SEPTEMBER 2018 M.Sc. in Biology NISREEN AL-DOORI

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INVESTIGATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) GENE PROMOTER MUTATIONS IN COLORECTAL CANCER

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NISREEN AL-DOORI

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I hereby declare that all information in this document has been obtained and presented according to the academic rules and appropriate conduct. Likewise, as required by these rules and conduct, I declare that, I have cited and referenced all material and results that are not original to this work.

Nisreen AL-DOORI

ABSTRACT

INVESTIGATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) GENE PROMOTER MUTATIONS IN COLORECTAL CANCER

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Among the different types of malignancies, colorectal cancer (CRC) is the third most common and also it is in the second place of the leading causes of cancer death in both men and women globally. In most of the cancer cells, telomere length is preserved by telomerase. Hence, telomere length and telomerase activity are essential for cancer initiation and the tumors' survival. In human, the promoter region of the TERT gene is considered to be the most important regulatory element for telomerase expression. TERT promoter mutations, such as the C228T and C250T, have been identified as important genetic events activating telomerase in different types of cancer. Consequently, these mutations may work as important markers in malignancies. In our study and for the first time in Turkey, the mutation analysis was performed in 43 pairs of tumor and matching normal tissues obtained from Turkish patients with colorectal cancers to determine the presence of mutations in the promoter region of the TERT gene and to investigate any possible association of the relevance of TERT promoter mutations to colorectal cancer. The mutations in the promoter region of TERT gene were examined in colorectal tumors and normal adjacent tissues by the Sanger-Bidirectional Sequencing technique. No mutations were observed in any of the specimens examined. We concluded from these results that the mutations in the promoter of the TERT gene may not be involved in CRC carcinogenesis and further investigations need to be performed for a stronger understanding.

Key Words: Telomerase Reverse Transcriptase (TERT), Colorectal Cancer, Promoter Mutation, C228T, C250T.

ÖZET

KOLOREKTAL KANSERDE TELOMERAZ TERS TRANSKRIPTAZ (TERT) GEN PROMOTÖR MUTASYONLARININ ARAŞTIRILMASI

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Yüksek Lisans Tezi, Biyoloji
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Malignitelerin farklı tipleri arasında, kolorektal kanser, hem erkek hem de kadınlarda küresel olarak kanser ölümlerinin en yaygın üçüncü nedenidir. Kanser hücrelerinin çoğunda telomer uzunluğu telomeraz ile korunur. Bu nedenle, telomer uzunluğu ve telomeraz aktivitesi, kanser başlangıcı ve tümörün hayatta kalması için gereklidir. İnsanda, TERT gen promotör bölgesi, telomeraz ekspresyonu için en önemli düzenleyici unsur olarak kabul edilir. C228T ve C250T gibi TERT promoter mutasyonları, farklı kanser türlerinde telomerazı aktive eden önemli genetik olaylar olarak tanımlanmıştır. Bu nedenle, bu mutasyonlar malignitelerde önemli belirteçler olarak çalışabilir. Türkiye'de ilk kez olarak, bizim çalışmamızda TERT geninin promotör bölgesinde mutasyonların varlığını belirlemek için ve TERT promoter mutasyonlarının kolorektal kanserle herhangi bir ilişkisi olup olmadığını araştırmak için kolorektal kanserli Türk hastalardan elde edilen 43 çift tümör ve normal komşu dokularında mutasyon analizi yapılmıştır. TERT geninin promoter bölgesindeki mutasyonlar, Sanger-Çift Yönlü sekanslama tekniği ile kolorektal tümörler ve normal komşu dokularında incelenmıştır. İncelenen örneklerin hiçbirinde mutasyon gözlenmemiştır. Bu çalışma ile TERT geninin promotör bölgesindeki mutasyonların kolorektal kanser ile ilişkili olmadığını sonucuna varılmıştır.

Anahtar Kelimeler: Telomeraz Ters Transkriptaz (TERT), kolorektal kanser, promotör mutasyonları, C250T, C228T.

To My Family

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
ATC	Anaplastic thyroid carcinoma
BCCs	Basal cell carcinomas
bp	Base pairs
℃	Celsius degree
CcRCCs	Clear Cell Renal Cell Carcinomas
CNS	Central Nervous System
CRC	Colorectal Cancer
dH2O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic Acid
EMT	Epithelial-mesenchymal transition
ETS	E-twenty-six
EWS	Ewing's sarcoma
g	Gram
GBM	Glioblastoma multiform
GI	Gastrointestinal system
HCC	Hepatocellular Carcinomas
HCV	Hepatitis C virus

HIF-1α	Hypoxia-inducible factor 1-alpha
HPV	Human Papillomavirus
HR	Hazard ratio
Kbs	Kilo bases
LNVUC	Large nested Variant of Urothelial Carcinoma
mad1	Mitotic arrest deficiency 1
mg	Milligram
ml	Milliliters
μM	Micro molar
mm	Millimetres
MUP	Metastases of an unknown primary tumor
N	Normal
NSCLC	Non-small cell lung cancer
OD	Optical Density
PCR	Polymerase Chain Reaction
POT1	Protection of Telomeres 1
PTs	Phyllodes tumors
Rb	Retinoblastoma protein
RBP2	Retinoblastoma binding protein 2
RMRP	RNA component of mitochondrial RNA-processing endoribonuclease
RNA	Ribonucleic acid
RTL	Relative telomere length
SP1	Specificity Protein1

SP/KLF	Specificity Protein/Krüppel-like Factor
STAT3	Signal transducer and activator of transcription 3

CHAPTER 1

INTRODUCTION

1.1 Biology of Cancer

For centuries, one of the most concerning health problems is cancer, and also it is one of the main causes that lead to death. Around (1,688,780) new incidences of cancer are anticipated to be diagnosed in 2017 and also approximately (600,920) cases are predicted to die of cancer in USA (Siegel et al., 2017). The definition of cancer can be referred to genetic illnesses that can result in wild cell growth and cell proliferation leading to form tumors in the body throughout a series of mutations which are acquired and/or inherited (Rieger, 2004).

There are two types of factors that can lead to cancer which can be divided into external factors and internal factors. The external factors are infectious diseases, radiation, tobacco, and irritating chemicals while the internal factors consist of mutations, hormones and immune conditions. Each of external and internal factors so-called carcinogens leads to initiation of carcinogenesis. Carcinogenesis is a procedure made of a cancer developmental requiring steps known as initiation, promotion, and progression (Gupta and Chaphalkar, 2015).

Tumors can be classified into benign and malignant. Benign is non-invasive and local hence it can be considered as the non-cancerous type. Yet, malignant is known to be invasive and metastatic thus it is defined as the cancerous type (Hanahan and Weinberg, 2011). Characteristically, malignant tumors act more aggressively through their development (Duesberg et al., 2000). The responsible genes for the occurrence of cancer are classified into proto-oncogenes and tumor-suppressing genes (Futreal et al., 2001). The majority of the genetic modifications found in cancer are divided to two categories: mutations that are responsible for function possession in proto-oncogenes, that stimulate cell growth, replication, and survival; and mutations in charge of the function loss in tumor-suppressing genes that ordinarily assist in

preventing unrestrained cellular growth, promoting DNA repair, and activating cell cycle checkpoint (Lee and Muller, 2010). Commonly the different types of cancer cells possess self-sufficiency in growth signals, also they are insensitive to anti-growth signals, avoiding apoptosis, possessing the possibility of replication unlimitedly, continuously angiogenic, tissue invader, and metastatic (Fouad and Aanei, 2017). The cancer types can be classified into six major groups according to the origin of the tumor formation (Cancer Research UK, 2017):

- Carcinoma develops from the skin cells or the epithelial tissue that sheathes the internal organs.
- Sarcoma is found in the bone tissue, cartilage tissue, fat, muscle, blood vessels, connective or supportive tissue.
- Leukemia originates from the marrow of bones.
- Lymphoma develops from the cells of the immune system.
- Myeloma usually is found in brain tissues or cells of the spinal cord.

1.2 Colorectal Cancer

Depending on Globocan, among the different types of malignancies, colorectal cancer (CRC) is in the third and in the second place of the causes that most commonly lead to cancer death globally in males and females respectively around 694,000 death cases of CRC have happened internationally in 2012 (Globocan, 2012). The rank of CRC is the third regarding the occurrence in men and women in Turkey (Hacıkamiloğlu et al., 2017). Despite the fact that CRC doesn't show signs in the primary stages, there are numerous indications witnessed in the moderate or the advanced stages; rectal bleeding, bloody stool, stool with dark- or black-color, changes in the stool shape, convulsive ache in the lower part of the stomach, losing weight unintentionally, a few days or more lasting constipation or diarrhea (American Cancer Society, 2016).

1.2.1 Anatomy and Function of Colon and Rectum

CRC takes place in the colon/rectum, the components of the gastrointestinal system (GI), which also known as the digestive system. There are 4 sections of the colon (Siegel and Jemal, 2011): the ascending colon, the transverse colon, the descending colon, and the sigmoid colon. The colon is around 5-6 feet in length, a muscular tube and it is the first part of the large intestine and the longest while the rectum is only its

final 6 inches. From inner to outer: colon and rectum are surrounded by the layers; mucosa (the epithelial cells on the internal surface), submucosa (a tissue of fibers), muscularis propria (a thick muscular layer), and serosa (a fatty and peritoneal layer) (Figure 1.1). Colon and rectum tracts are functioned to absorb the water and the nutrients from the digested food and to eliminate the rest of the undigested food as waste throughout the anus (Seeley et al., 2003).

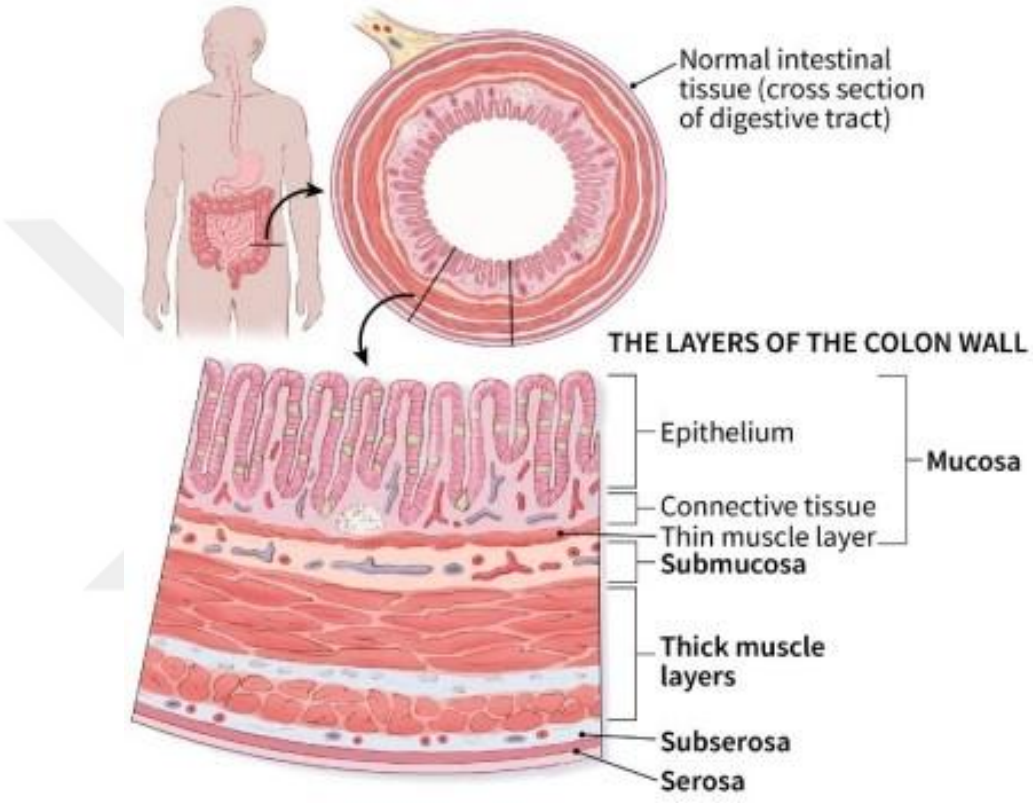

Figure 1.1 The colon and rectum in humans anatomically (American Cancer Society, 2018).

1.2.2 Staging of Colorectal Cancer

The classification (tumor, node, and metastasis) or system of TNM has been presented by the American Joint Committee on Cancer (AJCC) to be used for CRC staging. The TNM classification system basically depends on the tumor invasion level deeply inside the intestinal wall, the level of the tumor metastasis into lymph nodes, and the presence of distant metastasis towards any other organs. TNM staging delivers considerable information for the patients' prognosis (Wolpin and Mayer, 2008). Table 1.1 represents the TNM classification.

Table 1.1 TNM Classification System (Wolpin and Mayer, 2008).

Primary Tumor (T)		
T_x	Primary tumor cannot be assessed Tis Carcinoma in situ	
T_1	Tumor invades submucosa	
T_2	Tumor invades muscularis propria	
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 1–3 regional lymph nodes	
N_2	Metastases in ≥4 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 and 5-year survival		
Stage	TNM classification	5-year survival
I	T_{1-2}, N_0, M_0	>90%
IIA	T_3, N_0, M_0	80%–85%
IIB	T_4, N_0, M_0	70%–80%
IIIA	T_{1-2}, N_1, M_0	65%–80%
IIIB	T_{3-4}, N_1, M_0	50%–65%
IIIC	T_{1-4}, N_2, M_0	25%–50%
IV	T_{1-4}, N_{0-2}, M_1	5%–8%

1.2.3 The Fundamental Molecular Mechanisms for Colorectal Cancer

CRC is proposed to be generated from adenomas through stepwise volumetric and morphologic modifications in the colonic structure. A genetic model for CRC has been created by representing a stepwise gene imperfections accumulation. For that reason, CRC was recognized as one of the best genetic models for multistep tumorigenesis (Fearon and Vogelstein, 1989).

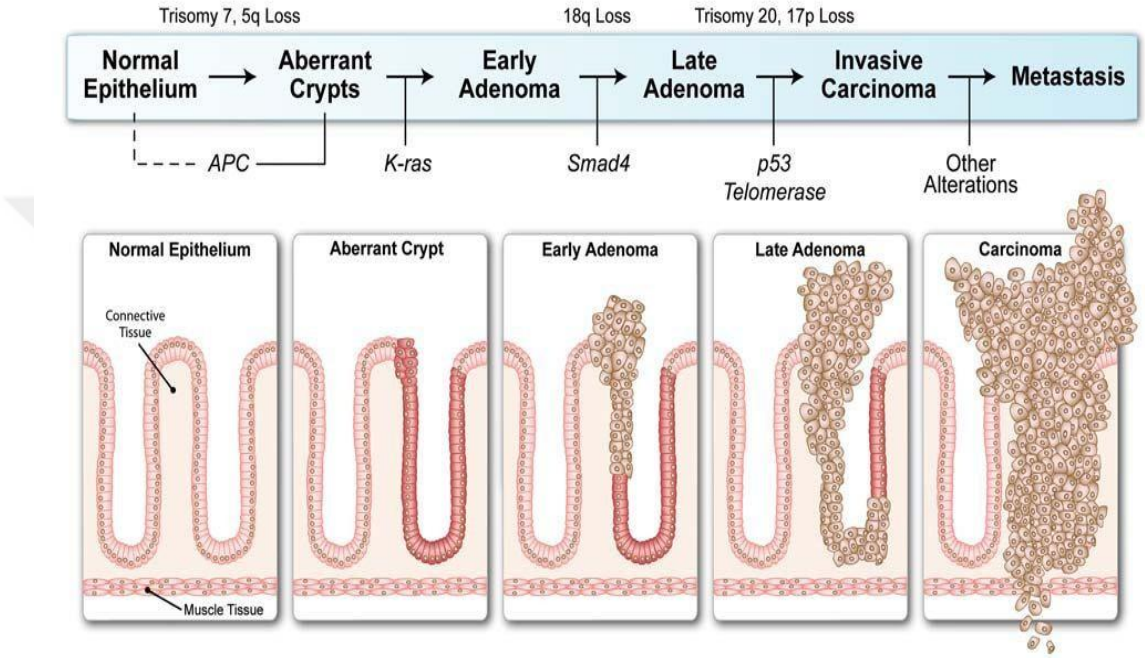

Figure 1.2 Multistep model of the developed colon cancer in human (Roig et al., 2009).

Adenomatous polyposis coli (APC) is a tumor suppressor gene that located on the chromosome 5q. The pivotal roles in controlling the cell cycle, apoptosis, migration, and adhesion have been reported to be played by the APC gene. APC controls the cell's shift into a hyper-proliferative state, and its inactivation which occurs from germline mutations results in 80% of colorectal cancers. For these reasons, APC is an early as well as the main event for developing the tumorigenesis of the colorectal (Morin and Weeraratna, 2003; Huang et al., 2004; Bush et al., 2013).

K-Ras mutations which are oncogenes commonly trigger the transition of cells from normal epithelium to adenoma (Kantarjian and Wolff, 2016). The absence of the

heterozygosity at chromosome 18q has been detected in around 50% of the CRCs in association with poor prognosis which is maybe due to the loss of the assumed tumor suppressor gene in CRC (Shibata et al., 1996). As labeled in Figure 1.2, other tumor suppressor genes' inactivation (p53, SMAD4 genes) and the telomerase gene's activation result in invasive carcinoma sequentially. As in the situation of the mutations' accumulation, mutations order has also been recommended to be a basic factor for colonic tumorigenesis (Knudson, 1993).

1.3 Telomere

1.3.1 Telomere Structure

The linear chromosomes end with structures that known as telomeres. All eukaryotic chromosomes end with guanine-rich tandem repeats. The human telomere is in dynamic homeostasis, and its sequence consists of (TTAGGG) repeats that range from 8 to 20 kb (Moyzis et al., 1988; Greider and Blackburn, 1989; Blackburn, 2001). There is a 3' overhang in telomere that usually loops back and inserts into the double strand of the telomere, thereby forming the t-loop and making the chromosome more stable (Griffith et al., 1999) as shown in Figure 1.3.

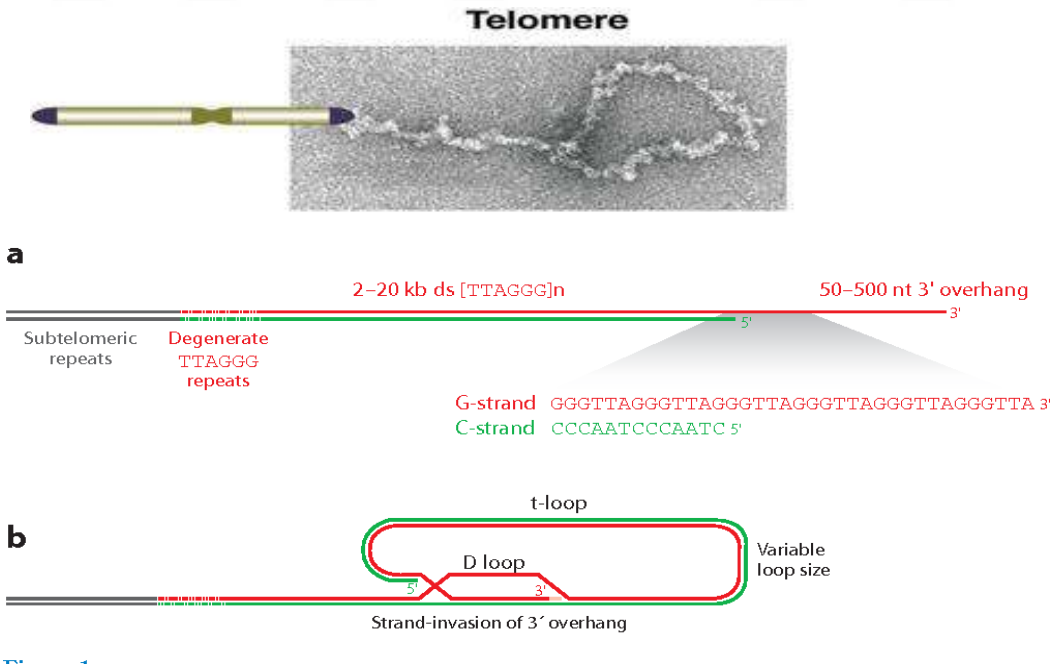

Figure 1.3 The telomeric structure in mammals (Nikitina and Woodcock, 2004).

1.3.2 Telomere-Binding Proteins

Telomeric DNA is associated with a specific protein complex, which is necessary for maintaining the structure and functionality of telomere. The most important protein complex structure is shelterin, which includes TRF1 (TTAGGG-repeat binding factor 1), TRF2, TIN2, RAP2, TPP1, and POT1. TRF1 and TRF2 bind the double-stranded telomere and combine with the rest proteins to form a special structure at the end of chromosomes. Shelterin regulates 3' overhang and t-loop formation (Lange, 2005) as shown in Figure 1.4.

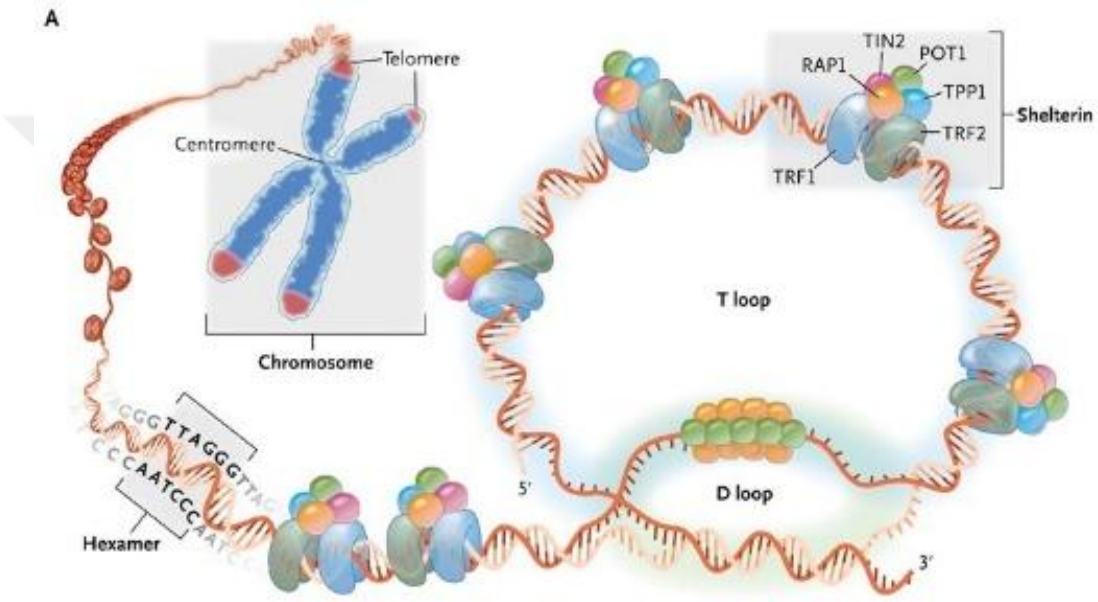

Figure 1.4 Structure of Shelterin complex (Calado and Young, 2008).

1.3.3 Telomere Shortens with Cell Division

In most normal human cells, the telomere length is shortened each time cell divides due to lack of telomere maintenance mechanism plus the end replication problem. The so-called 'end replication problem' was identified by Watson. During the DNA replication events DNA strands split into two types; leading strand is continuously synthesized, and lagging strand is synthesized as fragments that known as 'Okazaki fragments' which need RNA primer to be extended by DNA polymerase. Once the elimination of RNA primer at lagging strand happens, the gaps cannot be filled by DNA polymerase. Thus, telomere length is reduced step by step after each time the cell divides because of the incapability of DNA polymerase to reproduce the entire

lagging strand (Watson, 1972) (Figure 1.5). When telomere is shortened to a certain limit, cells will stop divide and undergo permanent growth arrest so-called 'replication senescence' (Wright et al., 1989; Harley, 1991). Therefore, most normal human cells are incapable of dividing indefinitely.

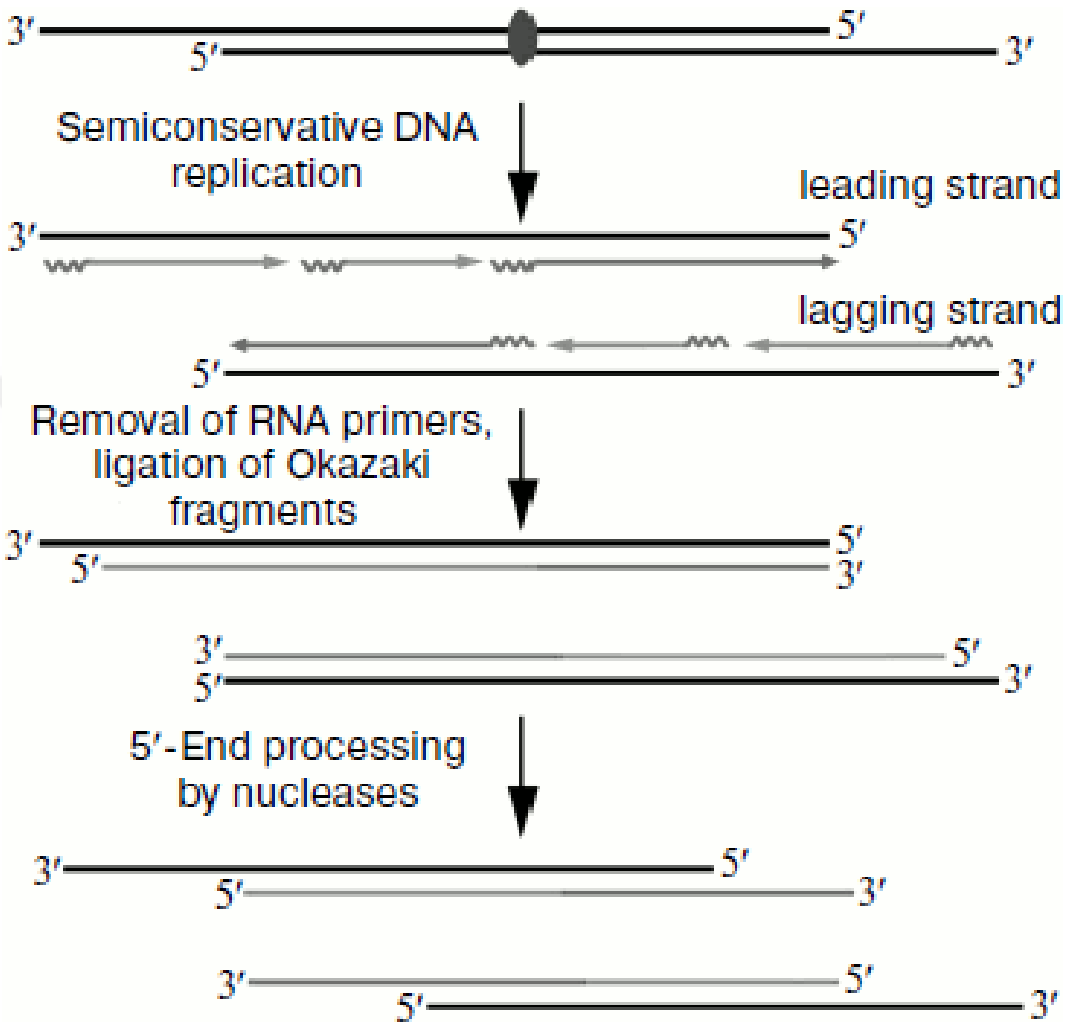

Figure 1.5 End replication problem (Zvereva et al., 2010).

1.3.4 Telomere Function

Telomere helps to maintain the stability and integrity of chromosomes. Telomere and their binding proteins form special structure at the end of chromosomes, thereby protecting chromosomes from recognition as double-stranded breaks or end-to-end fusion and suppressing DNA damage response. Telomere also prevents chromosome end degradation by an excessive nuclease. Importantly, progressive telomere shortening occurs as cells divide, which serves as a mitotic clock to control cell life-span and to prevent unlimited cellular proliferation (Cong et al., 2002; Kupiec, 2014).

1.4 Telomerase

Telomerase is a ribonucleoprotein enzyme that manufactures telomeric TTAGGG replications at the chromosomal ends of linear eukaryotes. Telomerase is an RNA-dependent DNA polymerase that consists of a catalytic subunit telomerase reverse transcriptase (TERT), including an RNA template, so-called Telomerase RNA component (TERC) and other components (Figure 1.6) (Greider and Blackburn, 1985; Greider and Blackburn, 1989; Morin, 1989). TERC is basically expressed in cells whereas TERT expression is tightly regulated in time, place and quantity, which makes TERT the rate-limiting subunit of telomerase (Feng et al., 1995; Weinrich et al., 1997). Normal cells stop divide at the hayflick-limit, but tumor cells need a telomere maintenance mechanism to evade the hayflick-limit or senescence. So telomerase activation is the major mechanism for maintaining telomere length in cancer cells.

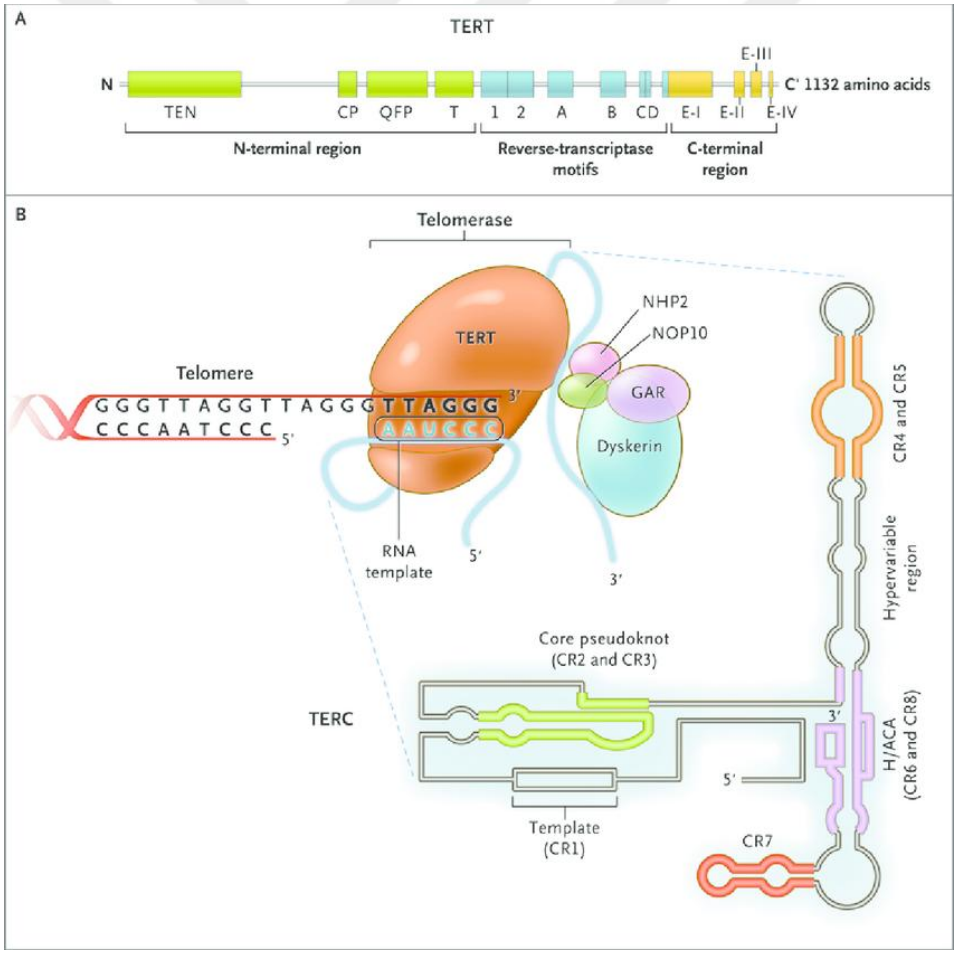

Figure 1.6 The telomerase complex; It consists of a rate-limiting, catalytic subunit TERT, an RNA template TERC, and other components. TERT and TERC are the core of the telomerase complex and sufficient to constitute telomerase activity (Calado and Young, 2009).

1.4.1 Telomerase Activity and Expression in Normal and Cancer Cells

Telomerase is a too large ribonucleoprotein complex with the key components of TERT and TERC Figure 1.6 (Venteicher et al., 2008). TERT and TERC are adequate to constitute telomerase activity in vitro. In most differentiated human somatic cells, telomerase activity is undetectable or very low (Shay and Bacchetti, 1997; Cong et al., 2002). However, human germline cells have telomerase activity and thus maintain a stable telomere length. Stem/progenitor cells, lymphocytes, and other cells with highly proliferative potentials express various levels of telomerase activity (Kim et al., 1994; Shay and Bacchetti, 1997). The expression of TERT is in general related to the acquirement of telomerase activity, and providing telomerase-deficient cells with TERT is adequate to activate telomerase, suggesting a key role of TERT in controlling telomerase activity (Bodnar et al., 1998). Telomerase activity is noticeable in around 90% of human malignant cells and the stimulation of TERT expression is a determinant step for gaining telomerase activity during the transformation of cells as described above (Kim et al., 1994; Shay and Bacchetti, 1997; Hahn et al., 1999; Freitas-Junior et al., 1999). One of the important hallmarks of cancer is limitless replication potential, which is mainly achieved by TERT expression and telomerase activation (Hanahan and Weinberg, 2000). Experimentally, TERT was shown to be one of the essential elements for the conversion of normal human cells into malignant ones (Hahn et al., 1999).

1.4.2 The Regulation of TERT Expression

The TERT gene was first cloned in 1997, (Kyo, Takakura, Fujiwara, & Inoue, 2008) and since then, its regulation has been exclusively explored. TERT is regulated at multiple levels by numerous factors (Kilian et al., 1997; Daniel et al. 2012).

1.4.2.1 Genetic Regulation of TERT Expression

1.4.2.1.1 Abnormal Gene Copy Numbers

The TERT gene spans 40 kbs and is localized at chromosome 5p15.33 (Meyerson et al., 1997). Normal somatic cells from healthy individuals carry 2 copies of the TERT gene, and loss or gain of the copy numbers causes abnormities. In Cri du chat syndrome, patients were found to have one TERT allele deletion, coupled with lower telomerase activity in their cells. TERT is haplo-insufficient for telomere maintenance

in these patients (Zhang et al., 2003; Du et al., 2007). The gain of TERT gene copies occurs in human cancers, either from a gain of 5p or gene amplification. Over 30% of human, malignancies carry more than 3 TERT copies, (Zhang et al., 2000; Hou et al., 2002) which contribute to telomerase activation in those malignancies.

1.4.2.1.2 TERT Gene Mutations

The TERT gene point mutation has been found to result in many degenerative diseases, such as aplastic anemia, idiopathic pulmonary fibrosis, and others. Non-synonymous TERT mutations cause stem cell dysfunction and lead to a low telomerase activity (Vulliamy et al., 2002; Armanios et al., 2007). Moreover, the polymorphism or mutation at the regulatory region of the TERT gene also affects TERT expression and telomerase activity.

1.4.2.2 Transcriptional Regulation of TERT Expression

The TERT promoter is GC rich and lacks both TATA and CAAT boxes. It includes five binding motifs for Sp1 in minimum, two E-boxes, and one start site for transcription which binds to multi-functional transcription factor (TFII-I) for gene expression. Numerous scientific articles have revealed that the TERT transcription machinery is controlled by various transcription and epigenetic factors (Figure 1.8) (Takakura et al., 1999; and Cong et al., 1999).

1.4.2.2.1 Positive Transcription Factors

1.4.2.2.1.1 c-Myc

The oncoprotein c-Myc is the well-characterized transcription factor that activates TERT transcription. Myc forms a dimer with Max that binds to the 5′- CACGTG-3′ consensus core sequence (E-box) in promoters, thereby trans-activating target genes (Wu et al., 1999). c-Myc is known to stimulate cell proliferation, block cellular differentiation, and promote tumorigenesis. Its role in trans-activation of the TERT gene is one of the important driving forces that transform cells (Xu et al., 2001).

1.4.2.2.1.2 Sp1 Family Transcription Factors

The Sp1 transcription factor belongs to the Specificity Protein/Krüppel-like Factor (SP/KLF) transcription factor family and its consensus binding sequence is GGGGCGGGG or GGGTGGG. There are at least 5 Sp1 binding GC-boxes in the TERT core promoter region (Takakura et al., 1999). Sp1 cooperates with c-Myc to activate the TERT transcription, and when all the Sp1 binding sites are disrupted, the TERT promoter activity is erased, and even over-expression of c-Myc is unable to activate the promoter any longer (Kyo et al., 2000).

1.4.2.2.1.3 Other Positive Regulators

Apart from the above two major factors, many other positive factors regulate TERT transcription directly or indirectly. Survivin increases the DNA binding ability of c-Myc and Sp1, thus augmenting TERT transcription (Endoh et al., 2005). HPV E6 oncoprotein forms a tertiary complex with E6AP and c-Myc, and binds to the E-box, thereby increasing TERT transcription. STAT3, EWS-ETS, and HIF- 1α have direct promoter interaction, stimulating TERT transcription (Nishi et al., 2004; Takakura et al., 2005).

1.4.2.2.2 Negative Transcription Factors

1.4.2.2.2.1 Mad/Max Heterodimer

Mad is expressed in non-proliferating cells, inhibits proliferation, promotes differentiation, and prevents malignant transformation. Mad and Max forms a heterodimer that binds the same E-box as Myc/Max in the TERT core promoter, and competes with Myc/Max heterodimer binding to the promoter, thus repressing TERT expression. During HL60 leukemic cell differentiation, there is a switch of E-box-binding from c-Myc/Max to Mad1/Max at the TERT promoter, and this switch is concomitant with shutting down of TERT transcription and silencing of telomerase (Hou et al., 2002; Lin and Elledge, 2003).

1.4.2.2.2.2 p53

p53 is a negative regulator of TERT expression, which is compatible with its tumor suppressive role. The p53 protein interacts with Sp1, a known TERT activator,

inhibiting Sp1 binding to the GC-box of the TERT promoter and repressing transcription. Expression of wild-type p53 in cancer cells causes a decrease in TERT expression and telomerase activity, while tumor cells with mutant p53 have high levels of telomerase activity (Xu et al., 2000; and Kanaya et al., 2000).

1.4.2.2.2.3 Other Negative Regulators

Many tumor suppressors are also negative transcription regulators of TERT expression. Wilms' tumor 1 (WT1) and menin can directly bind the promoter and repress TERT transcription (Oh et al., 1999; Lin and Elledge, 2003). Rb, TGF-β/Smad, and AP1 also contribute to TERT repression at the transcriptional level (Xu et al., 1997; Lin and Elledge, 2003; Takakura et al., 2005; Li et al., 2006).

1.4.2.3 Epigenetic Regulation of TERT Transcription

The transcriptional regulation by the above transcription factors is critical for TERT expression. However, there is an additional transcriptional controlling, the epigenetic mechanism, which also plays roles in regulating TERT expression. The epigenetic mechanism involves nucleosome/chromatin remodeling, DNA methylation, and histone modifications. For example, commonly histone acetylation/deacetylation is a fundamental merit to TERT transactivation/repression in human normal and malignant cells (Hou et al., 2002). Histone methylation transferase SMYD3 is required for best stimulation of c-MYC and Sp1 on the TERT promoter, while histone demethylase RBP2, can interact with Mad1, and be recruited to the TERT promoter, thereby inhibiting TERT transcription (Liu et al., 2007; Ge et al., 2010) (Figure 1.7).

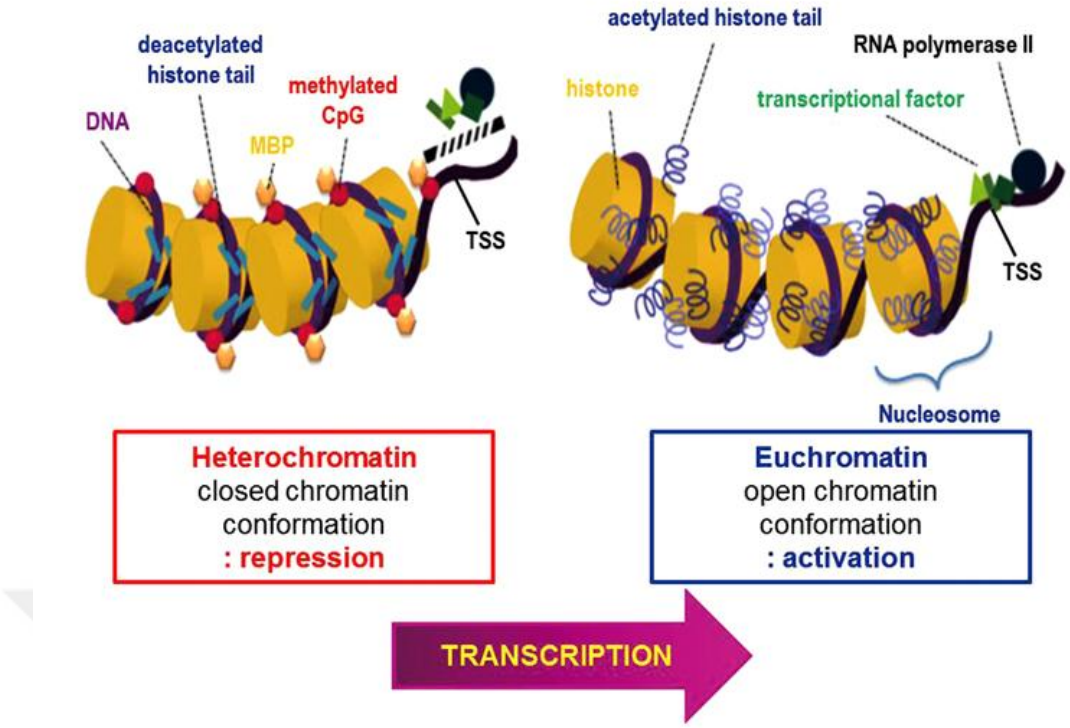

Figure 1.7 Histone Acetylation/Deacetylation (Kim, 2014).

1.4.2.4 Post-Transcriptional and Post-Translational Regulation of TERT Expression

Alternative splicing of TERT mRNA is the key post-transcriptional mechanism in the regulation of TERT expression. At least five TERT mRNA variants have been identified in human cells, but only the full-length transcript is capable of being translated into a catalytically active protein (Kilian et al., 1997; Ulaner et al., 1998; Yi et al., 2000). Post-translational regulation is involved in TERT protein modifications. Phosphorylated TERT protein has a longer half-life while its ubiquitination leads to a fast degradation.

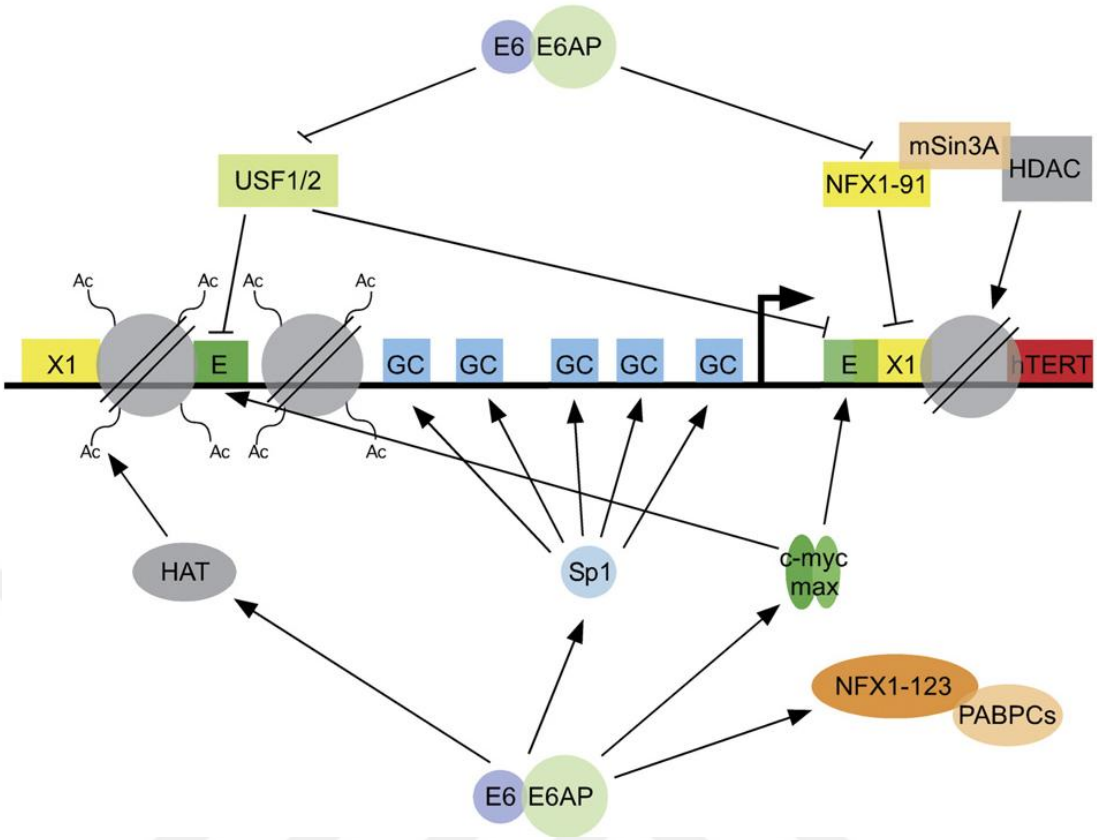

Figure 1.8 Structure and regulation of the TERT promoter. Proteins that activate TERT are located above the line; repressors are located below the line (Howie et al., 2009).

1.4.3 Telomerase Function Independent of Telomere-Lengthening

The canonical function of TERT is to maintain telomere length by synthesizing TTAGGG telomeric repeats at the chromosomal ends. Apart from its telomere lengthening function, TERT has many other activities in essential cellular processes, such as regulating gene expression, cell transformation, cell survival, mitochondrial function and epithelial-mesenchymal transition (EMT) (Ding et al., 2013). TERT has a transcriptional modulating utility. It occupies the Wnt target promoters, working as a co-factor in the Wnt-β-catenin transcriptional complex and regulating Wnt target gene expression (Park et al., 2015). The RNA component of mitochondrial RNA-processing endoribonuclease (RMRP) is a small non-coding RNA found in mitochondria. TERT has been shown to interact with RMRP and functions as RNA-dependent RNA polymerase that produces double-stranded RNAs. These RNAs are

extra processed into small interfering RNAs and regulate gene expression in a post-transcriptional way (Maida et al., 2010). TERT also binds to the p65 subunit of NF-κB and promotes the expression of NF-κB target genes, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Ghosh et al., 2012). EMT is an important player in cancer metastasis. Ectopic TERT expression promotes whereas its inhibition attenuates EMT in cancer cells (Liu et al., 2013).

1.4.4 TERT Promoter Mutations

Telomerase activation and TERT expression are important in cell immortalization and tumor progression. Although the regulation of TERT transcription has been extensively explored, the underlying mechanisms are still incompletely understood. More recently, TERT promoter mutations have been identified as important genetic events activating telomerase in different types of cancer (Wang et al., 2014).

1.4.4.1 TERT Promoter Mutations - Overview

Gene mutations are frequently related to tumorigenesis, and the cancer-specific mutations occur mostly in the protein-coding area, while rare in the gene regulatory area. Recently, however, somatic TERT promoter mutations have been detected, which are located within 200 base pairs (bp) upstream of the ATG translation start site, and these mutations stimulate the TERT transcription by creating *de novo* ETS binding motifs (Wu et al., 2014; Huang et al., 2013).

1.4.4.2 TERT Promoter Mutations and Their Functional Implication

Firstly, the TERT promoter mutations were reported by Huang and colleagues in 2013. This group identified recurrent somatic mutations in the TERT promoter region in malignant melanoma. Two major mutations, which caused a cytidine-to-thymidine (C >T) dipyrimidine transition, localized at - 124 and -146 bp (chromosome 5 1,295,228 and 1,295,250) from the ATG translation start site, and are named C228T and C250T, respectively. The two mutations are found to be mutually exclusive (Huang et al., 2013; Wu et al., 2014). The mutations exhibited the signature of an ultraviolet-induced DNA damage and were found to be more frequent in sun-exposed sites in primary cutaneous melanoma (Huang et al., 2013; Wu et al., 2014; Heidenreich et al., 2014). The above mutations in the TERT promoter region have functional implications. Both C228T and C250T create a new sequence 5′- CCCCTTCCGGG-3′, which is the E-

twenty-six (ETS) transcription factor binding motif (GGAA, from the reverse strand) (Figure 1.9). Huang et al. constructed a TERT promoter luciferase reporter and observed that the mutant TERT promoter activity increased by two to four-fold compared with the wild-type one in same cancer cells (Huang et al., 2013; Wu et al., 2014).

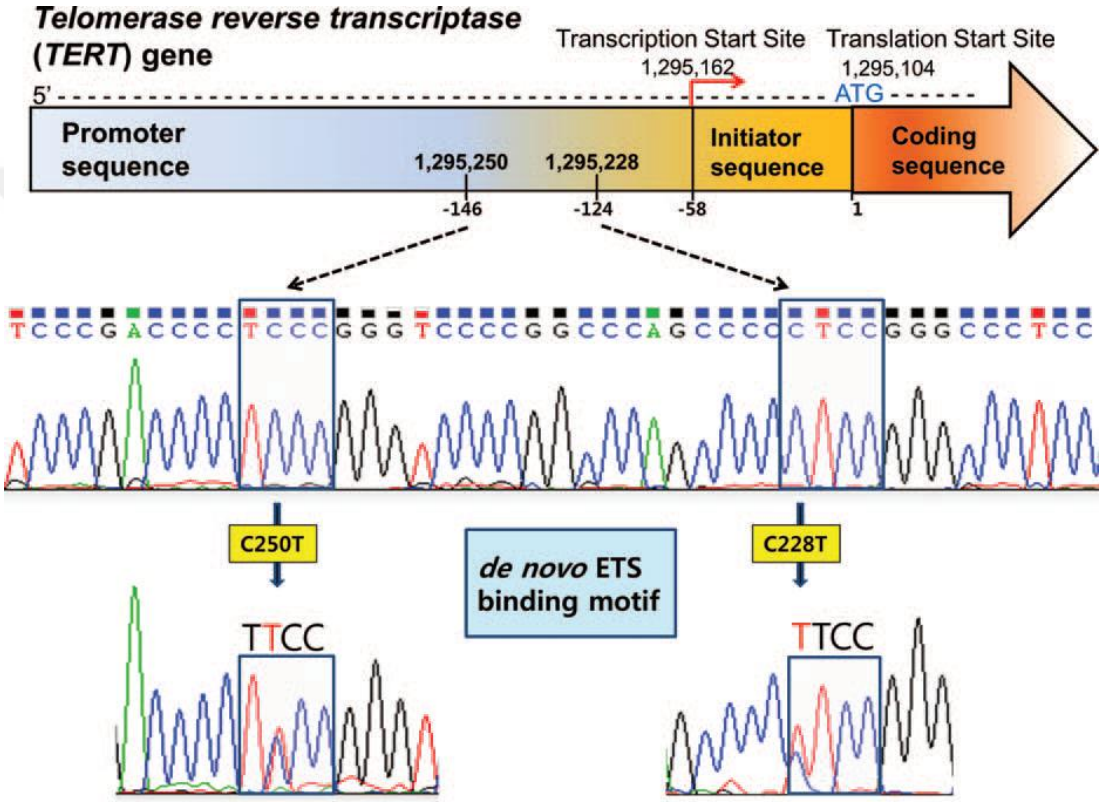

Figure 1.9 The structure of TERT promoter and the position of the two mutations: C228T and C250T. The mutation site is indicated above. C>T mutations lead to the generation of de novo ETS1 binding sites in the TERT proximal promoter region, thereby promoting TERT transcription (Lee et al., 2017).

In addition to these two major TERT promoter mutations, other mutations are also identified in that region. CC>TT tandem mutations at position -124/- 125bp and -138/-139, and C>T mutations at position -57 are found in a small proportion of cancer. The two tandem mutations also contributed to the generation of an extra ETS transcription factor-binding motif. Moreover, the mutation at position -57 creates a CCGGAA ternary complex factor (TCF) binding site, which also enhances TERT transcription (Horn et al., 2013).

1.5 Objectives and Aims of the Thesis

- Investigation of mutations' presence in a 163 bp-long region of the TERT gene promoter in the Turkish population.
- Analysis of the relationship between TERT mutations in tumor and normal tissue samples.
- Determination of the association between TERT mutations and all the clinicopathological characteristics of patients involved in this study.
- Exploration of the relevance between the TERT mutations and colorectal cancer development.

CHAPTER 2

LITERATURE REVIEW

2.1 TERT Promoter Mutations in Melanomas

In melanoma, the first reported mutations of TERT promoter were mentioned by Horn *et al.* in 2013. They observed the derived somatic mutations from metastatic melanomas in 125 of 168 (74%) of human cell lines, 45 (85%) of 53 metastatic tumor tissues, and 25 (33%) of 77 primary melanomas. In major, the locations of these mutations were 124 and 146 bp upstream of translation start site ATG, and they were referred to as C228T and C250T, correspondingly. The same two mutations were identified in 71% melanomas examined by Huang et al. The mutations mentioned above were suggested to produce *de novo* binding motifs for ETS transcription factors by both Horn *et al.* and Huang et al. (Horn et al., 2013; Huang et al., 2013). Soon after these initial discoveries, the earlier mentioned mutations were noticed in 12 (32%) of 38 conjunctival melanomas as well by Griewank et al. In accordance to their conclusions, there is a pathogenetic relationship between the occurrence of mutations and the cutaneous melanomas and their absence in uveal melanomas put emphasis on their genetic dissimilarity from cutaneous and conjunctival melanomas (Griewank et al., 2013). In 2014, another study on melanoma metastases of an unknown primary tumor (MUP) patients was accomplished by Egberts and his colleagues. 92 patients' specimens were screened for analyzing mutations. In total 33 patients (35.9%) with TERT promoter mutations were found and sub-grouped into 18 patients with 228 C> T, 11 patients with 250 C> T, 3 patients with 228–229 CC> TT, and finally one patient with 242–243 CC> TT. Significantly, the mutations were more common in MUP 26 (66.7%) than in mucosal melanomas 7 (13.2%); $P < 0.001$). and there was an association between the mutations occurrence and the gender of the patient 23 (51.1%) men, 10 (21.3%) women; P=0.004. Egberts *et al.* mentioned that the TERT promoter genotype of MUP pointed toward a cutaneous and not mucosal origin. Also they decided that the significant gender differences worth additional attention for the

probability of having therapeutic implications (Egberts et al., 2014). Subsequently, Liau et al. identified TERT promoter mutations in two of 32 patients (6%) of acral lentiginous melanomas and in 3 of 9 patients (33%) of non-acral cutaneous melanomas. and he disclosed that in the acral lentiginous melanoma, the fewness of TERT promoter mutation was along with the UV light's putative role in melanoma pathogenesis and reinforced the opinion that acral lentiginous melanomas have a diverse pathogenesis with melanomas from sun-exposed sites as well (Liau et al., 2014). Pópulo et al. inspected former mutations in [196 cutaneous basal cell carcinomas (BCCs), (102 X-irradiated tumors, 94 non-exposed to ionizing radiation treatment tumors), and 116 melanomas]. After evaluation, TERT mutations were distinguished in 27% of X-irradiated BCCs, 51% of non-irradiated BCCs, and 22% of melanomas. according to Pópulo et al., various carcinogenic factors could result in discrete TERT mutations in BCC, and conceivably TERT mutations were related to a poorer prognosis in melanoma (Pópulo et al., 2014). Gessi et al observed TERT mutations at position C228T and C250T in 8, 5 samples respectively, with a total of (13/23, 56%) melanoma brain metastases, while no mutations were shown in (25, 0.0%) primary tumorous melanomas of the central nervous system CNS. Their conclusion was that TERT mutations might come to be, in addition to BRAFV600E mutations, a further promising molecular indicator to discriminate metastatic from primary CNS melanoma (Gessi et al., 2014). Regarding the findings of Griewank *et al*, the TERT mutation presence is associated with BRAF or NRAS mutation, histologic type, and Breslow thickness, after recognizing TERT promoter mutations in 154 of 362 (43%) different subtypes of melanomas. Griewank et al confirmed that in nonacral cutaneous melanomas' patients, TERT mutations were autonomously concomitant with poorer overall survival (Griewank et al., 2014). In 2015, and after the analysis of 56 atypical Spitz tumor or spitzoid melanoma by Lee et al, only 4 mutations were found exclusively in the 4 samples with hematogenous metastasis, including 3 of 228 (C>T) and one of 242/243 (CC>TT). As a significant predictor of hematogenous metastasis, TERT promoter mutations were most ones among other variables analyzed. Lee et al concluded that TERT promoter mutations play the role of identification of a clinically high-risk subset of patients with spitzoid tumors (Lee et al., 2015). Finally, Nagore *et al* showed the existence of TERT mutations in 116 of 300 (38.7%) melanomas. Despite the different types of TERT promoter mutations found, the 250 C>T (58; 50.0%) and the 228 C>T (38; 32.8%) mutations were most

frequented. The result of data analysis showed that the existence of TERT mutations in melanoma was related to shorter disease-free survival. Nagore *et al* suggested that the data of their study provided primary proof of the ability for using the combination of (TERT promoter, BRAF, and NRAS) mutations to recognize patients who are at risk of aggressive cancer (Nagore et al., 2016).

2.2 TERT Promoter Mutations in Gliomas

Killela *et al* discovered TERT promoter mutations in (83%) of primary glioblastoma, and as a biomarker, they suggested that the TERT promoter mutations could be useful for the assistance in the brain tumors' classification and prognostication (Killela et al., 2013). Arita *et al* investigated the promoter mutations in the TERT gene in 546 gliomas. The C228T and C250T mutations were found in 55% of all glioma subtypes. Their final conclusion suggested that the TERT mutations' unique pattern and the other genetic modifications together play obvious roles in pathogenesis of oligodendroglial and astrocytic tumors (Arita et al., 2013). In 2014, both of the 228 C > T and 250 C > T mutations were detected in 143/178 (80.3%) primary glioblastoma multiform (GBM) and 4/14 (28.6%) secondary GBM by Simon *et al*. The results indicated that the presence of those mutations was related to poor overall survival (Simon et al., 2014). Subsequently, after sequencing 807 glioma DNAs for TERT promoter mutations by Labussière *et al*, mutations were observed in 60.8% of gliomas (491 out of 807), and they were universally associated with poorer outcome (Hazard ratio (HR)¼1.50) (Labussière et al., 2014). At the beginnings of 2015, in their report, Heidenreich *et al* showed the TERT promoter mutations frequented in 303 gliomas as 80% of glioblastomas, 70% of oligodendrogliomas, and 39% of astrocytomas. In comparison to TERT un-mutated tumors, TERT transcription was increased in the TERT mutated tumors. The worst survival was realized in only patients with TERT promoter mutations (Heidenreich et al., 2015). Later on that year, a similar study was conducted by Mosrati *et al* in which they screened 92 primary glioblastomas GBMs for TERT mutations. From the results of this study, 86% of the GBMs examined showed TERT mutations, and 75% of those mutations were C228T, while the rest 25% were C250T. A relation between the TERT mutations and shorter overall survival was noticed (Mosrati et al., 2015). In the fall of 2015, C228T and C250T mutations were shown in 17.0%; 11.8% of 389 gliomas, respectively by Gao *et al*. The two mutations were related with grades of tumors significantly. In Gao *et al*'s opinion, the TERT

mutations and the long relative telomere length RTL together were prognostic factors for poor clinical outcomes and predictors of the gliomas resistance toward radiotherapy (Gao et al., 2015).

2.3 TERT Promoter Mutations in Gallbladder

Kinde *et al* stated that 66% of bladder cancer specimens tested were harboring the C228T and C250T mutations. And the two mutations have a strong association with the recurrence of bladder cancer. More than that, these mutations are a probability of being a useful biomarker, for the early detection and the monitoring of the bladder cancer (Kinde et al., 2013). In 2014, after analyzing 154 gallbladder cancer tissues by Qu *et al*, 6 and 8 of 154 (3.9% and 5.2%) gallbladder cancer tissues were found harboring C228T and C250T mutations respectively, and they were mutually exclusive. Qu *et al* stated that the TERT mutations could play a restricted role in the tumorigenesis and pathogenesis of the gallbladder, due to the fact that no observation of any significant correlation was found between TERT promoter mutations and any clinicopathological characteristics in gallbladder cancer (Qu et al., 2014). On the contrary, the results from investigating TERT promoter mutations in different types of urothelial cancer by Zhong *et al* in 2014, intensely suggested that TERT promoter mutations are useful adjunct biomarkers for the differentiation of nested variant of urothelial carcinoma (NVUC) and Large Nested Variant of Urothelial Carcinoma (LNVUC) from benign mimickers (Zhong et al., 2015). In 2015, Hosen *et al* showed that only 12 cases of 188 Clear Cell Renal Cell Carcinomas (ccRCCs) (6.4%) were harboring TERT promoter mutations. According to their results, Hosen *et al* stated that TERT promoter mutations might be able to define a small subset of aggressive tumors (Hosen et al., 2015). Subsequently, in 2016, Cowan *et al* identified the C228T mutations in (57%; 4 of 7 non-enteric tumors but no mutations were detected in any of the 7 enteric tumors, collectively, TERT mutations were existed in 28.5%; 4 of 14 primary bladder adenocarcinomas. The findings of Cowan *et al* supported the potential utility of TERT mutation urine-based screening assay for bladder cancer (Cowan et al., 2016).

2.4 TERT Promoter Mutations in Liver

179 of 305 (59%) of Hepatocellular Carcinomas (HCCs) were found harboring the two most frequented mutations by Nault *et al* in 2013. In conclusion of their study, the most common hereditary alteration in hepatocellular carcinomas that were arisen from either cirrhotic or non- cirrhotic liver, is TERT mutation and it is also the earliest frequent genetic incident recognized in cirrhotic preneoplastic lesions so far (Nault et al., 2013). Thereafter, Chen *et al* identified TERT mutations in 57 of the 195 HCCs (29.2%). Those mutations were related with older ages, the existence of anti-hepatitis C, and lack of hepatitis surface antigen. this study showed that the TERT promoter mutation often happened in HCV- concomitant HCCs. The lack of Hepatitis B infection was significantly related with TERT promoter mutations. In accordance with these findings, Chen *et al* suggested that different etiological causes could be implicated in differing mechanisms to preserve the length of telomeres throughout the carcinogenesis of HCCs (Chen et al., 2014). Later in 2014 Quaas *et al* worked on analyzing the TERT promoter mutation in hepatocellular carcinomas, hepatocellular adenomas, and intrahepatic cholangiocarcinomas. And so, Quaas *et al* found out that the -124C>T mutation in 37 of 78 hepatocellular carcinomas (47%), whereas no mutations were diagnosed in the other two types. And an association between frequency of mutations and the differentiation grade was observed. That's how they concluded that the -124C>T mutation might turn out to be an indicative tool to distinguish hepatocellular adenoma from well-differentiated hepatocellular carcinoma (Quaas et al., 2014).

2.5 TERT Mutations in Thyroid Cancers

In thyroid cancers, Landa *et al* found the TERT mutations in 44% of samples. Their conclusion was that those mutations were very dominant in cancers with advanced stages, mostly in cancers with the BRAF or RAS mutations (Landa et al., 2013). Furthermore, Liu *et al* identified C228T or C250T mutation collectively in 37/152 of different subtypes of thyroid carcinoma (24%) (Liu et al., 2013). In the same year, there was a similar study by Liu and his colleagues. In which Liu *et al* showed the occurrence of the C228T and C250T mutations in 47 of 145 of different subtypes of thyroid carcinoma (32%) collectively. While no observations were witnessed in any of 85 benign thyroid tumors. The two studies suggested that TERT mutations might

play the role of a marker for disease aggressiveness and poor outcome (Liu et al., 2013; Liu et al., 2013). In 2014, depending on the results obtained from their study on Follicular Thyroid Adenomas, Atypical Follicular Thyroid Adenoma, and Follicular Thyroid Carcinoma, Wang *et al* stated that the existence of TERT mutations may possibly be a good indicator of TERT gene expression and telomerase activity, as well as its presence was associated with shorter survival of patient. Similarly, they mentioned that the occurrence of the TERT mutations could be as a primary genetic event in thyroid follicular tumors that have not demonstrated malignant features on routine histopathological workup (Wang et al., 2014). Another study performed by Xing *et al* on 507 patients with Papillary Thyroid Cancers, explained the cooperative presence of the BRAF V600E and the TERT C228T mutations in Papillary Thyroid Cancers relating with the worst clinic-pathologic outcomes, and the possibility of these two mutations being as a distinctive prognostic and therapeutic factors (Xing et al., 2014). In 2015, in their study on Anaplastic Thyroid Cancer (ATC) and after they examined 106 samples and found 41 TERT promoter mutations, Shi *et al* supported the TERT C228T mutation of being a possible new predictive marker and therapeutic target for ATC. Also, they provided a strong proof suggesting an important role of TERT promoter (C228T) in the tumorigenesis and aggressiveness of ATC (Shi et al., 2015). An additional opinion supporting the predictive role of the TERT mutations for the aggressive performance and their association with distant metastasis development was introduced by Gandolfi and his colleagues (Gandolfi et al., 2015).

2.6 TERT Mutations in Other Types of Cancers

The TERT mutations were examined in numerous cancer types;

In non-small cell lung cancer NSCLC, Ma *et al* detected a variety of TERT mutations in 12 of 467 patients. After the evaluation of these mutations was done, an association between the mutations and increased patient age was found. In conclusion, they suggested the TERT promoter mutations as a player of an important role in NSCLC pathogenesis and the probability of its being a therapeutic target (Ma et al., 2014).

Subsets of adrenal tumors (199 cases) have been assessed for TERT mutations by Liu *et al.* Overall, the C228T mutation was detected in only 3% while C250T was not observed in any of cases. Briefly, the C228T mutations seemed to be associated with disease aggressiveness, and this would provide a promising platform for further studies

assessing TERT promoter mutations as a possible malignancy indicator in ambiguous cases with unidentified malignant potential (Liu et al., 2014).

Differently, in laryngeal cancer tissues, TERT C250T and C228T mutations were existed in 24% and 3% respectively, of the samples obtained, but the two mutations were mutually exclusive in this cancer. As a final point, Qu and his colleagues concluded that TERT mutations occurred commonly in laryngeal cancers, specifically C250T being intensely related with the worse survival of patients with laryngeal cancer, and that those mutations could present important genetic driver events, and might be valuable prognostic indicators in this cancer (Qu et al., 2014).

Coming to Prostate Cancers, 167 tumors were evaluated for TERT mutations, but none were spotted in any of the samples. The results of this study indicated that the TERT mutations may not present any important role in prostate carcinogenesis (Stoehr et al., 2015).

The next year, 176 pairs of breast cancers known as Phyllodes tumors (PTs) including benign, borderline, and malignant tumors and their matched normal samples were collected and subjected to be sequenced. In total, the TERT promoter mutations observations were 18% of benign, 57% of borderline, and 68% of malignant PTs. Relying on these results, Piscuoglio *et al* suggested that TERT mutations may be a driver event for progression of PTs, and can help in the distinguishing diagnosis between PTs and fibroadenomas (Piscuoglio et al., 2016).

This year, the TERT mutations' presence in granulosa cell tumors of the ovary was identified by Pilsworth and his colleagues. By taking their data into consideration, Pilsworth *et al.'*s suggestion is that the C228T mutations may possibly have a significant role in the development of adult granulosa cell tumors (Pilsworth et al., 2018).

Finally, and for the first time, a study was achieved by Carloni and his colleagues on 123 cases of CRC. and no TERT promoter mutations were shown. As a result, the nonexistence of TERT mutations suggested that these mutations may not be implicated in CRC carcinogenesis (Carloni et al., 2018).

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

3.1.1 Patients

We collected 43 CRC tissue specimens and 43 adjacent non-CRC tissue samples from patients who were diagnosed and underwent surgery at Gaziantep University Hospital. All tissue specimens were fresh at time of collection and were stored at -80°C. Clinicopathological characteristic (age, gender, tissue type, stage, lymph node status, distant metastasis, other disease, and smoking habit) information were obtained from the Gaziantep University Hospital.

This study was permitted by the Ethics Committee of Clinical Research at University of Gaziantep (Decision No: 2017/192, Date: 08.05.2017) and was financed by the Unit of Scientific Research Projects at Gaziantep University (Project No: FEF.YLT.17.19). All consent forms were signed by the patients involved in this study.

3.1.2 Chemical Materials

3.1.2.1 DNA Extraction

For DNA extraction we used the PureLink Genomic DNA Mini Kit (cat # k1820-02) (Invitrogen, USA).

3.1.2.2 PCR Amplification

For PCR amplification we used

- Primers (Cat. # 10336-022) designed by (ThermoFisher, USA).

  hTERT Forward CAG CGC TGC CTG AAA CTC

  hTERT Reverse GTC CTG CCC CTT CAC CTT
- PCR Master Mix (2X) (Cat. # K0171) (ThermoFisher, USA)
- dH2O (ThermoFisher, USA)
- Hi-Di Formamide (cat # 4311320) (Applied Biosystems)

3.1.2.3 Gel Electrophoresis

For Gel Electrophoresis we used:

- Agarose powder (CAS # A9539) (SIGMA-ALDRICH, Germany)
- Tris (Hydroxymethyl) Aminomethane (Catalogue # TRS001) (Bioshop, Canada)
- Boric Acid (CAS # 10043-35-3) (MERK, Germany)
- EDTA (Disodium Salt, Dihydrate) (Cat. # EDT001) (Bioshop, Canada)
- Ethidium Bromide (CAS # 1239-45-8) (SIGMA-ALDRICH, Germany)
- DNA Gel Loading Dye (6X) (Cat. # R0611) (ThermoFisher, USA)
- Orange DNA Loading Dye (6X) (Cat. # R0631) (ThermoFisher, USA)
- O'RangeRuler 20-300 bp DNA Ladder (Cat. # SM1323) (ThermoFisher, USA)

3.1.2.4 Agarose and Gel Electrophoresis Buffers

- (Tris-Borate-EDTA) Buffer (10xTBE) was prepared by dissolving (108g) Tris Base, (55g) Boric Acid, and (8,3g) EDTA in sterile distilled water and completing the volume to one liter.
- Ethidium bromide: 10mg/mL
- (3.5%) Agarose Gel
- 1X TBE was prepared from diluting the 10x TBE.
- (0,30 mg/ml) Ethidium bromide

3.1.2.5 DNA Sequencing Kit

BigDye Terminator v3.1 Cycle Sequencing Kit (Cat. # 4337456) (Thermofisher, USA)

3.1.3 Machines Used

- Deep freezer (-80, -20)
- Vortex (Heidolph, Germany)
- Homogenizer (TissueLyser LT, Qiagen, Germany)
- Thermo-Shaker TS-100 (BioSan)
- Centrifuge (Hettich, Germany)
- Spectrophotometer (Nano MN-913, Maestrogen, Taiwan)
- ProFlex PCR System (Applied Biosystems, USA)
- Precise Balance (Dikomsan)
- Microwave oven (PROFILO)
- Autoclave (HICLAVE)
- The system of Agarose Gel Electrophoresis
- 3500 Genetic Analyzer (Applied Biosystems)

3.1.4 Software Used

- GeneMapper V4.1 Software (Applied Biosystems, USA).
- SeqTrace V0.9.0 Software (Brian J. Stucky)
- CLC Main WorkBench 8.0.1 (Qiagen, Germany).

3.2 Methods

3.2.1 Genomic DNA Extraction

1. The PureLink Genomic DNA Mini Kit (Invitrogen, USA) was used for the extraction of genomic DNA from the frozen tissue samples.
2. To prepare Lysate, we cut approximately (25-30 mg) of frozen tissue from each sample into small pieces on dry ice by using a sterile scalpel and collected into a (1.5 mL) sterile centrifuge tube.
3. Immediately after weight measurements, (180 µL) of Genomic Digestion Buffer was added to the sample tube and were homogenized at 50 Hz for 10 minutes by a homogenizer (Qiagen, Germany).

4. 20 µL of Proteinase K. was added to the previous mix, and we incubated the mix at 55°C with occasional vortexing overnight until lysis is done.
5. We centrifuged the lysate at (21.380 x g) maximum speed for (3 minutes) at room temperature.
6. The supernatant was transferred to a new sterile microcentrifuge tube.
7. Brief vortexing was done after adding (20 μL) of RNase A to the supernatant.
8. The mix was incubated at room temperature for 2 minutes.
9. 200 μL of Lysis/Binding Buffer was added to the mix and was mixed well by brief vortex.
10. 200 μL of absolute (100%) ethanol was added and mixed by vortex for 5 seconds.
11. ~640 μL of the Lysate which prepared with Lysis/ Binding Buffer and ethanol was transferred to spin column with collection tube and centrifuged at 10,000 x g for 1minute.
12. We discarded the collection tube and placed the spin column into a new collection tube.
13. 500 μL of Washing Buffer 1 (already prepared with ethanol) was added to spin column and centrifuged at 10,000 x g for 1 minute.
14. We discarded the collection tube and placed the spin column into a new one.
15. 500 μL of Washing Buffer 2 (already prepared with ethanol) was added to the spin column and centrifuged at maximum speed for 3 minutes, and we discarded the collection tube.
16. After placing the Spin Column into a 1.5 mL microcentrifuge tube, 50 μL of Elution Buffer was added to the spin column and incubated at room temperature for 1 minute then centrifuged at maximum speed for 1 minute.
17. We discarded the spin column but kept the microcentrifuge tube which contains the purified DNA.
18. DNA samples isolated were stored at -20° C.

3.2.2 Spectrophotometric Analysis

The DNA samples' concentration was determined by spectrophotometer at (260 nm and 280 nm) wavelengths. The DNA samples' purity was determined by calculating the optical density (OD) values from the proportion of (260/280).

3.2.3 PCR Amplification

The reaction contents used for the PCR process are given in Table 3.1

Table 3.1 PCR reaction contents

Reaction content	**Quantity**
PCR Master Mix (2X)	20 μL
Forward Primer	2.4 μL (10nm)
Reverse Primer	2.4 μL
dH2O	12.6 μL
Formamide	1 μL
Reaction Mix volume (38.4 μL)	
Genomic DNA	1.6 μL
Total volume (40 μL)	

The PCR reaction was run according to the settings in Table 3.2

Table 3.2 PCR conditions

Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95℃	5 min	
Denaturation	95℃	30 sec	40
Annealing	61℃	40 sec	
Extension	72℃	30 sec	
Final Extension	72℃	10 min	

3.2.4 Agarose Gel Electrophoresis

1. 3.5% of Agarose Gel was used to verify the required length of PCR amplicons.
2. The Agarose Gel was prepared by mixing 3.5g of Agarose Powder with (100 mL) 1xTBE buffer and by heating the mix in the microwave.
3. 30 μL of the Ethidium Bromide (10 mg/mL) was added to the Agarose Gel solution to view the DNA amplicons under UV light.
4. After the Agarose Gel solution was cooled down to 56°C, the gel was poured into a 5 mm thick gel plate.
5. After the gel was slightly stiffened, the ready Gel plate was transferred to an electrophoresis tank containing a 1xTBE buffer.

6. 5 μL of the PCR product was mixed with (3.5 μL) of the DNA Gel Loading Dye (1.5 X) to prepare the DNA loading mix.
7. The final DNA loading mix was loaded into gel holes.
8. By mixing an equal volume of Orange DNA Loading Dye (6X) and O'RangeRuler 20 bp DNA Ladder, and with a 50%s dilution, the ladder mix was prepared and loaded into one gel hole in order to validate amplicon length by comparison.
9. DNA Loading mix was run at (120 V) for about (25 minutes) and then examined under UV light.

3.2.5 Fragment Sequencing and Mutations Analysis

- After DNA fragments obtained from PCR products were verified by gel electrophoresis to ensure the desired fragment length is produced, DNA fragments were sequenced by Sanger-Bidirectional Sequencing performed by MEDSANTEK company.
- We processed all sequence data acquired from MEDSANTEK company by using SeqTrace V0.9.0 Software to create the consensus sequence from matching the Forward and Reverse Sequences of each sample separately.
- We got the Wild-Type sequence of TERT promoter region from the National Center for Biotechnology Information (NCBI) website.
- CLC Main Workbench 8.0.1 software was used to perform mutations analysis by aligning the consensus sequences (as FASTA files) obtained from SeqTrace software to the Wild-Type sequence as a reference.

CHAPTER 4

RESULTS

4.1 Clinicopathological Characteristics of Patients

This study included 43 tumor (T) and 43 matching normal (N) adjacent tissues for the detection of TERT mutations in CRC. Patients age data were divided into two groups in the current study, (1 = 50 years old patients or below, 2 = patients above 50 years old). The relationship between activity of telomerase and clinicopathological characteristics (such as age, gender, histological type of tissue, stage of disease, lymph node status, distant metastasis, chronic diseases, and smoking habit) was to be evaluated for this study. Regarding the TNM classification system, 20 out of 43 patients were assessed as advanced stage (stage III and IV) whereas 23 out of 43 patients were at premature stage (stage I and stage II) of CRC. About (44.2%) of patients showed lymph node metastasis. The status of distant metastasis (M) was also evaluated, and 10 out of 43 specimens were metastatic to different places in patients. All patients didn't receive any therapy before surgery. Almost half of the patients (48.8%) had different chronic diseases concomitantly with colorectal cancer. As a final point, (37.2%) of patients had the habit of smoking as seen in Table 4.1.

Table 4.1 All the clinicopathological characteristics of the involved patients in this study, (n) = number of samples.

Variables		***n***	**%**
Age	Mean 53.6 (range 26-84)	43	-
	50 years and below	17	39.5
	Above 50 years	26	60.5
Gender	Male	29	67.4
	Female	14	32.6
Tissue Type	Colon	26	60.5
	Rectum	17	39.5
Stage of disease	Early	23	53.5
	Late	20	46.5
Lymph node status	Yes	19	44.2
	No	24	55.8
Distant metastasis	Yes	10	23.3
	No	33	76.7
Other diseases	Yes	21	48.8
	No	22	51.2
Smoking habit	Yes	16	37.2
	No	27	62.8

4.2 DNA Concentrations

The purity and concentrations of DNA obtained from normal and tumorous tissues of patients with colorectal cancer were determined by spectrophotometer. The spectrophotometric measurement results are given in Table 4.2.

Table 4.2 Spectrophotometric measurements of DNA concentrations obtained from (N) normal and (T) tumor tissues of colorectal cancer patients, (OD: optical density).

Sample No		Concentration (ng/μL)	OD 260/280	Sample No		Concentration (ng/μL)	OD 260/280
1	**T**	100.66	2.168	**23**	**T**	946.62	2.057
	N	1149.56	2.104		**N**	1067.82	2.086
2	**T**	250	1.994	**24**	**T**	71.13	1.885
	N	107.15	2.052		**N**	1126.87	2.095
3	**T**	505.91	2.101	**25**	**T**	172.24	1.941
	N	181.12	1.884		**N**	136.78	1.665
4	**T**	225.9	1.95	**26**	**T**	559.35	2.027
	N	65.62	1.659		**N**	338.36	1.994
5	**T**	457.04	2.065	**27**	**T**	1361.5	2.06
	N	243.59	1.489		**N**	631.44	2.058
6	**T**	31.66	2.123	**28**	**T**	802.6	1.914
	N	140.25	2.087		**N**	576.43	2.014
7	**T**	1163.02	2.089	**29**	**T**	457.68	2.071
	N	546.75	2.098		**N**	621.21	1.783
8	**T**	1147.72	2.096	**30**	**T**	1890.08	2.053
	N	335.16	2.01		**N**	39.42	1.86
9	**T**	241.82	2.091	**31**	**T**	618.18	2.099
	N	231.72	2.061		**N**	632.01	2.101
10	**T**	1205.06	1.701	**32**	**T**	185.27	2.026
	N	1591.38	2.063		**N**	674.07	2.097
11	**T**	1278.69	2.059	**33**	**T**	710.02	2.055
	N	582.09	1.979		**N**	255.32	2.059
12	**T**	710.27	2.061	**34**	**T**	577.25	2.029
	N	162.14	1.221		**N**	313.36	1.996
13	**T**	2202.16	2.063	**35**	**T**	1071.69	1.474
	N	170.35	2.264		**N**	528.35	2.087
14	**T**	593.56	1.918	**36**	**T**	1085.59	2.09
	N	477.5	1.93		**N**	1529.95	2.063
15	**T**	70.09	1.976	**37**	**T**	121.59	2.008
	N	248.05	1.882		**N**	206.66	1.437
16	**T**	114.57	1.998	**38**	**T**	1536.02	1.986
	N	210.7	2.045		**N**	286.37	1.65
17	**T**	204.74	1.234	**39**	**T**	1625.11	1.993
	N	131.23	1.297		**N**	67.26	1.72
18	**T**	956.21	2.1	**40**	**T**	87.55	1.966
	N	81.46	2.102		**N**	564.28	2.06
19	**T**	1542.13	2.082	**41**	**T**	295.57	1.263
	N	533.99	2.102		**N**	105.65	1.936
20	**T**	88	2.167	**42**	**T**	125.23	1.753
	N	306.28	2.087		**N**	154.15	2.136
21	**T**	1138.69	2.08	**43**	**T**	130.96	1.982
	N	82.43	2.004		**N**	88.35	1.686
22	**T**	2142.49	2.057				
	N	682.68	2.087				

4.3 PCR and Gel Electrophoresis

We amplified a 163 bp-long fragment of the TERT promoter via polymerase chain reaction (PCR) from each sample using the primers previously mentioned in chapter 3, then we confirmed the quality of PCR products by gel electrophoresis.

The PCR products were run on 3% agarose gel at 120 V for 27 minutes, and images were taken under UV light as demonstrated in Figure 4.1

Figure 4.1 Showing (163 bp) TERT amplicons obtained from PCR and visualized under ultraviolet light after gel electrophoresis was done, M marker (20 – 300 bp), T amplicons obtained from tumor tissues, N amplicons obtained from normal tissues.

4.4 Sequencing and Mutation Analysis

After we analyzed the mutations for all sample sequences using the CLC Workbench software (version 8.0.1), no mutations were shown in any of the samples compared to the wild-type as shown in Figure 4.2

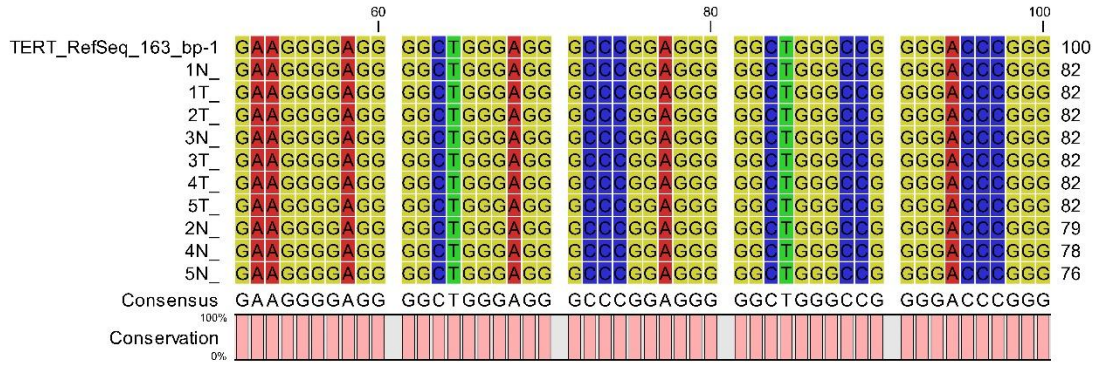

Figure 4.2 Results of the sample sequences alignment with the wild-type sequence of the TERT promoter region (163 bp), no mutations were detected.

CHAPTER 5

DISCUSSION

5.1 General

In this study, we investigated the existence of mutations in the TERT promoter region to determine the relationship between these mutations and the CRC. The results obtained from investigating whether there is an association between the mutations in the promoter region of the TERT gene and CRC or not, can be a useful indicator in the early detection of CRC and also these mutations may be accepted as a new marker to be used in determining the treatment strategy for the disease. As a result, we didn't find any mutations neither in the tumor nor in the normal specimens.

On the other hand, lately the two hotspot somatic mutations C228Tand C250T in the TERT promoter region have been labeled in a number of tumors, principally; skin, brain, thyroid and bladder cancers (Horn et al., 2013; Huang et al., 2013; Killela et al., 2013; Vinagre et al., 2013; and Heidenreich et al., 2014). The formation of novel binding motif sites (CCTT) for ETS transcription factors by these mutations leads to an increase in TERT activity and following telomere conservation (Horn et al., 2013; and Huang et al., 2013). Moreover, there was a pathogenetic relationship between the occurrence of mutations and the cutaneous melanomas (Griewank et al., 2013). Also, in another study, the TERT promoter genotype of MUP pointed toward a cutaneous and not mucosal origin, in addition to the obvious association between the mutations occurrence and the patients gender which may be having therapeutic implications (Egberts et al., 2014). Furthermore, the fewness of TERT promoter mutation found along with the UV light's putative role in melanoma pathogenesis reinforced the opinion that acral lentiginous melanomas have a diverse pathogenesis with melanomas from sun-exposed sites as well (Liau et al., 2014). Other than that the TERT mutations were related to a poorer prognosis in melanoma (Pópulo et al., 2014). While the occurrence of TERT mutations accompanying with BRAFV600E mutations might be a further promising molecular indicator to discriminate metastatic from primary CNS

melanoma (Gessi et al., 2014). Likewise, the presence of TERT mutation associated with BRAF or NRAS mutation, histologic type, and Breslow thickness, in different subtypes of melanomas, has confirmed that in nonacral cutaneous melanomas' patients, TERT mutations were autonomously concomitant with poorer overall survival (Griewank et al., 2014). TERT promoter mutations may play the role of identification of a clinically high-risk subset of patients with spitzoid tumors (Lee et al., 2015). Another study provided primary proof of the ability for using the combination of (TERT promoter, BRAF, and NRAS) mutations to recognize patients who are at risk of aggressive melanomas (Nagore et al., 2016). Also, those mutations were very dominant in cancers with advanced stages, mostly in thyroid cancers with the BRAF or RAS mutations (Landa et al., 2013). The cooperative presence of the BRAF V600E and the TERT C228T mutations in papillary thyroid cancers was found related with the worst clinicopathologic outcomes, and both mutations can possibly be as distinctive prognostic and therapeutic factors (Xing et al., 2014). The TERT promoter mutations could be useful for the assistance in the brain tumors' classification and prognostication (Killela et al., 2013). The TERT mutations' unique pattern and the other genetic modifications were also suggested to play obvious roles together in pathogenesis of oligodendroglial and astrocytic tumors (Arita et al., 2013). Other results indicated that the presence of TERT mutations was related to poor overall survival (Simon et al., 2014). The worst survival was realized in glioma patients with TERT promoter mutations (Heidenreich et al., 2015). As well as, a relationship was noticed between the TERT mutations and shorter overall survival of patients with glioma (Mosrati et al., 2015). Another study revealed that the TERT mutations and the long relative telomere length RTL together were prognostic factors for poor clinical outcomes and predictors of the gliomas resistance toward radiotherapy (Gao et al., 2015). Also, there was a strong association between TERT mutations and the recurrence of bladder cancer. More than that, these mutations are a probability of being a useful biomarker, for the early detection and the monitoring of the bladder cancer (Kinde et al., 2013). Furthermore, TERT promoter mutations may be useful adjunct biomarkers for the differentiation of nested variant of urothelial carcinoma (NVUC) and Large Nested Variant of Urothelial Carcinoma (LNVUC) from benign mimickers (Zhong et al., 2015). As well as, TERT promoter mutations might be able to define a small subset of gallbladder aggressive tumors (Hosen et al., 2015). The existence of TERT mutations in thyroid cancers may possibly be a good indicator of TERT gene

expression and telomerase activity, as well as its presence was associated with shorter survival of patient. Similarly, the occurrence of the TERT mutations could be as a primary genetic event in thyroid follicular tumors that have not demonstrated malignant features on routine histopathological workup (Wang et al., 2014). In addition to the predictive role of the TERT mutations for the aggressive performance and their association with distant metastasis development in thyroid cancers (Gandolfi et al., 2015). Furthermore, the TERT promoter mutations were suggested as a player of an important role in NSCLC pathogenesis (Ma et al., 2014). Also, the TERT C228T mutation of can be a possible new predictive marker and therapeutic target and may have an important role in the tumorigenesis and aggressiveness of ATC (Shi et al., 2015). The C228T mutations seemed to be associated with disease aggressiveness, and this would provide a promising platform for further studies assessing TERT promoter mutations as a possible malignancy indicator in ambiguous cases with unidentified malignant potential in adrenal tumors (Liu et al., 2014). The C228T mutations may possibly have a significant role in the development of adult granulosa cell tumors (Pilsworth et al., 2018). Whereas C250T mutations were intensely related with the worse survival and that those mutations could present important genetic driver events, and might be valuable prognostic indicators in this laryngeal cancer (Qu et al., 2014). TERT mutations may be a driver event for progression of PTs and can help in the distinguishing diagnosis between PTs and fibroadenomas (Piscuoglio et al., 2016). Moreover, the same mutations have been related with the advanced stages of the tumor and the patients' poor prognosis (Killela et al., 2013; Vinagre et al., 2013; Heidenreich et al., 2014).

The length of the telomere is a main tumor hallmark. Other than the TERT promoter hotspot mutations, additional pathways are implicated in telomere length increase (Heidenreich et al., 2014). The telomeres' alternative lengthening is one of these mechanisms (ATL) (Cesare and Reddel, 2010; Killela et al., 2013). Nevertheless, previously, another study has reported the lack of this mechanism in CRC (Heaphy et al., 2011). For that reason, the mechanisms of telomere length alteration in CRC are still anonymous.

Also, the suggestion of Chen et al in 2014, that different etiological causes could be implicated in differing mechanisms to preserve the length of telomeres throughout the carcinogenesis of HCCs (Chen et al., 2014) can be another justification for the

differences between our results and the earlier studies as other etiological causes were not investigated in our study.

In contrast with the previous results obtained from the former studies, it was mentioned earlier that the TERT mutations could play a restricted role in the tumorigenesis and pathogenesis of the gallbladder, due to the fact that no observation of any significant correlation was found between TERT promoter mutations and any clinicopathological characteristics in gallbladder cancer (Qu et al., 2014), which may support our results.

Another study also did not detect any TERT mutations in any of the samples examined. The results of this study indicated that the TERT mutations may not present any important role in prostate carcinogenesis (Stoehr et al., 2015), which may confirm the results of our study as well.

Recently, one study has examined the TERT promoter mutation frequencies in CRC, and mutations were not found in the colorectal adenocarcinomas (Cruvinel-Carloni et al., 2018). In both studies of ours and Cruvinel-Carloni et al. no mutations were witnessed in any of the samples. Even though the two studies were implemented in two different populations 'Brazilian and Turkish' the same results were seen. This emphasizes the fact that although the genetic differences existed between the two populations, we are still observing the same findings of no mutations which could be the proof to support our opinion that the TERT promoter might not harbor any mutations in regularly self-renewing cells due to the former epigenetic activation of the TERT gene. Also, we think that the genetic differences in populations may be playing an important role in the mutations occurrence in the region of TERT promoter. As well as, the total number of samples involved in our study may be another reason for our results. Likewise, uninspected presence of BRAF or RAS mutations which in our opinion may have a role in the formation of TERT mutations as they were observed in many other studies concomitantly (Griewank et al., 2014; Nagore et al., 2016; Landa et al., 2013; and Xing et al., 2014).

Despite the fact that it's the second globally, our study is the first study that investigated the frequency of TERT promoter mutations in a series of Turkish patients with CRC in Turkey.

5.2 Conclusion

As a result of the analysis of TERT promoter mutations in Turkish patients with CRC, the absence of TERT promoter mutations observed, is supporting our opinion that these modifications are not involved in CRC carcinogenesis and that cancers originated in tissues that are regularly self-renewing, such as cancers that originate from the epithelial cells of the gastrointestinal tract and from skin cells or from the bone marrow, might not harbor mutations, due to the previous epigenetic activation of the telomerase in their ancestor cells. Further investigations need to be performed for a stronger understanding.

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