REACTIVATION OF
TELOMERASE REVERSE TRANSCRIPTASE GENE
IN LIVER CANCER

A THESIS

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DOCTOR OF PHILOSOPHY

BY

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SEPTEMBER, 2014

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

REACTIVATION OF TELOMERASE REVERSE TRANSCRIPTASE GENE IN LIVER CANCER

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Ph.D. in Molecular Biology and Genetics

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September, 2014

Hepatocellular Carcinoma (HCC) is one of the major causes of cancer related deaths worldwide and its incidence has been increasing drastically, especially in western countries. HCC has a heterogeneous molecular and pathological background with various underlying risk factors and survival rate of HCC patients is very low due to late diagnosis and limited curative therapies. The mechanisms involved in hepatocellular immortality gains critical importance in order to develop preventive and therapeutic options against HCC. Telomerase reactivation is a keystone for HCC cells during transformation process. *TERT* promoter mutations activating its promoter by creating a novel activating motif were recently identified in different cancer types. In this study; we determined *TERT* promoter mutation frequency in HCC cell lines and tumors which are 67% (10/15) and 34% (15/44) respectively. High frequency of *TERT* promoter mutations in HCC indicated a possible functional role during hepatocarcinogenesis. We performed transcriptional factor search to find a candidate TF that could bind to mutant *TERT* promoter and STAT1 came out of that search. To study the role of STAT1 during reactivation of *TERT* expression, we activated STAT1 signaling by Interferon alpha (IFN-α) treatment and down regulated

STAT1 with RNA interference in several HCC cell lines. We have found that IFN-α was able to upregulate TERT expression in the HCC cell lines carrying a *TERT* promoter mutation and STAT1 knockdown was enough to eradicate this upregulation. In case of wild type cell lines, IFN-α treatment and STAT1 knock down had no effect on TERT expression. Our data delineates the contributions of *TERT* promoter mutations to hepatocellular immortality and gives insights into the potential use of *TERT* as a target for chemoprevention of hepatocarcinogenesis.

Keywords: Hepatocellular Carcinoma, TERT, promoter mutations, STAT1, Interferon alpha, IFN-α.

ÖZET

KARACİĞER KANSERİNDE TELOMERAZ REVERS TRANSKRİPTAZ GENİNİN REAKTİVASYONU

Dilek Çevik

Moleküler Biyoloji ve Genetik Doktora Tezi

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Eylül, 2014

Hepatoselüler Karcinoma (HSK) dünyada görülen kanser kaynaklı ölüm vakalarının ana sebeplerinden biridir. HSK farklı risk faktörlerine sahiptir ve heterojen bir moleküler ve patolojik temel üzerine oluşmaktadır. HSK' ya karşı daha etkili tanı ve tedavi seçenekleri geliştirmek için, hepatoselüler immortaliteye sebep olan mekanizmaların açıklanması önem kazanmaktadır. Telomeraz geninin yeniden aktive olması HSK hücreleri için kritik bir aşamadır. Yakın zamanda yapılan çalışmalarda, TERT geninin promotör bölgesinde mutasyonlara rastlanmıştır ve bu mutasyonların normalde var olmayan bir promotör motifi oluşturarak TERT geninin ifadesini arttırdığı düşünülmektedir. Bu çalışmada, HSK hücre hatlarındaki TERT promotör mutasyon oranı %67 (10/15), tümör örneklerindeki mutasyon oranı ise % 34 (15/44) olarak belirlenmiştir. TERT mutasyonlarının yüksek oranları, HSK oluşumunda önem taşıdıklarına işaret etmektedir. Yaptığımız analizlerde STAT1 transkripsiyon faktörü, TERT mutasyonlarının oluşturduğu yeni motife bağlanabilecek bir aday transkripsiyon faktörü olarak ön plana çıkmıştır. STAT1'in TERT gen ifadesi üzerine olan etkisini araştırmak için HSK hücreleri interferon-α ile muamele edildi ve bunun TERT promotör mutasyonu taşıyan HSK hücre hatlarında TERT gen ifadesini arttırdığı belirlendi. STAT1 gen ifadesi RNA interferaz yöntemi ile düşürülerek bunun TERT gen ifadesi üzerine yaptığı etki araştırıldı. TERT promotör mutasyonu

taşıyan HSK hücre hatlarında STAT1 gen ifadesi düşürüldüğünde, TERT gen ifadesinin azaldığı ve IFN-α muamelesinin TERT gen ifadesi üzerine olan etkisinin de yok olduğu gözlendi. Mutasyon taşımayan hücre hatlarında ise, IFN-α muamelesinin ve STAT1 gen ifadesinin düşürülmesinin TERT gen ifadesi üzerine etkilerinin bulunmadığı tespit edildi. Bu sonuçlar, TERT promotör mutasyonlarının hepatoselüler immortalite üzerinde önemli bir etkisinin olduğunu ve bu mutasyonların HSK oluşumunun önlenmesi amacıyla kullanılabileceğini göstermektedir.

Anahtar kelimeler: Hepatoselüler Karsinoma, TERT, STAT1, promotör mutasyonu, İnterferon alfa, IFN-α

TO MY MOTHER

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Abbreviations

AH	Alcoholic Hepatitis
ALD	Alcoholic Liver Disease
E-Value	Expected Value
HBV	Hepatitis B Virus
HBx	Hepatitis B virus X protein
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HSC	Hepatic Satellite Cells
IFN-α	Interferon alpha
NAFLD	Non Alcohol Fatty Liver Disease
ROS	Reactive Oxygen Species
STAT	Signal Transducers and Activators of Transcription
TERT	Telomerase Reverse Transcriptase

Chapter 1

Introduction

1.1 Hepatocellular Carcinoma

1.1.1 Epidemiology of Hepatocellular Carcinoma

Liver cancer is a global health problem since it is one of the most deadly cancers. Liver cancer has different hepatic neoplasms such as hepatocellular carcinoma (HCC), cholangiocarcinoma, hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma and epitheliod haemangioendothelioma[1]. Among these, HCC is the most commonly observed liver cancer type with 83% frequency and it is the sixth most common cancer worldwide. [2]. HCC is two times more common in men compared to women. 782,000 new HCC cases occurred in 2012 worldwide and male patients comprised 554,000 while females comprised 228,000 of all the cases. HCC shows a non-uniform distribution across different continents. In men and women together, Eastern and South-Eastern Asia are the regions with the highest incidences of HCC; Northern and Western Africa have the intermediate rates while Northern Europe and South-Central Asia are the regions with the lowest mortality rates. Mortality rates are very similar to incidence frequency of HCC since the survival rate is very low due to limited treatment options (Figure 1.1). HCC is the second leading cause of cancer related deaths worldwide with an estimated number of nearly 746,000 deaths in 2012 which is 9.1% of the total cancer related deaths.

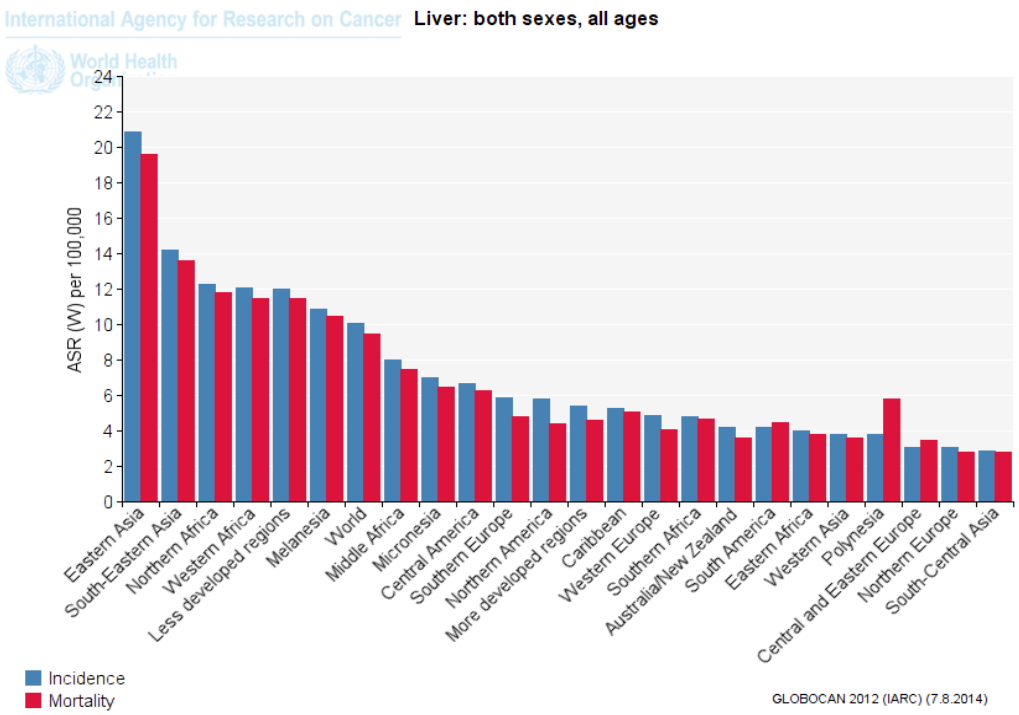


Figure1.1 Mortality and Incidence Rates of HCC across different geographical regions.

In all regions, incidence and mortality rates are quite similar. Eastern Asia has the highest incidence, Africa has the intermediate incidence and Europe and America has relatively lower incidences. Age standardized rate (W) per 100,000 cases. Adopted from GLOBOCAN.

Although HCC incidence is highest in China and other developing countries, the incidence of HCC has been increasing drastically in United States and Europe over the last decade [3]. In European countries like France, England and Spain; HCC incidence shows an increasing trend while in Slovakia and Denmark, HCC incidence is more stable. In Asian countries such as Japan, China, Philippines and Singapore; HCC incidence has been decreasing while in India there is an increased tendency. In United States, Canada and Australia, HCC incidence has been increasing, on the other hand, Colombia and Costa Rica has unstable changes in HCC rates. The increase of HCC incidence in Western countries is mostly caused by cirrhosis associated with alcohol abuse, fatty liver disease caused by obesity and type-2 diabetes [4].

1.1.2 Etiologies and Risk Factors of Hepatocellular Carcinoma

The most common risk factors of hepatocellular carcinoma include Hepatitis B and C viruses, Aflatoxin B exposure and alcohol abuse. Non Alcoholic Fatty Liver Disease (NAFLD), diabetes and haemochromatosis can be classified among less critical risk factors[5]. Mechanisms induced by the presence of the most common risk factors are shown in Figure 1.3. Aflatoxin B1 and HBV act together to inactivate p53 which increases cell proliferation. Viral hepatitis and alcohol abuse induced inflammation causes necrosis and regeneration cycles. Viral and alcohol induced oxidative stress causes mutagenesis and alters signaling pathways in favor of tumorigenesis. Finally, all risk factors lead to HCC formation after accumulation of genetic alterations in hepatocytes [2].

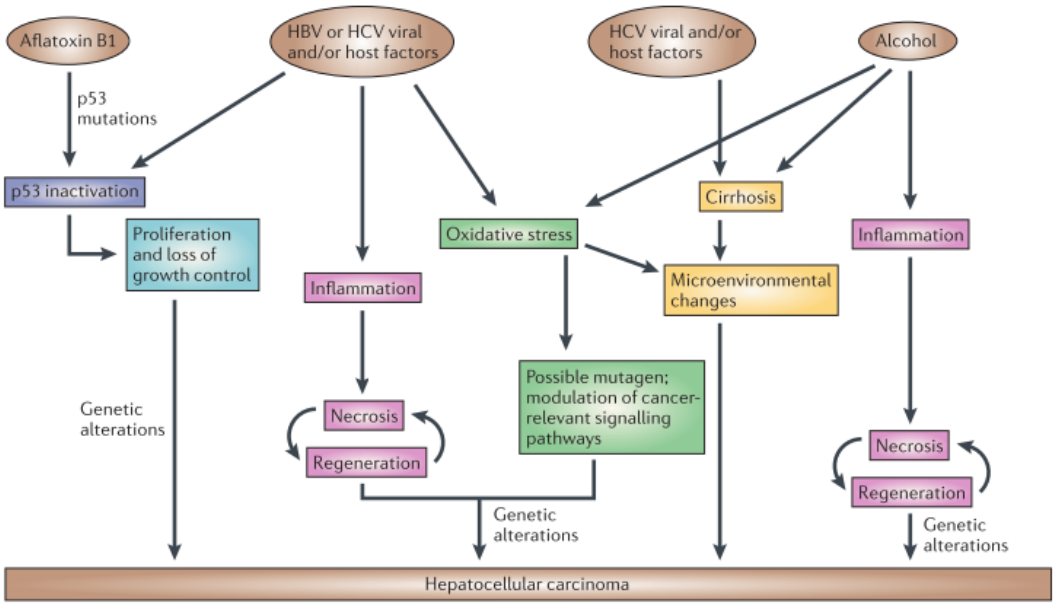


Figure1.2 HCC is induced by multiple risk factors.

Hepatitis B and Hepatitis C viruses, Aflatoxin B exposure and alcohol induce hepatocarcinogenesis by acting on intersecting mechanisms such as inactivation of tumor suppressor p53, chronic liver inflammation, cirrhosis, oxidative stress and mutagenesis. Adopted from [2].

1.1.2.1 Hepatitis B Virus

Hepatitis B virus (HBV) chronic infection is the primary cause of HCC worldwide since more than 50% of all HCC patients are infected with HBV [6]. However, there is a high geographical heterogeneity in HBV incidence [7]. In Asia and Africa, more than 70% of HCC cases are attributable to HBV due to high incidence of infection [8]. HBV enhances the effects of aflatoxin exposure to trigger HCC formation, thus patients with HBV carry a greater risk of getting HCC if they are exposed to aflatoxin [9]. In USA and Australia, HBV incidence is low in the general population, thus, 70–80% of the HCC cases that are linked to HBV are observed in immigrants from Asia [10]. In Europe, HBV incidence is very low compared to Asia or Africa, therefore, it is a low risk factor of HCC in European population [11]. Survival rates of HBV-related HCC is extremely low, thus HBV infection is a major public health problem [12]. Mechanisms behind the involvement of HBV in hepatocarcinogenesis have been discussed since the first time HBV was claimed to be related to HCC formation at seventies [13]. Most prominent effect of HBV during liver cancer progression starts with the integration of viral DNA into the host genome causing genetic instability and activation of oncogenes [14]. HBV DNA integration occurs in the early acute phase of the infection since it is critical for the persistence of the infection in hepatocytes even though it is not necessary for viral replication [15]. Apart from causing genetic instability, HBV also triggers hepatocarcinogenesis by synthesizing viral proteins such as HBx, PreS2/S, HBSP which will in turn cause genetic alterations, transactivation of oncogenic transcription factors, and deregulation of important cellular pathways such as cell proliferation, differentiation, and survival of hepatocytes [16]. HBx acts as a pro-apoptotic protein by creating reactive oxygen species (ROS) or as an anti-apoptotic protein by inhibiting p53, Fas, TNF and TGF-β induced apoptosis[17]. C terminal truncated version of HBx protein also contributes to hepatocarcinogenesis through different pathways; creating oxidative stress and mitochondrial damage; increasing invasiveness and metastatic potential of HCC; and causing over expression of Centromere protein A in HCC tissue [18]. HBx has another oncogenic effect by preventing repair of DNA damage by inhibiting base excision

repair system due to its structural similarity with human thymine DNA glycosylase , an important enzyme for the proper functioning of BER pathway [19].

1.1.2.2 Hepatitis C Virus

Hepatitis C Virus (HCV) is a positive RNA virus that belongs to the Flaviviridae family [20]. HCV causes a chronic infection in most of the infected individuals; it stays dormant for decades and then causes liver fibrosis and cirrhosis at the final stages of hepatitis [21]. There are 170 million people infected with HCV worldwide, thus, HCV is the second main risk factor for HCC and HCV related HCC cases comprise 10–20% of HCC cases worldwide [22]. Unlike HBV associated HCC, most HCV-related HCC cases develop after liver fibrosis and cirrhosis reach critical levels [23]. Chronic inflammation caused by chronic HCV infection creates a great risk of developing HCC, a risk higher than the one caused by any other non-viral risk factors [24]. HCV infection causes inflammation in the liver (hepatitis) and leads to infiltration of lymphocytes and Natural Killer cells [25]. Recognition of viral RNA by RIG-I (retinoic acid-inducible gene 1) and TLR-3 (Toll like receptor 3) triggers NF-κB pathway and cells start to secrete interferon and other pro-inflammatory cytokines. Moreover, HCV polymerase is found to activate inflammatory signaling pathways causing cytokine release [26]. Generation of ROS by HCV core protein leads to oxidative stress and DNA mutagenesis and acts as another mean of contribution to hepatocarcinogenesis [27]. Other than chronic inflammation, there are more direct effects of HCV on HCC. Both tumor suppressor genes and proto-oncogenes are subject to deregulation by viral proteins. HCV polymerase NS5B interacts with one of the most critical tumor suppressors, the retinoblastoma protein (Rb), causes its degradation favoring entry of cells into S phase [28]. HCV proteins interact with another critical tumor suppressor protein, p53, to inhibit apoptosis [29]. Furthermore, HCV targets the WNT/β-catenin pathway by causing stabilization of β-catenin and preventing it from functioning [30]. Transforming growth factor beta (TGF-β) is also a target of HCV proteins; HCV NS5A interacts with TGF-β receptor and prevents its signaling, leading to improper regulation of hepatocyte proliferation and contributes to further liver damage, fibrosis and transformation [31].

1.1.2.3 Alcohol Abuse

Severe alcohol consumption increases HCC risk up to 5 fold by a multistep process called Alcoholic Liver Disease (ALD) that leads to chronic liver disease, liver fibrosis and cirrhosis, and hepatocellular carcinoma [32,33]. Alcohol induced fatty liver disease, also called steatosis is the first pathology caused by chronic alcohol intake. As the name implies, it is caused by the accumulation of fat in liver cells. Alcohol increases the storage of triglycerides, phospholipids, and cholesterol by preventing their oxidation in mitochondria [32]. Another effect of alcohol on liver metabolism is to increase lipid uptake by the liver. Transcription factors regulating lipid metabolism are also subject to regulation by alcohol intake through induction of lipogenesis and inhibition of lipid oxidation. SREBP-1c (Sterol Regulatory Element-Binding Protein 1c) is a transcription factor favoring lipogenesis and it is upregulated by Ethanol in hepatocytes during severe alcohol intake [34]. Alcoholic Hepatitis (AH) is another pathology of alcohol abuse; it is marked with liver inflammation and hepatic injury caused by infiltration of inflammatory cells into the liver [35]. AH occurs after steatosis and is mostly accompanied by liver fibrosis in 10-35% of alcohol abusers. EtOH is metabolized in hepatocytes into acetaldehyde and acetate forms after acetaldehyde degradation [36]. Reactive oxygen species and acetaldehyde generated during ethanol metabolism in hepatocytes cause hepatocyte injury [37]. Acetate is not hepatoxic by itself but it upregulates pro inflammatory cytokine secretion triggering chronic inflammation in liver [38]. AH causes hepatocyte apoptosis, activation of innate immunity, infiltration by neutrophils, activation of adaptive immunity and inhibition of liver regeneration [39]. Liver fibrosis is the next step of alcoholic liver disease. It is a physiological healing process in response to chronic liver damage and it forms by accumulation of extracellular matrix proteins such as collagen, however excess amount of fibrotic liver tissue prevents liver from functioning [40]. Chronic liver damage stimulates Hepatic Satellite Cells (HSCs), fibroblasts and myofibroblasts to synthesize collagen and other extracellular matrix proteins [41]. Alcoholic cirrhosis leads to development of HCC as any other cirrhosis and it also increases the effects of HCV chronic infection [33].

1.1.2.4 Aflatoxin Exposure

Aflatoxins are main dietary risk factors triggering hepatocellular carcinogenesis; indeed 5 to 25% of HCC cases are associated with aflatoxin exposure [42]. Aflatoxins are produced by *Aspergillus flavus* and *Aspergillus parasiticus* and mainly found in contaminated food such as maize, rice, soy bean and nuts [43]. Aflatoxin is linked to hepatocellular carcinoma especially in sub-Saharan Africa, Southeast Asia and China where high exposure of this contaminant is observed in the general population [44]. Aflatoxin B1, the major hepatotoxic agent, metabolizes into mutagenic products in the liver. It is the main cause of the transversion mutation (G:C to T:A) found at codon 249 of the tumor suppressor *TP53* gene [45]. Since it is a well defined carcinogen, it was used as a biomarker in a study based in China and a very significant correlation was found between the urinary concentrations of aflatoxin metabolic by products and HCC occurrence [9]. To decrease HCC cases associated with aflatoxin exposure, aflatoxin content in food should be strictly controlled and any chemopreventive strategy should be applied in case of detection of the contaminant [44].

1.1.2.5 Non Alcoholic Fatty Liver Disease (NAFLD)

Non Alcoholic Fatty Liver Disease (NAFLD) is a disease marked with the accumulation of lipids in the liver of individuals who are not heavy alcohol drinkers [46]. NAFLD causes cirrhosis eventually, thus it is as dangerous as other triggers of cirrhosis that lead to HCC [47]. Unlike Asia where most HCC cases are related to HBV or HCV infections, in USA and Europe, it is NAFLD which establishes a huge risk of developing HCC [48]. As the percentage of people with obesity and metabolic syndrome increases, the incidence of NAFLD also increases accordingly [49].

1.1.2.6 Obesity and Diabetes Mellitus

Epidemiologic data suggests that both obesity and type 2 Diabetes Mellitus (T2DM) are associated with increased risk of developing HCC [50]. Metabolic disorders and increased fat tissue in liver are common observations in obesity and DM patients.

NAFLD is also quite frequent in these patients and there is a good correlation between Body Mass Index (BMI) and cancer risk [51]. Lipid peroxidation which occurs excessively as a result of NAFLD causes formation of mutagens from ROS and leads to both mutation accumulation and liver damage [49]. Moreover, obesity and diabetes are both associated with insulin resistance and increased IGF (Insulin-like growth factor) amount triggering cell proliferation and cancer progression [52].

1.1.3 Molecular Mechanisms of Hepatocarcinogenesis

Pathogenesis and molecular mechanisms of hepatocarcinogenesis are quite complex due to involvement of various factors during the development of HCC [2]. One of the first events through hepatic transformation is acquirement of mutations, genetic and epigenetic changes that lead to malignant transformation [53]. Other than hepatocytes, there are other cells that occupy the liver such as cholangiocytes, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells (HSCs). Exposure to hepatoxic substances such as aflatoxins and infection of the liver cells leads to inflammation in Kupffer cells and HSCs [54]. In case of severe chronic inflammation; necrosis, fibrosis and cirrhosis occur. The underlying reason for cirrhosis can be viral infections such as HBV and HCV, alcohol abuse or NAFLD. Once cirrhosis occurs, there is no going back in most cases and cirrhosis turns into HCC[55]. Hepatic cells enter into damage-repair-regeneration cycle and this increases chromosomal instability and sensitivity of the cells against carcinogens that cause formation of dysplastic nodules and HCC eventually [56]. Main pathways involved in hepatic transformation are as follows: tumor suppressor TP53, Retina Blastoma protein (Rb), Wnt-CDKN2A (β-catenin), IGF2R (Insulin-like growth factor receptor-2), PTEN (Phosphatase and tensin homolog), Axin1, PI3K (Phospatidiyl Inositol Kinase), JAK-STAT pathway, and K-ras/MAPK (K Rat Sarcoma) and TGF-β (Transforming Growth Factor Beta) [55]. A summary of these pathways is depicted in Figure 1.5. Each pathway is given together with the underlying risk factor that leads to HCC.

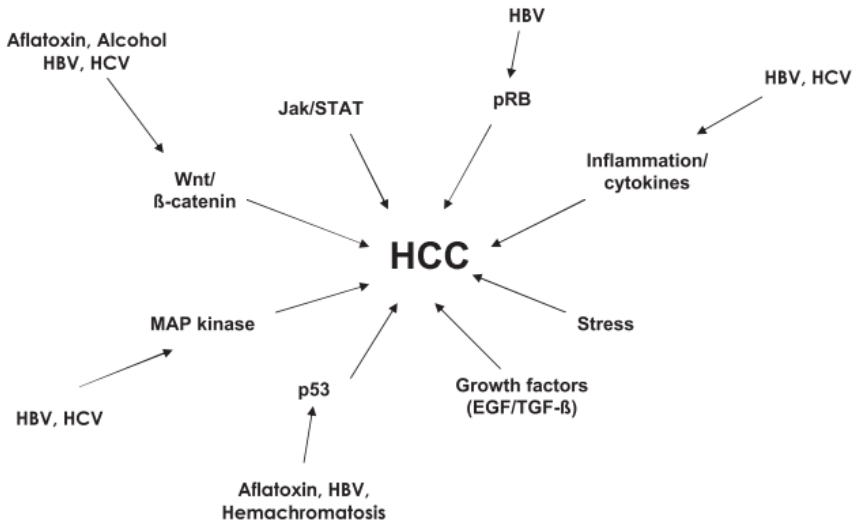


Figure1.3 Molecular Signaling Pathways Involved in Hepatocarcinogenesis

These pathways act together in a synergistic or additive manner during HCC development. Depending on the etiology, different combinations of these pathways can be observed in a patient specific manner. Adopted from [55].

1.1.4 Genetics and Epigenetics of Hepatocellular Carcinoma

Chromosomal aberrations in the form of deletions and copy number variations are commonly observed in HCC [57]. Most frequently amplified regions are the chromosomes 1q, 8q, 6p, 7, 8q, 17q and 20 while most frequently lost regions are 1p, 4q, 6q, 8p, 13q, 16, 17p and 21 chromosomal loci [58]. 13q and 4q are also lost, but only in poorly differentiated HCC tumors [59]. Chromosomal changes are not random but rather comprise the locations of critical genes such as p53 (17p) or Rb (13q) [60]. Moreover, there are genetic variations caused by integration of HBV DNA into the host genome in HCC patients with underlying HBV infection [61]. Integration of HBV DNA within or upstream of the *TERT* (telomerase reverse transcriptase) gene causes upregulation of TERT and provides cellular immortality in HCC patients [62]. TP53 was the first gene discovered to be mutated in HCC [63]. The two most frequently mutated genes are TP53 (coding for p53) with 35% of mutation frequency and CTNNB1 (coding for β-catenin protein) with close to 20% of mutation frequency [58,62,64]. Other than p53 and CTNNB1; AXIN1 and CDKN2A (p16INK4a) are

frequently mutated in HCC patients [65]. There are also a group of less frequently mutated genes that are involved in pathways critical for hepatocarcinogenesis such as Wnt/b-catenin, p53, PI3K/Ras signalling, oxidative and endoplasmic reticulum stress pathways [55].

In addition to genetic regulation of gene expression, epigenetic pathways regulating gene expression are also deregulated in HCC [66]. Like many other cancers, global hypomethylation and promoter hypermethylation are main epigenetic changes observed in HCC [67]. Mutations at the genes involved in epigenetic regulation are also reported in HCC and most frequent mutations were found in ARID1A, ARID1B, ARID2 genes [58,68].

1.1.5 Liver Cirrhosis

Liver cirrhosis is the term used for the last stage of chronic liver disease associated with regenerative nodules, fibrotic and necrotic liver tissue [69]. Regardless of the etiology behind it (HBV, HCV, alcohol abuse, toxic exposure etc.) liver cirrhosis is the major risk factor for hepatocarcinogenesis. The clinical complications of cirrhosis include impaired hepatocyte function, increased vascular pressure (portal hypertension), ascites formation [70]. Regenerative nodules are composed of disorganized hepatocytes, fibrotic tissue and bile duct cells and have occasional dysplastic nodules. Small and large cell dysplasias are two forms of dysplasia observed in liver. Small cell dysplasia (SCD) is characterized by hepatocytes with an increased nucleocytoplasmic ratio whereas Large Cell Dysplasia (LCD) is characterized by a decrease in nucleocytoplasmic ratio [71]. SCD is composed of cells with a high proliferation potential, and have common chromosomal alterations with neighboring HCC tissue thus it is considered to be the early precursor of HCC [72]. Unlike SCD, LCD is not considered to be the precursor of HCC since hepatocytes forming LCD have normal nucleocytoplasmic ratio and low proliferation capacity together with relatively high apoptosis. Another finding supporting this hypothesis is the fact that LCD forms as a result of hepatocyte senescence triggered by necrosis, inflammation and regeneration [73].

1.1.6 Diagnosis and Prognosis of Hepatocellular Carcinoma

Early diagnosis of HCC is not very common due to asymptomatic disease progression [74]. Late diagnosis is the reason of limited therapy options and low disease survival rate [75]. Ultrasonograpy is the most common surveillance method since it is non invasive and available [76]. Serum α-fetaprotein (AFP) is the most common serological test used for diagnosis of HCC; however, it has a low sensitivity [77]. Combination of ultrasound with AFP does not have a positive impact on diagnosis but increase false positive rates [78]. Based on available data, ultrasound screening of the patient twice a year is recommended [79]. When a nodule size exceeds 1cm in ultrasound, further diagnosis should be performed by biopsy or more accurate imaging methods such as CT (computer tomography) or MRI (magnetic resonance imaging) [80]. These methods are only applicable for patients with cirrhosis or viral infections (HBV or HCV) without cirrhosis; liver biopsy is necessary for other patients [75].

Once the patient is diagnosed with HCC, determination of prognosis becomes critical. Early diagnosis and proper treatment provides a 5 year of survival rate for HCC patients. During the assessment of prognosis, several factors such as tumor stage, liver function, and cancer-related symptoms should be considered for proper assessment [81].

1.1.7 Treatment of Hepatocellular Carcinoma

After the diagnosis and classification of HCC, treatment options should be determined by qualified experts. Early diagnosed cases have treatment options such as surgical resection of the tumor, liver transplantation and ablation with high curative potential [81]. Other therapeutic options are chemoembolisation and sorafenib; however, these treatments only increase survival, they are not curative for HCC [82]. Systemic chemotherapy or drugs such as tamoxifen, octreotide, or antiandrogens have no effect on HCC [83]. Surgical resection of the tumor is applicable to patients without cirrhosis and this group comprises a very low percentage of patients that is 5% in the USA and Europe and 40% in Asia [84]. Liver transplantation is the best option to treat patients with one HCC tumor of 5 cm or smaller and for patients

having up to 3 nodules of 3 cm or smaller without metastasis [85]. However, transplantation is limited with the number of donations [86]. Tumor ablation, injection of chemicals such as ethanol or acetic acid to induce tumor necrosis, is also an effective option for early stage HCC. Survival rates after tumor ablation and resection is 5 years in majority of the patients thus they are considered as interchangeable therapeutic options [87]. Chemoembolization (delivering a relatively large dose of chemotherapy directly to the liver tumor and cutting arterial blood supply) is only an option for patients with large tumors that are not suitable for resection or tumor ablation [88]. The only FDA approved drug for HCC is Sorafenib which is a multikinase inhibitor and it increases patient survival for only three months compared to placebo group [89]. In conclusion, there is an urgent need for novel biomarkers and therapeutic agents that can be used for HCC to increase the survival rate of both early and late diagnosed patients.

1.2 Telomerase and Cancer

Telomeres are repetitive DNA sequences (5′-TTAGGG-3′ in human) and a collection of proteins capping this DNA that are found at the end of chromosomes to protect chromosome ends from degradation and end-to-end chromosome fusions [90]. Human telomere length is between 5 and 15 kb and it is mostly double stranded except a 30–200 nucleotide GT-rich 3′ overhang [91] that forms a (T)-loop by annealing with a portion of double stranded region of the telomeric DNA. DNA polymerases cannot replicate the end of linear chromosomes due to lack of a proper primer sequence [92]. This problem is known as end of replication problem [93]. At the final stages of DNA replication from the lagging strand, a gap is formed after degradation of the very distal RNA primer. Moreover single stranded 3′ region of overhang of telomere has an intrinsic exonuclease activity and removes the 5′ end of the complementary strand [94]. At the end of each DNA replication, telomere DNA gets shorter due to these two mechanisms [94]. Telomerase is a ribonucleoprotein containing Telomerase Reverse Transcriptase (TERT) enzyme, the catalytic part of the complex and RNA component (TERC), the template used during synthesis of new telomeric DNA [95]. There are other proteins necessary to form the whole telomerase such as DKC

(dyskerin), (NOP10NOP10 ribonucleoprotein), GAR1 (GAR1 ribonucleoprotein homolog, NHP2 (NHP2 ribonucleoprotein), reptin and pontin [96]. Theoretically, telomerase can solve the end of replication problem by adding lost repeats to the chromosome ends, however, most normal somatic cells, except germ cells lose TERT expression [95]. Most tumor cells recover telomerase activity to overcome end of replication problem which will be discussed in detail in the next sections [97].

1.2.1 Replicative Senescence

The term of cellular senescence is first used by Hayflick and Moorhead in 1961 when they discovered that human fibroblasts cannot divide infinitely when they are continuously cultured [98]. However, what they had called cellular senescence is now called replicative senescence and described as a type of cellular senescence which is associated with telomere shortening [99]. Senescence phenotype is associated with growth arrest, apoptosis resistance, limited and altered gene expression, change in cell metabolism [100]. Senescent cells also carry one or more of senescence markers such as senescence associated β-galactosidase, p16 and senescence associated DNA damage foci (SDF) and senescence associated heterochromatin foci (SAHF) [101]. Oncogene induced senescence, ROS induced senescence, DNA damage induced senescence and replicative senescence are the main types of cellular senescence and they involve p53 and Rb pathways together with Cyclin dependent kinase inhibitors p16, p15, p21 and p27 [102].

Telomeric DNA gets shorter after each cell division due to end of replication problem, when shortening of telomeres reach a critical level, cells can no longer divide and enter replicative senescence [103]. Replicative senescence starts as critically short telomeres act like DNA double-stranded breaks that are sensed by 911 (RAD9/HUS1/RAD1) and MRN complex (MRE11–RAD50– NBS1), then DNA damage signal is sent to ATM-ATR and they activate CHK1 and CHK2 kinases which in turn activate p53 to make the cells enter a permanent cell cycle arrest [104].

Most tumor cells gain telomerase activity to bypass senescence; however, TERT expression can only overcome telomere induced senescence but not oncogene induced senescence or other stress induced senescence types [105]. The relationship

between telomere length, cell division and senescence is shown in Figure 1.6. In normal cells, there is no or very little TERT expression, that's why they enter into senescence when telomere length reaches a critically short level. In contrast to normal cells, germ cells and tumor cells have telomerase activity that keeps telomeric DNA length in the normal level, thus these cells continue dividing [100].

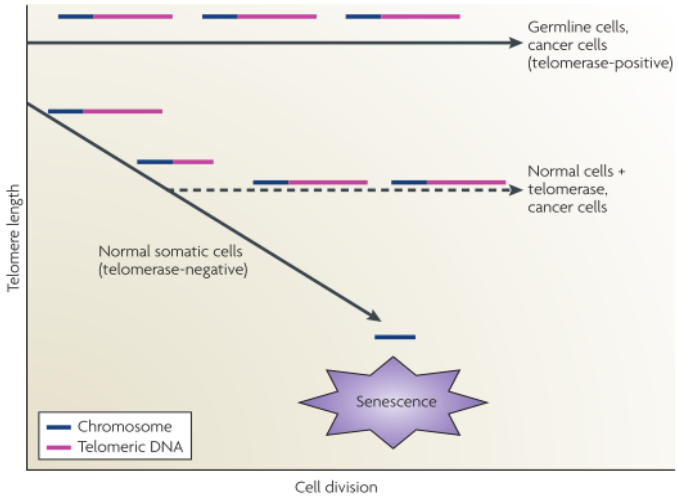


Figure1.4 Telomere induced senescence.

Normal somatic cells are telomerase negative, thus they enter into senescence as a response to shortened telomere length. Germ cells and cancer cells express telomerase and they can divide infinitely without entering into telomere induced senescence. Adopted from [100] .

Replicative senescence was studied in normal liver, cirrhotic liver and HCC and it was found that replicative senescence was a rare event in normal liver and common in cirrhotic liver. In HCC, medium levels of senescent cells were detected [106]. Since telomere length is an obstruction that leads to replicative senescence, it is expected to observe shorter telomere length in cirrhotic tissue that is already defined to have a high percentage of senescent cells. Indeed, telomere length was found to be significantly shorter in cirrhosis samples than that of the normal liver's regardless of the etiology behind the disease and degree of shortening of telomeres and senescence significantly correlated with increase in fibrosis and cirrhosis [107].

1.2.2 Cellular Immortality in HCC

Replicative senescence induced by the shortening of telomeres should be bypassed by HCC cells for cellular transformation. HCC cells also need to recover TERT expression in order to maintain telomere length and gain immortality. They achieve bypassing senescence by acquiring mutations in p53 gene and Rb pathway genes (p16 INK4a, p15INK4b or RB1 genes) [108]. p53 mutations are detected frequently in HCC [109]. Rb pathway genes are targets of mutations and are mainly silenced through epigenetic mechanisms such as promoter metylation[65].Acquiring telomerase activity is necessary and present in all kinds of cancers including HCC [110]. Indeed, 90% of HCC cases have increased telomerase activity [111,112]. One known mechanism to re-establish TERT expression in HCC is through integration of HBV viral DNA into TERT gene, however this is restricted to a very low number of HCC cases [113]. Viral proteins HBx and PreS2 also reported to de-repress TERT expression in HCC [114,115]. Oncogenes such as c-MYC, NF-κB and β- Catenin are transcription factors that induce TERT expression in several cancers [116]. In addition to re-gaining TERT expression, HCC cells also up-regulate expression of other telomeric proteins in order to preserve functional telomeres [117]. As an alternative to re-establishing TERT expression, tumor cells restore telomere length by a mechanism called Alternative Lengthening of Telomeres (ALT) that is similar to homologous recombination [118].

1.2.3 *TERT* Promoter Structure and Regulation

Telomerase reverse transcriptase (*TERT*) gene is a large gene (40 kb) located on chromosome 5p15.33 and it has 15 introns and 16 exons [119]. *TERT* expression is regulated tightly due to its protein's critical function. *TERT* core promoter is 260 base pairs and it does not contain TATA and CAAT boxes [120]. *TERT* promoter has binding sites for great number regulatory factors. It has E-boxes that are subject to binding and activation by c-MYC. BRCA1 is also known to down-regulate TERT transcription together with (Nmi) N-Myc interacting protein and c-Myc [121]. This inhibitory function of BRCA1 is compromised in some mutants [122]. TERT transcription is repressed by p53 tumor suppressor [123]. ETS transcription factor family members activate TERT transcription [124] and they are activated by several

oncogenes such as EGF, Her2/Nez, Ras and Raf [125]. One other property of *TERT* promoter is the fact that it is highly GC rich which makes it a target for zinc finger transcription factor, Sp1 [126] and this GC rich promoter is suitable for epigenetic regulations such as promoter methylation and chromatin remodeling [127]. Finally, TERT transcription is also controlled by hormones such as Estrogen and cytokines such as TGF-β [128]. During cancer formation, oncogenes are activated whereas tumor suppressors are repressed; these two events contribute to immortalization by regulating TERT expression in favor of tumor cells [129]. Moreover, recent reports discovered a critical relationship between obesity related hormone leptin and telomerase activity in breast cancer patients [130]. Leptin hormone is found to increase TERT expression in HepG2 cells (HCC cell line) and MCF-7 cells (breast cancer cell line) [131,132]. These results indicate that obesity may cause cellular immortality by increasing telomerase activity [127].

1.2.4 *TERT* Promoter Mutations in Different Cancers

Recently two groups detected highly recurrent germ line and somatic mutations in the promoter region of TERT gene in melanoma patients and cell lines [133,134]. Horn et al. first detected a single nucleotide mutation, A > C (T > G) at the -57 bp from transcription start site (Chromosome 5: 1,295,161) in a familial melanoma case. They detected this mutation to be in allelic linkage with the common polymorphism (rs2853669) found at the -245 bp from ATG start site in the TERT promoter [135]. Then they analyzed melanoma cell lines and other patients unrelated to the first family and detected two more mutations located at -124 bp (1,295,228; depicted as C228T) and -146 bp (1,295,250; depicted as C250T) of TERT promoter [133]. In an independent study by Huang et al. they have detected the same somatic promoter mutations at the *TERT* promoter as a result of whole genome sequencing analysis This group also discovered additional CC > TT mutations at the -124 and -146 bp [134]. These frequent promoter mutations are suggested to create a common binding motif (CCGGAA/T) for E-twenty six/ ternary complex factors (ETS/TCF) transcription factors thus increasing *TERT* expression which helps tumor cells during transformation proces[133,134]. Representative image of the TERT promoter including hotspot mutations is given in Figure 1.7.

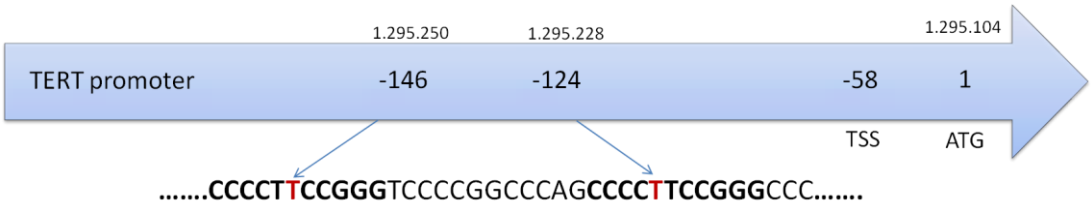


Figure1.5 Schematic representation of a part of the *TERT* promoter that contains hotspot mutations

Mutations are marked with red and new binding motif is marked with bold characters.

Discovery of frequent promoter mutations in *TERT* gene in melanoma led other scientists to search for the same mutations in different cancers, indeed they have found the same mutations [136]. Mutation frequency has been different in different types of cancers. Melanoma, pleomorphic dermal sarcoma, myxoid liposarcoma, glioma, urothelial cell carcinoma of bladder, basal and squamous cell carcinoma of skin and liver cancer are among the cancers with high frequency of *TERT* promoter mutations [136–146] In other cancers, TERT promoter mutations exist in a very low rate [136,139]. Two reports suggested that TERT promoter mutations are found at higher frequencies in tumors originated from tissues with low self renewal capacity [136,147]. TERT promoter mutations are high in frequency, they are suggested to be gain of function mutations and they are observed in many different cancers; thus, they are considered to be mutations driving cancerogenesis but not random mutations [129]. There is some evidence showing that these mutations are increasing promoter activity when tested in reporter assays together with controls which do not have mutated base [133,134,148]. In glioma and thyroid cancer, TERT promoter mutations have been associated with advanced disease and poor prognosis [136,143,149].

Other than promoter mutations, a T>C single-nucleotide polymorphism (SNP) also attracted attention of the scientists. This SNP is detected at position−245 bp from transcription start site of *TERT* gene (genomic loci 1295349) and it is represented by rs2853669 or T349C [133,134]. Hsu et al. reported that carriers of the *TERT* rs2853669 CC genotype had a significantly lower telomerase activity compared to ones with TT genotype in lung cancer since it disrupted an existing Ets2 transcription factor

binding site at TERT promoter [135]. In another study, *TERT* rs2853669 CC allele was found to be associated with a significantly increased risk of lung cancer [150]. In breast cancer, there is one study showing that there is no significant association between *TERT* rs2853669 SNP and breast cancer risk [151]. However, there is a more recent study demonstrating that the *TERT* rs2853669 CC genotype was correlated with a low risk of breast cancer [152]. There is also promising data about the possible use of *TERT* promoter mutations as biomarkers together with rs2853669 polymorphism in bladder cancer. It is found that a variant allele of rs2853669 polymorphism counteracts with the effect of *TERT* promoter mutations C228T and C250T in bladder cancer [148]. In the presence of the variant allele of rs2853669 polymorphism together with *TERT* promoter mutations; the patients have a higher survival rate compared to the patients with *TERT* promoter mutations but without the variant allele of rs2853669 polymorphism [148]. This data is supported with the observation that the variant allele of rs2853669 polymorphism prevents the binding of Ets2 to its non-canonical target site found in the vicinity of the polymorphism which is located near an E-box [135,153].

TERT promoter mutations provide an advantage to the bearing cells during transformation process provided that necessary transcription factors are available and functional to up-regulate TERT protein expression[154]. Increased expression of ETS transcription factors in many cancers [155,156] can be considered as a supporting event for the hypothesis that ETS transcription factor binding site is created by C228T and C228T mutations, however, there is no experimental data supporting this event yet.

1.3 Signal Transducer and Activator of Transcription 1 (STAT1) and Cancer

1.3.1 STAT Transcription Factor Family

The signal transducer and activator of transcription (STAT) transcription factors are composed of several members such as STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 [157]. STAT family member proteins have 5 functional domains composed of one amino-terminal domain, one coiled-coil domain, one DNA-binding

domain, one SH2 domain and one carboxy-terminal transactivation domain [158]. Phosphorylation of two amino acids inside the carboxy-terminal transactivation domain activates STAT transcription factors. One of these sites (tyrosine) is critical for the dimerization function while the other site (serine) is for activation of the transcriptional regulatory function of STATs [159]. STAT transcription factors regulate transcription with the signal coming from cytokines and growth factors. STAT transcription factor activation starts with the interaction of a ligand with its receptor. The next step is phosphorylation of the receptor and recruitment of STAT proteins to this site. Then, specific tyrosine kinases (growth factor receptors, Janus kinases (JAKs) or SRC family kinases) phosphorylate STATs on the tyrosine residue. Dimerization of two phosphorylated STAT proteins takes place and STAT dimers localize to the nucleus to bind to specific promoters and regulate gene transcription. STAT induced transcription activation is antagonized by SOCS (suppressors of cytokine signalling) and PTPs (protein tyrosine phosphatases) [160]. STAT proteins have major roles during normal cellular functioning such as cell growth and differentiation, development, apoptosis, immune responses and inflammation [161].

1.3.2 STAT1 and Interferon (IFN) Signaling

STAT1, the first member of STAT transcription factor family is activated by both types of Interferon (IFN). IFNs are important cytokines with critical functions such as antiviral signaling, prevention of cell proliferation, anti-tumoral activity and immunomodulation [162]. IFNs are classified into two groups; Type I and Type II IFNs. Type I IFNs are composed of a large group of molecules; IFN-alpha (which has 13 subtypes: IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17 and -α21), IFN-beta, IFN-omega, IFN-tau, IFN-kappa, IFN-lambda, and IFN-zeta. Type II interferon group however, only has one member which is IFN-gamma [163]. IFN signaling is the leading arm of STAT1 signaling pathway since STAT1 activation starts with binding of IFN to its receptor [162,164]. The fact that STAT1 knockout mice cannot respond to IFN [165] is critical for the establishment of the proof of concept that STAT1 is mediating antibacterial and viral functions of the cells through IFN signaling [166]. IFN mediated STAT1 signaling is schematized in Figure 1.8. [163] The first step of both type I and type II IFN signaling is the activation of JAKs through

autophosphorylation once dimerization of Interferon alpha receptor subunits 1 and 2 (IFNAR1 and IFNAR2) occurs. JAK1 and TYK2 then phosphorylate STAT1 and in low amounts STAT2, STAT3 and STAT5. STAT1 forms homodimers or heterodimers (with STAT2), translocates into the nucleus and starts transcription of target genes on promoters containing GAS (Gama activated sequence) or ISRF (Interferon stimulated response element). STAT1 and STAT2 dimers interact with another protein p48 or IRF9 to form a heterotrimeric transcription factor called ISGF3 (Interferon-stimulated gene factor 3) once they are in the nucleus [158].

1.3.3 STAT1 Signaling and HCC

Immune cell infiltration is a critical event in the continuance of hepatocyte injury during chronic liver disease induced by viral infections, alcohol or hepatoxins [167]. Activated T cells, both CD4+ and CD8+ are involved in liver injury during chronic hepatitis B or C [168,169] and their amount correlates with regenerating nodules, inflammation, and fibrosis in ALD [170]. STAT1 signaling is the key pathway for immune cell infiltration and induction of liver injury. Injured hepatocytes activate CD4+ and Natural killer T cells and stimulate secretion of interferon which in turn induces STAT1 phosphorylation through JAKs. pSTAT1 contributes to further liver injury by activating more CD4+ and NK T cells which will re-start the circle by secreting more interferon and inducing more damage [167]. Consistent with this phenomenon, STAT1 expression is found to be significantly higher in HCC tumors compared to its neighboring normal liver tissue. In parallel with the expression data, phosphorylated STAT1 level is higher in HCC samples with poor prognosis compared to the ones with good prognosis [171]. SOCS1 (suppressor of cytokine signaling) is the negative regulator of STAT1 signaling. Up-regulation of STAT1 activity and down regulation of STAT3 activity are the two reasons behind increased fibrosis and severe liver damage that are observed in SOCS1 knockout mice. This data provides another evidence for the contribution of STAT1 TF to hepatocancerogenesis [172].

1.3.4 IFN-α Therapy during HCC Treatment

IFN-α which is an antiviral cytokine, is used to treat Hepatitis infections which are main causes of HCC. Early diagnosed HCC cases are mostly treated by surgical resection, ablation or liver transplantation [173], however, nearly 20% of HCC cases reoccur in the first year after therapy and this value increases to 80–90% in a five year window [174]. Moreover, HCC recurrence is observed in a higher rate in the patients infected with Hepatitis B and C compared to those without a viral induced hepatitis. Thus, IFN-α is used as therapy in post operative HCC patients to decrease the risk of tumor recurrence[175]. IFN-α has also been shown to decrease proliferation and prevent angiogenesis hence IFN-α is suggested as an anticancer agent that will be able to decrease tumor recurrence rate in HCC patients [176]. There is controversial data about the efficacy of the interferon-α treatment for the patients with viral induced HCC. Some studies reported that IFN-α decreased tumor recurrence rate thus contributed to disease free survival [177,178]. On the other hand, others could not recapitulate the same kind of data after two randomized control trials (RCTs) and suggested that IFN-α does not have a significant effect on disease free survival and overall survival of the patients viral induced HCC after curative therapy [179,180]. In a recent report, Meta analysis of interferon therapy case studies is performed to determine the contributions of interferon-α on survival rate of HCC patients with underlying HBV or HCV induced hepatitis [181]. The results indicated that IFN-α therapy has improved survival of HCC patients with HCV induced hepatitis compared to the control group, however, there was no significant improvement in HCC patients with HBV induced hepatitis [181].

1.4 Aim and Strategy

Hepatocellular Carcinoma is one of the top reasons of cancer related deaths worldwide. HCC incidence has been increasing in USA and Europe, however, the highest incidence is observed in China, Middle Africa, and Japan [3]. HCC develops on a heterogeneous background with varying underlying risk factors in different geographical regions. Hepatitis B virus and Hepatitis C virus are considered as the main risk factors in Asia and Africa. In Europe and USA, alcohol abuse and obesity are the leading causes of HCC[2]. HCC patients are mostly diagnosed at later phases of cancer since HCC is mostly asymptomatic until the very end of tumorigenesis [74]. There are limited therapeutic options such as surgical resection, ablation and liver transplantation, but they are all suitable for early diagnosed cases and disease recurrence is very common after these therapies[75]. Due to late diagnosis and limited curative therapies, survival rate of HCC patients is very low [75]. Genetic mechanisms of hepatocarcinogenesis are very complicated yet critical to investigate in order to be able to find therapeutic targets against HCC. Unfortunately, most of the mutations discovered in HCC patients are "loss-of-function" mutations and they not appropriate therapeutic targets [57]. Sorafenib is the only FDA approved drug against HCC, however it only provides a three month of disease free survival compared to control group [89]. Telomere shortening problem is an obstacle for a cell during transformation process [103]. Tumor cells solve this problem by two ways; the first and most common way is re-gaining telomerase activity by up-regulating TERT expression [97] and the other is through ALT (alternative lengthening of telomeres), a mechanism similar to homologous recombination[118]. TERT up-regulation is very common and observed in 90% of HCC cases [111,112]; however molecular mechanisms behind TERT up regulation in HCC is still a mystery except for some cases with the involvement of Hepatitis B virus and loss of a region of chromosome 10p. HCC cells are reported to increase telomerase activity with allelic loss of chromosome 10p which codes for a telomerase repressor protein [113]. HBV involvement is more common than allelic loss; HBV DNA is integrated into TERT gene and increases its expression [113]. Yet, there is significant number of HCC cases which occur without an underlying HBV infection and mechanism of TERT activation is needed to be solved in those patients

for a better understanding of the telomerase activation in HCC. Recently, two frequent mutations in *TERT* promoter region have been reported in many tumors together with HCC [133,134,136–141,143,144,182,183]. And these mutations are suggested to have a functional role rather than being random genetic events. It is proposed that, the presence of *TERT* promoter mutations create a novel ETS transcription factor binding site and increase TERT expression through this mechanism [133,134]. Previously reported *TERT* promoter mutations in HCC were restricted to samples from USA[136] and France[146] with 44 % and 59% of frequencies. However, there was no data about the occurrence of TERT promoter mutations in different geographical regions such as China, Japan and Africa where HCC incidence is higher compared to USA and Europe. For this reason, we wanted to explore the frequency of TERT promoter mutations across the world, especially in Asia and Africa to have a deeper knowledge about this frequent genetic event.

The first objective of this study is to analyze our HCC cell line panel composed of 15 HCC cell lines together with 44 HCC tumors collected from different geographical regions to search for two common mutations in the promoter region of *TERT*. Next, we wanted to know if the presence of mutations in *TERT* promoter has a functional role on TERT expression or not. For this purpose, we have performed a bioinformatics analysis to search for possible transcription factors that can bind to mutant promoter sequence and we discovered that STAT1 is one of the candidate transcriptional factors. Considering the critical roles of STAT1 during liver carcinogenesis, we decided to continue analyzing this finding by designing experiments to see the effects of STAT1 activation or knockdown on the expression of TERT expression. Interferon alpha is the cytokine that triggers STAT1 signaling; therefore, we hypothesized that treatment of HCC cell lines with IFN-α would activate STAT1 signaling which will provide binding of activated STAT1 dimers onto mutant *TERT* promoter to increase expression of TERT enzyme. In conclusion, we analyzed *TERT* promoter mutations in HCC in a functional manner to unravel the molecular mechanism behind the possible upregulation of TERT expression by these mutations. This data will provide insides about the contributions of *TERT* promoter mutations into cellular immortality in hepatocellular carcinoma.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Laboratory Reagents

General laboratory reagents such as methanol, ethanol, and Bradford reagent were all analytical grade and were mainly purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Bradford reagent, methanol and ethanol were from Sigma-Aldrich (St. Louis, MO, USA). ECL Prime western blotting detection kit and Hybond nitrocellulose western blot membranes were from Amersham Pharmacia Biotech Company. DMSO and Ponceau S were purchased from Applied Biochemia (Darmstadt, Germany). Interferon alpha 2a human with catalog number SRP4594-100UG was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2 Tissue Culture Reagents and Materials

All cell culture media such as Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium and OptiMEM were purchased from GIBCO (Invitrogen, Carlsbad, CA, USA). Medium supplements like L-glutamine, Penicillin/streptomycin antibiotics, Non essential amino acids (NEAA), fetal calf serum (FCS) and Trypsin-EDTA were also purchased from the same company, GIBCO. Plastic materials used in cell culture such as Petri dishes, flasks, multiple well-plates, cryotubes were purchased from Corning Life Sciences Inc. (USA). Sterile serological pipettes were purchased from Costar Corporation (Cambridge, UK). Transfection reagents Lipofectamine 2000 and Lipofectamine RNAi Max were purchased from Invitrogen (Carlsbad, CA, USA).

2.1.3 Genomic DNA Isolation

Purelink Genomic DNA isolation kit (K1820-02) was purchased from Invitrogen (Carlsbad, CA, USA).

2.1.4 Polymerase Chain Reaction (PCR)

Recombinant Taq DNA polymerase enzymes (EP0401) were purchased from Thermo Scientific (MA, USA). AccuPrime GC-Rich DNA polymerase was purchased from Invitrogen (Carlsbad, CA, USA).

2.1.5 Primers

Primers for amplification of hTERT genomic DNA were the same as used by Horn et al, 2013[133].Apart from those, all primers were designed using Primer3 online tool (http://frodo.wi.mit.edu/primer3/input.htm) and shown in Table 2.1

Table2.1 Primers used in this study

Gene name	Forward Primer	Reverse Primer
TERT [133]	ACGAACGTGGCCAGCGGCAG	CTGGCGTCCCTGCACCCTGG
TERT	CGGAAGAGTGTCTGGAGCAA	GGATGAAGCGGAGTCTGGA
hIRF1	GAGGAGGTGAAAGACCAGAGCA	TAGCATCTCGGCTGGACTTCGA
STAT1	CACGCACACAAAAGTGATGA	AGAGGTCGTCTCGAGGTCAA
GAPDH	GGCTGAGAACGGGAAGCTTGTCAT	CAGCCTTCTCCATGGTGGTGAAGA

2.1.6 Agarose Gel Electrophoresis

Horizontal gel electrophoresis apparatus was Thermo EC Midicell Primo EC330 Electrophoretic Gel System and the power supply was EC250-90 both from Thermo Scientific (MA, USA). Other power supplies used were Power-PAC300 and Power-PAC200 which were from Bio Rad Laboratories (CA, USA).

2.1.7 Spectrophotometry

Spectrophotometer was from Beckman.

2.1.8 Determination of Gene Expression

2.1.8.1 Total RNA Isolation

Nucleospin RNA II total RNA isolation kit (740955.250) was from Macherey-Nagel (Duren, Germany).

2.1.8.2 First Strand cDNA Synthesis

RevertAid First Strand cDNAs Synthesis Kit (# 1622) was obtained from Fermentas-Thermo Scientific (MA, USA).

2.1.8.3 Quantitative Real Time PCR

Dynamo HS SYBR Green qPCR Kit (F-410L) was purchased from Thermo Scientific (MA, USA). The instrument used for gene expression studies was Stratagene Mx3000P qPCR System from Agilent Technologies (CA, USA).

2.1.9 Antibodies

Primary and secondary antibodies used in this study were purchased from different companies. All information including company name, catalog number and working conditions are shown in Table 2.2.

Table2.2 Antibodies used in this study

Antibody Name	**Catalog number**	**Western Blot Dilution**
Calnexin	Sigma-Aldrich, C4731	1:5000
α-tubulin	Calbiochem, CP06	1:5000
Anti-mouse-HRP	Sigma-Aldrich, A0168	1:5000

Anti-rabbit-HRP	Sigma-Aldrich, 6154	1:5000
STAT1	Cell Signaling, #9172	1:1000
pSTAT1(Tyr701)	Cell Signaling, #9171	1:1000

2.2 Solutions and Media

2.2.1 General Solutions

10X Phosphate Buffered Saline (PBS)	80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4 were mixed in 1 liter of ddH_2O Working dilution is 1X.
10X Tris buffered saline (TBS)	12.9g Tris base and 87.76g NaCl were mixed in 1 liter ddH_2O, pH is adjusted to 8.0. Solution is diluted to 1X before use.
1M Tris HCl	12.1 g of Tris in 100mL of ddH_2O. pH is adjusted to 6.8
1,5M Tris HCl	18.1 g Tris base in 100 mL of ddH_2O. pH is adjusted to 8.8.
50X Tris-Acetate-EDTA Buffer	242 g Tris base, 100 mL of 0.5 M EDTA (pH: 8.0), 28.55 mL of Glacial Acetic Acid were mixed and volume is brought to 1L with ddH_2O. Working dilution is 1X.

2.2.2 Bacterial solutions

Luria-Bertani medium (LB)	10 g bacto-tryptone, 5 g bacto yeast extract and 10 g NaCl were mixed in 1L of ddH_2O
Ampicillin	100 mg/mL solution is prepared in ddH_2O, sterilized by filtration and stored at -20°C. 100μg/mL is working dilution.

2.2.3 Tissue culture solutions

DMEM and RPMI media	10% FCS, 1% penicillin/streptomycin, 1% NEAA were added to prepare complete medium and it is stored at 4 ˚C.
10X Phosphate buffered saline (PBS)	80g NaCL, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4 in 1 litre ddH_2O, working dilution is 1X, stored at 4 ℃

2.2.4 Sodium Deodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) and immunoblotting Solutions

10X SDS Running buffer	144g Glycine, 30g Tris base and 50mL of 10% SDS were dissolved in 1L ddH_2O, working dilution is 1X.
Blocking solution	5% (w/v) non-fat dry milk was dissolved in 0.2% TBS-Tween 20, or 5% (w/v) bovine serum albumin (BSA) was dissolved in 0.2% TBS-Tween 20
10X Tris buffered saline (TBS)	12.9g Trisma base and 87.76g NaCl dissolved in 1L of ddH_2O and pH is adjusted to 8.0, working dilution is 1X.
TBS-Tween 20	(0.2%) Tween 20 in 1X TBS
Ponceau S	(0.1%) (w/v) Ponceau S and 5% (v/v) acetic acid in ddH_2O.
10X Transfer buffer	72g Glycine and 58g Trisma base and 2mL of 10% SDS in 1L ddH_2O, working solution is 1X plus 10-20% Methanol.

2.3 Methods

2.3.1 Tissue Culture Methods

2.3.1.1 Cell Lines and Growth Conditions of Cells

Several Hepatocellular carcinoma cell lines have been used in this study. Among all cell lines; Huh7, HepG2, Hep3B, Hep40, PLC/PRF/5, FOCUS, Mahlavu, FLC4 and SK-HEP-1 cells were grown in DMEM; while SNU182, SNU387, SNU398, SNU423, SNU449, and SNU475 cell lines were cultured in RPMI medium. Both DMEM and RPMI media were supplemented with 10% fetal calf serum, 2 mM of L-Glutamine, 1x NEAA and 100 units of penicillin/streptomycin. All cells were cultured at 37^0C in an incubator with 5% CO_2. All solutions and media used during cell culture studies were heated to 37^0C in a water bath before use.

2.3.1.2 Passaging Cells

HCC cell lines used in this study were all adherent cell lines and all cells were subcultured before they reached confluency. To begin, old cell culture medium was discarded by aspiration by the aid of sterilized glass pipettes, then the cells were washed with sterile 1X PBS and PBS was also discarded by aspiration. Enough amount of Trypsin-EDTA (enzymatic cell dissociation solution) to cover the plate surface was added onto the cells and trypsinized cells were kept at 37^0C for optimum trypsin. Cells were checked under microscope to control their detachment from the surface. It is important not to keep cells in trypsin solution for a long time. After all cells were dissociated from the surface, they were diluted in enough volume of fresh medium and seeded on new plates in desired dilutions.

2.3.1.3 Cryopreservation and Thawing of Cells

For cryopreservation of the cells, they were grown up to 60-70 % of confluency. Then, the cells were detached from culture plate with trypsinization, centrifuged at 1500 rpm for 3-5 min and supernatant was removed by aspiration and cell pellet was resuspended in 1ml of freezing medium containing 10% DMSO, 20% FCS was

prepared with complete medium (either DMEM or RPMI depending on the cell line). Cell suspension was placed in cryotubes and let freeze in a sequential manner. Cryovials containing cells were kept at -20 ^{0}C for 1-2 hours, then put into -80 ^{0}C for one day and lastly placed in liquid nitrogen tank for long term storage.

Cells that were conserved in liquid nitrogen should be thawed properly in order to prevent cell death. For that purpose, thawing process should be performed as fast as possible. First, one cryotube was taken from liquid nitrogen container and immediately placed on ice. Then the vial was kept in 37^{0}C water bath to let the cell suspension thaw quickly. After that, cells were carefully taken into a 15 mL Falcon tube containing 5 ml of fresh medium. The cells were mixed with this medium by pipetting. Lastly, all of the cell suspension was seeded into a cell culture plate and placed in CO_2 incubator.

2.3.1.4 Transfection of siRNA with Lipofectamine RNAi MAX

For transfection of siRNA into HCC cells, reverse transfection protocol has been used according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). All transfections were performed in a biosafety cabinet in multi well plates and cell numbers were adjusted accordingly. First of all, siRNA-lipofectamine RNAi max complexes were prepared in OptiMEM I reduced serum medium by gently mixing 50nM of siRNA of gene of interest or 50nM of control siRNA together with 500 µL of OptiMEM and 5 µL of lipofectamine RNAi max in the wells of a 6 well culture plate. This mixture was kept at room temperature (RT) for 10-20 minutes under the hood. In the mean time, cells were diluted in complete growth medium without antibiotics at a concentration 150.000-200.000 cells per 2 mL of medium if transfection was to be performed in a 6-well plate. Once incubation was completed, cells were added into the wells containing the siRNA-lipofectamine-OptiMEM mixture. The 6-well plate was then mixed back and forth gently and placed in CO_2 incubator for 48 of 72 hours depending on the following experiment.

2.3.1.5 Transfection of Plasmid DNA with Lipofectamine 2000

Transfection of plasmid DNA into HCC cells was also performed with the reverse transfection protocol in 6-well plates according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) with slight modifications. For each well of transfection, 0, 5 μg of plasmid DNA was mixed with 250 μL of OptiMEM and kept at RT for 15 min. In a different tube, 5 μL of lipofectamine 2000 was mixed with 250 μL of OptiMEM and again kept at RT for 15 min. In the mean time cells were diluted in complete growth medium without antibiotics so that 2 mL of medium contained 200.000-300.000 cells. Once incubation was over, plasmid-OptiMEM and lipofectamine-OptiMEM mixtures were mixed in wells and cells were added. The plate was rocked back and forth gently to mix the cells and the transfection complexes. Cells were placed in CO_2 incubator. Medium was changed 6 hours after the transfection to prevent lipofectamine 2000 reagent's toxicity. Then cells were incubated for 48 or 72 hours depending on the experiment.

2.3.1.6 Treatment of Cells with Interferon alpha

Interferon alpha 2a human (SRP4596-100UG) was reconstituted in 1000μL sterile ddH_2O at a stock concentration of 100ng/μL, aliquoted in very small amounts and kept at -20 ^{0}C for shorter or -80 ^{0}C for longer storage. Different concentrations of IFN-α was used depending on the cell line and experiment. Regardless of the experiment, cells were always sub-cultured in a new plate one day prior to the treatment. At the day of treatment, IFN-α was prepared in desired concentration in complete medium and added on the cells once the old culture medium was removed by aspiration. Cells were kept in IFN-α containing medium for short time periods such as between 10 to 60 minutes to detect phosphorylation of STAT1. In other experiments, incubation time changed between 24 to 72 h.

2.3.2 Isolation of Genomic DNA from Cultured Cells

Isolation of genomic DNA from HCC cell lines was performed with Purelink Genomic DNA isolation kit by following manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Cells were grown in 100 mm plates up to 70% of confluency

before the experiment; they were dissociated from plates by trypsinization, centrifuged at 1500 rpm for 5 min and resuspended in 200µL of 1X PBS. 20µL of Proteinase K was added into each tube containing cell suspension, then 20 µL of RNase A was added into the tubes and they were mixed well by vortex and kept at RT for 2 min. Then 200 µL of PureLink™ Genomic Lysis/Binding Buffer was added and mixed well by vortexing. To digest the proteins, samples were incubated at 55^0C in a water bath or a heat block. After that, 200 µl of 99% ethanol was added to the lysate, again mixed well by vortexing to have a homogenous solution. The lysate (~640 µl) was added to the spin column and column was centrifuged at 10,000 × g for 1 min RT. Collection tube was discarded and the spin column was placed into a clean collection tube. 500 µl Wash Buffer 1 was added to the column and it was centrifuged at 10,000 × g for 1 min RT. Again, the collection tube was discarded and the spin column was placed into a clean collection tube. 500 µl Wash Buffer 2 was added to the column and centrifuge at maximum speed for 3 minutes RT. Washing was completed after collection tube was discarded and the spin column was placed in a sterile 1.5-ml microcentrifuge tube. DNA was eluted by adding 50 µl of elution buffer to the column. Samples were incubated at RT for 1 min and centrifuged at maximum speed for 1 min. The purified genomic DNA was kept at -20°C.

2.3.3 Amplification of Genomic DNA

After genomic DNA was isolated with Purelink Genomic DNA isolation kit, DNA concentrations were measured with Nanodrop Spectrometer (Thermo Scientific). Once concentrations were determined, each DNA sample was diluted to have a concentration of 50ng/µL. AccuPrime GC-rich DNA polymerase kit was used to amplify genomic DNA. 50 µL of PCR reaction was prepared by mixing the following components: 10µM forward primer (final concentration of 10 pmoles) 10µM of reverse primer (final concentration of 10 pmoles), 10 µL of 5X Buffer A, 1 µl of AccuPrime GC-Rich DNA Polymerase (2 U/µl) and 100ng (2 µl) of DNA template, at the end complete the volume with sterile water to 50 µl. Then the tubes were flicked with finger to mix the components, spin down at maximum speed and placed in a thermal cycler programmed as follows: Initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation (95°C for 30 sec), annealing (68°C for

1min) and extension (72°C 1 min) steps finalized with final extention at 72°C for 10 min. After the program was over, 5 µL of each sample was loaded on agarose gel and the remaining 45µL was sent for Sanger sequencing by Genoks Biotechnology Company (Ankara Turkey).

2.3.4 Agarose Gel Electrophoresis

PCR products or plasmid DNA were analyzed on agarose gel by using horizontal gel electrophoresis. Agarose gel was prepared by melting agarose with different concentrations such as 1 and 2 % (w/v) in 1X TAE buffer. Percentage of agarose was determined based on amplicon size; smaller DNA fragments were run in high agarose concentration whereas larger DNA fragments were run in lower agarose concentrations. Once agarose was melted in microwave, solution was left for cooling for one minute, EtBr was then added a final concentration of 10µg/mL and agarose was poured into trays containing proper combs and kept under a fume hood until it was cooled and solidified completely. DNA samples were prepared by mixing with 6X DNA loading dye (30%glycerol in dH2O en bromophenol blue) and loaded into the gel. To determine the size of the DNA fragment properly, DNA weight marker was also loaded to be used as a reference. The gels were run at 100 V until the bands were separated properly. At the end of the run, the gels were visualized under UV light and their pictures were taken by the aid of Chemi-Capt software for image acquisition (Vilber Lourmat, Paris, France).

2.3.5 Total RNA Extraction from Cultured Cells

Total RNA extraction from cultured cells was performed according to manufacturer's protocol by using Nucleospin RNA II total RNA isolation kit. Cells were lyzed with 350µL of buffer RA1 and 3.5 µL Betamercaptoethanol in the culture plates and collected into a microcentrifuge tube and vortexed vigorously. To reduce viscosity, lysate was loaded into nucleospin filter placed in a collection tube and centrifuged for 1 min at 11.000g RT. After clearing the lysate, nucleospin filter was discarded and 350 µL of 70% EtOH was added and mixture was pipetted 4-5 times. To bind RNA, NucleoSpin RNA Column was placed into a clean collection tube and

lysate was added on top of the column, centrifuged for 30 seconds at 11.000g RT. Then collection tube was replaced with a clean one, 350µL of membrane desalting buffer was added for and centrifugation was performed for 1min at 11.000g RT. Next, DNA was removed by DNase digestion. For each sample, 10µL reconstituted rDNase was added to 90µL of reaction buffer for rDNase, mixed by flicking and applied onto the center of the silica membrane of the column. The column was then incubated at RT for 15 min. The next was washing protocol starting by adding 200 µL buffer RAW2 to the NucleoSpin RNA Column and centrifugation for 30 s at 11.000 g. Collection tube was discarded and column was placed into a new collection tube (2 mL). Buffer RAW2 was used to inactivate the rDNase. Second wash was performed by adding 600µL of buffer RA3 and centrifugation for 30 s at 11.000 g. Collection tube again was replaced with a new one and third washing was done by adding 250µL of RA3 and centrifugation for 2 min at 11.000 g to dry silica membrane completely. Then column was placed in nuclease free sterile collection tube and elution of RNA was performed by adding 30-60µL of RNase free water and centrifugation for 1 min at 11.000g. Eluted RNA concentration was measured with Nanodrop 2000 and RNA samples were used immediately or stored at -80°C.

2.3.6 First Strand cDNA Synthesis

Fermentas RevertAid cDNA synthesis Kit was used in cDNA synthesis experiment by following manufacturer's instructions. 1µg of total RNA was used as a starting material and reaction was started by adding 1 µL of Oligo(dT)18 primers and completing the volume to 12 µL. This mixture was incubated at 65 °C for 5 min, and then briefly centrifuged to spin down the solution, chilled on ice before the next reaction. Afterwards 4µL of 5X reaction buffer, 1µL of RiboLock RNase inhibitor (20 U/µL), 2µL of 10 mM dNTP Mix dNTPs and 1µL of RevertAid M-MuLV RT reverse transcriptase enzyme (200 U/µL) was added to this mixture in the stated order; mixture was then centrifuged briefly and cDNA synthesis reaction was performed for 60 min at 42 °C. At the end, reaction was terminated by incubation at 72 °C for 5 min. cDNAs were diluted 1/10 before use in qPCR experiments and stored at -80 °C.

2.3.7 Quantitative Real Time PCR (qRT-PCR)

Quantitative Real Time PCR (qRT-PCR) experiments were performed in Stratagene Mx 3005P qPCR System by using Dynamo HS SYBR Green qPCR Kit (F-410L) according to the manufacturer's protocol. Reaction mix was prepared by using 1 µl cDNA, 10µL of 2 X Master Mix and 0.5 µM of forward and reverse primers by completing total volume to 20µL with sterile water. Mixtures were added to 96-well qPCR plate in triplicates; plate was then spinned down and placed in the qPCR instrument. qPCR reaction composed of initial denaturation of samples at 95 °C for 10 minutes, followed by 40 cycles of denaturation (95 °C 30 s), annealing (55-60 °C 30 s) and elongation (72 °C 30 s) reactions. Gene expression analysis was performed by using Ct values of the gene of interest in comparison with Ct value of the reference gene (GAPDH) with the aid of the 2–[Delta][Delta]Ct method, where [Delta][Delta]Ct = [Delta]Ct, sample - [Delta]Ct, reference.

2.3.9 Total Protein Extraction from Cultured Cells

Cells were dissociated with trypsinization, centrifuged at 1500rpm for 5 min, washed with cold 1X PBS and put on ice directly. For cell lysis, Radio Immuno Precipitation **A**ssay buffer **(**RIPA) Buffer containing, 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate),50 mM Tris, pH 8.0. To prevent degradation of proteins, 1x Protease inhibitor cocktail was added extemporaneously to to RIPA buffer, for phospho proteins, phosphatase inhibitors were also added into the lysis buffer. Two volumes RIPA compared to cell pellet's volume was added on cells and resuspended carefully and vortexed. Cell lysis was performed on ice for 30 min, and samples were vortexed every 5 min. After 30 min, cell lysate was taken into 1.5 ml tubes and centrifuged for 45-60 min at 13.000 rpm at +4°C. After centrifugation, the supernatant containing proteins was transferred to pre-chilled 1.5ml tubes while the pellet containing cell debris was discarded. Sonication was performed to shear DNA and samples were stored at -20°C for short periods or -80°C for longer periods.

2.3.10 Western Blotting

Bradford assay was performed for quantification of total protein concentration by using BSA standard. Once protein concentration was determined, samples were prepared for loading accordingly. Typically 30-40 µg of total protein was loaded for a regular western blot with loading volume of 20 µL. Protein samples were mixed with 5X loading dye, volume was completed to 20 µL and proteins were denatured by heating the samples at 100°C for at least 10 min. After heating, samples were chilled on ice, spun down and loaded on the gel. Gel percentage was determined according to the size of protein of interest; for small proteins like 15-20kDa, 15 or 18% gels, for 50-90kDa proteins 10-12% gels and for big proteins like 150-200kDa 8% gels were used. Stacking and resolving gels were prepared according to the tables below. Bio-Rad Mini PROTEAN Tetra Cell system was used to prepare and run the gels as instructed by the manufacturer.

Table 2.3 Preparations of Stacking and Resolving Tris Glycine Gels

Ingredients	**7% Stacking Gel**	**10% Stacking Gel**	**12% Stacking Gel**	**15% Stacking Gel**
ddH_2O	5.1 ml	4.1 ml	3.4 ml	2.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
20% (w/v) SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2.3 ml	3.3 ml	4.0 ml	5.0 ml
10% (w/v) ammonium persulfate	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml
Total Volume	10.005 ml	10.005 ml	10.005 ml	10.005 ml

Ingredients	**5% Stacking Gel**
distilled H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide(30%/0.8% w/v)	0.67 ml
10% (w/v) APS	0.025 ml
TEMED	0.005 ml
Total Volume	5.05 ml

After the gels were ready, they were placed into mini tank and tank was filled with 1X Running buffer. Samples were loaded and gel was run at 80 V until the samples reached to resolving gel, afterwards voltage was increased up to 150V for the rest of the run. Once the running was completed, the gels were either directly stained with Coomassie Blue by incubating in Coomassie dye for 1 hour, and then de-staining the excess dye with acetic acid-methanol-water solution to visualize total proteins that were loaded in the gel or proteins were blotted with specific antibodies by following immune blotting protocol. Proteins were transferred on nitrocellulose membrane with wet transfer system by using Bio-Rad Mini Transblot Cell apparatus. 10X Transfer buffer was diluted to 1X containing 10-20% of MetOH by using cold ddH_2O. One piece of nitrocellulose membrane and four Whatman papers were cut with dimensions of 6.5 to -8.5 cm for each gel to be transferred. Two sponges were soaked in transfer buffer for 10 min while Whatman papers and membranes were soaked for 2-5 min. Then a transfer sandwich was prepared as depicted below:

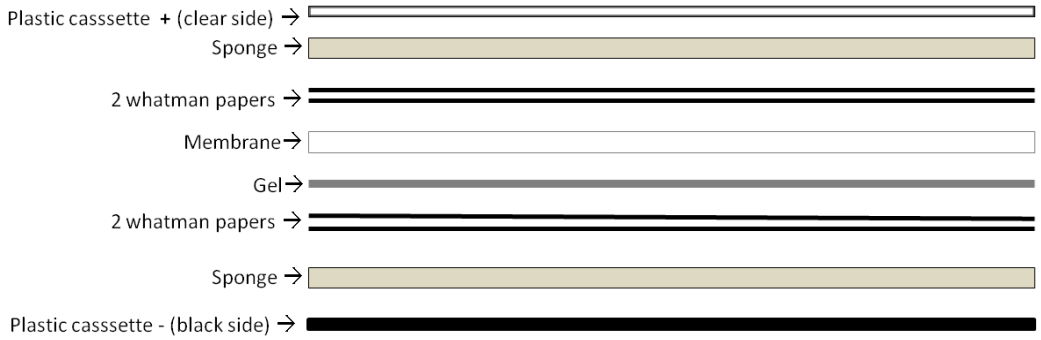


Figure 2.1 Placement of gel and membrane for blotting

The sandwich was carefully placed inside the transfer system; tank was filled with transfer buffer, covered with ice to prevent heating up. Then, transfer was performed for 75 min at 100 V (for big proteins more than 100kDa transfer time was increased). Ponceau Staining was performed after the transfer to check for bubbles and ensure that transfer was completed without problems. Membrane was washed with ddH_2O for 30 s and incubated with Ponceau Red for 1min on shaker; excess dye was removed by wash with ddH_2O to get the pink-red color. If there was no problem with the transfer, immunoblotting procedure was continued with blocking step. Depending on the antibody, blocking was performed with 5% (w/v) non fat dry milk or BSA is prepared in 1XTBS-0.2% Tween (TBS-T) for 1 hour at RT or overnight at +4 °C by slowly shaking. For phosphorylated proteins, blocking was always performed with BSA solution. After blocking was over, membrane was incubated with primary antibody for 1 h RT or overnight at +4°C slowly shaking. Antibody was prepared in blocking solution and its concentration was determined based on manufacturer's instructions and optimized according to the experiments. Unbound primary antibody was removed by washing the membrane with 1X -TBS-T for 30 min, changing washing solution every 10 min and shaking fast. Then membrane was incubated with 1:5000 diluted secondary antibody prepared in blocking solution for 1h at RT slowly shaking. Again excess antibody was removed washing the membrane with 1X -TBS-T for 30 min shaking fast. At the end, signal detection was performed with ECL Prime chemiluminescence detection kit. X-ray films were exposed to the membrane emitting chemiluminescent signal and developed in an X-ray developer with exposure times of 15 sec, 30 sec, 1 min and 5 min depending on the antibody.

2.3.11 Statistical Analysis and Bioinformatics Tools

Statistical significance between control and experimental groups was calculated using Student's T- test. $p<0.05$ was considered as significant (Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$).

2.3.11.1 DNA Dynamo Sequence Analysis Software

DNA Dynamo software from Bluetractor Software was used for sequencing analysis by using the manufacturer's guides. First of all, the file containing wild type *TERT* sequence downloaded from UCSC Genome Browser was opened in the main sequence window, then the .abi files representing our experimental sequencing results were opened from the 'Sequencing' menu, by selecting 'Open And Align Sequence Data Files to this Windows Sequence'. The sequences in the .abi files were aligned to the sequence of the 'reference *TERT* sequence' and appeared in a sequencing editor window. Compatible regions and discrepancies were highlighted by the software. Mutations could be detected by checking all discrepancies carefully because it was possible to have an incompatibility of reference and experimental sequence just because of low quality of the peaks.

2.3.11.2 STAMP DNA Motif Comparison Tool

The STAMP DNA motif comparison web tool [184] was used to search for candidate transcription factors that could bind to mutant TERT promoter sequence. DNA motif database of TRANSFAC (Transcription factor database) was searched via STAMP tool. Two analyses were performed by using the common mutation motif (CCCCTTCCGGG) generated at TERT promoter in the presence of C228T or C250T mutations. The first analysis aims to detect transcription factor families while the second analysis aims to determine individual transcription factors that might bind to the common mutant DNA sequence motif of the *TERT* gene promoter.

Chapter 3

Results

3.1 *TERT* Promoter Mutations in HCC

3.1.1 *TERT* Promoter Mutations in HCC Cell lines

We have tested 15 Hepatocellular Carcinoma cell lines, composed of six epithelial-like (Huh7, HepG2, Hep3B, Hep40, PLC/PRF/5, and FLC4) and nine mesenchymal-like (FOCUS, Mahlavu, SNU182, SNU387, SNU398, SNU423, SNU449, and SNU475, SKHEP1) cell types [185] to determine the mutation status of *TERT* gene promoter. For this purpose, we have PCR amplified a 474 base pair region of the *TERT* gene promoter flanking the 1,295,228 and 1,295,250 genomic sequences of chromosome 5 since recent studies reported a high mutation frequency at these regions that are found at the upstream of the transcription start site of *TERT* gene. These two mutations are also depicted as C228T for the one at the -124bp (G>A; C>T on opposite strand) and C250T for the one located at –146 bp (G>A; C>T on opposite strand) respectively[133,134]. Once PCR was completed, amplicons were sent for Sanger sequencing and the sequence data were analyzed using DNADynamo software taking wild type *TERT* gene sequence as reference. In Figure 3.1, alignment of the sequencing results of HCC cell lines against wild type *TERT* sequence is shown. Among fifteen HCC cell lines, only the Mahlavu cell line carried the C250T mutation while nine cell lines namely Huh7, HepG2, Hep3B, FLC4, FOCUS, SNU 387, SNU398, SNU 423 and SNU 475 carried the C228T mutation. All mutations were heterozygous and the two mutations were found in a mutually exclusive manner in all mutated cell lines.

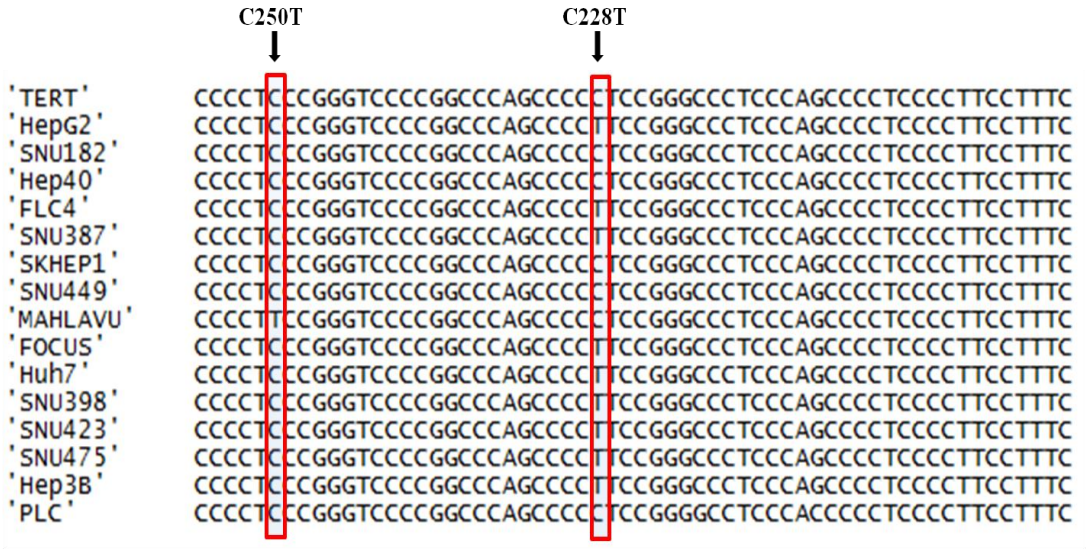
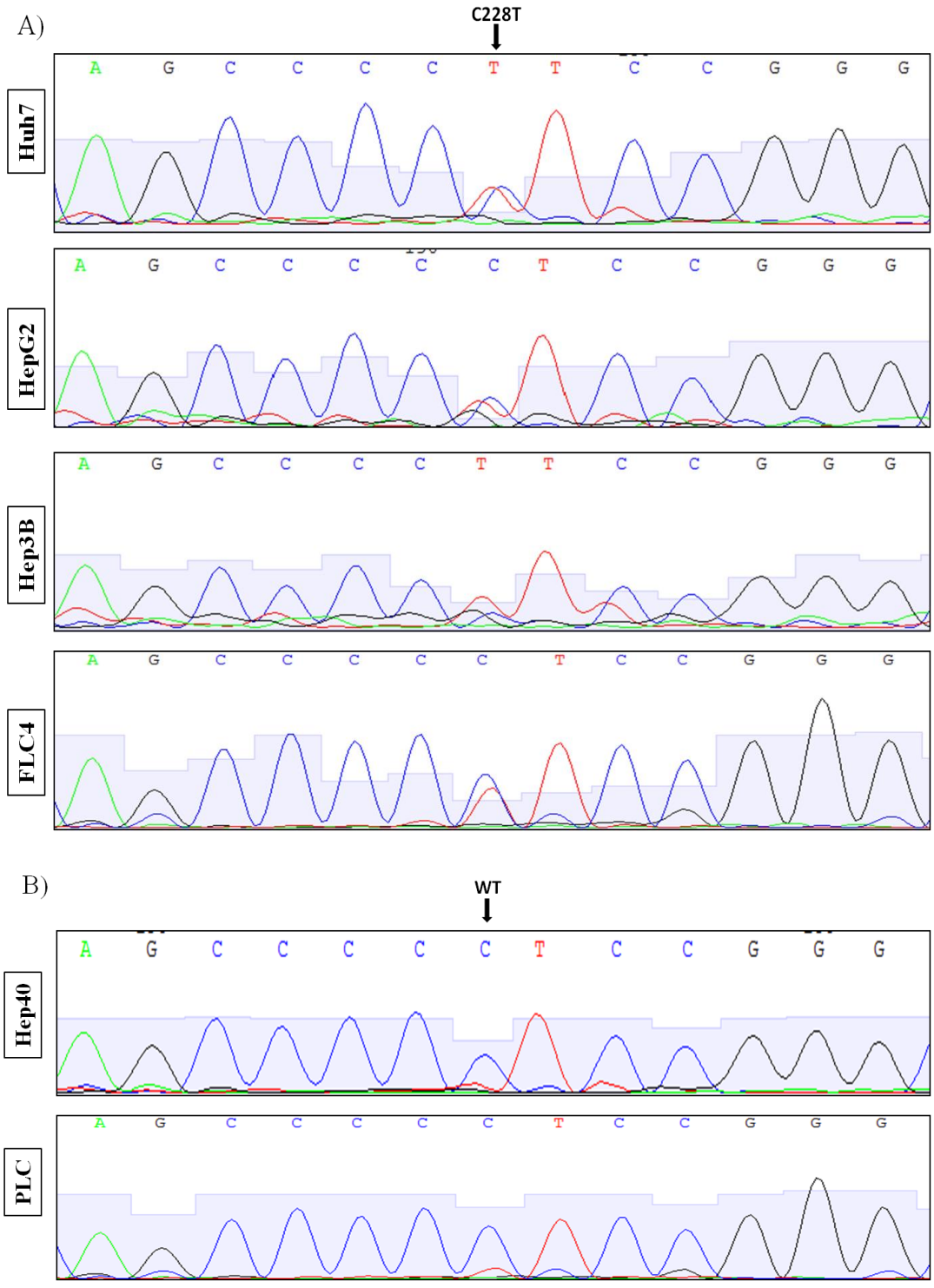


Figure 3.1 TERT sequencing alignment in HCC cell lines.

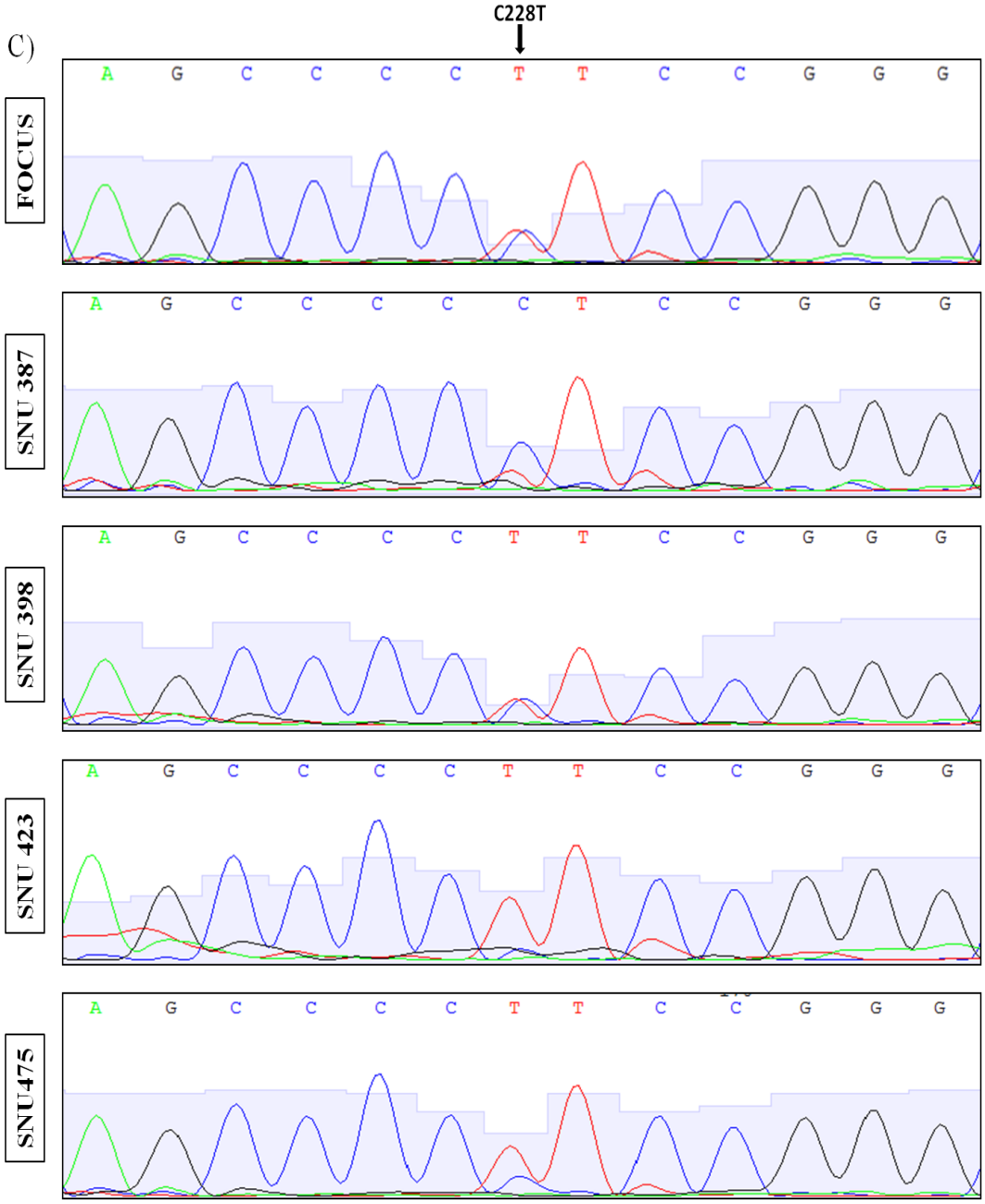
Sequencing results were analyzed with DNADynamo software and wild type *TERT* gene sequence is used as a reference.

Sequence chromatograms of all HCC cell lines are given in Figure 3.2. In Figure 3.2A, sequence chromatograms of epithelial-like cell lines bearing C2228T mutations are depicted; Huh7, HepG2, Hep3B, FLC4. In Figure 3.2B, chromatograms of epithelial-like cell lines which do not carry any mutation at the *TERT* promoter region, namely PLC and Hep40 are represented. In Figure 3.2C, mesenchymal-like cell lines carrying C228T *TERT* promoter mutations; FOCUS, SNU 387, SNU398, SNU 423 and SNU 475 are demonstrated. Figure 3.2D represents wild type mesenchymal-like cell lines in terms of TERT promoter mutations are shown; SNU 182, SNU 449 and SKHEP1. Sequence chromatogram of Mahlavu cell line which is the only cell line carrying a C250T mutation is given at Figure 3.2E. 10 over 15 HCC cell lines carry one of the *TERT* promoter mutations and the remaining 5 are wild type cell lines. Both epithelial-like and mesenchymal-like cells have a very similar frequency of mutation (4 out of 6, and 6 out of 9 respectively). This finding suggests that *TERT* promoter mutations occurred irrespective of the differentiation status of these cell lines. Considering the high frequency of these mutations among HCC cell lines, it is expected that these mutations have functional properties. Both mutations are mutually exclusive

meaning that having only one mutation is enough to accomplish the functions attributed to them in HCC cell lines. C228T mutation is observed in a much higher frequency compared to C250T mutation; however, the reason behind this bias is not clear yet.



C)



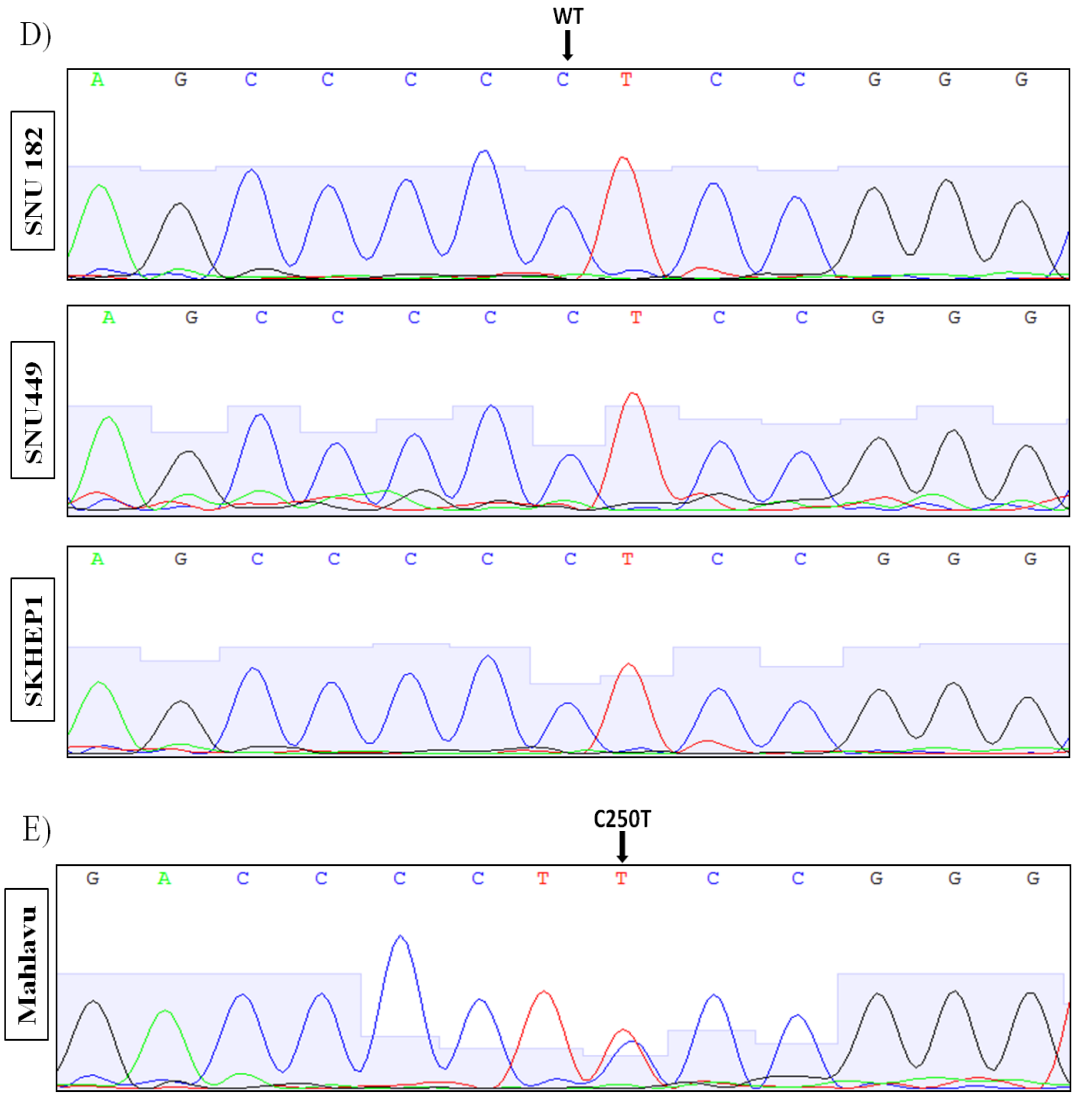


Figure 3.2 Sequence chromatograms of HCC cell lines comprising TERT promoter mutations C228T and C250T.

Sequencing results were analyzed with DNADynamo software by using wilt type TERT sequence as a reference. Arrows mark the mutations locations at the chromatograms. A: Epitheliel-Like Cells carrying C228T mutation; B) Epitheliel-Like Cells without promoter mutations; C) Mesenchymal- Like Cells carrying C228T mutation; D) Mesenchymal- Like Cells without promoter mutations; E) Mahlavu cell line with C250T promoter mutation.

3.1.2 *TERT* Promoter Mutations in HCC Tumors

Once we have detected a very high mutation frequency in HCC cell lines (67%), we decided to proceed with HCC tumor samples and searched for *TERT* promoter mutations in our archival HCC tumor collection. We had 44 archival HCC tumor DNA samples collected from different regions of the world; 11 were from Japan, 8 from China, 7 from Germany, 2 from France, 1 from Israel, 6 from Mozambique, 4 from Transkei, 2 from Lesotho, 1 from Swaziland and 2 samples from South Africa. The etiologies of 52.3% these tumors were Hepatitis B Virus infection that is evidenced by viral DNA testing [186,187]. The etiologies of the rest of the tumor samples are unknown.

Alignment results of HCC tumor samples compared to wild type *TERT* sequence is shown in Figure 3.3. Out of 44 tumors, 10 carried C228T mutation while 5 carried C250T mutation. Similar to the findings with HCC cell lines, tumor samples also had a more frequent C228T mutation rate (23%) compared to the frequency of C250T mutation (11%). These mutations are proposed to be somatic mutations since all previously reported TERT promoter mutations were also somatic. These two mutations were again detected in a mutually exclusive manner as in HCC cell lines. Overall *TERT* promoter mutation frequency in HCC tumor samples is 34%. If we compare mutation frequency of HCC cell lines (67%) and tumor samples (34%), it is clear that HCC cell lines have 2 times as much mutation as HCC tumor samples. We can explain this by hypothesizing that bearing *TERT* promoter mutations may have provided an advantage to the cells during establishment of cell lines. Thus, mutation frequency is much higher in established cell lines compared to primary tumors.

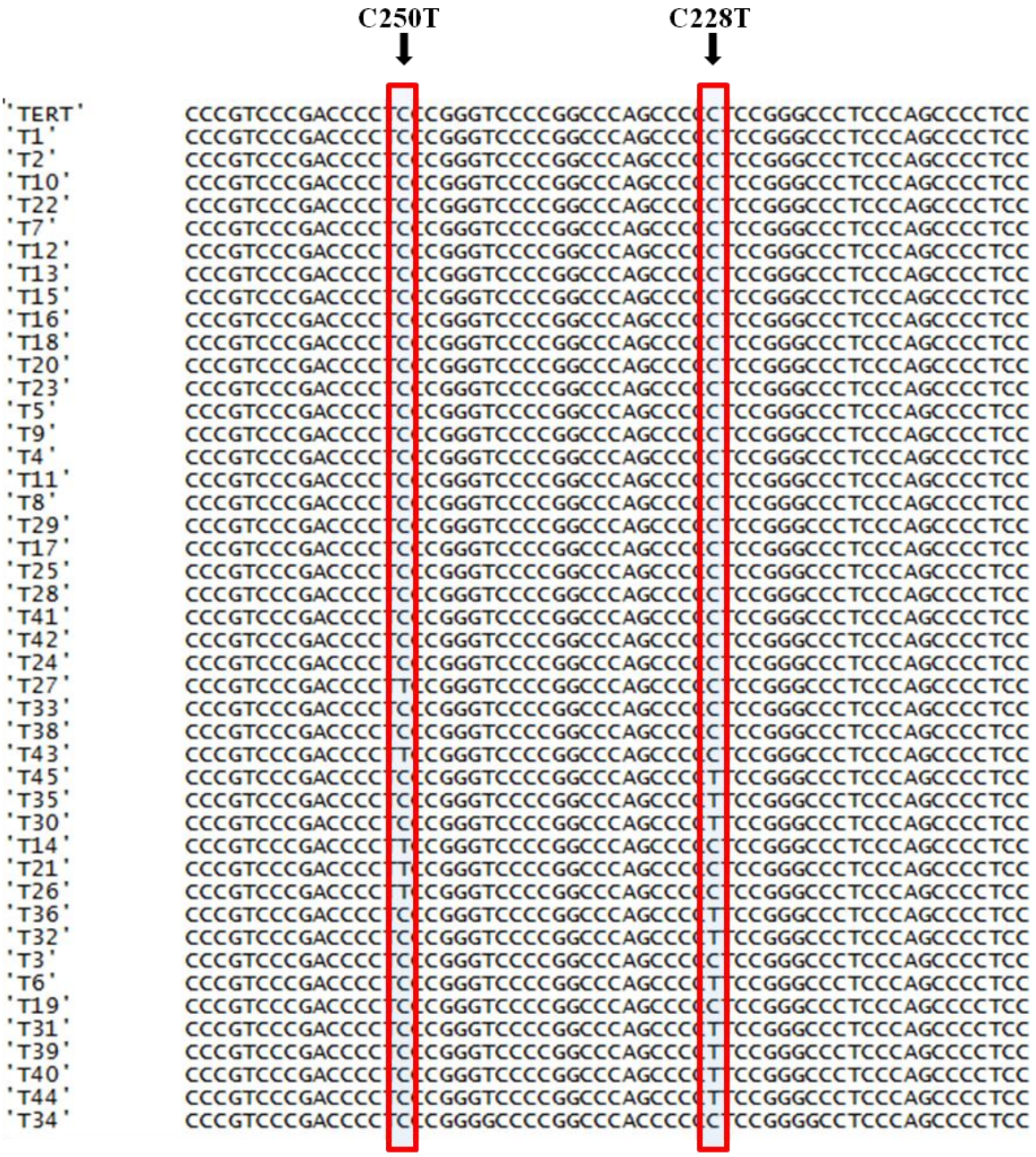


Figure 3.3 TERT sequencing alignment in HCC tumors.

Sequencing results were analyzed with DNADynamo software and wild type *TERT* gene sequence is used as a reference. Both C228T and C250T mutations are marked by arrows and red rectangles. C228T mutation is observed in 10 tumors out of 44, while C250T mutation is observed 5 tumors out of 44. Mutations frequencies of C228T and C250T in HCC tumor samples are 23% and 11% respectively.

Representative sequence chromatograms of the tumor samples carrying C228T or C250T mutations are given in Figure 3.4.

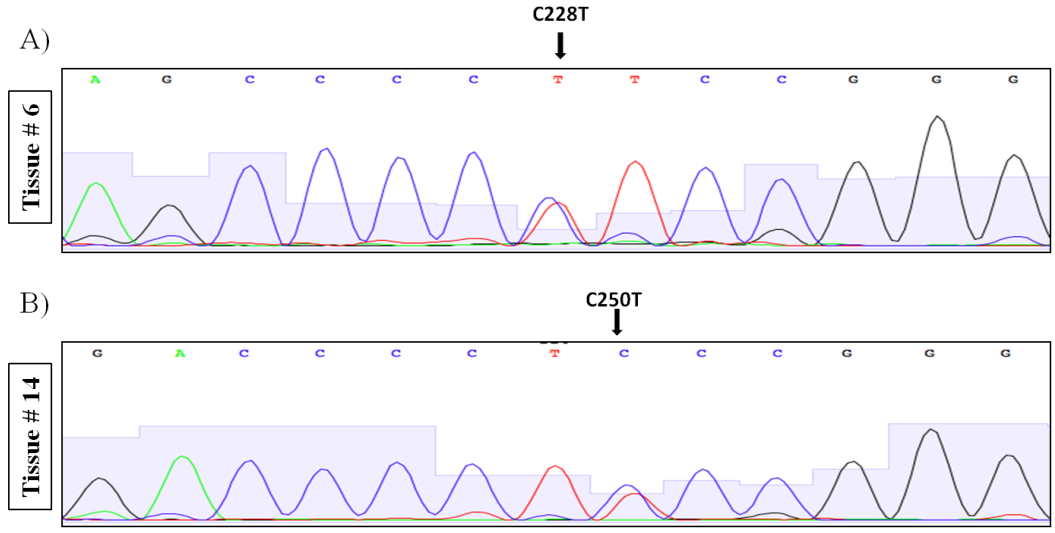


Figure 3.4 Representative sequence chromatograms of HCC tumor samples carrying TERT promoter mutations C228T and C250T

Sequencing results were analyzed with DNADynamo software by using wilt type TERT sequence as a reference. Arrows mark the mutations' locations at the chromatograms. A: Tumor # 6, C228T mutation; B) Tumor # 14, C250T mutation.

3.1.3 Geographic Distribution of *TERT* Promoter Mutations in HCC patients

We have analyzed TERT promoter mutation frequency of our archival HCC tumor DNA samples collected from different regions of the world (11 were from Japan, 8 from China, 7 from Germany, 2 from France, 1 from Israel, 6 from Mozambique, 4 from Transkei, 2 from Lesotho, 1 from Swaziland and 2 samples from South Africa) and found that *TERT* promoter mutation rate was 34% in total. Next, we wanted to group HCC tumor samples based on the continents where the patient samples had been collected. It is critical to estimate relative *TERT* promoter mutation rates in different geographical regions since HCC is a very heterogeneous disease with different underlying conditions depending on its origin. For example, HCC is mostly caused by viral infections and aflatoxin exposure in Asia and Africa whereas in Europe alcohol abuse and obesity are the main risk factors. Figure 3.5A displays geographic distribution of *TERT* promoter mutations grouped as origins of the

tumors such as Asia, Africa and Europe. If we consider the C228T and C250T mutation rates in three continents; the lowest overall mutation rate is observed in Asia with 4 over 19 samples (21%), it is followed by Europe with a mutation rate of 30% (3/10) and the highest mutation rate is observed in Africa with 53% (8/15) (Figure 3.5B). In all continents, C250T mutation rate is lower than C228T mutation rate and the ranking is the same as the overall mutation rate. The difference between mutations rates could be attributed to the underlying risk factors in different geographical regions, however the samples size is not large enough to make such as estimation.

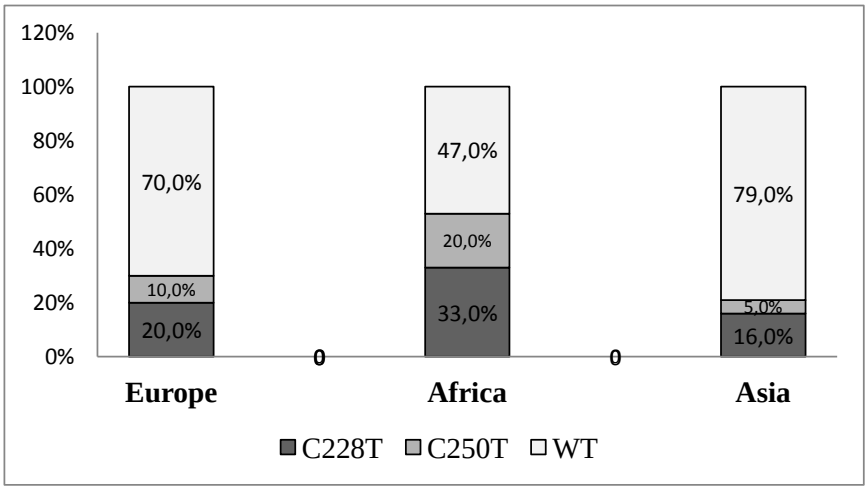


Figure 3.5 Geographic distibution of *TERT* promoter mutations across the world.

African tumor samples have the highest mutation frequency which is 53%. Europe has a mutation frequency of 33% and is second in the mutation rank and Asia has the least mutated tumor samples with 21% of frequency.

3.1.4 Association of *TERT* promoter mutations with patient characteristics

TERT promoter mutations have a very high frequency in HCC cell lines and tumors; thus, it is expected to find a correlation between tumor profiles and these mutations. For that purpose, we compared TERT promoter mutation status with patient characteristics such as sex, age, geographical origin, HBV positivity, *TP53* mutation status and MDM2 SNP 309 polymorphism and Table 3.1 revises the results of the association analysis. Patient age and gender was not correlated with TERT mutation

status at all; however there was a weak correlation between geographical origin of the tumors and TERT promoter mutations; patients from Africa had a 53% of mutation frequency while non-African patients had only 24% of mutation rate, therefore there was a weak correlation between African origin and presence of *TERT* promoter mutation with a *P* value of 0.056. Tumors without HBV DNA displayed *TERT* promoter mutations more frequently (39%) compared to the tumors containing HBV DNA (26%); but this difference was not in a significant level (*P* = 0.295). Considering *TP53*; although tumors with wild type *TP53* had a lower frequency of *TERT* promoter mutations (29%) compared to tumors carrying a *TP53* mutation (50%), there was no significant association between *TP53* mutation status (P = 0.280) and *TERT* mutation rate. *MDM2* SNP 309 polymorphism and *TERT* promoter mutations also displayed a weak association (P = 0.058). To sum up, we were not able to detect any significant association between *TERT* promoter mutation status and patient characteristics, this is probably due to the low number of available tumors for this analysis.

Table 3.1 Association of *TERT* promoter mutations with patient characteristics

Variable	Overall series (*n* = 44)	*TERT* promoter mutated (*n* = 15)	*TERT* promoter non-mutated (*n* = 29)	*P* value
Gender				
Male	27	10	17	0.2059
Female	1	1	0	
Age				
≥ 60 yr	9	3 (33)	6 (67)	0.6547
< 60 yr	19	8 (42)	11 (58)	
Geographical origin				
African	15	8 (53)	7 (47)	0.0528
Non-African	29	7 (24)	22 (76)	
HBV DNA				
Positive	23	6 (26)	17 (74)	0.2950
Negative	18	7 (39)	11 (61)	
TP53				
Mutated	6	3 (50)	3 (50)	0.6315
Wild-type	38	11 (29)	27 (71)	
MDM2 SNP 309				
TT	18	8 (44)	10 (56)	
TG	15	2 (13)	13 (87)	0.0528 (*vs* TT)
GG	11	4 (36)	7(64)	

3.2 *TERT* Promoter Polymorphism (rs2853669) Status in HCC

The 474 base pair region of TERT promoter sequence that is amplified for the detection of TERT promoter mutations also included a T>C single-nucleotide polymorphism (SNP) at position−245 bp (genomic loci 1295349). It is represented by rs2853669 or T349C and its variant allele is claimed to disrupt an existing ETS2 transcription factor binding site at TERT promoter [135]. However, there is controversial data about the function of this SNP in different cancer types. In a more recent study performed with urothelial cell carcinoma of bladder, they analyzed the effects of *TERT* mutations and rs2853669 polymorphism on patient survival. The results indicated that the patients carrying TERT promoter mutations showed poor survival and an increased rate of disease recurrence in the absence of the variant allele of the polymorphism and the patients with mutations had a better survival in the presence of the *TERT* rs2853669 variant allele [148]. In case of HCC, there is a report showing that there is no significant association between rs2853669 polymorphism and HCC risk [188]. However, there is no experimental data showing the relationship between telomerase activity and rs2853669 polymorphism. Here we determined rs2853669 polymorphism status in our HCC cell line panel and tumor samples to know whether the variant allele is commonly observed in HCC or not.

3.2.1 rs2853669 Polymorphism in HCC cell lines

Sequencing data used for *TERT* promoter mutation analysis is also used to determine rs2853669 polymorphism frequency in 15 HCC cell lines and the results are given Table 3.2. Over 15 cell lines, 6 carry variant allele (40%) of the rs2853669 SNP. Among these 6 cell lines carrying variant allele, only one is wild type in terms of promoter mutations and the remaining 5 cell lines carry both C228T mutation and rs2853669 variant TC allele. However, there is no correlation between the presence of the variant SNP allele and the presence of promoter mutations (P= 0.16).

Table 3.2 *TERT* promoter mutations and rs2853669 polymorphism in HCC cell lines

Cell lines	TERT Mutations		rs2853669
	C228T	C250T	T349C
Huh7	C228T	WT	TT
HepG2	C228T	WT	TC
Hep3B	WT	WT	TT
Hep40	WT	WT	TT
PLC	WT	WT	TT
FLC4	C228T	WT	TC
FOCUS	C228T	WT	TC
Mahlavu	WT	C250T	TT
SNU182	WT	WT	TT
SNU387	C228T	WT	TT
SNU398	C228T	WT	TT
SNU423	C228T	WT	TC
SNU449	WT	WT	TT
SNU475	C228T	WT	TC
SKHEP1	WT	WT	TC

WT: Wild Type

3.2.2 rs2853669 Polymorphism in HCC Tumors

SNP rs2853669 (T349C) status is also determined in 44 HCC tumor samples that were used for *TERT* promoter mutation analysis by using the same sequencing data. Among 44 tumors, 17 carried the variant allele (38.63%). Only 3 over 15 tumors with C228T or C250T mutations have the variant rs2853669 allele. On the other hand, 14 of 29 wild type tumors carried the variant allele. It could be interpreted as tumors with *TERT* promoter mutations tend to have a lower frequency of variant allele compared to wild type tumors but we only found a weak correlation to support this hypothesis (P=0.064). In Table 3.3, *TERT* promoter mutations and rs2853669 polymorphism in HCC tumors is shown together with some patient characteristics such as p53 mutation status, HBV status, tumor stage, sex and age. We could not find any association between rs2853669 status and patient characteristics that are represented in Table 3.3.

Table 3.3 *TERT* promoter mutations and rs2853669 polymorphism in HCC tumors

Country	TERT Mutations		rs2853669 T349C	p53 Mutations		MDM2	HBV	Stage	Sex	Age
	C228T	C250T		Amino Acid	Codon	SNP 309				
Japan	C228T	WT	TT	Del (AGCTAC)	6bpdel	G/G	minus	U	U	U
Japan	C228T	WT	TT	WT		T/G	minus	U	U	U
Japan	C228T	WT	TT	WT		T/G	minus	U	U	U
Japan	WT	C250T	TT	WT		G/G	minus	U	U	U
Japan	WT	WT	TC	WT		T/G	minus	U	U	U
Japan	WT	WT	TC	WT		T/G	minus	U	U	U
Japan	WT	WT	TC	WT		T/G	minus	U	U	U
Japan	WT	WT	TT	WT		T/G	minus	U	U	U
Japan	WT	WT	TT	WT		T/G	plus	U	U	U
Japan	WT	WT	TT	WT		G/G	minus	U	U	U
Japan	WT	WT	TT	WT		T/G	minus	U	U	U
China	WT	WT	TC	C>A Asp>Glu	281	T/T	plus	U	M	44
China	WT	WT	TC	WT		G/G	plus	U	M	58
China	WT	WT	CC	WT		T/G	plus	U	M	61
China	WT	WT	TT	WT		T/T	plus	U	M	56
China	WT	WT	TT	WT		G/G	plus	U	M	35
China	WT	WT	TT	WT		T/T	plus	U	M	67
China	WT	WT	TT	WT		G/G	plus	U	M	64
China	WT	WT	TT	WT		G/G	plus	U	M	45
Israel	WT	WT	TT	WT		T/G	minus	U	U	U
Mozambique	C228T	WT	TT	G>T Val>Phe	157	T/T	plus	Late	M	39
Mozambique	C228T	WT	TT	WT		T/T	plus	Late	M	26
Mozambique	WT	C250T	TT	WT		T/T	plus	Late	M	38
Mozambique	WT	C250T	TT	G>T Arg>Ser	249	T/T	minus	Early	M	27

Mozambique	WT	WT	TT	WT		T/T	plus	Late	M	36
Mozambique	WT	WT	TT	G>T Arg>Ser	249	T/T	plus	Late	M	36
Transkei	C228T	WT	TT	WT		T/T	NT	Late	M	27
Transkei	C228T	WT	TT	WT		T/T	NT	Late	M	50
Transkei	WT	WT	TC	WT		T/T	NT	Early	M	58
Transkei	WT	WT	TT	WT		T/T	plus	Late	M	49
Lesotho	WT	C250T	CC	WT		T/T	plus	Early	M	52
Lesotho	WT	WT	TC	WT		T/G	plus	Late	M	72
Swaziland	WT	WT	TT	WT		T/T	plus	Early	M	34
South Africa	C228T	WT	CC	WT		T/T	plus	Late	M	35
South Africa	WT	WT	TT	WT		T/G	plus	Late	M	14
Germany	C228T	WT	TC	WT		G/G	minus	Metastasis	M	83
Germany	C228T	WT	TT	C>T Arg>Cys	273	T/T	minus	HCC	F	70
Germany	WT	C250T	TT	WT		G/G	plus	HCC	M	83
Germany	WT	WT	TC	WT		G/G	minus	U	U	U
Germany	WT	WT	TC	WT		G/G	minus	Metastasis	M	83
Germany	WT	WT	TC	WT		T/T	plus	U	U	U
Germany	WT	WT	TC	WT		T/G	plus	HCC	M	87
France	WT	WT	TC	WT		T/G	minus	U	U	U
France	WT	WT	TC	WT		T/G	minus	U	U	U

[1]means reference [187]; [2] means reference [186]. WT: Wild type; Del: Deletion; HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism; MDM2: Murine double minute 2; HBV: Hepatitis B viral. U: Unknown.

3.3 STAT1 is a Candidate Transcription Factor for Mutant *TERT* Promoter

The STAMP DNA motif comparison web tool was used to search for candidate transcription factors that could bind to mutant *TERT* promoter sequence. DNA motif database of TRANSFAC (Transcription factor database) was searched via STAMP tool. Two analyses were performed by using the common mutation motif (CCCCTTCCGGG) generated at *TERT* promoter in the presence of C228T or C250T mutations. The first analysis aims to detect transcription factor families and the second analysis is performed to determine individual transcription factors that might bind to the mutant DNA sequence.

3.3.1 Transcription Factor Search for Mutant TERT Promoter

Transcription factor families that might bind to mutant TERT promoter are determined by STAMP tool and the results are shown in Figure 3.6. In this figure, transcription factor families are listed according to their motif binding score from top to bottom, meaning that the transcription family that is found at the top has the highest possibility of binding to common mutation motif CCCCTTCCGGG. STAT transcription factor family is found at the top of the list with the lowest *E value* and it is followed by ETS transcription factor family. Previous reports claimed that *TERT* promoter mutations created a new binding site for ETS TF family [133,134]; thus, it is the first time a new TF family, namely STAT TF family is considered to be able to bind to mutant promoter motif. To have a more detailed result, we have also performed a search for individual transcription factors that might bind to the mutant DNA sequence and the result is given in Figure 3.7. STATx (meaning any member of STAT TF), STAT1 and STAT3 transcription factors are found in the list together with other transcription factors. Similar to transcription factor family search result, STAT transcription factors are again found at the top of the list and ETS transcription factors come after STATs.

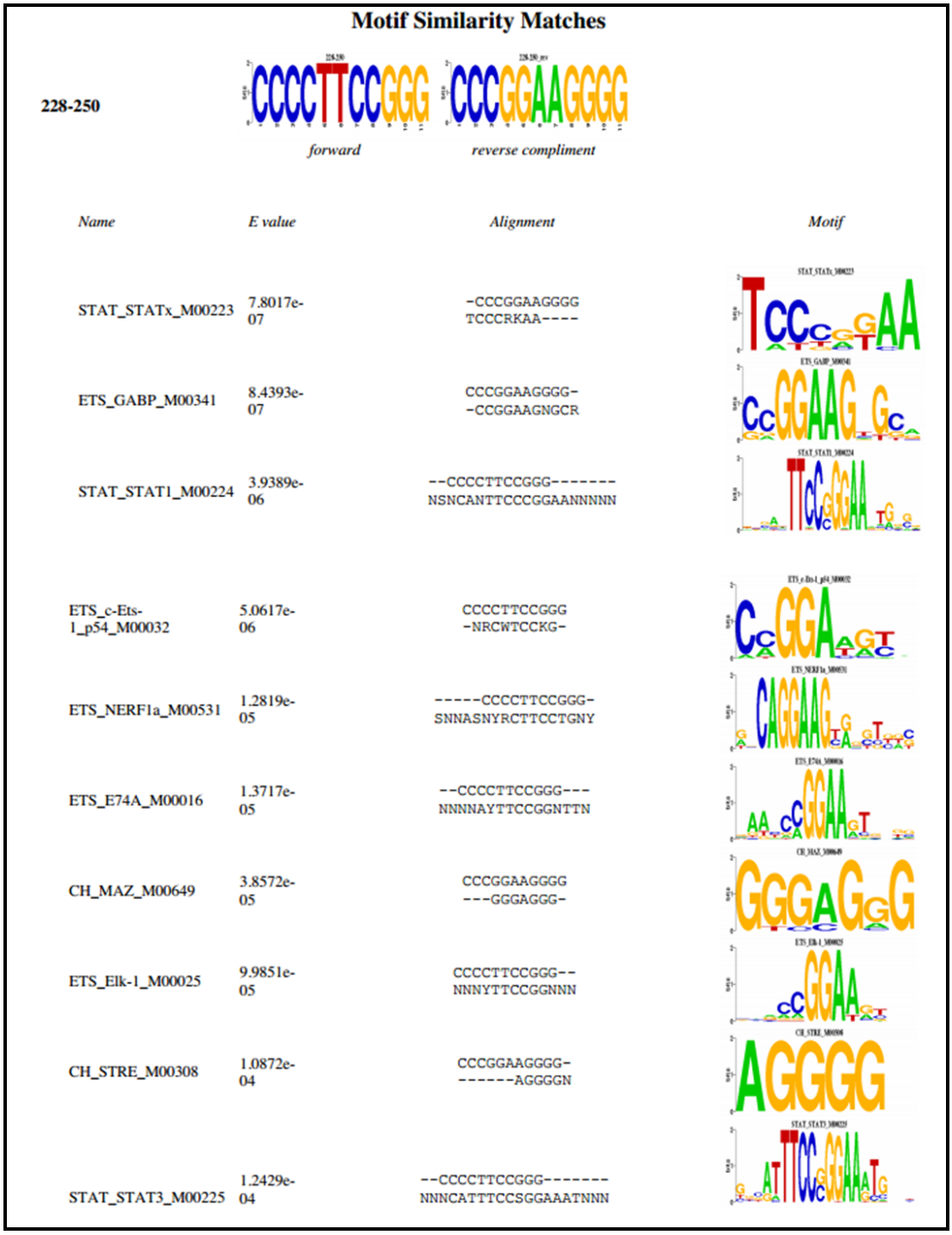


Figure 3.6 STAT Transcription Factor Family is a candidate TF family that might bind to common mutation motif.

Transcription factor families that might bind to CCCCTTCCGGG mutant TERT promoter motif are searched by STAMP tool and STAT transcription family is detected at the top of the list with the highest probability.

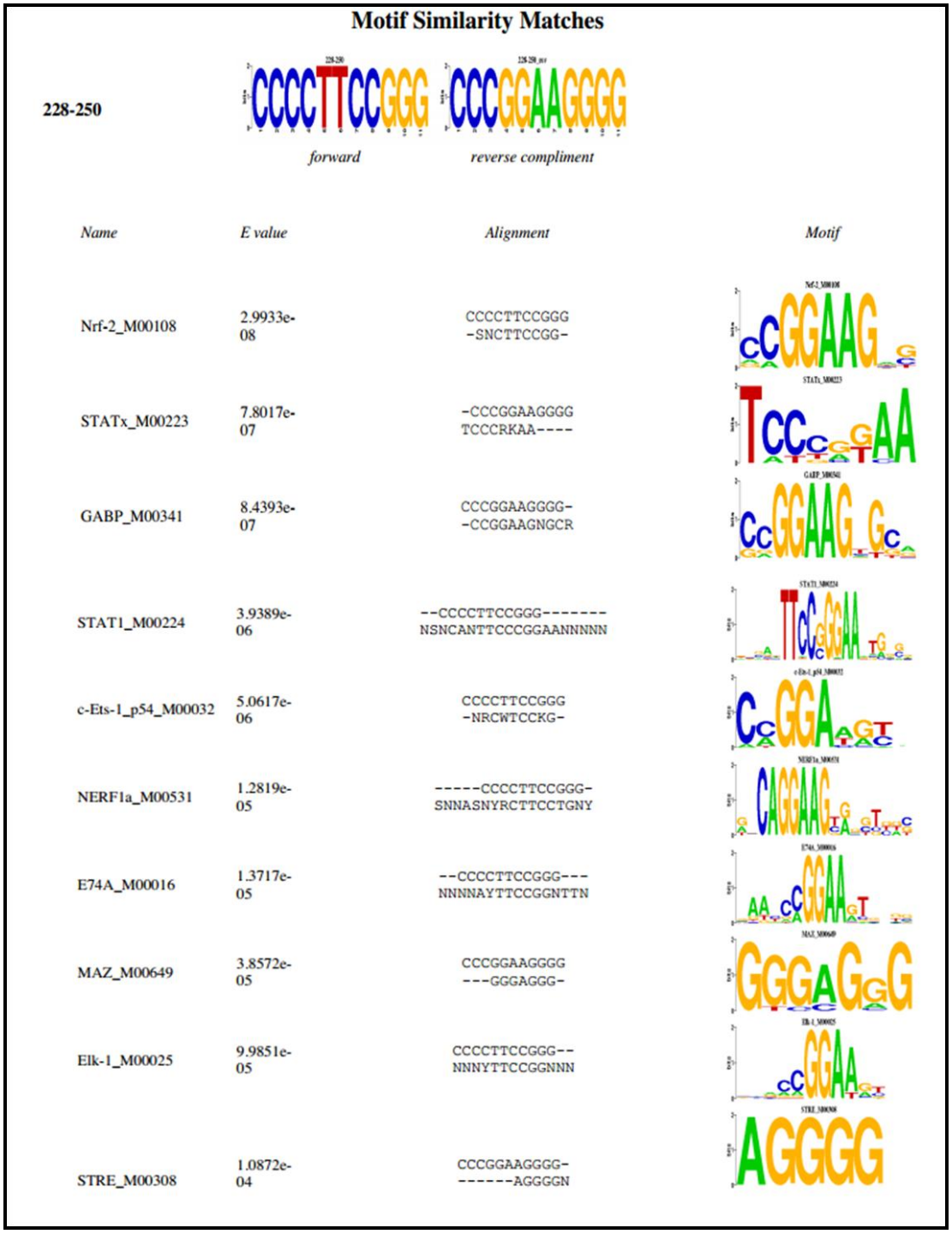


Figure 3.7 STAT1 is among individual Transcription Factors that might bind to common mutation motif

Individual TFs that might bind to CCCCTTCCGGG mutant TERT promoter motif are searched by STAMP tool and STAT1 transcription factor is detected in the list.

3.3.2 Detection of STAT1 protein level in HCC Cell Lines

Once we have found that STAT1 is a candidate transcription factor that might bind to mutant TERT promoter, we decided to determine STAT1 protein levels in different HCC cell lines. Western blot analysis of several HCC cell lines showed that STAT1 is differentially expressed in HCC cell lines (Figure 3.8). HepG2, Mahlavu and SNU 398 cell lines have high levels of STAT1 while Huh7 and Hep3B have lower expression and SNU 423 does not have a detectable amount of STAT1 protein. Among these cell lines, Huh7, HepG2, Hep3B, Mahlavu, SNU423 and SNU398 carry TERT promoter mutations while SNU 449 is wild type. For further analysis with STAT1; HCC cell lines that have high amounts of STAT1 will be studied to prevent low protein amount constraining our experiments.

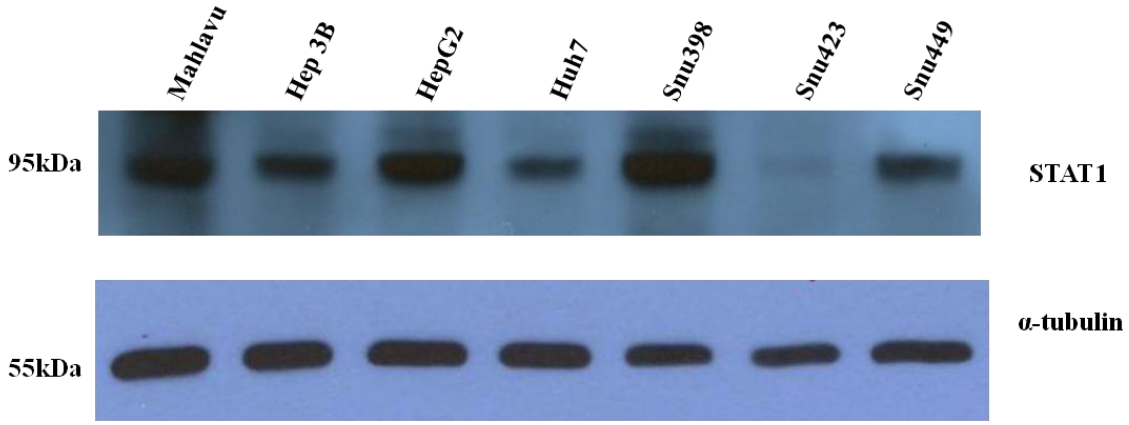


Figure 3.8 STAT1 protein is differentially expressed in HCC cell lines.

An equal amount of total cellular protein is loaded in each well and western blot analysis is performed with anti-STAT1 antibody to determine STAT1 protein level. α-tubulin is used for loading control.

3.3.3 Activation of STAT1 by IFN-α in HCC Cell Lines

Type I IFNs (primarily Interferon alpha and beta) bind to the Interferon alpha receptor (IFNAR) and they activate JAK1 and TYK2. Once JAKs are activated, they phosphorylate STAT1 and STAT2 transcription factors. Two STAT1 proteins form homodimers or STAT1 interacts with STAT2 and IRF-9 to bind to target genes promoters and start transcription [162,189]. Since STAT1 is activated by IFN-α and only

activated STAT1 can act as a transcription factor, we decided to trigger STAT1 activity by treating HCC cells with IFN-α. After IFN-α treatment, the effects of STAT1 on TERT expression was checked.

3.3.3.1 STAT1 is Phosphorylated by INF-α in HCC cell lines

HepG2 cell line which has C228T mutation was previously used to analyze the effects of TERT promoter mutations by reporter assay[134] and we detected a high STAT1 protein level in this cell line. For these reasons, we decided to start analyzing effects of IFN-α treatment on HepG2 cells to determine whether or not we can activate STAT1 this way. We tried different doses of IFN-α based on literature data [190–192] and performed treatments with 1ng/ml, 5ng/ml, 10ng/ml and 100ng/ml INF-α for 1 hour with HepG2 cells and harvested the cells, extracted total proteins and performed western blot analysis. As schematized in Figure 3.9 even 1ng/ml dose of INF-α is enough for the STAT1 phosphorylation required for STAT1 activation.

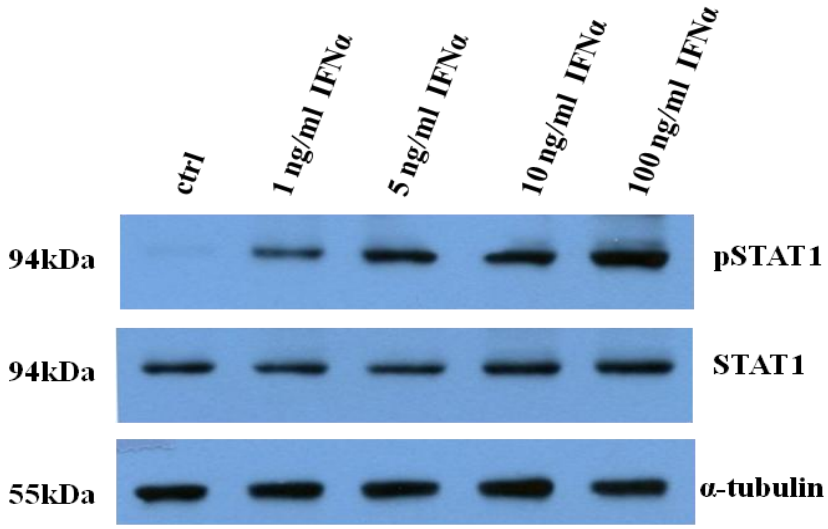


Figure 3.9 STAT1 is phosphorylated after IFN-α treatment in HepG2 cells.

Increasing doses of IFN-α was used to treat HepG2 cells for 1 h. Then total protein was extracted and an equal amount of it was loaded in each well. Western blot analysis was performed with anti-STAT1 antibody to determine STAT1 protein level. α-tubulin is used as loading control.

After detecting phosphorylation of STAT1 with different doses of IFN-α by a treatment of 1 h in HepG2 cell line, we decided to proceed with other cell lines. This time we picked up a single dose and treated HepG2, Mahlavu, PLC and SNU449 cell lines with 1ng/ml IFN-α for 1 h to detect pSTAT1 level. Once treatment was over, we extracted total proteins and performed western blot analysis with STAT1 and phosphoSTAT1 antibodies. The result is shown in Figure 3.10. In all cell lines tested, we managed to detect pSTAT1 after interferon alpha treatment as expected.

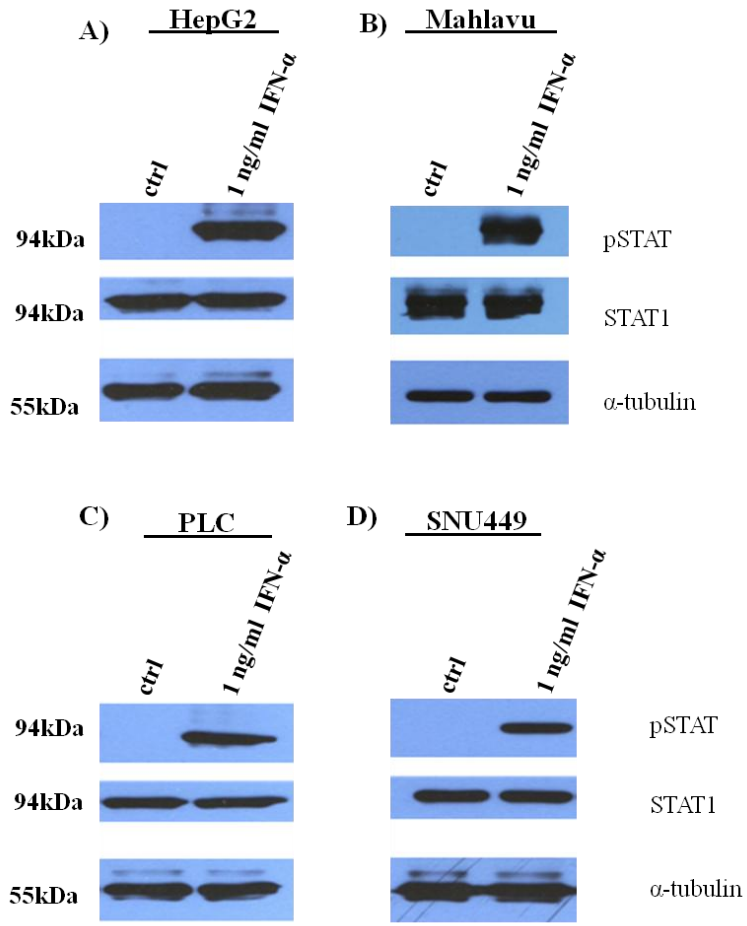


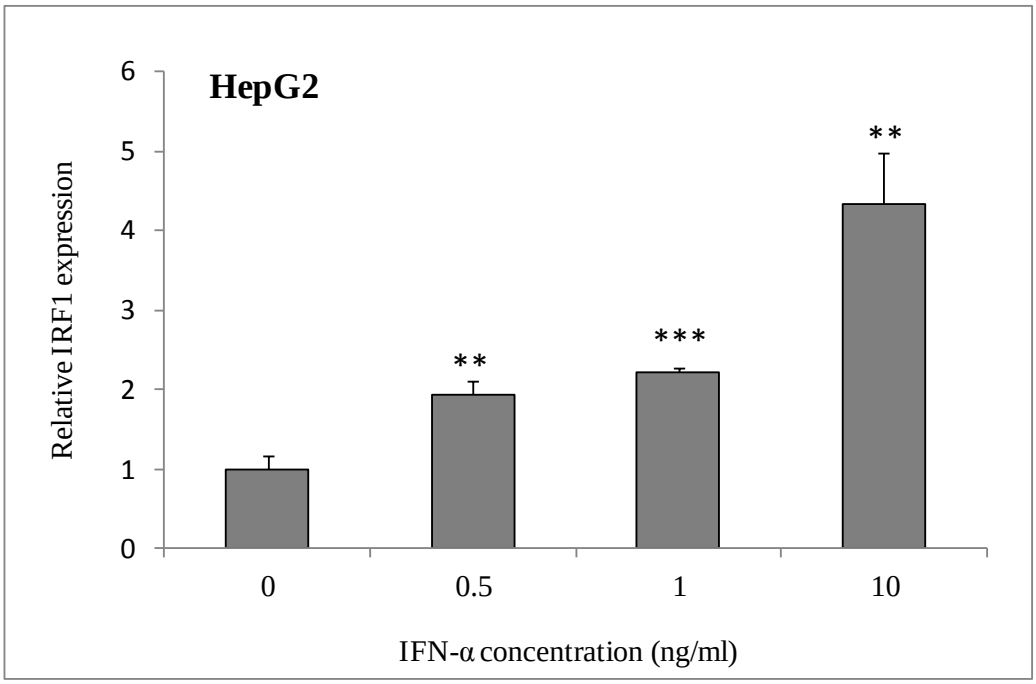
Figure 3.10 STAT1 is phosphorylated after IFN-α treatment in HCC cell lines.

1ng/ml of IFN-α was used to treat HepG2, Mahlavu, PLC and SNU449 cells for 1 h. Then total protein was extracted and an equal amount of it was loaded in each well and western blot analysis was performed with anti-STAT1 antibody to determine STAT1 protein levels. α-tubulin was used as loading control.A) HepG2, B) Mahlavu, C) PLC, D) SNU449

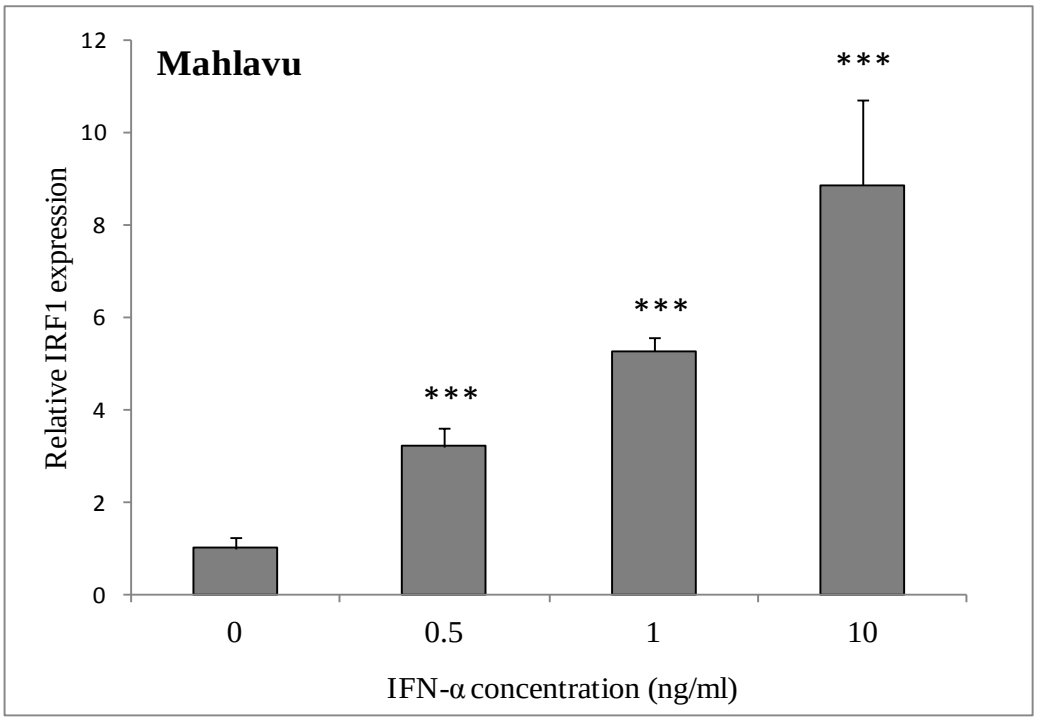
3.3.3.2 IRF1 is upregulated by IFN-α in HCC cell lines

We have shown that doses as low as 1ng/ml of IFN-α treatment could activate STAT1 evidenced by pSTAT1 levels in HCC cell lines (See Figure 3.9 and 3.10). But we had to make sure that STAT1 signaling is undisrupted, meaning that pSTAT1 can act as a functional transcription factor and upregulate expression of its known targets upon IFN-α treatment in HCC cell lines. For that purpose we decided to study the expression pattern of Interferon regulatory factor 1 (IRF1), a well-known STAT1 target that is upregulated through activated STAT1 transcription factor. Several HCC cell lines were treated with increasing doses of IFN-α such as 0.5 ng/ml, 1ng/ml and 10ng/ml for 48 hours, and then IRF1 expression was analyzed via qRT-PCR. In figure 3.11, relative IRF1 expression was calculated by using housekeeping gene GAPDH as a control. IFN-α treatment upregulates IRF1 expression in HepG2, Mahlavu, PLC and SNU 449 cell lines even in 0,5ng/ml concentration; however, IRF1 level does not change significantly in SNU423 cell line even at 10ng/ml of IFN-α concentration. HepG2 and Mahlavu cell lines carry *TERT* promoter mutations so it is advantageous to be able to show upregulation of a STAT1 target gene in response to IFN-α in these two cell lines. PLC and SNU449 cells are wild type for *TERT* promoter mutations and they have intact STAT1 signaling, thus we can use these two cell lines as controls in the following experiments. SNU 423 cell line is also mutated for TERT promoter but it does not have detectable amounts of STAT1 protein; thus, it is expected to see that IRF1 expression does not increase in response to IFN-α as STAT1 signaling is compromised by the absence of STAT1 protein.

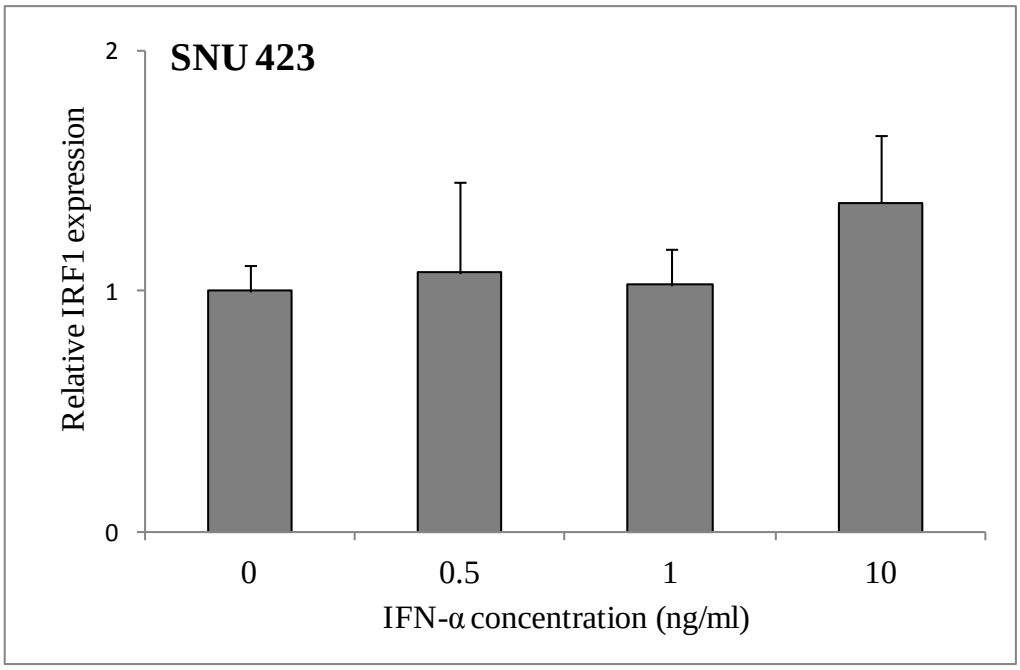
A)


HepG2
Relative IRF1 expression
0
1
2
3
4
5
6
**
***
**
0
0.5
1
10
IFN-α concentration (ng/ml)

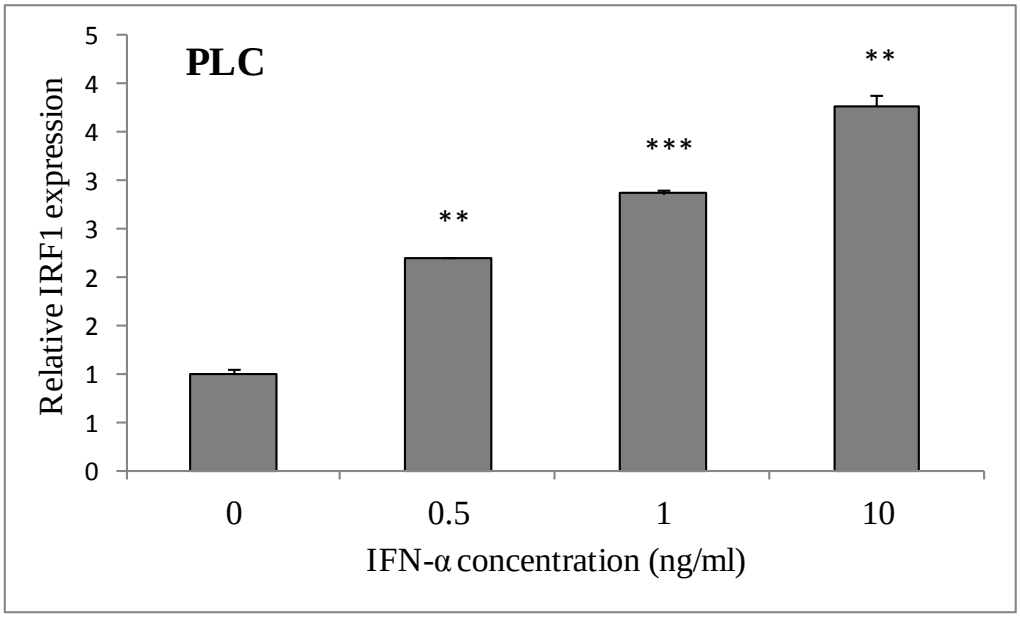
B)


Mahlavu
Relative IRF1 expression
0
2
4
6
8
10
12
***
***
***
0
0.5
1
10
IFN-α concentration (ng/ml)

C)


SNU 423
Relative IRF1 expression
2
1
0
0
0.5
1
10
IFN-α concentration (ng/ml)

D)


PLC
Relative IRF1 expression
5
4
4
3
3
2
2
1
1
0
**
***
**
0
0.5
1
10
IFN-α concentration (ng/ml)

E)

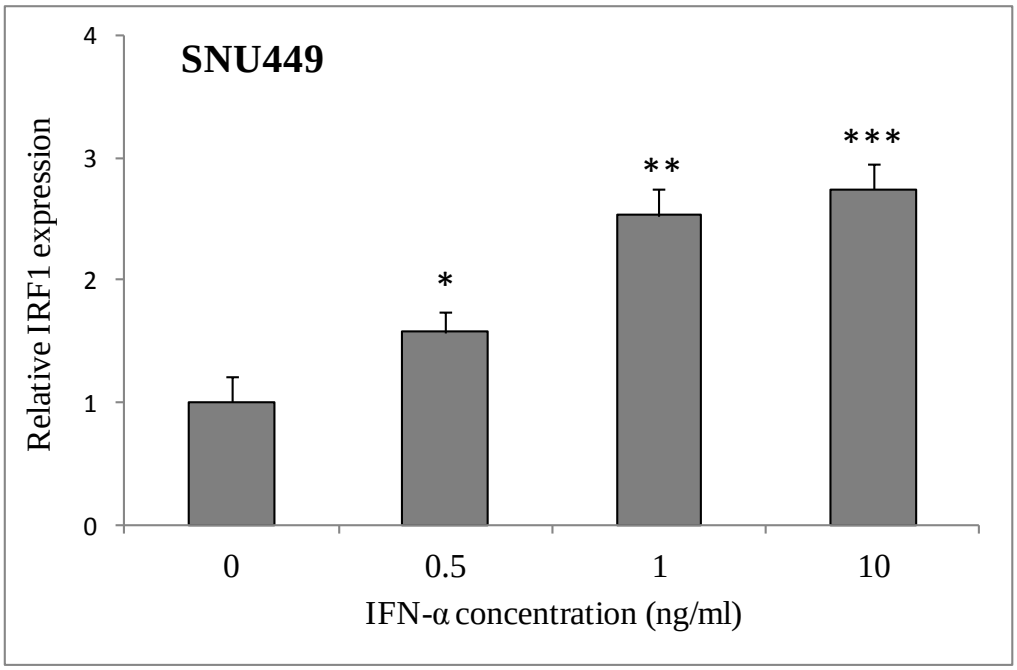


Figure 3.11 IRF1 expression is upregulated in response to IFN-α in HCC cells.

A) HepG2, B) Mahlavu, C) SNU423 and D) PLC and E) SNU 449 cells were treated with increasing doses of IFN-α for 48 hours in triplicates, then RNA was extracted, cDNA was synthesized and qPCR was performed with IRF1 or GAPDH specific primers. IRF1 expression level was calculated relative to GAPDH expression. All experiments were repeated at least 5 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

3.4 Regulation of *TERT* expression by IFN-α in HCC cell lines

We have found that STAT1 is a candidate transcription factor that might bind to mutant TERT promoter via bioinformatics analysis. Then we proceeded by showing that STAT1 is phosphorylated after IFN-α treatment in HCC cell lines and we showed that STAT1 activates transcription of IRF1 upon IFN-α treatment in HCC cell lines. Overall, these results demonstrated that STAT1 signaling is intact and can be triggered by IFN-α in HCC cell lines and prepared us to continue with testing TERT expression through activation of STAT1 via IFN-α.

3.4.1 *TERT* is upregulated by IFN-α in mutant HCC Cell Lines

STAT1 signaling is initiated by IFN-α through several phosphorylation events and the last step of this signaling is activation of transcription of target genes. We already showed that IRF1 is upregulated after 48 hours of treatment even with 1ng/ml of IFN-α, but it is not clear whether STAT1 can upregulate *TERT* expression again in 48 hours. HepG2 cell line which carries C228T mutation is used for the first analysis of TERT expression after interferon treatment. We treated HepG2 cells with 1ng/ml IFN-α for different time points such as 1h, 2 h, 6h, 24h, 48h and 72h. The results showed that TERT expression is upregulated significantly in HepG2 cells upon IFN-α treatment starting from 24 hours and it reaches its top level at 48 h (Figure 3.12).

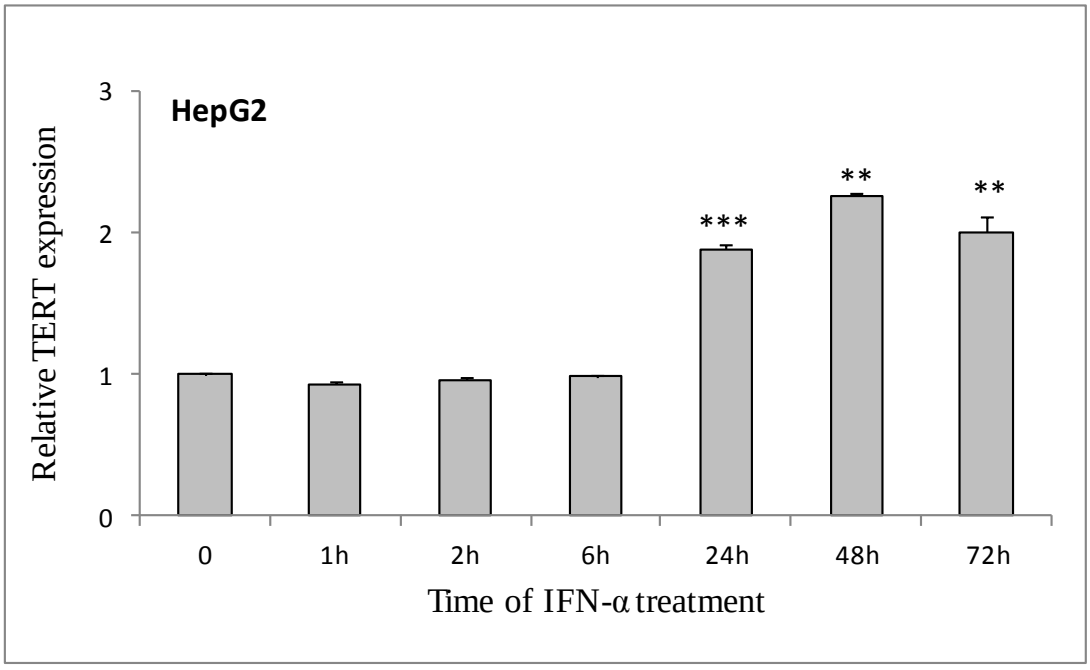


Figure 3.12 TERT expression is upregulated by IFN-α starting from 24h.

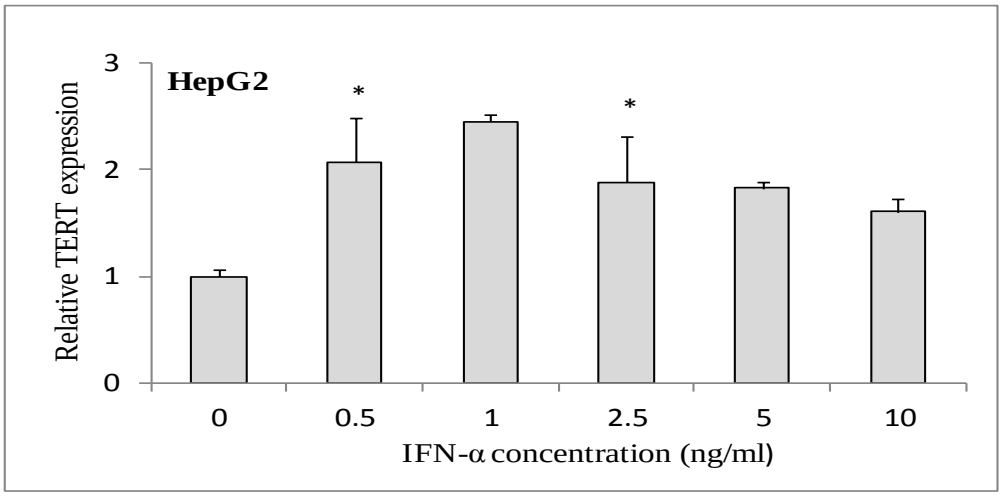
HepG2 cells were treated with 1ng/ml of IFN-α in triplicate, cells were harvested after 1h, 2h, 6h, 24h, 48h and 72h; RNA is extracted, cDNA was synthesized and qRT-PCR was performed and results were normalized with GAPDH. Experiment was repeated 3 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

After performing time dependent treatments of 1ng/ml IFN-α in HepG2 cells, we decided to perform the following IFN-α treatments for 48h since the highest TERT expression is observed at this time point. As a next step, we tested different concentrations of IFN-α starting from 0.5ng/ml up to 10ng/ml in HepG2 and Mahlavu cell lines. qRT-PCR results showing relative TERT expression of these cell lines after IFN-α treatment are given at Figure 3. 13. TERT expression increased nearly 2 fold in a significant manner in response to 0.5 and 1ng/ml of IFN-α in both HepG2 (C228T) and Mahlavu (C250T), cells. However, when we continued increasing the IFN-α dose, TERT expression started to decrease. At 2.5ng/ml IFN-α, TERT fold change was still significantly higher compared to no-interferon control in both cell lines. However, TERT expression continued to decrease as IFN-α dose was increased to 10ng/ml. We expected to see an increase in the expression level of TERT gene in response to IFN-α in cell lines carrying either C228T or C250T mutations. Indeed, Figure 3.13 A and B verifies our hypothesis for HepG2 (C228T) and Mahlavu (C250T) cells that were subjected to 0.5, 1 and 2.5ng/ml of IFN-α; however, the results we observe at 5 and 10ng/ml of IFN-α treatments for these two cell lines are unexpected. SNU423 cells have C228T mutation and it was expected that they would have similar responses to IFN-α treatment as HepG2 and Mahlavu cells, but this is not the case. As discussed in previous sections, SNU423 cells do not have detectable amounts of STAT1 protein and expression of IRF1 (a known target of STAT1) does not change in response to IFN-α. This data displays that SNU423 cells do not have an intact STAT1 signaling; hence it is not surprising that TERT expression is not effected by IFN-α in these cells (Figure 3.13 C).

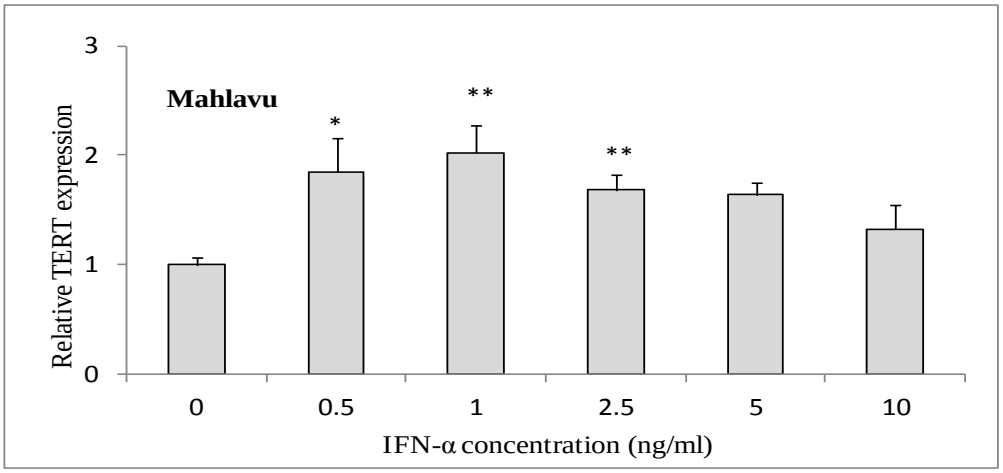
Figure 3.13 TERT expression is regulated by IFN-α in HCC cell lines.

IFN-α treatment was performed in different concentrations such as 0.5ng/ml, 1ng/ml, 2.5ng/ml, 5ng/ml and 10ng/ml for 48h in four HCC cell lines. A) HepG2, B) Mahlavu, C) SNU 423. All experiments were repeated 3 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

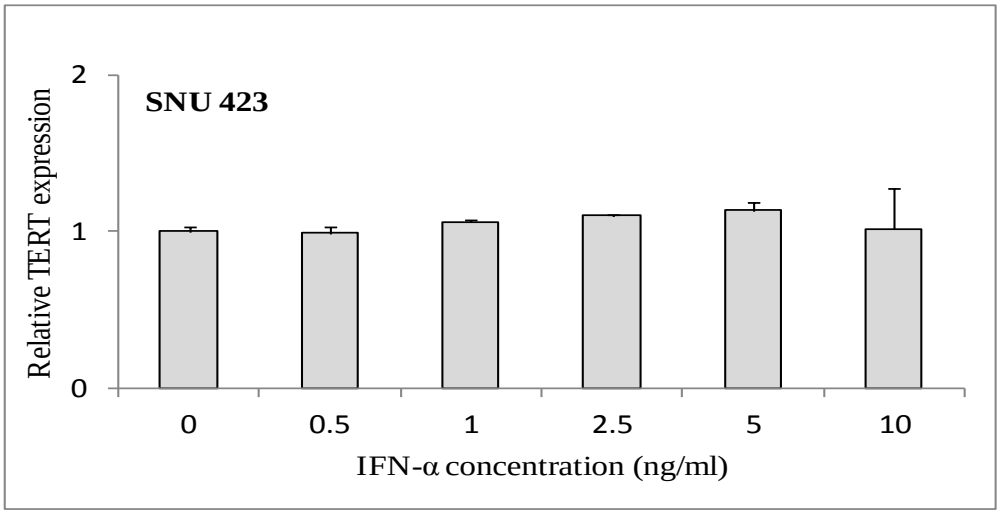
A)


HepG2
Relative TERT expression
3
2
1
0
*
*
0
0.5
1
2.5
5
10
IFN-α concentration (ng/ml)

B)

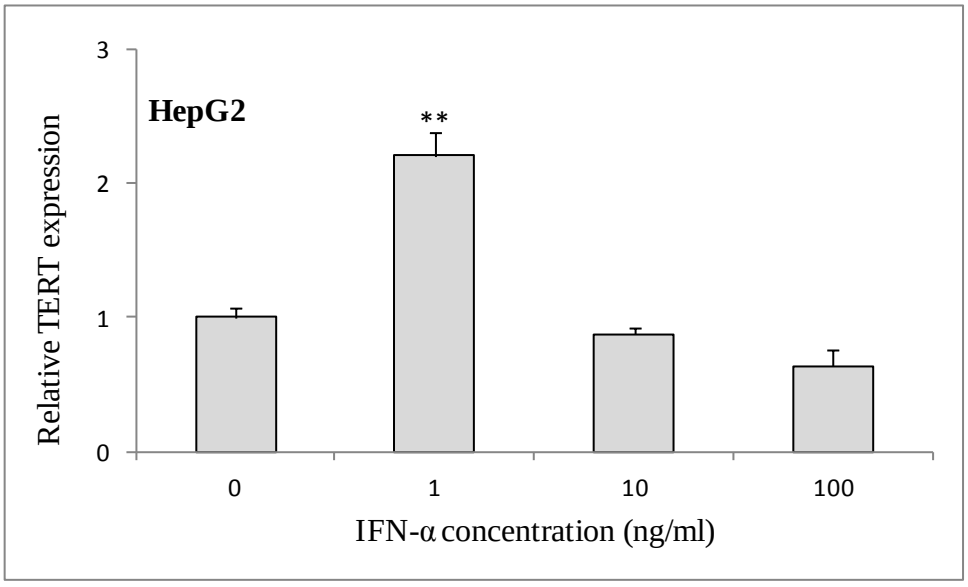

Mahlavu
Relative TERT expression
3
2
1
0
*
**
**
0
0.5
1
2.5
5
10
IFN-α concentration (ng/ml)

C)

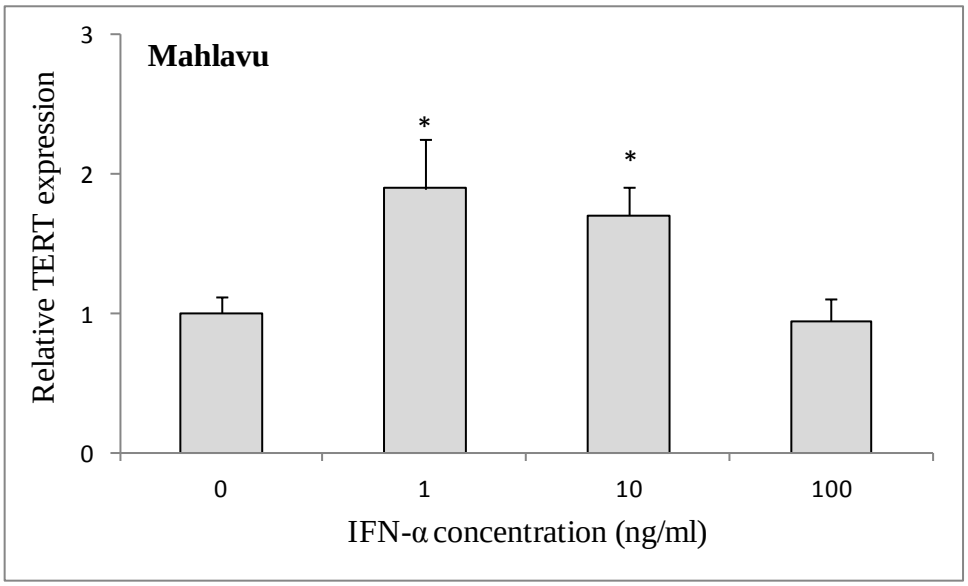

SNU 423
Relative TERT expression
2
1
0
0
0.5
1
2.5
5
10
IFN-α concentration (ng/ml)

Next, we wanted to analyze the effect of high concentrations of IFN-α on TERT expression in HepG2, Mahlavu and SNU423 cells to confirm the results shown in Figure 3.13. For this purpose, we performed treatments with 1ng/ml, 10ng/ml and 100ng/ml concentrations of IFN-α for 48 hours in these cell lines and checked TERT expression via qRT-PCR. The results are given at Figure 3.14 and they are consistent with the previous experiment's results. TERT expression again increased significantly in response to 1ng/ml IFN-α in both HepG2 and Mahlavu cells (Figure 3.14A and B). Upregulation of the TERT expression was lost as we increased IFN-α dose to 10ng/ml and 100ng/ml and expression level decreased to its basal level. SNU423 cells again did not respond to IFN-α treatment and TERT expression stayed stable in all doses of IFN-α (Figure 3.14C). The reason behind the effects of high doses of interferon alpha on TERT expression is not clear for the moment. One possibility could be the presence of a negative regulator or a feedback loop that is activated at high concentrations of IFN-α which in turn down regulates TERT expression.

A)



B)



C)

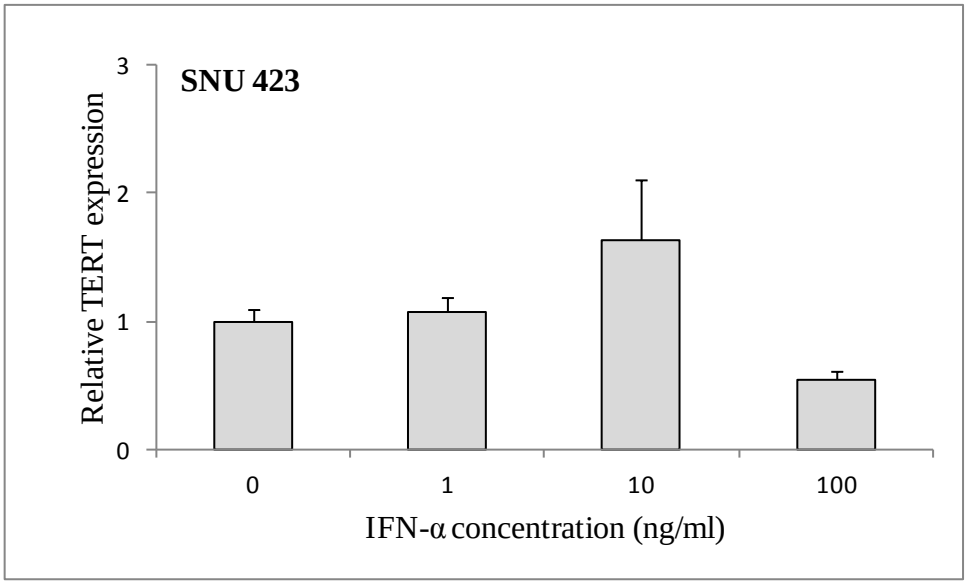


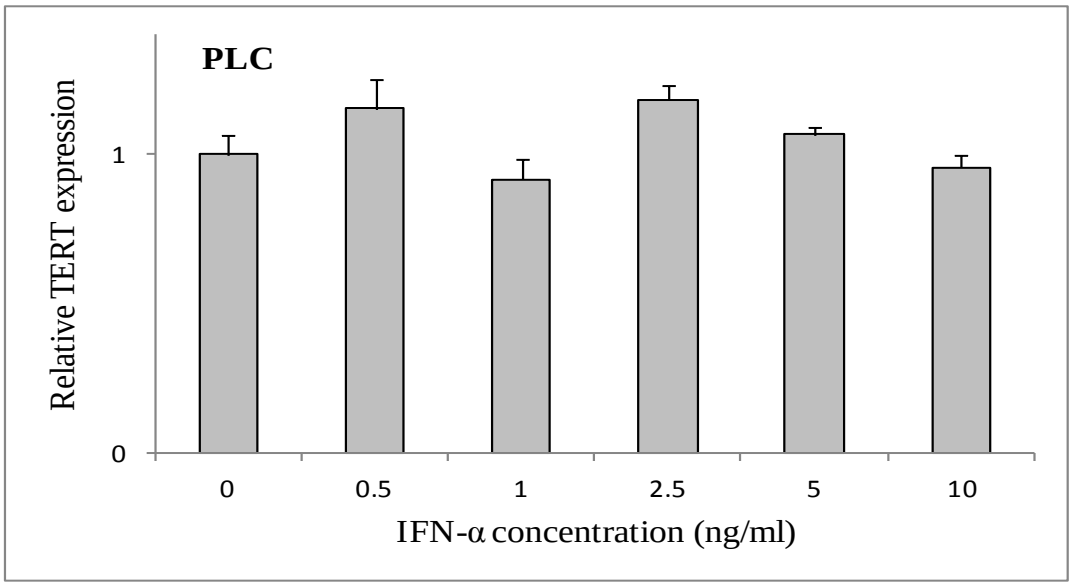
Figure 3.14 TERT expression is regulated by IFN-α in HCC cell lines.

IFN treatment was performed in different concentrations such as 1ng/ml, 10ng/ml and 100ng/ml for 48h in four HCC cell lines. A) HepG2, B) Mahlavu, C) SNU423. All experiments were repeated 3 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

3.4.2 *TERT* expression is not regulated by IFN-α in WT HCC cell lines

HepG2 cells carry the C228T mutation and Mahlavu cells carry the C250T mutation and *TERT* expression is upregulated by IFN-α in both cell lines. Since we hypothesized that STAT1 would bind only to mutant *TERT* promoters, we need to test wild type HCC cell lines and show that TERT expression does not change by IFN-α treatment in WT cells. We chose one epithelial-like (PLC) and one mesenchymal-like (SNU449) wild type cell line and tested the effects of IFN-α treatment by performing the same experiments as performed with mutant HCC cell lines; HepG2, Mahlavu and SNU423. First, we treated PLC and SNU449 cells for 48 hours with different concentrations of IFN-α starting from 0.5ng/ml up to 10ng/ml and performed q-RT-PCR analysis to have comparable results with the previous experiments. Relative TERT expression results are given in Figure 3.15 for PLC and SNU449 cells. Since these two cell lines are wild type in terms of *TERT* promoter mutations, there is no significant change in TERT expression levels in response to all doses of IFN-α as expected.

A)



B)

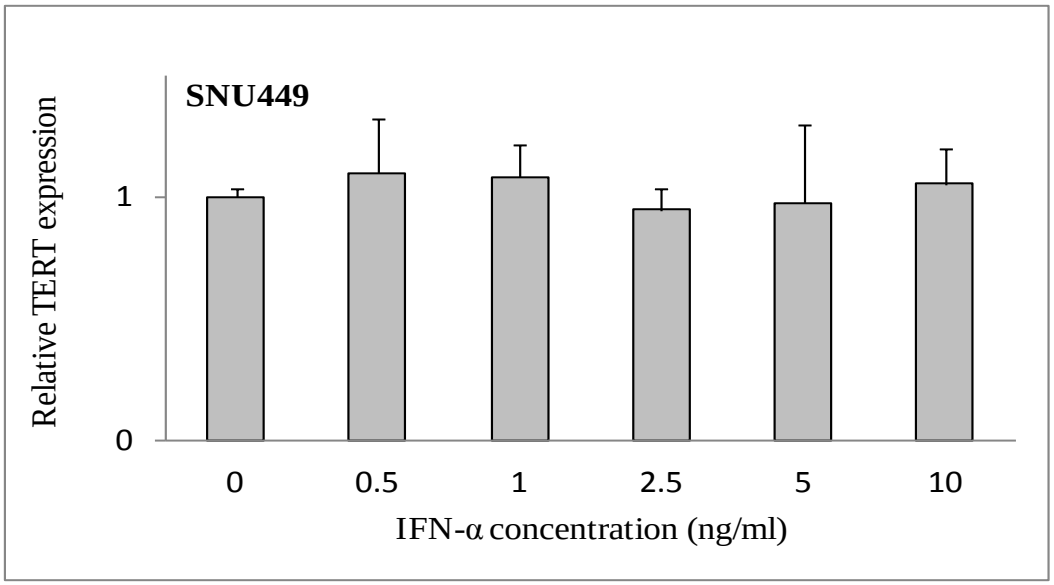
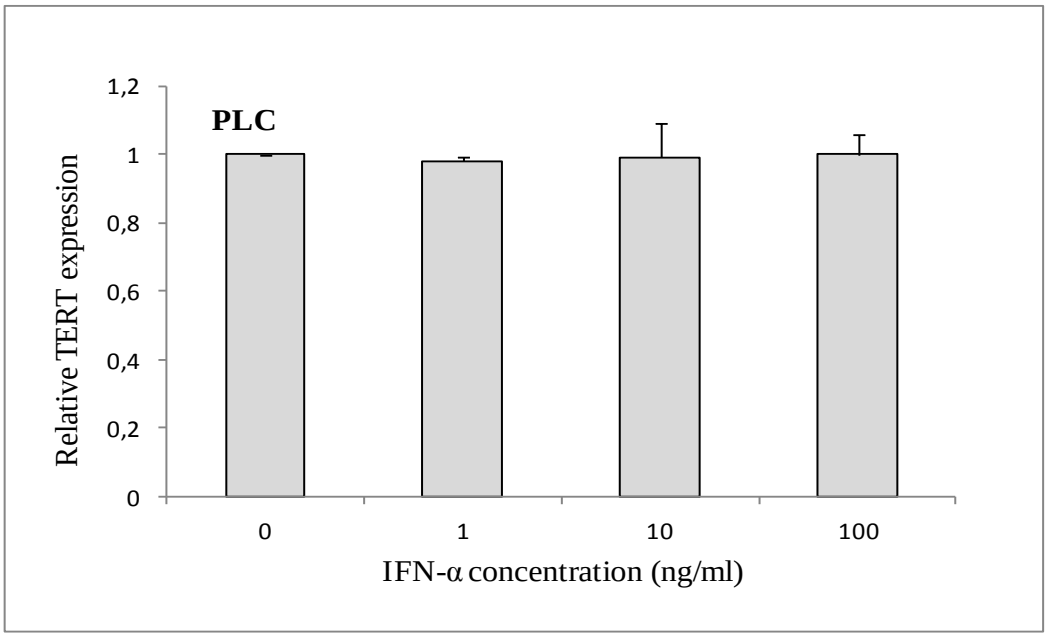


Figure 3.15 TERT expression is not regulated by IFN-α in wild type HCC cell lines.

IFN-α treatment was performed in different concentrations such as 0.5ng/ml, 1ng/ml, 2.5ng/ml, 5ng/ml and 10ng/ml for 48h in two HCC cell lines. A) PLC, B) SNU 449. All experiments were repeated 3 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

Secondly, we analyzed the effect of high concentrations of IFN-α on wild type cell lines by treating PLC and SNU449 cells with 1ng/ml, 10ng/ml and 100ng/ml concentrations of IFN-α for 48 hours. In the previous section, we showed that TERT expression was increased by small doses of IFN-α and brought to its basal level with high doses of IFN-α in HepG2, and Mahlavu cells which carry a *TERT* promoter mutation. When the results of high doses of IFN-α in wild type cells PLC and SNU 423 (Figure 3.16) was checked, it was clear that TERT expression was not affected by high doses of IFN-α neither. In conclusion, IFN-α regulates TERT expression in only mutant cells but not in wild type cells.

A)



B)

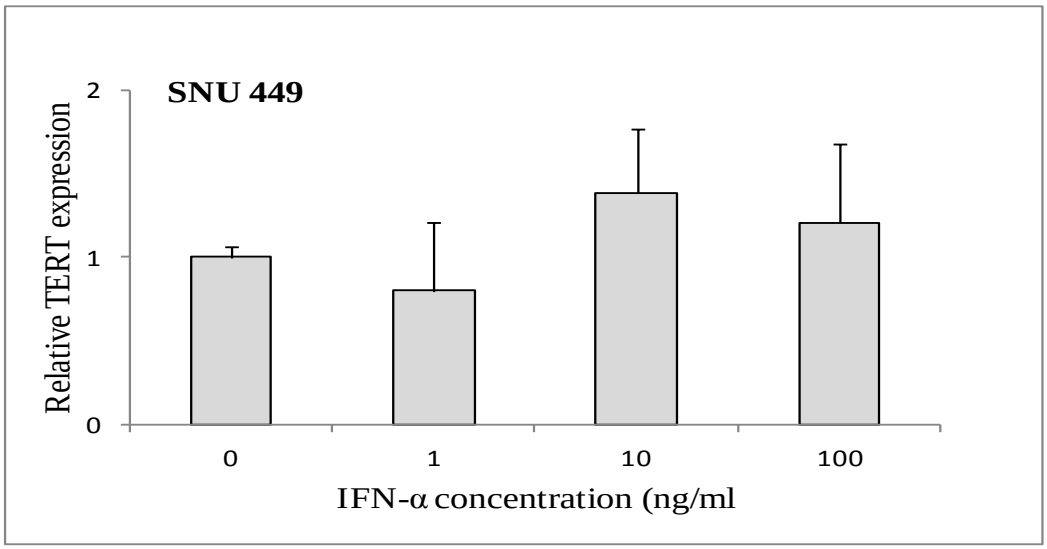


Figure 3.16 TERT expression is not regulated by IFN-α in wild type HCC cell lines.

IFN treatment was performed in different concentrations such as 1ng/ml, 10ng/ml and 100ng/ml for 48h in four two cell lines. A) PLC, B) SNU 449. All experiments were repeated 3 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

3.5 Regulation of *TERT* expression by STAT1 knockdown in HCC cell lines

In the previous sections; we first showed that STAT1 signaling was intact in HepG2, Mahlavu, and PLC and SNU449 cell lines. We treated these cell lines with different doses of IFN-α and showed that we can trigger phosphorylation of STAT1which leads to an increase in expression levels of IRF1, a known target of STAT1 TF. Second, we showed that TERT expression is upregulated in two HCC cell lines which carry TERT promoter mutations, namely HepG2 and Mahlavu, when treated with 1ng/ml IFN-α. Next, we displayed that upregulation of TERT expression by IFN-α was not observed in HCC cell lines without TERT promoter mutations, PLC and SNU449, meaning that this upregulation is unique to mutant cell lines and does not exist in wild type ones. However, it is not clear whether the intermediate regulator between IFN-α and *TERT* gene is the STAT1 transcription factor. To search the link between IFN-α and *TERT* gene, we decided to downregulate STAT1 and check the expression of TERT.

3.5.1 TERT is down regulated by STAT1 knock down in HepG2 cell line

If upregulation of TERT expression occurs through activation of STAT1 by IFN-α, STAT1 knock down is expected to abolish this upregulation. To confirm this hypothesis, we knocked down STAT1 by transfecting HCC cells with STAT1 specific siRNA and checked TERT expression. First of all, we showed that STAT1 knock down was successfully performed. We treated HepG2 cells with 50nm of siSTAT1 or siCTRL (non targeting siRNA) for 72 hours and performed western blotting with anti-STAT1 antibody. STAT1 protein level is diminished after siSTAT1 treatment compared to no treatment and siCTRL treated samples; thus we conclude that STAT1 knock down is successful (Figure 3.17).

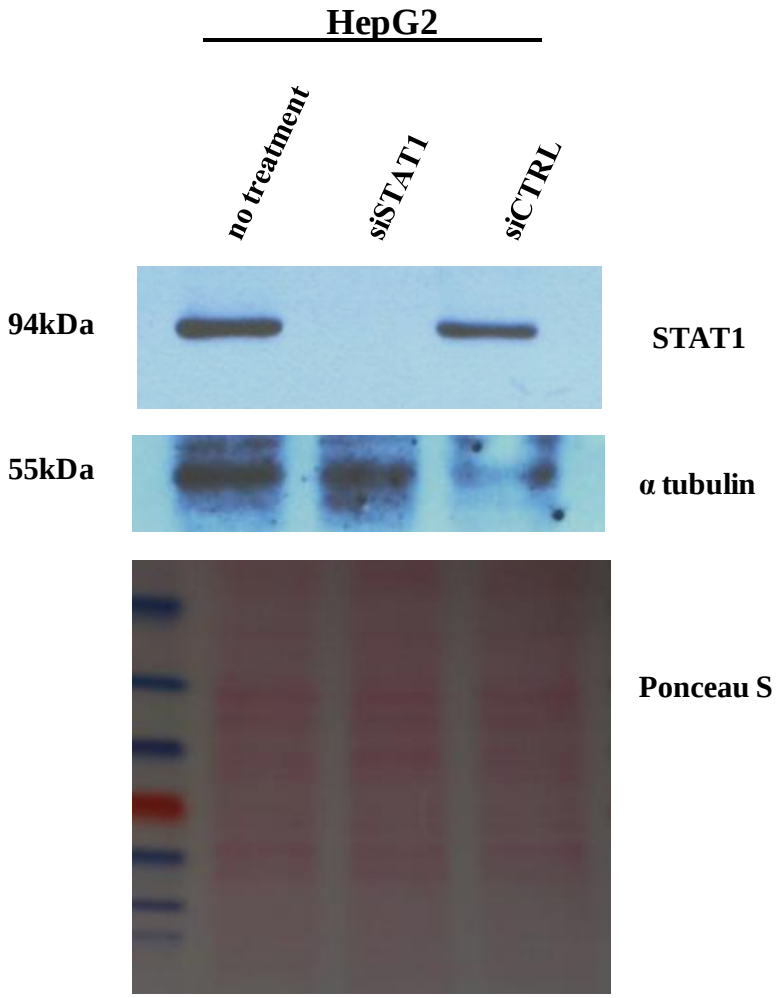


Figure 3.17 STAT1 is down regulated by STAT1 specific siRNA in HepG2 cells.

50nm of siSTAT1 or siCTRL was transfected into HepG2 cells with lipofectamine siRNA mix and cells were harvested after 72 hours for protein extraction. An equal amount of total protein was loaded into each well and western blotting was performed with STAT1 antibody. α- tubulin and Ponceau S. staining were used as loading controls.

Once we showed that we could effectively down regulate STAT1 with STAT1 specific siRNA, we can proceed with the functional analysis of this knock down starting with HepG2 cells. We performed the same knock down experiment in triplicates, harvested the cells for RNA extraction, synthesized cDNAs and performed qRT-PCR with STAT1 and TERT specific primers to determine TERT expression after STAT1 knockdown. As displayed in Figure 3.18, TERT expression decreased significantly in response to STAT1 knock down in HepG2 cells. This result indicates that STAT1 is critical for TERT transcriptional regulation.

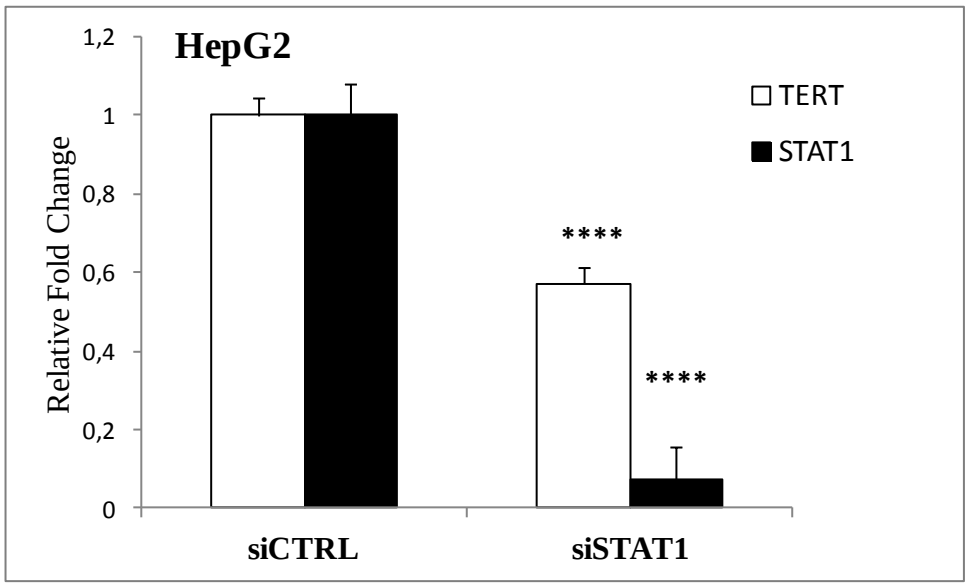
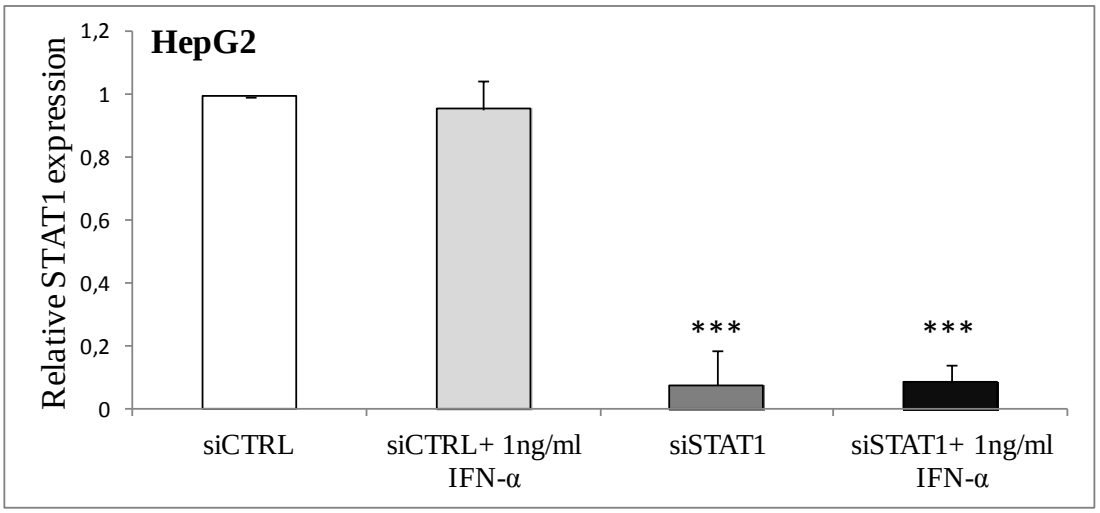


Figure 3.18 TERT is downregulated after STAT1 knockdown in HepG2 cell line.

Cells were transfected with 50nm of STAT1 specific siRNA or non-targeting (CTRL) siRNA with lipofectamine RNAimax for 72 hours, cells were harvested and qRT-PCR was performed with STAT1, TERT and GAPDH specific primers and results were normalized with GAPDH. All experiments were repeated 6 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

After showing that STAT1 knock down causes a significant (40-50%) decrease in HepG2 cells, we need to show that upregulation of TERT expression through IFN-α will be abolished in response to STAT1 knock down. Intending to produce that result, we designed an experiment to determine the effects of IFN-α treatment and STAT1 knockdown at the same time in HepG2 cells. In the first day of the experiment, we performed reverse transfection using siSTAT1 and siCTRL. One day later, we treated the transfected cells with 1ng/ml of IFN-α since we had already determined this dose to upregulate TERT expression. 48 hours later, we harvested the cells, extracted RNA and performed q-RT-PCR experiment. In Figure 3.19, relative STAT1 and TERT expression levels after STAT1 knockdown and IFN-α treatment are displayed. STAT1 expression was reduced significantly after siSTAT1 transfection compared to siCTRL transfection and IFN-α treatment did not affect STAT1 expression level (Figure 3.19 A). TERT expression was again down regulated significantly after STAT1 knock down compared to siCTRL sample. TERT expression was upregulated by 1ng/ml of IFN-α treatment when the cells were transfected with siCTRL. This shows that the transfection does not affect IFN-α responsiveness of the cells. However, the same dose of IFN-α (1ng/ml) could not upregulate TERT expression when the cells were transfected with siSTAT1 (Figure 3.19 B). These results demonstrate that STAT1 protein is necessary for the upregulation of TERT expression by IFN-α in HepG2 cell line (C228T).

A)



B)

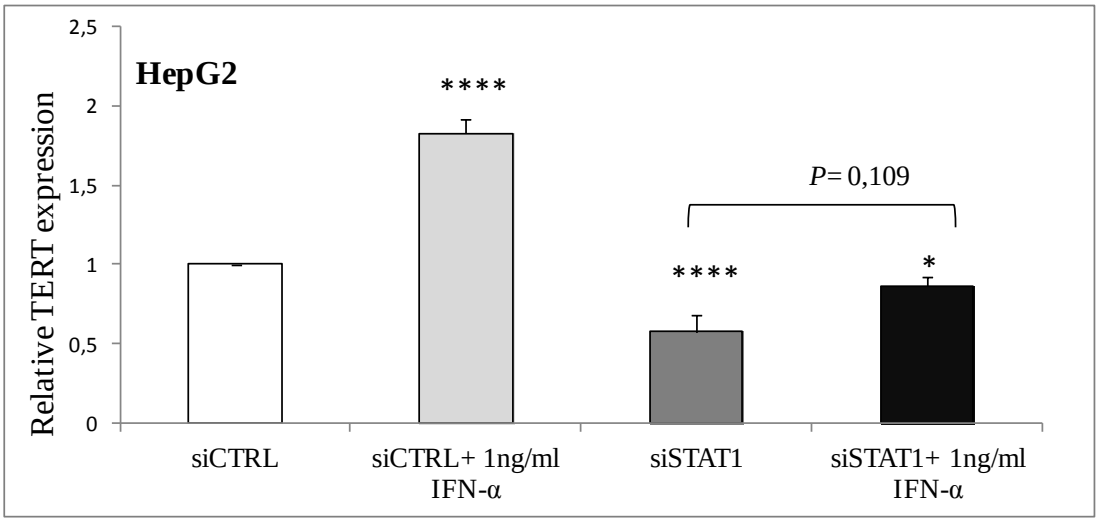


Figure3.19 TERT expression is regulated by STAT1 knock down and IFN-α treatment in HepG2 cell line.

HepG2 cells were transfected with 50nm of STAT1 specific siRNA or non-targeting (CTRL) siRNA. 24 h post transfection, cells were treated with 1ng/ml of IFN-α and incubated for another 48 hours. After 72h, cells were harvested and qRT-PCR was performed with STAT1, TERT and GAPDH specific primers and results were normalized with GAPDH. All experiments were repeated 6 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

3.5.2 TERT is downregulated by STAT1 knock down in Mahlavu cell line

STAT1 knock down causes a significant decrease in TERT expression in HepG2 cells and upregulation of TERT expression through IFN-α is eradicated when STAT1 is knocked down. Since HepG2 cells carry C228T mutation, we need to show that the same results can be obtained in the presence of a C250T mutation and Mahlavu is the only cell line bearing this mutation. Therefore, we performed the same experiments in this cell line as well. To begin with, we transfected Mahlavu cells with STAT1 speific siRNA and checked TERT expression (Figure 3.20). TERT expression is significantly diminished in response to STAT1 knockdown compared to siCTRL transfection in Mahlavu cells as observed in HepG2 cells.

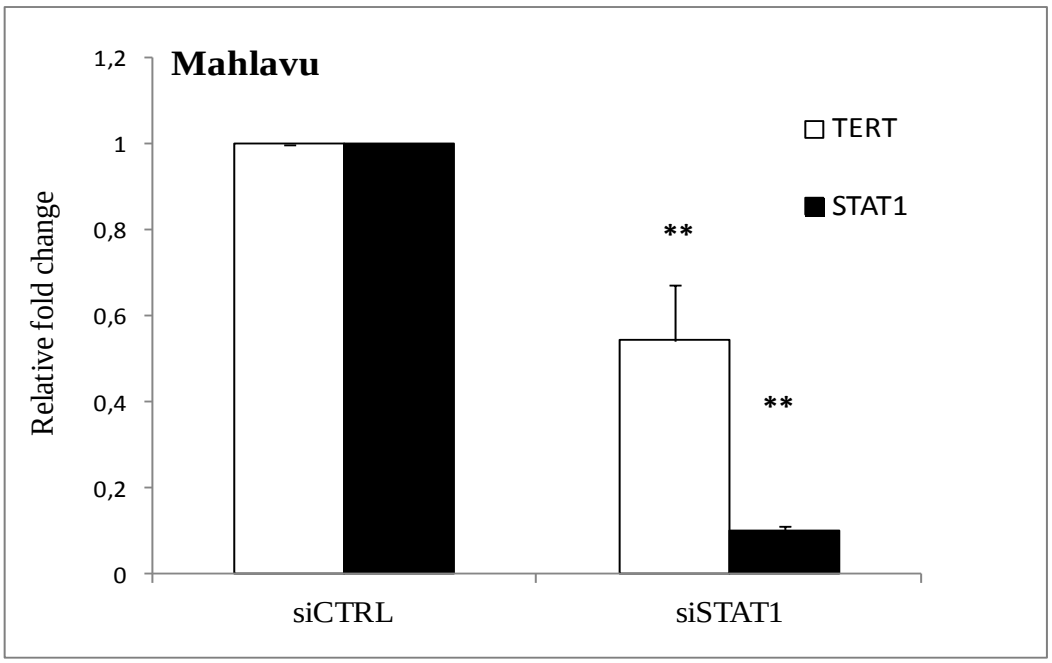
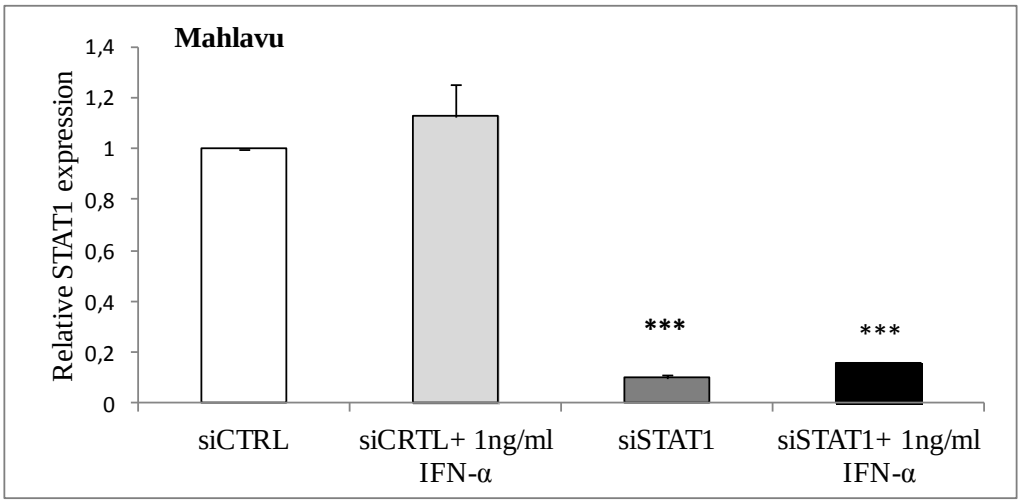


Figure 3.20 TERT is downregulated in response to STAT1 knockdown in Mahlavu cell line.

Cells were transfected with 50nm of STAT1 specific siRNA or non-targeting (CTRL) siRNA for 72 hours, cells were harvested and qRT-PCR was performed with STAT1, TERT and GAPDH specific primers and results were normalized with GAPDH. All experiments were repeated 6 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

As a next step, we again tested the effects of STAT1 knockdown together with IFN-α treatment in Mahlavu cells. We performed the same experimental set-up as described before. We started with reverse transfection using siSTAT1 or siCTRL and we treated the transfected cells with 1ng/ml of IFN-α the next day. Then we harvested the cells 48 hours later, extracted RNA and performed q-RT-PCR experiment. In Figure 3.20 the results of this experiment are displayed. Similar to HepG2 cells, STAT1 expression decreased significantly after STAT1 knock down and IFN-α treatment had no effect on STAT1 expression level (Figure 3.21 A). TERT expression was again down regulated significantly after STAT1 knock down compared to siCTRL sample. Considering the effects of IFN-α treatment; 1ng/ml of IFN-α could upregulate TERT expression in siCTRL transfected samples meaning that the transfection does not affect the IFN-α response of Mahlavu cells. On the other hand, TERT expression is not upregulated by 1ng/ml of IFN-α when STAT1 is downregulated (Figure 3.21 B). In conclusion; we had very similar results with HepG2 and Mahlavu cells in terms of STAT1 knock down and IFN-α treatment. Both cells showed a significant downregulation in TERT expression after STAT1 knock down and IFN-α treatment could not upregulate TERT expression when STAT1 is downregulated.

A)



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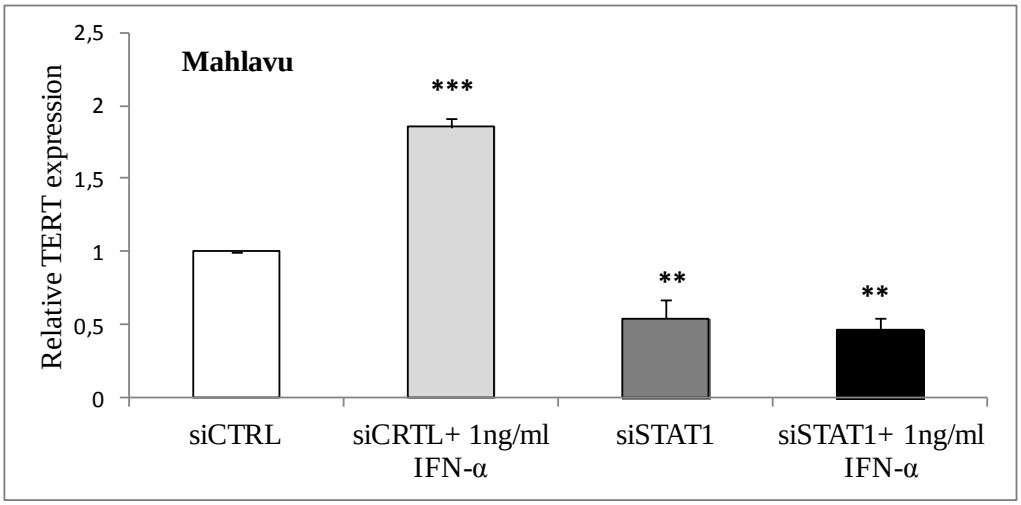

Figure3.21 TERT expression is regulated by STAT1 knock down and IFN-α treatment in Mahlavu cell line.

Mahlavu cells were transfected with 50nm of STAT1 specific siRNA or non-targeting (CTRL) siRNA. 24 h post transfection, cells were treated with 1ng/ml of IFN-α and incubated for 48 hours. After 72h, cells were harvested and qRT-PCR is performed with STAT1, TERT and GAPDH specific primers and results are normalized with GAPDH. All experiments were repeated 6 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

3.5.3 TERT is not regulated by STAT1 knock down in PLC cell line

TERT expression is downregulated upon STAT1 knockdown in HepG2 (C228T) and Mahlavu cells (C250T). And these cells fail to increase TERT expression when treated with 1ng/ml INF-α after STAT1 knockdown. Now, we will use the PLC cell line, WT for *TERT* promoter mutations to see whether STAT1 knockdown effects TERT expression in this cell line or not. We already showed that IFN-α treatment did not change *TERT* expression in PLC cell line because of the wild type *TERT* promoter, here we will try to recapitulate this finding by showing that TERT expression will not change after STAT1 knock down as well. We performed STAT1 knock down in PLC cells and checked TERT expression via q-RT-PCR. In Figure 3.22, relative expression levels of STAT1 and TERT are shown. STAT1 knockdown is performed successfully and TERT expression does not change upon STAT1 knock down. Since PLC is WT, STAT1 cannot bind to *TERT* promoter thus it is expected that TERT expression is not affected by the downregulation of STAT1.

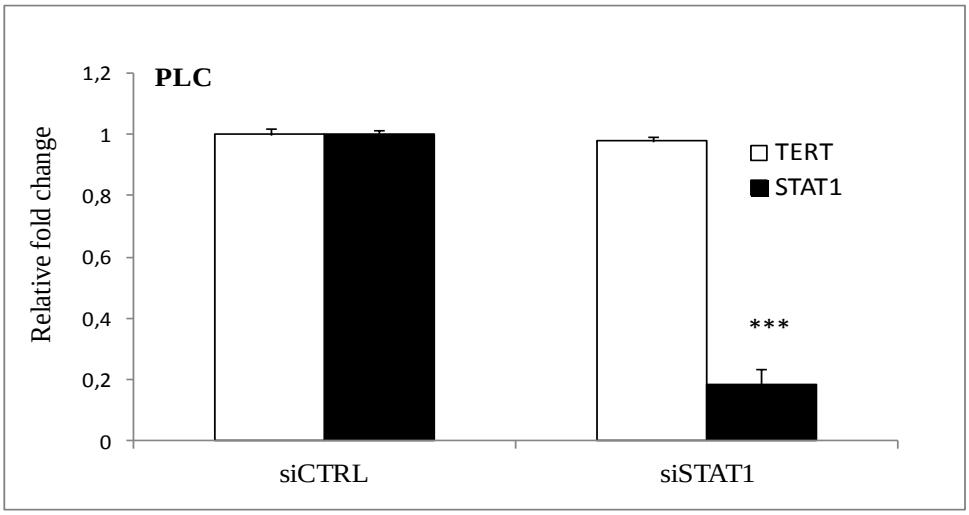

Figure3.22 TERT expression does not change in response to STAT1 knockdown in PLC cell line.

Cells were transfected with 50nm of STAT1 siRNA or CTRL siRNA for 72 hours, cells were harvested and qRT-PCR was performed. Results were normalized with GAPDH. All experiments were repeated 6 times and P-values were calculated by comparing experimental group with the control. Not significant if $P > 0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; *** if $P \leq 0.001$ and **** if $P \leq 0.0001$.

Chapter 4

Discussion

Telomerase reverse transcriptase (*TERT*) gene encodes for the catalytic subunit of telomerase enzyme which is responsible for the replacement of telomeric DNA repeats by using its RNA subunit of telomerase (TERC) [103]. *TERT* expression is critical for the maintenance of telomeres, yet it is not expressed in most somatic cells including hepatocytes[193]. In somatic cells, telomeres get shorter during successive cell divisions; once they become critically short, telomeres trigger replicative senescence which is a permanent cell cycle arrest. *TERT* expression is a constraint for the immortality of cancer cells, they need to overcome the telomere shortening problem and avoid replicative senescence to be able to divide infinitely. Most HCC cells (80%-90%) reactivate *TERT* gene expression[108,110] to bypass senescence; however, mechanisms behind the activation of *TERT* gene expression are not well defined. Involvement of HBV in activation of *TERT* expression through integration of viral DNA near the *TERT* gene is reported, but it is a rare event and it only provides evidence for HCC cases with underlying HBV infection[62]. For the majority of HCC cases, the mechanism behind *TERT* reactivation is still an issue to be solved. Recently, two frequent *TERT* promoter mutations (C228T and C250T) have been detected in many cancers, melanoma being the first one to be reported [133,134,136]. *TERT* promoter mutations are counted as the most frequent genetic aberrations in many tumors; and they are suggested to have functional roles during reactivation of TERT expression in the tumors bearing them. Our work with HCC cell lines and tissues is in accordance with the previous reports showing high frequency of these mutations. *TERT* mutation frequency is 60% in HCC cell lines and this high frequency makes *TERT* one of the most frequently mutated genes in HCC together with *TP53* [27]. Therefore, it is a strong possibility that *TERT* promoter mutations

provides selectable advantage to HCC cells during the establishment of cell lines to bypass replicative senescence caused by telomere shortening during *in vitro* cell culture. Epithelial-like and mesenchymal-like HCC cell lines have very similar mutation frequencies, thus we conclude that *TERT* promoter mutations occur independently of the cells' differentiation state. Epithelial-like cell lines are representative of early HCC whereas mesenchymal-like cell lines are similar to advanced HCC cases [185,194]. High mutation rates observed in epithelial-like cell lines may indicate that *TERT* mutations occur in the early phases of hepatocarcinogenesis; this finding is also confirmed by another report [146]. *TERT* promoter mutation frequency is 34% in primary HCC tumors. HCC cell lines have a higher mutation frequency compared to HCC tumor samples. The difference between the mutation rates of cell lines and primary tumors could be caused by the selective advantage mentioned above. Moreover, the fact that *TERT* promoter mutations are heterozygous mutations could make it difficult to detect them in tumor samples because of contaminating DNA of non-cancerous tissue. Previous studies in our group identified *TERT* as one of the critical genes involved in the immortalization of hepatocytes during hepatocellular carcinogenesis [195,196]. This present study recapitulates our previous findings and defines *TERT* promoter mutations as a hallmark of hepatocarcinogenesis. Considering incidence of *TERT* promoter mutations across the world, our results indicate that *TERT* mutations are observed in HCC tumor samples independent of the geographical origin of HCC tumors. Furthermore, we have detected a higher incidence of *TERT* promoter mutations in HCC tumor samples collected from Africa. Our findings related to geographical origins of HCC tumors provide evidence about not only the suitability of these mutations to be used as universal biomarkers all over the world but also possibility of using them in high risk populations such as African an Asian populations. We also searched for a correlation between several patient characteristics and the presence of *TERT* promoter mutations in HCC tumors. HCC tumors with HBV involvement carried less *TERT* promoter mutations compared to the ones without HBV, but this difference did not reach significance, Tumors carrying *TP53* mutations had a higher *TERT* promoter mutation frequency (50%), compared to the tumors with wild type

TP53 (29%); however, this correlation was not significant as well (P = 0.280). Finally, we checked *MDM2* SNP 309 polymorphism and *TERT* promoter mutation correlation and this correlation was not significant (P = 0.058). Although patient characteristics seemed to have tendencies to correlate with *TERT* promoter mutations, we could not detect any significance, probably due to low sample size. Next, we determined rs2853669 SNP status (located at -245 bp from TSS in *TERT* promoter) in HCC cell lines and tumors. There is a study showing that the variant allele (C) disrupts an existing ETS2 transcription factor binding site at the *TERT* promoter [135]; however, data about the functionality of rs2853669 is controversial [151,152]. A recent study searched for the relationship between the presence of the variant allele of rs2853669 with *TERT* promoter mutations and concluded that the presence of the variant allele neutralized the negative effects of C228T and C250T mutations on patient survival in urothelial cell carcinoma of bladder [148]. There is only one publication related to the functionality of this SNP in HCC and no significant association was detected between rs2853669 variant allele and HCC risk [188]. We detected the variant allele of this polymorphism in 40% of the HCC cell lines and there was no correlation between the presence of the variant SNP allele and *TERT* promoter mutations (*P*= 0.16). Variant allele frequency was 38.63% in the patient tumors. Among tumors carrying variant allele, 3 carried C228T or C250T mutation; while 14 of them were wild type for *TERT* promoter mutations. This result could indicate that tumors with *TERT* promoter mutations have a lower frequency of rs2853669 variant allele, but we could not find a significant correlation to support it (*P*=0.064). Patient survival data was not available for the tumors tested, thus we could not perform an analysis to determine the risk associated with the presence of the variant allele with or without the C228T or C250T mutations in our samples. In summary, we found that C228T and C250T *TERT* promoter mutations were frequent in both HCC cell lines and primary tumors; this high frequency could be the reflection of the critical role of telomerase during hepatocellular immortality and further analysis is needed to reveal the functionality of these mutations for the reactivation of TERT expression.

TERT promoter mutations are claimed to create a new binding site for ETS/TCF transcription factors and they are suggested to increase promoter activity and upregulate TERT expression by doing so [133,134]. This hypothesis is considered as the evidence for the functionality of these promoter mutations; however, there is still no experimental data confirming it. We aimed to search the presence of *TERT* promoter mutations in a functional manner in HCC. For this purpose, we used STAMP tool to determine the potential transcriptional factors that could bind to the mutant promoter motif and discovered STAT1 as a candidate TF. Then, we wanted to confirm our bioinformatics analysis to determine the role of STAT1 on transcriptional regulation of mutant *TERT* promoter. First, we determined STAT1 protein levels in several HCC cell lines and found that it is differentially expressed. HepG2, Mahlavu and SNU 398 cell lines had high levels of STAT1 while Huh7 and Hep3B had a lower expression and SNU 423 did not have a detectable amount of STAT1 protein. Next, we wanted to see if we could activate STAT1 signaling through IFN-α treatments in HCC cell lines. We treated HepG2 (C228T), Mahlavu (C250T), PLC (WT) and SNU 449 (WT) cells with different IFN-α concentrations and showed that even doses as low as 1ng/ml of IFN-α could trigger phosphorylation of STAT1 in these cell lines. In order to show that STAT1 was transcriptionally active in these cell lines we determined the variation in expression levels of a well known STAT1 target, IRF1, in several HCC cell lines after different doses of IFN-α. Our results showed that IRF1 expression was upregulated upon IFN-α treatment in HepG2, Mahlavu, PLC and SNU 449 cells regardless of their *TERT* promoter mutation status, but we could not show an upregulation of IRF1 in SNU423 cells. This result was due to undetectable levels of STAT1 protein.

After showing that we were able to activate STAT1 signaling, we decided to study the effects of STAT1 activation on *TERT* transcription in HCC cell lines with or without promoter mutations. We hypothesized that STAT1 would only bind to mutant *TERT* promoter. Therefore, we expected to see an increase in the expression level of *TERT* gene in cell lines carrying a C228T of C250T mutation but not in the cells with wild type alleles when we activated STAT1 signaling with IFN-α treatment. We started by treating HepG2, PLC, Mahlavu, SNU423, SNU449 cells

with different doses of IFN-α for different times and checked TERT expression levels via qRT-PCR. After IFN-α treatment, *TERT* gene expression was upregulated in mutant cell lines, HepG2 and Mahlavu cells even with 1ng/ml of IFN-α; whereas expression level stayed unchanged in PLC and SNU449 cell lines since they are wild type for *TERT* promoter mutations. *TERT* expression was not regulated by IFN-α in SNU423 cells although this cell line carried C228T mutation. This result was expected due to undetectable amounts of STAT1 and unresponsiveness of this cell line to IFN-α treatment evidenced by stable IRF1 levels. In summary, TERT expression is regulated by IFN-α in mutant cell lines with intact STAT1 signaling, but not in wild type HCC cell lines. Once the critical role of IFN-α in the regulation of *TERT* gene expression in mutant HCC cells was demonstrated, we went on to establish that this finding was dependent on STAT1. For this purpose, we down regulated STAT1 with STAT1 specific siRNA and checked for *TERT* expression. We were able to show that STAT1 knockdown caused a significant down regulation (40-50%) in *TERT* expression in mutant HepG2 and Mahlavu cell lines; however, there was no change in *TERT* expression level in wild type PLC cells. We also studied the effects of IFN-α and siSTAT1 in one experimental set-up in HepG2 and Mahlavu cells. In case of siCTRL transfection, IFN-α was able to upregulate TERT expression in both cells. On the other hand, when HepG2 and Mahlavu cells were transfected with siSTAT1, *TERT* expression stayed unchanged upon IFN-α treatment. These findings indicated that STAT1 is the critical transcription factor for the regulation of *TERT* expression by IFN-α in HCC cells carrying C228T or C250T mutations. To our knowledge, we are the first to work on the TERT promoter mutations from a functional point of view and define a certain transcription factor that reactivates *TERT* expression provided that there is the C228T or the C250T mutation at its promoter region. Moreover, it is highly possible that STAT1 is not the only transcription factor in the picture since its down regulation only caused a 50% reduction in TERT transcription. Thus, there is still a need for further research to define any partners of STAT1 during regulation of *TERT* gene expression.

There is a very recent study demonstrating that *TERT* promoter mutations are very early genetic events in the course of hepatocarcinogenesis [197]. In this study, they

detected *TERT* promoter mutations occurred in 6 % of LGDN and 19 % of HGDN. The rate of *TERT* promoter mutations increased to 61% in early HCC and this high mutation rate was also detected in progressed HCC and in advanced HCC. They suggest that *TERT* mutations are key events during transformation of premalignant lesions to hepatocellular carcinoma[197]. These findings are very critical for the interpretation of our results about the effects of IFN-α on *TERT* expression in mutant HCC cells. IFN-α is used as a therapeutic agent against HBV and HCV infections before the onset of HCC in the cases with viral involvement. In conjunction with the findings of the recent study discussed above; we hypothesize that rare random mutations occur in *TERT* core promoter in some cells of the LGDNs and presence of IFN-α contributes to *TERT* reactivation in those cells by activating STAT1. The cells bearing a *TERT* promoter mutation escape senescence and start the transformation process. As they continue to divide, they accumulate more mutations and occupy a higher portion of the dysplastic nodules. This way, the percentage of cells with *TERT* promoter mutations increases as dysplastic nodules turn into early HCC. In the absence of IFN-α or STAT1 protein, alternative pathways take on their part and *TERT* is reactivated by unknown transcription factors. ETS transcription factor could be one candidate, however there is still no data proving its involvement in this process. To sum up, *TERT* promoter mutations are critical in the early genetic alterations during hepatocarcinogenesis. This fact makes them great candidates for biomarkers for the screening of patients with cirrhosis or LGDNs to determine the predisposition for HCC. Moreover TERT should be considered for targeted therapy for the prevention of liver carcinogenesis at the very early phases. IFN-α therapies should also be performed carefully considering its positive role on *TERT* transcriptional regulation. In conclusion, we analyzed *TERT* promoter mutations in HCC in a functional manner to unravel the molecular mechanism behind the possible upregulation of *TERT* expression by these mutations. This data provides insight to the contributions of *TERT* promoter mutations to cellular immortality in hepatocellular carcinoma and its potentials to be used for chemoprevention of hepatocarcinogenesis.

Chapter 5

Future Perspectives

We studied *TERT* promoter mutations in several HCC cell lines and tumor samples from different geographic regions and detected a high mutation frequency. However, we could not relate any patient characteristics such as geographical origin, sex, age, HBV involvement, *TP53* mutation status etc. with the presence of *TERT* promoter mutations. The low number of available tumor samples is the main constraint behind this. Our first perspective is to increase tumor samples for a more detailed analysis between *TERT* mutations and patient characteristics to have a deeper knowledge about any predispositions to bear these mutations. We can also analyze variant allele rs2853669 in this larger tumor sample pool to determine the relationship between *TERT* mutations, rs2853669 variant allele and patient survival. Secondly, we aim to analyze the involvement of Interferon and STAT1 in *TERT* transcriptional regulation in more detail. We may evaluate the remaining five HCC cell lines by performing the same experiments performed in our present study and compare their results with our current data. Moreover, we may treat HCC cell lines with different interferon types and study their effects on *TERT* expression accordingly. STAT transcription factor family members could be studied together with STAT1 to detect the involvement of another family member during *TERT* expression regulation. *TERT* transcriptional regulation by STAT1 will also be analyzed in more detail. Chromatin IP will be performed by using STAT1 antibody to show the binding of STAT1 on TERT promoter. This step is the ultimate step necessary for the completion of this analysis.

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Appendix A

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Curriculum Vitae and Publications

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EDUCATION

Bilkent University, Ankara, Turkey
Department of Molecular Biology and Genetics 2009-Present
PhD in Molecular Biology and Genetics

Middle East Technical University, Ankara, Turkey
Department of Biological Sciences 2004-2009
BSc in Biology

CHO Anatolian High School Mersin, Turkey
High School Diploma in Science Department 1999-2003

RESEARCH EXPERIENCE

Bilkent University, BilGen Genetics and Biotechnology Center Ankara, Turkey
2009-Present

PhD Fellow

Advisor: Prof. Mehmet OZTURK

Thesis Title: Reactivation of Telomerase Reverse Transcriptase (TERT) Gene in Liver Cancer

Institut Albert Bonniot, (INSERM/UJF U823), Team 4-Chromatin and Epigenetics; Visiting Research Fellow — Grenoble,France — 28.09.2011-

Advisors: Prof. Mehmet OZTURK and Prof. Stefan DIMITROV — 31.07.2012

Project 1: Role of H3 Histone Variant CENPA in Hepatocellular Carcinoma — 01.07.2013-

Project 2: Analysis of Histone Variants in Male Gamete — 31.11.2013

Middle East Technical University, Department of Biological Sciences
Undergraduate Researcher — Ankara,Turkey
Advisor: Prof. Inci TOGAN — 01.10.2008-
Project: TURKHAYGEN Project — 01.06.2009
(In Vitro Conservation and Preliminary Molecular Identification of Some Turkish Domestic Animal Genetic Resources-I)

TUBITAK Marmara Research Center, Genetic Engineering and Biotechnology Institute**,** Summer Intern — Gebze,Turkey, Summer 2008
Project: Evaluation of Cytotixic effects of Novel Nanotubes in Human Fibroblasts

GRANTS AND AWARDS

The Scientific and Technological Research Council of Turkey (TUBITAK) — 2009-Present
2211 - National Ph.D. Fellowship Program

French Government, Ministry of Foreign Affairs — 01.07.2013-31.11.2013
Research Fellowship — 28.09.2011-31.07.2012

WORKSHOPS AND COURSES

EBI-The Bioinformatics SLING Roadshow, Sabanci University — Istanbul, Turkey 2011
The Novartis International Biotechnology Leadership Camp (BioCamp) 2010, Novartis — Basel, Switzerland 2010
Laboratory Animals Course, Bilkent University — Ankara, Turkey 2010
Workshops on Current Trends in Molecular Nanobiosciences, Bilkent University — Ankara, Turkey 2010
Winter School on Frontiers in Nanomedicine and Nanobiotechnology, Bilkent University — Ankara, Turkey 2010
Population Genomics Workshop, Middle East Technical University — Ankara, Turkey 2008

TEACHING EXPERIENCE

Bilkent University, — Ankara, Turkey
Department of Molecular Biology and Genetics — 2012-2013 Spring
Teaching Assistant, Molecular Biology of the Cell — 2010-2011 Fall
Teaching Assistant, Biology — 2009-2010 Spring
Teaching Assistant, Fundamentals of Molecular Genetics

CONFERENCE PRESENTATIONS

Cevik, D., Yildiz, G., and Ozturk, M. Detection of Telomerase Reverse Transcriptase Promoter Mutations in Hepatocellular Carcinoma. EACR-Sponsored 2nd Anticancer Agents Congress. Poster presentation. Bodrum, Turkey, April, 2014

Cevik, D., Kutlu, F., Ozen, C., Ozturk M and Bozdag-Dundar,O. Novel piperazine containing flavone derivatives have anticancer activities evidenced by histone deacetylase inhibition. 2nd International Congress of the Molecular Biology Association of Turkey. Poster presentation. Istanbul, Turkey, November, 2013.

PUBLICATIONS

Cevik, D., Yildiz, G., and Ozturk, M. Common TERT promoter mutations in hepatocellular carcinomas from different geographical locations. *World J. Gastroenterol*. In Press

Kutlu, F.*, **Cevik, D**.*, Ozen, C., Ozturk M and Bozdag-Dundar,O. Investigation of anticancer potential and histone deacetylase inhibitory activities of novel piperazinyl flavone derivatives. In Preparation, * Authors contributed equally

Ozen, C. Yildiz, G. Dagcan, A. **Cevik, D**. and Ozturk, M. Genetics and epigenetics of liver cancer. *N. Biotechnol*. 30, 381–4 (2013).

REFERENCES

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