients did not take any therapy before surgery.

Table 2.1 Clinicopathological features of patients

	No.Cases
Gender	
Woman	11
Man	9
Age	
Young (<40)	3
Old (40<)	17
Histological Type	
Usual (Adenocarcinoma)	18
Unusual (Mucinous adenocarcinoma)	2
Tumor Site	
Right Colon	7
Left Colon	13
Rectum	7
Others	13
Stage	
Early	
I	1
II	5
Late	
III	12
IV	2
Grade	
I	0
II	15
III	5
Tumor size	
≤2 cm	4
2 cm >	16

Table 2.2 Results of TNM staiging of patients

TNM STAGING	**No.Cases**
Tumor status (T)	
Intramural	
pT1	0
pT2	1
Extramural	
pT3	17
pT4	2
Lymph Node Status (N)	
Positive	
N1	8
N2	5
Negative	
N0	7
No. Metastatic Lenf Node	
0-5	18
5-10	3
10>	2
M (Distant Metastasis)	
Positive	
M1	2
Negative	
M0	18

2.1.3 Primers

hTERT, h POT 1 and GAPDH primers were designed with the help of Primer 3 (Massachusetts Institute of Technology) and PrimerBLAST (National Center for Biotechnology) software programs and obtained from Alpha DNA, Canada.

Primers used in quantitative real-time polymerase reaction (qPCR) are listed below in the Table 2.3:

Table 2.3 Primers used in quantitative real-time polymerase chain reaction (qPCR)

Primer	**Sequence**	**Amplicon Size**
h TERT Sense	5'GCATTGGAATCAGACAGCAC3'	165 bp
h TERT Antisense	5'CCACGACGTAGTCCATGTTC3'	
h POT 1 Sense	5'GAAGTGGACGGAGCATCATT3'	127 bp
h POT1 Antisense	5'TTTGTAGCCGATGGATGTGA3'	
GAPDH Sense	5'ATGGGTGTGAACCATGAGAA3'	79 bp
GAPDH Antisense	5'GTGCTAAGCAGTTGGTGGTG3'	

2.2 METHODS

2.2.1 Staging and Classification of Patients

The tissue specimens were fixed in 10% formalin solution and embedded in parafin for the staging, histopathological analysis and analysis of clinicopathological parameters. Tumors were staged using TNM system endorsed by American Joint Committee on Cancer (AJCC, Version7).

Hematoxylin and eosin staining were performed for TNM staging, The histopathological examinations of tumor tissues were performed by Assist. Prof. Dr. Armağan Günal (Department of Pathology, Gülhane Millitary Academy of Medicine).

2.2.2 Telomeric Repeat Amplification Protocol

2.2.2.1 Protein Extraction from Tissue Specimens

Pipette tips, mortars and pestles, eppendorf tubes and other glasswares were incubated overnight with active DEPC treated distilled H_2O in order to inactivate RNAses, then all equipments were autoclaved at 121 °C for 20 min. Fresh frozen tissue specimens was placed in a sterile mortar and liquid nitrogen was poured onto the tissue until it covered whole tissue. The frozen tissue was homogenized well with liquid nitrogen to a fine powder with a matching pestle. The frozen tissue powder was transferred with a sterile surgical blade to a RNAse free 1.5 mL microcentrifuge tube containing 40 unit/uL of RNAse inhibitor and 250 uL 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), a zwitterionic detergent allowing the extraction and solubilization of proteins, provided by the TRAPeze® telomerase detection kit.

The homogenate was incubated on ice for 30 min and then spun in a benchtop microcentrifuge at 12.000 X g for 20 min at 4 °C. About 160 uL of supernatant was collected in a sterile RNAse free plastic tube, then stored at -80 °C for further use.

2.2.2.2 Determination of Protein Concentration

Protein concentrations of each samples were determined by Bradford assay which is a colorimetric assay. As the concentration of protein extract increases, the color of the sample becomes darker. Since coomassie dye binds to proteins proportionally. Serial dilutions of bovine serum albumin (BSA) were prepared in triplicate to prepare standards as shown in the Table 2.4.

Table 2.4 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 standard tubes and sample tubes were vortexed for 5 seconds, and then incubated at room temperature for 10 min. Sample tubes contained 10 µL of protein extract diluted in 490 µL dH2O and mixed with 1X coomassie brillant blue solution (Appendix A).

The absorbance of each sample tube was measured against the blank. Standard curve was prepared by plotting absorbance at 595 nm versus protein concentration. Trendline equation of the standard curve was used to calculate protein concentration according to formula below (Equation 2.1) :

$y = mx + b,$

where ;

y = absorbance at 595 nm and x = protein concentration. (2.1)

Protein concentration of extracts was determined according to following formula (Equation 2.2) :

[Protein concentration] (μg/mL) = [(y-b)/m] X dilution factor. (2.2)

2.2.2.3 Telomerase Assay

TRAP assay is a PCR-based assay which is performed through two steps. In the first step, telomerase in tissue extracts adds telomeric repeats (TTAGGG in human) to the 3' end of telomerase substrate (TS) primer which is a substrate oligonucleotide provided by the kit. In the second step, extended products are amplified using TS and RP (reverse primer) by PCR. This process generates a ladder of products with 6 base increments beginning at 50 nucleotide (Figure 2.1).

The TRAPeze Telomerase Detection kit provides a primer mix inluding TSK1 and K1 primers to eliminate false negative results. TSK1 and K1 primers produce a 36 bp internal control (IC) band. This 36 bp internal control band also serves for the detection of amplification efficieny in each reaction. Telomerase assay was performed according to manufacturers instructions.

CHAPS lysis buffer only and heat inactivated protein extracts of tissue samples were used as negative controls. In addition, a positive control was also included in each reaction.

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). Reaction mix was given in the Table 2.5 below:

Table 2.5 Reaction mix for TRAP Assay

Components	**Amounts**
10X TRAP Buffer	5 uL
50X dNTP Mix	1 uL
TS Primer	1 uL
Primer Mix	1 uL
Taq Polymerase	0.4 uL
PCR Grade Water	39.6 uL
Tissue extract	2 uL
Total	50 uL

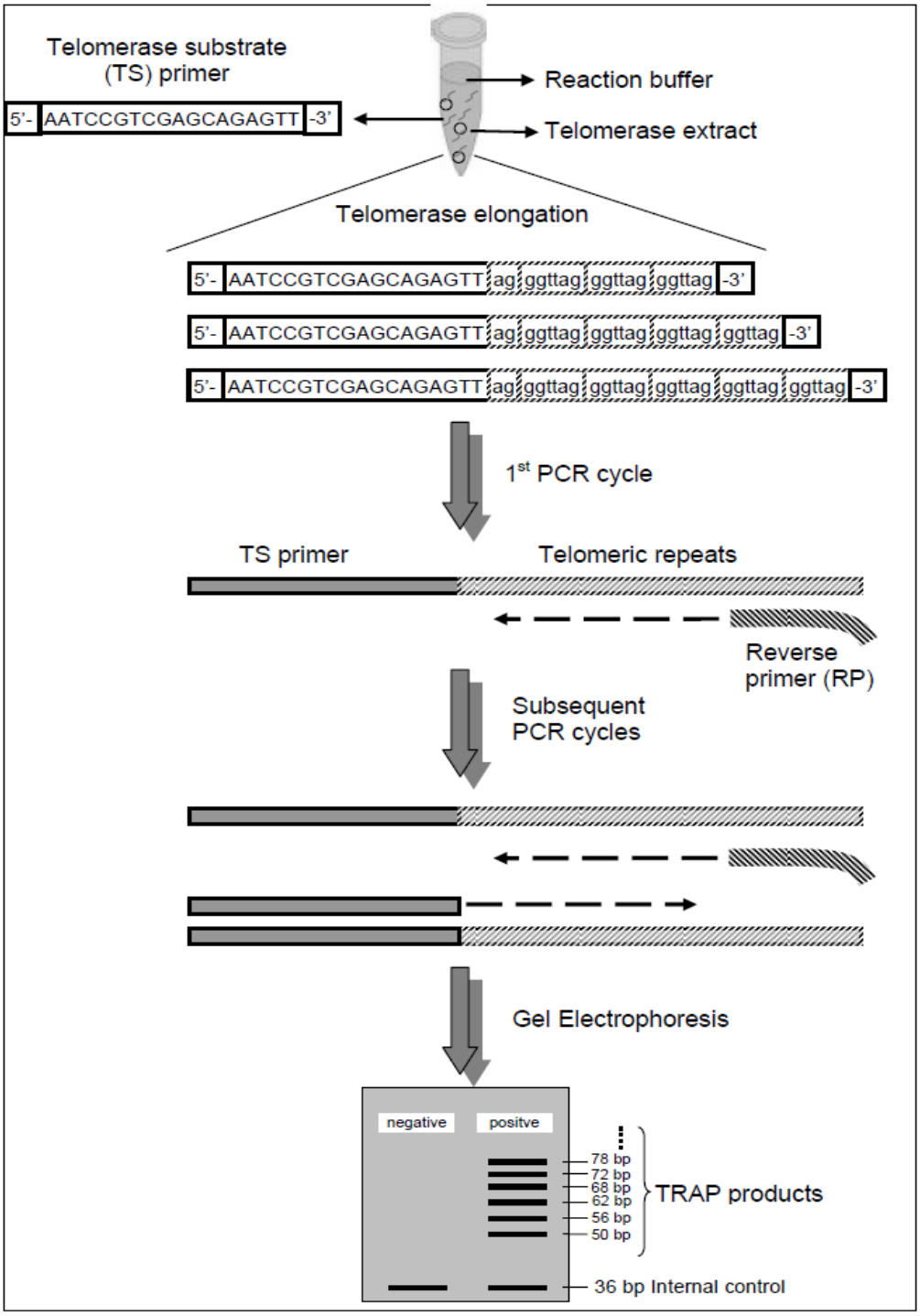


Figure 2.1 Schematic illustration of TRAP assay

2.2.2.3.1 Polyacrylamide Gel Electrophoresis

TRAP assay products were run in a 12.5 % non denaturating PAGE. 50 mL gel was prepared by using 49.5 mL, 12.5 % Polyacrylamide stock in 0.5 X TBE (Appendix A) and 0.5 mL 10 % APS. 3 mL of this solution was mixed with 15 µL TEMED and poured between glasses. After formation of a layer at the bottom of the glasses that blocks leakage, remaining gel solution was mixed with 35 µL TEMED and poured between glasses. The comb was placed and the gel was left for polymerization. The gel was fixed into vertical gel electrophoresis unit (Thermo Scientific) and the middle and lower reservoirs were filled with 0.5 X TBE buffer. 5 µL of loading dye (Appendix A) was mixed with each TRAP product. Twenty five µL of each reaction was loaded into each lane and the gel was electrophoresed at 300 V for 65 min.

2.2.2.3.2 Silver Staining

PAGE gel was carefully transfered to a plastic folder containing 300 mL of fixation solution which prevents macromolecules from diffusing out of the gel (Appendix A). The gel was put on to a shaker around 5 min for incubation and then the solution was poured. This step was repeated one more time with fresh fixation solution. After the first step of staining, the gel was rinsed with ddH2O. Secondly, the gel was then incubated in silver impregnation solution for 20 min on the shaker in order to inhibit reduction of silver to metallic silver (Appendix A)**.** After removal of silver nitrate solution, the gel was rinsed again with distilled water. Lastly, the gel was incubated in developing solution for 35 min which reduces silver ion to metallic silver (Appendix A).

2.2.2.3.3 Densitometric Analysis of Polyacrylamide Gels

The gel images were analysed with Image J software. Relative telomerase activity (RTA) was calculated by the following formula (Equation 2.3) :

$$RTA = \frac{X_s / X_{IC}}{X_q / X_{qIC}} \quad (2.3)$$

where;

X_s : Densitometric intensity of TRAP products $_{(sample)}$

X_{IC}: Densitometric intensity of internal control$_{(sample)}$

$X_{p\ :}$ Densitometric intensity of TRAP products $_{(positive\ control)}$

$X_{pIC:}$Densitometric intensity of internal control $_{(positive\ control)}$

Band intensity was measured from 50 bp containing all the bands of 6 bp increment ladder for TP whereas band intensity for IC was obtained from the 36 bp band.

2.2.2.3.4 Statistical Analysis

Relative telomerase activity (RTA) was calculated for each sample and RTA results were evaluated by two-tailed t tests to compare two groups using GraphPad Prism 5.0 Software (GraphPad Inc, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL) to determine significant difference between means of different groups at the 0.05 level.

2.2.2.4 Reverse Transcription-Quantitative PCR (qPCR)

2.2.2.4.1 Isolation of Total RNA

Fresh frozen tissue sample, approximately 100 mg, was put into ice for 5 min, then spun down briefly in a microcentrifuge to remove RNAlater. Tissue specimen was then placed in a sterile RNAse free mortar and liquid nitrogen was poured onto the tissue. The tissue was grinded with a pestle rigorously until it totally became a fine powder. With the help of a sterile surgical blade, the grinded tissue was transfered to a RNAse free 2 mL Eppendorf tube containing 1 mL TRIzol Reagent (Sigma Aldrich, USA) and 1 ul glycogen (Roche, Germany). Tissue powder was homogenized well in TRI Reagent by pipetting and incubated at room temperature for 5 min. After incubation, chloroform was added in 1/5 volume of TRIzol Reagent and mixed for 15 sec, then incubated on ice for 15 min. The tube was centrifuged at 12000 X g at 4°C for 15 min. Phase separation was seen and the upper aqueous phase containing RNA was taken carefully to a new RNAse free tube. Isopropanol was added onto the aqueous phase in 1/5 volume of TRIzol Reagent, incubated then at room temperature for 10 min. Following incubation, tube was centrifuged at 12000 X g at 4°C for 10 min. Isopropanol was removed carefully and 75 % ethanol was added in 1/1 volume of TRIzol Reagent. The tube was then incubated at -20°C for two days. The tube was centrifuged 12000 X g at 4°C for 15 min and supernatant was discarded. The remaning ethanol was removed by air drying. Onto the pellet, appropriate amount of nuclease free water was added and incubated at 55°C for 10 min to solubilize RNA, subsequently stored at -80°C.

2.2.2.4.2 Quantification of isolated RNA

The concentration and purity of isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using NanoDrop 2000 C spectrophotometer (Thermo Fischer Scientific, USA). Absorbance at 260 nm is used to calculate RNA concentration while ratio of absorbance at 260 nm to that of 280 nm indicates purity of RNA.

The concentration of RNA was calculated using formula below (Equation 2.4) :

$$[\text{RNA}]\ \mu g/mL = A_{260} \times 40.0 \qquad (2.4)$$

where ;

A_{260} = absorbance at 260 nm,

40.0 = average extinction coefficient of RNA.

Pure RNA sample should have an A_{260}/A_{280} ratio of 2.0±0.1.

2.2.2.4.3 Agarose Gel Electrophoresis of RNA

The intactness of isolated RNA and DNA contamination were checked by agarose gel electrophoresis. 1g of agarose was weighed and dissolved in 50 mL of 1X TAE buffer. The mixture was boiled in microwave oven until agarose completely melted. After cooling of gel solution, 4 µL of ethidium bromide solution was added and mixed. The gel solution was poured into electrophoresis apparatus and comb was placed. After the gel solidified, 4 µL of RNA sample was mixed with 4 µL of 2X loading dye and loaded. The samples were run on at 80V for 60 min and visualized by UV gel acquisition system (Vilber Lourmat, Marine la Valee, France).

2.2.2.4.4 cDNA Synthesis

cDNA synthesis was performed with 1ug total RNA and 25 pmol of gene specific primers, either of GAPDH, h TERT, h POT 1, together with other components at a final volume of 20 uL.

QuantiTect Reverse Transcription Kit (Qiagen, Germany) was used for cDNA synthesis which requires 3 steps. According to manufacturers instructions, firstly, genomic DNA elimination step was performed at 42°C for 2 min by using 2 ul genomic DNA wipeout buffer, 1 ug RNA to a final volume 11ul. Secondly, reverse transcription reaction was performed at 42°C for 15 min by the addition of 4 ul RT buffer, 1 ul reverse primer or primer mix and 1 ul reverse transcriptase. Finally, the reverse transciption was terminated by incubation at 95°C for 3 min to inactivate reverse transcriptase. cDNA was stored at -20°C. Reaction mix is shown in Table 2.6 below:

Table 2.6 Reaction mix for cDNA synthesis

Component	**Amount**
Genomic DNA Wipeout Buffer	2uL
RNA (up to 1 ug)	variable
RNAse free water	variable
RT Buffer	4 uL
Reverse transcriptase	1uL
Primer	1 uL

2.2.2.4.5 Quantitative Real Time Polymerase Chain Reaction

Quantitative real time PCR (qRT-PCR) was performed in Rotor-Gene 6000 (Corbett Research, Australia). Light-Cycler-FastStart SYBR Green I DNA master mix kit was used. SYBR green is a DNA intercalating agent that binds to all double stranded DNA and accumulates during PCR cycles.

Before the reaction set up, all plastic and glasswares were autoclaved and dried in the oven for 24 hours. The mix was composed of 10 ul SYBR Green 2X master mix, 2.8 uL of cDNA, 0.15 uM of reverse and forward primers and appropriate amount of nuclease free water to a total volume of 20 ul.

All samples were run as triplicates in each run and a negative control was also included which did not contain cDNA template to check background signal. Amplification plots were shown by plotting fluorescence versus threshold cycle number. Melt curve analysis was also perfomed to detect whether only the expected product was amplified. Amplification conditions of each gene were represented below:

Table 2.7 qPCR conditions of GAPDH, hPOT1, hTERT genes

	GAPDH	**hPOT1**	**hTERT**
Activation	95°C,10 min	95°C,10 min	95°C,10 min
Denaturation	95°C,30 sec	95°C,30 sec	95°C,20 sec
Annealing	56°C,35 sec	61°C,30 sec	62°C,30 sec
Extention	72°C,45 sec	72°C,45 sec	72°C,45 sec
Melting	50°C -99°C	50°C -99°C	50°C -99°C
Cycle number	45	50	45

2.2.2.4.6 Quantification of Q-RT-PCR Products

Relative quantification of qPCR products was perfomed by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). According to delta delta Ct ($2^{-\Delta\Delta Ct}$) method, the fold change in gene expression is found which is determined by the normalization to a internal control and relative to reference group The analysis in gene expressions of hPOT1 and hTERT was achieved by following equation (Equation 2.5) :

Fold change = $2^{-\Delta\Delta Ct}$

$$\Delta\Delta Ct = (Ct_{target} - Ct_{internal\ control})_{tumor} - (Ct_{target} - Ct_{internal\ control})_{normal} \quad (2.5)$$

Fold changes of *hPOT1* and *hTERT* genes were normalized to the internal control gene *GAPDH* and calculated for each sample relative to normal tissue sample.

2.2.2.4.7 Statistical Analysis

All experiments were repeated three times and each run was perfomed in triplicates. The data were expressed as mean ± standard error of the means (SEM). The results were analysed by one-way ANOVA test by using GraphPad Prism 5.0 Software (GraphPad Software Inc, USA). Tukey's Multiple Comparison Analysis was applied to compare different groups. The mean differences were significant at the 0.05 level.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Determination of Protein Concentrations in Tissue Specimens

Biopsy samples from 23 patients with colorectal cancer were obtained from GATA. Clinicopathological features of patients were given in section 2.1.2.1. Three out of twenty three samples were found to have very low amount of protein concentration due to degradation. Therefore, they were excluded from further analysis.

Protein extraction from tumor and adjacent normal tissues was carried out succesfully according to procedure described in *section 2.2.2.2.*

Sufficient amounts of protein concentrations were obtained from each tissue sample. Serial dilutions of protein extracts were made for optimization of TRAP-Silver Staining Assay. Table 3.1 represents the protein concentrations of the samples used in the current study.

Table 3.1 Protein concentations of tissue specimens

Tumor	**Concentration (ug/mL)**	**Normal**	**Concentration (ug/mL)**
1	2000	**1**	2102
2	546	**2**	540
3	640	**3**	1018
4	619	**4**	1196
5	1394	**5**	1962
6	852	**6**	286
7	3661	**7**	479
8	815	**8**	124
9	1822	**9**	290
10	284	**10**	523
11	4500	**11**	1587
12	2147	**12**	1790
13	1682	**13**	1500
14	2063	**14**	3110
15	722	**15**	1184
16	762	**16**	909
17	2836	**17**	1876
18	500	**18**	1243
19	1741	**19**	1273
20	4547	**20**	1211

3.2 TRAP-Silver Staining Assay

Telomerase activity could be detected by four different methods which are TRAP aasay, terminal rectriction fragment length analysis, Real-time PCR and immunohistochemistry. Each of these methods is used for detection of a different aspect of telomerase activity. Therefore, all four methods are not homogenous in research meaning (Smith and Lam, 2009).

TRAP assay is the best analysis method of enzymatic activity. In this assay, specimens were considered as positive regarding telomerase activity showing six base pair incremental bands which starts from 50 bp (i.e, 50, 56, 62, 68, 74,etc) which are called also regular TRAP products (Figure3.1, Lane 1 and 2). Beside regular TRAP products, 36 bp internal control (IC) band also is seen. In contrast, telomerase negative specimens show only 36 bp IC band (Figure3.1, Lane 5 and 6).

Some lanes may exhibit weak IC band due to high telomerase activity. Representative results of both tumor and normal samples were illustrated in Figure 3.1.

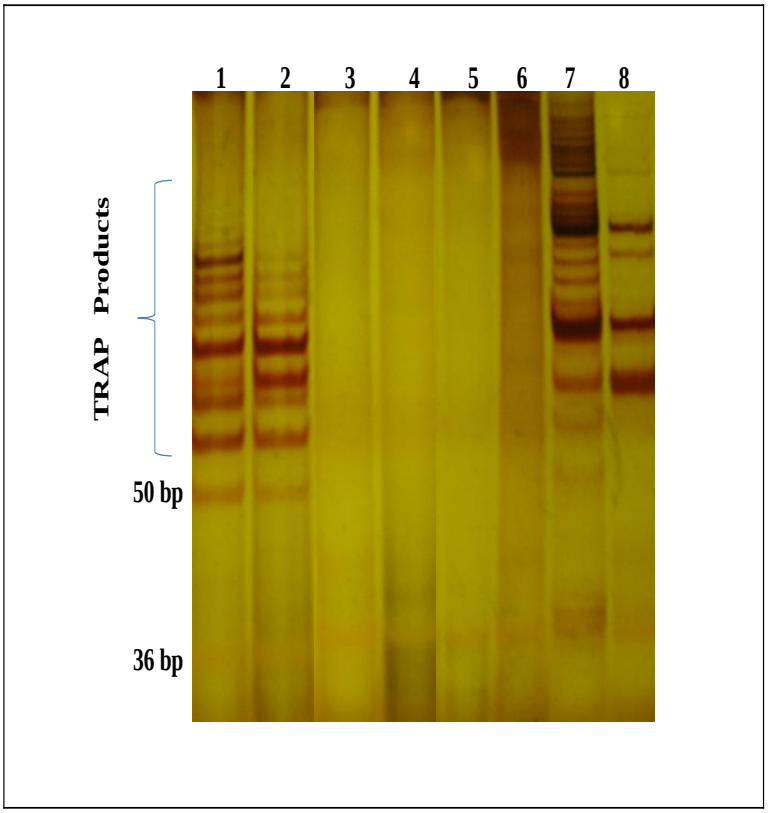


Figure 3.1 Representative results of TRAP-Silver Staning Assay. Lane 1 and 2: tumor specimens, Lane 3 and 4: normal specimens, Lane 5: CHAPS only, Lane 6: heat inactivated control, Lane 7 & 8: positive controls at different concentrations.

Since there is a competition for amplification between internal control and TRAP products. A heat-treated sample extract was used as a negative control for each assay because telomerase is a heat sensitive enzyme. To check contamination or primer dimer artifacts, CHAPS lysis buffer was used instead of sample extract in each experimental setup. Besides negative controls, a positive control was also included in the TRAP assay.

3.2.1 Optimization of Protein Content and PCR Cycle Number

Protein extracts of tissue specimens from colorectal cancer patients were diluted several folds to find out the optimal protein concentration for the TRAP-Silver Staning Assay.

Figure 3.2 demonstrated telomerase activity at different protein concentrations. It was seen that a linear correlation was found with and a r value of 0.996 between 100 ng to 800 ng according to RTA values. In Lane 6, where 1000 ng of protein extract was used, however no telomerase activity was observed. In the manufacturer's instructions, it is stated that high amount of protein does not correlate with high telomerase activity. There might ve some inhibitors in the protein extracts which can impair the results at high protein concentrations.

In colorectal cancer samples, the optimum amount of protein extract was determined to be 600 ng in order to get a significant band intensity in the TRAP-Silver Staning assay (Figure 3.2).

Since TRAP Assay is a PCR-based assay, PCR cycle number was optimized. Same extract and same amount of protein concentration was used in all tubes to detect optimum cycle number.

It was observed that RTA value increases exponentially as the cycle number increases (Figure 3.3B). From Figure 3.2 B, it was seen that 33 cycle seems to be optimal for the assay which is also consistent with manufacturers instructions for non-isotopic assay.

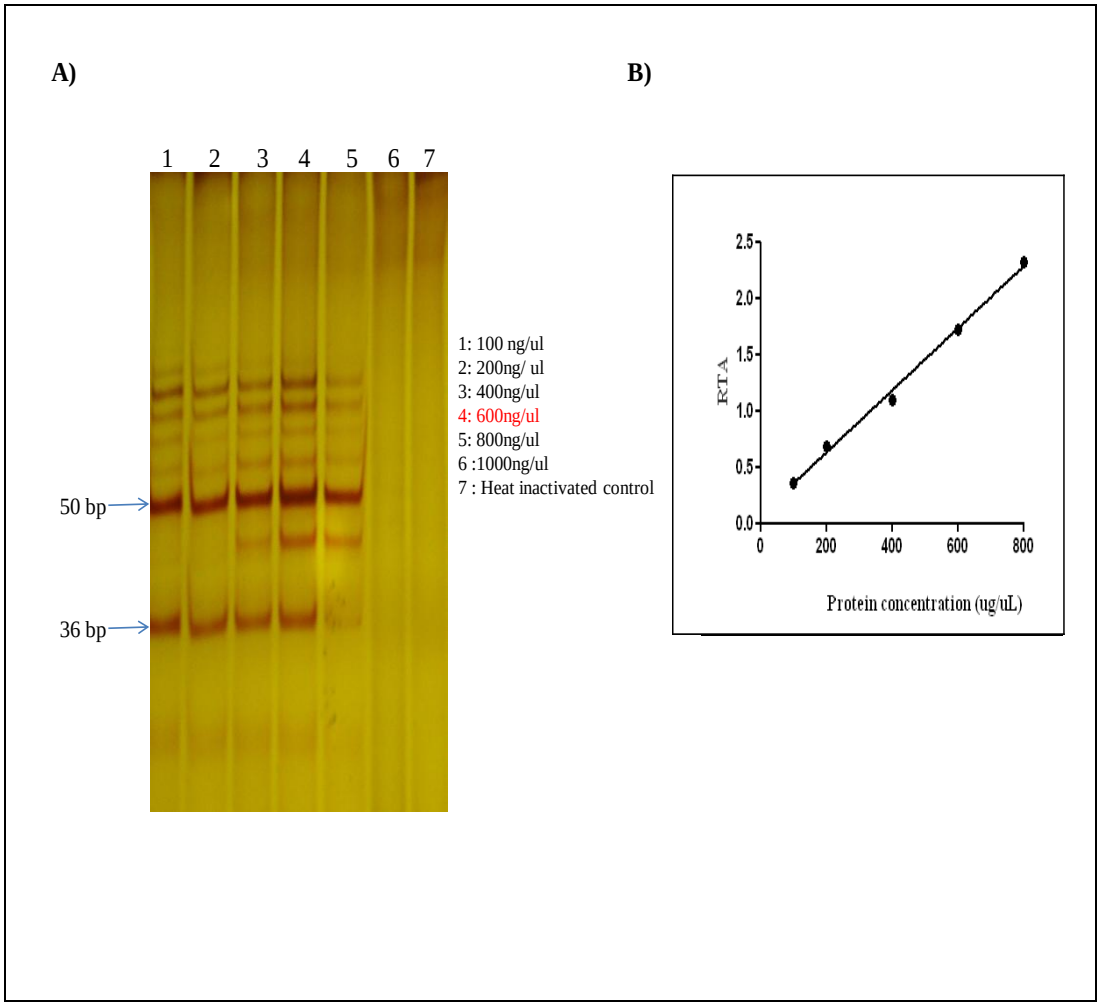


Figure 3.2 Optimization of protein content for TRAP Assay

Figure 3.3 represents PAGE-silver staining result for PCR cycle optimization (Figure 3.3 A) and statistical analysis (Figure 3.3 B).

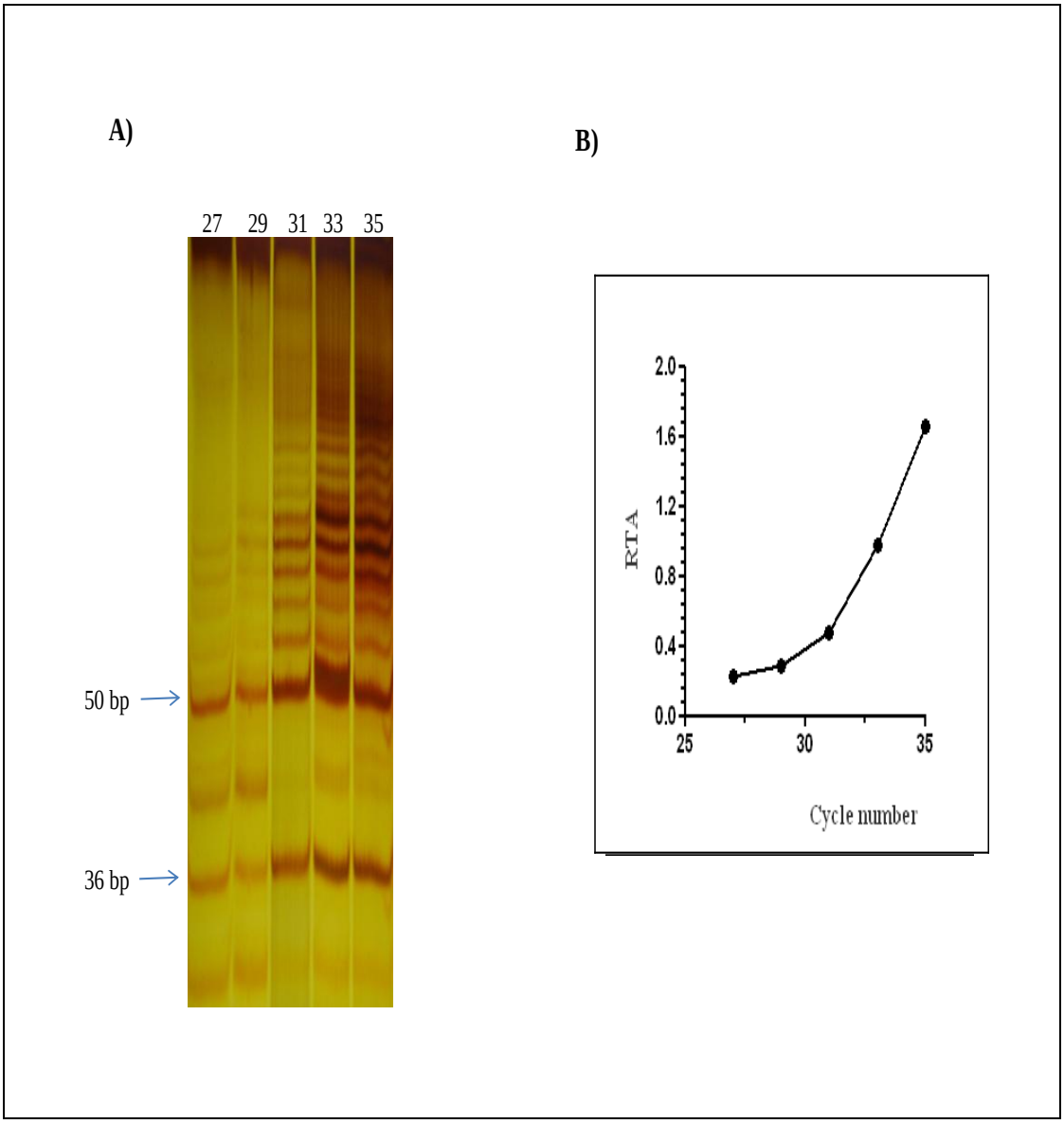


Figure 3.3 Optimization of PCR cycle number for TRAP assay

3.3 Telomerase Activity

A variety of studies have investigated the enzymatic activity of telomerase in colorectal cancer (Roig *et. al*, 2009). However, whether the activity of telomerase is in association with clinicopathological features of patients still controversial.

In this study, all 20 of colorectal cancer cases showed telomerase activity at different levels, whereas non of the normal tissues were telomerase positive. 3 of 23 cancerous tissues were found to be useless due to degraded RNAs and protein structures. These tissues showed neither detectable telomerase activity nor intact RNAs. This could be related with inappropriate storage conditions. These tissues were not included for the statistical analysis. A study conducted by Yan and colleagues reported the importance of tissue qualitiy for detection of telomerase activity (Yan *et. al*, 1998).

Although some of the previous studies reported that 15% of normal colonic mucosa specimens (Ghori *et. al*, 2002), 14% of normal colon tissues (Yoshida *et. al*, 1997) showed telomerase activity, the current study demonstrated no telomerase activity in normal colonic tissues which is consistent with other studies which showed telomerase activity in normal colon tissue 0/22 (Tang *et. al*, 1998), 0/15 (Yan *et. al*, 1999).

Telomerase activity was found 100% in colorectal cancer in this study which is similar to previous studies which detected telomerase activity 98 of 122 (80%; Tabata *et. al*, 2002), 60 of 60 (100%; Shoji *et. al*, 2000), 18 of 22 (96%; Tang *et. al*,1998).

As shown in Figure 3.4, telomerase activity has a tendency to be higher in young ages compared to old ages, however, this is not statistically significant ($p>0.05$). This could be an indicator of poor prognosis.

Likewise, the difference between man and woman regarding telomerase activity was not significant ($p> 0.05$).

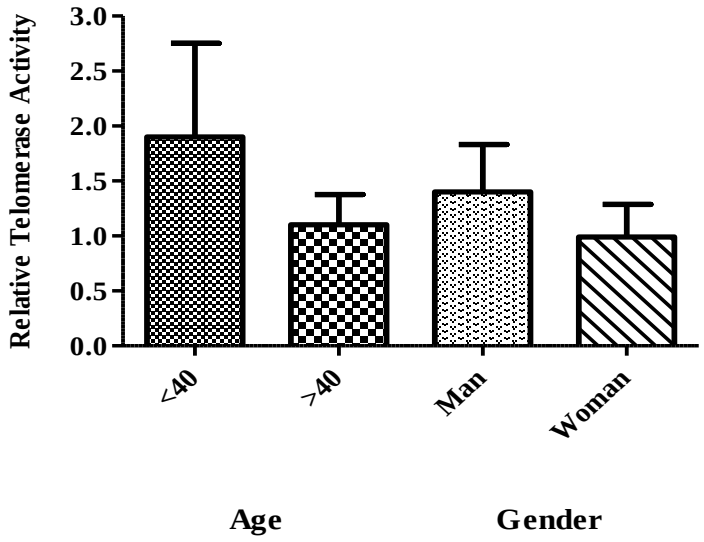


Figure 3.4 Relation between telomerase activity and age and gender parameter

In the current study, a northworty difference was observed between tumor site and telomerase activity. Tumors located at left colon had significantly higher telomerase activity compared to right colon as shown in Figure 3.5.

Proximal and distal colon carcinogenesis were found to have distinct biological characterictics which have been reported to be acquired during embryogenesis or postnatal development. Moreover, it was stated that different procarcinogenic pathways are responsible for development of carcinogenesis in right and left colon (Glebov V. et. al,2003). This may be related with the difference in the relative telomerase activity between right and left colon.

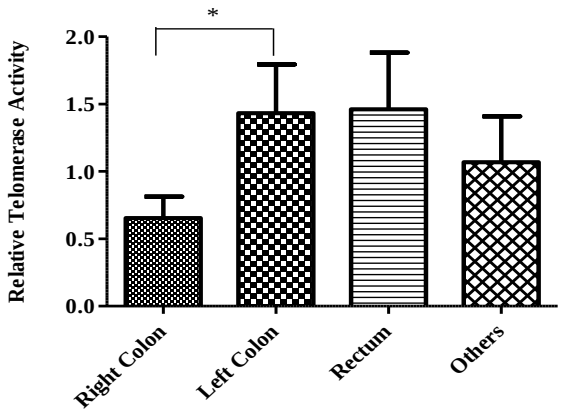


Figure 3.5 Relation between tumor site and telomerase activity. Others include tumors located at hepatic flexure, ascending colon, cecum, sigmoid colon. (* Results were signifcant with a $p<0.05$)

Furthermore, telomerase activity observed in rectum was slightly higher than other parts of colon but this difference was not significantly.

In previous studies conducted by Engelhardt et al.,1997 and Ghori et al.,2002, a correlation was found between pathological staging and telomerase activity (Engelhardt, Drullinsky, Guillem, & Moore, 1997). It was suggested that telomerase activity could be an indicator of the degree of aggressiveness of malignancies (Ghori *et al*.,2002).

In this study, 6 out of 20 patients in stage I and II were compared with 14 patients out of 20 in stage III and IV regarding telomerase activity. Telomerase activity increased significantly with advanced stages (Stage III and Stage IV) compared to early stages (Stage I and Stage II) (Figure 3.6). This could be related with accumulation of genetic alterations and mutations during carcinogenesis.

High telomerase activity with advance stages indicates agressiveness of cancer. (Chadeneau *et. al*, 1995).

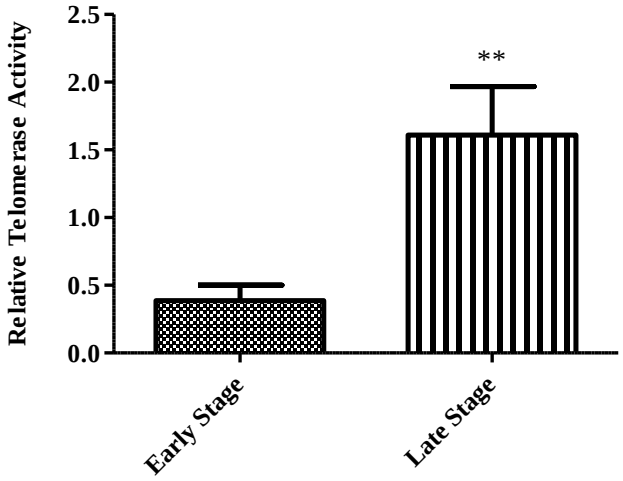


Figure 3.6 Association between stage of colorectal tumors and relative telomerase activity (** Results with a $p < 0.01$)

A comparison was made between histopathological types of the colorectal cancer tumors and their relative telomerase activities.

Although the major and usual histopathological type, colorectal adenocarcinomas, were also positive with respect to telomerase activity, the unusual histological type, mucinous adenocarcinomas, showed significantly higher telomerase activity compared to usual colorectal adenocarcinomas ($p<0.05$).

Generally, tumors with Grade 3 had slightly higher relative telomerase activity than tumors with Grade 2, however, the difference was not found to be statistically significant as shown in Figure 3.7.

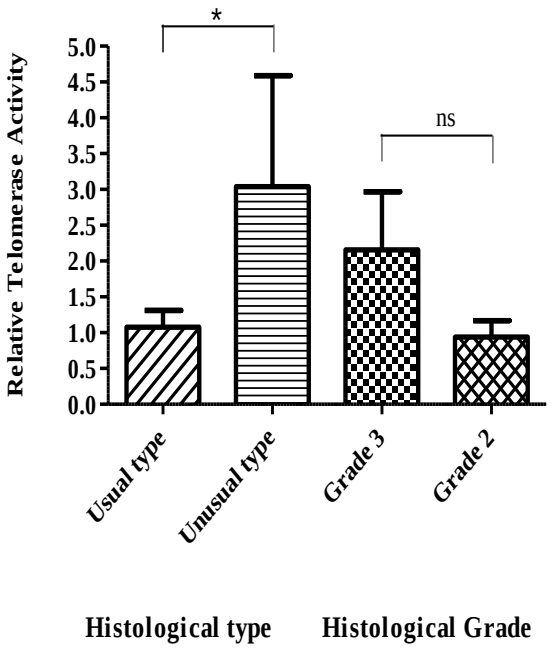


Figure 3.7 The association among relative telomerase activity, histopathological features, and grade of tumors (* Results were signifcant with a $p<0.05$)

TNM classification parameters were defined in section 1.2.3. Tumors with distant metastasis (M1) and lymph node metastasis revealed high telomerase activity when compared to tumors without distant metastasis (M0) and lymph node metastasis (N0) respectively (Figure 3.8).

Metastatic tumors were shown to have high telomerase activity. High telomerase activity may contribute tumor cells to proliferate fast and help escaping from apoptosis due to shortened telomeres. However, this may not be necessarily a requisite for metastasis (Saito *et. al*, 1997)

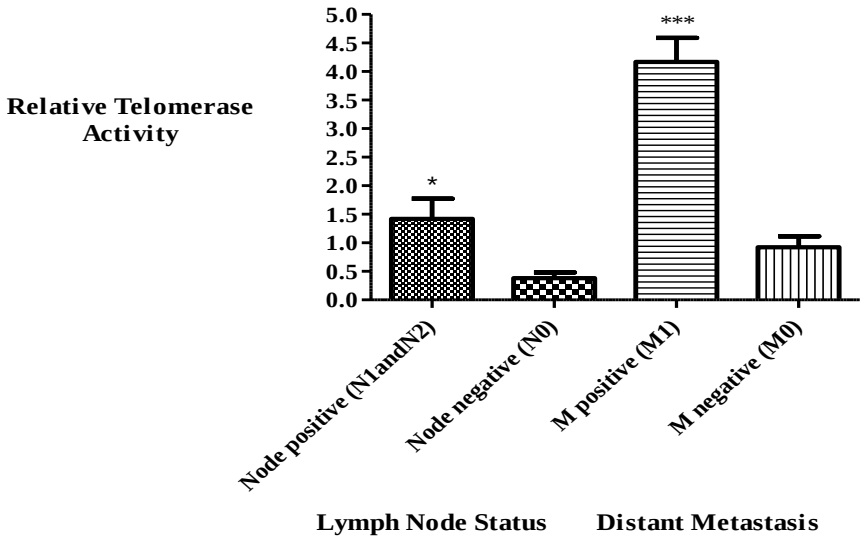


Figure 3.8 Evaluation of RTA in different TNM classification parameters (* Results were signifcant with a $p<0.05$ and *** results with a $p<0.0001$)

In this study, there is only one tumor sample determined with pT1; therefore, statistical analysis between RTA and tumor depth could not be perfomed. Similarly, in a study by Shoji *et. al*, 1999 found a relationship between telomerase activity and depth of invasion of tumors (pT) (Shoji *et. al*, 2000).

In two cases out of twenty, liver metastasis was observed with high telomerase activity. In addition, these two cases with liver metastasis were classified as pT3 and pT4, respectively.

The number of metastatic lymph node ranged from 0 to 13 among node positive (N1 and N2) tumors. Table 3.2 summaries RTA values (mean±sem**)** of clinicopathological factors obtained from Mann Whitney's U- test.

The correlation analysis was also performed with Spearman's rank correlation test. Lymph node status (positivity), the number of metastatic lymph node and stage were correlated with telomerase activity positively (Appendix B).

Additionaly, tumor size was found to be correlated with telomerase activity but was not statistically significant. Interestingly, age correlated negatively with telomerase activity in the current study.

Linear regression analysis was also performed to demonstrate the association clinicopathological factors effecting on RTA values. Analysis showed that among the all clinicopathological parameters, age, localization, stage of tumor and metastatis status were determined to have effect on telomerase activity significantly (Appendix B)

Table 3.2 Comparison of telomerase activity with clinicopathological factors

Factors	**No. Cases (percentage)**	**RTA (Mean ± SEM)**	**p**
Gender			
Woman	%55	0.99 (±0.98)	0.080
Man	%45	1.87 (±1.44)	
Age			
Young (<40)	%15	1.90 (±1.48)	0.145
Old (40<)	%85	1.30 (±1.24)	
Histological Type			
Usual	%10	1.26 (±1.04)	0.031*****
Unusual	%90	2.54 (±2,09)	
Localization			
Right	%35	0.69 (±0.37)	0.039 *****
Left	%65	1.76 (±1.42)	
Rectum	%35	1.68 (±1.12)	0.154
Colon	%65	1.23 (±1.35)	
Stage			
Early			
I	%30	0.52 (±0.26)	0.003 ******
II			
Late			
III	%70	1.78 (±1.34)	
IV			
Grade			
Low (I,II)	%75	1.13 (±0.96)	0.095
High (III,IV)	%25	1.16 (±1.83)	
N			
Negative (N0)	%35	0.76 (±0.36)	0.032 *****
Positive (N1, N2)	%65	1.419 (±0.35)	
M			
Negative (M0)	%90	1.08 (±0.83)	<0.0001 *******
Positive (M1)	%10	4.17 (±0.59)	

3.4 Gene Expression Analysis by qRT-PCR

3.4.1 Isolation of Total RNA

After isolation of RNA from colorectal cancer and their adjacent normal tissues, the intactness of RNAs were checked on 2% agarose gel. 28S and 18S rRNAs were visualized by agarose gel indicate the intactness of isolated RNA through two sharp and distinct bands. Furthermore, the purity and amount of RNA was checked by Nanodrop 2000C (Thermo Fischer Scientific, USA). All RNA samples involved in the current study had 1.8-2.1 (A260/280) ratio corresponding nucleid acid/ protein ratio in the sample (Table 3.3).

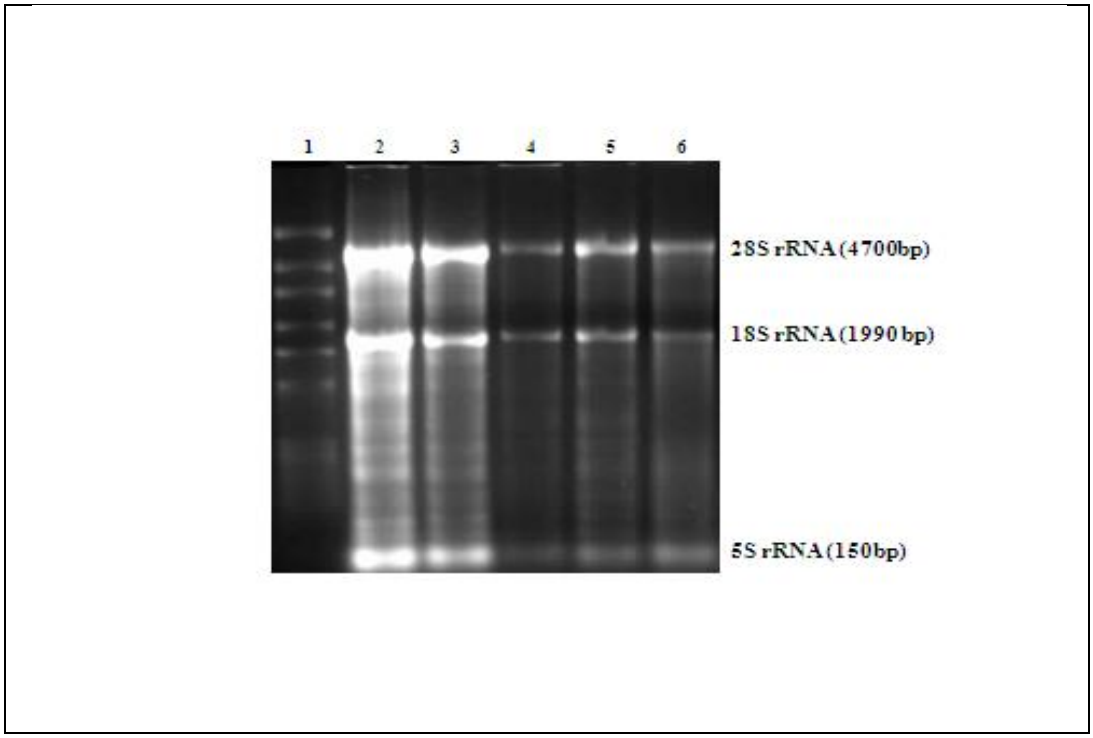


Figure 3.9 Total RNA isolation from tumor (Lane 2,3) and normal tissues samples (Lane 4-6) (Lane 1: RNA ladder)

Table 3.3 Purity and concentration of RNAs of tissue specimens

Tumors	**Amount (ng/ul)**	**Purity (A260/280)**	**Normals**	**Amount (ng/ul)**	**Purity (A260/280)**
1T	1440	1.95	**1N**	150	1.99
2T	871	1.99	**2N**	304.56	1.89
3T	382.6	1.93	**3N**	135	1.94
4T	400	1.85	**4N**	505	1.88
5T	304.9	1.82	**5N**	732	1.90
6T	450.9	1.89	**6N**	1011	1.85
7T	731.4	1.93	**7N**	566	1.98
8T	1657	1.92	**8N**	738	1.95
9T	2002	1.99	**9N**	191.1	2.00
10T	274.7	1.93	**10N**	2378	1.94
11T	859.8	1.99	**11N**	500	1.95
12T	2001	1.99	**12N**	435	1.94
13T	678	1.98	**13N**	650	1.89
14T	1582.7	1.79	**14N**	180	1.95
15T	1519	1.95	**15N**	196	1.97
16T	782	1.85	**16N**	258	1.93
17T	1859	1.80	**17N**	450	1.80
18T	658	1.94	**18N**	159	1.89
19T	1852	2.02	**19N**	693	1.92
20T	589	1.84	**20N**	458	1.93

3 out of 23 tissue specimens were excluded for further analysis due to degraded RNAs. Colorectal cancer and their adjacent tissues samples were fatty tissues. Therefore, the removal of fatty content from tissues specimens caused to get lower concentrations of RNAs especially from normal adjacent tissues (Figure 3.9, Lane 4-6).

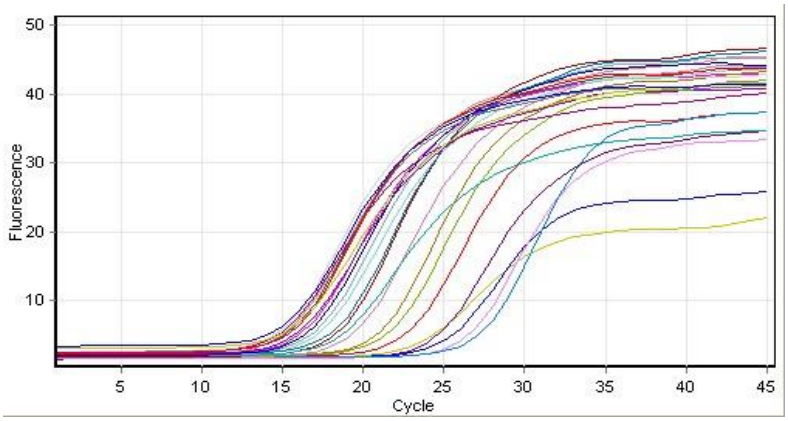
3.4.2 Quantitative Real-Time Polymerase Chain Reaction Analysis

Gene expression analysis was performed to determine the difference of expression patterns of hTERT (human telomerase catalytic subunit), hPOT 1 (human protection of telomeres 1), GAPDH (Glyceraldehyde 3 phosphatase dehydrogenase) in tumor and their adjacent normal tissues. GAPDH was used as internal control gene.

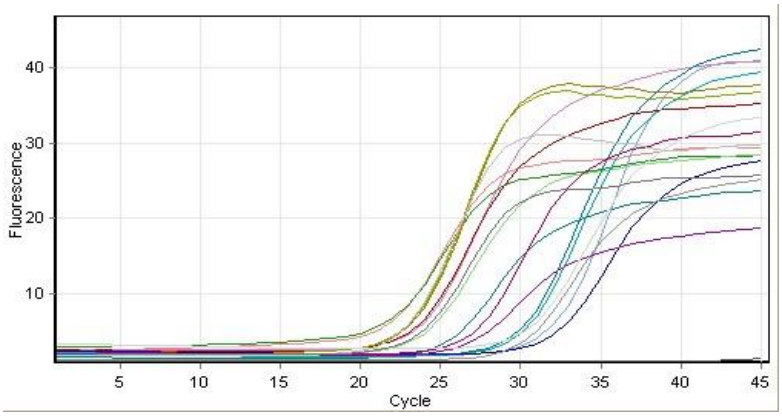
Amplification curves were displayed as fluorescence vs threshold cycle number (Figure 3.10).

Melt curve analysis was also perfomed after each run in order to check the presence of nonspecific amplification by monitoring the dissociation kinetics of the qPCR products (Figure 3.11).

a)



b)



c)

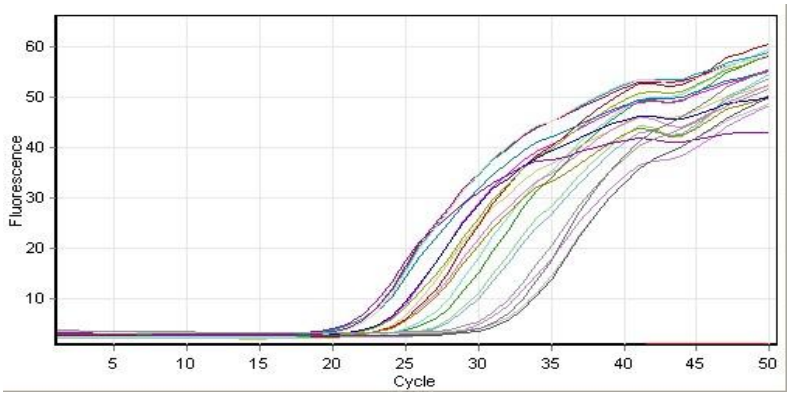
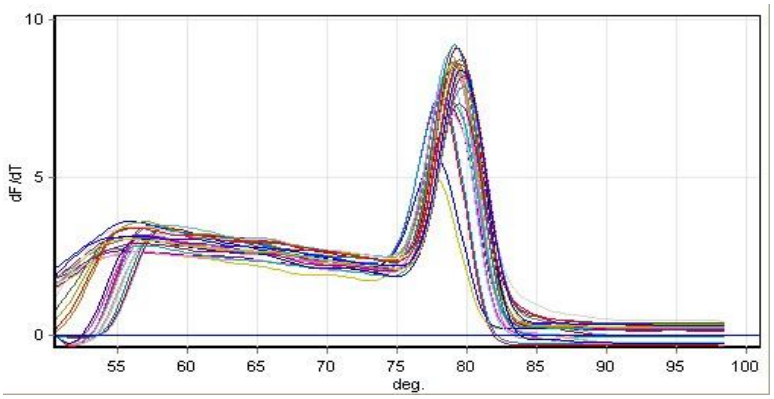
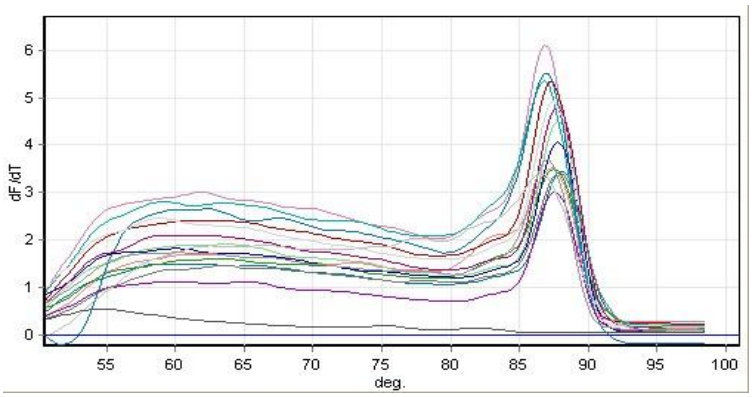


Figure 3.10 Amplification curves for a) GAPDH, b) hTERT, c) hPOT 1 in tumor and normal tissues.

a)



b)



c)

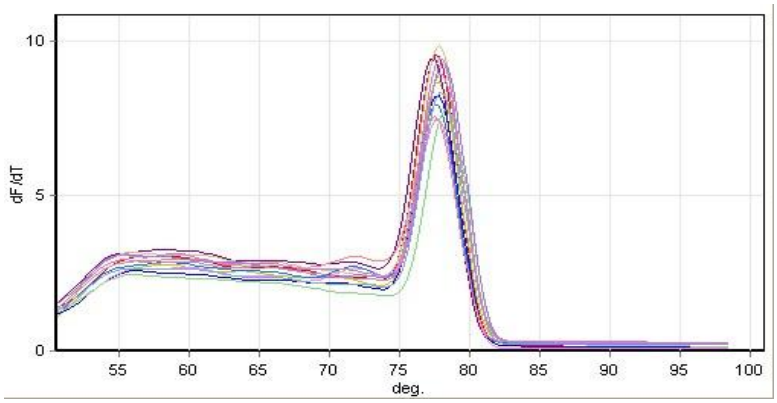


Figure 3.11 Melt curve analysis for a) GAPDH, b) hTERT, c) hPOT1 genes in tumor and normal tissues

Melt curves of GAPDH, hTERT, POT1 genes demonstrated that gene-specific products were generated which had melting peaks at the same temperature.

The quantitation data of hPOT1 and hTERT of tumor samples were normalized with respect to internal control gene GAPDH. The fold changes in the expression levels of GAPDH, hPOT1 and hTERT were determined relative to adjacent normal tissue specimens by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The results were interpreted as relative fold changes and demonstrated as bar graphs.

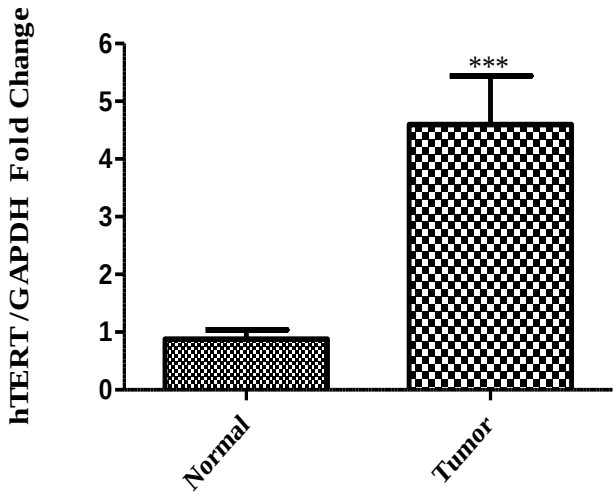


Figure 3.12 Expression of hTERT gene in tumor and normal tissues (*** Results with a $p<0.001$)

All of the tumor and adjacent normal tissues were found to express hTERT as displayed in Figure 3.12, however, tumor tissues showed 3.8 fold more hTERT expression compared to their normal adjacent tissues which is in accordance with previous results which revealed also increased expression of hTERT in colorectal tumors compared to corresponding normal tissues (Gertler *et. al*, 2002).

Similary, tumor tissues showed high telomerase activity compared to their adjacent non-canceorus tissues in this study. These results indicated that there was a close relation between telomerase activity and the level of hTERT expression.

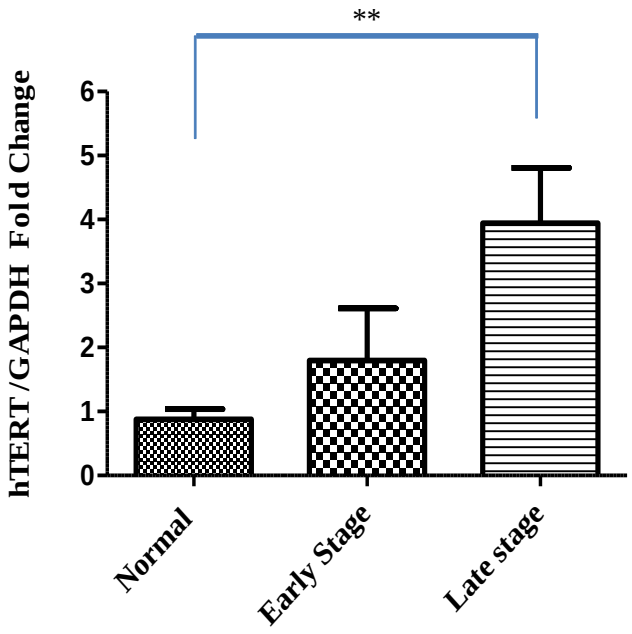


Figure 3.13 The association of hTERT expression with stage of tumors (** Results with a $p < 0.01$)

In the current study, gene expression of hTERT was found to increase as the cancer progressed (Figure 3.13). Tumors at early stage expressed high level of hTERT compared to normal tissues but statistically not significant. On the other hand, tumors in late stages showed 3.19 fold more expression level of hTERT compared to normal tissue samples which is consistent with other studies showing an association between telomerase catalytic subunit expression and stage of tumor (Ghori *et. al*, 2002). The comparison between early and late stage tumors with respect to telomerase activity revealed paralel pattterns. Late stage tumors displayed higher telomerase activity than early stage tumors.

The association between expression level of hTERT and clinicopathological parameters were also analysed as shown in Figure 3.14.

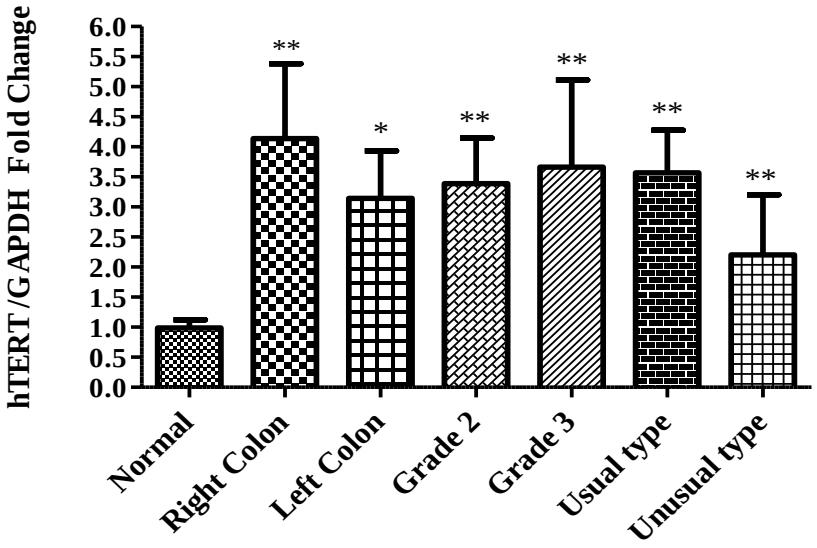


Figure 3.14 The assocation of hTERT expression with clinicopathological parameters (** Results with a $p < 0.01$)

The location (right and left colon), histology (usual and unusual types) and grade (II and III) of colorectal cancer tissues did not differ from each other significantly with respect to hTERT expression in the current study. But their expression was significantly higher than normal tissues.

Some of previous studies reported a correlation of telomerase catalytic subunit expression with depth of tumor (Shoji *et. al*, 2000) and tumor grade (Gertler *et. al*, 2002).

Even though a significant association was not found in the expression of hTERT among clinicopathological parameters (Figure 3.14), interestingly, the activity of telomerase was found to be higher significantly in left colon tumors compared to right colon tumors. This result may be related with different biological features and responses and/or acquired genetic alterations between right and left colon.

Additionally, histologically unusual type tumors showed higher telomerase activity than histologically usual type tumors.

According to Figure 3.15, lymph node metastasis status (positive or negative) showed no significant difference with respect to hTERT expression but their hTERT expression was found to be significantly high compared to normal tissues.

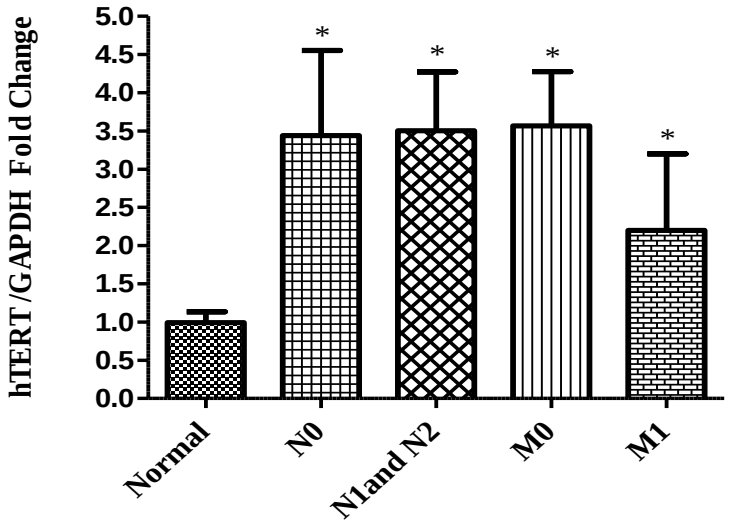


Figure 3.15 The relation between hTERT expression with N and M status (*Results wit a $p<0.05$, ** results with a $p < 0.01$)

Likewise, in tumors with distant metastasis, hTERT expression did not differ from tumors without distant metastasis but hTERT expression in tumors with or without distant metastasis was higher significantly than normal tissues.

Conversely, these two parameters, namely, lymph node status and distant metastasis, showed significant difference regarding telomerase actitivity in this study.

Fold changes in the expression of hTERT were represented in Table 3.4.

There are a number of studies which are controversial showing the association of expression levels of hTERT with clinicopathological parameters.

Some of earlier results were consistent with the results of current study which were unable to demonstrate a correlation among the expression of telomerase catalytic subunit and clinicopathological characteristics of tumors such as tumor site, lymph node involvement and distant metastasis (Gertler *et. al*, 2002).

In contrast, a study revealed a significant difference between expression of hTERT and tumor site. Moreover, another study demonstrated that hTERT expression correlated with tumor grade (Terrin *et. al*, 2008).

In the current study, normal tissues were telomerase negative, however, they were shown to express hTERT gene. This may be due to posttranscriptional splicing of hTERT or posttranslational regulation of hTERT gene. Kilian et. al,1997, reported that posttranscriptional splicing of hTERT products may lead to production of different proteins with different functions (Kilian *et. al*, 1997). Alternatively, alternative splicing of hTERT variants may cause the deletion in the protein structure (Yi *et. al*, 2000).

Table 3.4 Fold Changes in the expression of hTERT gene and relative telomerase acitivity (RTA)

Groups	**Fold Change (hTERT)**	**RTA**
Normal	0.99± 0.13	0.000
Tumor	4.59± 0.86	2,987 ± 0,015
Early Stage	1.79± 0.81	0,387 ± 0,113
Late Stage	3.94± 0.31	1,609 ± 0,358
Right Colon	4.13± 0.74	0,653±0,159
Left Colon	3.14± 0.78	1,432 ± 0,362
Grade 2	3.38± 0.74	0,941 ± 0,228
Grade 3	3.66± 0.94	2,156 ± 0,814
Usual Type	3.56± 0.71	1,078 ± 0,232
Unusual Type	2.20± 0.95	3,038 ± 1,551
Lymph node metastasis negative (N0)	3.56± 0.91	0,382 ± 0,100
Lymph node metastasis positive (N1 and N2)	3.50± 0.58	1,419 ± 0,353
Distant metastasis negative (M0)	3.56± 0.62	0,922 ± 0,191
Distant metastasis positive (M1)	2.20 ± 0.89	4,169 ± 0,420

Fold changes were represented as 'mean± SEM'. SEM values were determined from three independent experiments, each run in dublicates.

All tumor tissues showed telomerase activity together with significantly high level of hTERT expression. Telomerase activity is seemed to be correlated with the expression of its catalytic subunit (Appendix B).

hPOT1 which is a telomere binding protein is thought to effect on the activity of telomerase. The expression of protection of telomere 1 gene was also studied in the current study. Tumor tissues showed 2.6 fold more expression of hPOT1 than normal tissues (Figure 3.16).

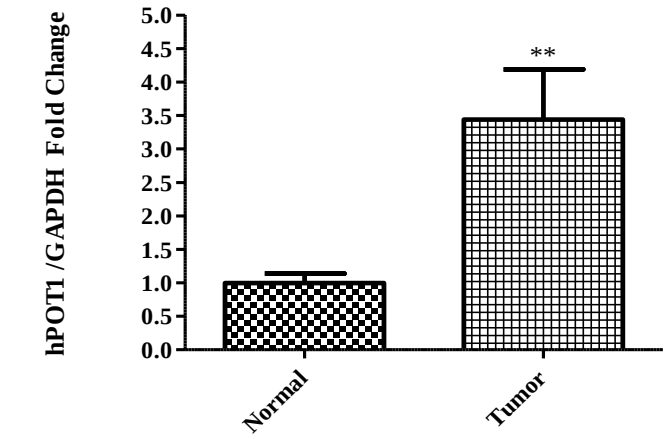


Figure 3.16 Expression of hPOT1 in tumor and matched normal tissues (** Results with a $p < 0.01$)

The relative fold change of hPOT1 gene expression was compared among different tumor parameters.

Early stage tumors were found to express slightly higher level of hPOT1 compared to normal tissues but not statistically significant. However, tumors at late stage expressed 2.55 fold more hPOT1 compared to normal tissues. The difference in the expression level of hPOT1 was not statistically significant between late stage tumors and early stage tumors.

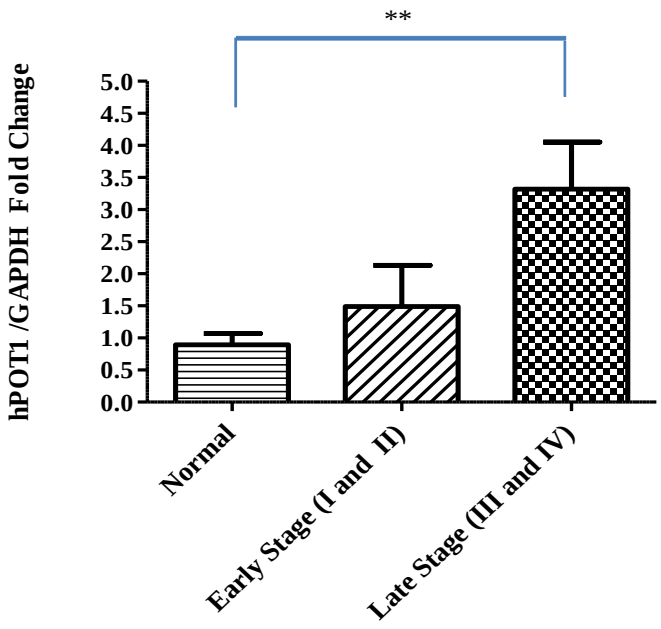


Figure 3.17 The hPOT1 gene expression at different stages of tumors (** Results with a $p < 0.01$)

A study conducted in gastic carcinoma revealed similar results that POT1 expression was found to increase with tumor stage. Tumors in stage III and IV, POT1 expression was found to be upregulated (Kondo *et. al*, 2004).

In addition, the tumor site showed significant difference wih respect to expression of hPOT1. Tumors located at left colon showed notably high level of hPOT1 expression compared to tumors at right colon and normal tissues as shown in Figure 3.18.

Moreover, tumors with grade II and both usual and unsual type tumors showed significant difference regarding the expression level of hPOT1compared to normal tissues in the current study. However, the difference among histological type and grade of tumors in the level of hPOT1 expression was not statistically significant.

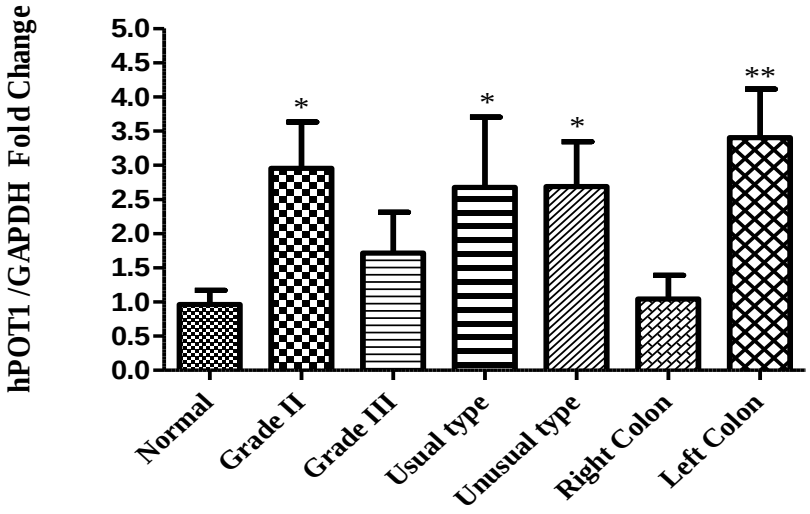


Figure 3.18 The relation of the expression of hPOT1 with histopathological and clinicopathological features (*Results with a $p<0.05$, ** results with a $p < 0.01$)

According to Figure 3.19, among TNM parameters, tumor with lymph node metastasis, tumors with and without distant metastasis demonstated significantly high level of hPOT1 expression when compared to normal tissues. However, the difference regarding hPOT1 expression between tumors with distant metastasis and tumors without distant metastasis was not found to be statistically significant.

Tumors with lymph node metastasis showed significanlty high level of hPOT1 expression than tumors without lymph node metastasis and normal tissues.

Similar pattern was observed regarding telomerase activity in tumors with lymph node metastasis which demonstrated higher activity of telomerase compared to lymph node metastasis negative tumors.

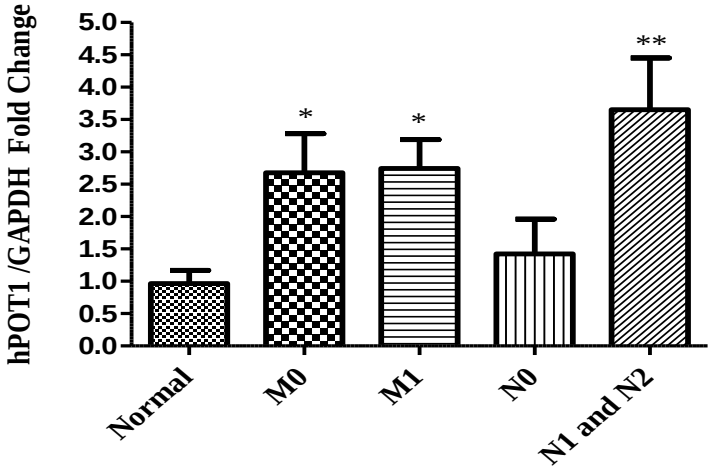


Figure 3.19 Expression of hPOT1 and TNM parameters (Distant metastasis (M) and lymph node metastasis (N)) (*Results wit a $p<0.05$, ** results with a $p < 0.01$)

Fold changes in the expression of hPOT1 are represented in Table 3.5.

Previous studies regarding the expression of hPOT1 and its relation with telomerase acitivity and telomere elongation were controversial. In the current study, a significant correlation was found between relative telomerase activity and the expression of hPOT1 (Appendix B).

Table 3.5 Fold Changes in expression of hPOT1 and relative telomerase activity (RTA)

Groups	**Fold Change (hPOT1)**	**RTA**
Normal	0.99 ± 0.14	0.000
Tumor	3.43 ± 0.75	2,987 ± 0,015
Early Stage	1.49 ± 0.64	0,387 ± 0,113
Late Stage	3.32 ± 0.73	1,609 ± 0,358
Right Colon	1.40 ± 0.34	0,653±0,159
Left Colon	3.40 ± 0.71	1,432 ± 0,362
Grade 2	2.95 ± 0.67	0,941 ± 0,228
Grade 3	1.71 ± 0.59	2,156 ± 0,814
Usual Type	2.67 ± 0.96	1,078 ± 0,232
Unusual Type	2.68 ± 0.58	3,038 ± 1,551
Lymph node metastasis negative (N0)	1.42 ± 0.43	0,382 ± 0,100
Lymph node metastasis positive (N1 and N2)	3.65 ± 0.78	1,419 ± 0,353
Distant metastasis negative (M0)	2.67 ± 0.60	0,922 ± 0,191
Distant metastasis positive (M1)	2.74 ± 0.44	4,169 ± 0,420

Fold changes were represented as 'mean± SEM'. SEM values were determined from three independent experiments, each run in dublicates.

Considering that all tumors in the current study were telomerase positive together with elevated expression of hPOT1 may indicate the recruitment of telomerase to the end of the telomeres by hPOT1.

hPOT1 may provide more open configuration for the access of telomerase by displacing T-loop structure (Blackburn, 2001).

Another study also confirmed these findings and stated that both overexpression and knockdown of hPOT1 gene led to telomere elongation. It was proposed that there was a homestatic control for the control of telomere length in telomerase positive cells for which a certain level of hPOT1 expression is responsible. Overexpression or downregulation of hPOT1 gene may result in telomere elongation depending on the activity of telomerase (Yang *et. al*, 2007).

Colgin *et. al*. suggested that hPOT1 acts as telomerase dependent, positive regulator of telomere lenght. Because they found that cells with the overexpression of hPOT1 gene were also telomerase positive and had longer telomeres (Colgin *et. al*, 2003).

The results in the current work were consistent with previous studies which have found a positive correlation with telomerase activity and hPOT1 gene expression in colorectal cancer.

CHAPTER 4

CONCLUSION

1. All of the samples obtained from colorectal cancer patients showed high telomerase activity, whereas their normal adjacent tissues showed no detectable telomerase activity. This could indicate that telomerase activity may serve as a diagnostic or prognostic marker in colorectal cancer.

2. Telomerase activity did not differ in genders; however, the activity of telomerase was found to be higher at young age.

3. Tumors located at left colon demonstrated higher telomerase activity compared to tumors located at right colon.

4. Tumors at late stage showed significantly high telomerase activity compared to tumors at early stage. This result indicated that telomerase activity increased with cancer progression. However, such an association was not found between telomerase activity and tumor grade.

5. Histopathologically unusual tumor types, mucinous adenocarcinomas, showed significantly higher telomerase activity than usual type of tumors, adenocarcinomas.

6. Tumors with lymph node metastasis and distant metastasis demonstrated statistically significant telomerase activity than tumors negative regarding lymph node metastasis and distant metastasis, respectively.

7. Tumor size, lymph node metastasis, distant metastasis and stage of tumor were found to correlate with telomerase activity positively, while age was found to correlate with telomerase activity negatively.

8. Gene expression analysis demonstrated that both hTERT and hPOT1 were overexpressed in tumor tissues compared to normal tissues. The expression levels of hTERT and hPOT1 were found to increase with the stage of tumor. Late stage tumors expressed significantly high level of hTERT and hPOT1 compared to early stage tumors which is paralel with telomerase activity.

9. Localization, grade, histological type, distant metastasis and lymph node status of tumor did not demonstrate significant difference in the expression of hTERT.

10. Expression of hTERT was correlated with telomerase activity. The activity of telomerase is regulated in a number of way. This could be explained by alternative splicing variants of hTERT.

11. Left-sided tumors, tumors with lymph node metastasis and distant metastasis expressed hPOT1 gene higher than right-sided tumors, tumors negative for lymph node metastasis and distant metastasis, respectively.

12. Expression of hPOT1 was significantly correlated with telomerase activity positively.

In conclusion, the absence of telomerase activity in normal adjacent tissues may indicate that the enzymatic activity of telomerase and hTERT expression may be clinically useful as a diagnostic or prognostic tool.

Moreover, the activity of telomerase and its catalytic subunit expression was found to correlate significanlty in colorectal cancer in the current study. These results may indicate that telomerase could be a promosing marker for colorectal cancer in the future.

In this study, some relations or/and associations found was statistically non-significant. This could be related with a limited number of patients categorized in different clinicopathological parameters. Thus, it may be useful to demonstrate these results in a larger number of patients' pool.

In addition, the activity of telomerase was shown to correlate with the expression of human protection of telomere 1 gene which is a single stranded telomere binding protein. Overexpression of protection of telomere 1 protein in tumor tissues may lead to association of POT1 protein with telomere 3' overhang more internally, thus, more 3' overhang is accessible for telomerase to bind to telomeres. This correlation indicates that the regulation of telomerase activity is a complex process than anticipated.

REFERENCES

American Cancer Society.2011. Cancer Facts & Figures 2011. Atlanta; American Cancer Society; 2011.

Aisner, D. L., Wright, W. E. and Shay, J. W. (2002). Telomerase regulation: not just flipping the switch. *Curr. Opin. Genet. Dev.* 12, 80-85.

Autexier, C., & Lue, N. F. (2006). The Structure and Function of Telomerase Reverse Transcriptase. Annu Rev Biochem.75:493-517.

Baumann, P., Podell, E., & Cech, T. R. (2002a). Human Pot1 (Protection of Telomeres) Protein: Cytolocalization, Gene Structure, and Alternative Splicing, *22*(22), 8079-8087.

Baumann, P., Podell, E., and Cech, T.R. (2002). Human POT1 (protection of telomeres) protein: cytolocalization, gene structure, and alternative splicing. Mol. Cell. Biol. *22*, 8079–8087

Blackburn, E. H. and Gall, J. G. (1978). A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena.. J. Mol. Biol. 120, 33-53.

Blackburn EH (1990) Telomere structure and synthesis. *J Biol Chem* 265: 5919-5921.

Blackburn, H., & Biology, C. (1990). Minireview Telomeres and Synthesis, *265*(11), 5919-592

Blackburn EH (1991) Structure and function of telomeres. *Nature* 350: 569-573.

Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell*, *106*(6),66-173.

Blasco, M.A., M. Rizen, C.W. Greider, and D. Hanahan.1995.Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nature Genet.12: 200 -204.

Carroll, K. a, & Ly, H. (2009). Telomere dysfunction in human diseases: the long and short of it. *International journal of clinical and experimental pathology*, *2*(6), 528-43.

Chadeneau, C., Hay, K., Hirte, H. W., Gallinger, S., & Bacchetti, S. (1995). Advances in Brief Telomerase Activity Associated with Acquisition of Malignancy in Human Colorectal Cancer1. *Medicine*, 2533-2536.

Chai, W., Du, Q., Shay, J. W., & Wright, W. E. (2006). Human telomeres have different overhang sizes at leading versus lagging strands. *Molecular cell*, *21*(3), 427-35.

Chen, J.-L., and Greider, C.W. (2003) Template boundary definition in mammalian telomerase. Genes Dev. 17: 2747-2752.

Colgin, L. M., Baran, K., Baumann, P., Cech, T. R., & Reddel, R. R. (2003). Human POT1 Facilitates Telomere Elongation by Telomerase. *Current*, *13*, 942-946.

Colgin, L., & Reddel, R. (2004). Telomere biology: a new player in the end zone. *Current biology: CB*, *14*(20), R901-2.

Collins, K. (2008). Physiological assembly and activity of human telomerase complexes. *Mechanisms of ageing and development*, *129*(1-2), 91-8.

Cong, Y.-sheng, Wright, W. E., & Shay, J. W. (2002). Human Telomerase and Its Regulation, *66*(3), 407-425.

Dahse, R., Fiedler, W., & Ernst, G. (1997). Telomeres and telomerase: biological and clinical importance. *Clinical chemistry*, *43*(5), 708-14.

de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes & development*, *19*(18), 2100-10.

de Lange T., Shiue L., Myers R.M., Cox D.R., Naylor S.L.,Killery A.M., and Varmus H.E. 1990. Structure and variability of human chromosome ends. *Mol. Cell. Biol.* 10: 518.

Duesberg P, Li R, Rasnick D, Rausch C, Willer A, Kraemer A, Yerganian G, Hehlmann R (2000) Aneuploidy precedes and segregates with chemical carcinogenesis. Cancer Genetics and Cytogenetics, 119(2):83-93.

Elkak, a, Mokbel, R., Wilson, C., Jiang, W. G., Newbold, R. F., & Mokbel, K. (2006). hTERT mRNA expression is associated with a poor clinical outcome in human breast cancer. *Anticancer research*, *26*(6C), 4901-4.

Engelhardt, M., Drullinsky, P., Guillem, J., & Moore, M. a. (1997). Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *3*(11), 1931-41.

Fearon E.R, Vogelstein B.(1990) A genetic model for colorectal tumorigenesis, Cell,61, 759-767.

Forsythe, H. L., Jarvis, J. L., Turner, J. W., Elmore, L. W. and Holt, S. E. (2001). Stable association of hsp90 and p23, but Not hsp70, with active human telomerase. *J. Biol. Chem.* 276, 15571-15574.

Fu, D. and Collins, K. (2003). Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. *Mol. Cell* 11, 1361-1372.

Futreal P. A., Kasprzyk A, Birney E, Mullikin J.C, Wooster R, Stratton M.R (2001) Cancer and Genomics, *Nature* 409, 850-852.

Gertler, R., Rosenberg, R., Stricker, D., Werner, M., Lassmann, S., Ulm, K.,Nekarda, H., et al. (2002). Prognostic potential of the telomerase subunit human telomerase reverse transcriptase in tumor tissue and nontumorous mucosa from patients with colorectal carcinoma. *Cancer*, *95*(10), 2103-11.

Ghori A.,Usselmann B.,Ferryman S.,Morris A., Fraser I.(2000). Telomerase expression of malignant epithelial cells correlates with Dukes' stage in colorectal cancer. Colorectal Disease 4(6), 441–446.

Greenberg, R. a, & Rudolph, K. L. (2005). Telomere structural dynamics in genome integrity control and carcinogenesis. *Advances in experimental medicine and biology*, *570*, 311-41.

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell 97, 503–514.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674. Elsevier Inc.

Hanahan, D., Weinberg, R. A., & Francisco, S. (2000). The Hallmarks of Cancer Review University of California at San Francisco, *100*, 57-70.

Haendeler, J., Hoffmann, J., Brandes, R. P., Zeiher, A. M. and Dimmeler, S. (2003). Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol. Cell. Biol. 23, 4598-4610.

Henderson ER, Blackburn EH.(1989). An overhanging 3' terminus is a conserved feature of telomeres. Mol Cell Biol.,9(1):345-8.

Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, Shay JW (1995) Correlating telomerase activity levels with human neuroblastoma outcomes.Nat Med. 1(3):249-55.

Hockemeyer, D., Daniels, J.-P., Takai, H., & de Lange, T. (2006). Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres. *Cell*, *126*(1), 63-77.

Huang, J., Zheng, S., Jin, S.-H., & Zhang, S.-Z. (2004). Somatic mutations of APC gene in carcinomas from hereditary non-polyposis colorectal cancer patients. *World journal of gastroenterology: WJG*, *10*(6), 834-6.

Kawanishi-Tabata, R., Lopez, F., Fratantonio, S., Kim, N., Goldblum, J., Tubbs, R., Elson, P., et al. (2002). Telomerase activity in stage II colorectal carcinoma. *Cancer*, *95*(9), 1834-9.

Kelleher, C., Kurth, I., & Lingner, J. (2005). Human Protection of Telomeres 1 (POT1) Is a Negative Regulator of Telomerase Activity In Vitro. *Society*, *25*(2), 808-818.

Kilian, A., Bowtell, D.D.L. and Abud, H.E. (1997) Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex slicing patterns in different cell types. *Hum. Mol. Genet.*, 6, 2011–2019.

Kim, N. W., Piatyszek, M. a, Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., et al. (1994, December 23). Specific association of human telomerase activity with immortal cells and cancer. *Science* 266 (5193), 2011-2015

Kinzler, K. W., & Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell*, *87*(2), 159-70.

Klingelhutz, A. J., Normal, A., Abbreviation, C., & Human, H. P. V. (1997). Telomerase activation and cancer, 45-49.

Knudson, A. G. (1993). Antioncogenes and human cancer. Proc Natl Acad Sci U S A, *90*, 10914-10921.

Kondo, T. (2004). Expression of POT1 is Associated with Tumor Stage and Telomere Length in Gastric Carcinoma. *Cancer Research*, *64*(2), 523-529.

Lei M, Podell ER, Cech TR (2004) Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. *Nat Struct Mol Biol* 11: 1223–1229

Le S, Zhu JJ, Anthony DC, Greider CW, Black PM(1998). Telomerase activity in human gliomas. Neurosurgery 42(5):1120-4; 1124-5

Lei, M., Podell, E.R., and Cech, T.R. (2004). Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. Nat. Struct. Mol. Biol. 11, 1223–1229.

Lei, M., Zaug, A. J., Podell, E. R., & Cech, T. R. (2005). Switching human telomerase on and off with hPOT1 protein in vitro. *The Journal of biological chemistry*, *280*(21), 20449-56.

Livak, K J.,Schmitthen, T.D.(2001).Analysis of relative gene expression data using realtime quantitative PCR and the 2(Delta Delta C(T)) Methods,25(4),402-408.

Londoño-Vallejo, J. A. (2008). Telomere instability and cancer. *Biochimie*, *90*(1), 73-82.

Moon, I. K., & Jarstfer, M. B. (2007). University of North Carolina, School of Pharmacy, Division of Medicinal Chemistry and Natural Products Chapel Hill, North Carolina 27599-7360. *Medicinal Chemistry*, 4595-4620.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW (1997) Activation of β-Catenin-Tcf Signaling in Colon Cancer by Mutations in β-Catenin or APC, Science, 275(5307):1787-90.

Muñoz-Jordán JL, Cross GA, de Lange T, Griffith JD.(2001) T-loops at trypanosome telomeres. EMBO J.20(3), 579-88.

National Cancer Institute. (2011) Colon and rectal Cancer. http://www.cancer.gov/cancertopics/types/colon-and-rectal. Last accessed on 12.01.12.

Poole, J. C., Andrews, L. G., & Tollefsbol, T. O. (2001). Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene*, *269*(1-2), 1-12.

Rhodin Edsö, J., Gustafsson, C., & Cohn, M. (2011). Single- and double-stranded DNA binding proteins act in concert to conserve a telomeric DNA core sequence. *Genome integrity*, *2*(1), 2.

Rieger, P. T. (2004). The biology of cancer genetics. *Seminars in Oncology Nursing*, *20*(3), 145-154.

Roig, A. I., Wright, W. E., & Shay, J. W. (2009). Is Telomerase a Novel Target for Metastatic Colon Cancer, Current Colorectal Cancer Reports, 5:203–208

Saito Y, Kosugi S, Wakabayashi Y, Mishima Y, Hatakeyama K, Kominami R (1997)Telomerase activity and metastasis: expansion of cells having higher telomerase activity within culture lines and tumor tissues, Jpn J Cancer Res.,88(8)-732-7.

Sawyers CL, McLaughlin J, Goga A, Havilik M, Witte O (1994). The nuclear tyrosine kinase c-Abl negatively regulates cell growth. *Cell*,77:121-131.

Sauer R., Becker H, Hohenberger W.,Rödel C.,Wittekind C.,Fietkau R.,Martus P.,Tschmelitsch J.,Hager E.,Hess F.C., Karstens J-H., Liersch T.,Schmidberger H., Raab R., (2004).Preoperative versus postoperative chemoradiotherapy for rectal cancer N Engl J Med 2004; 351:1731-1740.

Shay JW, Bacchetti S. (1997) A survey of telomerase activity in human cancer.EurJCancer, 33(5),787-791.

Shay, J. W., Zou, Y., Hiyama, E., & Wright, W. E. (2001). Telomerase and cancer, Human Molecular Genetics *10*(7), 677-686.

Shoji Y., Yoshinaga K., Inoue A., Iwasaki A.,Sugihara K.,2000. Quantification of telomerase activity in sporadic colorectal carcinoma. Cancer 88(6), 1304–1309.

Smith R. A Lam A. K-Y (2009) Telomerase activity: detection and clinical implications in human cancers. Clinical oncology and search; 2009-24614.

Stansel, R.M., de Lange, T., and Griffith, J.D. (2001). T-loop assembly in vitro involves binding of TRF2 near the 3_ telomeric overhang EMBO J. *20*, E5532–E5540

Takai, K. K., Kibe, T., Donigian, J. R., Frescas, D., & de Lange, T. (2011). Telomere protection by TPP1/POT1 requires tethering to TIN2. *Molecular cell*, *44*(4), 647-59.

Tang, R., Cheng, a J., Wang, J. Y., & Wang, T. C. (1998). Close correlation between telomerase expression and adenomatous polyp progression in multistep colorectal carcinogenesis. *Cancer research*, *58*(18), 4052-4.

Terrin, L., Rampazzo, E., Pucciarelli, S., Agostini, M., Bertorelle, R., Esposito, G., DelBianco, P., et al. (2008). Relationship between tumor and plasma levels of hTERT mRNA in patients with colorectal cancer: implications for monitoring of neoplastic disease. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *14*(22), 7444-51.

Theimer, C. a, & Feigon, J. (2006). Structure and function of telomerase RNA. *Current opinion in structural biology*, *16*(3), 307-18.

Wang, F., Podell, E. R., Zaug, A. J., Yang, Y., Baciu, P., Cech, T. R., & Lei, M. (2007). The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature*, *445*(7127), 506-10.

Watson, J. D. (1972). "Origin of concatemeric T7 DNA." *Nat New Biol* 239(94):197-201.

Wolpin, B. M., Meyerhardt, J. a, Mamon, H. J., & Mayer, R. J. (n.d.). Adjuvant treatment of colorectal cancer. *CA: a cancer journal for clinicians*, *57*(3), 168-85.

Wick M, Zubov D, Hagen G (1999). Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT).Gene 232, 97-106.

Wright,W.E. and Shay,J.W. (1992) The two-stage mechanism controlling cellular senescence and immortalization. Exp. Gerontol., 27,383-389.

Wright WE, Pereira-Smith OM, Shay JW. (1989) Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. Mol Cell Biol. 9(7):3088-92.

Wright, W. E., Tesmer, V. M., Liao, M. L., & Shay, J. W. (1999). Normal human telomeres are not late replicating. *Experimental cell research*, *251*(2), 492-9.

Urquidi, V., Tarin, D., Goodison, S. Role of telomerase in cell senescence and oncogenesis. Annu. Rev. Med., *51:* 65–79, 2000.

Yan, P., & Bosman, F. T. (1998). Diagnostic Techniques Tissue Quality is an Important Determinant of Telomerase Activity as Measured by TRAP Assay. *Biotechniques*, *25*(4), 660-662.

Yang, Q., Zhang, R., Horikawa, I., Aaltonen, L. A., & Harris, C. C. (2007). Functional Diversity of Human Protection of Telomeres 1 Isoforms in Telomere Protection and Cellular Senescence, 11677-11686.

Ye, J.Z., and de Lange, T. (2004). TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. Nat. Genet. 36, 618–623.

Ye, J. Z.-sheng, Hockemeyer, D., Krutchinsky, A. N., Loayza, D., Hooper, M., Chait, B. T., & De, T. (2004). POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2 / TRF1 complex, (212), 1649-1654.

Yi X, White D.M, Aisner D.L, Baur J.A, Wright W.E, Shay J.W (2000) An Alternate Splicing Variant of the Human Telomerase Catalytic Subunit Inhibits Telomerase Activity. Neoplasia 2(5): 433–440.

Zhong, Z., Shiue, L., Kaplan, S. & de Lange, T. (1992). A mammalian factor that binds telomeric TTAGGG repeats in vitro. Mol. Cell. Biol. 13, 4834-4844.

APPENDIX A

BUFFERS AND SOLUTIONS

Polyacrylamide solution, %40

38 % Acrylamide

2 % Bisacrylamide

Store at 4 °C in dark.

Non-denaturating Polyacrylamide stock solution, %12.5 (400mL)

40% Polyacrylamide solution (19:1)	125 mL
5X TBE Buffer	40 mL
Deionized Water	235 mL

5X Tris-Boric acid-EDTA (TBE) Buffer (1L)

Tris Base	54 g
Boric acid	27,5 g
0.5 M EDTA, pH 8.0	20 mL

Loading dye solution (Non-Denaturating Gel) (5mL)

Glycerol	2,5 mL
1.25% Bromophenol Blue	1mL
1.25% Xylene Cyanol	1mL
0.5 M EDTA, pH 8.0	0.5 mL

Fixation solution (600mL)

10% EtOH	600 mL
98% Acetic acid	3 mL

Impragnation Solution(300mL)

0,1% $AgNO_3$ (w/v)	300 mL

Developing Solution (300mL)

1.5% NaOH (w/v)	300 mL
35% Formaldehyde	1.2 mL

Ethidium bromide (Etbr) solution

Etbr	10 mg
dH_2O	1 mL

Store at 4 °C in dark.

50x Tris-Acetate-EDTA (TAE) Buffer (1L)

Tris base	242 g
Acetic acid	57.1 mL
0.5 M EDTA	100 mL

Diethylpyrocarbonate (DEPC) treated water (1L)

DEPC	1 mL
dH_2O	1L

2X RNA loading dye (Fermantas, Lithuania)

0.5 mM EDTA

95% Formamide

0.025% SDS

0.025% Bromophenol blue

0.025% Xylene cyanol FF

0.025% Ethidium bromide

6x DNA loading dye (Fermantas, Lithuania)

60 mM EDTA

10 mM Tris-HCl (pH 7.6)

0.03 % Xylene cyanol FF

60% Glycerol

APPENDIX B

CORRELATION AND LINEAR REGRESSION ANALYSIS

Table B.1 Correlation analysis between RTA values and clinicopathological findings

	Age	**Tumor Size**	**N (lymph node status)**	**No. Metastatic Lymph node**	**Stage**
r value	-0.485	+0.345	+0.455	+0.467	+0.770
p value	0.030	0.136	0.044	0.038	<0.001

Table B.2 Linear regression analysis of clinicopathological findings with telomerase activity

Variable	**B Value**	**p**
Age	0.259	0,033
Localization	0.275	0,025
Tumor Grade	0.228	0,044
M status (Distant Metastasis)	0.756	<0.001

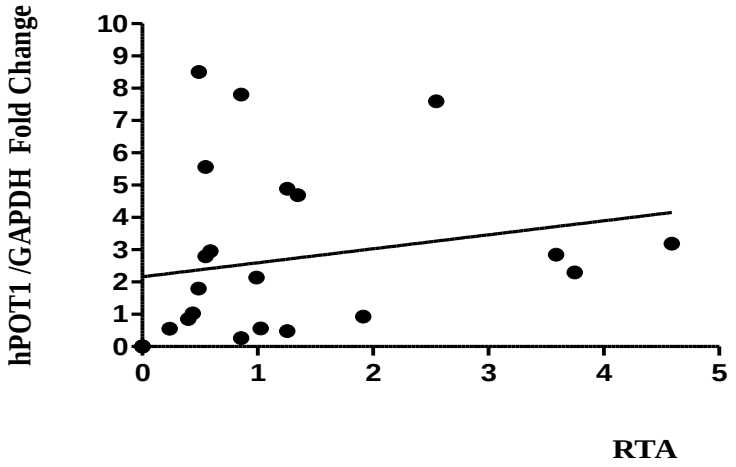


Figure B.1 Correlation between relative telomerase activity and expression of hPOT1

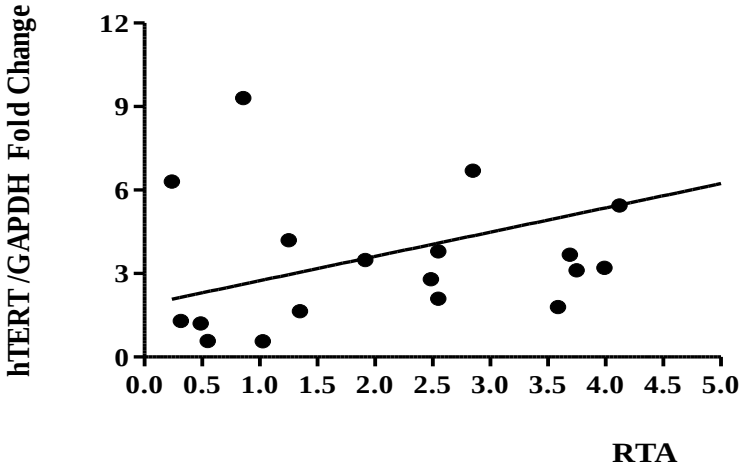


Figure B.2 Correlation between relative telomerase activity and expression of hTERT