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Development of panel of urine DNA Biomarkers for Hepatocellular Carcinoma screening

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This is to certify that the thesis prepared by Maria Degef T/mariam, entitled: Development of panel of urine DNA Biomarkers for Hepatocellular carcinoma screening and submitted in fulfilment of the requirement for the Degree of Doctor of Philosophy complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

AFP	Alpha-fetoprotein
ALT	Alanine transaminase
APC	Adenomatous polyposis coli
ASR	Age standardised rate
AST	Aspartate transaminase
AUROC	Area under the receiver operating Characteristic curve
Axin1	Axis inhibition protein-1
BNA	Bridged nucleic acid
bp	Base pair
CBD	Carboxylated metallic beads
CK-1	Casein Kinase 1
ctDNA	Circulating tumor DNA
CTNNB1	Catenin (cadherin- associated protein) Beta -one
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
EDTA	Ethylenediamine tetra acetic acid
GSK3β	Glycogen synthase kinase- β
GSTP1	Glutathione-S-transferase pi one
HBV	Hepatitis B Virus
HBx	Hepatitis BX gene
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HMBS	Human methylated bisulfite standards
HMW	High molecular weight
hTERT	Human telomerase reverse transcriptase
LMW	Low molecular weight
LNA	Locked nucleic acid
MAPK	Mitogen activated protein kinase
MSP	Methylation Specific Polymerase Chain Reaction
NADH	Nicotinamide adenine dinucleotide

NS3	Non structural gene 3
NS5A	Non structural gene 5A
PCR	Polymerase chain reaction
Tp53	53KDa nuclear phosphoprotein
qPCR	Quantitative Real- time Polymerase chain reaction
RASSF1A	Ras associated domain containing protein one
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
SEPT 9	MLL septin-like fusion protein
SNP	Single nucleotide polymorphism
TASH	Tikur Anbessa Specialized Hospital
Tp53	Tumor protein P53
Tr-DNA	Trans renal DNA
WNT	wingless integrated

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Abstract

Background: - Hepatocellular carcinoma (HCC) is one of the most common types of cancer with an increasing incidence rate and high mortality globally. It is a malignant tumor that arises from the hepatocytes. The principal risk factors are Hepatitis B and C viruses. The five year survival rate of HCC is very low and can be maximized by early detection of the disease. The current and most widely used biomarker for HCC screening is serum Alpha Fetoprotein (AFP). However, due to the low sensitivity (40 - 60%) of this biomarker, the need to develop better and effective assays, especially for early detection of HCC is crucial. It must be noted that this study on urine DNA biomarkers is the first of its kind that was conducted in Ethiopia.

Objective:- The aim of this study was to investigate the potential use of circulation derived urine DNA biomarkers for HCC-associated mutations in the human telomerase reverse transcriptase (h*TERT)*, Tumor protein P53 (*Tp53*) and Catenin (cadherin-associated protein) Beta-one *(CTNNB1)* genes and HCC associated hypermethylation in Glutathione-S-transferases (*GSTP1),* Ras associated domain family one A (*RASSF1A*) and *SEPT9* genes detected by short amplicon quantitative polymerase chain reaction (qPCR) based assays for HCC screenings.

Materials and Methods: - Urine samples were collected from patients with HCC, cirrhosis and viral hepatitis attending Tikur Anbessa Specialized Hospital from May 2016 - May 2017. DNA was extracted using guanidine thiocyante and was further fractionated into high and low molecular weight (LMW). The LMW DNA was quantified by qPCR using human genomic DNA as standard. The mutations in the *hTERT, Tp53* and *CTNNB1* genes were quantified by qPCR using fluorescent hybridization probes. The hypermethylation of *GSTP1, RASSF1A* and *SEPT9* genes were analyzed using methylation specific PCR (MSP). Alpha fetoprotein (AFP) the gold standard serum biomarker for HCC, ALT (alanine transaminase) and AST (Aspartate transaminase) were also measured. Area under receiver operating characteristic (AUROC) curve analysis was used to assess the predictive value of the six biomarkers for HCC detection.

Result: - The AUROC curve analysis for mutation assay of *Tp53, CTNNB1* and *hTERT,* to distinguish HCC from cirrhosis and hepatitis were 0.495, 0.553 and 0.561, respectively. The

AUROC curve for the hypermethylation assay of *RASSF1A, GSTP1* and *Sptin9* were shown to be 0.535, 0.544 and 0.405, respectively. *hTERT, CTNNB1*, *RASSF1A* and *GSTP1* had a better differentiating capacity since they have 50% AUROC whereas *Tp53* and *SEPT9* had less AUROC. The distinguishing power of HCC from cirrhosis was 0.484, 0.575, 0.605, 0.546, 0.581, and 0.420 for *Tp53, CTNNB*, *hTERT, RASSF1A, GSTP1* and *SEPT9,* respectively implicating that *hTERT, RASSF1A, CTNNB1and GSTP1* had better differentiating capacity. The result obtained from the distinguishing capacity of HCC from hepatitis with the AUROC curve have also showed 0.455, 0.538, 0.529, 0,518, 0.567, and 0.392, for *Tp53, CTNNB1* and *hTERT*, *RASSF1A, GSTP1* and *SEPT9,* respectively indicating relatively better predictive potential of *CTNNB1* and *hTERT*, *RASSF1A, GSTP1* biomarkers. In parallel study using samples from United States *RASSF1A*, *GSTP1* and SEPT9 differentiated HCC from Cirrhosis and hepatitis with AUROC curve value 0.740, 0.628 and 0.559, respectively. The predictive value of HCC from cirrhosis with AUROC curve analysis was 0.737, 0.604 and 0.587 for *RASSF1A*, *GSTP1*, and *SEPT9*, respectively. The predictive values of HCC from hepatitis were also 0.746, 0.672 and 0.529 for *RASSF1A*, *GSTP1* and *SEPT9*, respectively. In all cases, *Tp53* and *SEPT9* shown to have relatively low differentiating capacity as indicated by the AUROC. AFP in all the cases had around 70% ability in distinguishing HCC from Hepatitis and Cirrhosis. This study therefore indicated that the biomarkers from US had better predictive potential than Ethiopian Biomarkers.

Conclusion: - Most of the biomarkers used in this study were able to differentiate HCC from cirrhosis and hepatitis with close performance to samples from the US. The result obtained from this study is a proof of concept for potential use of these biomarkers in the Ethiopian population. This might require further investigation on large set of samples and may require customized approach of the assay that would fit best to the Ethiopian populations in order to get a better sensitivity, specificity and predictive potential. The knowledge gained from such study could be employed in the early detection, screening, diagnosis, treatment and counselling of hepatitis, cirrhosis and liver cancer patients.

CHAPTER ONE

1. Introduction

Cancer is a disease in which cells lose their normal checks on cells proliferation. It is one of the deadliest diseases in humans in which cells undergo multiple genetic and epigenetic changes leading to uncontrolled growth (Jones and Thompson, 2009). Among the diverse cancer types, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer related death (GLOBOCAN, 2012). The incidence of HCC is rising in alarming rates and has become a public concern internationally. Its incidence and mortality rates are more frequent in developing countries than the developed ones with respect to geographical variation, ethnic disparities and socioeconomic status (Hamid *et al.,* 2013). According to the Recent data from Centre for Disease Control and Prevention (CDC, 2018), liver cancer deaths in adults have shown to reach 43% between 2000 and 2016.

Many risk factors have been identified as capable elements for the development of HCC including chronic infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV), in which these viral infections attributed to HCC development in more than 80% of cases worldwide. The proportion of cases of HCC associated with HBV infection has been estimated, and in Africa and East Asia alone, the largest attributable fraction is due to hepatitis B (60%), whereas in the developed western world, approximately 20% of cases are attributed to HBV infection. The other factors are cirrhosis, heavy alcoholism, cigarette smoking, non-alcoholic fatty liver disease, presence of diabetes, obesity, dietary aflatoxin B1, some inherited disorders like hemochromatosis, alpha1-antitrypsin deficiency and glycogen storage disease type 1 and host genetic factors (Hussain *et al*., 2007; Adrian and Bisceglie, 2009; Hennedige, and Venkatesh, 2012; Mittal and El-Serag, 2013).

Aflatoxin B1 is a carcinogens produced by *Aspergillus* species. In the liver, cytochrome p450 enzymes metabolizes aflatoxin into a mutagenic intermediates namely aflatoxin B1-8, 9 - exo-epoxide which is converted to 8,9-dihydroxy-8-(N7) guanyl-9-hydroxy aflatoxin B1 adduct which in turn hydrolyzed and converted to aflatoxin B1-formaminopyrimidine adduct This adduct reacts with tumor suppressor gene *Tp53* and causes guanine (G) to thymine (T) transversion mutation and contribute to hepatocarcinogenesis. Aflatoxin acts also synergistically with HBV and cause HCC (Kew, 2013; Chawanthayatham *et al.,* 2017).

In regard to gender related studies, men are at approximately three times higher risk of devloping the disease than women. These differences might be explained by behavioral and environmental exposures such as alcohol use and level of consumptions. Additionally, the level of male and female sex hormones (estrogens prevent and androgens promote liver cancer) have shown to have considerable association with the disease. Estrogens may act as general suppressors of HCC through its anti inflammatory effect whereas androgen pathway has shown to increase the transcription of HBV genes by binding directly to viral genome (Keng *et al.,* 2011; El-Serag and Kanwal, 2014).

Alcohol consumption will lead to alcoholic liver disease that is characterized by fat accumulation and inflammation which lead to cirrhosis which consequently leads to HCC. Metabolism of alcohol by alcohol dehydrogenase and microsomal cytochrome P450 leads to enhanced production of reactive oxygen species (ROS) that affect various signaling pathways (Osna, 2011). Chronic inflammation also results in increased production of ROS which can cause DNA damage and leads to gene mutations. Increased intracellular ROS levels can also activate several signal transduction pathways that regulate proliferation, differentiation and apoptosis. Inflammation induced oxidative stress and influx of kupffer cells can promote the activation of stellate cells that produces extracellular matrix in the liver. Their persistent activation can finally lead to cirrhosis, which is characterized by the co-existence of regenerative nodules, irreversible fibrosis and severe liver injury. Cirrhosis is an important predisposing state for HCC development. About 80 - 90% of HCCs of all etiological backgrounds arise in cirrhotic livers (Chemin and Zoulim, 2009; El-Serag and Davis, 2011; Fox, 2013; Nault *et al.,* 2013). In HBV endemic areas, infected patients with cirrhosis have an approximately 3-fold higher risk for HCC than those with only chronic hepatitis B, and a 16-fold higher risk than asymptomatic carriers. The incidence of HCC development is approximately 3–5% per year in HBV associated cirrhosis (Chemin and Zoulim, 2009).

With the standardization of effective methods or assays, liquid biopsy (plasma, serum, urine) biomarkers alone or in combination with conventional serum biomarkers like AFP might serve as a promising diagnostic, prognostic, therapeutic monitoring and risk assessment for HCC (Rojas *et al.,* 2018). However, it must be noted that this research on urine DNA biomarkers is the first of its kind that was conducted in Ethiopia and the aim of this study was to investigate panel of urine DNA biomarkers using *Tp53*, h*TERT*, *CTNNB1, RASSF1A,*

*GSTP1*and *SEPT9* genes for early detection and screening of HCC. Therefore, it has been hypothesized that tumor specific mutation and hypermethylation can be detected in the circulatory urine DNA of patients with hepatitis, Cirrhosis and HCC.

1.1 Literature review

1.1.1 Hepatocellular Carcinoma and Hepatitis

Hepatocellular carcinoma is a malignant tumor that arises from the hepatocytes, with one of the highest mortality rates and shortest survival period of all cancers. Hepatitis is an inflammation of the liver, most commonly caused by a viral infection. There are at least 10 different genotypes of hepatitis named from A to J (Suhail *et al*., 2014). Of these genotypes, HBV and HCV infections account for a substantial proportion of liver diseases in the world. These viruses are responsible for liver damages ranging from minor disorders to liver cirrhosis and HCC (Ayele and Gebreslassie, 2013).

Hepatitis B virus is a partially double stranded DNA virus and is a member of the Hepadnaviridae virus family. The viral genome is 3.2kb in length and contains a small covalently closed circular DNA (cccDNA) that is transcribed to generate four known transcripts of 3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb size. These transcripts encode to generate Polymerase, HBcAg, HBeAg, and HBsAg and hepatitis BX gene (HBx) with a defined role in HBV life cycle and liver injury (Suhail *et al*., 2014).

Hepatitis B virus can deregulate the proliferation, differentiation and viability of liver cells by synthesizing some of its own proteins and inducing genetic alterations in the host. It also inhibits immunological surveillance and mediate low activity of intrahepatic natural killer cells. HBV protein S, C, P and X are responsible for viral replication and activation of several cell signaling pathways. Of all the HBV genes, HBx is the most critical carcinogenic component. HBx comprises 452 nucleotides that encode 154 amino acids with a mass of 17KDa. It is a multifunctional regulatory protein that regulates and modulates transcription of several genes involved in signal transduction pathways, cell cycle progression, protein degradation, apoptosis and genetic stability by interacting with host transcription factors. Activation of these signaling pathways may contribute to HBx protein-mediated hepatocellular carcinogenesis (Aguilar-Olivos *et al*., 2013).

Researchers have revealed two possible mechanisms by which HBV contributes to the development of HCC. The first one is the integration of the viral genome into the host chromosome that results in silencing of tumor suppressor gene functions and activation of oncogenes. The other mechanism engages the expression of trans-activating factors derived from the HBV genome, which have the potential to influence intracellular signal transduction pathways and change host gene expression (Chemin and Zoulim, 2009; Aguilar-Olivos *et al*., 2013).

When it comes to HCV the virus has little potential for integration into the host genome, but it is more associated with chronic inflammation, fibrosis, cirrhosis and subsequently to HCC. The mechanism of HCV associated HCC occurs by activation of oncogenes and inactivation of tumor suppressor genes (Selimovic *et al.,* 2012). In Addition, HCV proteins interfere with the intracellular signal transduction pathway and also encodes a single polyprotein that can be cleaved into 10 mature proteins; among which the core, non structural gene 3 (NS3) and non structural gene 5A (NS5A) have shown to possess an oncogenic potential that result from interference of viral proteins with cellular proteins (Selimovic *et al.,* 2012; McGivern and Lemon, 2011).

Direct binding of HBx has also been reported with p53, DNA repair protein DNA damage binding protein-1 (DDB1), transcription factor II H (TFIIH) which in turn affects DNA repair functions and may allow the buildup of genetic mutation (Suhail *et al*., 2014). For example, the HBx gene expression is associated with activation of the Ras-Raf-Mitogen activated protein kinase signaling pathway that is essential cellular pathway that has been implicated in hepatocarcinogenesis (Figure 1) (Adrian and Bisceglie, 2009). Abnormal activation of many molecules in various signalling pathways contributes to the progression of liver cancer. There are many other cell signaling pathways involved in liver cancer, including Notch signaling pathway, IGF/IGFR signaling pathway, HGF/c-Met signaling pathway and EGFR signaling pathway (Aravalli *et al*; 2013).

Epigenetic modifications induced by HBx protein consist of changes in DNA methylation pattern. Inactivation of tumor suppressor genes occurs as a result of hypermethylation of DNA. Malignant transformation may also result from chromosomal instability caused by overall hypomethylation of DNA. Telomerase activity appears to play an important role in HCC since 80% of HCCs show high levels of telomerase activity because of expression of

telomerase reverse transcriptase that result in chromosomal instability and malignant transformation (Aguilar-Olivos *et al*., 2013).

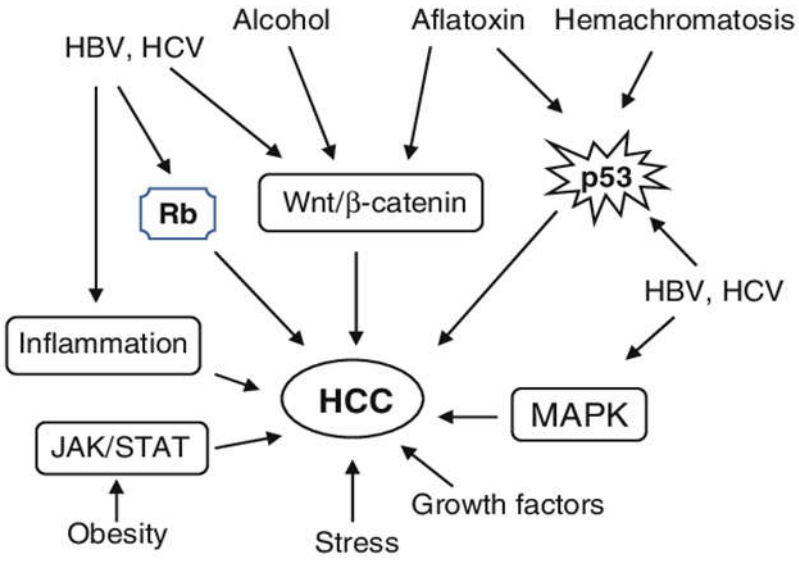

Figure 1. Signal transduction pathways shown to be involved in HCC with associated risk factors. Mitogen activated protein kinase (MAPK), Janus Kinase /Signal transducer and activator transcription proteins (JAK/STAT), the p53 pathway that play a role during oxidative stress, the retinoblastoma (Rb) pathway that control cell cycle and the wnt/β-catenin pathway (Aravalli *et al*; 2013).

Inflammatory processes and epigenetic deregulation are early events in hepatocarcinogenesis. Epigenetic alterations might occur as early events in carcinogenesis and even in precancerous stages and might precede genetic alterations during oncogenic transformation. Aberrant hypermethylation have been found in tumor suppressor genes or other cancer associated genes in precancerous lesions in liver, such as fibrosis and cirrhosis. The cellular pathways that are activated during a prolonged inflammatory response may trigger a wide range of potentially harmful processes, such as induction of DNA damage through ROS accumulation and hence acquisition of mutations and cancer cell growth advantages, that promote initiation and progression of HCC (Martin and Herceg, 2012).

1.1.2 Detection of HCC using biomarkers

The diagnosis of HCC is commonly performed using the serum biomarker AFP, radiographic imaging, and liver biopsy. AFP, the current gold standard serum biomarker, is a 70-kD

glycoprotein consisting of 591 amino acids with carbohydrate residues. There are 11 AFP isoforms identified based on variations in the terminal carbohydrate residue. It is highly elevated in the circulation of newborns with concentrations decreasing in the next 12 months to 10-20 μg/L. Serum elevations after that suggest underlying pathology which may be malignant. The cut off value of AFP for a positive test result of HCC is > 20 μg/L. AFP is also found to be raised in 20% of chronic hepatitis and 40% of cirrhosis patients (Bialecki and Di Bisceglie, 2005). Another researcher also mentioned that AFP is elevated in about 60% of patients with HCC, and in 10% to 33% of patients with chronic liver disease without HCC (Davis, 2011). The sensitivity of the AFP for detection of HCC reported by many researches varies around a value of approximately 40 - 65%, hence, limiting it applicability for diagnosis (Bialecki and Di Bisceglie, 2005; El-Houseini *et al.,* 2005; Jain *et al.,* 2012).

The current imaging modalities such as ultrasound (US), computed tomography (CT)-scan and magnetic resonance imaging (MRI) are very expensive. Moreover, these instruments are capable of picking the HCC only when the cancer is at least 2 cm. Additionally, there is a huge risk factor of cumulative radiation exposure from multiple scans (Hennedige, and Venkatesh, 2012). When it comes to tumor tissue biopsy which is meant to be the gold standards to assess the molecular alteration has also its own limitations. One reason is that it doesn't reveal the complete genomic landscape of the whole patient's tumor cell population because of the intra-tumoral genetic heterogeneity. It might also result in complication in some advanced stage cancer and it might not allow repeated assessment of genomic profile (Vendrel *et al.,* 2017; Rojas *et al;* 2018).

Advances in molecular sciences and technologies provide tools needed for investigation of tumor biology and develop molecular biomarkers that can be used to provide accurate diagnosis, prognosis and response to therapy. Currently, cancer is a global burden and assessment of biomarkers is crucial for early diagnosis and it is a huge concern in the medical society. One of the current molecular techniques used in the detection of cancer is Real-time quantitative PCR that play an increasingly important role in clinical testing due to its efficacy in providing information about gene expression, gene amplification or loss, point mutations and helps in investigating the genetic and epigenetic causes of cancer. Moreover, the technique is efficient in analyzing multiple genes simultaneously within a single reaction. In addition, it can be applied to detect and quantify viral causes of cancer, such as Hepatitis

virus and human papillomavirus. The use of real-time PCR for molecular diagnosis is attractive because it is rapid, simple, versatile, and cost-effective and can be multiplexed and can be performed on small amount of blood, tissue and urine samples (Bernard and Wittwer 2002).

In the past, the molecular technique used to detect mutation had shown to have a number of inconveniences such as difficulty in designing the assay, time consuming and mostly gel based methods which are labour intensive. Recently a simple, sensitive and accurate PCR based high resolution melting curve analysis has been developed which is a very beneficial technique in molecular diagnostic and genetic analysis (Tindall *et al.,* 2009). Mutation analysis that uses fluorescently labelled oligonucleotide probes is performed immediately after PCR amplification followed by monitoring of the change in fluorescence by real time PCR quantification (Pryor and Wittwer, 2006).

The current molecular targeted therapy for HCC is Sorafenib, which was the only Tyrosine kinase inhibitor approved by the United States (US) Food and Drug Administration and have been used for years. Previously many molecularly targeted Biomarkers that had been at different stages of clinical trials for HCC therapy have failed. However, in the past couple of years, Sorafenib, with limited efficacy, still remained as the chemotherapy for HCC after its approval almost 10 years ago. But, recently in April 2017, Sorafenib has been followed by approval of other drug. The U.S. Food and Drug Administration approved another drug regorafinib and Nivolumab to include treatment of patients with hepatocellular carcinoma. Sorafenib is a multikinase inhibitor targeting the Raf serine/threonine kinases that regulate cell proliferation, the vascular endothelial growth factor receptor 1-3 (VEGFR 1-3) that regulate angiogenesis and platelet-derived growth factor receptor (PDGFR)-b, stem cell factor receptor (c-Kit), fms-like tyrosine kinase-3 (FLT-3), and p38 tyrosine kinases. It was the first approved molecular targeted agent that demonstrated survival benefits in patients with advanced HCC in 2007 (Zhu, 2012; Ma *et al*., 2014; Deng *et al*., 2015; Colagrande *et al.,*2016; Bruix *et al.,* 2017).

As a result of physiological and pathological processes, an estimated amount of 10^{11} - 10^{12} cells die in human body per day. Cell death is normally accompanied by DNA degradation, and part of the degradation products gets into the blood stream and named as cell free circulating DNA (cfDNA). The presence of cell-free DNA in blood plasma was first

discovered in 1948 by Mnadel and Metais. Recent advances in the field have indicated that circulating tumor DNA (ctDNA) has huge potential to serve as a biomarker for early detection and precision treatment as well as prognosis of cancer like HCC. ctDNA carries tumor-specific genetic or epigenetic alterations offering a unique opportunity for monitoring tumor genomes in a non-invasive, convenient and accurate manner. These circulating DNA fragments from blood cross the kidney barrier and gets into the urine. This Transrenal DNA (Tr-DNA) contains sequences from cells that have been degraded and circulated throughout the body, including tumor cells (Su *et al.,* 2004; Lichtenstein *et al*., 2006; Tang *et al* 2016; Rojas *et al;* 2018).

Many researchers used to detect cancer specific mutation from tissue and blood. But recently there is an increasing interest in detection of tumor specific mutation in DNA that is isolated from urine. Urine contains circulation-derived low molecular weight (LMW) DNA fragments that are mostly between 150 to 250 base pairs (bps) and high molecular weight (HMW) DNA, greater than 1000 bps that comes from the urinary tract. The LMW urine DNA contains DNA from tumor tissues when tumors are present. The tumor derived DNA fragments called circulating tumor DNA (ctDNA) offers the potential to develop urine tests for the detection of the cancer with known DNA biomarkers. The LMW urine DNA has a better assay sensitivity than total urine DNA for detection of circulation derived DNA biomarkers for cancer (Su *et al.,* 2004; Lin *et al*., 2011).

Tumor specific sequences were detected in DNA isolated from urine. Sample collection of urine is non-invasive, and isolation of DNA from urine is technically easier than from serum or plasma, due to the low protein content of urine. Thus, urine could be a useful source of circulating DNA for molecular diagnosis and prognosis. Hence it is very important to characterize the nature of the nucleic acid recovered from urine, as compared to circulating DNA in the bloodstream (Su *et al,* 2004).

Analysis of circulation derived urine DNA has many advantages over existing technologies like plasma DNA analysis. It is absolutely noninvasive, it can be easily obtained for analysis, Isolation of DNA is much more easier, as a whole urine DNA analysis might have ranges of potential applications in a very broad area of molecular and diagnostic testing including prenatal detection of inherited disease, tumor diagnostic, therapeutic monitoring, and

transplant medicine (Botezatu *et al.,* 2000; Umansky and Tomei, 2006; Su *et al.,* 2008; Bauer and Pertl, 2009).

Different researchers have shown that the potential use of urine DNA as a biomarker for detection of mutation that are responsible for the development of cancer such as liver cancer (Lin *et al.,* 2012), colorectal cancer (Su *et al,* 2004) and pancreatic cancer (Botezatu *et al.,* 2000).

1.1.3 Genetic and Epigenetic alterations in HCC

The molecular mechanisms leading to the development of HCC are extremely complicated and involve prominent genetic and epigenetic alterations (Pogribny *et al.,* 2012). Genetic and epigenetic studies may help to understand the mechanism of hepatocarcinogenesis better and provide new tools for early detection, diagnosis and therapy. Genetic alterations in specific genes in HCC have been identified with the most frequently mutated genes to be *hTERT*, *CTNNB1* and the tumor suppressor *Tp53* genes. Recently, in addition to the well known genes, next generation sequencing studies with whole genome sequencing and whole exome sequencing approaches revealed a large number of genetic alterations. These are 11731 nucleotide substitutions, 670 insertion and deletions, and 22 chromosomal rearrangement (Chaiteerakij and Roberts, 2012; Kan *et al*, 2013; Nault *et al.,* 2013; Ozen *et al.,* 2013). Epigenetic alteration is an important mechanism of carcinogenesis that involves both global hypomethylation and gene specific DNA hypermethylation (Um *et al.,* 2011). It is now believed that epigenetic defects emerge at an early stage during hepatocarcinogenesis (Nishida and Goel, 2011).

Aberrant methylation of CpG islands has been documented as a relatively frequent event in human cancer and has been associated with transcriptional inactivation of tumor suppressor genes. Changes in DNA methylation is an emerging molecular markers for tumor diagnosis, prediction of tumor progression and prognosis. In HCC aberrant hypermethylation occurs in genes such as *GSTP1*, *RASSF1A*, Adenomatous polyposis Coli (*APC)* and others have been detected using MSP, combined bisulfite restriction analysis, and bisulfite sequencing techniques (Song *et al,* 2013). MSP is a sensitive and specific technique that has become an important tool for understanding of both normal and pathologic gene expression events by mapping of methylation patterns of CpG Islands. It needs bisulfite-modified DNA and

primers to distinguish methylated from unmethylated DNA and taking advantage of the sequence differences resulting from bisulfite modification (Herman *et al.,* 1996). Combinations of different methylation markers are proposed by researchers to increase the sensitivity and specificity of the diagnosis of HCC (Hua, 2011) and his colleagues reported that combination of genes *RASSF1A,* cyclin D2 (CCND2) and serine peptidase inhibitors, kunitz type 2 (SPINT2) showed a sensitivity of 89% and specificity of 90% for discrimination of HCC from non-HCC tissues. The other important issue is epigenetic abnormalities that are reversible compared to gene mutation and can be used in designing of epigenetic therapy of cancer by using small molecular inhibitor like demethylating agents (Perret, 2011).

1.1.3.1 Genetic alterations

A. *Tp53* gene and HCC

The gene *Tp53*, located on 17p13.1, is a transcription factor that functions in a complex signalling network to mediate cellular adaptation to stress (Figure 2). It can be induced by diverse cellular injury including genotoxic damage, activation of oncogenes, inactivation of tumor suppressor genes, telomerase erosion, loss of stromal support and nutrient and oxygen deprivation resulting in expression of genes that induce apoptosis, senescence, cell cycle arrest and changes in metabolism. *Tp53* also acts as antioxidant by *Tp53* dependent activation of the expression of genes that helps to lower intracellular ROS levels protecting cells from cancer development and aging. It is the most common cancer associated gene that is mutated in about half of all human cancer (Gottlieb and Vousden, 2010; Jones and Thompson, 2009). Liver carcinogenesis is a multistep and multifactorial process driven by genetic or epigenetic damage in susceptible cells. Mutation of the *Tp53* gene has been shown to be a major factor leading to the development of HCC. However, the *Tp53* mutation spectrum is significantly different in HCCs for different geographic regions (Qi *et al.,* 2013).

The carcinogenic component, HBx has been shown to interact with *Tp53;* thereby inactivating several critical *Tp53*-dependent activities, including *Tp53* mediated apoptosis, transactivation properties of *Tp53*, regulation of cell cycle, DNA repair genes and tumor suppressor genes. Apoptosis is one of the mechanisms by which HBx protein induces HCC, as HBx has both antiapoptotic and proapoptotic effects. It inhibits *Tp53*-mediated apoptosis

by forming a complex with *Tp53* protein in the cytoplasm, preventing *Tp53* entry into the nucleus and disrupting protein–protein interactions with other factors in the apoptotic pathway. HBx protein also prevents *Tp53* induction by inactivating caspase 3 or in association with the HRas oncogene by activation of the phosphatidyl inositol-3 kinase and the serine threonine kinase (Akt) pathway. The antiapoptotic protein, survivin expression is also upregulated by the HBx protein (Kremsdorf *et al.,* 2006; Aguilar-Olivos *et al.,* 2013). Researchers pointed out that HBx protein works in collaboration with cellular transcription factors and induce transformation in liver cells causing tumors or cancer. Thus, expression of HBx gene in hepatocytes is a positive hallmark of HCC (Ayub *et al*., 2013).

Even if there are many different mutations that are found in *Tp53* gene of HCC patients, there is a hotspot mutation that is found in almost all of them. This mutation is a missense that results from a guanine to thymine (G-T) transversion at the third position of codon 249 (249T) of the exon 7 of the gene. Approximately 50% of patients with HCC have this *Tp53* hotspot mutation; in some patients with HCC, this *Tp53* hotspot mutation is also found in the circulation. Hence, the *Tp53* 249T hotspot mutation could possibly be a DNA marker for HCC screening (Bressac *et al.,* 1991; Jackson *et al.,* 2003). Qi and his colleagues (2013) pointed out that there is significant association between *Tp53* mutation and carcinogenesis of HCC even they associated it with aflatoxins. They sequenced the eleven exon of *Tp53* and got mutation in exon 4, 5, 6, 7, 8 and 9 with the most frequently occurring mutation in exon 7 in codon 249 that changes Arginine to serine (R249S).

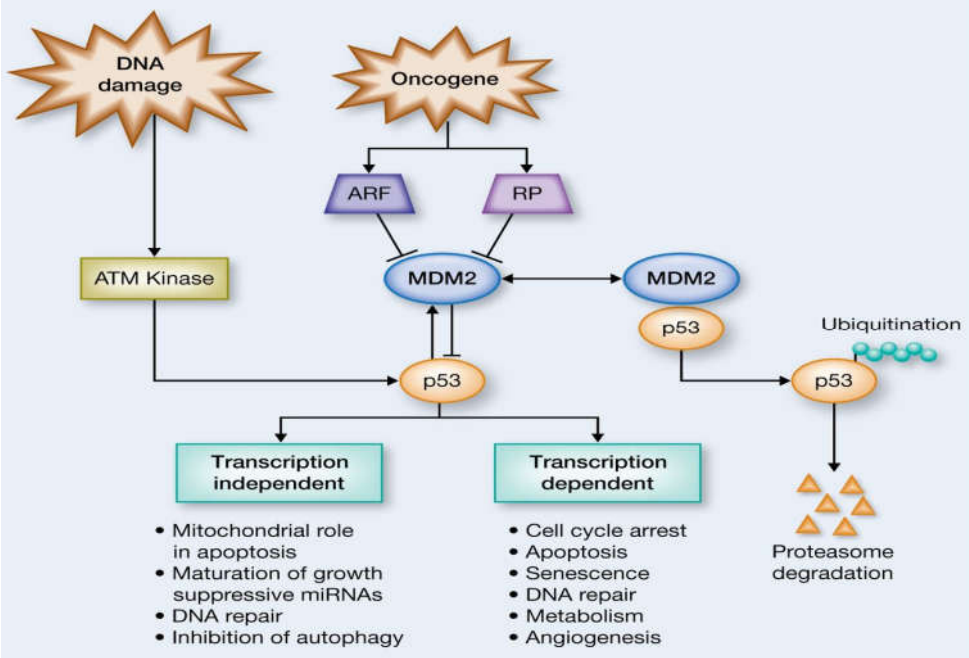

Figure 2. p53 - MDM2 pathway

Mouse double minute 2 homolog (MDM2) inhibits p53 by stimulating its degradation and blocking its transcriptional activity and in turn p53 activates the expression of MDM2. Oncogenes can activates p53 through p14ARF and/or Ribosomal protein (RP) form complex with MDM2 initiating p53 dependent cell cycle arrest. DNA damage induces Ataxia Telangiectasia mutated (ATM) serine/threonine kinase which in turn inhibits MDM2 and activate p53 (Meng *et al.,* 2014).

B. *CTNNB1* gene and HCC

The gene that codes for the β-catenin protein, *CTNNB1*contains 16 exons and spans 23.2kb and it has 781 amino acids. It is a downstream transcriptional activator of the Wnt/ β-catenin signalling pathway and has been mutated in about 20 - 40% of HCC. Wnt/β-catenin signalling pathway plays integral roles in the development of the embryo and as well as in the maintenance, rejuvenation and differentiation of adult mammalian tissues and deregulation of this pathway results in the development of cancer (Huang *et al.,* 1999; Chen *et al*., 2017).

Without the ligand, Wnt β-catenin is continuously degraded by a multifactor destruction complex mainly composed of the scaffolding proteins Axis inhibition protein-1 (Axin1) and Axin2, Adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). The captured β-catenin will be sequentially phosphorylated by (CK1), followed by GSK3β. The phosphorylated β-catenin in destruction complex will be recognized

by the β transducin repeat-containing protein (β -TrCP)-dependent E3 ligase, and be degraded by the ubiquitin-proteasome pathway (Chen *et al*., 2017).

When Wnt binds to β-catenin, the dishevelled protein inhibits GSK3β phosphorlyation activity, and the destruction complex becomes disintegrated. Subsequently, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it interacts with the transcription factor T-cell factor (TCF) 4/lymphoid enhancer-binding factor (LEF) to transcriptionally activate downstream target genes like c-MYC, cyclin D1, cytochrome-C-oxidase subunit 2 (COX2), and matrix metalloproteinase seven (MMP7) involved in the cell cycle control and proliferation (Tornesello *et al.,* 2013; Ma *et al*., 2014).

Hyperactivation of the Wnt/β-catenin signalling pathway (Figure 3) is a major contributor to the initiation and development of HCC and approximately one third of HCC patients show mutation in *CTNNB1* gene. In HBV related HCC *CTNNB1* gene mutation was found in 16% of the patients, primarily located on exon 3 on the Serine/Threonine residues that provide β-catenin phosphorylation by GSK3β at Serine 37 and Serine 33 and CK1 at Serine 45 and Threoinine 41 (Taniguchi *et al.,* 2002; Ma *et al*., 2014).

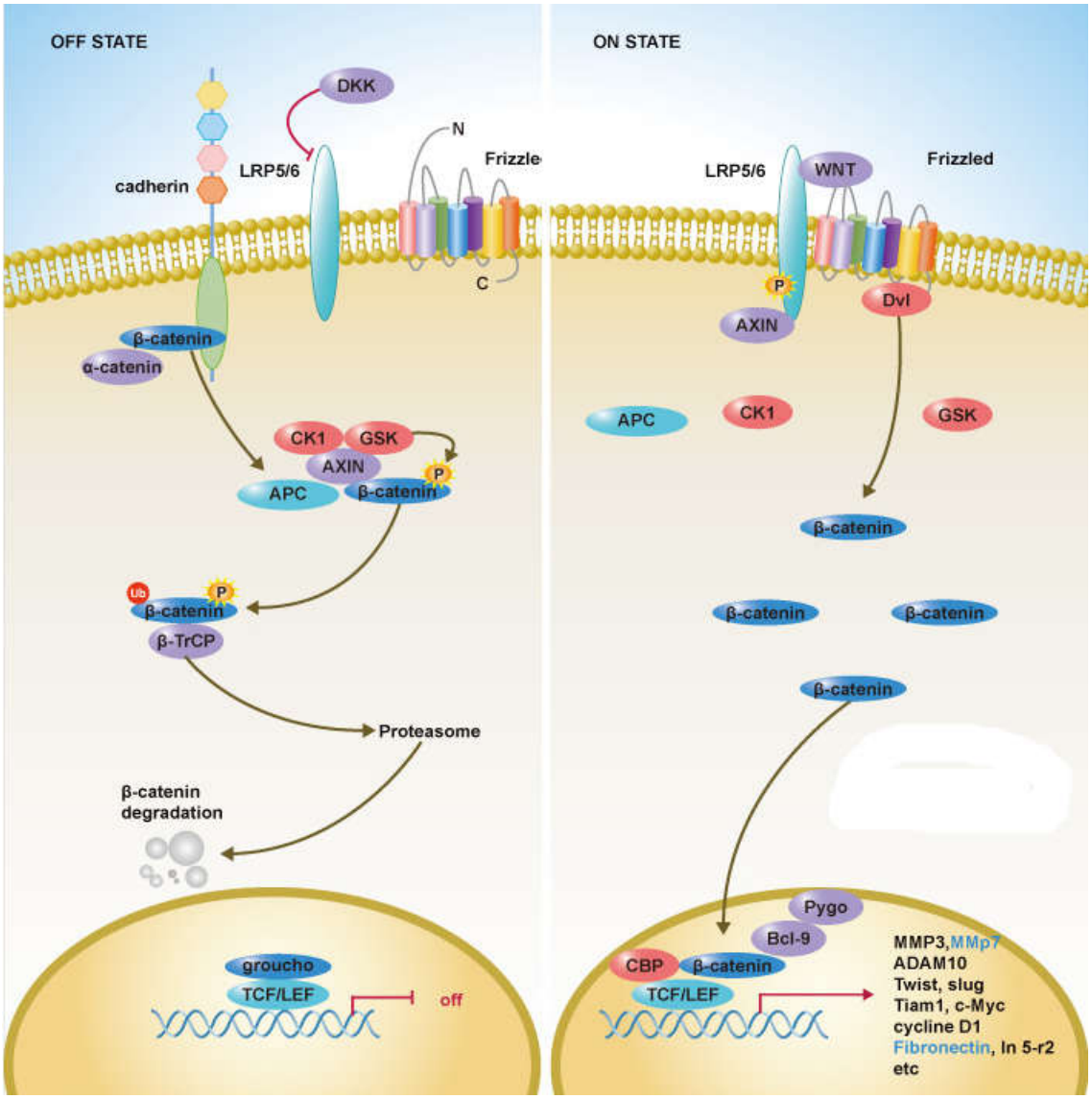

Figure 3. Wnt/β catenin signaling pathway

Wnt proteins bind to the Frizzled (Fz) receptors and low density lipoprotein receptor related protein 5/6 (LRP5/6) co-receptors, and result in destabilization of Axin and activation of β-catenin activity. In parallel, Wnt binding to Fz protein results in hyperphosphorylation of the Disheveled (Dvl) protein, which, through its association with Axin and APC, prevents GSK3β from phosphorylating β-catenin. Unphosphorylated β-catenin is stabilized by escaping the recognition of by β-TrCP, a component of an E3 ubiquitin ligase, and eventually translocates to the nucleus where it engages transcription factors LEF/Tcf-4 (lymphoid enhancing binding factor/T cell factor-4) to activate expression of downstream genes like matrix metalloproteinases (MMP3,7), ADMA10, Cyclin D1, cMyc (http://www.sinobiological.com/).

C. *hTERT* and HCC

Telomerase are proteins that are responsible for the maintenance of chromosomal integrity and genome stability. It is ribonucleoprotien that synthesize the hexameric Tandem repeats 5'-TTAGGG-3' and add them catalytically on to the 3' hydroxyl end of human chromosome to cap and protect the chromosome ends. Telomere shortens 50-200bps with each cell division resulting from incomplete DNA replication of the lagging strand and other end processing events. The shortening can be overcome by the expression of telomerase. The core enzyme contains two essential components; one of them is the catalytic subunit in human telomerase reverse transcriptase (*hTERT*) and telomerase RNA component. The *hTERT* gene consists of 16 exon and 15 introns spanning 35kb (Cong *et al.,* 1998).

In normal somatic cells *hTERT* is inactive, or has very low activity. At least 85% of human cancer cells constitutively express *TERT* that confers them an unlimited growth potential via telomerase dependent telomere elongation (Sunami *et al*., 2014). Telomere shortening has been described as a key feature of chronic hyperproliferative liver disease that contributes to the induction of genomic instability that result in HCC. Integration of HBV viral DNA into *TERT* gene has been also shown in human HCCs, indicating that the viral enhancers might increase *TERT* gene expression (Farazi and DePinho, 2006; Cevik *et al.,* 2015). *TERT* promoter mutations may work together with other oncogenic mutations, such as *CTNNB1*, to induce hepatocellular malignant transformation (Pinyol *et al*, 2014).

Mutation in the promoter region of the telomerase reverse-transcriptase and activating mutations in *CTNNB1* are significantly associated with HCC (Villanueva and Llovet, 2014, Pinyol *et al*, 2014). *TERT* promoter mutations are found in 59% of HCC and in 25% of cirrhotic macronodules with or without dysplasia. It is also the most frequent somatic genetic alteration in HCC. It is the first recurrent gene somatically mutated in preneoplastic cirrhotic lesions. The most frequent mutations were located at -124 bp from the ATG start site, they consist of G to A (124G>A, or G to T (124G>T) substitutions and the second hot spot was situated at -146 bp from the ATG and characterized by G to A substitutions 146G>A, (Figure 4) (Nault *et al.,* 2013; Quaas *et al*., 2014). Other study on *TERT* promoter from HCC have showed that tumors from Africa has the highest mutation frequency (53%) followed by tumors from Europe (30%) and Asia (21%) but they have seen mostly C228Tand C250T mutations. *TERT* promoter mutation is the most frequent genetic alteration in HCC that may

serve not only as novel diagnostic biomarkers but also as potential therapeutic targets for HCC (Cevik *et al.,* 2015). Jiao and colleagues have also found *hTERT* promoter mutation in circulating cfDNA of HCC and cirrhosis patients and showed its promising risk prediction potential for HCC (Jiao *et al.,* 2018).

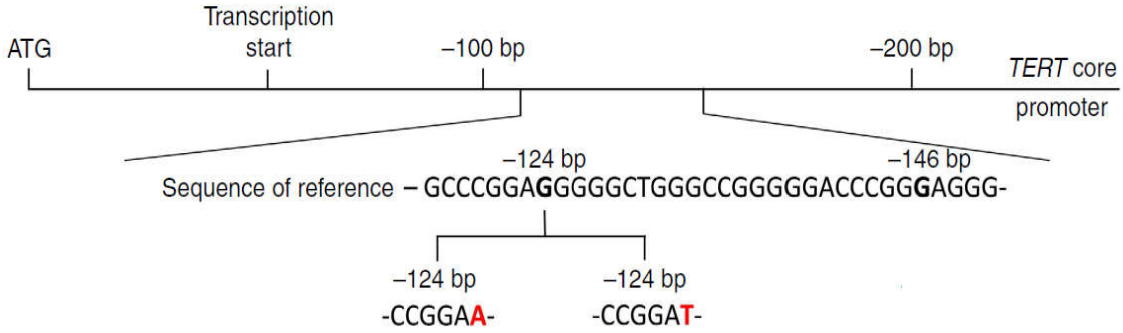

Figure 4. Schematic representation of *hTERT* promoter mutation located in -124 and -146 bp upstream from the transcription start site in human liver carcinogenesis (Nault *et al.,* 2013).

1.1.3.2 Epigenetic alterations

A. *RASSF1A* gene and HCC

Ras associated domain family one A (*RASSF1A*) is a multifunctional tumor suppressor gene located on chromosome 3. It is one of the eight (A-H) isoforms of *RASSF1*gene and it spans around 11Kb and has eight exons and two different promoters with two associated CpG islands. The protein contains 340 amino acids with cysteine rich domain. *RASSF1A* has a major effect on many cellular processes that are important for normal growth control. Such as cell cycle progression and apoptosis (Figure 5). It also protects cells from genomic instability and transformation by stabilizing the microtubules. Its expression is frequently silenced and epigenetically inactivated in cancer and is associated with increased metastasis (Donninger *et al.,* 2007; Arnette *et al.,* 2014). *RASSF1A* has a Ras association domain like that of Ras effectors; it heterodimerizes with the Ras-GTP binding protein Nore1; and it is predicted to exert its function through a Ras signal transduction pathway (Mathe, 2004).

In the cell cycle, at the G1-S checkpoint *RASSF1A* modulates the levels of cyclin D1. It could also affect the G1 transition by interacting with the transcription factor $p120^{E4F}$ which can negatively regulate the transcription of cyclin A2, leading to cell cycle arrest in G1 phase.

RASSF1A can also modulate with tubulin dynamics that can help to control cell motility and invasion. It also modulate apoptosis and interact with Microphge stimulating protein (MST1) and MST2 that are pro-apoptotic serine/threonine kinases and activate the SAPK-JNK signaling pathway and phosphorylate histone H2B. They bind directly to *RASSF1A* and other RASSF family members via their SARAH motifs (Donninger *et al.,* 2007).

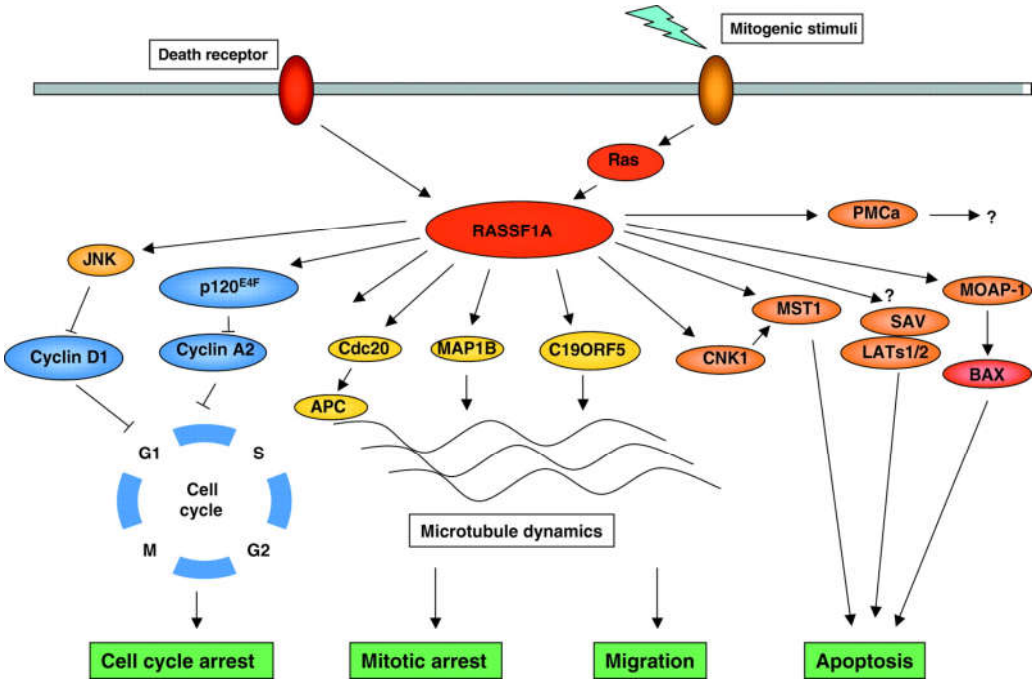

Figure 5. *RASSF1A* Signalling Pathways.
RASSF1A binds to microtubule-binding proteins (MAP) complexes and regulates mitosis, the cell cycle and apoptosis in response to mitogenic or apoptotic stimuli. *RASSF1A* also interacts with Cyclin D1 and A through JNK and p120 and inhibit cell cycle. Microphge stimulating protein (MST1), Salvador (SAV) and modulator of apopotosis-1 (MOAP1) interact with *RASSF1A* and modulate apoptosis (Donninger *et al.,* 2007).

An aberrant promoter methylation was detected in about 50% of malignant tumors. In HCC, hypermethylation occurred in approximately 54% to 95%, whereas HBV-associated HCC showed higher levels of *RASSF1A* methylation compared to HCC without risk factors. Hypermethylation of *RASSF1A* occurs not only in HCC, but also in non-neoplastic precancerous conditions like cirrhotic liver and chronic hepatitis as a result of this epigenetic changes it might be used as early stage biomarker for screening of patients with an increased

risk for HCC (Tischoff and Tannapfel, 2008; Hua *et al.,* 2010). Another study detected aberrant promoter hypermethylation of *RASSF1A* gene by MSP and found frequent methylation of the gene (86%) in liver tissue of HCC patients (Saelee *et al.,* 2010). *RASSF1A* gene promoter hypermethylation in urine of HCC and non-HCC (cirrhosis and hepatitis) samples have also evaluated by (Surbhi, *et al,* 2014) and found that at 90% sensitivity, specificity for the hypermethylated promoter was 72.9% which showed the potential use RASSF1A as biomarker for early detection of HCC.

B. *GSTP1* gene and HCC

Glutathione-S-transferases (*GSTs)* are a family of isoenzymes that play an important role in protecting cells from cytotoxic and carcinogenic agents. GSTs catalyze the conjugation of glutathione with electrophilic compounds, including carcinogens and exogenous potential toxins, resulting in less toxic and more readily excreted metabolites. Several GST variants are principally expressed in the liver, an organ whose primary functions include detoxification and metabolism. *GSTs* are encoded by different genes at different loci and are categorized into eight classes: alpha (α), mu (μ), pi (π), and theta (θ), kappa (k), omega (Ω), sigma (σ), and zeta (ζ). These enzymes may help defend hepatocytes against a variety of potentially promutagenic stresses, including ROS associated with chronic hepatic inflammation and reactive electrophilic compounds associated with the hepatic metabolism of dietary carcinogens. GST has been proposed to protect the liver from HBV related injury, which is mostly manifested as extensive oxidative DNA damage (Zhong *et al.,* 2002; White *et al.,* 2008).

The GST's enzymes are involved in many signal transduction pathway without direct involvement and among them *GSTP1* may be the most peculiar GST with its inhibitory role in various signaling pathways implicated in apoptosis and proliferation. GSTs are able to interact with different signal transduction pathway such as MAPK:- c-jun N-terminal kinase (JNK) and apoptosis signal-regulating kinase 1 (ASK1) in a raf independent manner in response to oxidative stress. S-glutathionylation is a posttranslational modification of proteins characterized by the conjugation of glutathione allowing a protection against oxidative stress. *GSTP1* could influence the rate of this reaction under stress conditions, *GSTP1* can mediate a self S-glutathionylation and that these modifications by interfering with the GSTP1/JNK complex lead to *GSTP1* aggregate's formation and JNK activation (Pajaud *et al.,* 2012).

GSTP1 found on chromosome 11 and contains 7 exons and spans approximately 2.8kb. It is one of the detoxification enzymes that have been considered as tumor suppressor gene and shows reduced expression in many types of cancer including HCC. HBx protein plays the key role in repressing *GSTP1* expression by inducing hypermethylation of the promoter regions. *GSTP1* DNA hypermethylation changes have been detected in HCC (Tchou *et al.,* 2000; Zhong *et al.,* 2002; Niu *et al.,* 2009). In HCC, methylation of the *GSTP1* gene occurred in 41% to 85% (Tischoff and Tannapfel, 2008). Jain (2012) and her colleagues detected *GSTP1* gene promoter hypermethylation and pointed out that its potential use as biomarker for HCC. There are also common polymorphisms in the GST gene leading to absent or reduced enzymatic activity that result in HCC development (White *et al.,* 2008)

C. *SEPT9* and HCC

Cell division is very important for multicellular organisms development, it produces many different cells in a very complex and precise way, defects in this process have been linked to many types of cancer. Septins are filament-forming guanosine triphosphatases (GTPases) required for cell division (Estey *et al.,* 2013). Initially Septins was known to function in cytokinesis. Recently they were found to be involved in the biogenesis of mitochondria, in the formation of multi-vesicular bodies, autophagosomes and in lysosomal homeostasis (Angelis and Spiliotis, 2016).

In the cytoplasm, the rod-shaped heteromeric septin complexes associate with the actomyosin and microtubule cytoskeletons**.** Septin–anillin complexes are enriched at the cleavage furrow during cytokinesis and act as membrane anchors for the midbody ring (MBR) The organization and contractile properties of actin-myosin filaments are modulated by septins, which cross-link and bend actin filaments into functional structures such as the cytokinetic contractile ring, (Figure 6) the actin stress fibers that regulates cell migration, and cellular protrusions such as filopodia, pseudopodia and lamellipodia. Septins also affect the organization, dynamics and post-translational modifications of the microtubule cytoskeleton, influencing the morphogenesis of epithelia and neurons. Microtubule-associated septins are also essential for proper chromosome alignment and segregation during mitosis, and the cytoskeleton-dependent transport of membrane vesicles in interphase cells (Angelis and Spiliotis, 2016).

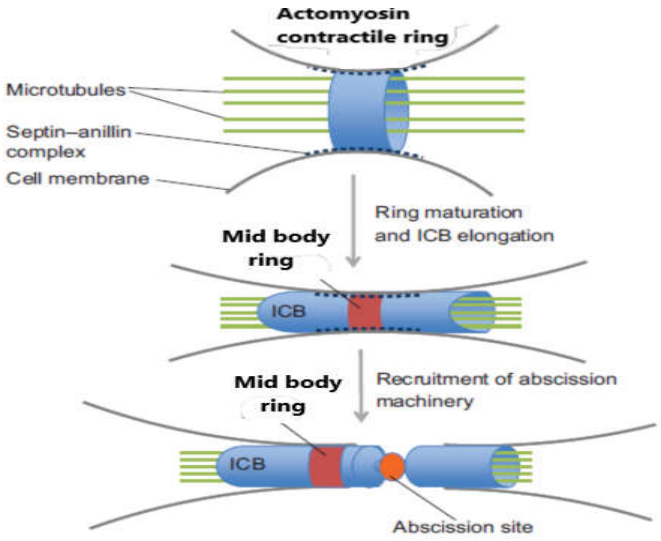

Figure 6. Septin-dependent cytokinesis of mammalian cells

An actomyosin contractile ring (ACR) is formed in the anaphase of mitosis. During telophase, ACR ingression leads to the development of an intracellular bridge (ICB) in which a midbody (MD) containing a midbody ring (MBR) is observed. Septin–anillin complexes are enriched at the cleavage furrow and act as membrane anchors for the MBR. Finally, on the flank of the midbody ring, the abscission site is generated which mediates complete separation of both sister cells (Menon and Gaestel, 2015).

In human, cells express multiple Septin family that comprises of 13 genes, and encode 13 different septins (SEPT1- SEPT12, SEPT14) ubiquitous and tissue-specific with multiple isoform variants. The core GTP binding structure of septin consists of alternating α- helix and β-sheets and loops that come in contact with the phosphate group of GTP (Menon and Gaestel, 2015).

Among all septins, *SEPT9* was the first *Septin* that has been extensively implicated in human cancer. *SEPT9* contains an elongated N-terminal tail, which is enriched with prolines and interacts with microtubules and actin filaments. *Septin 9* gene is mapped to chromosome 17 and it spans 266 kb and contains at least 17 exons, including 4 alternative first exons (Angelis and Spiliotis, 2016). *SEPT9* is phosphorylated by the cell cyclin-dependent kinase 1 (Cdk1) at threonine 24, which controls entry into mitosis and promotes cell proliferation and survival (Estey *et al.,* 2013). Altered expression of *SEPT9* gene was observed in almost every cancer types like colon, prostate, breast, ovary, pancreas, lung, kidney, liver, thyroid, and esophagus

and epigenetic changes have also been reported (Powrozek *et al.,* 2014; Angelis and Spiliotis, 2016).

1.2 Epidemiology

Liver cancer is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total) It is the fifth most common cancer in men with estimated number of cases 554,000 (7.5% of the total) and 521,000 deaths. It is the ninth in women with estimated number of cases 228,000 (3.4% of the total) and 224,000 deaths. Liver cancer is largely a problem of the developing countries where 83% of the estimated 782,000 new cancer cases worldwide occurred in 2012 due to late stage diagnosis, lack of timely and standard treatment and also lack of screening and public awareness (GLOBOCAN, 2012).

In Ethiopia also there are some studies that showed HBV, HCV and HCC prevalence. The prevalence of HBV was 7%, higher in males 9% than females 5% (Abebe *et al,* 2003) and in another study the overall prevalence rate of HBV was 9.02% (Kefenie *et al,.* 1989). Tsega and his colleagues found that 112 had HCC among 334 patients with chronic liver disease (Tsega *et al.,* 1992).

1.3 Significance of the study

The use of circulation derived urine DNA biomarkers for HCC has the potential to support in the design of new strategies to prevent or cure the cancer and screen family member who are at risk. It requires very imperative strategy to prevent or cure liver cancer by screening for cancer or pre-cancer states by making use of strong, constant, and well organized advocacy programs.

In general, it is less prevalent in women; it is 43 in developed and 182 in less developed countries per 100000. As studies indicated, estimated number of incidence of liver cancer in Ethiopia is 2 age standardise rate (ASR) per 100,000 and estimated deaths is 1.8 per 100,000 for both sexes. The mortality rate in men is 1.7 where as in woman is 2 ASR/100000 (GLOBOCAN, 2012). HCC continues to cause major public health problems, unless new methods of cancer screening and prevention are made available and accessible. Although

large studies carried out in liver cancer in the world, currently studies on HCC in Ethiopia on biomarkers that can be used for early detection for the cancer have not yet been conducted.

Assessing tumor derived circulatory mutated and hypermethylated urine DNA as biomarker is very interesting since urine is non-invasive, easy to collect and isolate the DNA and has high sensitivity and specificity. Moreover, urine has become for the first time a body fluid that is non invasive, inexpensive and easily applied for DNA analysis. There is no doubt that this study would make significant contribution for the scientific world since it provides information for understanding of the pathogenesis of the liver cancer by looking through the *hTERT, Tp53, CTNNB1*, *GSTP1, SEPT9* and *RASSF1A* genes in the urine that are responsible for development and progression of liver cancer. The other crucial aspect of this study is its contribution in identifying different factors that would help to make early diagnosis in its early state prior to metastasis and choose appropriate treatment for it. Furthermore, it improves survival and quality of life. Due to the limitations of existing HCC biomarkers, the search for new biomarkers is crucial. Several markers are currently under evaluation for their potential to predict disease prognosis and for their possible roles as targets for therapeutic interventions in the future and this study will be part of the progress for development of biomarkers for early detection and therapy and it can potentially be used as screening test to diagnose the onset of HCC in HBV and HCV and cirrhosis patients. When it comes to the local populations, the immediate benefits of the research result will be used for treatment, follow up and diagnose the patients especially those with hepatitis and cirrhosis. Since HCC develops as a late complication of chronic hepatitis and cirrhosis. The long term benefits are to improve the understanding of the diagnosis, prognosis and treatment of liver cancer.

1.4 Hypothesis

Circulation derived tumor specific mutation and hypermethylation can be detected in the urine of patients with hepatocellular carcinoma.

CHAPTER TWO

2. Objectives

2.1 General objective

To investigate the potential use of panel of urine DNA biomarkers for early detection of HCC by detecting mutation of *hTERT*, *Tp53* and *CTNNB1* genes and hypermethylation of *RASSF1A, SEPT9 and GSTP1* genes alone or in combination with serum AFP, ALT and AST.

2.1.1 Specific Objectives

- To detect HCC associated mutations in h*TERT, Tp53* and *CTNNB1* genes from DNA in urine
- To detect HCC associated hypermethylation of *RASSF1A, SEPT9* and *GSTP1* genes from DNA in urine
- To assess the association of *hTERT, Tp53* and *CTNNB1* mutation with the HCC, cirrhosis and hepatitis.
- To assess the association of *RASSF1A, SEPT9* and *GSTP1* hypermethylation with HCC, Cirrhosis and hepatitis
- To compare the mutation and hypermethylation of the above genes in Ethiopian with USA samples.

CHAPTER THREE

3. Materials and Methods

3.1 Study area

This study was conducted in the Department of Biochemistry, Tikur Anbessa Specialized Hospital (TASH), Internal Medicine, Division of Gastroenterology, School of Medicine, Addis Ababa University in collaboration with the Hepatitis B Foundation/Baruch S. Blumberg institute, Philadelphia from May 2016 - May 2017. TASH is a tertiary teaching University Hospital located in Addis Ababa, the capital city of Ethiopia. The hospital is the leading and the oldest general tertiary referral hospital in the country. This referral hospital is the main teaching hospital for both clinical and preclinical training for most disciplines. Additionally, it provides training services for undergraduate and postgraduate medical students, dentists, nurses, midwives, pharmacists, medical laboratory technologists, radiology technologists, and others disciplines which are under the College of Health Sciences.

Hepatitis B Foundation/Baruch S. Blumberg institute is a research institute located in Philadelphia, USA. It is a world class research institute that is renamed in 2013 as the Baruch S. Blumberg institute in honour of its co-founder Dr. Baruch S. Blumberg who won the noble Prize for his discovery of the hepatitis B virus. It is an organization working on the problem of hepatitis B and liver cancer dedicated to finding a cure and eliminating HBV worldwide through research, education and patient care advocacy.

3.2 Study Design

This study was designed to be a cross sectional comparative study and planned to compare urine DNA biomarkers of Ethiopian samples with that of the US samples. The samples used in this study were obtained by voluntarily selected participants from eligible population

3.3 Study Populations

The study in this project included chronic hepatitis, cirrhosis and liver cancer patients from Tikur Anbessa Specialized Hospital and from Baruch S. Blumberg institute. Each study participants that had been recruited in this study has been diagnosed and selected by

Gastroenterologists/surgeons/residents. A Questionnaire was prepared and used to investigate the relationship of HCC with that of family health background/status, age and gender.

3.3.1 Inclusion criteria

- Hepatocellular carcinoma, cirrhosis and Hepatitis patients.

3.3.2 Exclusion criteria

- Hepatocellular carcinoma and Hepatitis patients with kidney problem.
- Patients < 18 years old.
- Pregnant woman.
- Patients who fail to give consent.

3.4 Sample size

The sample size was calculated according to (Hanely and McNeil, 1982; www.medical Biostatistics.com/ROCcurve) based on 85% specificity and 9% prevalence of HBV (Kefenie *et al.,* 1989). The calculated sample size was 215. Among these 110 is HCC, 53 Hepatitis and 52 cirrhosis patients.

3.5 Specimen collection and handling

Fifty ml of urine from hepatitis, cirrhosis and liver cancer patients was collected in each tubes containing Ethylelenediamine tetra acetic acid, (EDTA) and five ml of blood was collected in serum separator tube for AFP, ALT and AST analysis. As soon as the collection was completed the sample were placed in ice box and transported to the laboratory. The urine samples were stored at -20^{o}C until DNA isolation was performed and the blood in the serum separator tube have been centrifuged for 10 minute and serum was collected in an eppendorf tube and stored in freezer at -80^{o}C until it was taken for biochemical analysis.

3.6 Biochemical analysis

3.6.1 Determination of serum α-fetoprotein

Alpha-fetoprotein was determined using Human ELISA kit and according to the manufacturer protocol with Vidas (Vitek Immunodiagnostic Assay System) (Mini

Vidas.com). AFP was determined using automated quantitative enzyme linked fluorescent assay (ELFA) at wavelength of 450nm. The principle combines enzyme immunoassay with a final fluorescent detection. The serum sample was transferred to a well containing anti-AFP antibody conjugated with alkaline phosphatase. The sample/conjugate mixture was cycled in and out of the Solid phase Receptacle several times. The antigen was bound to antibodies coated on the Solid phase Receptacle forming a sandwich. The remaining free AFP (unbound) components was washed out. Finally, the fluorescence of the substrate, 4-Methyl-umbelliferyl phosphate, the conjugate enzyme that catalyzed the hydrolysis of the substrate into a fluorescent product, 4-Methyl-umbelliferone was measured. The intensity of the fluorescence is proportional to the concentration of AFP present in the serum. The normal concentrations in the blood are <10 IU/ml according to the manufacturers protocol (Vidas.com)

3.6.2 Determination of Serum ALT

Mindray BS-200E Chemistry analyzer was used to determine concentration of serum ALT. ALT can be used in combination with other enzymes to monitor the course of various liver disorders. The normal concentrations in the blood are from 5 to 40 U l^{-1} for AST and from 5 to 35 U l^{-1} for ALT. The pyruvate formed in the reaction by the action of ALT is reduced to L-Lactate by Lactate dehydrogenase (LDH) with the Oxidation of nicotinamide adenine dinucleotide (NADH).

$$\text{Alanine} + \alpha\text{- ketoglutarate} \xrightarrow[\text{ALT}]{} \text{Pyruvate} + \text{glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{LDH}]{} \text{L-Lactate} + NAD^+ + H_2O$$

The rate of change in absorbance resulting from the oxidation of $NADH/NAD^+$ was monitored kinetically at a wavelength of 340nm that was equivalent to the concentration of ALT.

3.6.3 Determination of serum AST

Mindray BS-200E Chemistry analyzer was used to determine serum AST. ALT and AST are enzymes found mainly in the liver, but they are also found in red blood cells, heart cells, muscle tissue and other organs, such as the pancreas and kidneys. AST catalyzes the transfer of the amino group from asparate to α-ketoglutarate with the formation of glutamate and

oxaloacetate. The latter is reduced to malate by malate dehydrogenase (MDH) in the presence of reduced NADH.

$$\text{Aspartate} + \text{Ketoglutarate} \xrightarrow[\text{AST}]{} \alpha\text{–Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow[\text{MDH}]{} \text{L-Malate} + NAD^{+}$$

The reaction is monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD^{+} and it is proportional to the activity of AST

3.7 Molecular analysis

3.7.1 Isolation of DNA from urine

Total DNA was isolated from urine of HCC, hepatitis and cirrhosis using study participants Urine was mixed with 6M guanidine thiocyanate and resin from Wizard® Plus Minipreps DNA purification system, US (www.Promega.com) and incubated at room temperature overnight with mixing on roller shaker. Resin-DNA complex was centrifuged, transferred to a mini-column, filtered and washed with wash buffer. DNA was eluted with distilled and autoclaved water. Electrophoresis was run to validate the quality of the isolated DNA. 1µl DNA and 0.5µl loading dye was mixed and loaded with DNA marker (Su *et al.*, 2004).

3.7.2 Fractionation of low and high molecular weight DNA

To the isolated DNA, Polyethylene Glycol Molecular weight 8,000, 5M sodium chloride and prewashed AMPure carboxylated metallic beads (CBD) was added and mixed well and incubated for an hour at room temperature on a rotor. The mixture was held on magnetic holder. The LMW DNA solution was taken out from the eppendorf tube leaving the HMW DNA that was bound on the bead. Ice cold isopropanol and unwashed beads was added to the LMW DNA and mixed well and incubated overnight at $4^{o}C$ on rotor. After incubation the tube with the LMW DNA was centrifuged and was placed on magnetic holder for five minutes. The LMW DNA in solution was removed and collected in eppendorf tube and stored at $-20^{o}C$ (Su *et al.,* 2004; Su *et al.,* 2008).

3.7.3 Quantification of wild type DNA by real time PCR (qPCR)

The amount of DNA was measured using qPCR (Su *et al.,* 2004). To quantify the DNA, a master mix that contains primers of each gene (Table 2) (Actin, *Tp53, hTERT, CTNNB1, RASSF1A* and *SEPT9*), LC 480 SYBR green, dNTPs, hot start DNA polymerase, double distilled water and the DNA was prepared. Successively diluted Human genomic DNA (5 copies (0.015ng), 50 copies (0.15ng), 500 copies (1.5ng) and 5000 copies (15ng)) have been used as standards (Figure 7). Aliquot of the master mix was mixed with the DNA in a 1:9 ratio in a 96 well qPCR plate. Similar experimental procedure was also used when preparing the standards and measured the concentration by qPCR. Each wild type gene was quantified with qPCR using the primers mentioned in the table below (Table 1). The actin gene (NG_007992) was used to evaluate the quantity of DNA isolated. Standard curves (Figure 7) and amplification curves (Figure 8) of the wild type DNA quantification assay that used human genomic DNA as standard and water as negative control are depicted below.

Table 1. Sequence of primers used for wild type gene quantification with qPCR (Prof. Su Lab.)

Primers	Sequences 5' -3'	Tm
Actin Fwd	GAGATGTATGAAGGCTTTTGGTC	62°C
Actin Rev	GCTGCCTCCACCCACT	61°C
***Tp53* Fwd**	CTGCATGGGCGGCATG	66°C
***Tp53* Rev**	AACCGGAGGCCCATCCTCA	68°C
***CTNNB1* Fwd**	GCAGCAACAGTCTTACCT	60°C
***CTNNB1* Rev**	GGTGCCACTACCACAG	60°C
***hTERT* Fwd**	AGGGGCTGGGAGGGCCC	75°C
***hTERT* Rev**	GGGGACCCGGGAGGGGT	72°C
***RASSF1A* Fwd**	AAATACGGGTATTTTCGC	56°C
***RASSF1A* Rev**	GTCGTCGTCGTTGTGGTC	78°C

For quantification of the wild type gene the qPCR was set at:-

For Actin:-

95°C - 10min (95°C - 10sec, 58°C - 10sec, 72°C - 10sec) x 40 cycles, melt cool.

For P53:-

95°C - 5min (95°C - 10sec, 58°C - 20 sec, 72°C - 10sec) x 45cycles, melt, cool.

For CTNNB1:-

95°C - 5 min (95°C - 10 sec, 60°C - 10sec, 72°C - 10 sec) x 50cycles, melt, cool.

For *hTERT*:-

95°C - 5min (95°C - 10sec, 61°C - 20sec, 72°C - 10sec) x 45 cycle, melt cool.

For *RASSF1A*:-

95°C - 5min (95°C - 10sec, 64°C - 10sec, 72°C - 10sec) x 45 cycle, melt, cool.

For SEPT9:-

95°C - 5min (95°C - 10sec, 62°C - 10sec, 72°C - 10sec) x 45 cycle, melt cool.

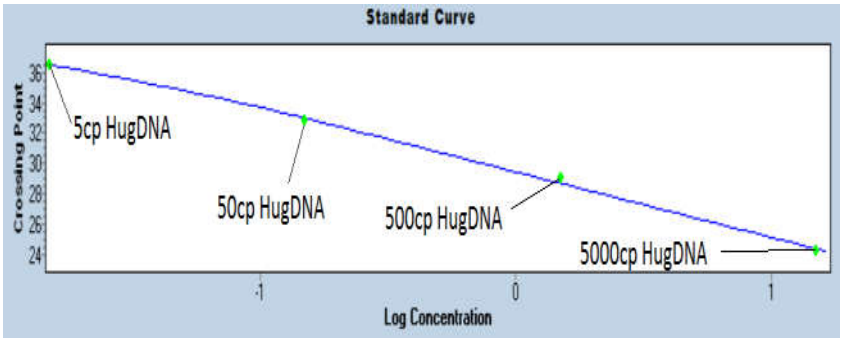

Figure 7. Standard Curve of the wild type DNA quantification assay using Human genomic DNA as standards

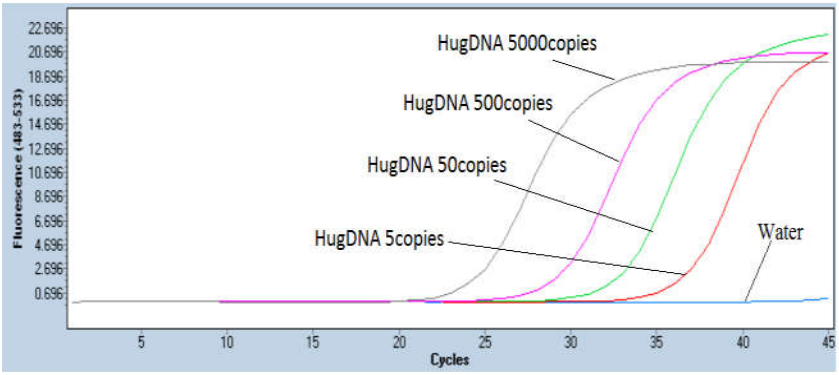

Figure 8. Amplification curve of human genomic DNA standard

3.7.4 Mutation quantification using qPCR

3.7.4.1 Mutation quantification of *Tp53* gene codon 249

The *Tp53* gene Codon 249 mutation was quantified using short amplicon PCR based assay that has a 41 bp product size and a two step PCR reactions. Hot start PCR was run after preparing a master mix (PCR reactions) that contain, 5U/µl hot start Taq Polymerase, dNTP, 10x PCR buffer, double distilled water, forward and backward primers of the genes, Locked nucleic acid (LNA) clamp of the genes was mixed with the DNA in 1:9 ratios. PCR was set and run. The LNA is synthetic RNA analogue with a methylene bridge that helps to increase specificity of the target DNA and competes for binding with the detection probe, enhancing the detection of the mutant variant (Lin *et al*., 2012; Pryor *et al.,* 2013). Serially diluted mutant plasmid *Tp53* (10 copies, 100 copies, 1000 copies and 10000 copies) was used as standards. The primers, LNA and probes are mentioned in (Table 2) below.

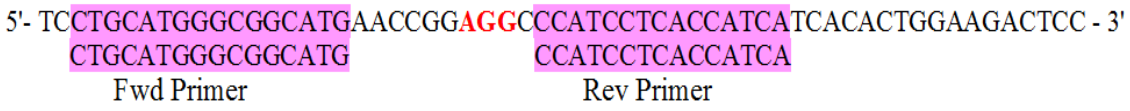

Figure 9. Schematic diagram of primer position of *Tp53* for first step PCR reactions.

The first step PCR setting is touchdown PCR with a setting:-

95^{o}C - 5min (95^{o}C - 30sec, 64^{o}C - 30 sec (-1^{o}C/cycle), 72^{o}C - 30sec) x 10cycles (95^{o}C - 30sec, 54^{o}C - 30 sec, 72^{o}C - 30sec) x 15cycles, 72^{o}C - 4min, 4^{o}C - hold

In the second step the mutations of the amplified PCR products of the *Tp53* gene was quantified using real time PCR (LightCycler® 480 system) in the presence of fluorescently labelled oligonucleotide simple probes and Light Cycler 480 genotyping master mix with a qPCR setting:- 95^{o}C - 10min (95^{o}C - 10sec, 54^{o}C - 15sec, 72^{o}C - 10sec) x 35cycles.

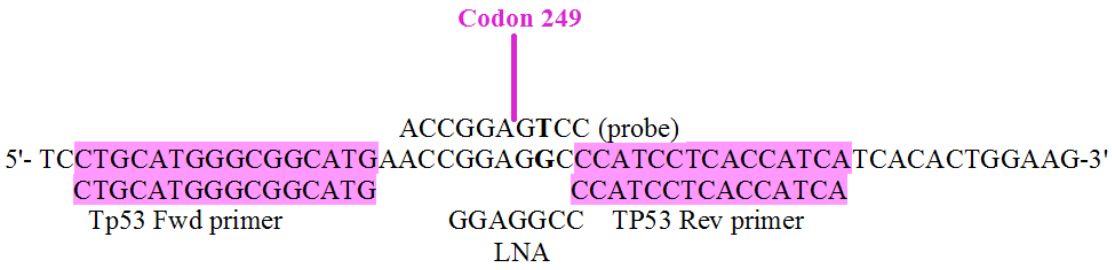

Figure 10. Schematic diagram of primer and probe position of *Tp53* for mutation quantification.

Table 2. Sequence of primers, probe and LNA used for quantification of the *Tp53* gene mutation (Prof. Su Lab.).

Primers	Sequences 5'-3'	Tm
1st step		
***Tp53* Fwd**	CTGCATGGGCGGCATG	66°C
***Tp53* Rev**	CCATCCTCACCATCA	55°C
2nd Step		
***Tp53* Fwd**	CTGCATGGGCGGCATG	66°C
***Tp53* Rev**	CCATCCTCACCATCA	55°C
LNA	GGAGGCC	67°C
***Tp53* probe**	ACCGGAG**T**CCCA	64°C

3.7.4.2 Mutation quantification of *CTNNB1* gene codons 32-37

The *CTNNB1* gene (NG_013302.1) mutation (codons 32-37) has a 53bp amplicon size and it is a three step reaction. Hot start PCR was run after preparing a master mix (PCR reactions) that contain, 5U/µl hot start Taq Polymerase, dNTP, 10x PCR buffer, double distilled water, forward and backward primers of the genes, BNA clamp of the genes was mixed with the DNA in 1:9 ratios. PCR was set and run. Serially diluted *CTNNB1* mutant plasmid (10 copies, 100 copies, 1000 copies and 10000 copies) was used as standards. The primers, LNA and the Light cycler 480 probe master mix are mentioned in (Table 3) below.

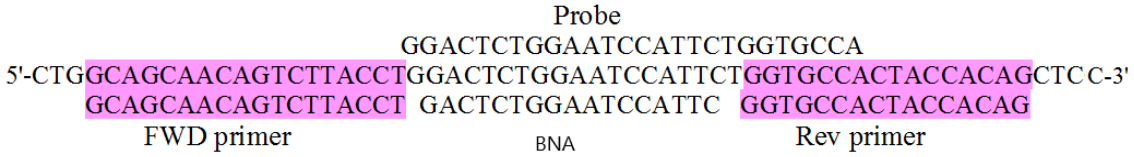

Figure 11. Schematic diagram of primer, LNA/BNA and probe position of *CTNNB1*gene for the PCR reaction.

The first step PCR setting was :-

95°C - 5min (95°C - 30sec, 70°C - 20sec, 60°C - 30sec) x 15 cycle, 72°C-4min, 4°C-hold

The mutations of the amplified PCR products of the *CTNNB1* genes was quantified using real time PCR in the presence of fluorescently labelled oligonucleotide probes (Lin *et al*., 2012; Pryor *et al.,* 2013).

The Second step is treatment of 1st step PCR product with Exonuclease VII and run it at a PCR setting of :- 37°C - 60min, 95°C - 10min, 60°C - 1min (2% ramp). 4°C – hold

The third step was run with qPCR (LightCycler® 480 system) in the presence of Light cycler 480 probe master mix at qPCR conditions:- monocolor hydrolysis

95°C - 5min (95°C - 10sec, 80°C - 5sec, 60°C - 1sec) x 50 cycle

Table 3. Sequence of primers, probe and LNA used for quantification of the *CTNNB1* mutation.

Primers	**Nucleotide Sequences 5' -3'**	**Tm**
1st Step		
***CTNNB1* Fwd**	GCAGCAACAGTCTTACCT	60°C
***CTNNB1* Rev**	GGTGCCACTACCACAG	60°C
2nd Step		
***CTNNB1* Fwd**	GCAGCAACAGTCTTACCT	60°C
***CTNNB1* Rev**	GGTGCCACTACCACAG	60°C
LNA	GACTCTGGAATCCATTC	71°C
Probe	GGACTCTGGAATCCATTCTGGTGCCA	70°C

The amplification curves (Figure 12) of the *CTNNB1* gene mutation quantification assay that used *CTNNB1* mutant plasmid as standard and water as negative control are depicted below.

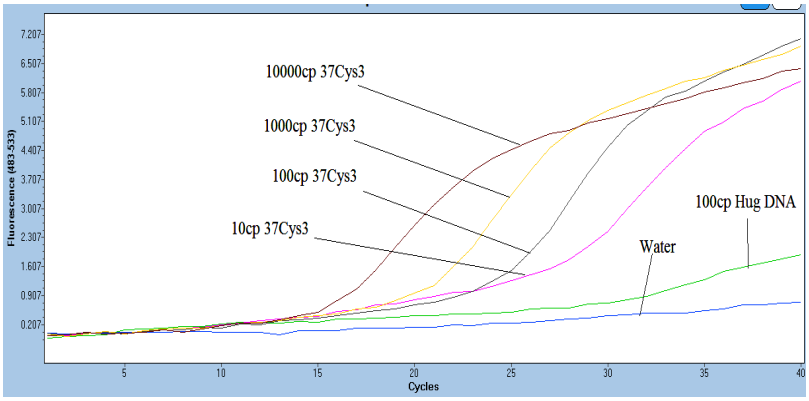

Figure 12. Amplification curve of the *CTNNB1* mutant plasmid standards

3.7.4.3 Mutation quantification of *hTERT* gene promoter

The *hTERT* 124 hot spot promoter mutation (-124 G>A) quantification is a two step qPCR assay. Hot start PCR was run after preparing a master mix (20 µl PCR reactions) that contain, 5U/µl hot start Taq. Polymerase,10x PCR buffer, 2.5mM dNTP, double distilled water, 10µM forward and backward primers, 1µM *hTERT* Bridged nucleic acid (BNA) clamp of the genes, Dimethyl sulphoxide (DMSO) and was mixed with the DNA in 1:9 ratios. Serially diluted *hTERT* mutant plasmid (10 copies, 100 copies, 1000 copies and 10000 copies) was used as standards and 1000 copies of human gnomic DNA was used as negative control then PCR was set and run.

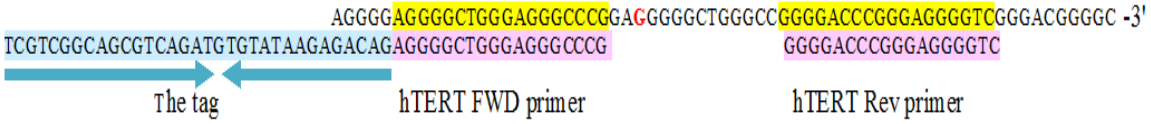

Figure 13. Schematic diagram of primer and probe position for *hTERT* first step PCR reaction

The mutations of the amplified PCR products of the *hTERT* genes was quantified using qPCR (LightCycler® 480 system) in the presence of 3µM *hTERT* TQ1 fluorescently labelled oligonucleotide probes (Lin *et al*., 2012; Pryor *et al.,* 2013).

PCR setting: - hot start PCR

95°C -5min (95°C - 30 sec., 80°C - 20sec., 72°c - 45sec) x 35 cycles, 72°C 4min, 4°c - hold

The assay format is Monocolor hydrolysis probe with qPCR conditions:-

95°C -5min (95°C - 10 sec, 61°C - 10sec, 72°c - 10sec) x 40 cycles

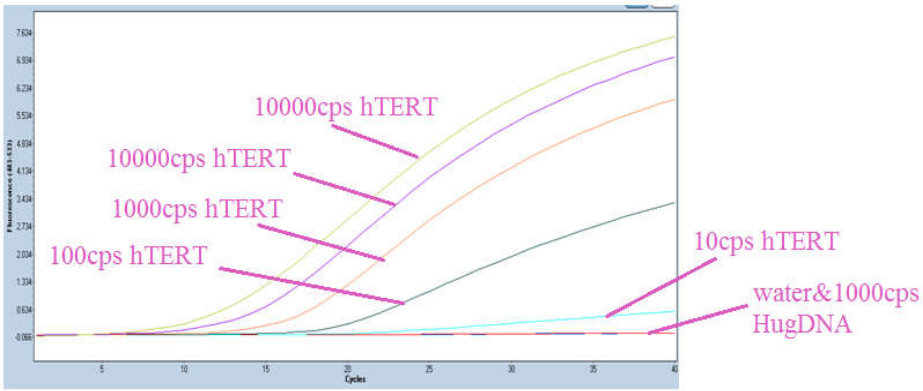

Figure 14. Amplification curves of the *hTERT* plasmid standards

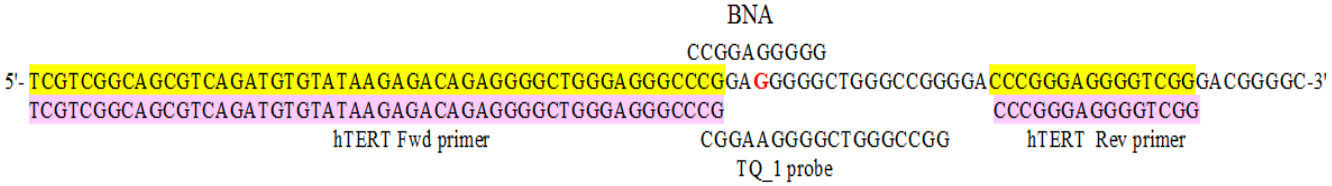

Figure 15**.** Schematic diagram of primer, BNA and probe position of *hTERT* for second step PCR reaction

Table 4. Sequence of oligonucleotides primers, probe and BNA used for quantification of *hTERT* 124 mutations

Primers	Sequences 5' -3'	Tm
1st Step		
***hTERT* Fwd**	AGGGGCTGGGAGGGCCC	75°C
***hTERT* Rev**	GGGGACCCGGGAGGGGT	72°C
2nd Step		
***hTERT* Fwd**	TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGAGGGGCTGGGAGGGCCCG	83°C
***hTERT* Rev**	CCCGGGAGGGGTCGG	68°C
BNA	CCGGAGGGGGC	91°C
Probe	CGGAAGGGGCTGGGCCGG	74°C

3.7.5 Bisulfite conversion of DNA to methylated form

Bisulfite modification of DNA was performed using EZ DNA Methylation kit according to manufacturer's instructions (www.zymoresearch.com). LMW DNA was added to PCR tube and also lightning conversion buffer to it. It was mixed and centrifuged and placed in a thermal cycler with a set up of 98^{o}C for 8min, 54^{o}C for 60 minutes and 4^{o}C for hold. M-binding buffer was added to a zymo-spin column and placed on a collection tube. The PCR product was loaded into the column and centrifuged at full speed. M-wash buffer was added to the column and centrifuged at high speed. Desulphonation buffer was added to the column, incubated for 20 minutes and centrifuged. The column was washed twice with wash buffer and centrifuged and transferred into a 1.5ml microcentrifuge tube and eluted with double distilled water. These have been done on *RASSF1A*, *SEPT9* and *GSTP1* DNA. The eluted DNA was used for quantification of hypermethylation using qPCR.

3.7.5.1 Quantification of methylated *RASSF1A, GSTP1* and *SEPT9* by MSP

Methylated *RASSF1A, SEPT9* and *GSTP1* genes were determined using bisulfite modification of DNA and Methylation specific PCR (MSP) (Hu *et al.,* 2010). The DNA underwent bisulfite conversion of cytosine to uracil and then the methylated sequences were selectively amplified with primers specific for methylation.

3.7.5.2 Quantification of methylated *RASSF1A* promoter (P1) region with MSP

Promoter methylation status of *RASSF1A* (Gene Bank No: DQ444319.1) DNA was determined by two step MSP assay (Hu *et al.,* 2010). For this hot start PCR with 20µl PCR reaction was run using the bisulfite treated DNA of *RASSF1A* in the presence of 10µM primers, 10x PCR buffer, 2.5mM dNTP and 5U/µl hot start Taq polymerase. The methylation of the amplified PCR products of the genes was quantified using qPCR. Serially diluted Human Methylated Bisulfite standards (HMBS) was used as a standard (10, 50, 250 and 1250 copies) and 200 copies HeLa cell DNA was used as negative control and double distilled water as negative control. List of the sequences of primers and annealing temperature are shown in the table 5 below.

First step PCR settings was:- 95°C - 5min (95°C - 30sec, 57°C - 30 sec, 72°C - 30sec) x 25cycles, 72°C -4min, 4°C – hold.

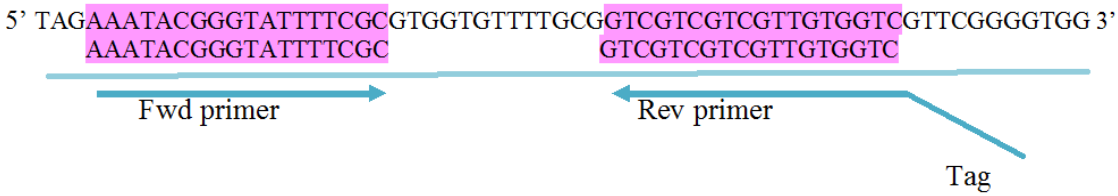

Figure 16. Schematic diagram of primer position of *RASSF1A* for the first step PCR reactions

In the second step a master mix was prepared that contain the first step PCR reaction products (1:10 diluted), primers specific for methylated bisulfate treated DNA and 2x LC 480 SYBR green and run with qPCR (LightCycler® 480 system).

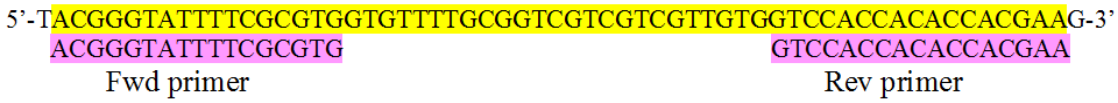

Figure 17. Schematic diagram of primer position *RASSF1A* of the second step qPCR reactions.

The qPCR condition was :-

95°C - 5min (95°C - 10sec, 58°C - 30 sec, 72°C - 30sec) x 40cycles.

Table 5. Sequence of oligonucleotide primers used for quantification of methylated *RASSF1A*

Primers	Sequences 5' -3'	Tm
1st step		
***RASSF1A* Fwd**	AAATACGGGTATTTTCGC	56°C
***RASSF1A* Rev**	GTCGTCGTCGTTGTGGTC	78°C
Tag	GCTCTTCGTGGTGTGGTG	
2nd Step		
***RASSF1A* Fwd**	ACGGGTATTTTCGCGTG	61°C
***RASSF1A* Rev**	GTCCACCACACCACGAA	62°C

3.7.5.3 Quantification of *mGSTP1* by MSP

Methylated *GSTP1* gene (Gene Bank No: M24485) promoter was determined by two steps MSP (Hu *et al.,* 2010). Hot start PCR with 20ml PCR reaction was run using the bisulfite treated *GSTP1* DNA in the presence of 10µM LNA primers, 10x PCR buffer, 2.5mM dNTP and 5U/µl hot start Taq polymerase. The methylation of the amplified PCR products of the genes was quantified using qPCR. Serially diluted HMBS (10, 50, 100 and 500 copies) was used as a standards and 200 copies SW480 Cell DNA was used as positive control and double distilled water as negative control.

First step PCR settings was:- 95°C - 5min (95°C - 30sec, 59°C - 30 sec, 72°C - 30sec) x 33cycles, 72°C - 4min, 4°C – hold.

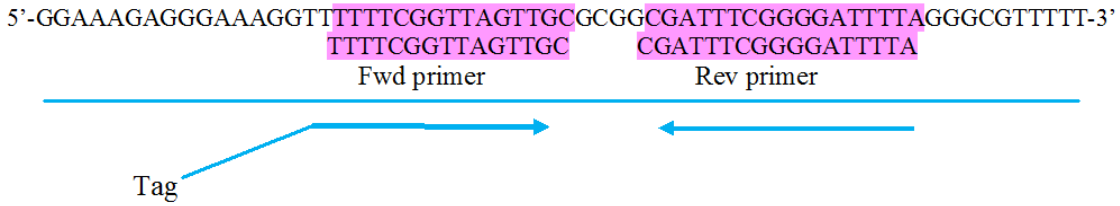

Figure 18. Schematic diagram of primer position of *GSTP1*gene for the first step PCR reaction.

In the second step a master mix was prepared that contained the products of the first PCR reaction products, 10µM primers and 3µM GSTP1 TQ3 probe, 2x LC probe master mix and run with qPCR (LightCycler® 480 system) with assay format of monocolor hydrolysis probe and qPCR conditions was :-

95°C - 5min (95°C - 10sec, 58°C - 30 sec, 72°C - 30sec) x 40cycles, cool.

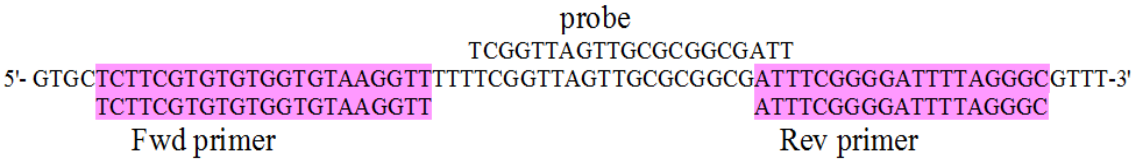

Figure 19. Schematic overview of primer position of *GSTP1*gene promoter for the second step qPCR reactions

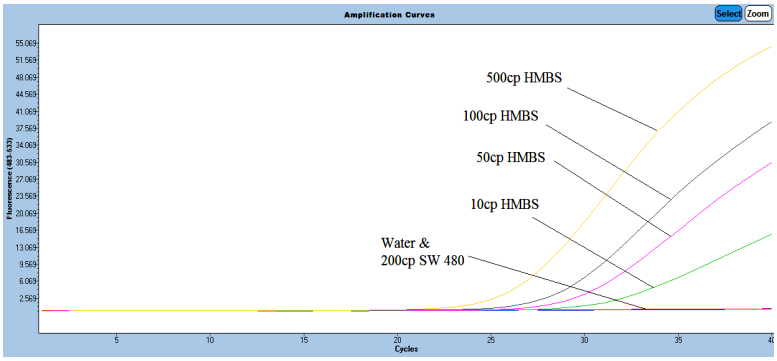

Figure 20. Amplification curve of HMBS standards for the MSP assay of *GSTP1*

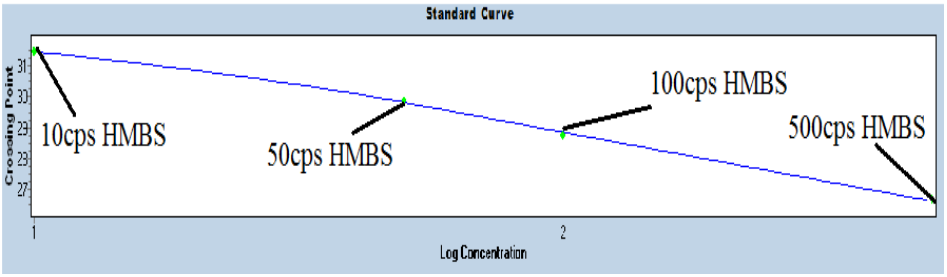

Figure 21. Standard curve for the MSP assay of *GSTP1* using HMBS as standards

Table 6. Sequence of primers and probes used for quantification of hypermethylation of *GSTP1* gene promoters (Prof. Su Lab.).

Primers	Sequences 5' -3'	Tm
1st step		
***GSTP1* Fwd**	TTTTCGGTTAGTTGC	57°C
***GSTP1* Rev**	CGATTTCGGGGATTTTA	62°C
2nd Step		
***GSTP1* Fwd**	TCTTCGTGTGTGGTGTAAGGTT	60°C
***GSTP1* Rev**	ATTTCGGGGATTTTAGGGC	70 °C
Probe	TCGGTTAGTTGCGCGGCGATT	70 °C

3.7.5.4 Quantification of *SEPT9* by MSP

Methylated *SEPT9* gene (NM_006640) was determined by MSP (Hu *et al.,* 2010). Hot start PCR was run using the bisulfite treated DNA of *SEPT9* in the presence of primers, 10xPCR

buffer, dNTP and5U/µl hot start Taq polymerase. The methylation of the amplified PCR products of the genes was quantified using qPCR. Serially diluted Human methylated bisulfite standards (HMBS) (10, 100, 500, and 1000copies) was used as standards and HeLa cell DNA as positive control and double distilled water was used as negative control.

qPCR conditions:-

$95^{o}C$ - 5min ($95^{o}C$ - 10sec, $58^{o}C$ - 30 sec, $72^{o}C$ - 30sec) x 40cycles

3.8 Data processing and statistical analysis

Laboratory results and data obtained from the questionnaires were entered in excel and were analyzed using the statistical package SPSS software version 25 (SPSS Inc, USA). The association between the mutations and hypermethylation biomarkers with the disease was performed using Pearson Correlation analysis and the specificity and sensitivity was analyzed by Receiver operating characteristics (ROC) curves analysis. The distribution of the biomarkers among the different groups (HCC, Cirrhosis and Hepatitis) have been analyzed by Kruskal Wallis Test which is a non parametric statistical test for independent samples

3.9 Data Quality Assurance

The quality of the data was checked starting from sample collection. Samples were collected using standardized techniques and procedures. Investigators scrutinized the appropriateness of methodologies and proper functioning of equipments and supplies form the best manufacturer and suppliers for the intended research work. All the samples taking procedures and analyses of samples were carried out by professionals or under the guidance of qualified investigators. Negative and positive controls and standards were done for each protocol and indiscriminate results were repeated.

3.10 Ethical consideration

This study was designed to investigate the presence of mutation and hypermethylation in circulating DNA in the urine of hepatitis, cirrhosis and HCC patients. Urine samples were collected in sterile falcon tubes. Blood samples were collected with appropriate and disposable sterile needle and syringes in order to avoid blood transmitted diseases.

The participants were informed about the purpose of the study. They were given written informed consents and signed the consents and filled the questionnaires. In addition, they were made aware of the right to keep hold of information, decline to cooperate and with draw from the study. They were also informed that their participation had valuable contribution to the future efforts in the design and implementation of appropriate monitoring in the treatments of liver cancer patients. Only patients who are volunteered to give informed consents were included in the study (see annex IV and V). The study participants were informed that access to information was restricted to the research study team. For the purpose of confidentiality, any information related with the analyses of the results were identified using codes and data were also entered with codes.

This study was approved by Department of Biochemistry Research and Ethics committee, CHS, Addis Ababa University, the College of Health Sciences Institutional Review Board, AAU and Baruch S. Blumberg Institute and in addition, the National Institutional Review Board, Science and Technology Ministry of Ethiopia.

3.11 Operational Definitions

1. **Hepatocellular carcinoma (HCC)** - a malignant tumor arising from hepatocytes and present as a single mass, as multiple nodules, or as a diffuse liver involvement. It is diagnosed by clinical and biochemical test specifically by ultrasound and serum alpha-fetoprotein respectively.
2. **Cirrhosis** – widespread disruption of normal liver structure by fibrosis and formation of regenerative nodules.
3. **Hepatitis** – inflammation of the liver and liver disease involving degenerative or necrotic alterations of hepatocytes. Blood (serology) test is used to detect for antibodies to each of the hepatitis virus.
4. **Chronic Hepatitis** – long lasting inflammation of the liver due to virus in which symptoms of hepatitis continue for several months and may increase in severity
5. **Acute Hepatitis** – liver inflammation of abrupt onset which may be due to a viral infection or toxins
6. **Locked nucleic acid (LNA)** – is a new class of bicyclic high affinity DNA analogs. LNA-containing oligonucleotides confer significantly increased affinity against their

complementary DNA targets, increased mismatch discrimination and allow full control of the melting point of the hybridization reaction.

7. **Methylation Specific Polymerase Chain Reaction** – A technique used for qualitative DNA methylation analysis that has high sensitivity to detect small quantities of methylated DNA.

CHAPTER FOUR

4. Results

4.1 Socio-demographic and Clinical characteristics of the study participants

In the present study, we showed the feasibility of early detection of HCC by quantifying the mutation and hypermethylation of six known HCC associated genes (*Tp53, CTNNB1, hTERT, GSTP1, RASSF1A* and *SEPT9*) by qPCR. In this study a total of 163 study participants have been involved of which 67/163 (41%) of them were females and 96/163 (59%) of them were males with a mean age of 40. From the total study participants 51 of them had HCC, while 53 of them had cirrhosis and the remaining 59 had Hepatitis. In HCC study participants 35/51 (68.6%) of them were male and 16/51 (31.4%) of them were female this shows that the male were more susceptible to develop HCC than female. Most of the study participants came from Addis Ababa 88/163(54%) and the remaining 75/163(46%) were from regions. The ethnic proportion of the study participants is indicated in table 7.the highest proportion was from Amhara and the lowest from Somalia.

Table 7. Ethnic groups of the study participants

Ethnic Group	Hepatitis (n=59)	Cirrhosis (n=53)	HCC (n=51)
Amhara (n)	29	24	27
Oromo (n)	17	13	16
Tigre (n)	1	4	0
Debub (n)	7	5	4
Gurage (n)	5	6	4
Somalia (n)	0	1	0

n = number

As the values in table 8 indicated the mean age of HCC (48) study participants was found relatively higher than that of cirrhosis (37) and hepatitis (34). In addition to age, this study

has seen the association of various substance uses to HCC. For instance, among the 51 HCC study participants 2/51 (3.9%) of them were smokers, 6/51 (11.76%) of them were alcohol users, 2/51 (3.9%) of them was chat edulis user, 2 (3.9%) of them used a combination (one used alcohol and chata edulis and the second one alcohol and smoking) of the above substances whereas 39 (76.5%) of them had no history of substances use. For all the study participants the substance use is presented in (Table 8). The proportion of the substance use of the study participants with cirrhosis was higher than the hepatitis and HCC group (50%). As for cirrhosis, the higher values obtained for the alcohol users which was 8/53 (15.1%), as compared to the other two types of substance users (smokers 3/53 (5.66%) and chata edulis (Khat) 4/53 (7.54%)) whereas the combined users were 4/53 (7.54%). As the data in table 7 showed most of the study participants that were involved in this project had no history of substance uses.

Table 8. Socio-demographic and behavioural characteristics

Characteristics	Hepatitis (n=59)	Cirrhosis (n=53)	HCC (n=51)
age (years) Mean±SD	34 ±11.8	37 ±10.2	48 ±11.8
Male (n)	33	28	35
Female (n)	26	25	16
Substance use n / (%)			
Smoking	1/1.7	3/5.66	2/3.9
Alcohol	4/6.8	8/15.1	6/11.76
Catha edulis	1/1.7	4/7.54	2/3.9
Combined user	1/1.7	4/7.54	2/3.9
Non user	52/88.1	34/64.15	39/76.5

n = Number

When the family history of liver disease for all study participants was assessed only 16/163 (9.8%) of them had family history. In regard to HCC only 4/51 (7.8%) of the study participants had family history of liver disease. On the contrary, 30/51 (58.8%) of the

participants did not exhibit any family history of liver disease while 17/51 (33.3%) didn't know the existence of this disease in their family.

As indicated in table 9 below, the median level of AFP (2.5), is not that much higher than the other groups. The median level of ALT (34.5) and AST (69) values were higher in HCC group compared to cirrhosis (ALT = 28, AST = 40) and hepatitis (ALT = 25.5, AST = 31).

Table 7. Biochemical Biomarkers /serum biomarkers

Characteristics	Hepatitis (n=59)	Cirrhosis (n=53)	HCC (n=51)
AFP (IU/ml) Median/IQR	2.4 (1.66 - 3.59)	2.3 (1.21- 4.57)	2.5 (0.77 - 32.7)
ALT (IU/ml) Median/IQR	25.5 (18.3 - 51.8)	28.0 (21.5 - 47.5)	34.5 (22.3 - 64.3)
AST (IU/ml) Median/IQR	31.0 (23 - 57)	40.0 (31.5 -70.5)	69.0 (36 - 143)

IQR - interquartile range

Although, the median level of AFP was not that much higher in HCC patients, among the 51 HCC study participants 19 (40.4%), of them have low AFP (<10 IU/ml) that are false negatives. The normal concentrations in the blood is <10 IU/ml according to the manufacturers protocol (Vidas.com). In contrast, in non-HCC study participants (hepatitis and cirrhosis), 6 (3.7%) of them had high AFP i.e. false positives and all of them had cirrhosis except one. In this study 25/51 (49%) of the HCC study participants have underlying hepatitis (15 HBV, 8 HCV and 2 of them have co-infection). 21/51 (41.2.1%) of them have underlying cirrhosis, among these 6 (15.7%) of them without hepatitis and/or cirrhotic liver. Again from these HCC 13 (25.5%) of them had both hepatitis and cirrhosis.

4.2 Wild type LMW DNA quantification Data

The circulating cell free DNA concentration in this case the wild type LMW DNA was measured. Wild type LMW DNA quantification of each gene in the urine was performed for all samples (n=163) and the median and interquartile range (IQR) of the HCC, cirrhosis and

hepatitis study participants have been shown in Table 10. These showed how much cell free circulating LMW DNA (150 - 250bps) was found in the urine of the study participants. The median level of LMW DNA (cfDNA) in *Tp53, CTNNB1, hTERT, GSTP1, SEPT9* was higher in HCC study participants followed by Cirrhosis and then hepatitis whereas the level in *RASSF1A* gene was a bit lower than the HCC and instead it was higher in Hepatitis. The median of Actin that was used as internal control was 85.9 for 59 of the samples out of 163.

Table 8. Quantification of wild type LMW DNA of the cfDNA in the urine

Gene Name	Hepatitis (n=59)	Cirrhosis (n=53)	HCC (n=51)
Tp53 (Copies/ml) Median (IQR)	204 (118.7-1003) n = 50	356 (188.2-125.6) n = 47	546 (223 - 2066) n = 48
CTNNB1 (Copies/ml) Median (IQR)	168 (76 - 1304) n = 52	245 (76 - 1117) n = 46	678 (75.8 -1631) n = 42
hTERT (Copies/ml) Median (IQR)	1094 (410-3420) n = 50	1245 (179.5-2719) n = 40	3607 (713.7-11581) n = 45
RASSF1A(Copies/ml) Median (IQR)	879.5 (387.9 -2795) n = 52	1810 (882 - 3121) n= 43	1742 (799 - 3924) n = 45
SEPT9 (Copies/ml) Median (IQR)	330 (123.3 -1417) n = 50	187 (47.9 - 802) n = 45	550 (38.9 -1741) n = 50
Actin (Copies/rxn) Median (IQR)		85.9 (28.5 -170) n = 59	

IQR = interquartile range, n = number of study participants

4.3 LMW DNA (wild type DNA) distribution among HCC

We have seen the distribution of the LMW DNA among HCC, Cirrhosis and Hepatitis with Box plot at significance level of $p < 0.05$. In All type of disease (HCC, Hepatitis and Cirrhosis) the LMW DNA (wild type DNA) distribution was higher in HCC study participants.

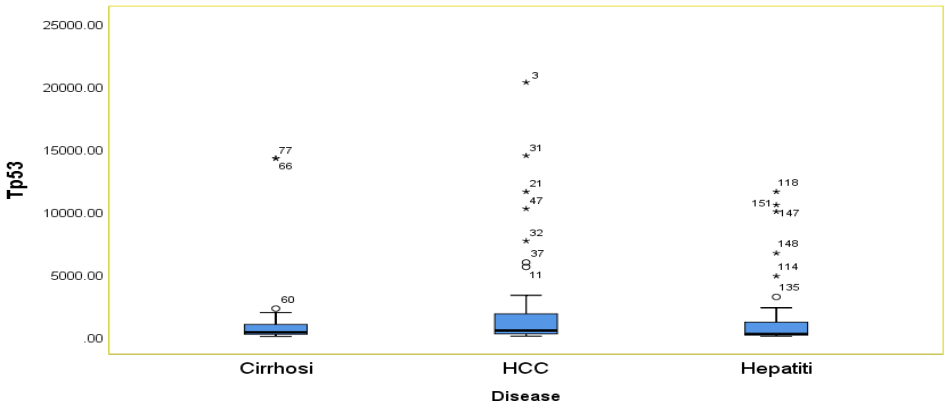

Figure 22. The distribution of the *Tp53* LMW DNA (copies/ml) among Cirrhosis (n = 47), HCC (n =47), and Hepatitis (n = 48) with Box plot.

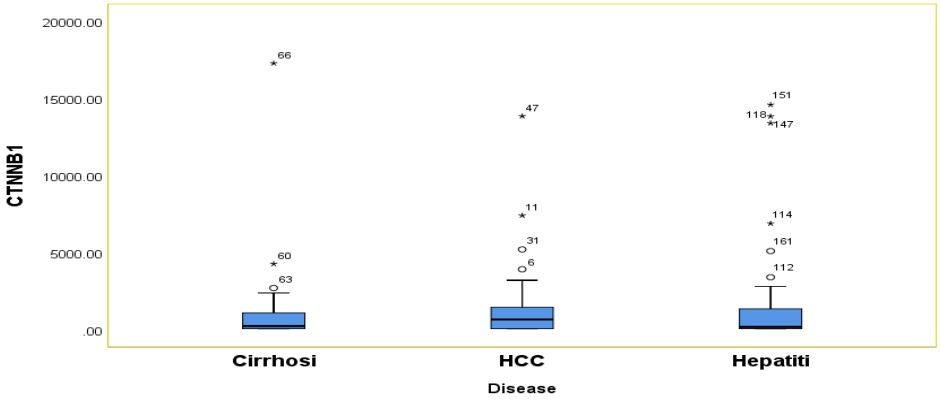

Figure 23. The distribution of the *CTNNB1* LMW DNA (copies/ml) among Cirrhosis (n =46), HCC (n =42), and Hepatitis (n = 50) with Box plot.

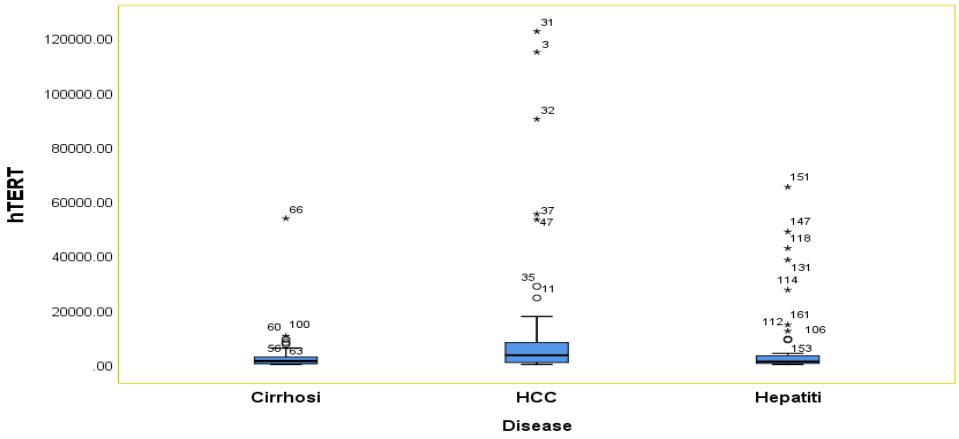

Figure 24. The distribution of the *hTERT* LMW DNA (copies/ml) among Cirrhosis (n = 40), HCC (n =44), and Hepatitis (n = 49) with Box Plot.

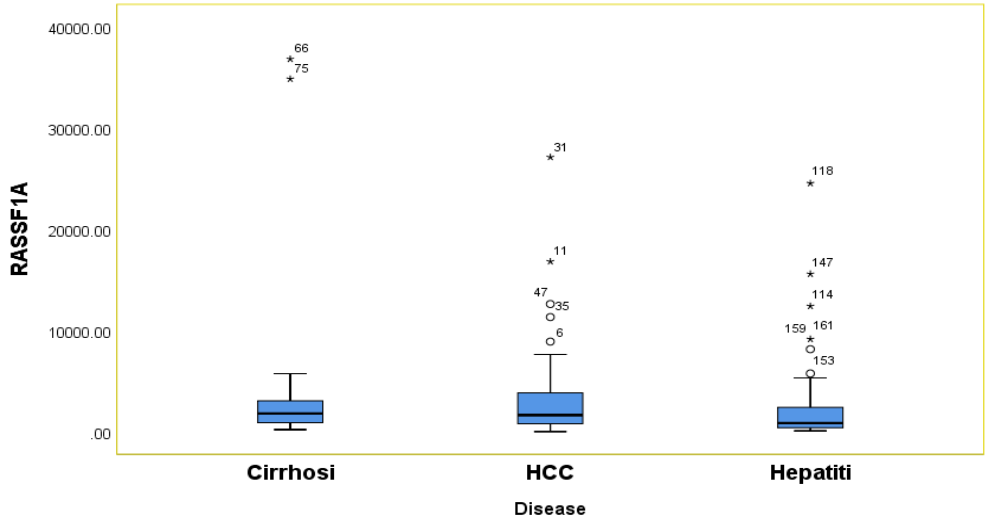

Figure 25. The distribution of the *RASSF1A* LMW DNA (copies/ml) among Cirrhosis (n= 43), HCC (n =44), and Hepatitis (n = 49) with Box Plot.

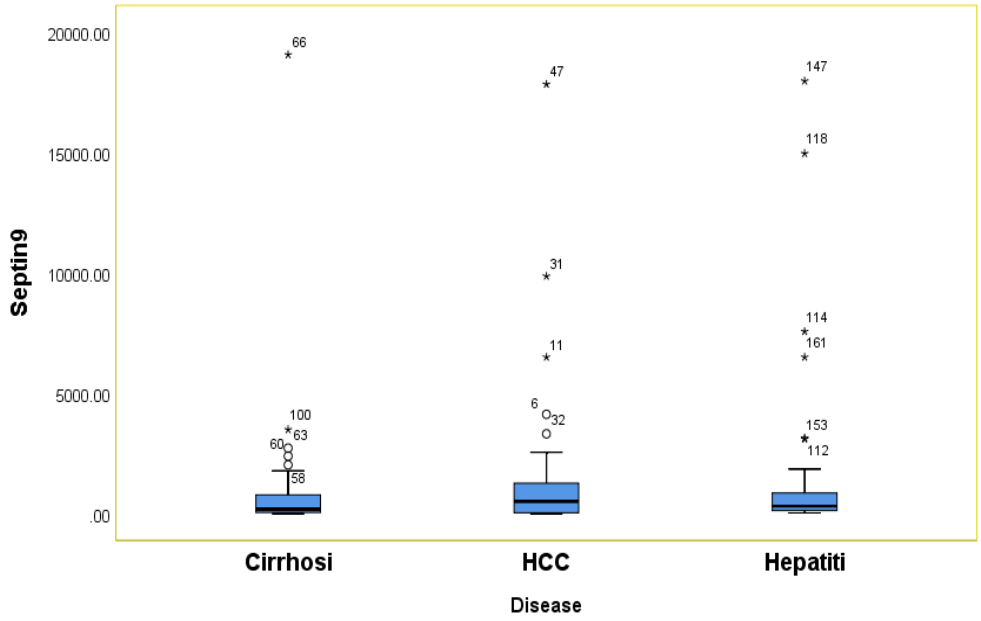

Figure 26. The distribution of the *SEPT9* LMW DNA (copies/ml) among Cirrhosis (n = 45), HCC (n =47), and Hepatitis (n = 47) with Box

4.4 Mutation of *Tp53, CTNNB1* and *hTERT* and hypermethylation of *GSTP1, RASSF1A* and *SEPT9* data analysis

Mutation and hypermethylation analysis were carried out with simple, sensitive and accurate qPCR based quantification assays that used fluorescently labelled oligonucleotide probes, standards and negative controls. Our findings showed that the mean level of mutation of each gene in the urine samples were higher in HCC compared to cirrhosis and hepatitis (Table 11). But generally, the level was low and in some cases below the level of detection.

Table 9. Quantification of mutation of *Tp53, CTNNB1* and *hTERT* genes

Gene Name	Hepatitis (n=59)	Cirrhosis (n=53)	HCC (n=51)
Tp53(Copies/ml) Mean (Range)	19.1(0.0-542.0) n =58	2.6(0.01-133.0) n =53	25.9(0.01-1139) n =51
CTNNB1 (Copies/ml) Mean (Range)	1.8 (0.2-24.9) n =40	1.6 (0.2-12.7) n =29	2.6 (0.21-50.8) n =35
hTERT (Copies/ml) Mean (Range)	11.6 (1.0-379.2) n =40	11.2 (0.42-297.6) n =29	15.6 (1.0-238.5) n =35

n = Number of Study Participants

Gene Name US sample	Hepatitis (n=49)	Cirrhosis (n=91)	HCC (n=73)
RASSF1A(Copies/ml) Mean (Range)	7.7(76.2)	10.9(244)	94.3(1003.9)
GSTP1 (Copies/ml) Mean (Range)	8.1(115)	60.7(745)	36335
AFP (ng/ml) Mean (Range)	3.78(12.6)	6.6(219.2)	61840(147214.8)

n= Number of Study Participants

This study also showed that the mean hypermethylation of the *RASSF1A* and *GSTP1* genes is higher in HCC compared to Hepatitis and cirrhosis however, when it comes to *SEPT9* it is higher in hepatitis (Table 12).

Table 10. Quantification of hypermethylation of *GSTP1*, *RASSF1A* and *SEPT9* genes

Gene Name	Hepatitis (n=59)	Cirrhosis (n=53)	HCC (n=51)
GSTP1(Copies/rxn) Mean (Range)	3.0 (0.0-5.0) n =22	2.9 (0.0-5.0) n =26	3.8 (0.0-5.0) n =16
RASSF1A(Copies/ml) Mean (Range)	6.7 (0.3-32.8) n =58	6.3 (0.6-35.0) n =53	8.3 (1.0-56.8) n =51
SEPT9 (Copies/ml) Mean (Range)	4.9 (0.01-37.0) n =58	4.1 (0.2-5.0) n =53	3.4 (0.0-11.4) n =51

n = Number of Study Participants

In AFP negative (<10 IU/ml) HCC study participants, 4 out of 19 were positives for *Tp53* gene mutation (21%), 6 out of 13 were positives for *CTNNB1* gene mutation (46%), 3 out of 13 was positive for *hTERT* gene mutation (23%), 6 out of 19 was positive for *RASSF1A* gene hypermethylation (31%) and 7 out of 19 was positive for *SEPT9* gene hypermethylation (36.8%).

4.5 Analysis of the Biomarkers with AUROC curve

This study was designed to assess the performance of six biomarkers for HCC by the receiver operating characteristic ROC curve. ROC curve is a graphical display of the true-positive rate (Sensitivity) and the false-positive rate (1-specificity) from multiple classification rules.

The present study showed the predictive value of the DNA mutation and hypermethylation biomarkers with the ROC curve analysis. The AUROC curve of each gene that showed the distinguishing capacity of HCC from Cirrhosis and hepatitis (combined) were as follows: for *Tp53*, 0.495 (49.5%), for *CTNNB1,* 0.553 (55.3%) and 0.560 (56%) for *hTERT* at 95% confidence interval (Figure 22 a, b, c).

The predictive values of these biomarkers were also shown in HCC and cirrhosis as shown in (Figure 24 a, b, c). The AUROC curve were 0.484 (48.4%) for *Tp53*, 0.575 (57.5%) for *CTNNB1* and 0.605 (60.5%) for *hTERT* at 95% confidence interval. For HCC from hepatitis the AUROC curve were 0.455 (45.5%) for *Tp53,* 0.538 (53.8%) for *CTNNB1* and 0.529 (52.9%) for *hTERT* at 95 % confidence interval (Figure 25 c, d and Figure 26 a).

The analysis of the hypermethylation biomarkers using ROC curve to distinguish HCC from Cirrhosis and hepatitis were 0.532 (53.2%) for *RASSF1A,* 0.574 (57.4%) for *GSTP1* and 0.405 (40.5%) for *SEPT9* at 95% confidence interval (Figure 22d and Figure 23a, c). When we evaluated the discriminating capacity of HCC from cirrhosis we got 0.546 (54.6%) for *RASSF1A*, 0.581 (58.1%) for *GSTP1* and 0.420 (42%) for *SEPT9* (Figure 24d and 25 a, b). When it comes to discriminating ability of HCC from hepatitis, the AUROC curve was 0.518 (51.8%) for *RASSF1A*, 0.567 (56.7%) for *GSTP1* and 0.392 (39.2%) for *SEPT9* (Figure 26 b, c, d).

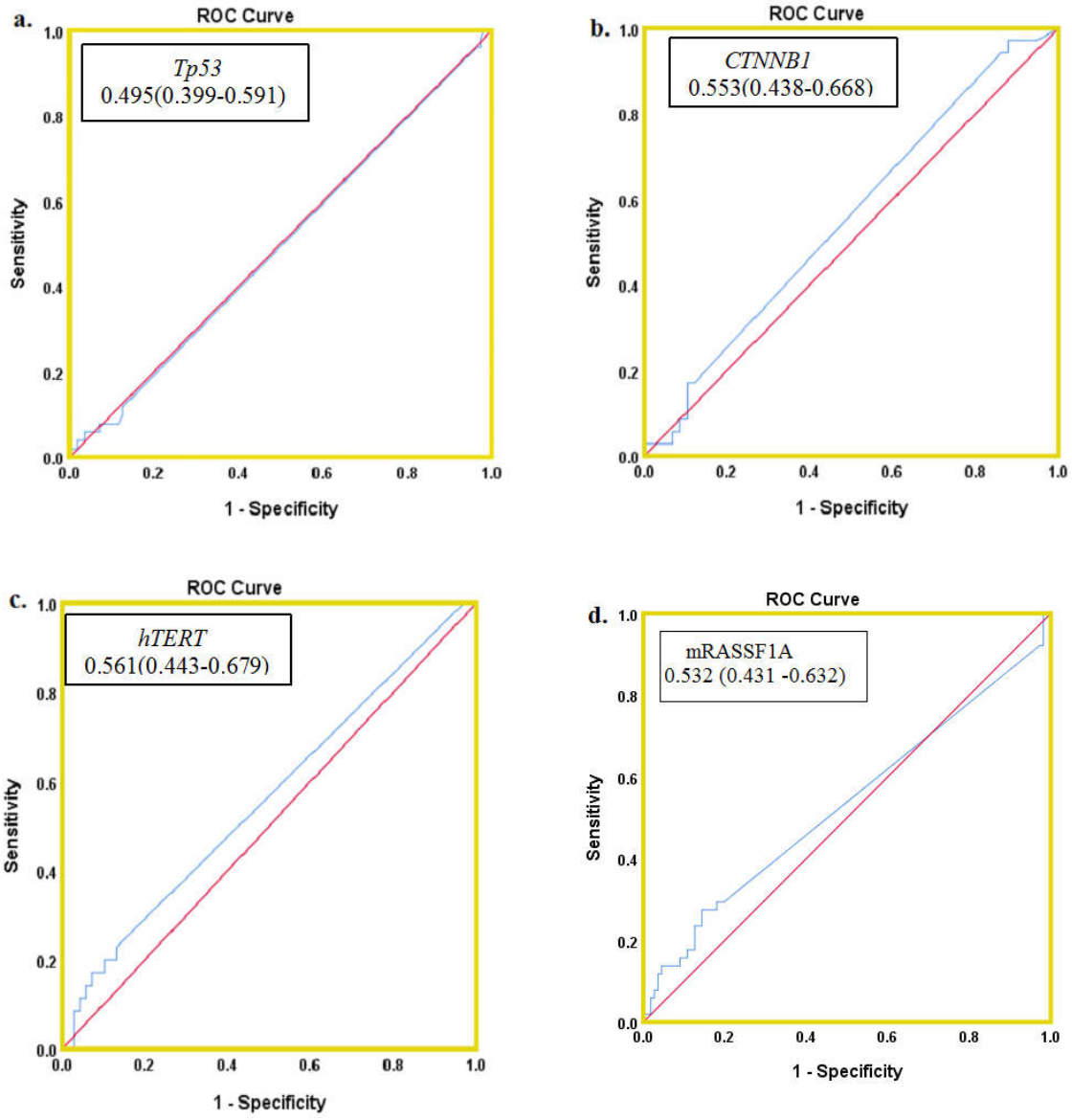

Figure 27. ROC curve generated to distinguish HCC from cirrhosis and hepatitis.

a. *Tp53* gene mutation assay (HCC n=51, Cirrhosis, n=53 and hepatitis, n=58). **b**. *CTNNB*1 gene mutation assay (HCC, n=35 Cirrhosis, n=29 and hepatitis, n=40). **c**. *hTERT* gene mutation assay (HCC n=35, Cirrhosis, n=29 and hepatitis, n=40). **d.** *mRASSF1A* gene methylation assay (HCC, n=51, Cirrhosis, n=53 and hepatitis, n=58). The AUROC curve shown below the name of the genes. Y-axes indicated the sensitivity and the x-axes 1-specificty. The red line represents the chance line and the blue lines area under the curve.

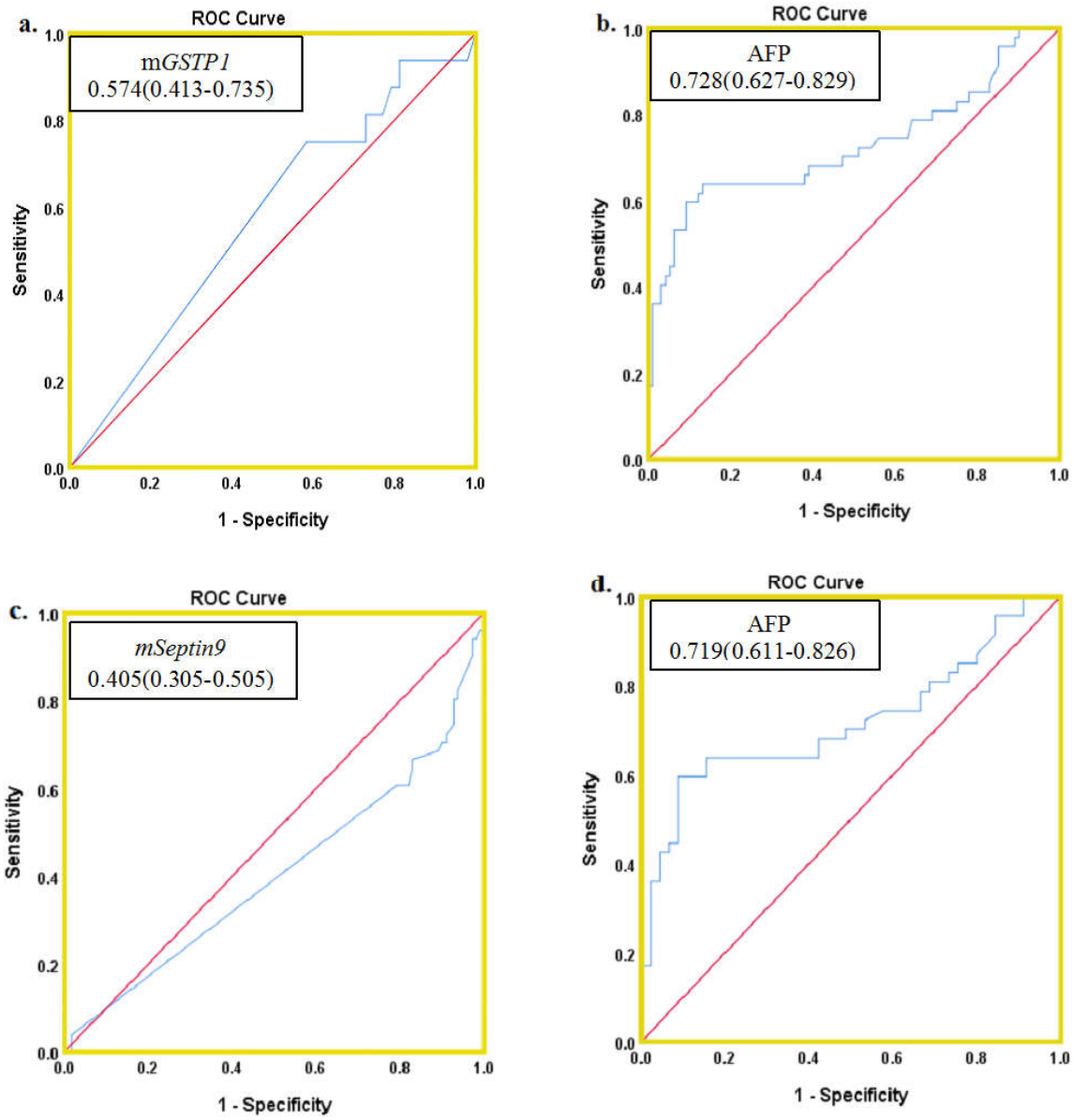

Figure 28. ROC curve generated to distinguish HCC from cirrhosis and hepatitis.

a. *mGSTP1* gene HCC (n=16) from cirrhosis (n=26) and hepatitis (n =22). **b.** Serum AFP biomarker (HCC n= 47 from cirrhosis n= 44 and hepatitis n= 56). **c.** *SEPT9* gene MSP assay (HCC n=50 from cirrhosis (n=53) and Hepatitis (n=58)). **d**. Serum AFP biomarker (HCC n=47 from Cirrhosis, n= 44). AUROC curve shown below the name of the biomarkers. Y-axes indicated the sensitivity and the x-axes 1-specificty. The red line represents the chance line and the blue lines area under the curve.

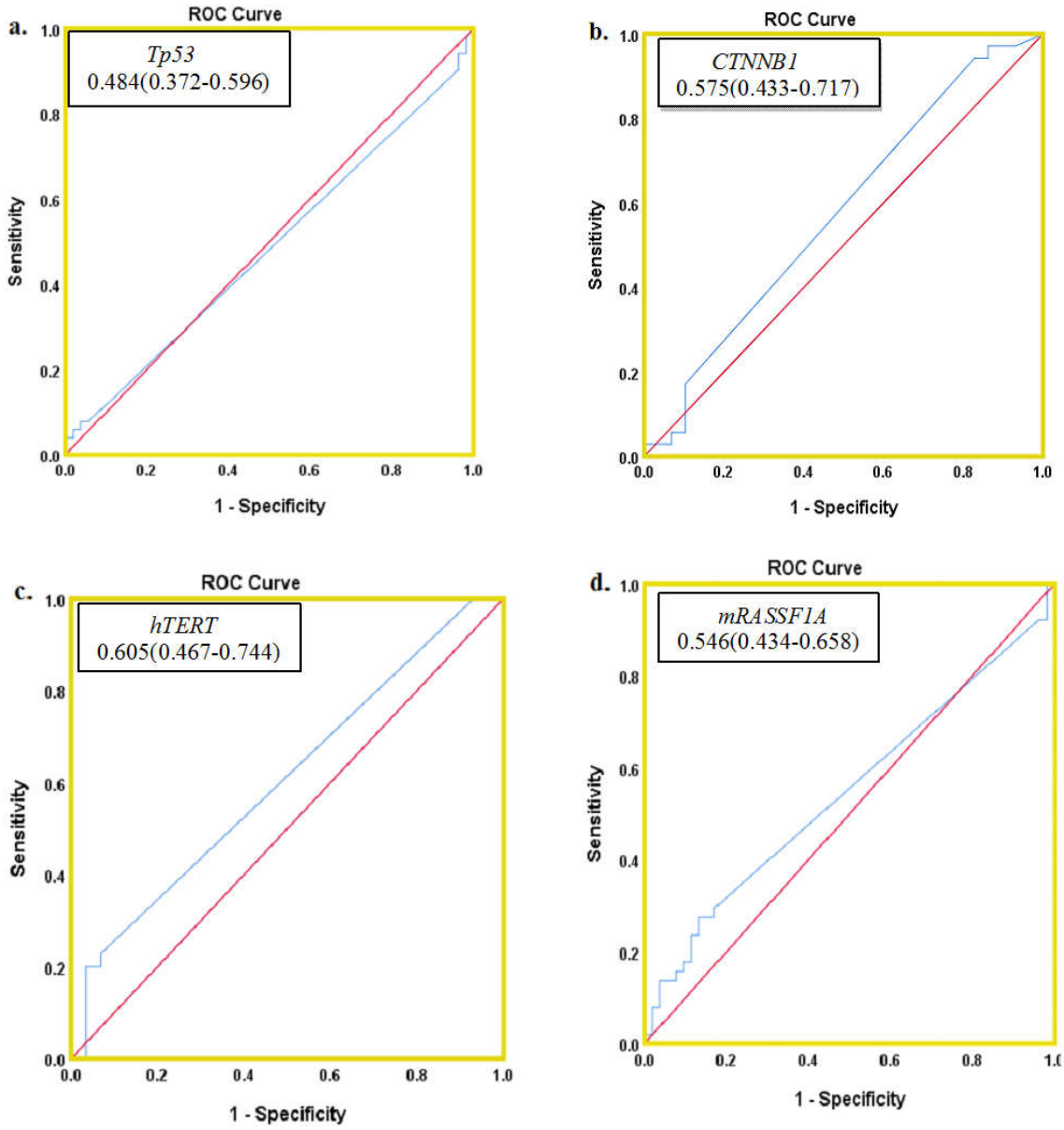

Figure 29. ROC curve generated to distinguish HCC from Cirrhosis.

a. *Tp53* gene mutation assay (HCC, n=51 from Cirrhosis, n=53). **b**. *CTNNB1* gene mutation assay (HCC, n=35 from Cirrhosis, n=29). **c**. *hTERT* gene mutation assay (HCC, n=35 from Cirrhosis, n=29). **d.** *mRASSF1A* gene methylation assay (HCC, n=51 from Cirrhosis, n=53). The AUROC curve value shown below the name of the genes**.** Y-axes indicated the sensitivity and the x-axes 1-specificty. The red line represents the chance line and the blue lines area under the curve.

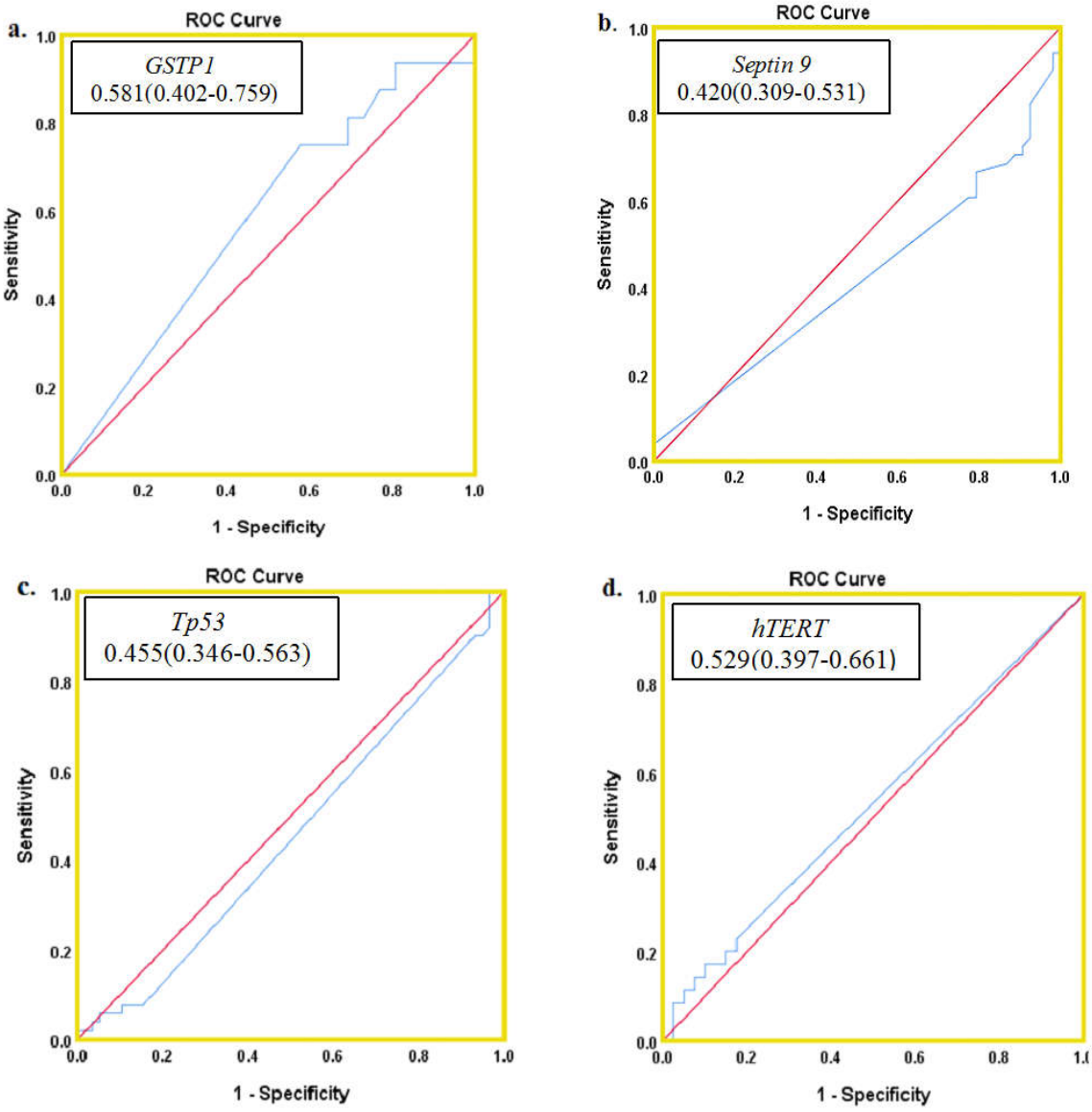

Figure 30 ROC curve generated to distinguish HCC from cirrhosis and also hepatitis.

a. *mGSTP1* gene MSP assay (HCC, n=16 from cirrhosis, n=26). b. *mSEPT9* gene MSP assay (HCC, n=51 from cirrhosis, n=53). c. *Tp53* gene mutation assay (HCC, n=51 from hepatitis, n=58). d. *hTERT* gene mutation assay (HCC, n=51 from hepatitis, n=40). The AUROC curve values shown below the name of the genes. Y-axes indicated the sensitivity and the x-axes 1-specificty. The red line represents the chance line and the blue lines area under the curve.

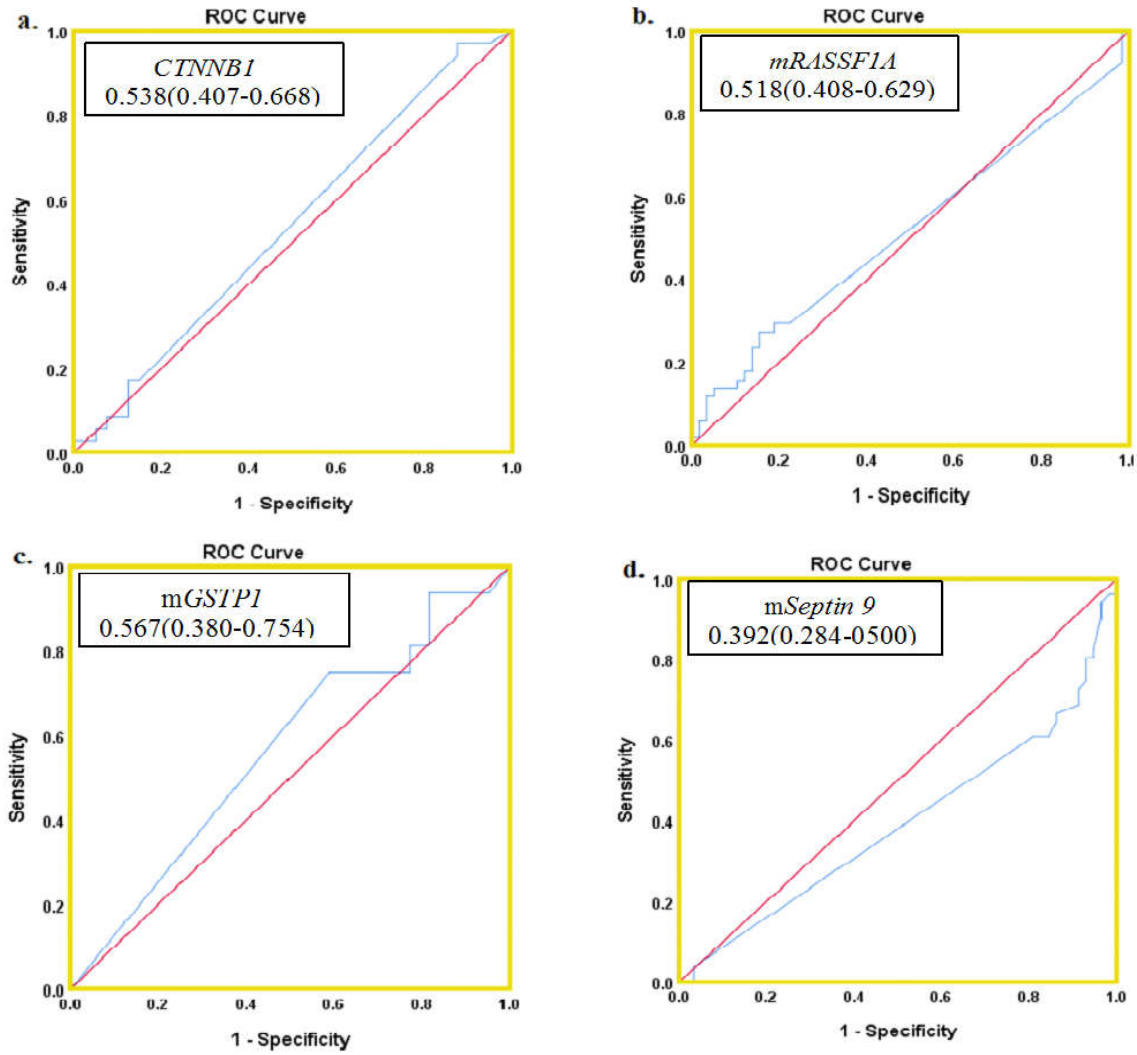

Figure 31. ROC curve generated for *CTNNB1,* m*RASSF1A, mGSTP1*and *mSEPT9*

a. *CTNNB1* gene mutation assay used to distinguish HCC (n=51) from hepatitis (n=40). **b**. m*RASSF1A* gene MSP assay used to distinguish HCC (n=51) from hepatitis (n=58). **c**. *mGSTP1* gene MSP assay used to distinguish HCC (n=16) from hepatitis (n=40). **d.** *mSEPT9* gene methylation assay used to distinguish HCC (n=51) from hepatitis (n=58). The AUROC curve values shown below the name of the genes. Y-axes indicated the sensitivity and the x-axes 1-specificty. The red line represents the chance line and the blue lines area under the curve.

Table 11. Summary of AUROC curve data for all the Biomarkers of Ethiopian samples

Genes and biomarkers	**AUROC curve**
Tp53	0.495(0.399 - 0.591)
HCC vs Non HCC (Cirrhosis, Hepatitis)	
HCC vs Cirrhosis	0.484(0.372 - 0.596)
HCC vs Hepatitis	0.455(0.346 - 0.563)
CTNNB1	
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.553(0.438 - 0.668)
HCC vs Cirrhosis	0.575(0.433 - 0.717)
HCC vs Hepatitis	0.538(0.407 - 0.668)
hTERT	
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.561(0.443 - 0.679)
HCC vs Cirrhosis	0.605(0.467 - 0.744)
HCC vs Hepatitis	0.529(0.397-0.661)
RASSF1A	
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.532(0.431-0.632)
HCC vs Cirrhosis	0.546(0.434-0.658)
HCC vs Hepatitis	0.518(0.408-0.629)
GSTP1	
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.574(0.413-0.735)
HCC vs Cirrhosis	0.581(0.402-0.759)
HCC vs Hepatitis	0.567(0.380-0.754)
Septin 9	
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.405(0.305-0.505)
HCC vs Cirrhosis	0.420(0.309-0.531)
HCC vs Hepatitis	0.392(0.284-0.500)
AFP	
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.728(0.627-0.829)
HCC vs Cirrhosis	0.719(0.611-0.826)
HCC vs Hepatitis	0.719(0.612-0.826)

4.6 Correlation of AFP with the biomarkers

We used Spearman's rho correlation to see the association of the DNA biomarkers with Age and serum AST, ALT, and AFP. The spearman's rho (ρ) revealed a statistically significant relationship between age and AFP and AST at $p < 0.01$. AFP was significantly correlated with age ($\rho = 0.424$, $p = 0.001$), ALT ($\rho = 0.445$, $p = 0.001$) and AST ($\rho = 0.374$, $P = 0.005$). Similarly, *hTERT* was correlated with *CTNNB1* ($\rho = 0.374$, $p = 0.017$), *RASSF1A* with *SEPT9* ($\rho = -0.298$, $p = 0.024$) and GSTP1 with ALT ($\rho = 0.506$, $p = 0.027$). Statistically significant correlation was not detected between the other biomarkers.

4.7 Distribution of the biomarkers among HCC, cirrhosis and hepatitis

We have seen the distribution of the biomarkers among HCC, Cirrhosis and Hepatitis with Kruskal Wallis Test at significance level of $p < 0.05$. In half of the biomarkers the distribution is the same for all diseases but serum AFP (Figure 27), *mRASSF1A* (Figure 31), *mGSTP1* ($P = 0.564$) (Figure 32) and *mSEPT9* (Figure 33) showed a better distribution (higher level) in HCC.

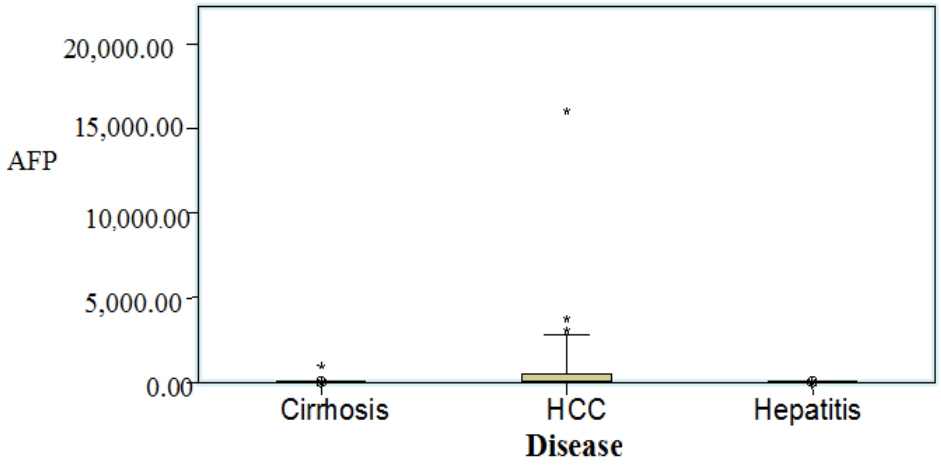

Figure 32. Independent samples Kruskal Wallis Test that showed the distribution of serum AFP (IU/ml) in HCC (n =47), Cirrhosis (n = 44), and Hepatitis (n = 56). The stars indicate outliers in the groups.

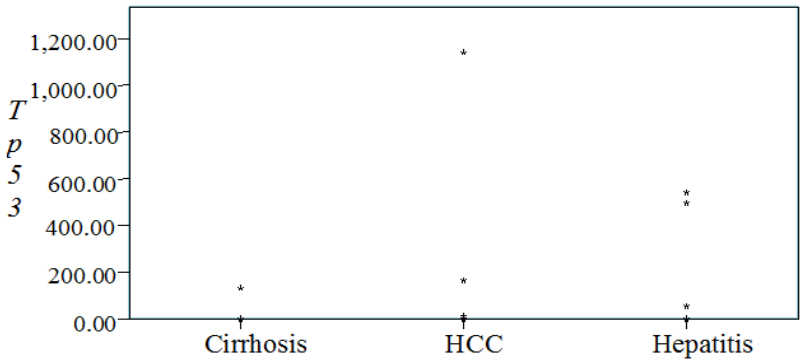

Figure 33. Independent samples Kruskal Wallis Test that showed the distribution of *Tp53* 249T (copies/ml) mutation in HCC (n =51), Cirrhosis (n = 53), and Hepatitis (n = 58). The stars indicate outliers in the groups.

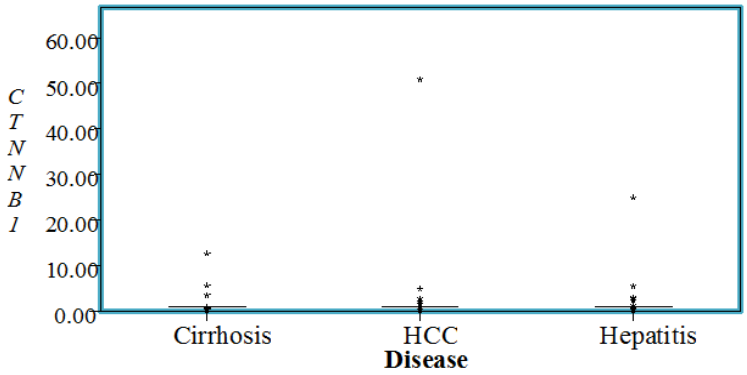

Figure 34. Independent samples Kruskal Wallis Test that showed the distribution of *CTNNB1* (copies/ml) mutation in HCC (n = 35), Cirrhosis (n = 29), and Hepatitis (n = 40). The stars indicate outliers in the groups.

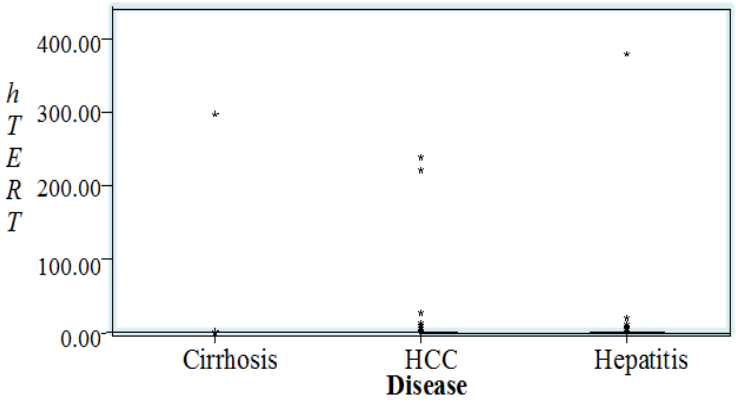

Figure 35. Independent samples Kruskal Wallis Test that showed the distribution of *hTERT* (copies/ml) Hypermethylation in HCC (n = 35), Cirrhosis (n = 29), and Hepatitis (n = 40). The stars indicate outliers in the groups.

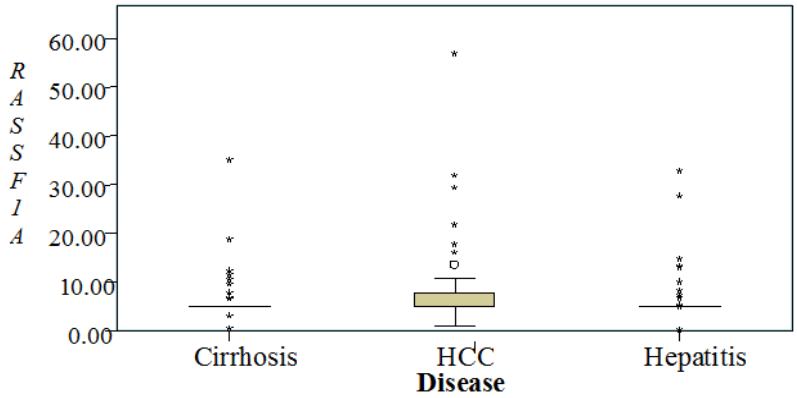

Figure 36. Independent samples Kruskal Wallis Test that showed the distribution of *mRASSF1A* (copies/ml) hypermethylation in HCC (n =51), Cirrhosis (n = 53), and Hepatitis (n = 58). The stars indicate outliers in the groups.

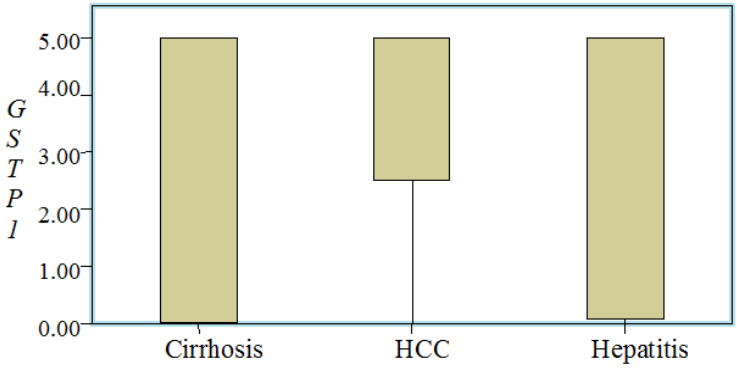

Figure 37. Independent samples Kruskal Wallis Test that showed the distribution of *mGSTP1* (copies/rxn) Hypermethylation in HCC (n = 16), Cirrhosis (n = 26), and Hepatitis (n = 22).

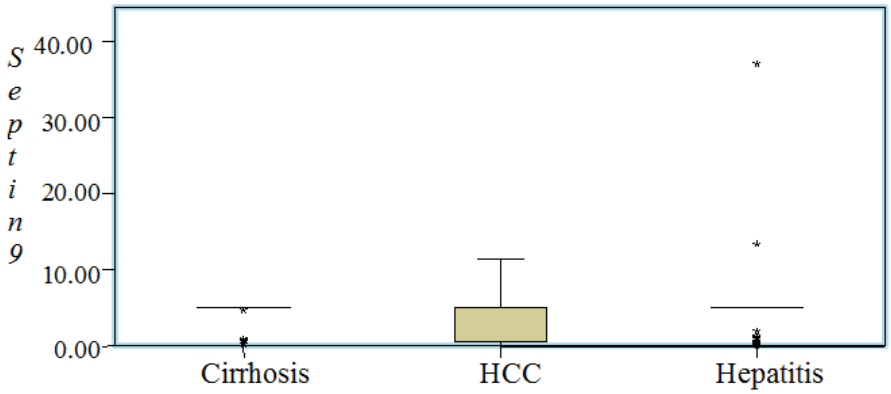

Figure 38. Independent samples Kruskal Wallis Test that showed the distribution of *mSEPT9* (copies/ml) Hypermethylation in HCC (n = 51), Cirrhosis (n = 53), and Hepatitis (n = 58). The stars indicate outliers in the groups.

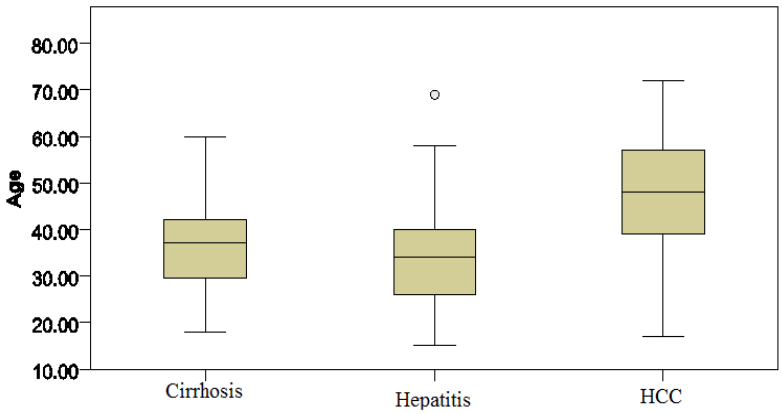

Figure 39. Independent samples Kruskal Wallis Test that showed the distribution of age in HCC (n = 51), Cirrhosis (n = 53), and Hepatitis (n = 59).

4.8 Comparison of the data with the US samples using ROC curve

Table 12. Socio-demographic characteristics of US Study participants

Characteristics	Hepatitis (n=49)	Cirrhosis (n=91)	HCC (n=73)
age (years) Mean ± SD	53 ±10.1	58 ±10.6	59 ±11.9
Male (n)	29	62	54
Female (n)	20	29	19

The data of the present study was then compared with the US samples that had 73 HCC, 91 cirrhosis and 49 hepatitis study participants for the four of the biomarkers namely *RASSF1A*, *GSTP1, SEPT9* genes, and serum AFP. The mean age of HCC, Cirrhosis and hepatitis was similar in all groups that is 53, 58 and 59 respectively. The mean age in HCC (59) of study participants was higher than the Ethiopian one (48) and the proportion of male to male was also higher in US study participants.

The AUROC curve generated for AFP to distinguish HCC (n=73) from Cirrhosis (n=91) and Hepatitis (n=49) was 0.811(81.1%) at 95% confidence interval (Figure 34). With the same token for *mRASSF1A* (Figure 34) and *mGSTP1* (Figure 34) values of 0.740 (74.0%) and 0.628 (62.85%) was obtained at 95% confidence interval. For *mSEPT9* (Figure 37) as a

biomarker to distinguish HCC (n=46) from Cirrhosis (n=18) and Hepatitis (n=16) the AUROC curve was 0.559 (55.9%) at 95% confidence interval.

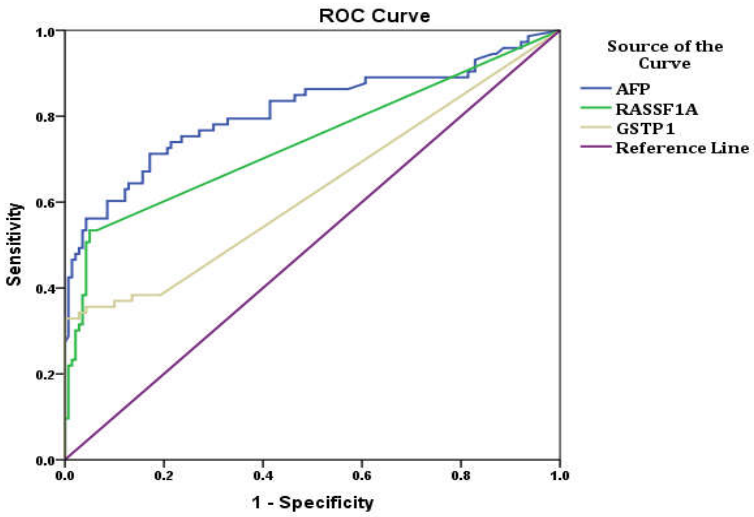

Figure 40. ROC curve generated for the biomarkers AFP, *RASSF1A* and *GSTP1*

AUROC curve for serum AFP biomarker was 0.811(0.742 - 0.880), for m*RASSF1A* gene MSP assay was 0.740(0.663 - 0.818), for *mGSTP1* gene MSP assay was 0.628(0.543 - 0.713) to differentiate HCC (n=73) from cirrhosis (n= 91) and hepatitis (n=49).Y-axes indicated the sensitivity and the x-axes 1-specificty.

The AUROC curve generated for the biomarkers was elucidated as follows:-

- For AFP, *mRASSF1A, mGSTP1* to differentiate HCC (n=73) from Cirrhosis (n=91) the AUROC curve values were 0.805 (80.5%), 0.737 (73.7%) and 0.604 (60.4%), respectively (Figure 35).
- For *mSEPT9* as a biomarker to distinguish HCC (n=44) from Cirrhosis (n=18) the AUROC curve value was 0.587 (58.7%) (Figure 38).
- Accordingly, for biomarkers AFP, *mRASSF1A,* and *mGSTP1* to differentiate HCC (n = 73) from hepatitis (n= 49) the AUROC curve were 0.823(82.3%), 0.746 (74.6%), and 0.672 (67.2%) respectively at 95% confidence interval (Figure 36).
- For *mSEPT9* as a biomarker to distinguish HCC (n=44) from hepatitis (n=16) the AUROC curve was 0.529 (52.9%) (Figure 39).

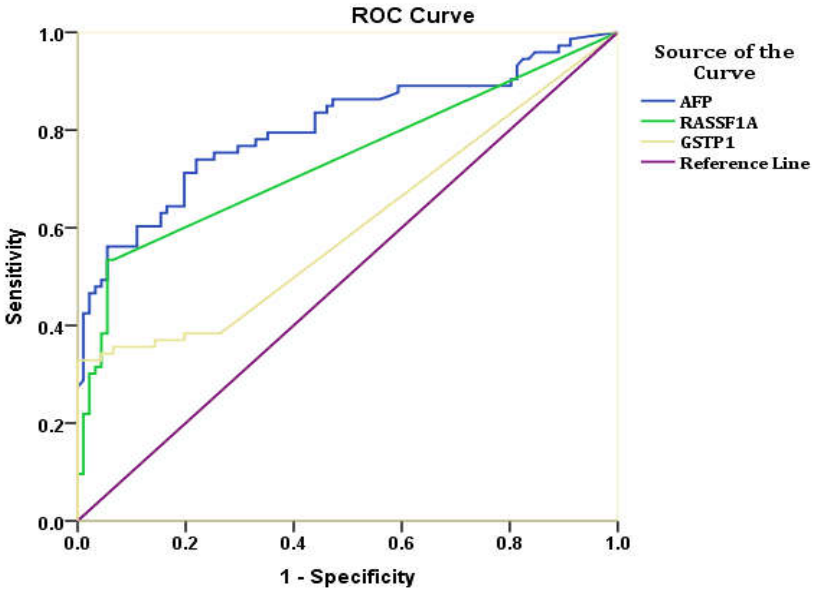

Figure 41**.** ROC curve generated for the biomarkers AFP, *RASSF1A* and *GSTP1*.

Serum AFP, *RASSF1A* and *GSTP1* biomarker used to distinguish HCC (n=73) from cirrhosis (n=91). The AUROC curve for AFP is 0.805(0.734-0.875), *RASSF1A* 0.737 (0.656-0.817) and *GSTP1* 0.604(0.514-0.695). Y-axes indicated the sensitivity and the x-axes 1-specificty.

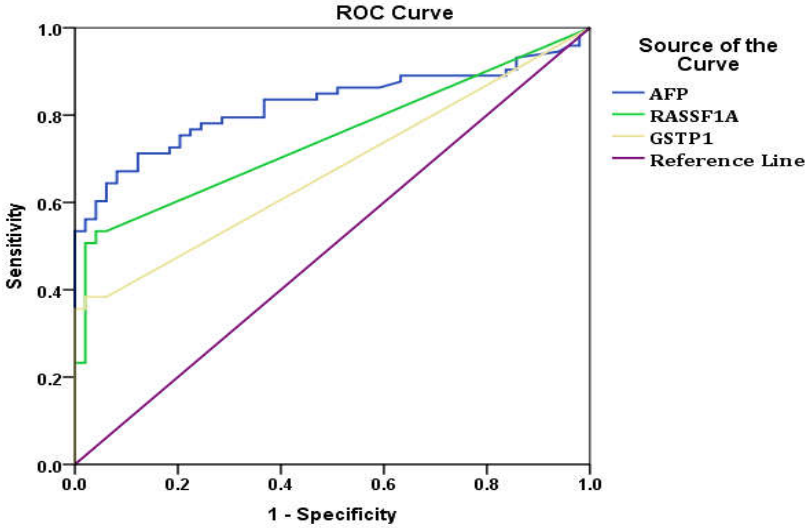

Figure 42 ROC curves for biomarkers to distinguish HCC from hepatitis

RASSF1A, *GSTP1* and Serum AFP biomarkers used to distinguish HCC (n=73) from hepatitis (n= 49). The AUROC curve for:- AFP 0.823 (0.748- 0.898), *mRASSF1A* 0.746 (0.661 - 0.766) and *mGSTP1* 0.672 (0.579- 0.766). Y-axes indicated the sensitivity and the x-axes 1-specificty.

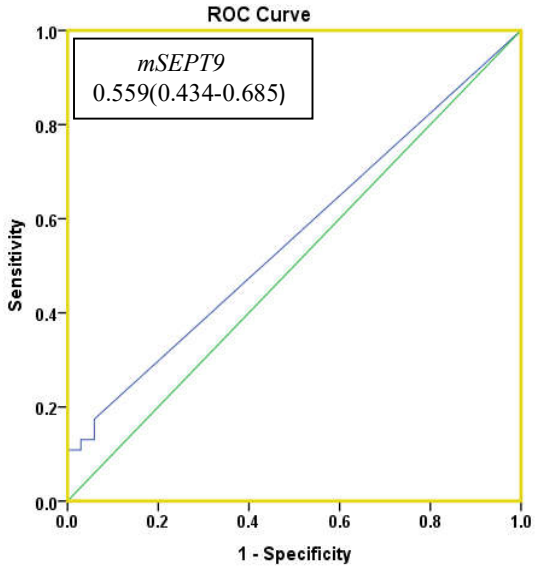

Figure 43. ROC curves for biomarker *mSEPT9*

mSEPT9 gene MSP assay used to distinguish HCC (n=46) from cirrhosis (n=34) and hepatitis. The AUROC curve values shown below the name of the genes. Y-axes indicated the sensitivity and the x-axes 1-specificty. The green line represents the reference line and the blue lines area under the curve.

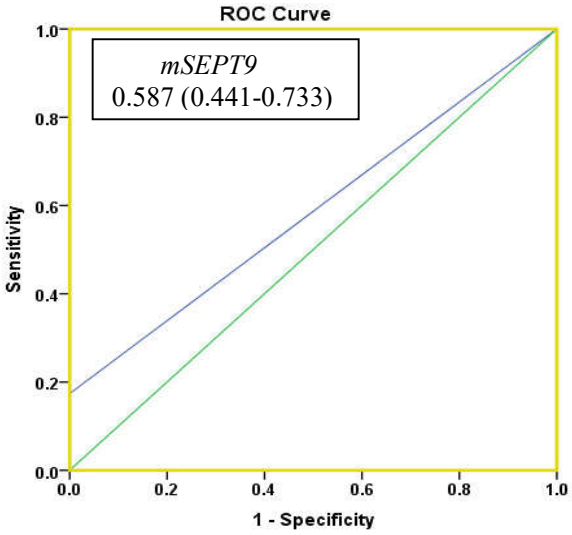

Figure 44. ROC curves for biomarker *mSEPT9* used to differentiate HCC from Cirrhosis

mSEPT9 gene MSP assay for HCC (n=44) and cirrhosis (n=18). The AUROC curve values shown below the name of the genes. Y-axes indicated the sensitivity and the x-axes 1-specificty. The green line represents the reference line and the blue lines area under the curve.

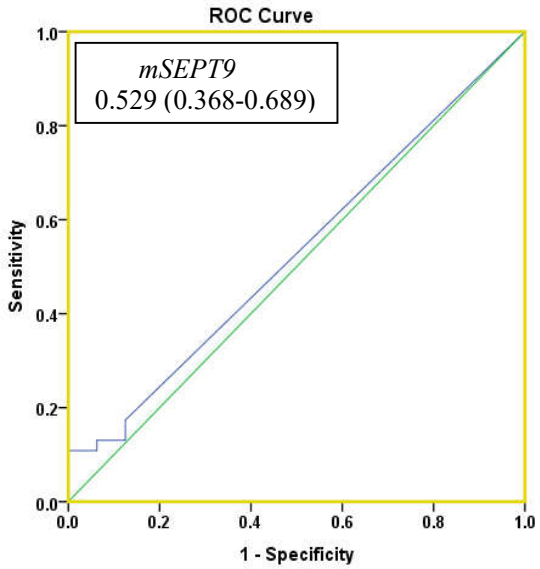

Figure 45. ROC curves for biomarker *mSEPT9* to differentiate HCC from Hepatitis

mSEPT9 gene MSP assay that had HCC (n=44) and hepatitis (n= 16). AUROC curve shown below the name of the gene. Y-axes indicated the sensitivity and the x-axes 1-specificty. The green line represents the reference line and the blue lines area under the curve.

Table 13. Comparison of AUROC curve data for all the Biomarkers of Ethiopian and USA samples

Genes and biomarkers	**AUROC curve for USA samples**	**AUROC curve for Ethiopian**
AFP		
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.811(0.742 - 0.880)	0.728(0.627-0.829)
HCC vs Cirrhosis	0.805(0.734 - 0.875)	0.719(0.611-0.826)
HCC vs Hepatitis	0.823(0.748 - 0.898)	0.719(0.612-0.826)
RASSF1A		
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.740(0.663 - 0.818)	0.532(0.431-0.632)
HCC vs Cirrhosis	0.737(0.656 - 0.817)	0.546(0.434-0.658)
HCC vs Hepatitis	0.746(0.661 - 0.832)	0.518(0.408-0.629)
GSTP1		

HCC vs Non HCC (Cirrhosis, Hepatitis)	0.628(0.543 - 0.713)	0.574(0.413-0.735)
HCC vs Cirrhosis	0.604(0.514 - 0.695)	0.581(0.402-0.759)
HCC vs Hepatitis	0.672(0.579 - 0.766)	0.567(0.380-0.754)
SEPT9		
HCC vs Non HCC (Cirrhosis, Hepatitis)	0.559(0.434 - 0.685)	0.405(0.305-0.505)
HCC vs Cirrhosis	0.587(0.441 - 0.733)	0.420(0.309-0.531)
HCC vs Hepatitis	0.529(0.368 - 0.689)	0.392(0.284-0.500)

The distribution of the three biomarkers among the three types of liver diseases (HCC, Cirrhosis and hepatitis) has also shown below in the diagram using Kruskal Wallis Test at significance level of $P < 0.05$. In AFP (Figure 40) and *mSEPT9* (Figure 43) the distribution of the biomarkers value among the three diseases was the same. As for *SEPT9* it was comparatively similar with the Ethiopian samples but somewhat different for AFP. *mRASSF1A*, (Figure 41) and *mGSTP1* (Figure 42) showed a better distribution (higher level) in HCC.

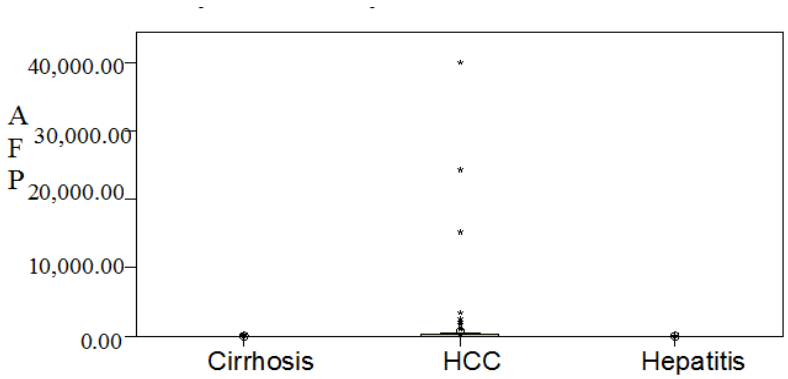

Figure 46. Independent samples Kruskal Wallis Test that showed the distribution of serum AFP (ng/ml) in HCC (n = 73), Cirrhosis (n = 91) and Hepatitis (n =49). The stars indicate outliers in the groups.

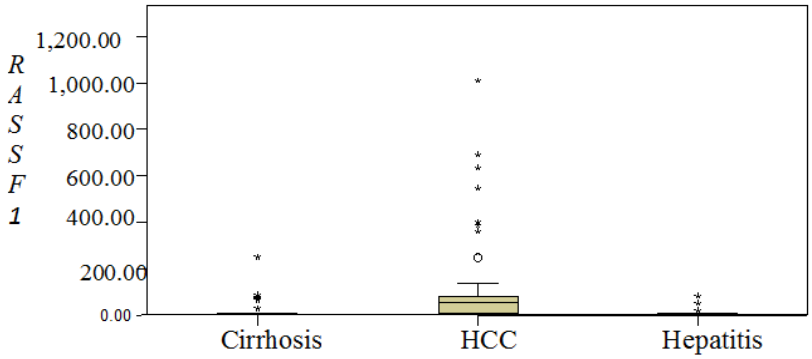

Figure 47. Independent samples Kruskal Wallis Test that showed the distribution of Urine mRASSF1A (Copies/ml) in HCC (n = 73), Cirrhosis (n = 91), and Hepatitis (n = 49). The stars indicate outliers in the groups.

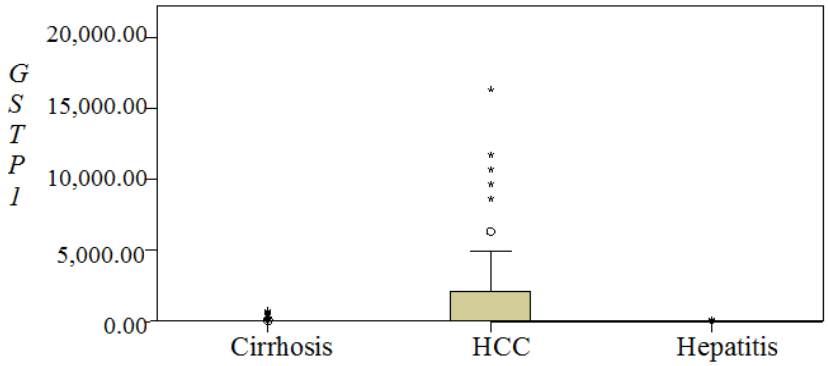

Figure 48 Independent samples Kruskal Wallis Test that showed the distribution of urine *mGSTP1* (Copies/ml) in HCC (n = 73), Cirrhosis (n = 91), and Hepatitis (n = 49). The stars indicate outliers in the groups.

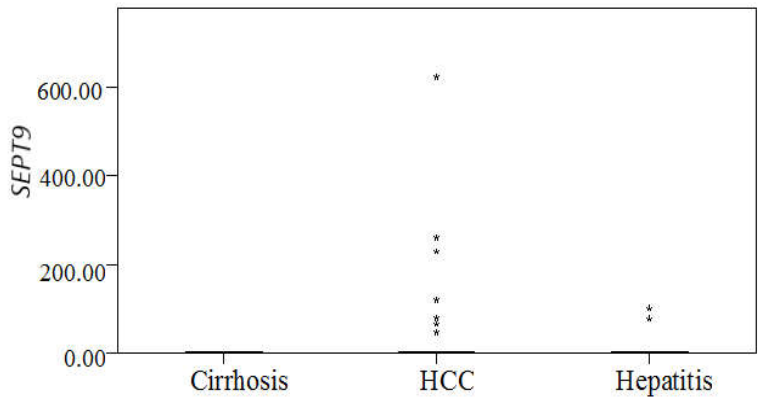

Figure 49. Independent samples Kruskal Wallis Test that showed the distribution of *mSEPT9* (Copies/ml) in HCC (n = 46), Cirrhosis (n = 34), and Hepatitis (n = 16). The stars indicate outliers in the groups.

CHAPTER FIVE

5. Discussion

Currently, evaluation of biomarkers for early detection of a cancer is a crucial and a serious concern of the modern medical society not only for confirming the presence of the disease but also to rule out the disease in healthy individuals and also for real time monitoring of a tumor response to therapy**.** Hence, advancements of molecular techniques in biomedical research pave a way for detecting tumor specific genetic and epigenetic alteration in ctDNA and transforming the diagnosis, prognosis and treatment of cancer to a higher level and helped us to reach the era of precision medicine.

The present study evaluated six urine DNA biomarkers from Ethiopian HCC, Cirrhosis and hepatitis patients and compared with the USA ones. The data was analyzed with ROC curve to see the differentiating capacity of the biomarkers for HCC from non-HCC. The USA urine DNA biomarkers showed higher differentiate capacity of HCC from non-HCC with a better AUROC curve analysis results.

In this study six biomarkers in Ethiopian samples were evaluated to see the differentiating capacity of HCC from cirrhosis and hepatitis and also to detect serum AFP negative HCC patients Since the sensitivity of the AFP for detection of HCC limits its applicability that is around of 40 - 60% and we believe that those that are not diagnosed with AFP can be diagnosed with the DNA biomarkers. Hence, we quantified the level of mutation and hypermethylation in the DNA biomarkers and in an effort to enhance specificity and sensitivity of the assay panel of six biomarkers have been used in this study.

There are many risk factors that contribute for the development of HCC, among which are alcohol, tobacco, age and gender. Considering this significance, this study investigated the frequency of substance users (alcohol, Chata edulis, and Smoking) and their influence on patients who had been diagnosed with the disease. As the result indicated on this particular study, the contribution of the substance use in disease progression or development of HCC was found to be insignificant. It is noteworthy to state that even the values obtained from the combined substance user have showed only negligible contribution as risk factors towards the development of HCC. However, taking the number of study participants into consideration, additional study must be conducted on large group of study participants to validate the result.

In contrast to this study, a research done on American HCC and cirrhosis patients by Marrero and his colleagues (2005) had pointed out that there was significant relationship between alcohol and tobacco with HCC. Other researchers also showed the risk of alcohol and tobacco intake for the development of HCC among HBV patients (El-Serag and Kanwal, 2014).

From the result that was obtained the age of the study participants with the HCC one's had higher mean age (48 years) compared to cirrhosis and hepatitis. This output thus indicated the considerable contribution of age in the development of HCC. This is somewhat in agreement with El-serag (2012) that indicated the mean ages of diagnosis with HCC were 55–59 years in China. This little difference might be explained by the variation in geographical location.

Gender has been also noted to be another risk factor for the development of HCC. Similar to other studies ours has showed that about 70 % of the HCC study participants were male. Male are at higher risk than women in developing the disease and this might be explained by behavioural and environmental exposures like alcohol and tobacco. In addition to substance use, other factor is the level of sex hormones:- estrogens prevent and androgens promote liver cancer. Moreover, the androgen pathway has shown to increase the transcription of HBV genes by binding directly to viral genome and on the contrary, HBx protein can increase the transcription of androgen receptors. Estrogens may act as general suppressors of HCC through reduction in the proinflammatory effects or through its anti-inflammatory effect (Keng *et al.,* 2011; El-Serag and Kanwal, 2014). Chiu and colleagues (2007) was also showed that males are more susceptible than females to HBV related HCC with their cell lines study and showed the HBx mediated activation of c-Src kinase and in turn the downstream MEK and AKT which is involved in HBx mediated Androgen receptor activation.

In the HCC study participants AFP was measured and found to be negative (<10 IU/ml) in 40.4% of the study participants. As a result of this incidence, and also being a chief objective of this project the need for searching alternative better biomarkers capable to exhibit higher sensitivity and specificity than AFP to detect those false negatives (AFP negative HCC) was pivotal. It is known that not all cancer cells secrete specific protein biomarkers like AFP; but only a subset of them (Jin *et al*., 2012). In a study conducted on 112 study population it was found out that 61 (54%) of the study participants with HCC had serum AFP levels <20 ng/ml, and considered to be AFP-negative. However, with DNA biomarkers analysis 56 (92%) of the 61 study participants of the AFP-negative HCC tissues were found to be positive for the

HCC, This assay strongly justified that the potential use of the DNA biomarkers for detecting of AFP negative HCC samples. it has been also mentioned that here is no biochemical biomarker that can detect AFP-negative HCC (Su *et al*., 2014).

In non-HCC (hepatitis and cirrhosis), we found high AFP in 3.7% hence indicating the presences of false positives. This is in line with other researchers findings that pointed out that the occurrence of elevated level of AFP in 10% to 40% of patients with chronic liver disease without HCC (Bialecki and Di Bisceglie, 2005; Davis, 2011). Gomaa (2009) and his colleagues have also found elevated level of AFP in some patients with cirrhosis and/or hepatic inflammation even without the presence of a liver cancer. This might be the reason for the elevated values of AFP level in non HCC patients in this study.

We have analyzed total LMW DNA (150 - 250bp) in the urine of the study participants that had helped us to see how much circulating cell free DNA was there. The tumor DNA (ctDNA) is a subset of this and its concentration is much higher than the ctDNA. As Su (2004) and her colleagues pointed out that the LMW DNA has a better sensitivity than total urine DNA for detection of the mutation and hypermethylation in the DNA biomarkers in the urine of the cancer patients (Botezatu *et al*., 2000; Su *et al.,* 2004).

The mean level of LMW DNA (cfDNA) in the DNA biomarkers *Tp53, CTNNB1, hTERT, GSTP1, SEPT9* was higher in HCC study participants followed by cirrhosis and hepatitis except RASSF1A which was higher in hepatitis. This is in line with Jin and his colleagues (2012) that mentioned the amount of cfDNA in the plasma and serum of healthy individuals is low but the concentration is significantly increased in cancer patients.

In this study significantly higher (49%) HCC study participants have underlying hepatitis. This findings is in line with other studies that illustrated 80% of HCC cases worldwide are attributed to infection with HBV and HCV even if there are a number of risk factors (Hussain *et al*., 2007; Hennedige, and Venkatesh, 2012; Mittal and El-Serag, 2013). Adrian and Bisceglie (2009) also mentioned that the largest attributable fraction of HCC is due to hepatitis B (60%) in Africa and East Asia. There were also 41.2% of HCC had underlying cirrhosis. Many researchers have indicated that cirrhosis is an important predisposing condition for the development HCC and approximately 80 - 90% of HCCs arise in cirrhotic livers (Chemin and Zoulim, 2009). El-Serag and Davila (2011) had also pointed out that the

major risk factors for the development of HCC are HBV, HCV and alcoholic liver diseases. Premalignant lesions developed on cirrhotic liver that has high risk of transformation to HCC (Nault *et al.,* 2013). Again from these HCC 25.5% of them had both hepatitis and cirrhosis that is in agreement with (Chemin and Zoulim, 2009) that said in HBV infected patients with cirrhosis there was approximately 3-fold higher risk for HCC development than those with only chronic hepatitis B. Adrian and Bisceglie (2009) had also said approximately 70% of cases of HBV-related HCC occur in association with cirrhosis,

With regard to quantification of mutation and hypermethylation of the six biomarkers our findings showed that the mean level of mutation and hypermethylation of each gene in the urine samples is higher in HCC compared to cirrhosis and hepatitis except *SEPT9* which is a somewhat higher in hepatitis. But generally, the level of mutation and hypermethylation was low and in some cases below level of detection compared to other studies. As it was explained by other researchers ctDNA may be quite low and therefore below the limit of detection, especially in early stage of cancer that is positively associated with degree of malignancy, size of tumor and vascular invasion, they also indicated that the proportion of HCC patients with detectable ctDNA varies widely between studies as well (Tang *et al*., 2016; Rojas *et al*., 2018). Jin and colleagues also mentioned that the amount of mutant gene fragments is small compared to the of normal circulating DNA fragments that makes it difficult to detect and quantify with sufficient sensitivity and specificity for meaningful diagnostic purpose (Jin *et al*., 2012).

In AFP-negative HCC, mutation was found in 4 out of 19 for *Tp53*, 6 out of 13 for *CTNNB1*, 3 out of 13 for *hTERT* and hypermethylation was found 6 out of 19 for *RASSF1A* and 7 out of 19 in *SEPT9* of the study participants. This showed that the potential use of the biomarkers to detect HCC in AFP-negative. When it comes to *Tp53,* Lin and her colleagues detected mutation in 9 out of 17 of HCC from urine (Lin *et al.,* 2011; Hann *et al.,* 2017). The potential use of these biomarkers to detect AFP negative HCC tissue Samples have been shown on 61 AFP negative HCC samples out of which 56 (92%) of them became positive for the DNA biomarkers (m*APC*, *Tp53249T*, m*GSTP1* and m*RASSF1A*) (Su *et al*., 2014). Jain also showed this on 62 AFP negative tissue samples and found 33.8% (21/62) of them positive for m*GSTP1* biomarker (Jain *et al.,* 2012).

Advances in biomarker development for early detection of cancer have taken more prominent role in cancer research and recently urine based biomarker development have attracted more attention as an easily administered since it is noninvasive, easy to collect and isolate the DNA. An important and useful technique for evaluating and assessing the performance of biomarkers or diagnostic medical tests is the AUROC curve (Baker, 2003; Mandrekar, 2010).

The receiver operating characteristic (ROC) curve is a graphical display of the true-positive rate (Sensitivity) and the false-positive rate (1-specificity) from multiple classification rules. For biomarkers to be used as diagnostic test AUROC curve should be > 0.50. A value 0.5 for AUROC curve that fall on the diagonal line suggests that the diagnostic test has no discriminatory ability of diseased from the non-diseased. AUROC curve 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent and more than 0.9 is outstanding (Hanely and McNeil, 1982; Mandrekar, 2010). We have used AUROC curve for the analysis of the six biomarkers from urine to assess their predictive value and the power of differentiating capacity of HCC from non-HCC (cirrhosis and Hepatitis).

In all the cases, (the first case is biomarkers that can make a distinction of HCC from hepatitis and cirrhosis, the second one is HCC from cirrhosis, and the last one HCC from hepatitis) *CTNNB1* and *hTERT* mutation biomarkers have better predictive value since they have >0.5 AUROC curve and *Tp53* showed relatively low AUROC curve. Of the six biomarkers *hTERT* had better predictive value in differentiating HCC from cirrhosis followed by *CTNNB1*. In contrast to our study Wang and his colleagues have shown better performance of the urine biomarker *Tp53* to distinguish HCC from non-HCC and had found 0.56 (56%) AUROC curve (Lin *et al.,* 2011; Hann *et al.,* 2017; Wang *et al.,* 2018). Other researchers have found that *hTERT* promoter mutation in circulating cfDNA of HCC and cirrhosis and showed its promising risk prediction potential for HCC (Jiao *et al,.* 2018). Nault and his colleagues also detected *hTERT* promoter mutation on panel of 10 genes on HCC and cirrhosis tissue samples and showed that the *hTERT* promoter mutation as an early event in liver carcinogenesis (Nault *et al;* 2014). Yang and his colleagues sequenced 276 HCC tissue samples for *hTERT* promoter and found in 85 (31%) of the study participants the hot spot mutation (Yang *et al.,* 2016).

The result showed that *SEPT9* had relatively low capacity in distinguishing HCC from non-HCC in all the cases. These findings suggest that promoter hypermethylation of

the *RASSF1A,* and *GSTP1* genes were good since they had more than 50% value. The biomarkers had also showed better distinguishing capacity for HCC and cirrhosis group. This is also in contrary to the findings of (Wang *et al.,* 2018) that showed AUROC curve for *RASSF1A* and *GSTP1* as 0.69 (69%) and 0.62 (62%) respectively that showed better predictive value of HCC from non-HCC compared to our study. In another study conducted on tissue samples by Jain and her colleagues, hypermethylation of *GSTP1* gene promoter region has been reported to be a potential biomarker to distinguish HCC from other liver diseases with AUROC 0.76 (Jain *et al*., 2012). In a similar pattern, again on tissue samples Jain and her colleagues have also showed in their findings that AUROC for RASSF1A is 0.697 to distinguish HCC from cirrhosis and hepatitis and 0.881 to distinguish hepatitis from cirrhosis (Jain *et al*., 2015). Again on tissue samples other researchers have also showed the association of *RASSF1A* hypermethylation with HCC (Saelee *et al.,* 2010). In another findings, the AUROC curve result that have found by them were 0.733 for *Tp53* 249T, 0.887 for *mRASF1A* and 0.756 for m*GSTP1* that showed significantly higher distinguish potential of HCC (n=120) from cirrhosis (n= 35) and hepatitis (n = 35) (Su *et al,* 2104).

Most of the studies that have been done by other researchers were on plasma, serum and tissue. The data of another researcher on ctDNA specifically from plasma DNA also showed the discriminating ability of HCC from normal controls with 90.2% sensitivity and 90.3% specificity and AUROC was 0.949 (95% Confidence interval) measured by qPCR quantitative method (Huang *et al*., 2008). *RASSF1A* was found hypermethylated in serum of 93% HCC patients compared to healthy people and this frequency was similar to the one found in HCC tumor tissues (Chan *et al.,* 2008). These two studies had shown the importance of ctDNA for diagnosis of HCC. Another study who have seen the mutation profile of biopsy and plasma samples in HCC showed that the ctDNA as a very importance tool to overcome tumor heterogeneity and also real time monitoring of the tumor response to therapy (Cai *et al.,* 2017).

In our findings:- serum AFP in all the cases has about 70% capacity in distinguishing HCC from other liver diseases (Hepatitis and Cirrhosis) that is a bit less than Wang and his colleagues which is 0.88 (88%) AUROC curve (Wang *et al.,* 2018). Another one showed that AFP had the best AUROC curve which is 0.80 at 95% confidence interval for early stage HCC (Marrero *et al*. 2009).

The distribution of the biomarkers among HCC, Cirrhosis and Hepatitis with Kruskal Wallis Test showed that *Tp53*, *CTNNB1*and *hTERT* have somewhat similar distribution level across the groups as compared to the non-HCC group in *mRASSF1A, mGSTP1, Tp53* and Serum AFP (Wang *et al.,* 2018).

Generally, in this study the hypermethylation biomarkers are better indicators than the mutation ones. Although the level of mutation were expected to have a higher predictive value for the HCC, the result obtained in this study have shown to have relatively low mutation than the data obtained from USA. This occurrence may have attributed to the methods employed in the isolation of cfDNA from urine which can affect DNA yield and low and high molecular weight fractions recovery. This hypothesis can be supported by the study conducted by Perez-Barrios which have taken into account this observation for cfDNA analysis (Perez-Barrios *et al.,* 2016). Additionally, other findings also indicated that the levels of urine DNA Biomarkers can fluctuate due to hydration of the patient at time of urine collection which can result in diluted DNA in the urine (Hann *et al.,* 2017).

6. Conclusion

It is clear that HCC screening is very beneficial to reduce mortality rate by detecting the disease at its earliest stages. This has significant advantages from the prospective of disease control and treatment. However, as the current working efficiency of available biomarkers indicated, they have shown to lack in addressing the required sensitivity and specificity for effective and reliable detection of disease state. Such precise analyses of genetic and epigenetic profiling have huge benefit for the development of molecular biomarkers that support the discovery and advancement of diagnostic and predictive biomarkers for HCC diagnosis. Taking this point into consideration, the present study has attempted to show the potential use of panel of circulatory urine DNA biomarkers for early detection and screening of HCC by assessing mutation and hypermethylation of commonly defective genes. ctDNA are a prominent tool for early detection and diagnosis of cancer specifically when it becomes from urine.

The interests of molecular biomarkers in this study were *Tp53, CTNNB1, hTERT, GSTP1, RASSF1A,* and *SEPT9* which have analyzed *by* the qPCR assay. From the findings *hTERT, CTNNB1*, *RASSF1A* and *GSTP1* have exhibited better differentiating capacity of HCC from other liver diseases. On the contrary *Tp53* and *SEPT9* did not display the expected and desired predictive potential. Although the data obtained from the US have shown a slightly better predictive potential as compared to the data obtained in this study, it must be underscored that the findings in this project are highly valuable and capable to be used as biomarkers for early detection and screening of HCC. Moreover, environmental variation, geographical setting, race, genetic makeup and related elements could be contributing factors for obtaining different data in the current study.

From this study it can be concluded that the utilization of PCR based techniques that applied liquid biopsies such as urine DNA biomarkers in combination with other biochemical parameters has better diagnostic efficiency as assessed from its enhanced sensitivity and specificity detection potential. In future outlook and in an effort to enhance the predictive potential with improved sensitivity and specificity, it is important to stress the need for considering some factors such as recruiting large group of study participants, the use of standardized procedures along with customized methodology that can fit best to the Ethiopian populations.

Generally, these urine DNA biomarkers may be regarded as new tool and may even have most promising and potentially useful diagnostic marker to be applied in the early detection and screening of HCC. Most importantly, development of biomarkers for cancer diagnosis from circulating nucleic acids from plasma serum and urine has produced many stimulating developments that have the potential to transform cancer diagnosis. Hence, it should be taken as a promising diagnostic, prognostic, and risk assessment of HCC to significantly improve the lives of HCC patients.

7. Limitation of the Study

Many challenges exist that hinder to do a research in a very organized, timely and smooth way. It was impossible to get different molecular reagents and chemicals in the country for some of laboratory analysis to be performed. As a result of this difficulty, the materials had to be shipped from overseas which consequently cost significant time and energy. The unavailability of well-organized and well-equipped molecular laboratories to conduct the research was the other difficulty that had been encountered. The samples size had been also another issue. Although the sample collection took a year, it was difficult to get the required amount and this might had an effect on the research findings. This was due to lack of awareness among HCC patients who have been willing to participate in this study. Lack of enough research budgets network with clinical departments. So there is a need to establish a very good network with clinical departments in order to have well organized and smooth way of sample collection, access to get patient data and others which can benefit all of us. This also limits the usefulness of the research findings in clinical applications. Recruiting research participants was a difficult task and it is important to work on creating awareness to change the attitudes of the people towards research, this will benefit the society and the country at large. Last but not least, as stated above this research is the first in its kind in Ethiopia, hence it has a great potential/capacity to be further investigated.

8. Recommendations

In order to improve the predictive value of the biomarkers utilized in this study and others a bigger study might be required. This might require further investigation on large set of samples and may require customized approach of the assay which includes different primer and probe design that would fit best to the Ethiopian populations in order to get a better sensitivity, specificity and predictive potential. Furthermore, a better sample collection scheme that control the volume of fluid taken prior to the sample collection might be needed.

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Annex I Questionnaire

1. Introduction

1.1 Study participant identification number_____

1.2 Date of interview_____

1.3 Place of interview_____

2 Age (in year)_____

3 Gender 1. Male ☐ 2. Female ☐

4 Ethnicity _____

5 Address _____

6 Diagnosis _____

7 History of other diseases/infection with HBV or HCV for the HCC patients

a. _____

b. _____

c. _____

8 Age at which the participant had the liver cancer _____

9 Does his/her family have history of Liver cancer and/or other liver diseases?

1.Yes ☐ 2. No ☐ 3. Unknown ☐

If yes, what type of cancer _____

If yes, who Father ☐ Sister ☐

Mother ☐ Brother ☐

Grandmother ☐ Grandfather ☐

10 Would the person use the following items, if so how frequent?

a. Peanut frequently ☐ Sometimes ☐ Never ☐

b. Alcohol frequently ☐ Sometimes ☐ Never ☐

c. Smoking frequently ☐ Sometimes ☐ Never ☐

d. Chat Edulis frequently ☐ Sometimes ☐ Never ☐

Annex II Information Sheet

To the participant of the study

Principal investigator Maria D. T/mariam

Name of Sponsors Addis Ababa University and Hepatitis B foundation/Baruch S. Blumberg institute, Philadelphia.

1. **Aims of the study**

   This study will be conducted on liver cancer, hepatitis and cirrhosis patients. The aim of the study is to detect the abnormal genes that are responsible for the development of the liver cancer and also look their association with the disease.

2. **Role of the participants in the study**

   If he/she agrees to participate in the study, he/she will cooperate during questionnaire filling and he/she will give 5millilitre or 3 spoon full blood and 50 millilitre urine specimens. The specimen will be collected using sterile medical instruments.

3. **Risks associated with the specimen collection**

   Collection of the specimen would follow the routine procedure for laboratory investigation but there might be some pain that is associated with the specimen collection specifically the blood.

4. **Benefits**

   The study result will be beneficial for liver cancer patients for early detection, improve the diagnosis and to give appropriate treatment. In the future it is used for the development of drugs for the treatment of the disease. It will be also beneficial for the researcher who works in this area to understand the disease and give better treatment.

5. **Rights**

   The study participant have a right to keep hold of information and decline to cooperate in the study. This would have no bearings at all on their health benefits. The study participants have full right to ask information about the research before they decide to participate.

6. Confidentiality

All the information contained within the questionnaire is to be kept confidential and the specimen taken will be used only for the purpose of the research and the name of the participant will not be mentioned.

7. Approval

This research project gets approval from Institutional Review Board, College of Health Sciences, Addis Ababa University. It is also approved by School of Medicine, Postgraduate program and Department of Biochemistry.

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  Tel. 251-118 96 13 96

Annex III Information sheet (Amharic version)

በጉበት ካንሰር ሕመምተኞች ላይ ለሚደረገው ጥናት የማብራሪያ ቅፅ

<u>ለጥናቱ ተሳታፊዎች</u>

ዋና ተመራማሪ	ማሪያ ደገፍ
ምርምሩን የሚያካሔዱበት ተቋም	አዲስ አበባ ዩኒቨርስቲ የጤና ሳይንስ ኮሌጅና ሄፕታይተስ ፋውንዴሽን/ብሉምበርግ እስንቲትዩት

1. **የጥናቱ አላማ**

ጥናቱ የሚካሄደው በጉበት ካንሰርና በሌላ አይነት የጉበት ሕመም ባላቸው ሕመምተኞች ላይ ሲሆን አላማውም ይህን በሽታ ወደአደገኛ ደረጃ ያደርሳሉ ተብለው የሚገመቱን ዘረመሎች (Genes) ያሉበትን ሁኔታ ማጥናት፡፡ በተጨማሪም የእነዚህን ዘረመሎች ሁኔታ በማጥናት ከህመሙ ጋር ያላቸውን ዝምድና ማየት፡፡ ከሌሎች ለህመሙ አስተዋጽዖ አላቸው ተብለው ከሚገመቱት ነገሮች ጋር ያላቸውንም ዝምድና ማጥናት፡፡

2. **የተሳትፎ ሁኔ**

በጥናቱ ውስጥ ለመሳተፍ ከተስማሙ ተመራማሪው ለሚጠይቁት ጥያቄዎች መልስ መስጠት ለምሳሌ የራሳዎን እድሜ፤ በበሽታው መቼ እንደተያዙና ሌሎችም፡፡ ለጥናቱ የሚያስፈልገውን 5 ሚሊ ሊትር (3 የሻይ ማንኪያ) የደምና ሀምሳ ሚሊ ሊትር የሽንት ናሙና መስጠት፡፡ ናሙናው የሚወሠደው ንጹህ በሆኑ የህክምና መሳሪያዎች ነው፡፡

3. **ሊከሰቱ ስለሚችሉ ስጋቶችና የምቾት መጓደሎች**

ለጥናቱ በሚወሠደው ናሙና ምክንያት የሚፈጠር የተለየ ችግር የለም፡፡ ምክንያቱም የናሙናው አወሳሰድ ከወትሮው በሽተኛው ለራሱ ህመም ክትትል ተብሎ ከሚሠጠው የተለየ አይደለም፡፡ ናሙና መውሠድ የተለመደ የላቦራትሪ የዕለት ተዕለት ስራ ነው፡፡ ነገር ግን መጠነኛ የህመም ስሜት ደም ሲወሰድ ሊኖር ይችላል፡፡

4. **ጥቅሞች**

የጥናቱ ውጤት ለሕመምተኞቹ የሚሠጠውን የህክምና ዘዴ እንዲሻሻል ያደርጋል፤ለወደፊት የተሻለ መድሀኒት እንዲሠራ ይረዳል፡፡ በተጨማሪም በዚህ ምርምር ሥራያ ለሚሰሩ ተመራማሪዎች (የህክምና ባለሙያዎች) በሽተኛውን በተሻለ መልኩ እንዲረዱትና የተሻለ ህክምና እንዲሰሩ ይረዳቸዋል፡፡

5. በጥናቱ ያለመሳተፍ ወይም ራስን የማግለል መብት

የእርስዎ ተሳትፎ በፈቃድኝነት ላይ የተመሠረተ ነው። በጥናቱ ውስጥ አለመግባት ከፈለጉ ያለመሳተፍ ይችላሉ ነገር ግን በዚህ ጥናት ውስጥ መሳተፍ አለመፈለግዎ የጤናዎ እንክብካቤ ክትትል ላይ ምንም አይነት ተፅዕኖ አይኖረውም። ጥያቄ የመጠየቅም ሆነ ስለምርምሩ የማወቅ የፈለጉትን ማብራሪያ የመጠየቅ መብት አለዎት ። እርስዎ በጥናቱ ላይ መሳተፍዎን ለመሠረዝ ከፈለጉ በማንኛውም ሠዓት መሠረዝ ይችላሉ።

6. ሚስጥር ስለመጠበቅ

ለሁሉም ጥያቄዎች የሠጡንን መልሶች በሚስጥር እንደምንይዝ ሊያውቁ ይገባል።
የተወሠደውም ናሙና ለተጠቀሠው ጥናት ብቻ የሚውል ነው። የእርስዎ ማንነት በስም አይገለፅም።

7. ጥናቱን የማፅድቅ ሁኔታ

ይህ ጥናት በሥነ ምግባር ቅኝትና ግምገማ ኮሚቴ በአዲስ አበባ ዩኒቨርሲቲ፤ የጤና ሳይንስ ኮሌጅ፤ የህክምና የትምህርት ክፍል፤ ድጋፍ አግኝቷል። በድህረ ምረቃ ትምህርት ክፍልና በባዮ ኬሚስትሪ ትምህርት ክፍልም ድጋፍ አግኝቷል።

የተመራማሪው አድራሻ - ማሪያ ደገፍ
የጤና ሳይንስ ኮሌጅ
አዲስ አበባ ዩኒቨርስቲ
ስልክ ቁጥር 251 0911 68 33 98
የጤና ሳይንስ ኮሌጅ የሥነ ምግባር ቅኝትና ግምገማ ኮሚቴ አድራሻ -
ስልክ ቁጥር 251-118 96 13 96

Annex IV Consent form

Name of study participants_____

I have been informed about a study plans that deals with Hepatitis, cirrhosis and liver cancer patients for this purpose urine and blood sample will be taken from me. The aims of the study were explained to me. Collection of the specimen would follow the usual procedure for laboratory investigation but there might be some pain that is associated with the blood collection.

I am also informed that all the information contained within the questionnaire is to be kept confidential. The collected urine and blood specimen will be used only for this study purpose. Treatment will continue to be given to me whether I agree to participate in this study or not**.** Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and make myself withdraw from the study. I have been informed that laboratory results will be disclosed to me whenever the result is ready.

It is therefore with full understanding of the situation that I gave the informed consent voluntarily to the researcher to use the specimen taken from me for the investigation. Moreover, I have had the opportunity to ask questions about it and received clarification to my satisfaction.

Signature _____ Signature _____

(Participant) (Investigator)

Date _____

Witness name _____ Signature _____

Annex V Consent form (Amharic version)

የስምምነት ቅጽ

የተሳታፊው ሙሉ ስም----------

እኔ ስሜ ከላይ የተጠቀሰው የጥናቱ ተሳፊ የጉበት ካንሰር ሕመምተኞች ላይ እና ሌሎችም የጉበት ሕመሞች ላይ ሊደረግ ለታሰበው ጥናት መረጃ አግኝቻለሁ። ለዚህ ይረዳ ዘንድ ከእኔ ላይ የሽንትና የደም ናሙና መውሰድ እንደሚፈለግ ተረድቻለሁ። ስለጥናቱ አላማ፤ ከተመራማሪው ገለጻ ተረድቻለሁ። እንዲሁም ናሙና ሲወሰድ ከወትሮው ለበሽተኛው ክትትል ተብሎ ከሚሠጠው የተለየ አለመሆኑ ተነግሮኝ የደም ናሙና ሲወሰድ ግን መጠነኛ የህመም ስሜት ሊያስከትል እንደሚችል ከተመራማሪው ገለጻ ተረድቻለሁ።

በተጨማሪም መጠይቁ ውስጥ በተካተቱት ጥቄዎች መሰረት የምሰጣቸው መረጃዎች በጠቅላላ በሚስጥር እንደሚጠበቁ ተገልጾልኛል። ከእኔ የሚወሰደው ናሙና ለዚህ ምርምር ብቻ እንደሚውል ተረድቻለሁ። እንዲሁም እኔን በተመለከተ የምጠየቀውን መረጃ ያለመስጠት፤ በጥናቱ ያለመተባበርና ከጥናቱ በማናቸውም ጊዜ ራሴን የማግለል መብቴ የተጠበቀ መሆኑ ተገልጾልኛል። ነገር ግን በዚህ ጥናት ውስጥ መሳተፍ አለመፈለግ የጤናዬ እንክብካቤ ክትትል ላይ ምንም አይነት ተፅዕኖ እንደማይኖረው ተረድቻለሁ። የምርምሩ ውጤት ምርምሩ ሲያልቅ እንደሚገለጽልኝ ተነግሮኛል።

ስለዚህ ለተመራማሪው መረጃ እና የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍጹም ፈቃደኝነት ነው። በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለግኩትን ያህል ማብራሪያ አግኝቻለሁ።

የጥናቱ ተሳታፊ ፊርማ ---------- የተመራማሪው ፊርማ ----------

ቀን----------

የእማኝ ስም _____ **ፊርማ** _____

Annex VI Material Transfer Agreement (MTA)

This Material Transfer Agreement (MTA) has been prepared for use by

Department of Biochemistry, Addis Ababa University and Hepatitis B Foundation/ Baruch S. Blumberg institute.

in all transfer of research material (samples, derivatives, and specimens) related to the protocol: **"Development of panel of urine DNA Biomarkers for Hepatocellular carcinoma screening and early detection"**

Provider: Department of Biochemistry, Addis Ababa University

Recipient: Hepatitis B Foundation/Baruch S. Blumberg institute, Philadelphia.

1. Provider agrees to transfer to recipient's designated (human biological sample) the following research materials (specimen) **Human urine and blood.**

   The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.

   a) Are the Research materials of human origin?

      Yes [X] No [ ]

   b) If yes, will they be collected according to the details in the protocol and in adherence to National Health Research Ethics Review Committee (NERC) and Addis Ababa University, College of Health Sciences, School of Medicine Institutional Review Board recommendations and their approval?

      Yes [X] No [ ]

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows "Development of panel of urine DNA biomarkers for Hepatocellular carcinoma screening"

3. In all presentations or written publications concerning the research projects, recipient will seek agreement of provider and acknowledge provider's contribution of this research material unless requested otherwise.
4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the research Material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of completion of the project.
5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the Recipient under conditions agreed to in the protocol on shipment of the samples. This Research Material is provided as a service to research community. It is being supplied to recipient with no warranties, express or implied, including any warranty of merchantability or fitness for a particular purpose. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right or third parties.
6. The recipient shall notify the provider in writing of any intention, improvement, modification discovery or development to the material or the information made by Recipient or parities, collaborating with Recipient, herein after referred to an "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
7. The under-signed provider and Recipient expressly certify any affirm that the contents of any statements made herein are truthful and accurate.
8. Any additional terms (use an attached page if necessary)
9. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise.
10. The provider will retain a copy of every sample sent abroad as much as possible for local research needs.

Material transfer agreement

Signature Page

AGREED:

AAU responsible:	Recipient Scientist:
_____	_____
Dr. Daniel Seifu	Dr. Timothy Block
Department of Biochemistry	Hepatitis B foundation/ Baruch S. Blumberg Institute
Date:- _____	Date:- _____
Tel: _____	Tel: _____
Mailing Address	Mailing Address

Annex VII Protocols

Urine DNA Isolation

Reagents that we need - a. 6M Guanidine Thiocyanate
b. Promega DNA purification Resin
c. Column wash buffer
d. double distilled and autoclaved water.

1. Put the guanidine thiocyanate on an incubator shaker set at 190rpm and $37^{O}C$ (to dissolve the crystal or precipitate).
2. Thaw urine (if it is frozen)
3. In 50ml falcon tube add 10ml 6M Guanindine thiocyanate + 10ml urine + and add 1ml resin (for each 10ml of urine 1ml of resin) shake well the resin before you use it using hand and vortex mixer.
4. Leave it overnight on a roller mixer (balance it).

Next Day

5. Remove the tubes from the mixer and centrifuge it for 5 min at a speed of 15000rpm.
6. Suck out the upper liquid with suction filtration (vacuum) without disturbing the lower resin until 1.5 to 2ml solution left.
7. The remaining solution (with a resin) will be filtered by Vacuum manifold.
8. Connect the Vacuum manifold with vacuum.
9. Take out the column on a clean towel paper using tweezers and label it with sample number (code). Don't touch the columns with hands it is only with the tweezers or forceps.
10. Once you labeled it fix it on the vacuum manifold together with the barrel on it. Fix two columns for one sample.
11. Mix the solution with the resin and pipette out using 1 ml micropipette and dispense it in the column. Make the volume equal that you pipette out for each column (duplicates).
12. Open the valve and filter it.
13. Make ready the wash buffer by diluting with 170ml alcohol
14. Wash the column with the wash buffer three times filtering each wash

Remove the column with tweezers and put it on a round bottom eppendorf tube and centrifuge it at a speed of 12000rpm for 2 minutes.

15. Transfer the column to a labeled 1.7ml eppendorf tube. Discard the liquid in the collection tube.
16. Prepare preheated water on a water bath at $65^{O}C$
17. Add 40µl of the preheated ddwater directly on to the filter.
18. Incubate at room temperature for 2 minutes.
19. After incubation, centrifuge the filter with microcentrifuge tube at 13000rpm for 2min.
20. Add 30µl of the preheated ddwater again.
21. Incubate at room temperature for 2minutes.
22. Centrifuge at 13000 rpm for 2min and discard the column after double checking the elution volume. Combine the entire DNA from the same sample back into one.
23. Store labeled samples in $-20^{o}C$.

Fractionation of HMW DNA from LMW DNA

Reagents:-

a. Ampure beads
b. 20% polyethylene glycol (PEG) Molecular weight 8000
c. 5M NaCl
d. Ice cold isopropanol
e. 80% ethanol
f. 1 x TE buffer
g. Double distilled and deionized water

1. Take 48µl DNA + water in eppendorf tube.
2. Add 30 µl 20% PEG, 12µl of 5M NaCl and 10 µl of AMPure beads the AMPure beads has to be prewashed with 1 x TE buffer twice.
3. Make the volume to 100 µl
4. Mix well (pipette for at least 10 times).
5. Incubate for 1 hour in dark at room temperature.
6. Set the mix in a magnetic holder for 5 min.
7. Take out the unbound solution ie. the LMW DNA. The beads have the HMW DNA.

Low MW DNA binding mix

a. 100 ul unbound portion
b. 85ul ice cold isopropanol (-20°C)
c. 20 ul of Ampure Beads (not pre-washed) that makes total of 195µl.

8. Mix well by pipetting for at least 10 times.
9. Incubate overnight at 4°C in dark on rotator.
10. After incubation; briefly centrifuge tube to remove liquid from cap
11. Set the sample in magnetic holder for 5 min.
12. Remove the solution.

Wash beads—both High and low DNA bound beads

1. Add 200 µl of 80% ethanol (made fresh), incubate for 30 sec and Set in magnetic holder for 5 min, remove the wash solution (2X)
2. Let the beads dry at RT for 15-20 min. Do not over-dry.
3. Elute the DNA in H_2O incubate for 5 min, and set in the magnetic holder for 10 minutes and transfer the DNA solution to another tube.

Protocol for Bisulfite Treatment of the DNA

1. Add 84.5 µl of Lightning Conversion Reagent to 13µl of a DNA sample in a PCR tube Mix, then centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.
Note: If the volume of DNA is less than 20 µl, compensate with water.
2. Place the PCR tube in a thermal cycler and perform the following steps:
    1. 98°C for 8 minutes
    2. 54°C for 60 minutes
    3. 4°C storage for up to 20 hours

Note: The 4°C storage step is optional.
3. Add 400 µl of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided Collection Tube.
4. Load the sample (from Step 2) into the Zymo-Spin™ IC Column containing the M-binding buffer. Close the cap and mix by inverting the column several times.
5. Centrifuge at full speed (> 10,000 x g) for 30 seconds. Discard the flow-through.
6. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.
7. Add 200 µl of L-Desulphonation Buffer to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.
8. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Repeat this wash step.
9. Place the column into a 1.5 ml microcentrifuge tube and add 11 µl of ddH_2O directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA. repeat with another 11µl of ddH_2O to make the total elution 22 µl.
The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C. We recommend using 1-4 µl of eluted DNA for each PCR, however, up to 10 µl can be used if necessary. The elution volume can be > 10 µl depending on the requirements of your experiments, but small elution volumes will yield higher DNA concentrations.
Note if 13 µl DNA is not there you can add water to adjust the volume. Eg 8µl DNA +5 µl water. 4ml equiv (0.5ml/ µl)

TP53 249T hot spot mutation quantification assay protocol

First step Master mix 18:2

Reagent	Stock	Final conc.	One reaction	N x Rxn
Water			12.2µl	
PCR buffer	10x	1x	2µl	
dNTP	2.5mM	200µM	1.6µl	
P53FwdA/RevB primer	10µM	0.5µM	1µl	
P53 LNA clamp	20µM	1µM	1µl	
Hot start Taq polymerase	5U/µl	0.25U/µl	0.2µl	
DNA template	2µl			

PCR settings:- 95°C - 5min (95°C - 30sec, 64°C - 30 sec (-1°C/cycle), 72°C - 30sec) x 10cycles

(95°C - 30sec, 54°C - 30 sec, 72°C - 30sec) x 15cycles, 72°C - 4min, 4°C - hold

Standards.

1. Water
2. 1000cp P53 WT plasmid
3. 10cp P53 249T plasmid + 1000cp P53 WT plasmid
4. 100cp P53 249T plasmid + 1000cp P53 WT plasmid
5. 1000cp P53 249T plasmid + 1000cp P53 WT plasmid

Second Step

Master mix 9:1

Reagent	**stock**	**Final conc.**	**One Rxn.**	**N x Rxn**
Water			5µl	
P53FwdA primer	1µM	0.1µM	1µl	
P53RevB primer	10µM	0.5µM	0.5µl	
P53 249T simple probe	3µM	0.15 µM	0.5µl	
LC genotyping master mix	5x	1x	2µl	
DNA template (first step PCR product diluted to 1:20)	1µl			

qPCR settings :- simple probe

95°C - 10min (95°C - 10sec, 54°C - 15sec, 72°C - 10sec) x 35cycles, melt

hTERT 124 hotspot mutation quantification assay

It is a two step assay

Step 1.

The first step - is a 20µl reaction

Master mix 18:2

S/n	Stock conc.		Final conc.	One Rxn	N x Rxn
1		Water		11.7µl	
2	10x	PCR buffer	0.5x	2µl	
3	2.5mM	dNTP	0.125 mM	1.6µl	
4	10µM	*hTERT*124Fwd1_AMP/*hTERT*124Rev1 primer	0.5µM	0.5µl	
5	1 µM	*hTERT* Bridged nucleic acid (BNA)	0.05 µM	1µl	
6		Dimethyl sulphoxide (DMSO)		1µl	
7	5units/µl	Hot start Taq polymerase		0.2µl	
8		DNA template (first step PCR product)		2µl	

N = No. of samples

PCR setting : -

95^{o}C -5min (95^{o}C - 30 sec., 80^{o}C - 20sec., 72^{o}c - 45sec) x 35 cycles , 72^{o}C 4min, 4^{o}c - hold

Standards

1. Water
2. 1000cp HugDNA
3. 10cp *hTERT* plasmid + 1000cp HugDNA
4. 100cp *hTERT* plasmid + 1000cp HugDNA
5. 1000cp *hTERT* plasmid + 1000cp HugDNA
6. 10000cp *hTERT* plasmid + 1000cp HugDNA

The second step of *hTERT* assay

Master mix 9:1 (9µl master mix and 1µl DNA template)

S/n	Stock conc.		Final conc.	One Rxn	N x Rxn
1		Water		2µl	
2	10µM	*hTERT*124Fwd_Amp/*hTERT* Rev primer	1µM	1µl	
3	3µM	*hTERT* TQ_1 probe	0.3µM	1µl	
4	2x	LC 480 probe master mix	1x	5µl	
5		DNA template (first step PCR product)		1µl	

N= No. of samples

qPCR settings :- Monocolor hydrolysis probe

95^{o}C -5min (95^{o}C - 10 sec, 61^{o}C - 10sec, 72^{o}c - 10sec) x 40 cycles, cool.

B-catenin 32-37 Codon mutation quantification with Taqman probe

1st step PCR 7:3 for sample and 9:1for standards

Stock conc.	Reagents	Final Conc.	One Rxn. stds	6 x Rxns. Stds	One Rxn. samp.+ w	NxRxn. Samp+water
	Water		3.5ul	21ul	2.5	
10X	PCR buffer	1x	1ul	6ul	1ul	
2.5uM	dNTP	200uM	0.8ul	4.8ul	0.8ul	
10uM	β-Cat Fwd32A/Rev37A primer	0.5uM	0.5ul	3ul	0.5ul	
10uM	β-Cat BNA 32	2uM	2ul	12ul	2ul	
3ng	Hug DNA		1ul	6ul	----	
5units/ul	Hot start Taq polymerase		0.2ul	1.2ul	0.2ul	
DNA Template		2ul				

PCR settings:- N = number of samples

95°C - 5min (95°C - 30sec, 70°C - 20sec, 60°C - 30sec) x 15 cycle, 72°C-4min, 4°C-hold

Standards

1. water
2. 1000cp HugDNA
3. 10cp B-cat 37Cys3 + 1000cp HugDNA
4. 100cp B-cat 37Cys3 + 1000cp HugDNA
5. 1000cp B-cat 37Cys3 + 1000cp HugDNA
6. 10000cp B-cat 37Cys3 + 1000cp HugDNA

2nd step PCR

Treatment of 1st step PCR product with Exonuclease VII

The Exo mix	10ul rxn	5.5x
1. Exonuclease VII	2ul	38.5ul
2. 10xPCR buffer	1ul	5.5ul
3. Water	7ul	11ul

Add 1ul of the mix into each PCR tubes and put it back in to PCR

PCR settings:- 37°C - 60min, 95°C - 10min, 60°C - 1min (2% ramp). 4°C-hold

3rd step PCR master mix 9:1

Stock conc.	Reagents	Final Conc.	One reaction	**56 xrxns**
	Water		2ul	112ul
10uM	B-cat F32A/R37A primer	1uM	1ul	56ul
2uM	B-cat TQ 32 probe		1ul	56ul
2x	LC 480 probe master mix	1x	5ul	280ul
DNA template (first step PCR product)			1ul	

qPCR setting:- monocolor hydrolysis

95^{o}C - 5min (95^{o}C - 10sec, 60^{o}C - 10sec, 72^{o}C - 10sec) x 40 cycle, cool

GSTP1 Short Amplicon Assay (m*GSTP1*)

Master mix 18:2

Stock	Reagents	Final conc.	One rxn.	N x rxn.
	Water		13.2µl	
10x	PCR buffer	1x	2µl	
2.5mM	dNTP	200µM	1.6µl	
10µM	*GSTP1*_F1/45R_LNA primer	0.5µM	1µl	
5U/µl	Hot start Taq polymerase	0.05U/µM	0.2µl	
	DNA template		2 µl	

N = No. of samples

PCR settings:- 95^{o}C - 5min (95^{o}C - 30sec, 59^{o}C - 30 sec, 72^{o}C - 30sec) x 33cycles,

72^{o}C - 4min, 4^{o}C - hold

Standards

1. water 2µl
2. 200cp SW 480 + 1µl water
3. 10cp HMBS + 200cp SW 480
4. 50cp HMBS + 200cp SW 480
5. 100cp HMBS + 200cp SW 480
6. 500cp HMBS + 200cp SW 480

2nd step

master mix 9:1

Stock	Reagents	Final conc	One Rxn.	59 x rxn.
	Water		2µl	118 µl
10µM	*GSTP1* S2F1/R primer	1µM	1µl	59 µl
3µM	GSTP1 Sh.Amp.TQ3 probe	0.15µM	1µl	59 µl
2x	LC probe master mix	1x	5µl	295µl
	First step PCR product		1µl	

N = No. of samples

qPCR setting:- Monocolor hydrolysis probe

95^{o}C - 10min (95^{o}C - 10sec, 56^{o}C - 10 sec, 72^{o}C - 10sec) x 40cycles, cool.

RASSF1A **Short Amplicon Assay (*mRASSF1A*)**

First step

Master mix 18:2

Stock	Reagents	Final conc.	One rxn.	N x rxn.
	Water		13.2µl	
10x	PCR buffer	1x	2µl	
2.5mM	dNTP	200µM	1.6µl	
10µM	*RASSF1A* P1_SMF2/TMR1 primer	0.5µM	1µl	
5U/µl	Hot start Taq polymerase	0.05U/µM	0.2µl	

N = No. of samples

PCR settings:- 95°C - 5min (95°C - 30sec, 57°C - 30 sec, 72°C - 30sec) x 25cycles, 72°C - 4min, 4°C - hold

Standards

1. water 2µl
2. 200cp HeLa + 1µl water
3. 10cp HMBS + 200cp HeLa
4. 50cp HMBS + 200cp HeLa
5. 500cp HMBS + 200cp HeLa
6. 1000cp HMBS + 200cp HeLa

2nd step

Master mix 9:1

Stock	Reagents	Final Conc.	One rxn.	N x rxn.
	Water		3µl	
10µM	*RASSF1A* P1_S2F1/S2R primers	1µM	1µl	
2x	Light Cycler 480 SYBR Green	1x	5µl	
	First step PCR product 1:10 diluted		1µl	

N = No. of samples

qPCR setting:- SYBR green

95°C - 5min (95°C - 10sec, 58°C - 30 sec, 72°C - 30sec) x 40cycles, melt, cool.

Declaration by the candidate

I hereby declare that this thesis is my own work and effort. Where other sources of information have been used, they have been indicated or acknowledged

Maria Degef Teklemariam Signature :- _____