Study of p53 gene polymorphism and risk of hepatocellular carcinoma: in a Bangladeshi cohort

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To Whom It May Concern

We hereby declare in accordance with the by-law of the University of Dhaka, that the thesis work entitled **"Study of p53 gene polymorphism and risk of hepatocellular carcinoma: in a Bangladeshi cohort"** describe here is entire own by Md. Abdur Rahman. This work was conducted and carried out under our supervision while he enrolled in the degree of Master of Philosophy in Biochemistry and Molecular Biology in the Faculty of Biological Sciences at the University of Dhaka. All information in this document has been obtained and presented in accordance with academic rules and ethical conduct and have been recommended for the award of M.Phil. of Biological Sciences. To the best of our knowledge no part of the research work has been submitted for other degree or qualification in an institution at home or abroad.

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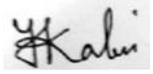

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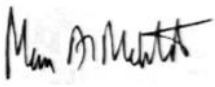

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Dedicated

to

My Parents &Teachers

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The Author
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List of Abbreviations

Name	Details
AFP	Alphafeto protein
AJCC	American Joint Committee on Cancer
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ASIR	Age-standardised incidence rates
ATM	Ataxia telangiectasia mutated
BCLC	Barcelona clinic liver cancer
C	Cytosine
Cdk	Cyclin-dependent kinases
Cip	CDK interacting protein
CT Scan	Computed tomography
DBD	DNA binding domain
DENA	Diethylnitrosamine
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
FADD	Fas-associated protein with death domain
FLICE	Femtosecond laser irradiation and chemical etching
G	Guanine
GADD45	Growth arrest and DNA damage-inducible gene 45
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric Acid
HCV	Hepatitis C virus
IGF2R	Insulin-like growth factor 2 receptor
IKK-β	Inhibitor of nuclear factor kappa-B kinase
IL-1α	Interleukin-1α
kDa	Kilo Dalton

Kip	Kinase inhibitory protein
MDM2	Mouse double minute 2
MgCl	Magnesium Chloride
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NASH	Non-alcoholic steatohepatitis
NER	Nucleotide excision repair
NF-κB	Nuclear factor-kappa B
PBC	Primary biliary cirrhosis
PCR	Polymerase chain reaction
PCT	Porphyria cutaneatarda
pRb	Retinoblastoma
PT	Prothrombin time
PTEN	Phosphatase and TENsin homolog
PVC	Polyvinyl chloride
PXXP	Proline rich domain
RB1	Retinoblastoma protein
SNP	Single nucleotide polymorphisms
T	Thymine
TAD	The amino-terminal transactivation domains
TERT	Telomerase Reverse Transcriptase
TNF-α	Tumour necrosis factor-α
TNM	Tumour-Node-Metastasis
TP53	Tumor protein 53
UICC	International Union Against Cancer
VEGF	Vascular endothelial growth factor
WHO	World health organization
VIP	Vasoactive intestinal peptide
WNT	Wingless-related integration site
ZBP-89	Zinc binding protein 89

Abstract

Hepatocellular carcinoma (HCC) is the number one malignancy of the liver, the 5th leading cancer death throughout the world. Most liver cancers are related to gene polymorphism, specifically the p53 tumor suppressor gene (TP53). The study aims to investigate the association of TP53 gene polymorphism with the risk of liver cancer in the Bangladeshi cohort. It was a case-control study with 119 HCC patients and 150 healthy controls. The genetic polymorphism of TP53 was determined by polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method using restriction enzyme digestion with *BustUI* for exon 4 and *HaeIII* for exon 7. Appropriate statistical analysis was performed to determine the association of genotypes with gender, smoking status, tumor stage, tumor grade, and family history of cancer. The demographic data showed a significant difference between the age of HCC and control subjects (49.8±1.2 and 35.8±1.1, respectively). Among the patients, males were much higher than females (83.2% vs 16.8% respectively). Smoking status was significantly higher in the HCC group than in the control group. In contrast, there was no significant difference in the family history of cancer and alcohol consumption between the two groups. Among the hepatocellular carcinoma subjects (n=119), the homozygous wild type was 20.17%, the homozygous mutant was 49.58%, and the heterozygous mutant was 30.25% at codon 72 in exon 4. Homozygous mutant variants (CC) and heterozygous mutant (GC) of TP53 at 72 position were substantially related to liver cancer risk when compared with the controls (OR=0.17; 95% CI=0.09-0.31; $p<0.001$ and OR =0.11; 95% CI =0.06-0.23; $p<0.001$) considering GG as reference group. On the other hand, at codon 249 in exon 7 genotype, the homozygous wild type was 48.74%, the heterozygous mutant was 42.86 %, and the homozygous mutant was 8.40%. Heterozygous mutant (GT)of TP53 at 249 positions were significantly associated with liver cancer risk when compared with the controls (OR=0.19; 95% CI=0.10-0.35; $p<0.001$), whereas no association was found with homozygous mutant variants (TT) (OR=0.43; 95% CI=0.18-1.17; $p=0.124$) when GG was considered as the reference group. TP53 genome analysis of exon 4, an association of CC and GC variation with the risk of cancer was found when GG genotype was considered as a reference group. On the other hand, the TP53 genome analysis of exon 7, GT, and TT variants was significantly associated with male cancer patients. The genetic linkage evaluation of the TP53 gene (exon 4 and exon 7) suggested that polymorphism of G to C and G to T showed a high risk for HCC in the study subjects. Therefore, this study can conclude that TP53 gene polymorphisms are highly associated with hepatocellular carcinoma in the Bangladeshi cohort.

Introduction

1.1 Background

Hepatocellular Carcinoma (HCC) is the most common primary liver malignancy. Frequently, chronic liver disease patients infected with hepatitis B or C are liable for hepatocellular carcinoma. Globally, the prevalence of HCC is fifth for all cancer and the third leading mortality rate after lung and stomach cancers [1]. Cancer-related death was progressively increasing due to the yearly occurrence of HCC, about 900,000 cases [2]. Due to the high mortality rate of HCC, the incidence and mortality rates of HCC are approximately equal [3], although they differ among various countries. The highest HCC cases are found in Asia [4]. In Asia and sub-Saharan Africa, where many people are infected by hepatitis B from their families and hepatitis B is an epidemic, most HCC cases occur. Due to hepatitis C virus infection, the prevalence of HCC is increasing in the United States and other developing countries. Because of unknown causes, it is observed to be higher in males than females [5]. In Eastern Asia, South-Eastern Asia, Mid-Africa, and West Africa, about 80% of HCC infections are caused by viral hepatitis [6-8]. Although hereditary hemochromatosis and α1-antitrypsin deficiencies are genetic aetiologies for HCC [9-11]. and different foreign factors like viral hepatitis, aflatoxin B1 (AFB1), polyvinyl chloride (PVC), and few diseases like non-alcoholic fatty liver disease (NAFLD), and excess iron exposure are most common factors for liver cancer [12,13]. Many countries have taken various steps to prevent liver cancer like vaccinations for hepatitis B virus (HBV), inventing new management for hepatitis C virus (HCV) infections, controlling PVC production, and monitoring AFB1 contamination of food products to avoid this food [13,14].

The significant risk factors for HCC were chronic hepatitis B infection and aflatoxin B1 exposure, shown in different studies in China [15]. Besides hepatitis C virus infection, smoking and alcohol consumption are also associated risk factors. Genetic factors may assist in the carcinogenic mechanism because hepatocellular carcinoma (HCC) develops in a small group of hepatitis B viruses.

The molecular etiology of HCC includes multiple genetic and metamorphic changes, WNT signaling pathway activation and p53 tumor suppressor gene (TP53) somatic mutations. AFB1 exposure and HCC risk may be worked as biomarker in plasma TP53 mutant DNA identification. The DNA may be damage by generating reactive oxygen/nitrogen species by HBV and HCV viruses be subject to chronic infection, and oxyradical disorders. A gain of oncogenic function may be shown by specific mutant p53 proteins. Present of oxidative and nitrosative stress may be responded by p53 biological netting. Counting on the expanse of the DNA damage, p53 regulates the transcription of protective antioxidant genes and transactivates pro-oxidant genes, also controlling extensive DNA damage that contributes to apoptosis. The extensive DNA damage that contributing to the apoptosis was regulated by p53 transcription of protective antioxidant genes and transactivates pro-oxidant genes depending on DNA damage. The tumor suppressor gene (OMIM no. 191170) of TP53 located in chromosome 17p13.1, and codes a 53-kDa nuclear phosphoprotein plays a primary role in protection the integrity of the human genome [16]. The TP53 protein binds to the DNA within the cell and stimulates the production of a protein, p21. p21 interplays with a cell division-stimulating protein (cdk2), which plays the role of a tumor suppressor protein. The TP53 protein binding with DNA and and stimulating the production of a protein, which interactions with a protein (cdk2) of cell division-stimulating that works as tumor suppressor protein. The P53 gene mutations and polymorphisms are usually linked with cancer [17]. Loss of TP53 has been showed to be the greatest noteworthy step in multistage hepatocarcinogenesis, TP53 is a possible contender to decline the risk of HCC [18]. The codon 72 polymorphism is originating in exon 4 of the TP53 superiority, this locale covering as it were some changes. The TP53 protein uncovers a strategies polymorphism at amino acid position 72, motivating to the replacement of an Arginine (Arg) buildup (CGC) with Proline (Professional) buildup (CCC), allocated as TP53 Arg72Pro (pro no dbSNP ID: rs1042522) [19].

Alteration of *p53* expression has been declared to associate with progression of tumor grade as well as therapy, and survival from cancer [20-23]. On the other hand, a four-zinc finger transcription expression factor, ZBP-89, that controls the expression of cell growth genes that binding to GC-rich DNA elements [24], has not been described in HCC. HCC is a particularly aggressive disease, and therefore the death rate is extremely high. A surgical cut of the tumor is presently the only effective treatment for HCC. In initial diagnosis, the short-term prediction has

been enhanced. Anyhow, recurrent tumor after surgical resection remains a severe impediment to improve the projection of patients with HCC. Moreover, recurring tumors generally grow faster and have lower chemo- or radiosensitivity, which depends on the status of the *p53* gene [25, 26].

The whole length p53 isoform proteins are often subdivided into various protein domains. To induce a subgroup of p53 target genes, transactivated of amino-terminal domains (TAD 1, TAD 2) with N-terminus opening was required. This domain is followed by the Proline-rich domain (PXXP), where the motif PXXP is repeated (P is proline and X are often any amino acid) and needed for p53-mediated apoptosis [27]. The diversifying activities of the TP53 gene was indicated the missing of involvement of p53 in apoptosis such as Proline-rich domain, like Δ133p53β,γ, and Δ160p53α,β,γ [28]. Ultimately, the DNA binding domain (DBD) permits the proteins to the sequence-specific binding. Since of the isoforms nature of p53 proteins, there is numerous indication presentation that mutations within the TP53 gene giving rise to mutated isoforms are determinative factors of numerous cancer phenotypes.

Based on previous findings and the association between*p53* gene polymorphism and risk of hepatocellular carcinoma, we hypothesized a link between mutation of p53 gene and hepatocellular carcinoma. Therefore, the goal of the current study will be to assess the correlation between p53 gene polymorphism the hepatocellular carcinoma in a Bangladeshi cohort.

1.2 Etiology of Cancer

Various changes within the genomes of cancer cells assist in the base for several oncogenic procedures. Cancer cells benefit from raised rates of transformation in arrange to gather a few mutations needed to nourish tumorigenesis. They are doing this through [29].

- Elevated sensibility to mutagenic factors.
- Breakdown in 1 or more of the cell's DNA repair pathway mediated by genes such like Tp53 susceptivity protein.
- A coalition of those factors.

Aggregation of those mutations is forwarded by changing the DNA-maintenance system or "caretaker" genes. Those genes are liable for-

- Identifying DNA damage and activating the DNA repair system.
- Straightly recovering damaged DNA.
- Inactivating or breaking mutagenic particles.

Cancer cells are aneuploid cells, a product of failed mitosis resulting in an odd number and composition of chromosomes in the cells. Interpretation of neoplastic cell genomes also shows function-changing mutations and reveals that genomic instability rises at tumor development [30]. Point mutations are single variations in a base pair in the DNA sequence. A point mutation can be synonymous with an exon, meaning that the codon is changed, but there is no change in what amino acid it is translated into protein. On the other hand, a non-synonymous point mutation changes the DNA sequence in an exon so that the amino acid sequence changes when a protein-coding gene is translated. Nonsense mutations are point mutations that change a codon from an amino acid coding codon into a stop codon. In most cases, the change in a gene's copy number is not reflected in the amount of gene product [31]. The epigenetic changes in cancer, such as hypermethylation of CpG islands in promoter regions, can silence tumor suppressor genes. Hypomethylation, be that as it may, can activate the expression of oncogenes. Depending on the modification, modifications of histones can either increase the expression of oncogenes or quiet tumor suppressor genes.

The generation of the latest blood vessels or angiogenesis pathway in neoplastic cells is risky for sustained tumor progression and metastasis. It’s a multistep procedure that associates with signaling input from more pro-angiogenic growth factors [32].The "angiogenic switch" is usually mentioned when the tumor begins to overexpress pro-angiogenic factors, like vascular endothelial growth factor (VEGF), which is usually mentioned because the “angiogenic switch."

Nonstop angiogenesis permits tumor propagation and native incursion through-

- Parturition of oxygen and nutrients.
- Formation of growth factors that help the neoplastic cells [33]

For the treatment of metastases, the molecular cancer researcher also advises that it can be possible through the new tumor vasculature into the circulation.

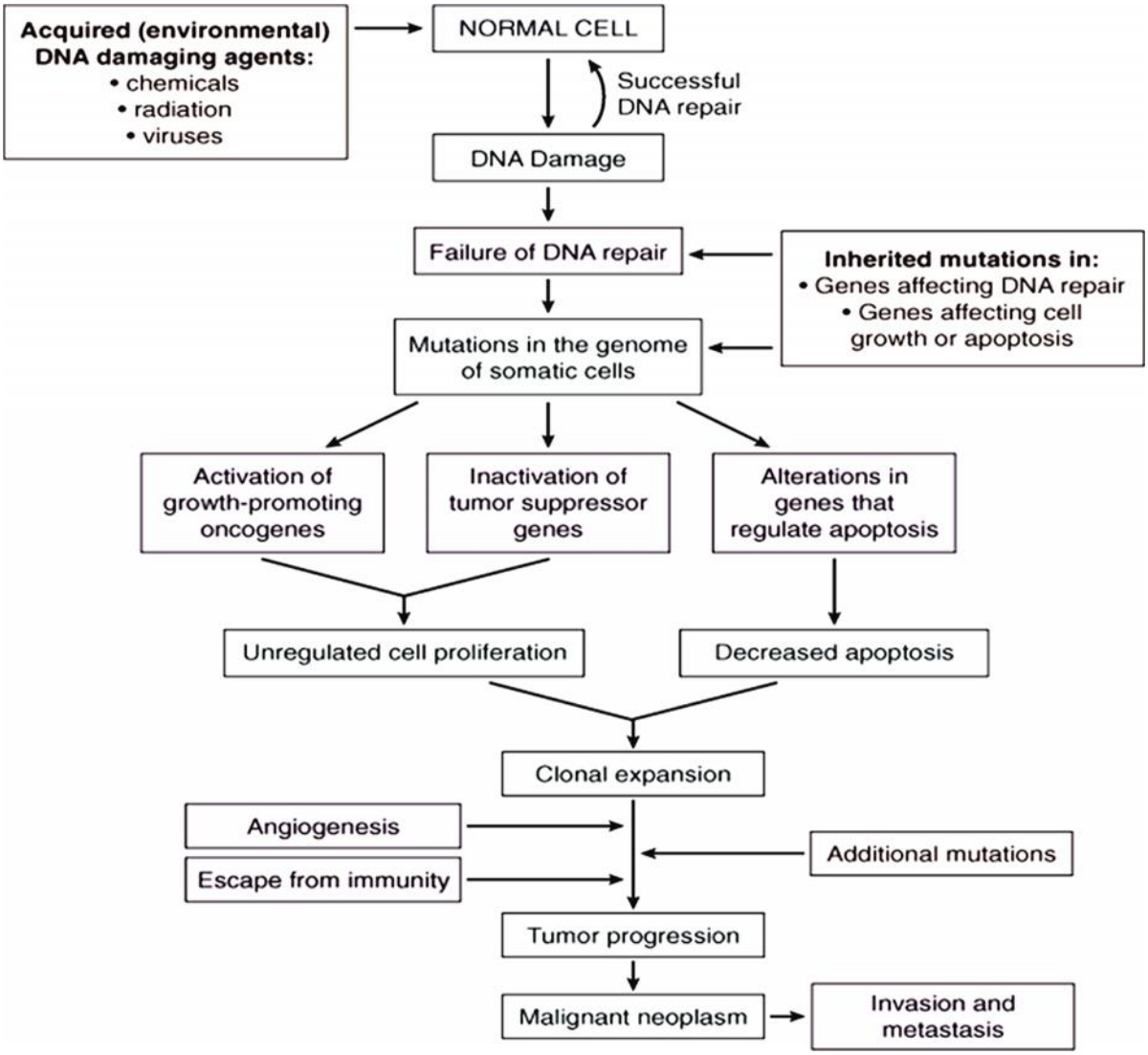


Figure 1.1: Molecular basis of cancer [34]

1.3 Liver Cancer

1.3.1 Liver

The liver is that the organ which maximum in size within the body. It weighs 1200-1500 gm. and includes one-fiftieth of the entire adult human body weight [35]. It's comparably bigger in the newborn (one-eighteenth of the total body weight) than the adult.

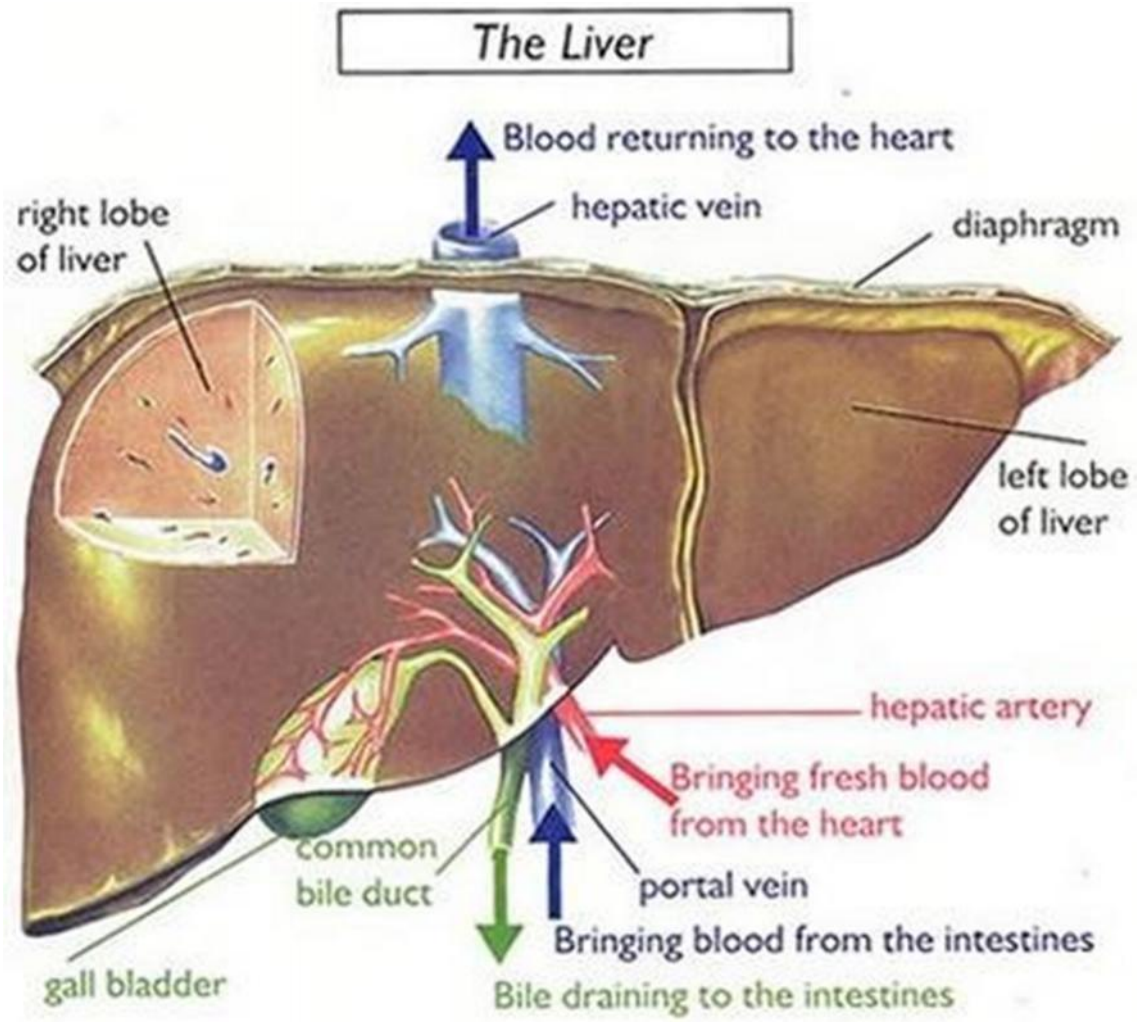


Figure 1.2: Structure of Liver

1.3.2 Epidemiology of Live Cancer

The topmost pervasive rate of liver cancer is found in Mongolia within East Asia [36]. The common incidence of liver cancer was 15.3 in males, 5.3 in females, and 10.1 per 100,000 people. The maximum fatality rates occurred noticed within the East Asian countries. The standardized fatality percentage for liver cancer of the liver were 14.3 in males and 5.1 in females, and 9.5 per 100,000 people. An entire of 782,451 new cases of HCC and 745,533 similar deaths were guessedin 2012, [37]. The total incidence is 95% in developing countries, and the death rate is 96%,

respectively. Worldwide, North America and Southern Europe had the best prevalence to fatality ratios. The frequency rates of liver cancer varied quite nine-fold worldwide in 2012. In men, the utmost was found in Eastern Asia (ASR 31.9 per 100,000), South-Eastern Asia (22.2), Northern Africa (18.0), and Western Africa (16.4), and thus rock bottom were recorded in South-Central Asia (3.7), Eastern Africa (4.9) and Western Asia (5.0). The fatality rates of liver cancer varied by quite eight-fold worldwide in 2012, [37]. In men, the exceptionally high death rates were reported within the Eastern Asia (29.9), South-Eastern Asia (21.4), and Northern Africa (17.4); while in women, the exceptionally high fatality was also observed in these three regions (ASR mortality = 9.6, 6.8 and 7.7 per 100,000, respectively). The lowest lethality rates were reported in South-Central Asia (3.6), northern Europe (4.0), and Eastern Africa (4.6) in men. For women, northern Europe (1.8), Australia/New Zealand (2.0), Western Europe (2.1), and South-Central Asia (2.1) reported the minimum fatality rates. The highest incidence of mortality ratios in men was found in North America (1.39), Southern Europe (1.27), and Western Europe (1.23), and thus the ratios were the utmost for girls in North America (1.17), Southern Europe (1.16) and Central America (1.08), [38]. In Bangladesh, liver cancer death is 2.68 per 100,000 population, and the death rank is 170 globally [39].

1.3.3 Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma (HCC) is known as primary liver cancer. Generally, it develops in patients suffering from chronic liver disease and cirrhosis. Therefore, approximately 25% of patients haven’t a chance to HCC from liver cirrhosis. Other factors for developing HCC include chronic liver inflammation, chronic hepatitis by hepatitis B or C virus, exposure to alcohol, aflatoxin, or pyrrolizidine-like toxin. Hemochromatosis and alpha 1-antitrypsin deficiency is also associated and significantly increase the chance of developing hepatocellular carcinoma. On the other hand, different metabolic disorders and NASH also accelerate the risk factors for HCC [40].

1.3.4 Symptoms of HCC

In early-stage have not developed any symptoms in the patients of hepatocellular carcinoma. When cancer develops, they may have shown one or more symptoms like these:

- Pain in the upper right part of the belly
- Feeling of heaviness in the upper belly
- Swelling the belly
- Feelings of the fullness of the stomach and loss of appetite
- Weight loss
- Deep fatigue
- Vomiting and nausea
- Yellow eyes and skin
- Chalky bowel motilities, pales, and dark urine
- Fever

1.3.5 Risk Factor of HCC

Factors that can develop an individual's possibility of hepatocellular carcinoma (HCC) [41- 46].

1.3.5.1 Gender

Hepatocellular carcinoma is the commonest in men than the female. But the fibrolamellar subtype of HCC is more familiar among females.

1.3.5.2 Ethnicity

The maxima rates of liver cancer are found in the race followed by Hispanics/Latinos, American Indians/Alaska Natives, African Americans, whites in the United States, Asian Americans, and Pacific Islanders.

1.3.5.3 Chronic Viral Hepatitis

Hepatitis B virus (HBV) or hepatitis C virus (HCV) induced chronic infection is the utmost dangerous factor for liver cancer. Hepatitis B virus and hepatitis C virus-dependent liver cirrhosis

are liable for cancer of the liver. This sort of cancer is a more common cancer of the liver within the world.

In the US, infection by HCV is that the utmost usual explanation for HCC, while HBV infection is more popular in Asia and developing countries. People who are infected with hepatitis B and hepatitis C viruses have a great chance of developing chronic hepatitis, cirrhosis, and cancer of the liver. Frequent alcoholic drinkers are highly at risk for HCC.

The route for spreading HBV and HCV from person to person through using contaminated needles like drug use, unprotected sex, or childbirth. These viruses also may be passed on by blood transfusions, and if blood is taken from virus carriers, this is mostly very rare nowadays. In most developing countries, children are infected contract with HBV-infected persons.

HBV is shown common symptoms like a flu-like illness and jaundice. However, most people get well thoroughly from HBV infection within a few months. The percentage of developing liver cancer among adults is very small, and infected infants and little children have a better risk of becoming chronic carriers.

On the other hand, HCV infection shows mild symptoms. But HCV infection leads to chronic infection. As a result, liver damage in infected people and lead to cancer.

Other causes of developing hepatitis are the infection by the hepatitis A virus and hepatitis E virus. But those viruses don’t cause permanent injury in the hepatocytes by the advancement of chronic hepatitis or cirrhosis and, in this case, have a chance of cancer.

1.3.5.4 Cirrhosis

In cirrhosis, the liver, and the hepatocytes become injured and are replaced by connective scar tissue. Cirrhotic patients have a great chance of increasing the risk of liver cancer.

Though there are many reasons for cirrhosis, in the US, alcohol abuse and chronic HVB or HCV have been reported for hematoma.

1.3.5.5 Non-alcoholic Fatty Liver Disease

Bloated people have a chance of fatty liver. NAFLD is a common reason to be a cirrhotic patient. It is also used with the term NASH (non-alcoholic steatohepatitis), leading to the progression of cirrhosis.

1.3.5.6 Primary Biliary Cirrhosis

Sometimes few categories of autoimmune diseases affect the liver, which may cause liver cirrhosis. Primary biliary cirrhosis (PBC) is the disease of the bile ducts, but in many cases, the liver is injured or even blasted, which may cause cirrhosis. In advanced PBC, patients have an utmost chance of liver cancer.

1.3.5.7 Inherited Metabolic Diseases

Particular hereditary diseases can cause cirrhosis. Genetically hemochromatosis patients absorb too much iron from their eating food. These iron deposits in various tissues throughout the body and the liver. If enough iron builds up in the liver, it can cause cirrhosis, the precursor of liver cancer.

1.3.5.8 Heavy Alcohol Use

Heavy alcohol drinking is a primary cause of cirrhosis in the United States, and it is one of the factors that increase the chance of liver cancer.

1.3.5.9 Tobacco Use

The risk of liver cancer is also increased by smoking. Current smokers are at a lower risk than ex-smokers, but both groups are at a higher risk than individuals who have never smocked.

1.3.5.10 Obesity

Overweight people are more likely to acquire liver cancer. Obesity leads to fatty liver. Fatty liver can result in cirrhosis.

1.3.5.11 Type 2 Diabetes

The factors associated with liver cancer, type 2 diabetes is one of these factors, and if the patients also suffer or are infected with chronic viral hepatitis or alcohol. People with type 2 diabetes are more chance of being overweight or obese. Obesity is a factor that increases the risk of liver cirrhosis.

1.3.5.12 Certain Rare Diseases

The following are examples of rare disorders that raise the risk of liver cancer:

- Deficiency of alpha1-antitrypsin
- Glycogen storage diseases
- Porphyria cutaneatarda
- Tyrosinemia
- Wilson disease

1.3.5.13 Aflatoxins

A fungus found in peanuts, wheat, soybeans, groundnuts, corn, and rice can cause cancer. The growth of this fungus can occur if those foods are stored in a moist, warm atmosphere. It is more frequent in tropical and warmer climates, but it can occur practically anywhere. Foods are tested for aflatoxins in developed nations like the United States and Europe.

1.3.5.14 Vinyl Chloride and Thorium Dioxide

When the liver is exposed to these chemicals, the chance of angiosarcoma increases. As a result, it contributes to an increased risk of cholangiocarcinoma and hepatoma, albeit only to a minor extent. Vinyl chloride is used in the production of several polymers. Thorotrast is a substance that was once administered to some patients as part of x-ray procedures. When these chemicals were discovered to be cancer-causing substances, actions were taken to eliminate or reduce their exposure. Now a day's, thorotrast is not used, and vinyl chloride use is strictly regulated to avoid exposure of workers.

1.3.5.15 Anabolic Steroids

Anabolic steroids are used as male hormones, and some athletes utilize them to boost their strength and muscular mass. Long-term usage of these hormones may increase the chance of HCC by a small amount.

1.3.6 Molecular Mechanism of Hepatocellular Carcinoma

Different etiological factors for HCC include HBV, HCV, alcohol, aflatoxins, inborn and acquired metabolic illnesses, and inborn and acquired metabolic disorders. It's possible that the cancer started in mature liver cells or progenitor cells. As a result, the molecular basis of HCC growth may vary based on various circumstances, and several mechanisms may be implicated [47]. A few key pathways linked to hepatocellular carcinogenesis are discussed here.

1.3.6.1 Loss of Cell Cycle Control

The loss of cell cycle regulation is a frequent hallmark of malignant cells of all types. This accelerates the multiplication tendency, hyperplasia, and the creation of tumors development. Normal liver cells are mainly in the G0 (quiescent) cell cycle phase and are progressively awakened. Whatever the case may be, they have active regeneration abilities, and after receiving mitogenic signals, they enter the cell cycle and proceed to cell division [48]. The combined actions of cyclins and cyclin-dependent kinases drive eukaryotic cell cycle progression (Cdk). Growth factors and cytokines stimulate the production of the cyclin D1 gene, which is responsible for the cell cycle transition of inactive hepatocytes [49, 50]. Several regulatory checkpoints keep unfettered proliferation under control and keep dormant hepatocytes from entering the cell cycle (Fig. 1.3). Retinoblastoma (pRb) and other proteins attach to and seize E2F transcription agents, as shown in **Fig. 1.3**, inhibiting their function [51]. The Ink4 family of Cdk inhibitors (p15/16/18/19) similarly prevents entry into the cell cycle by binding to Cdk4/6 kinases and blocking the formation of the cyclin D-Cdk4/6 complex [52]. The CDK interacting protein (Cip)/Kinase inhibitory protein (Kip) group inhibitory proteins p21/27/57 bind to Cdk/cyclin complexes and inactivate them, preventing cell cycle upgrading [53]. In gene expression profiles

of hepatocellular carcinoma samples, a "proliferation cluster" was discovered, which was evaluated for enhanced expression of proliferation-related genes [54, 55].

Because the p16/pRb pathway controls cell cycle entry, abnormalities that lower the expression levels of p16/pRb genes or activate their protein eventually lead to cancer. The expression of the pRb gene is changed in HCC, and this is a common occurrence [56]. According to research, the expression levels of CIP/KIP family member proteins p21/27 are frequently reduced in HCC samples [57].

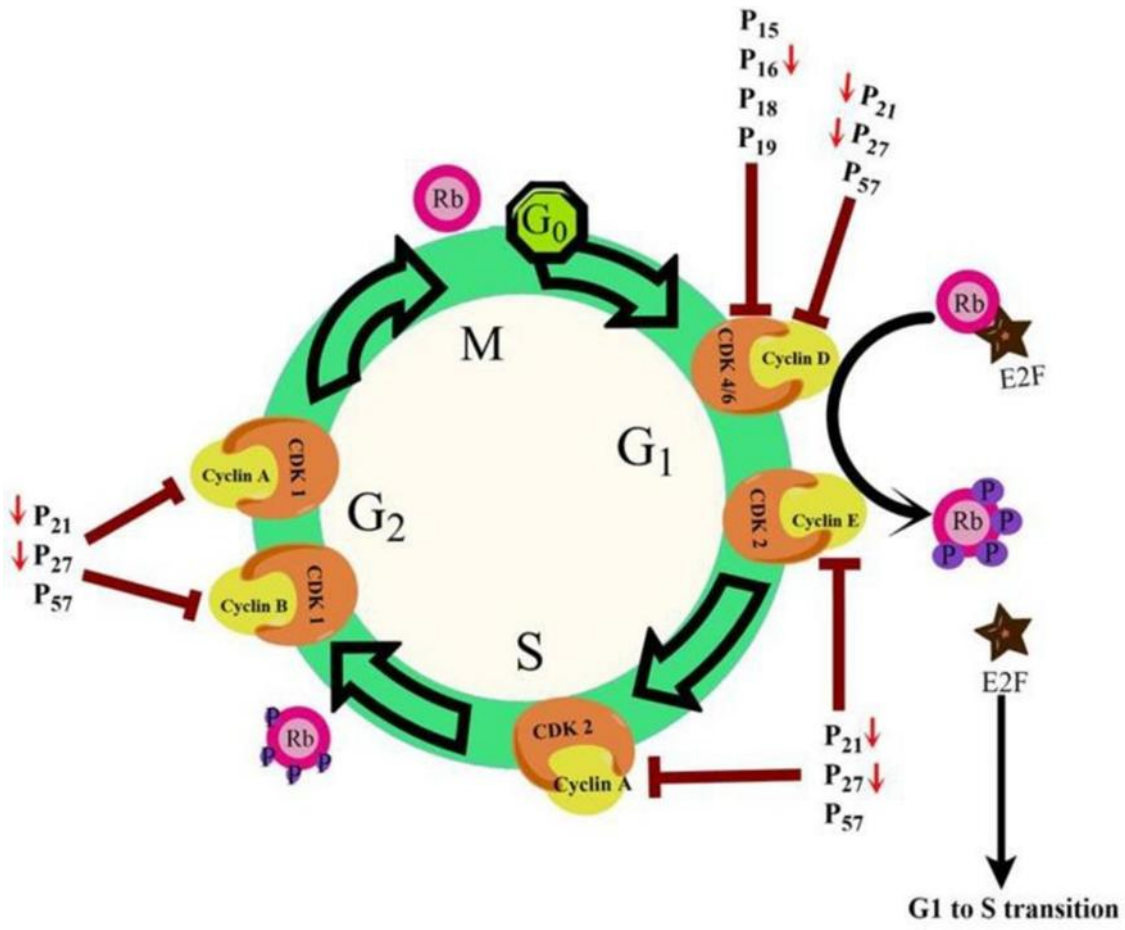


Figure 1.3: The cell cycle pathway with common checkpoints

At the period of hepatocarcinogenesis, the expression of Ink4 group Cdk inhibitor (p16) and Cip/Kinase inhibitory protein (Kip) family inhibitor (p21/27) was reduced. The G1 to S transition is triggered by the discharge of the E2F transcription factor.

1.3.6.2 Loss of Senescence Control

Senescence is a cell culture growth inhibition characterized by specific morphological abnormalities [58]. The mechanism of senescence in hepatocytes is not well known. The telomeric region of liver cells is continuously reduced by replicative senescence, which restricts partial proliferative capability [59]. Telomere-independent pathways for hepatocyte senescence have been proposed, and they include free radical and oncogene-dependent senescence, which are both monitored in significant chronic liver disorders. The ensuing DNA damage triggers the ATM/Chk/p53 pathway, preventing cells from progressing to the G1 phase. The p16/pRb pathway, on the other hand, performs a similar purpose. Abnormalities in the DNA damage checkpoint and cell cycle regulatory pathways allowed genetically engineered hepatic cells to proliferate indefinitely throughout the senescent phase, eventually leading to malignant transformation **(Fig.1.4).**

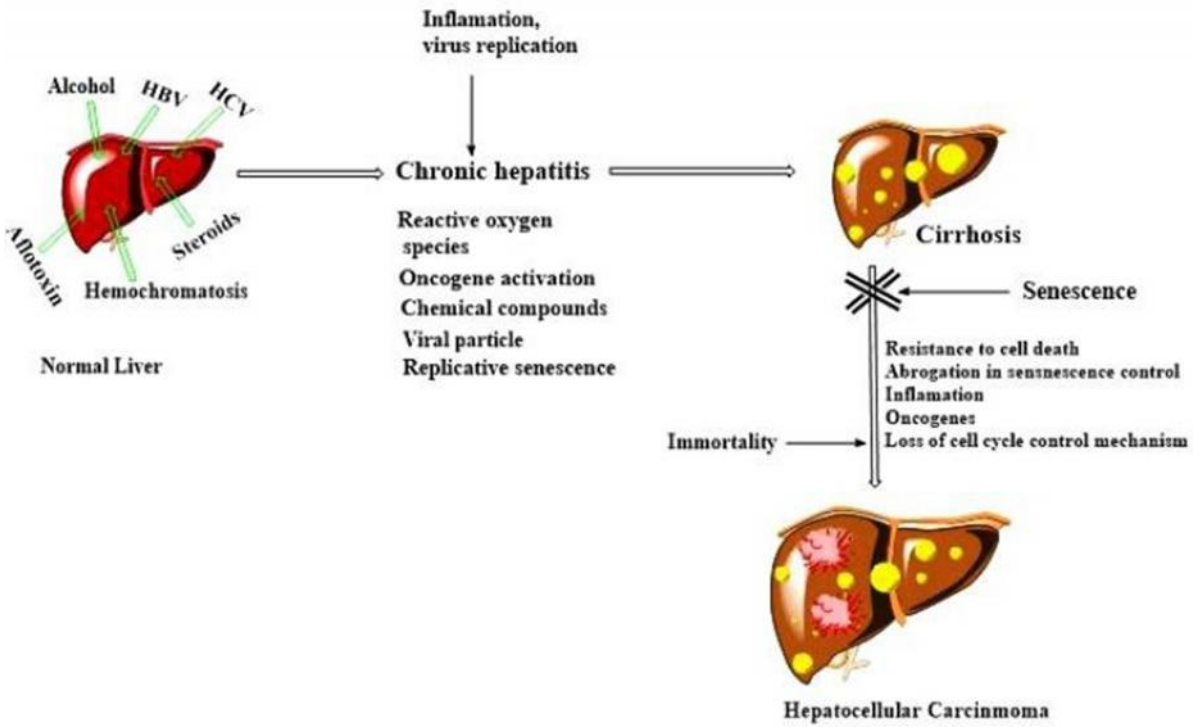


Figure 1.4: The suggested model of hepatocellular carcinoma development

In human HCC, the p53 pathway has an impact on many levels: (a) about 50% of aflatoxin-mediated HCC cases have p53 mutations, whereas 20–30% of non-aflatoxin-mediated HCC cases

have p53 mutations; (b) microdeletions of p14ARF are rare in HCC with p53 mutations but are reported in 15-20% of human HCC; (c) human HCC also has elated Mdm2 expression; (d) overexpression of gankyrin, an oncoprotein, is frequently observed in human hepatocellular carcinoma, which enforces a restriction on the pRb and p53 [59].

More than 80% of human HCC has anomalies in the pRb pathway (p16, p15, or RB1 genes). The p16/15 promoter is methylated, and the RB1 gene is deleted or mutated. The p16 suppression caused by promoter methylation is a well-known oddity [60]. Telomerase activation occurs with the progression of precancerous lesions to HCC. Cirrhosis is associated with telomere-dependent senescence arrest in hepatocytes. Telomerase Reverse Transcriptase (TERT) reactivation plays a function in HCC development that should not be overlooked. TERT is not found in normal hepatocytes, but 90 percent of human HCC have telomerase activity, a rate-limiting stage in the onset of cell immortality [61]. Integration of HBV DNA into the TERT gene causes deregulation of TERT expression, which is a rare occurrence [56]. On the other hand, HBV surface proteins (viral X and PreS2) and HCV core proteins can increase telomerase activity [62]. During hepatocarcinogenesis, defects in telomerase function and senescence-dominating genes (p53) worked together to cause hepatocarcinogenesis.

1.3.6.3 Dysregulation of Apoptosis

Cell death might be necrotic, apoptotic, or uncontrolled in the case of liver injury. Caspases 3, 6, and 7 are activated by intrinsic and extrinsic mechanisms, resulting in programmed cell death [49, 63]. If you're looking for a unique way to express yourself. In hepatocellular carcinoma (HCC) cells, death receptors mediate resistance to apoptosis. Most HCCs have one or more mutations in Fas pathway components, which inhibit Fas-mediated apoptosis. Downregulating Fas expression results in increased expression of cellular FLICE/caspase-8-inhibitory protein (cFLIP) or reduced FADD or FLICE by upregulating nuclear factor-kappa B (NF-B), Bcl-2 or Bcl-XL, and Mcl-1 in HCC cells or tissues [64-66].

HCC is down regulated by pro-apoptotic proteins (Bax or Bcl-XS). At the cirrhosis stage, the TGF-β pathway is regularly stimulated and boosts apoptosis by switching Smad3 mediated Bcl2 downregulation and, as a result of this, decreasing the sensitivity towards HCC development [67]. Activation of the PI3K-Akt pathway and insulin-receptor signaling might also be associated with

resistance towards apoptosis [68]. The (IGF2R) decreases cell division by inciting TGF-β signaling and the breakdown of the IGF2 mitogen [69]. At the initial phase of human hepatocarcinogenesis, heterozygosity in IGF2R locus is frequently lost [70]. Pro-apoptotic proteins inhibit HCC growth (Bax or Bcl-XS). The TGF- pathway is frequently boosted during the cirrhosis stage, and it enhances apoptosis by switching Smad3-mediated Bcl2 downregulation, lowering the sensitivity to HCC development [67]. Resistance to apoptosis may also be linked to activation of the PI3K-Akt pathway and insulin-receptor signaling [68]. The (IGF2R) inhibits cell division by inducing TGF signaling and IGF2 mitogen breakdown [69]. Heterozygosity at the IGF2R locus is frequently lost during the early stages of human hepatocarcinogenesis [70]. Loss of IGF2R and overexpression of IGF2 growth factor are principal characteristics of human HCCs.In 40-60% of HCC cases, increased Akt signaling and decreased expression of a negative regulator of Akt, phosphatase, and tensin homolog (PTEN) have been found [71].

1.3.6.4 Liver Inflammation and Hepatocarcinogenesis

Most researchers have found that liver damage in viral hepatitis is induced by the viral protein-mediated host immune response rather than the direct cytopathic effects of hepatitis viruses [72]. On the other hand, several animal studies have revealed that viral hepatitis is triggered by an antigen-specific intrahepatic cellular response that initiates a cascade of antigen-nonspecific cellular and molecular effector mechanisms. The cellular and humeral limbs of the defense system work to eliminate viruses through three different mechanisms: first, virus-specific T-cell-mediated direct eradication of injured hepatocytes; second, antibody-mediated removal of free viral molecules from circulation; and third, non-cytopathic viral inactivation in infected hepatocytes by some inflammatory cytokines produced by activated mononuclear cells [73]. According to a recent study, NF-B signaling-mediated inflammation plays an essential role in tumor initiation, progression, and growth [74].

1.3.6.5 Cytokines

Interleukin-1 (IL-1), IL-1, IL-6, IL-8, and tumor necrosis factor (TNF-) are inflammatory cytokines that play a role in chronic hepatic inflammation. In chronic hepatitis, activated kupffer cells produce IL-6, an essential interleukin group. It generates a local inflammatory response and activates hepatocyte proliferation, which leads to the development of malignant hepatocytes,

mainly to the activation of kupffer cells [75]. Chronic liver disorders, such as HBV and HCV-induced hepatitis, alcoholic hepatitis, and non-alcoholic steatohepatitis, have increased blood IL-6 levels. These findings highlight the pivotal function of IL-6 in human hepatocarcinogenesis. The IL-6 mutant animals showed a significant reduction in HCC progression induced by diethylnitrosamine (DENA), suggesting a direct link between IL-6 signaling and experimental hepatocarcinogenesis. IL-6 generation via activation of Toll-Like Receptor (TLR) mediated through MyD88 has also been reported to perform inborn immune response in hepatocarcinogenesis [76].

1.3.6.6 NF-κB Pathway

NF-B is a transcription factor involved in inborn immunity and inflammatory signaling in the liver [77, 78]. It is unique to express that Interleukins or cytokines such as TNF-, IL-6, and IL-1, viral and bacterial DNA and RNA, and pathogen-mediated lipopolysaccharides activate this. After stimulation, NF-B forms a dimer, travels to the nucleus, binds to a specific DNA region, and activates the transcription of genes involved in immunological responses, inflammation, cell proliferation, and survival [79, 80]. All chronic liver disease is mediated by NF-B, which is triggered by alcoholic/non-alcoholic/biliary liver disease and viral hepatitis [81]. In the animal HCC model, it was discovered that inducible IκB great-repressor mediated NF-B inhibition slows the progression of hepatic tumors in Mdr2 mutant mice with chronic inflammation [82, 83]. The liver tumor-promoting activity of NF-B has been verified in another inflammatory HCC model using a hepatocyte-specific lymphotoxin transgenic mice model. This model also demonstrated that NF-κB was suppressed by hepatocyte-specific IKK-β -remotion, resulting in a complete reduction in HCC growth, as shown in **Fig. 1.5** [84].

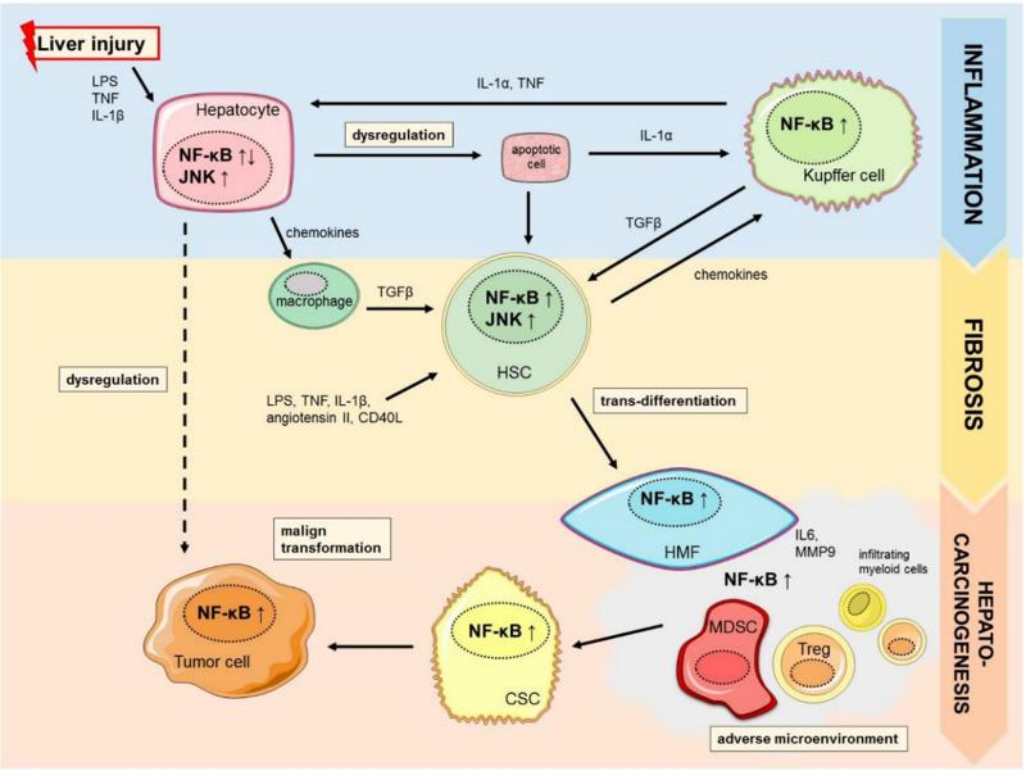


Figure 1.5: NF-κB Pathway of hepatocellular carcinoma development

1.4 Prevalence of HCC

Liver cancer is caused by a lethal tumor usually diagnosed late in its progression and has a high degree-to-mortality ratio [85]. In 2015, around 854,000 new instances of liver cancer were reported, with an estimated 810,000 liver cancer-related fatalities [86]. Hepatocellular carcinoma (HCC) accounts for 75–85 percent of all primary liver malignancies [87] and is a significant public health concern worldwide.

Because of the varying occurrence of significant risk factors, the global prevalence of hepatocellular carcinoma is diverse. According to estimates, 72 percent of instances occur in Asia (with China accounting for more than half of all cases), 10% in Europe, 7.8% in Africa, 5.1 percent in North America, 4.6 percent in Latin America, and 0.5 percent in Oceania [88]. The expected age-standardized incidence rates (ASIRs) for liver cancer worldwide in 2018 are shown in Figure 1.6. Eastern Asia (17.7) has the most ASIRs per 100,000, with Mongolia (93.4) having the highest ASIR in this region and globally, followed by South-East Asia (13.3) and Africa (8.4),

with Egypt (32.2) and Gambia (23.9) having the highest ASIRs in Africa. South-Central Asia has the lowest ASIR (2.5), followed by Central and Eastern Europe and Western Asia (equally about 4.0) [89].

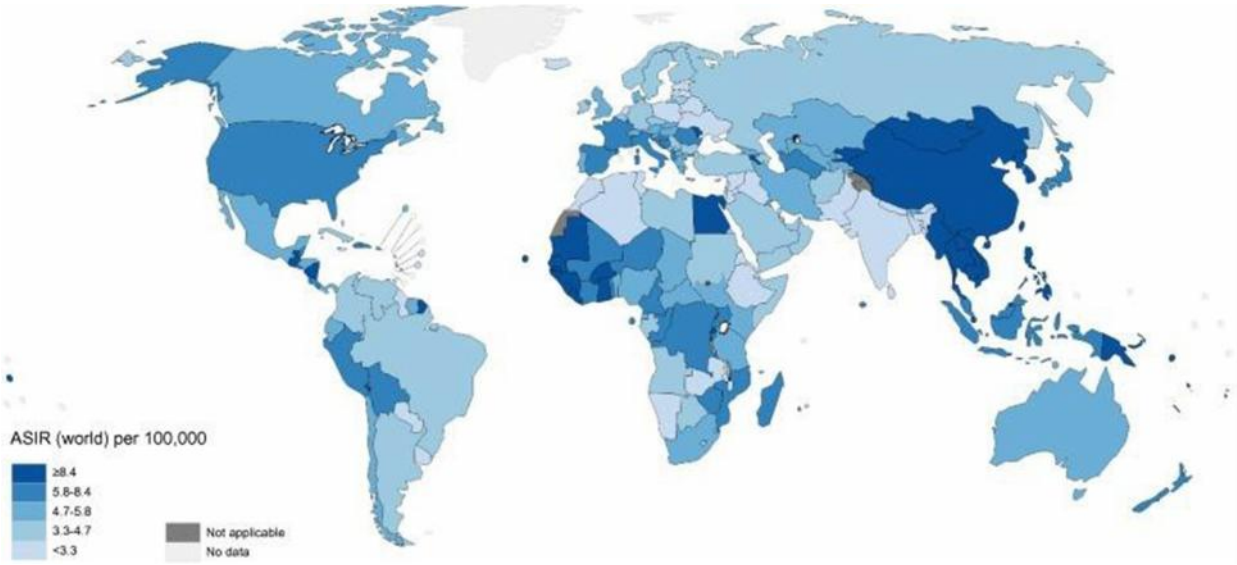


Figure 1.6: Worldwide age-standardized HCC incidence rates, 2018 [90].

1.5 Aetiology of HCC

The majority of hepatocellular carcinoma (HCC) instances occur in chronic liver disease, with cirrhosis being the most common risk factor for HCC, regardless of the cause of liver disease. In their lives, one-third of cirrhotic people would acquire liver cancer [91]. Long-term follow-up studies have indicated an annual incidence of (1–8) percent (for example, 2 percent in HBV-infected cirrhotic patients and 3–8% in HCV-infected cirrhotic patients) [92]. Hepatocellular carcinoma (HCC) is more common in alcohol-related and non-alcohol steatohepatitis (NASH)-related cirrhosis than inactive viral hepatitis, with more than 1.5 percent across all cirrhosis etiologies.

1.6 HCC Surveillance Data

Cancer monitoring programs aim to discover tumors at an early stage so that they may be treated with curative therapy, which has been shown to enhance survival [93]. The evidence pointing to a survival advantage linked with HCC screening in cirrhotic individuals is still debatable [94].

Aside from the several methodological flaws noted below, a literature review reveals that negative studies frequently suggest faulty or unimportant screening procedures rather than a failure of monitoring programs to convert into a survival advantage [95]. A randomized controlled trial supporting HCC monitoring with 6-monthly abdominal ultrasonography was conducted in over 18,000 Chinese patients and found a 37 percent reduction in death risk in those who were checked [96].

This study was conducted on HBV-infected individuals. It is unclear if the findings would apply to cirrhotic patients due to increased modularity, affecting surveillance efficiency, and a higher risk of liver-related death. Because using trials to compare screening to no monitoring would be unethical, [97] the quality of evidence is generally based on reflective experimental studies that show that surveillance for HCC is an independent predictor of survival [98-102]. Patients with recompensed viral cirrhosis who were advised for screening after a 6-month gap had a more significant probability of HCC detected at an early stage. This assistance resulted in a survival advantage due to the recurring execution of first-line curative therapies [103]. Whatever the case may be, there are several flaws and biases in experimental cancer screening trials that must be noted. Lead-time bias, for example, suggests that a certain percentage of the survival benefit can be attributable to earlier diagnosis owing to monitoring. Furthermore, a sizeable temporal bias means that tumors found early in a monitoring program may have a different prognosis than tumors diagnosed later. In recent trials examining the impact of HCC screening on outcomes, these biases were considered to strengthen the results [103]. Based on HCC volume doubling-time, estimated to be about six months, Western recommendations advocate screening after a 6-month interval [104]. Japanese guidelines have advocated a 3-month gap for select categories regarded at greater risk to reduce the probability of detecting HCC at a late stage [105].

But a French randomized trial experiment observed there is no surveillance benefit to screening capered intervals of 3 and 6 months. This experiment had done on more than 1,200 cirrhotic patients. More small-size focal lesions were found compared to ultrasound every six months [106]. In addition, significant retrospective research looked at the effects of varied monitoring intervals in HCC patients [107]. Shorter ultrasound screening intervals were associated with lower overall mortality in these individuals, and the 6-month screening interval was widely regarded as the ideal cut-off for HCC monitoring.

1.7 Diagnosis of HCC

HCC is a disease that often develops and progresses invisibly. It is challenging to identify before the onset of symptoms, and most cases are diagnosed at a later stage of the disease. There are a variety of clinical manifestations that are typically linked to the degree of the hepatic reserve at the time of diagnosis. Cirrhotic individuals have a lower tolerance for malignant aggressiveness in the liver and commonly appear with nonspecific hepatic decompensation signs and symptoms such as jaundice, hepatic encephalopathy, and anasarca. Ascites, variceal hemorrhage, or other signs compatible with portal hypertension are examined in the early stages of HCC. Abnormal laboratory results in chronic liver disease are vague, and they may represent the effects of routinely used cirrhotic medicines like spironolactone. Hepatocellular carcinoma in non-cirrhotic people manifests in various ways, and it is commonly observed in Sub-Saharan Africa and other high-incidence countries. Their malignancies are frequently permitted to develop unrestricted. Long-term malignancy and tumor development are widely associated with malaise, anorexia, wasting, right upper quadrant abdominal discomfort, and distension [108]. An abdominal mass or hepatomegaly with thick and irregular boundaries and avascular bruit may be discovered on physical examination [109].

Painless obstructive jaundice can sometimes indicate malignant invasion of extrahepatic biliary structures [110]. Tumor burst is a deadly complication of HCC when a sizeable vascular tumor on the liver's periphery outgrows its blood supply [111]. Patients with this condition have intense stomach discomfort, peritoneal irritation, and hypotension. An abdominal laparotomy or peritoneal lavage can confirm the diagnosis. However, these results and problems are not necessarily limited to any patient setting, and there is some overlap.

Hepatocellular carcinoma (HCC) has several extrahepatic manifestations, some of which lead to subsequent paraneoplastic symptoms or metastases. HCC can migrate to any organ system by hematogenous or lymphatic channels in the metastatic stage, and it usually spreads to bone, lung, and abdominal viscera [112]. The first indicator of HCC to metastasis may be bone pain or other consequences related to hepatitis. Hypoglycemia, hypocalcemia, polycythemia, and feminization syndrome are all unusual paraneoplastic symptoms of HCC [113]. Watery diarrhea is one of the most prevalent and severe symptoms, and it has been linked to cirrhosis and HCC. A probable reason has been suggested: increased synthesis of intestinal secretory chemicals like gastrin and vasoactive intestinal peptide (VIP) [114,115]. HCC has various cutaneous characteristics, including the Leser-Trelat sign, dermatomyositis, pemphigus foliaceous, and pityriasis rotunda, which are not disease-specific [116]. Porphyria cutaneatarda (PCT) has been linked to chronic hepatitis C. A few studies have linked the show to an increased risk of hepatocellular cancer [117].

I've seen that routine surveillance of high-risk individuals has increased the likelihood of finding asymptomatic HCC. Patients whose tumors are detected early, before hepatic decompensation or other problems such as those described above, are more likely to be candidates for invasive procedures that have been shown to improve survival. Above all, HCC has been diagnosed by:

- Estimating Serum AFP level
- Angiography
- Liver biopsy
- Diagnose by imaging
    - Ultrasonography
    - CT Scan
    - MRI

1.8 Staging of HCC

Staging determines the extent of a tumor's burden in the main organ and its dissemination all over the body.

They play an essential role in treating all malignancies, including the precise tumor prognostic categorization and the selection of the best treatment strategy depending on the stage.

Furthermore, it is essential to consistently categorize patients for clinical trials and scientific research and compare patients from different clinical studies.

In most solid tumors, just the degree of the tumor size within the initial primary organ and its invading nearby tissues is appropriate for staging. Various cancers, including hepatocellular carcinoma (HCC), typically occur in the context of a liver problem, raising the amount of regulatory complexity above and beyond any other malignancy.

It has been discovered that, regardless of tumor grade, the functional loss of the underlying liver disease significantly influences prognosis [118]. Processes that only consider the physical properties of the tumor, such as the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) staging system, which stratifies patients using a Tumor-Node-Metastasis (TNM) classification, do not have a good predictive capability in and of themselves [119, 120]. BCLC staging is preferred and used by the majority of physicians.

The BCLC staging method is the only one now in use. It involves an integrated assessment of liver damage, tumor extent, the existence of constitutional symptoms, and a sign of first-line therapy. It groups the patient into five categories based on illness severity or tumor features, ranging from 0 to D, each with its treatment and prognosis [121].

The BCLC staging technique takes into account the number and size of the liver tumors, as well as liver function. Doctors routinely use the Child-Pugh score to evaluate liver disease, and **figure 1.7** depicts the phases used to determine the severity of the liver disease.

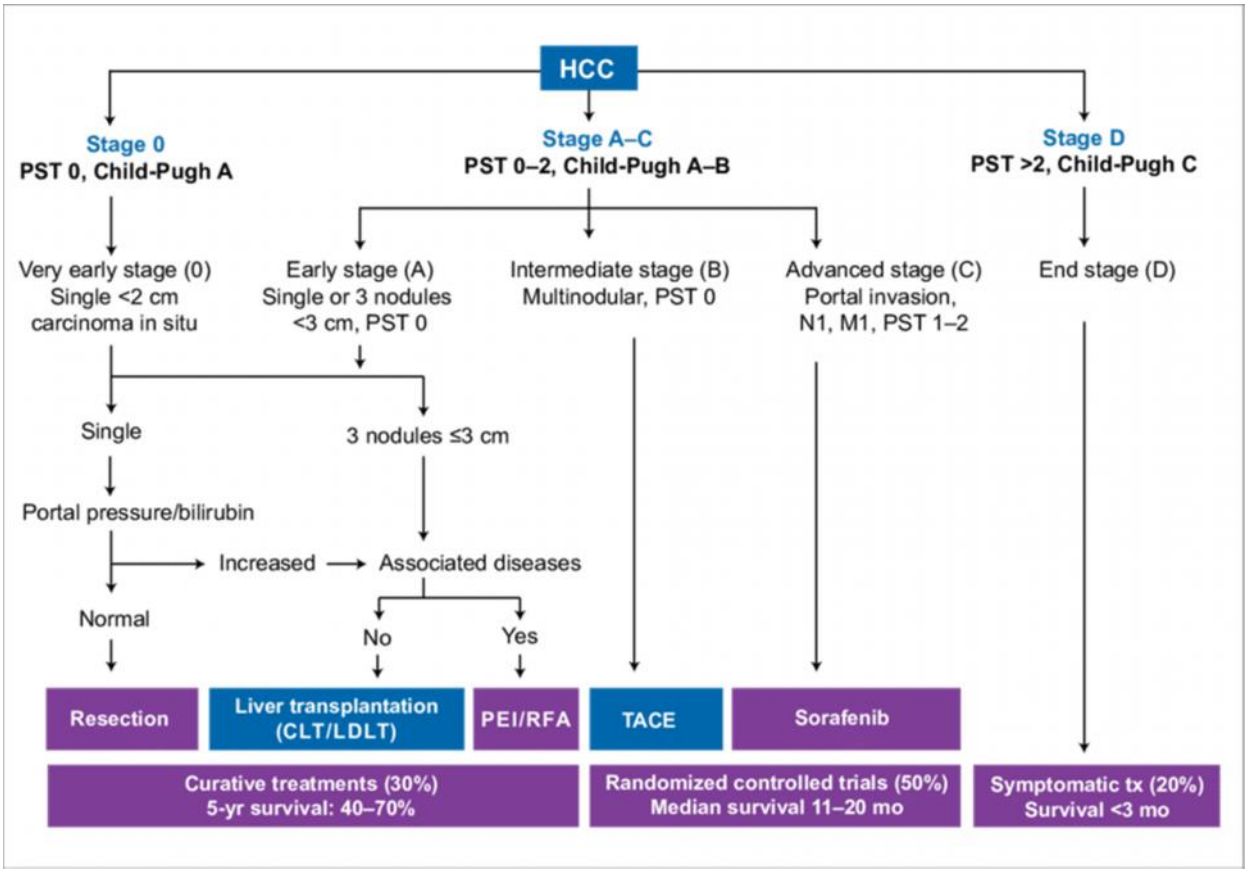


Figure 1.7: BCLC staging system and the Child-Pugh system; TNM

There are five stages to the Barcelona Clinic Liver Cancer (BCLC) staging system.

Stage 0 (Very early stage)

Stage 0 is defined as a tumor diameter of less than 2 cm (PS 0) with regular liver activity (Child-Pugh A).

Stage A (Early stage)

A single or up to three tumors might be seen in stage A. However, all tumors are less than 3 cm in diameter and proliferative (PS 0), but the liver is functioning normally (Child-Pugh A or B).

Stage B (Intermediate Stage)

There are more tumors inside the liver at this stage. On the other hand, patients are in better spirits (PS 0), and their livers usually function (Child-Pugh A or B).

Stage C (Advanced stage)

Stage C indicates that cancer has spread to all of the body's organs. Whatever the case may be, the patients appear to be in good health and are less active (PS 1 or 2). The liver continues to function normally. (A or B Child-Pugh)

Stage D

This is referred to as the final stage. It denotes serious liver injury (Child-Pugh C), and the liver is not functioning correctly and requires assistance to care for it (PS 3 or 4).

1.9 Treatment of Liver Cancer

HCC is the principal reason for a cancer-related fatality that is particularly refractory to be had chemotherapeutic drugs [122]. In most instances, HCC is associated with continual liver harm because of viral infections or with the aid of using different agents or carcinogens. Hepatitis B virus contamination is maximum not unusual place viral etiology many of the diver's etiology of HCC. Viral infections, alongside alcoholic liver sicknesses and non-alcoholic steatohepatitis (NASH) are fundamental hazard elements for developing cirrhosis and its pre-level for HCC. Liver cirrhosis and continual liver sicknesses related to HCC bills for approximately 90% of all HCC instances [123].

In most instances, HCC is detected late, limiting therapy options, and currently available medications are only beneficial in a small percentage of patients. Patients with complicated pathophysiology of HCC have a tough time deciding on a treatment plan. Several recommendations for treating HCC are published and revised daily across the world. In terms of monitoring and treatment allocation, the varied recommendations throughout eastern and western nations have harmony and variations [124]. The differences are mostly determined by the etiologies of hepatocellular carcinoma and the available resources for HCC therapy; nonetheless, the goals of all of these recommendations are to aid healthcare practitioners in making treatment

decisions and determining prognosis. The stage-wise treatment pattern has illustrated in **Figure 1.8**

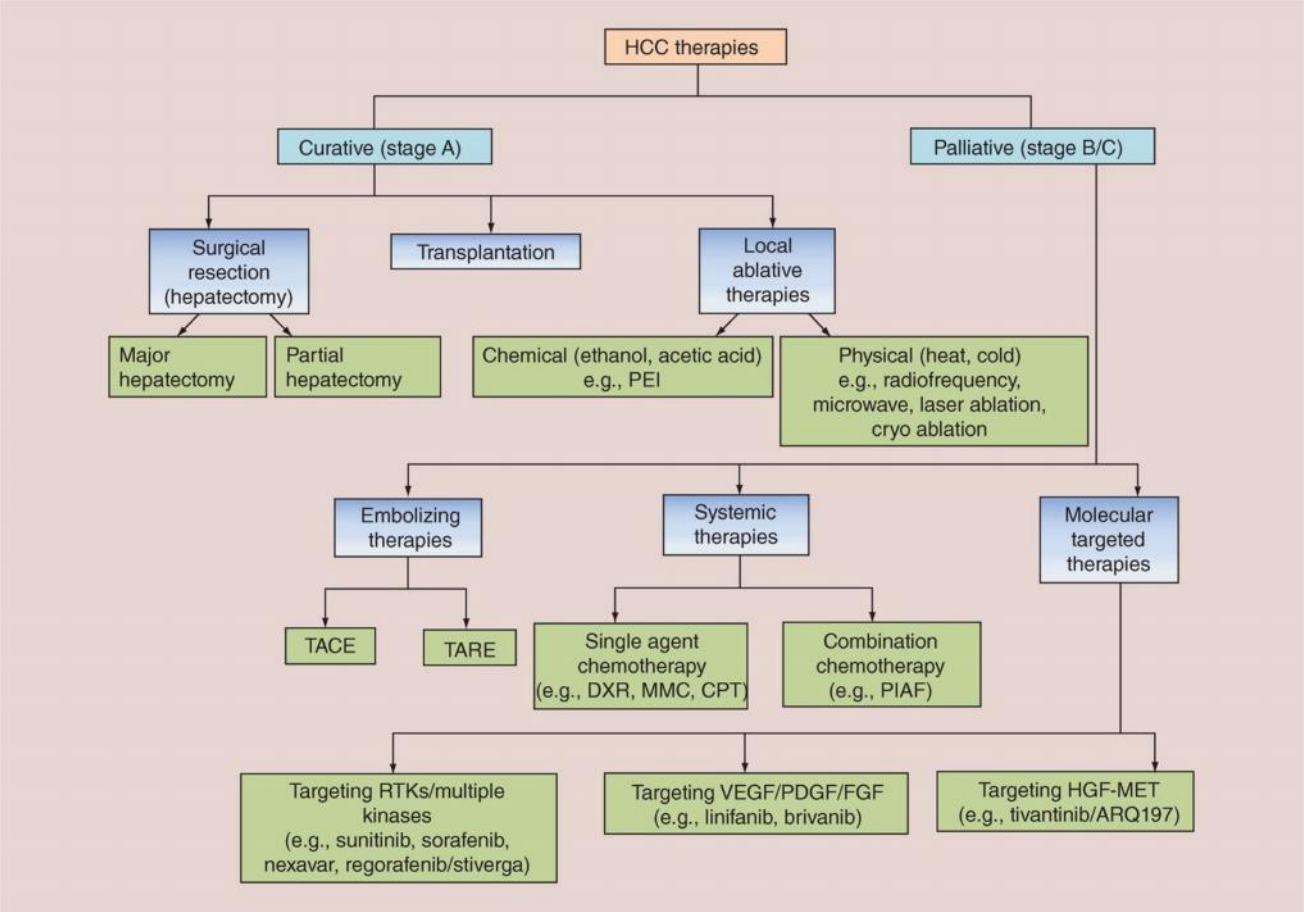


Figure 1.8: Stage-wise treatment for liver cancer

1.10 TP53 Gene

TP53 is a gene that releases a protein, and that is located inside the nucleus of the cell and controls cell growth and division with cell mortality. Cancer cells produce and circulate throughout the body due to a mutation mostly in the p53 gene. The TP53 gene performs as a tumor suppressor. This gene is also described as the tumor protein p53 gene. Tumor protein (EC: 2.7.1.37) is a gene that codes for a protein that modulates the cell cycle and inhibits tumors. This is incredibly significant to multicellular creatures' cells to inhibit malignancy. TP53 has been dubbed "the genome's guardian," owing to its function in maintaining genomic integrity by avoiding mutation

[125]. It has a molecular weight of 53 kilo Daltons [126]. Wild-type p53 is a labile protein, and it is made up of folded and unstructured areas that work together synergistically [127].

10.1 Location

The Tp53 gene has been detected on the 17th chromosome of humans (17p13.1). The location of the Tp53 gene has been revealed in **Figure 1.9** [128].

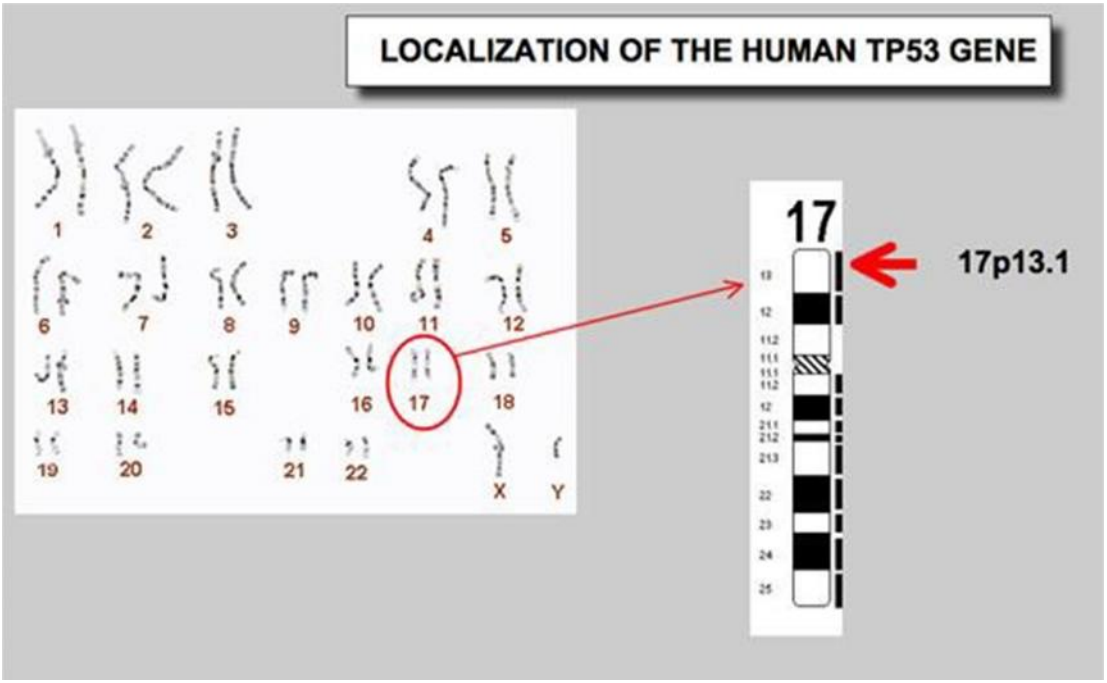


Figure 1.9: Localization of TP53 gene

10.2 TP53 Gene Structure

The TP53 protein is a phosphoprotein. It's made up of 393 different amino acids. It is made up of four units or domains, as shown in Figure 1.10: [129].

• A transcription factor-activating domain.

• A domain that can recognize specific DNA sequences (core domain).

• A region that is responsible for the protein's tetramerization.

• A domain for detecting DNA damage includes mismatched base pairs or single-stranded DNA.

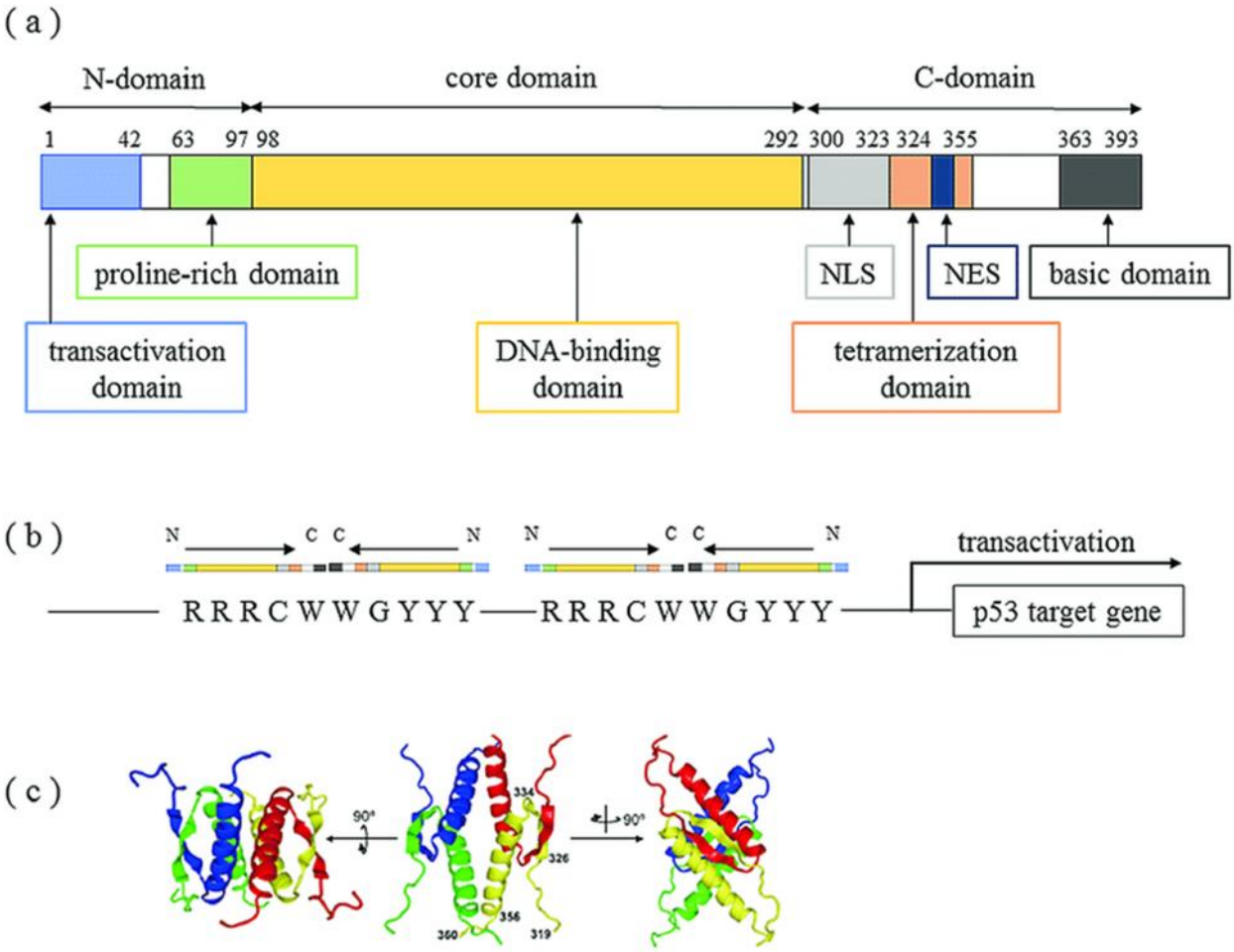


Figure 1.10: Structure of TP53 gene: a) Domain structure of TP53. b) Transcriptional activation mechanisms of target genes by p53 protein. c) NMR structure of tetramerization domains [130].

10.3 Role of TP53

Figure 1.11 depicts the activities of TP53 in cell cycle arrest and apoptosis. TP53 is directly engaged in DNA repair. P53R2, which encodes ribonucleotide reductase and is critical for DNA replication and repair, is one of its transcriptional target genes. DNA polymerase and AP endonuclease, both engaged in base excision repair, bind to TP53 [129, 131].

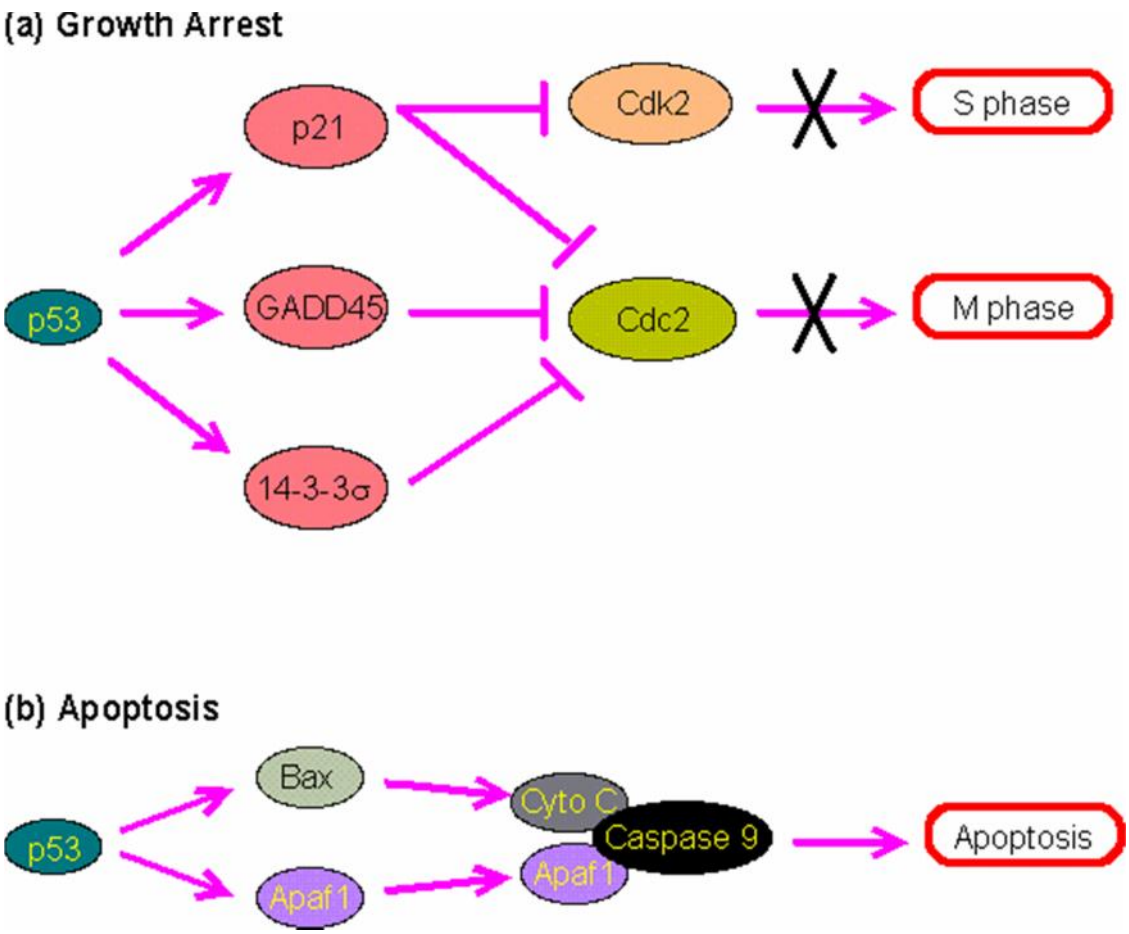


Figure 1.11: The roles of p53 in growth arrest and apoptosis

(a) The progression of the cell cycle in phase s requires Enzyme CDK2, blocked by P21. CDC2 needs to evolve in the M stage, which P21, GADD45, or 1433 can stop. To arrest growth, TP53 controls the production of these inhibitory proteins.

(b) Apoptosis can be activated by the Caspase 9 link at Cytochrome C and APAF1. APAF1 and BAX expressions can be started by TP53. May then trigger mitochondrial cytochrome c release.

1.11 TP53 Gene and Cancer

In humans, a frequent polymorphism is the replacement of arginine for proline at codon position 72. Numerous investigations have shown a genetic connection between this polymorphism and cancer risk, although the findings have just been mixed. A meta-analysis from 2009, for example,

was unable to establish a relation between cervical cancer and smoking [132]. The TP53 proline mutation did have a dramatic influence on pancreatic cancer risk in men, according to research published in 2011[133]. Another study on Arab women discovered that proline homozygozygosity at TP53 codon 72 is linked to a lower incidence of breast cancer [134]. According to one research, TP53 codon 72 polymorphisms, MDM2 SNP309, and A216G are connected to non-oropharyngeal cancer risk, and MDM2 SNP309 combined with TP53 codon 72 may enhance the risk of non-oropharyngeal cancer in women [135]. The TP53 codon 72 polymorphism has been associated with an increased risk of lung cancer, according to a 2011 research [136].

A meta-analysis from 2011[137] showed no significant relationships between TP53 codon 72 polymorphisms and colorectal cancer risk or endometrial cancer risk [138]. Researchers discovered a link between non-mutant arginine TP53 and those without a family history of cancer in a Brazilian birth cohort analysis [139]. The p53 homozygous (Pro/Pro) genotype was associated with a considerably higher risk of renal cell cancer in another study published in 2011[140].

1.12 TP53 Gene Mutation and Liver Cancer

HCC is among the most common and dangerous cancers on the globe. Around 560 000 new people have been diagnosed every year, making cancer the third leading cause of death [141, 142]. Hepatocellular carcinoma (HCC) is a popular topic of cancer-linked death in these countries because less than 3% of these patients survive more than five years [143]. The positions and features of DNA base alterations differ amongst human tumor types, according to TP53 mutations. An important molecular link between carcinogenic exposure and cancer has been observed in the mutational spectrum of human hepatocellular carcinoma.AFB1 dietary exposure (a mycotoxin found in foods such as maize, rice, and peanuts) is affected by geographical locations and chronic viral hepatitis, such as in China and Africa, where a point mutation at codon 249 ser results in a G:C to T:G change. In HCC, transversion was prevalent [144, 145]. Others have corroborated and expanded on these findings [146-148]. The most recent investigations show that the AFB1-induced TP53 249ser mutation, as measured by plasma TP53 249ser, and chronic viral hepatitis have a multiplicative effect on HCC risk [149].

HBV, HCV, and other hepatitis viruses cause liver injury, liver mortality, and the most infected liver. HBV is a Hepadna virus. It is also a type of DNA virus about 3.2 KB long and consists of four open reading frames, encrypted envelopes and nucleocapsid proteins (core), HBV X (HBX) proteins, and the process of the reverse virus. HBX is essential for the transcription of the virus genome. On this, HBX protein has been concentrated because it claims to play an important role in HCC [150]. The integrated HBV gene is most commonly known as the HBX gene [151]. It seems to come from the expression profile of the essential protein genes of VHC, 2A, and 4D genes in HEPG2 and HH7 cells, indicating that each main protein has a self-expression and related records to copy VHC as well as growth and oncogenesis [152]. To pay more attention to the role of P53 in HCC. Some cases of Mutant TP53 and TP53 wild-type HCC cases have been tested for a microstructure of 83 P53 Linked genes in HCC mutant TP53 when they are correlated with HCCs wild-type TP53[153]. Chronic infections and irritation are often involved in increasing cancer risk, while exceptions can be considered, including papillomavirus infections in humans and rheumatoid arthritis [154]. Virus infections of VHB and HCV cause inflammation with a free radical release, chemokine, and cytokine, causing DNA damage, cells of cells, fibrosis, and arising. How P53 is an essential response to inflammatory stress [155]. In the Human Alenic TP53, more than 50% of the tumor has a mutant of Missense, and the other person often comes with deletion (loss of heterozygosity, LOH) [156]. Many current mutations are placed in DBD, mainly leading to the helplessness of Mutant P53 (MUTP53) to link P53E and thus transactivate to the P53Target genes, which is illustrated in **Fig. 1.12**. The codon 249 mutation represents a particular case among hotspots, as it is over-represented in HCC. However, when wtp53 is expressed and encoded from the unmodified resting allele, it forms foreign bodies or aggregates. It thus eliminates wtp53's ability to organize genomic integrity through a negative effect to distribute [157].

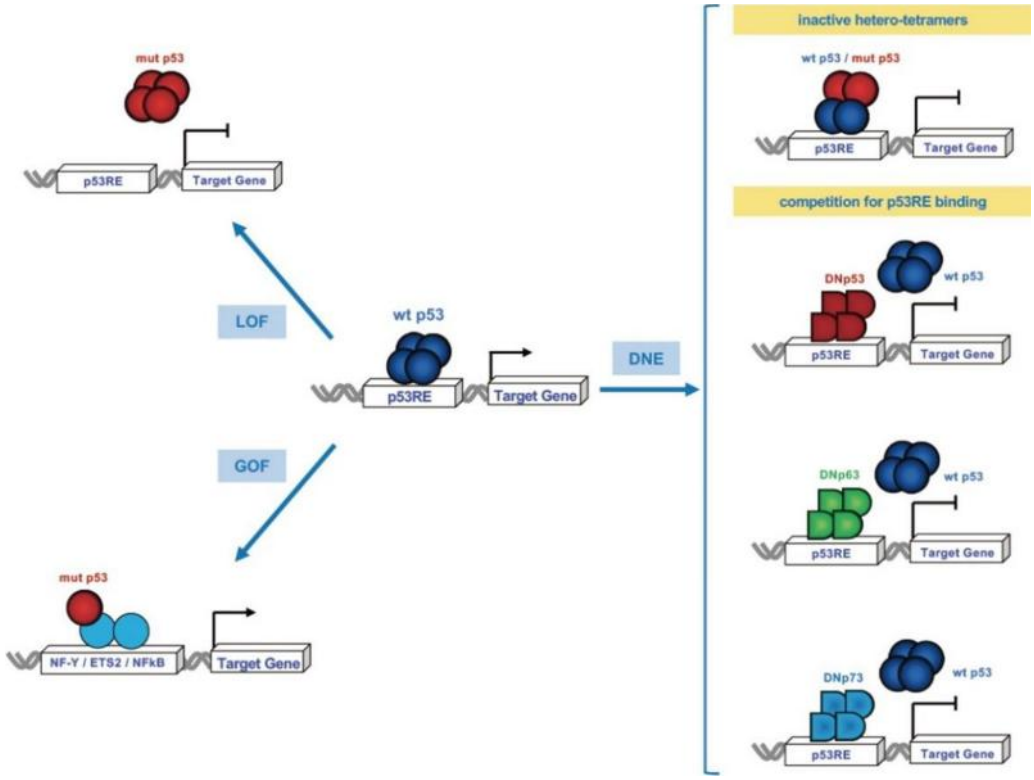


Figure 1.12: Wtp53 inactivation by genetic mutations and dominant-negative p53 family proteins

1.13. Purpose and Specific Objectives of the Study

The process of polymorphisms in DNA repair genes may change DNA repair ability; as a result, it leads to genetic instability and carcinogenesis. SNPs in the nucleotide excision repair (NER) pathway genes are increasingly thought to play a role in the genesis of liver cancer. The concept is clear that the interaction between genetic and environmental factors accounts for the several levels of capabilities to develop liver cancer. Many investigators have proposed the involvement of free radicals in carcinogenesis, mutation, and transformation. Therefore, this investigation aims to study polymorphisms of TP53gene to determine whether there might be an increase in liver cancer incidence in individuals with polymorphism of this gene or not.

Specific objectives of our study were as follows-

I. To investigate the association of TP53 (exon 4 and exon 7 at codon GG 72 GC and GG 249 GT respectively) genotype with thesusceptibility and aggressiveness of liver cancer in the Bangladeshi population.
II. To identify the differences in the frequency distribution of TP53 (exon 4 and exon 7) gene variants between liver cancer patients and healthy individuals.
III. To investigate the risk of liver cancer associated with TP53 genotype according to gender, smoking status, family history of cancer, HVB, and HCV infections.

1.14. Significance of the Study

Liver cancer begins in the liver cells. Hepatocellular carcinoma is the most frequent kind of liver cancer, and it starts in the primary types of liver cells, although it has no symptoms in its early stages. As a result, diagnosing it at an early stage is challenging. So, liver cancer remains one of the most frequent cancers worldwide. The Bangladeshi populace is also more susceptible to liver cancer and its effects. Increased prevalence is posing a severe threat to Bangladesh's healthcare system. Both environmental and genetic elements are accused of this incidence. Different epidemiological studies suggest that TP53 gene polymorphism modifies the predisposition of hepatocellular carcinoma. Therefore, the current case-control study will aid us in determining the impact of TP53 gene polymorphisms on the risk of HCC in the Bangladeshi community. As there is little or no information availableonthedistributionofTP53 genotype in the Bangladeshi population, this study will provide information about the status of genetic risk. It is more apparent that identifying risk factors should also help understand disease etiology with the chance of offering lifestyle advice to those at highest risk. This will also help take protective and preventive measures to develop liver cancer.

Materials and Methods

2.1 Study Design

The study was designed as a case-control study.

(i) Case: liver cancer patients (n=119)

(ii) Control: healthy subjects who had no history of liver cancer or any chronic diseases (n=150)

Liver cancer was confirmed by liver biopsy, CT Scan, MRI reports of the liver, AFP level, and pathological examination and graded according to the standard method [158-161].

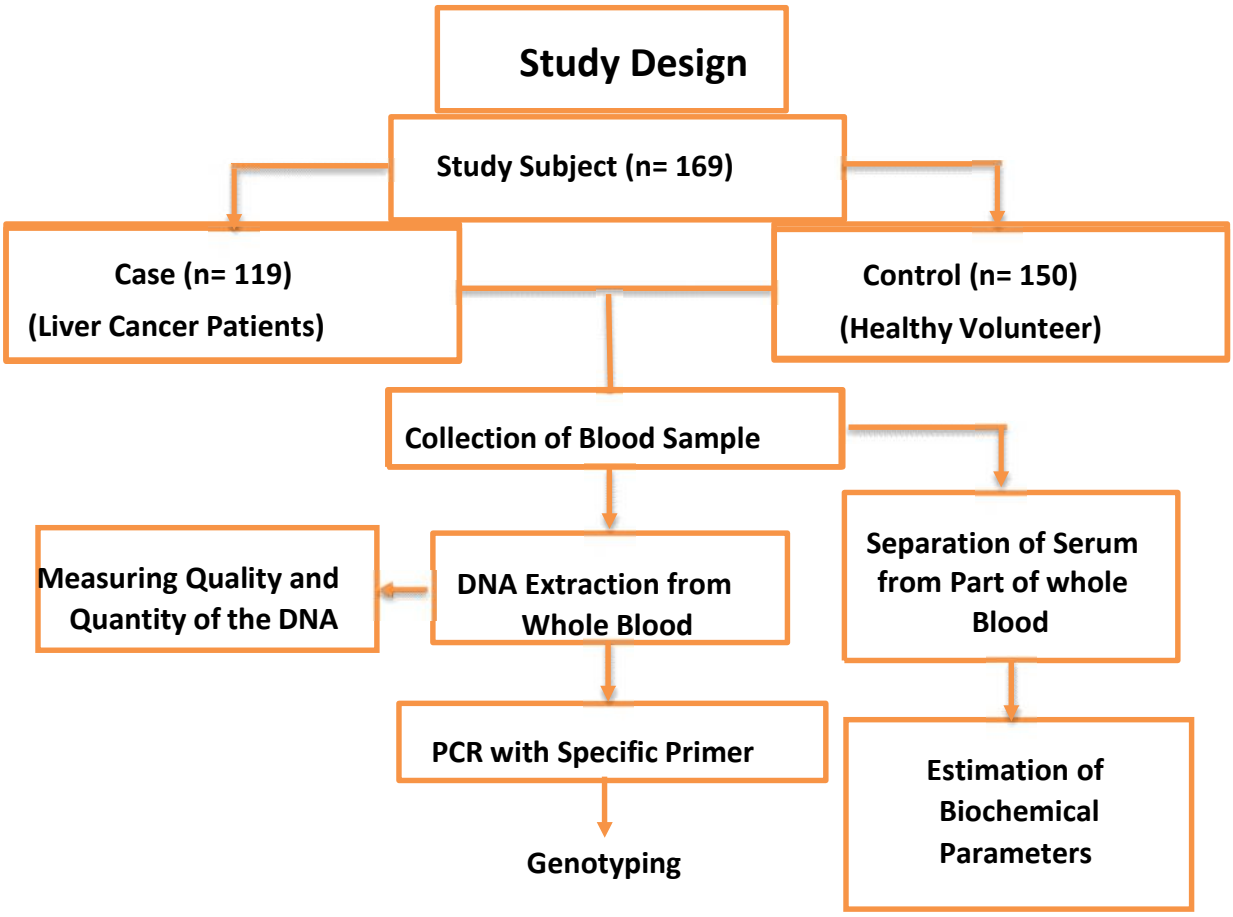


Figure 2.1: Schematic Diagram of Study Design

2.1.1 Estimation of sample size

The sample size was calculated following the below case-control study formula:

$$n = (\frac{r+1}{r}) \frac{\pm^2 (Z \quad + Z_{\sim/2})^2}{(d\text{ifference})^2}$$

- For 80% power, Zβ=0.84
- For 0.05 significance level, Zα=1.96
- r=1 (equal number of cases and controls)
- σ=10.0 (Standard deviation of the outcome variable)
- Difference = 5.0

$$n = (2) \frac{10^2 (7.84)}{(5)^2} = (2)2^2 (7.84) = 63$$

Thus, incorporating the above formula, a total of 63 subjects was needed. To allow for the fact that some subjects fail to complete the protocol, an allowance (50%) has been made. Hence, 95 subjects were recruited for each group to give a total study population of 190 (95 cases, 95 controls) subjects. In this study, 119 cases and 150 controls were included.

2.2 Subjects: Patient and Control Population

The case-control study was performed from January 2017 to March 2018. One hundred nineteen liver cancer patients were selected from the Dept. of Hepatology, Bangabandhu Sheikh Mujib Medical University (BSMMU) based on medical and liver biopsy, CT Scan, MRI, and AFP level records. All cases of liver cancer were between 16 and 85 years of age. The control group consisted of 150 subjects and was recruited from OPD of the Dept. of Hepatology in the Bangabandhu Sheikh Mujib Medical University (BSMMU) and had no history of liver cancer or chronic diseases.

2.3 Eligible Criteria for Patient and Control Subjects

For patients: Confirmed liver cancer patients by Liver biopsy, CT Scan, MRI, and AFP level.

For controls: Healthy subjects who had no liver cancer history or chronic diseases.

2.4 Questionnaire

All study subjects completed a questionnaire covering gender, age, occupational history, medical history, family history of liver cancer, and smoking history. All the information was noted by an interview. The questionnaire was added in the Appendix.

2.5 Blood Samples Collection and Storage

After obtaining consent from participants, blood samples were collected from patients and the control subjects. By using a disposable syringe, about six (6.0) mL of venous blood was drawn from each individual following all aseptic precautions with a trained person. Three (3.0) mL of blood samples were taken for serum isolation (collected in a plain vacutainer tube), and three (3.0) mL of whole blood were kept for isolation of genomic DNA (collected in EDTA containing tube). The blood-containing tubes were kept in an icebox and immediately transferred to the laboratory. Serum was isolated from collected blood after centrifugation for 10 minutes at 3,000 rpm. Whole blood samples were stored at -20°C and serum was stored at -80°C until further use.

2.6 DNA Extraction and Quantification

2.6.1 Extraction of DNA

Genomic DNA was extracted from peripheral blood using the method described by Bailes et al.[131].

2.6.1.1 Chemicals and Reagents

EDTA (0.5 M), pH 8.0

18.61gm of anhydrous EDTA was added to 80.0 mL of distilled water. Then adjusted pH up to 8.0 with NaOH. By adding distilled water, the solution was made up to 100 mL. Then it was autoclaved for 15 min. at 15p.s.i.

Tris-HC1 (1 M), pH 7.6

12.11gm of Tris base was dissolved in 80.0 mL of distilled H2O, and the solution was cooled at room temperature before adjusting the pH, then added concentrated HCl to adjust pH. After adjusting pH, the solution's volume was made up to 100 mL with distilled water and autoclaved for 15 min. at 15p.s.i.

Red Blood Cell Lysis Buffer

10.954 gm of sucrose, 0.101 gm MgC12, 1.0 mL of Tris (1 M), and finally 1.0 mL of Triton X-100 were taken into 80 mL of distilled water, and then its pH was adjusted at 8.0. Then distilled water was added to this solution to make up to 100 mL and autoclaved for 10 min at 15 p.s.i.

Nucleic Acid Lysis Buffer

In a volumetric flask, 100 ml distilled was taken and added 1.0 mL of Tris-HC1 (1 M, pH 7.6), 0.375 gm of anhydrous EDTA (pH 8.0), 1.0 gm of SDS, and 0.294 gm of sodium citrate. Then its pH was adjusted to 8.0, and it was autoclaved at 15 p.s.i. for 15 min.

TE Buffer, pH 8.0

A flask was taken with 100 mL distilled water and added 0.5 mL of Tris-HCl (1 M, pH 7.6) and 0.2 mL of EDTA (0.5 M, pH 8). Then the pH was adjusted up to 8.0 and then autoclaved it at 15. p.s.i for 15 min.

Chloroform (Roth), Prechilled to 4 °C

Ethanol (Merck) (100%), Prechilled to -20 °C.

2.6.1.2 Extraction procedure

Five hundred (500) µl of blood was taken into a 1.5 mL Eppendorf tube, then added 1000 µL of red cell lysis buffer. It shook mildly (up to homogenizing), then it was sewn for 2 minutes at 7,000 rpm. After spinning, the supernatant was discarded, and the above procedure was repeated 2 or 3 times to remove hemoglobin. For a few seconds, the tube was placed downward on tissue paper. Then 400µL of nucleic lysis buffer, 100 µL of saturated NaCl (5M), and 600µL of chloroform were added into the Eppendorf tube and mixed well. Then it spins at 7000 rpm for 2 min. Then 400µl supernatant was separated into a new 1.5 mL Eppendorf tube. Then 800µL of cold (–20°C) absolute ethanol was added, and it was shaken smoothly first, and it was vortex. DNA appeared in the solution phage as a mucus-like strand. The Eppendorf tube was spun at 12,000 rpm for one minute to precipitate the DNA. After the supernatant was discarded carefully, the tube was dried entirely at room temperature, placing the tube downward on tissue paper. Finally, 50 µl of TE buffer was added to the containing DNA tube, and tube was kept at –20°C for later uses.

The steps of DNA extraction are shown in **Figure 2.2**.

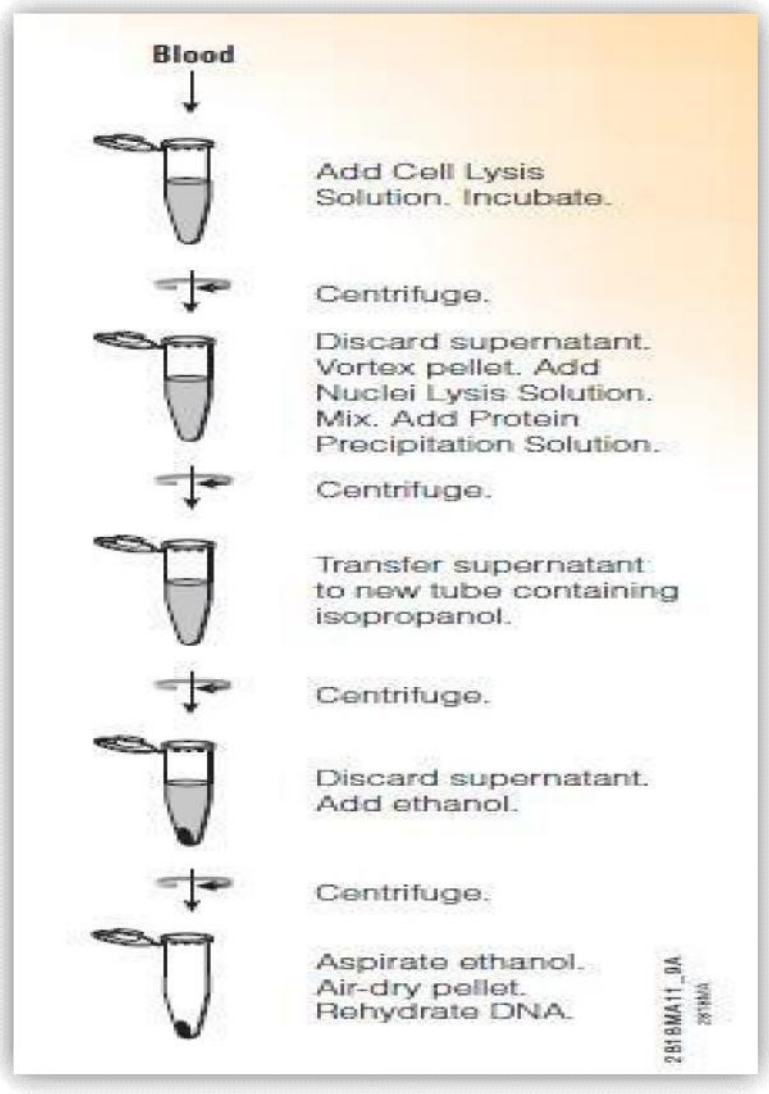


Figure 2.2: Steps of the DNA Extraction Procedure

2.6.2 Quantification of DNA

The quantity of the extracted genomic DNA in the elute was determined by NanoDrop 1000 spectrophotometer (NanoDrop 1000, US). Extracted DNA was diluted, and absorbance was measured at 260 nm and 280 nm. The consistency of DNA elutes (5 µl) of the samples was also evaluated by agarose gel electrophoresis. A gel containing 0.5% agar was used for this purpose. The electrophoresis was performed at low voltage (40 V) for an hour and visualized using a gel picture analyzer (AlphaImager mini, USA).

2.7 PCR-RFLP

The PCR-RFLP method was used for genotyping of TP53 (exon-4 and exon-7) gene.

Reagents

- Go Star Taq Polymerase (Promega, USA)
- dNTPs (New England Biolabs, USA)
- Primers (New England Biolabs, USA)
- Agarose (Sigma Chemical Co. (U.S.A.)
- Ethidium bromide (Sigma Chemical Co. (U.S.A.)
- TAE (Tris-Acetate EDTA) buffer
- 100 bp DNA ladder (Promega)

2.8 Identification of TP53 Genotyping

2.8.1 Exon-4 Genotyping

The total reaction volume of polymerase chain reaction (PCR) was 25 µL. Product size is 416bpfor the primer in this PCR reaction. The following primer set was used for PCR:

TP53 (exon-4)	Primer
Forward primer	5′-TGAGGACCTGGTCCTCTGACT-3′
Reverse primer	5′-AAGAGGAATCCCAAAGTTCCA-3′

PCR primer was designed according to Mitra et al.[159]. The primer sequences were verified using NCBI BLASThttp://blast.ncbi.nlm.nih.gov/Blast.cgi

2.8.2 PCR Condition for TP53 (Exon-4) Gene Application

Go Taq polymerase was used for PCR reaction. PCR conditions for the amplification were in the initial step for denaturation at 95oC for 5 minutes, followed by 35 cycles of denaturation at 95oC for 30 seconds, and annealing was carried out at 55°C for 60 seconds, where elongation was completed at 72oC for 30 seconds, and followed final elongation at 72oC for 10 minutes. A DNA thermal cycler (Applied Biosystems) was used for performing PCR assays. Reagent blank was

used as a negative control, which contained all components of the reaction ingredients without the DNA sample. Stage-wise PCR procedure is illustrated in **Figure 2.3**.

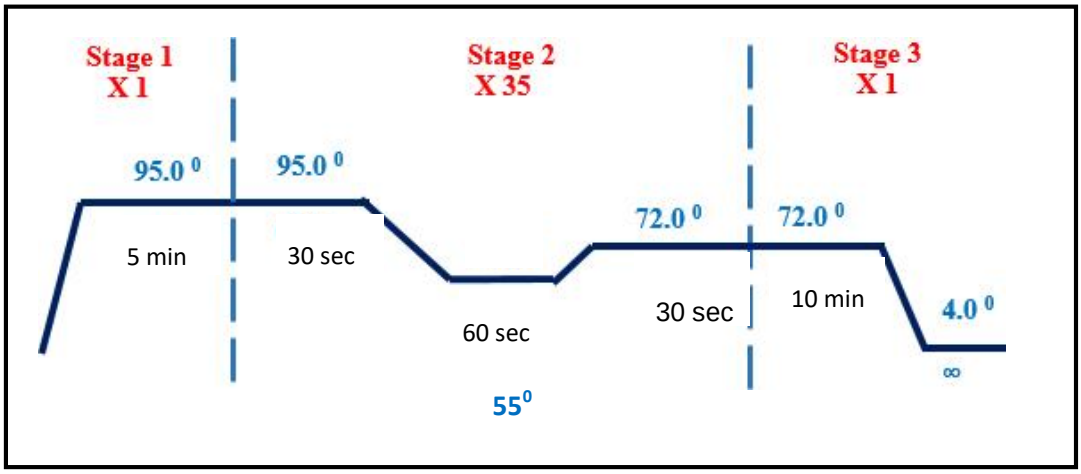


Figure 2.3: PCR condition for TP53 (Exon-4) gene application

2.8.3 Exon-7 Genotyping

The total reaction volume of polymerase chain reaction (PCR) was 25 µL. PCR product size is 254bp for the primer in this PCR reaction. The following primer set was used for PCR:

TP53 (exon-7)	Primer
Forward primer	5′-CTT GCC ACA GGT CTC CCC AA-3′
Reverse primer	5′-AGG GGT CAG CGG CAA GCA GA-3′

PCR primer was designed according to Mitra et al [159]. The primer sequences were verified usingNCBI BLASThttp://blast.ncbi.nlm.nih.gov/Blast.cgi

2.8.4 PCR Condition for TP53 (Exon-7) Gene Application

Go Taq polymerase was used for PCR reaction. PCR conditions for the amplification were in the initial step for denaturation at 95oC for 5 minutes, followed by 35 cycles of denaturation at 95oC for 30 seconds, and annealing was carried out at 60°C for 30 seconds, where elongation was completed at 72oC for 30 seconds, and followed final elongation at 72oC for 10 minutes. A DNA

thermal cycler (Applied Biosystems) was used for performing PCR assays. Reagent blank was used as a negative control, which contained all components of the reaction ingredients without the DNA sample. Stage-wise PCR procedure is illustrated in **Figure 2.4**.

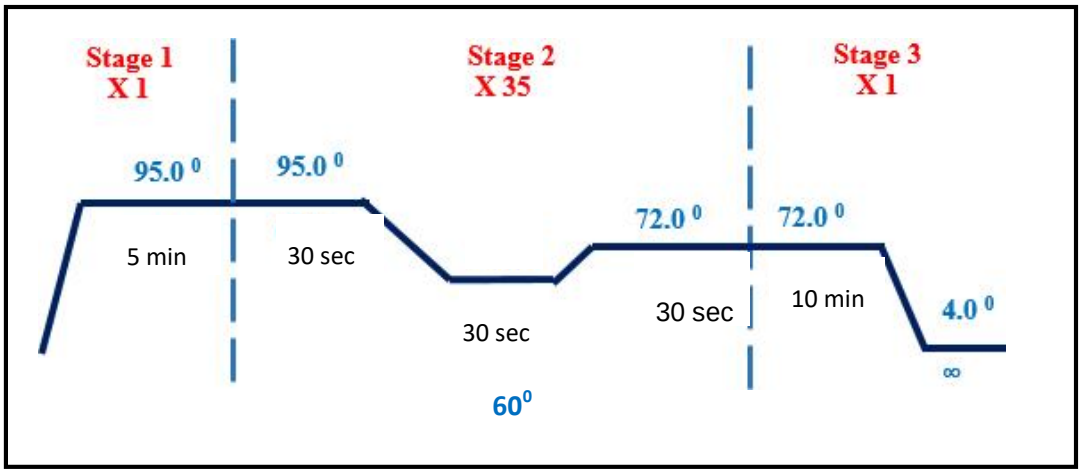


Figure 2.4: PCR condition for TP53 (Exon-7) gene application

2.8.5 Composition of PCR Product

Table 2.1: Composition of 25 µL Reaction Mixture for a PCR

Name of the components	Volume («L)
DNA	5.0
Buffer	2.5
dNTPs	0.2
Forward Primer (F)	1.0
Reverse Primer (R)	1.0
Taq Polymerase	0.175
ddH_2O	15.12
Total	**25.00 «L**

2.8.6 Evaluation of PCR

Five µL PCR product was taken on a 2% agarose gel run at 100V 300 mA for 1 hour to check amplification. The optional product size was ascertained by comparing it with the 100 bp DNA ladder. For documentation, the amplified DNA product was visualized under UV light, and a gel image was taken **(Figure 3.1)**.

2.8.6.1 RFLP Analysis of Exon- 4 at Codon 72

The restriction enzyme *BstUI* was taken for PCR-RFLP analysis using NEBcutter V2.0, an online tool. The 254 bp PCR product was subjected to restriction digestion by using *BstUI* following optimum reaction conditions as per the manufacturer's protocols.

Table 2.2: Composition of the reaction mixture for *BstUI* restriction enzyme digestion

Name of the Component	Volume (µL)
PCR H_2O	8.2
10X Buffer (Supplied with enzyme)	1.5
BstUI	0.3
PCR Product	5.0
Total Volume	**15.0**

BstUI restriction enzyme digestion was carried out at 37°C for 5-15 minutes in a water bath. The RFLP products were separated in 2% agarose gel and visualized using a gel documentation system following ethidium bromide staining for 15 minutes. The gel was used to separate bands for genotyping. A homozygous wild type (GG) genotype generated a single band of 416 bp in restriction digestion; the homozygous mutant genotype (CC) produced a single band of 161 bp. In comparison, the heterozygous genotype (GC) was fixed by the presence of all the two bands (416 bp and 263 bp) upon visualization on an agarose gel following restriction digestion using the enzyme *BstUI*.

2.8.6.2 RFLP Analysis of Exon- 7 at Codon 249

For SNP-RFLP analysis, the *Hae III* was selected for PCR-RFLP studies with the help of the online tool NEBcutter V2.0. After restriction digestion using *Hae III*, the 254 bp PCR products were targeted by following the manufacturer's reaction protocols.

Table 2.3: Composition of the reaction mixture for *Hae III* restriction enzyme digestion

Name of the Component	Volume (µL)
PCR H_2O	8.2
10X Buffer (Supplied with enzyme)	1.5
Hae III	0.3
PCR Product	5.0
Total Volume	**15.0**

The restriction enzyme *Hae III* digestion was conducted at 37°C for 15 minutes in a water bath. Then the digested PCR products were resolved in 2% agarose gel which was visualized in a gel documentation system by staining ethidium bromide. Genotypes were analyzed by observing the band number in the gel. Two bands of 92 bp and 66 bp was generated by the homozygous wild type (GG) genotype in digestion products; the homozygous mutant genotype (TT) produced another undigested of 158 bp fragment. The heterozygous genotype (GT) was identified by the presence of the three bands (158 bp 92 bp and 66 bp) consequent to visualization in agarose gel by using the same restriction enzyme.

2.9 Estimation of Serum Albumin (S. ALB)

Serum Albumin was estimated using the Bromcresolgreen albumin assay kit [162].

Principle

The Bromcresol green albumin assay kit is designed to measure albumin directly without any pretreatment of serum or plasma samples. The albumin's spectrophotometric determination in human plasma is done based on the dye bromocresol green, which forms a colored complex specifically with albumin in serum or EDTA plasma. The intensity of the color, measured at 630 nm is directly proportional to the albumin concentration in the sample.

Reagents

1. Monoreagent

   - Succinatebuffer,pH4.2 75.00mmol/l

- ❖ Bromcresolgreen 0.12 mmol/l
- ❖ Tensioactive2g/l

2. Standard 5g/l

Pipette into Cuvette

	Blank	Standard	Sample
Reagent	2000 µL	2000 µL	2000 µL
Standard	-	10 µL	-
Serum	-	-	10 µL

Procedure

1. Samples were taken in a well cup, then samples.
2. Reagents were delivered to instruments with a reagent cartridge holder.
3. Select ALB from the instrument monitor and Run the test.

After 5 minutes of incubation, the absorbance of sample A(S) and the standard (STD) against the reagent as blank A (BL) was taken at 630 nm.

Calculation

$(\mathbf{S})$ = Absorbance of sample; $(\mathbf{STD})$ = Absorbance of standard

$$\textbf{Albumin (g/dL)} = \frac{(\mathbf{S})}{\delta A_{(\mathbf{STD})}} \times 4$$

2.10 Estimation of Serum Alanine Aminotransferase (S.ALT)

Using Automatic Analyzer (Dimension® Siemens, Healthcare, Diagnostics. Inc), Serum ALT was estimated using the enzymatic colorimetric method.

Principle:

Alanine aminotransferase (ALT) catalyzes the transamination of L-alanine to α-ketoglutarate (α-KG), producing L-glutamate and pyruvate. The pyruvate formed is reduced to lactate by lactate dehydrogenase (LDH) with simultaneous oxidation of reduced nicotinamide adenine

dinucleotide (NADH). The optical density change is directly proportional to the ALT activity, measured by the bichromatic (340, 700 nm) rate technique.

$$\text{L-alanine} + \alpha\text{-KG} \xrightarrow[\text{P5P, Tris, PH 7.4}]{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH(H+)} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD+}$$

Reagents

- Tris buffer,pH4.2 100.00mmol/l
- Alanine 260 mmol/l
- α-KG 20 mmol/l
- P5P 0.15 mmo/l
- NADH 0.22 mmol/l
- LDH 3000 u/l

Pipette into Cuvette

	Blank	Standard	Sample
Reagent 1	30 µL	30 µL	30 µL
Reagent 2	-	80 µL	-
Diluent			
Serum	-	-	35 µL

Procedure

1. Samples were taken in well cup, then samples.
2. Reagents were delivered to instruments with a reagent cartridge holder.
3. Select ALT from the instrument monitor and Run the test.

After 10 minutes of incubation, the absorbance of the sample was taken at 700 nm.

Calculation

ALT activity was calculate by using the following formula:

ALT activity, U/L = ΔA /min × 1/6.22 × 0.200 ml/0.010 ml × 1000

= ΔA /min × 3215

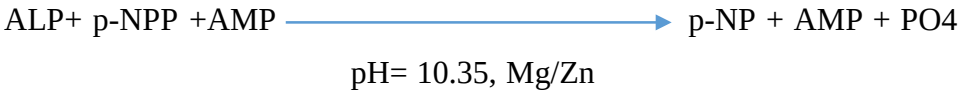
Where, ΔA /min is the absorbance change per minute, 6.22 is the mmol/L absorptivity of NADH at 340nm, 0.200 ml is the total volume in the cuvette, 0.010 ml is the volume of a serum sample, and 1000 is the factor to convert millimolar absorptivity to micromolar.

2.11 Estimation of Serum Alkaline Phosphatase (S.ALP)

By using Automatic Analyzer (Dimension® Siemens, Healthcare, Diagnostics. Inc), Serum ALP was estimated in the colorimetric method.

Principle:

Alkaline phosphatase catalyzes the transphosphorylation of p-nitrophenyl phosphate (p-NPP) to p-nitrophenol (p-NP) in the presence of the transphosphorylating buffer, 2 amino-2-methyl-1-propanol (AMP). Magnesium and zinc ions work as a catalyst in this reaction. The formation of p-NP is directly proportional to the ALP activity and is measured using a bichromatic (405, 510) rate technique.



Reagents

- Tris buffer,10.35 100.00mmol/l
- P-NPP 20.50mmol/l
- AMP 0.50 mmol/l
- MgSO4 2.30 mmo/l
- ZnSO480 µmol/L

Procedure

1. Samples were taken in a well cup, then samples.
2. Reagents were delivered to instruments with a reagent cartridge holder.
3. Select ALP from the instrument monitor and Run the test.

After10minutesincubation, the absorbance of the sample was taken at 510 nm.

Pipette into Cuvette

	Blank	Standard	Sample
Reagent 1	14 µL	14 µL	14 µL
Reagent 2	-	45 µL	-
Reagent 3	-	45 µL	-
Diluent	-	239 µL	-
Serum	-		7 µL

Calculation

ALP activity was calculate by using the following formula:

ALP activity, U/L = ΔA/min × 1/6.22 × 0.200 ml/0.010 ml × 1000

= ΔA /min × 3215

2.12 Estimation of Plasma Prothrombin Time (PT)

Principle:

The coagulation action is activated by incubating plasma with the ideal amount of thromboplastin and calcium; as a result, a fibrin clot is formed, then it is measured.

Reagent

- Thromborcel100 µL

Procedure

1. 50 µL samples were taken in a sample cup with a magnet piece
2. Then it is placed into the incubation hole in the instrument.
3. 100 µL reagent was added after 60 seconds and started counting the time until the magnet stopped moving.

Pipette into Cuvette

	Blank	Standard	Sample
Reagent	-	-	100µL
Plasma	-		50 µL

Calculation

PT= Required time to stop magnet in the sample cup when sample clotted.

2.13 Estimation of Serum Alphafeto Protein (S.AFP)

Serum AFP was estimated Enzymed-Linked Immunosorbent Assay (ELISA) method with the Automatic Analyzer, Vitros 7600, Ortho Diagnostic Clinic.

Principle:

The Test Kit is designed for AFP quantitative assay based on a solid phase enzyme-linked immunosorbent assay. In this assay method, one anti-AFP antibody is used for solid phase (microtiter wells) immobilization and mouse monoclonal anti-AFP antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The specimen (serum) for the test is added to the AFP antibody-coated microtiter wells and incubated with the Zero Buffer. If the specimen contains AFP, it will combine with the antibody on the well. Then the well is washed to discharge any additional test specimens. AFP antibodies labeled with conjugate (horseradish peroxidase) are added. The conjugate and the AFP bind immunologically on the well; as a result, the AFP molecules are sandwiched between the solid phase and enzyme-linked antibodies. Then it incubates at room temperature. After incubation, the wells are washed with washing buffer to remove unbound labeled antibodies. Then substrate solution (chemiluminescence) is added, and

optical density (OD) is taken in the appropriate Luminometers. The intensity (OD) is proportional to the amount of enzyme present and is directly related to the amount of AFP in the sample.

Reagents

- Antibody-coated microtiter well 96 wells
- Zero buffer 12 ml
- Enzyme conjugate reagent 18 ml
- 50X wash buffer concentrate 15 ml
- Chemiluminescence reagent A 6.0 ml
- Chemiluminescence reagent B 6.0 ml

Reagents Preparation

1. Before use, all reagents were confirmed to reach room temperature (18-25°C).

2. Reagent A mixed with Reagent B by ration1:1 to prepare substrate solution.

3. Diluted 1 volume of Wash Buffer Concentrate (50x) was diluted 1:49 ratio with distilled water.

Assay Procedure

1. The desired number of coated wells loads to the analyzer with the holder.

2. Twenty (20) µl of standard samples and controls were taken into appropriate wells.

3. 100 mL of zero buffer was added to each well.

4. It was mixed thoroughly for 10 seconds.

5. Then, it was incubated at (18-25°C) for 30 minutes.

6. After incubation, it was flicked on the plate to remove.

7. The microtiter wells were rinsed and flicked 5 times with washing buffer (1X).

8. The wells zapped sharply onto absorbent paper for removing all residual water droplets.

9.150 µl of Enzyme Conjugate Reagent was added into each well and smoothly mixed for up to 5 seconds.

10. It was incubated at (18-25°C) for 30 minutes.

11. Incubation mixture was removed by flicking plate contents into a waste container.

12. The microtiter wells were rinsed 5 times with washing buffer (1X) and flicked.

13. Again, the wells were zapped sharply onto absorbent paper to remove residual water droplets.

14. 100µl Chemiluminescence substrate solution was added into each well and smoothly mixed for 5 seconds.

15. OD was taken by a chemiluminescence microwell reader after 5 minutes.

Calculation

The average read relative light units (RLU) was calculated by comparing with standards and control for each set of unknown samples.

2.14 Determination of HBsAg

Principle

To detect HBsAg, the double-antibody kit uses the "sandwich" ELISA method, in which polystyrene microwell strips are pre-coated with antibodies specific to HBsAg. The tested samples are added into the microwells with HRP-Conjugate and directed as opposed to a different epitope of HBsAg. At the time of incubation, if HBsAg is present, a specific immunocomplex is formed. During incubation, a particular immunocomplex is produced if HBsAg is present in the sample and it is captured in the solid phase. Then it washes to remove other serum proteins and unbound HRP-conjugate. After washing, chromogen solution (ng tetramethyl-benzidine (TMB)) and urea peroxide are added to each well. In the presence of the antibody-antigen-antibody (HRP) "sandwich" immunocomplex, the colorless chromogen is hydrolyzed by the bound HRP-conjugate to produce a blue-colored product, and the reaction is stopped by adding sulfuric acid; as a result, the blue color turns to yellow. The color intensity can be measured, and it is directly proportional to the amount of the sample respectively. If the sample is negative for HBsAg, the respective wells do not produce any color.

Ab(p) +Ag(s) + (Ab)ENZ → [Ab(P)-Ag(s)-(Ab)ENZ]. → blue color → yellow color (+)

Ab(p) + (Ab)ENZ → [Ab(P)- → no color (-)

Incubation	Immobilized Complex	Coloring	Result
60 min	15 min		

Procedure

Step1. Reagent preparation: Before use, all reagents were confirmed to reach room temperature (18-25°C). Wash Buffer was diluted 50 times with deionized water.

Step2.Numbering Wells: Taken strips in a strip-holder and numbered required wells with negative and positive controls and one blank with HRP-conjugate.

Step3.50 µl of samples, positive control, and negative control was pipetted into respective wells, and then 50 µl of HRP-Conjugate was added to each well except the blank. After pipetting, it was mixed by tapping the plate smoothly.

*Step*4. After shaking, the plate was covered and incubated at 370C for 60 min.

Step5. After incubation, the plate cover was removed and discarded. Then each well was washed with wash buffer at least 5 times and allowed the microwells to soak for 30-60 seconds. After final washing, the plate was placed onto blotting paper to remove any residues.

Step6. Then 50 µl of Chromogen A and 50 µl of Chromogen B solutions were added to each well and mixed by tapping gently. Then the plate has incubated the plate for 15 minutes at 37°C, avoiding light. In this step, the enzymatic reaction occurs between chromogen and HRP-conjugate and produces blue color if HBsAg is positive.

Step7. Then 50 µl stop solution was added to each well and mixed smoothly. HBsAg positive wells appeared yellow in color.

S*tep8*.The plate reader was calibrated with a blank well, and OD was taken at 450 nm.

Calculation

(The individual specimen optical density =S)

Negative Result (S/C.O. < 1): If the sample's absorbance value is less than the cut-off value, it is considered a negative result for the hepatitis B virus.

Positive Result (S/C.O. ≥1): If the sample's absorbance is greater than or equal to the cut-off value is considered positive for hepatitis B virus.

2.15 Determination of HCV

Principle

The Advanced HCV detection method is EIA. A specimen diluent is used to dilute human serum or plasma, and then it incubates with microtiter wells coated with recombinant HCV antigen (CORE/NS3/NS4/NS5). If the sample is positive with antibodies, then the patients' sample's immunoglobulins bind to the coated wells. The antigen-antibody complex with a solution containing horseradish peroxidase-labeled goat antibodies is controlled against human immunoglobulins heavy and light chains.

After removing the unbound enzyme conjugate, the microwells are washed. Then incubates by adding enzyme-substrate, tetramethylbenzidine (TMB). If HCV-specific antibodies are presented, the bound enzyme hydrolyzes the substrate and forms blue or blue-green color in the respective wells. Then, sulphuric acid was added to stop the enzymatic reaction. The color density is measured spectrophotometrically at 450 nm, and the color density is proportional to the number of antibodies present in the samples.

Reagent preparation

1. Enzyme conjugate working solution:

a) Allowed conjugate and diluent to room temperature before use.

b) The conjugate was diluted 1:20 ratio with a conjugate diluent, as shown in the following table. Swirled gently to mix thoroughly without foaming.

Enzyme Conjugate Table

Number of tests	16	24	32	40	48	56	64	72-80	88-96
Enzyme Conjugate Diluent (ml)	2	3	4	5	6	7	8	9	10
Enzyme Conjugate Concentrate (µl)	100	150	200	250	300	350	400	450	500

2. Wash buffer

a) Concentrated wash buffer was diluted with deionized at a ratio 1:19.

b) Mixed wells

Procedure:

1. Before use, it was confirmed all reagents reached room temperature (18-25°C). It was swirled smoothly before use.
2. Numbered required wells with one well for blank, and two wells for negative and positive controls, respectively.
3. In each well, 100 µl sample diluent
4. Was taken and added 100 µl Specimen, Negative control, Positive control to each appropriate well according to the datasheet. (Reserve 1 well for blank).
5. Tabbed the plate to mix.
6. Incubated the plate in a 370C water bath and incubated for 30 min.
7. Each well is washed five times with wash buffer by the following protocol.
    a. The well contents were discarded completely and filled the wells with 350 µl wash buffer but avoided overflow.
    b. It was ensured that no fluid remained on the strip holder.
8. Then added 100 µl enzyme conjugate solution into the wells avoiding the blank well.
9. The microwell plate was covered by the plate sealer. Then it was incubated for 30 min. at 370C.
10. Each well was washed five times using a wash buffer flowing the washing procedure,

11. 50 µl color A and 50 µl color B were added to every well and tapped to the plate for mixing.
12. The plate was incubated for 10 min at 370C in the water bath.
13. 50 µl stopping solution was added to each well for stopping the reaction and tapped on the plate to mix.
14. Then absorbance of the solution was taken in each well at 450 nm (single wavelength) or 450 and 600 nm as reference (dual-wavelength) within 30 min.

Calculation

The presence or absence of antibodies for HCV is identified by measuring the absorbance value of the sample by comparing cut-off values.

1. For test validity, the following conditions were observed:

b. The blank well absorbance value was<0.100

c. absorbance of negative control was<0.100 after minimizing with the blank.

d. Absorbance of positive control was >0.800 after minimizing with the blank.

2. Calculation of control

Mean of the Negative controls NCx = (NC1 + NC2 + NC3)/3 Eliminate any NC greater than (>) 0.100

3. Calculation of the cut-off Value

Cutoff value = 0.120 + NCx

Calculation the cutoff value as shown in example below: NCx = 0.020 cutoff value = 0.120 + 0.020 = 0.140

4. The sample value calculation

The sample value was calculated by subtracting the blank absorbance from the sample absorbance value.

2.16 Statistical Analysis of Data

Results were expressed as Mean ± SEM. Data were analyzed by using GraphPad Prism (version 8.0). Odds ratios (OR) were measured relative risk at 95% confidence intervals (95% CI) using logistic regression models. The statistical methods used were the student's t-test (two-tailed), Fisher's exact test, and Chi-square test. More than two variables were analyzed by performing a one-way ANOVA test. A p-value less than 0.05 was considered statistically significant.

Results

3. Result

The results of the study on the association of G>C located at 72 positions of TP53 (exon 4) and G>T located at 249 positions of TP53 (exon7) gene polymorphism with liver cancer are presented in this chapter. This study was conducted with 119 hepatocellular carcinomas (HCC) cases and 150 controls.

3.1 Baseline Characteristics of the Study Subject

The baseline characteristics of study subjects according to age, gender, smoking status, and family history of cancer are presented in **Table 3.1.** The result of basic demographic data shows significant differences in age, gender, smoking status, and family history of cancer.

Table 3.1: Baseline Characteristics of the study Subject

Variables	Patient (n= 119) n (%)	Control (n=150) n (%)	*p*-value
Age (Yrs.)			
16 – 40	31 (26.05)	108 (72.00)	
41 – 60	65(54.62)	35 (23.33)	<0.001
61 – 80	22 (18.49)	06 (4.00)	
≥ 81	01 (0.84)	01 (0.67)	
Age (Mean± SEM)	49.76±1.16	35.78±1.10	<0.001
Gender			
Male	99 (83.19)	91 (60.67)	<0.05
Female	20 (16.81)	59 (39.33)	
Smoking Status			
Smoker	65 (54.62)	06 (4.00)	< 0.001
Nonsmoker	54 (45.38)	144 (96.00)	
Family History of Cancer			
Yes	25 (17.71)	09 (14.00)	< 0.003
No	94 (82.29)	141 (86.00)	

Results are expressed as a number (percentage). Fisher's test was performed to Calculate the statistical significance. A $p<0.05$ was taken as the level of significance.

The mean ± SEM age of HCC case and control subjects were 49.76 ±1.160 and 35.78 ± 1.098, respectively. Males were more than females in cancer patients (83.19% vs16.81% respectively). Smoking history was found to be significantly higher in the HCC group than control subject. Smokers comprised 54.62% of the cases and 4.00% of the controls. The number of patients having a family history of cancer was higher than the control subject. Alcohol consumption history was not found significant in the study groups.

3.2 Polymorphism Study of TP53 Gene

3.2.1 PCR Product for Detecting the SNP GG72GC (Arg/Pro)

A segment of the TP53 gene was amplified by using specific primers, as shown in **Figure 3.1**. The length of the PCR product was 416 bp. The amplified PCR products were evaluated by running them on 2% agarose gel, and the product size was determined by comparing them with a 100 bp DNA ladder.

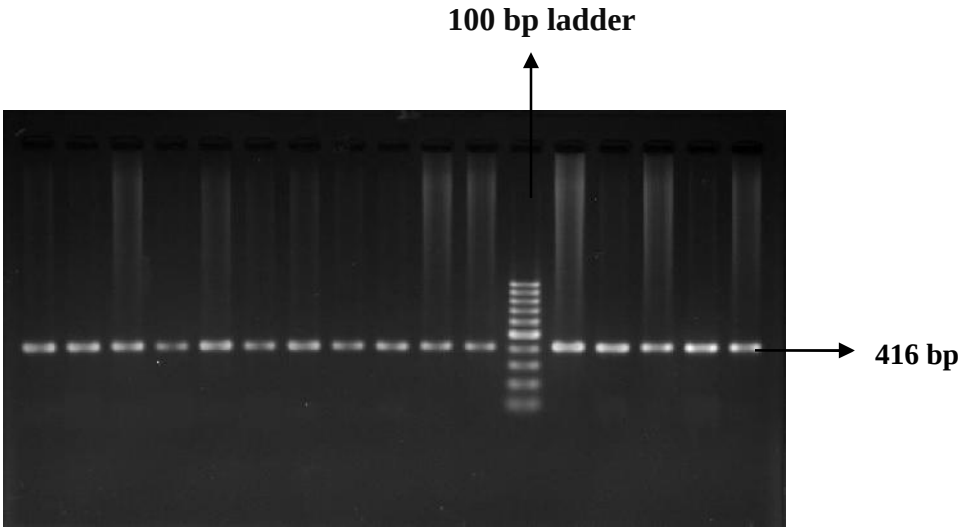


Figure 3.1: Representative PCR products of TP53 gene exon 4 in 2% agarose gel

3.2.2 Restriction Digestion of the PCR product of TP53 Gene Exon 4

Restriction digestion enzyme *BstUI* digested the 416 bp PCR product. The digested PCR product was evaluated by running them on2% agarose gel. As shown in **Figure 3.2**, the homozygous wild type (GG) genotype generated a single band of 416 bp upon restriction digestion (lane 3, 7 and 8), and the heterozygous mutant genotype (GC) produced a three bands of 416 bp , 263 bp and 161 bp (lane 6). The homozygousg mutant genotype (CC) had two bands of 263 bp and 161 bp (lane 4 and 5). For visualizing restriction digestion enzyme product 50 bp ladder was used.

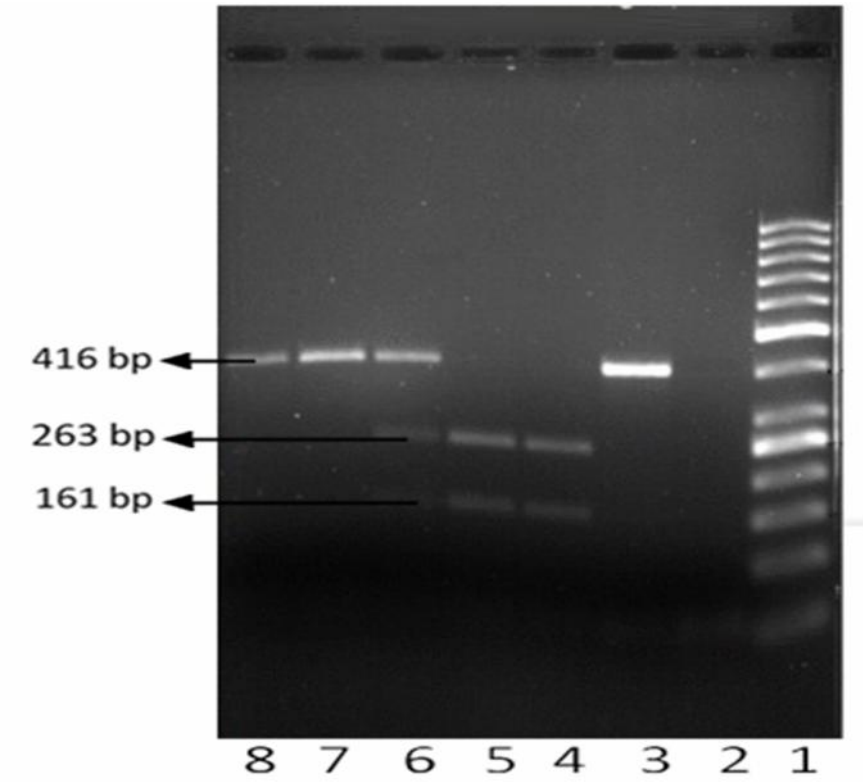


Figure 3.2: Representative digestion products of TP53 exon 4 PCR products in 2% agarose gel

3.2.3 PCR Product for Detecting the SNP GG249GT (Arg/Ser)

A segment of theTP53 gene was amplified by using specific primers, as shown in **Figure 3.3**. The length of the PCR product was 254 bp. The amplified PCR products were evaluated by running them on 2% agarose gel, and the product size was determined by comparing them with a 100 bp DNA ladder.

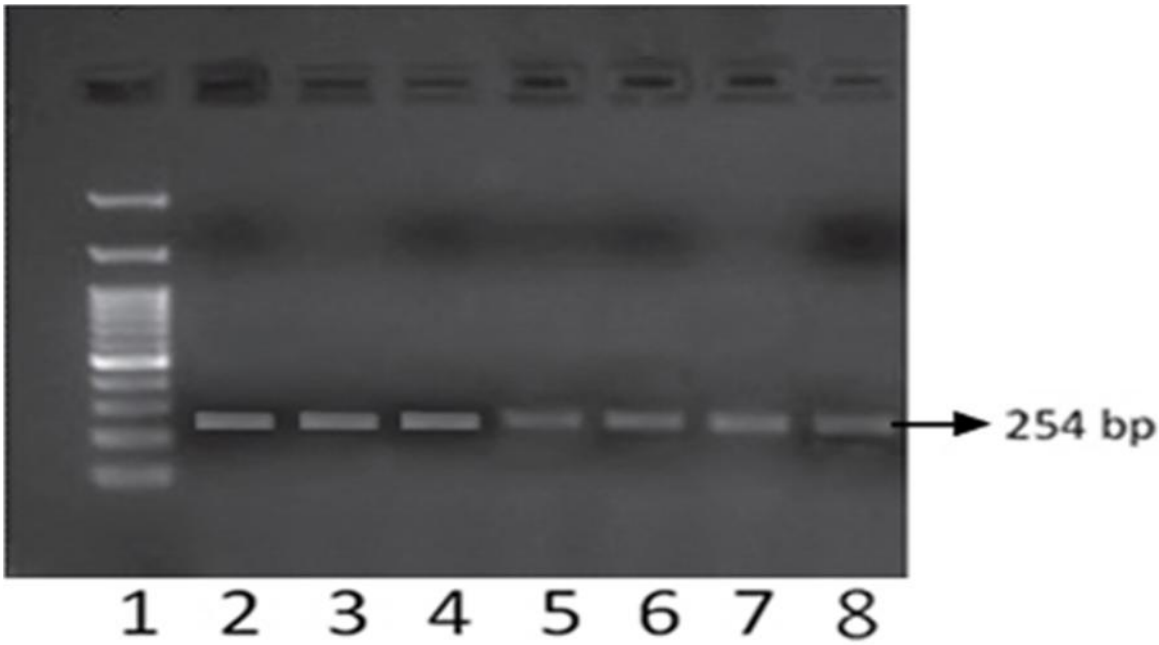


Figure 3.3: Representative PCR products of TP53 gene exon 7 in 2% agarose gel

3.2.4 Restriction Digestion of the PCR Product ofTP53 exon 7Gene

Restriction digestion enzyme *HaeIII* digested the 254 bp PCR product. The digested PCR product was evaluated by running them on 2% agarose gel. As shown in **Figure 3.4**, the homozygous wild-type (GG) genotype generated two bands of 92 bp and 66 bp (lane 8) upon restriction digestion; the heterozygous mutant genotype (GT) produced three bands of 158 bp, 92 bp and 66 bp (lane 2, 4 6, 7 and 9). In comparison, the homozygous mutant genotype (TT) produced of single undigested 158 bp fragment (lane 5). For visualizing restriction digestion enzyme products 50 bp ladder was used.

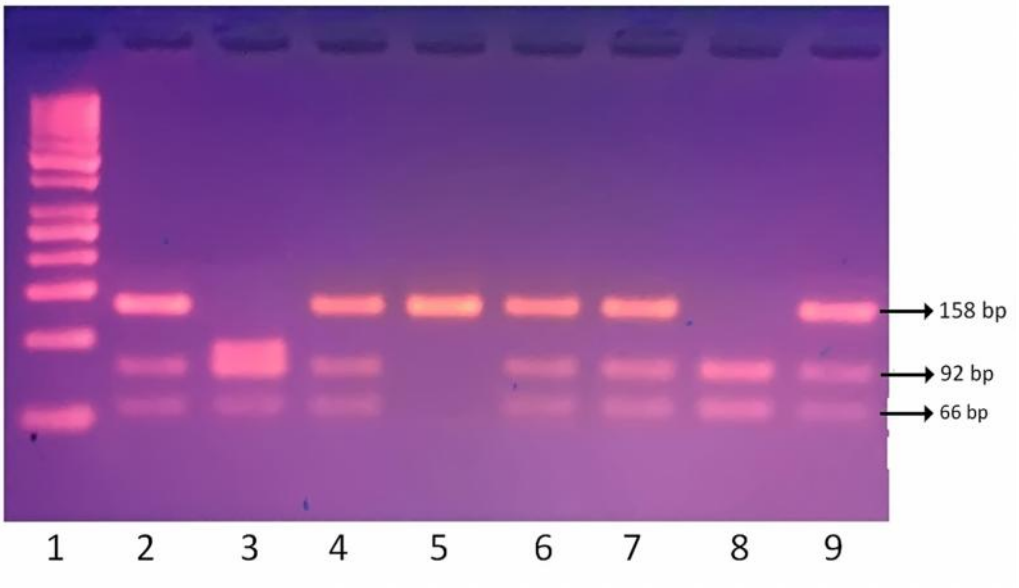


Figure 3.4: Representative digestion products of TP53 exon 7 PCR products in 2% agarose gel.

3.3 Frequency Distribution of TP53 Genotypes of Codon 72 and 249 and Risk of Hepatocellular carcinoma

Frequency distribution of TP53gene codon 72 and 249 in exon 4 and exon 7 genotype in patient and control was shown in **Table 3.2.** Among 119 hepatocellular carcinoma patients, the homozygous wild type was 20.17%, the homozygous mutant was 49.58%, and the heterozygous mutant was 30.25% compared to control at codon 72 in exon 4. Homozygous mutant variants (CC) and heterozygous mutant (GC) of TP53 at 72 position were significantly associated with liver cancer risk when compared with the controls (OR=0.17; 95% CI=0.09-0.31; $p< 0.001$ and OR =0.11; 95% CI =0.06-0.23; p<0.001) when GG was considered as the reference group. Frequency distribution of TP53 gene codon 249 in exon 7 genotype in patient and controlsamong 119 patients of hepatocellular carcinoma, homozygous wild type was 48.74%, theheterozygous mutant was 42.86 %, the homozygous mutant was 8.40% compared to control.

Heterozygous mutant (GT) of TP53 at 249 positions were significantly associated with liver cancer risk when compared with the controls (OR=0.19; 95% CI=0.10-0.35; $p<0.0001$), but there was no association found with homozygous mutant variants (TT) (OR=0.43; 95% CI=0.18-1.17; $p=0.124$) when GG was considered as the reference group. Comparison between observed and expected genotypic frequencies in **Tables 3.2** and Hardy Weinberg Equilibrium isshown in **3.3 and 3.4.**

Table 3.2: The allele and genotypic distribution of TP53 gene codons 72 and 249 in the study subject

Gene	Exon	Genotype	Patient (n=119) (n,%)	Control (n=150) (n, %)	OR (95% CI)	*p* value
TP53	Exon 4	GG/GG	24 (20.17)	95 (63.33)	1 (Ref.)	-
		GG/GC	59 (49.58)	39 (26.00)	0.167 (0.09-0.31)	< 0.001
		GG/CC	36 (30.25)	16 (10.67)	0.112 (0.06-0.23)	< 0.001
	Exon 7	GG/GG	58 (48.74)	121 (80.67)	1 (Ref.)	-
		GG/GT	51 (42.86)	20 (13.33)	0.19 (0.10-0.35)	<0.0001
		GG/TT	10 (8.40)	09 (6.00)	0.43 (0.18-1.17)	NS
	Exon4	GG allele frequency	0.45	0.76	1 (Ref.)	-
		CC allele frequency	0.55	0.24	0.26 (0.14-0.47)	<0.0001
	Exon 7	GG allele frequency	0.53	0.87	1 (Ref.)	-
		TT allele frequency	0.47	0.13	0.21 (0.11-0.41)	<0.0001

Results are expressed as a number (percentage). Fisher's test was performed to calculate the statistical significance. A $p<0.05$ was taken as the level of significance.

Table 3.3: Genotypic frequency distribution of TP53 gene codon 72 (exon 4) in the study subject according to Hardy Weinberg Equilibrium

Variables	Patient (n=119)			Control (n=150)		
Genotype	GG/GG	GG/GC	GG/CC	GG/GG	GG/GC	GG/CC
Observed Value	24	59	36	95	39	16
Expected Value	24.05	58.90	36.05	87.40	54.20	8.40
***X*2 value**		0.0004			11.79	

Table 3.4: Genotypic frequency distribution of TP53 gene codon 249 (exon 7) in the study subject according to Hardy Weinberg Equilibrium

Variables	Patient (n=119)			Control (n=150)		
Genotype	GG/GG	GG/GT	GG/TT	GG/GG	GG/GT	GG/TT
Observed Value	58	51	10	121	20	09
Expected Value	58.59	49.82	10.59	114.40	33.19	2.41
***X*2 value**		0.67			23.68	

3.4 TP53 Gene Codon 72 (exon 4) and Codon 249 (exon 7) Genotypes on Risk of HCC According to Gender

The frequencies of TP53 codon 72 genotypes between male and female liver cancer patients and controls are presented in **Table 3.5.** The frequency of CC was significantly higher in both male and female patient (OR=0.09, 95% CI=0.04-0.23, $p<0.0001$; OR=0.15, 95% CI=0.04-0.63, $p=0.156$ respectively) compared with GG genotype of control. There was no significant association in the frequency of GC in females, but in the males, a significant association was found (OR=0.12, 95% CI= 0.06-0.24, p<0.0001). A significant association was found between

gender and liver cancer risk based on the TP53 gene codon 72 genotypes in the Bangladeshi cohort. The frequency of TP53 codon 249 genotypes in male and female liver cancer patients and control are presented in **Table 3.5.** The frequency of GT and TT were significantly higherin male patient (OR=0.14, 95% CI=0.07-0.29, p<0.0001; OR=0.23, 95% CI=0.06-0.80, p=0.049 respectively) compared with GG genotype of control. There was no significant association in the frequency in females. There was a significant association between gender and liver cancer risk based on TP53 gene codon 249 genotypes in the Bangladeshi cohort.

Table 3.5: TP53 gene codon 72 (exon 4) and codon 249 (exon 7) genotypes on risk for HCC according to gender

Gene	Gender	Genotype	Patient (n=119)	Control (n=150)	Odd ratio (95% CI)	*p* value
TP53 Exon 4	Male	GG/GG	20	64	1(Ref.)	-
		GG/GC	48	18	0.12 (0.06-0.24)	<0.0001
		GG/CC	31	09	0.09 (0.04-0.23)	<0.0001
	Female	GG/GG	04	31	1(Ref)	-
		GG/GC	10	21	0.27(0.09-1.03)	NS
		GG/CC	06	07	0.15 (0.04-0.63)	0.0156
TP53 Exon 7	Male	GG/GG	47	78	1 (Ref.)	-
		GG/GT	44	10	0.14 (0.07-0.29)	<0.0001
		GG/TT	08	03	0.23(0.06-0.80)	0.049
	Female	GG/GG	11	43	1 (Ref.)	-
		GG/GT	07	10	0.37 (0.13-1.15)	NS
		GG/TT	02	06	0.77 (0.15-4.17)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. p<0.05 was taken as the level of significance

3.5 TP53 Codone72 (exon 4) and Codon 249 (exon 7) Genotypes on Risk of HCC According to Smoking Status

Table 3.6 represents the frequency of TP53 codon 72 and codon 249 genotypes with a possible risk of liver cancer and control according to smoking. In the smoker's case, the frequency of genotypes was non-significant compared to the control. Overall, there was found no significant association between smoking and liver cancer risk based on TP53 codon 72 and codon 249 genotypes in the Bangladeshi population.

Table 3.6: TP53 codone72 (exon 4) and codon 249 (exon 7) genotypes on risk of HCC according to smoking status

Gene	Smoking Status	Genotype	Patient (n=119)	Control (n=150)	OR (95% CI)	p value
T53 Exon 4	Smoker	GG/GG	15	03	1(Ref.)	-
		GG/GC	34	02	0.29 (0.05-1.60)	NS
		GG/CC	16	01	0.31 (0.23-2.37)	NS
	Nonsmoker	GG/GG	09	89	1 (Ref)	-
		GG/GC	24	39	0.16(0.07-0.39)	<0.0001
		GG/CC	21	16	0.08(0.03-0.20)	<0.0001
T53 Exon 7	Smoker	GG/GG	32	06	1 (Ref.)	-
		GG/GT	28	00	0.00 (0.00-0.94)	NS
		GG/TT	05	00	0.00 (0.00-5.29)	NS
	Nonsmoker	GG/GG	26	121	1 (Ref.)	-
		GG/GT	23	20	0.19 (0.09-0.40)	<0.0001
		GG/TT	05	09	0.39 (0.12-1.11)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. A $p<0.05$ was taken as a level of significance.

3.6 TP53 Gene Codon 72 (exon 4) and Codon 249 (exon 7) Genotypes on Risk of HCC with a Family History of Cancer

Table3.7 represents the frequency of TP53 codon 72 and codon 249 genotypes with a possible risk of liver cancer and control according to familial cancer history. There was no significant association between patients with a family history of cancer compared with control groups.

Table 3.7: TP53 gene codon 72 (exon 4) and codon 249 (exon 7) genotype on risk of HCC according to family history of cancer

Gene	Family history of cancer	Genotype	Patient (n=119)	Control (n=150)	OR (95% CI)	p value
T53 Exon 4	Yes	GG/GC	05	03	1 (Ref.)	-
		GG/GC	12	03	0.42 (0.08-2.37)	NS
		GG/CC	08	01	0.21 (0.15-1.91	NS
	No	GG/GC	19	92	1 (Ref.)	-
		GG/GC	46	36	0.16 (0.08-0.31)	<0.0001
		GG/CC	29	15	0.11 (0.05-0.23)	<0.0001
T53 Exon 7	Yes	GG/GC	12	06	1 (Ref.)	-
		GG/GT	11	01	0.18 (0.02-1.68)	NS
		GG/TT	02	09	9.00 (1.59-47.58)	NS
	No	GG/GC	46	92	1 (Ref.)	-
		GG/GT	40	03	0.04 (0.01-0.12)	<0.0001
		GG/TT	08	36	2.25 (1.01-4.93)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. A $p<0.05$ was taken as a level of significance.

3.7 TP53 Gene Codon72 (exon 4) and Codon 249 (exon 7) Genotypes on Risk of HCC with HBV

Table 3.8 represents the frequency of TP53 genotype among liver cancer and HBV infection. There was no significant association found with HBV in this study.

Table 3.8: TP53 gene codon 72 (exon 4) and codon 249 (exon 7) genotype on risk of HCC with HBV

Gene	Genotype	HBV Positive (n=87)	HBV Negative (n= 32)	Odds ratio (95% CI)	*p* value
T53 Exon 4	GG/GG	20	04	1(Ref.)	-
	GG/GC	43	15	1.74 (0.55-5.29)	NS
	GG/CC	24	17	2.71 (0.77-8.43)	NS
T53 Exon 7	GG/GG	41	16	1(Ref.)	-
	GG/GT	39	12	0.79 (0.35-1.89)	NS
	GG/TT	07	04	1.46 (0.43-5.85)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. A $p<0.05$ was taken as a level of significance.

3.8 TP53 Gene codon 72 (exon 4) andTP53 Gene Codon 72 (exon 4) and Codon 249 (exon 7) Genotypes on Risk of HCC with HCV

Table 3.9 represents the frequency of TP53 genotype among liver cancer and HCV infection. There was no significant association found with HCV in this study.

Table 3.9: TP53 gene codon 72 (exon 4) and codon 249 (exon 7) genotype on Risk of HCC with HCV

Gene	Genotype	HCV Positive (n=09)	HCV Negative (n= 110)	Odds ratio (95% CI)	*p* value
T53 Exon 4	GG/GG	01	23	1(Ref.)	-
	GG/GC	04	54	0.59 (0.05-3.93)	NS
	GG/CC	04	33	0.36 (0.03-2.47)	NS
T53 Exon 7	GG/GG	02	54	1(Ref.)	-
	GG/GT	06	45	0.28 (0.84-18.03)	NS
	GG/TT	01	11	0.41 (0.05-6.40)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. $p<0.05$ was taken as the level of significance

3.9 BCLC Staging in Percentage in the Case Subject

According to BCLC staging, there was no patient in very early (0) but 31.09% in staging A, 37.82% in staging B, 29.41% in staging C, and 1.68% in staging D among the study case subjects.

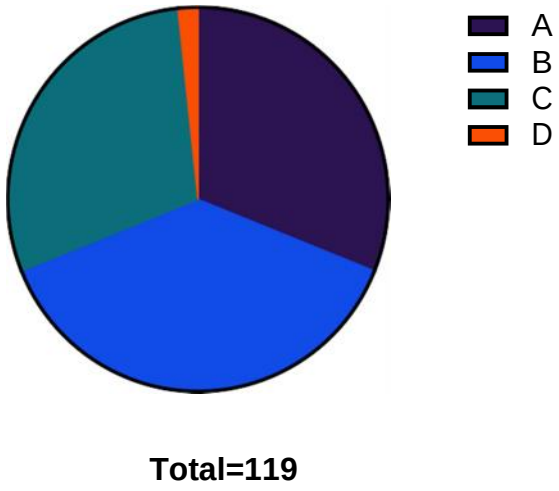


Figure 3.5: Frequency distribution of BCLC staging in case subjects

3.10 Frequency Distribution of HBV Positive and Negative in Case Subject

Among the total study subjects (n= 119), 73.11% (n=87) subjects was HBsAg positive, and 26.89% (n=32) was negative.

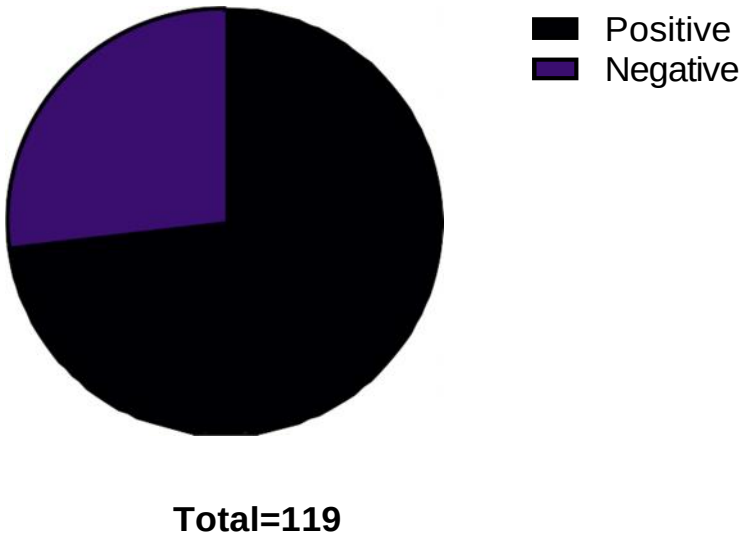


Figure 3.6: Frequency distribution of HBV positive and negative in case subject

3.11 TP53 Codon 72 (exon 4) and Codon 249 (exon 7) Genotype on Risk of Hepato-cellular Cancer According to HBV Infected and Non-infected with Gender

Table 3.10 represents the frequency of TP53 genotype among liver cancer and HBV infected and non-infected. There was no significant association with hepatitis B virus (HBV) infection in this study.

Table 3.10: TP53 codon 72 (exon 4) and codon 249 (exon 7) genotype on risk of hepatocellular cancer according to HBV infected and non-infected with gender

Gene	Gender	Genotype	HBV Positive (n=87)	HBV Negative (n=32)	Odds ratio (95% CI)	*p* value
T53 Exon 4	Male	GG/GG	17	03	1 (Ref.)	-
		GG/GC	33	11	1.69(0.47-6.18)	NS
		GG/CC	24	11	3.12(0.82-11.56)	NS
	Female	GG/GG	03	01	1 (Ref.)	-
		GG/GC	06	04	2.00(0.21-31.72)	NS
		GG/CC	04	02	1.50(0.12-28.22)	NS
T53 Exon 7	Male	GG/GG	35	12	1(Ref.)	-
		GG/GT	33	11	1.03 (0.39-2.64)	NS
		GG/TT	06	02	0.97(0.18-4.93)	NS
	Female	GG/GG	07	04	1 (Ref.)	-
		GG/GT	06	01	0.29 (0.02-3.24)	NS
		GG/TT	00	02	8.33 (0.32-215.70)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. $p<0.05$ was taken as the level of significance

3.12 Frequency Distribution of HCV Positive and Negative in Case Subject

Among the total study subjects (n= 119), 7.56% (n=09) subjects was HCV positive, and 92.44% (n=110) was negative.

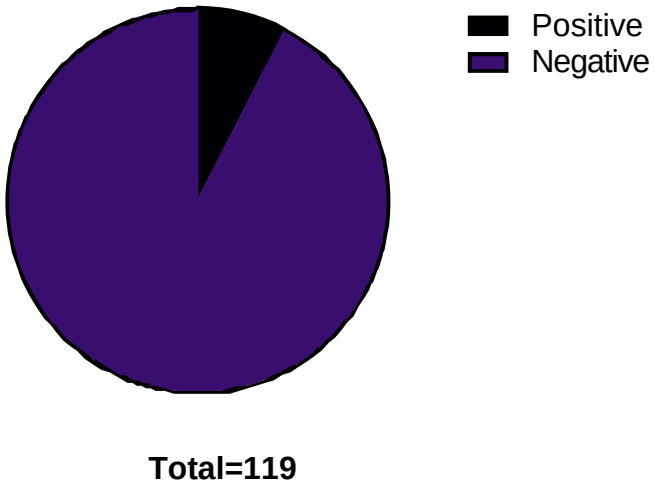


Figure 3.7: Frequency distribution of HCV positive and negative in case subject

3.13 TP53 Codon 72 (exon 4) and Codon 249 (exon 7) Genotype on Risk of Hepato-cellular Cancer According to HCV Infected and Non-infected with Gender

Table 3.11 represents the frequency of TP53 genotype among liver cancer and HCV infected and non-infected. There was no significant association with HCV in this study subjects.

Table 3.11: TP53 codon 72 (exon 4) and codon 249 (exon 7) genotype on risk of hepatocellular cancer according to HCV infected & non-infected with gender

Gene	Gender	Genotype	HCV Positive (n=09)	HCV Negative (n=110)	Odds ratio (95% CI)	*p* value
T53 Exon 4	Male	GG/GG	00	20	1(Ref.)	-
		GG/GC	03	45	0.00(0.00-2.70)	NS
		GG/CC	03	28	0.00 (0.00-1.75)	NS
	Female	GG/GG	01	03	1 (Ref.)	-
		GG/GC	01	09	3.00 (0.12-62.29)	NS
		GG/CC	01	05	1.66 (0.07-37.27)	NS
T53 Exon 7	Male	GG/GG	00	47	1 (Ref.)	-
		GG/GT	05	39	0.00 (0.00-61.00)	NS
		GG/TT	01	07	0.00 (0.00-1.53)	NS
	Female	GG/GG	02	09	1(Ref.)	-
		GG/GT	01	06	1.33 (0.13-22.21)	NS
		GG/TT	00	02	∞ (0.07- ∞)	NS

Results are expressed as a number. Fisher's test was performed to calculate the statistical significance. $p<0.05$ was taken as the level of significance

3.14 Clinical Characteristics in the Study Subjects

A biochemical marker taken to confirm hepatocellular carcinoma was compared with controls subjects. The biochemical parameters in this study subjects were serum albumin, serum ALT, serum ALP, Serum AFP, and plasma Prothrombin Time (PT), that presented in **Table 3.12.** The mean serum albumin level in liver cancer patients and controls was 2.88±0.61 g/dL and 3.98±0.57 g/dL, respectively. In liver cancer patients, serum Albumin level was significantly lower than in controls ($p<0.05$).

In HCC patients and control groups, serum alkaline phosphatase (S. ALP) mean level was 265.10±210.80 IU/L and 35±35.04 IU/L, respectively. In HCC patients, serum alkaline phosphatase level was significantly higher than in control groups ($p<0.05$).

The mean level of serum Alanine Aminotransferase (S.ALT) was 78.25±96.35 IU/L and 327±13.82 IU/L in HCC patients and control groups, respectively and there was strongly higher compared with controls. p-value was <0.05.

Serum Alphafeto Protein (S.AFP) was another parameter compared with HCC patients and control groups. The mean was 44601±66659 ng/ml and 2.36±0.95 ng/ml, respectively. This parameter also found a significant association, and the p-value was <0.05.

The mean Prothrombin Time (PT) level in the HCC patients and control groups was 15.51±3.42 Sec. and 12.09±2.87 Sec. respectively. It was significantly higher than in the control groups, and the p-value was <0.05.

Table 3.12: Clinical Characteristics among the total study subjects

Biochemical Marker	Study Subjects	Number	Mean	Std. Deviation	Std. Error of Mean	p-value
S. Albumin	Case	119	2.88	0.606	0.056	<0.05
	Control	150	3.98	0.567	0.046	
S. ALP	Case	119	265.10	210.80	19.33	<0.05
	Control	150	83.22	35.04	2.86	
S. ALT	Case	119	78.25	96.35	8.83	<0.05
	Control	150	27.16	13.82	1.13	
S. AFP	Case	119	44601	66659	6111	<0.05
	Control	150	2.36	0.95	0.08	
PT	Case	119	15.51	3.42	0.28	<0.05
	Control	150	12.09	2.87	0.14	

3.15 Frequency of Serum Albumin (S.ALB) Level between Liver Cancer Patients and Control in Study

The frequency distribution of S.ALB levels in HCC patients and the control group was represented in **Figure 3.8**. Serum Albumin level was significantly lowers in HCC patients than incontrol groups.

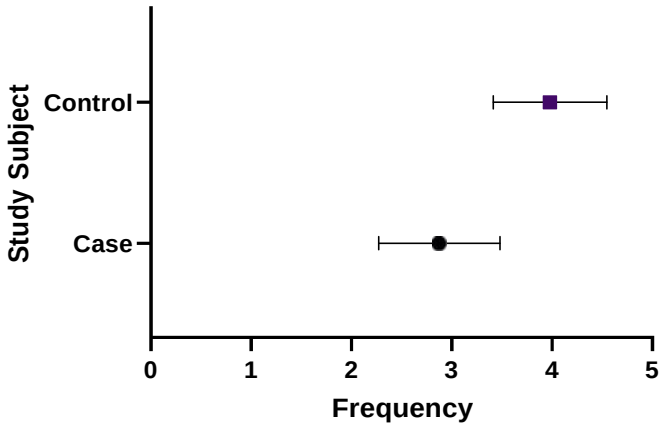


Figure 3.8: Frequency of serum albumin (S.ALB) level between liver cancer and healthy subjects

3.16 Frequency of Serum Alkaline Phosphatase (S.ALP) Level between Liver Cancer Patients and Control in the study.

The frequency distribution of S.ALP levels in the liver cancer patients and healthy groups are represented in **Figure 3.9**. Serum alkaline phosphatase was significantly higher in HCC groups than in control groups.

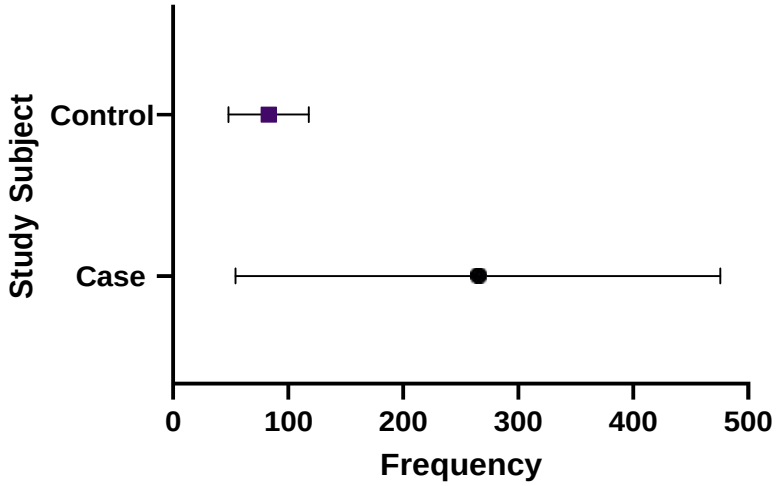


Figure 3.9: Frequency of serum alkaline phosphatase (S.ALP) level between liver cancer and healthy subjects

3.17 Frequency of Serum Alanine Aminotransferase (S.ALT) Level between Liver Cancer Patients and Control in the study

The frequency distribution of S.ALT levels in the liver cancer patients and healthy groups are represented in **Figure 3.10**. Serum Alanine Aminotransferase was significantly higher in HCC patients than in healthy control groups.

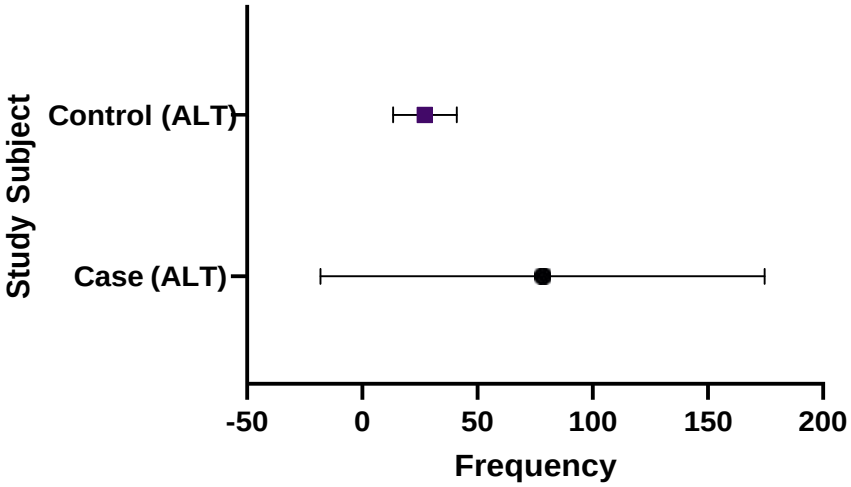


Figure 3.10: Frequency of serum alanine aminotransferase (S.ALT) level between liver cancer and healthy subjects

3.18 Frequency of Serum Alphafeto Protein (S.AFP) Level between Liver Cancer Patients and Control in the study

The frequency distribution of S.AFP levels in the HCC patients and healthy control groups are represented in **Figure 3.11**. Serum Alphafeto Protein was significantly lower in the healthy groupsthan in HCC patients.

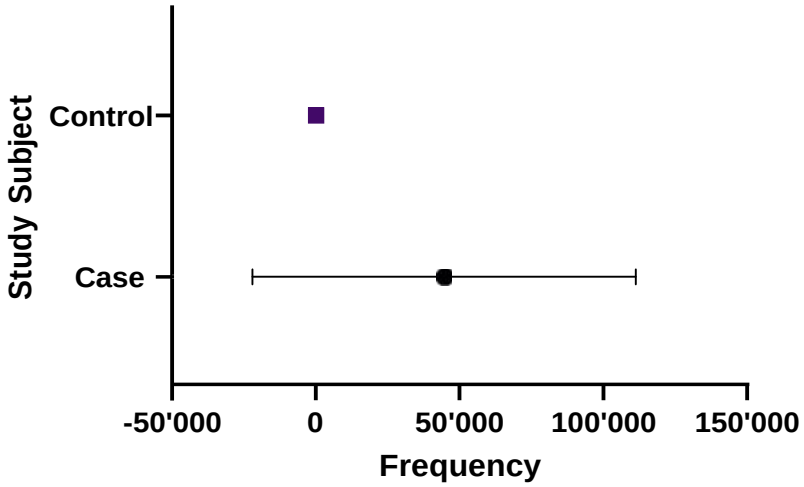


Figure 3.11: Frequency of serum alpha feta protein (S.AFP) level between liver cancer and healthy subjects

3.19 Frequency of Plasma Prothrombin Time (PT) Level between Liver Cancer Patients and Control in the study.

The frequency distribution of PT levels in the liver cancer patients and healthy groups are represented in **Figure 3.12**. Plasma Prothrombin Time was significantly higher in the HCC patients than in healthy groups.

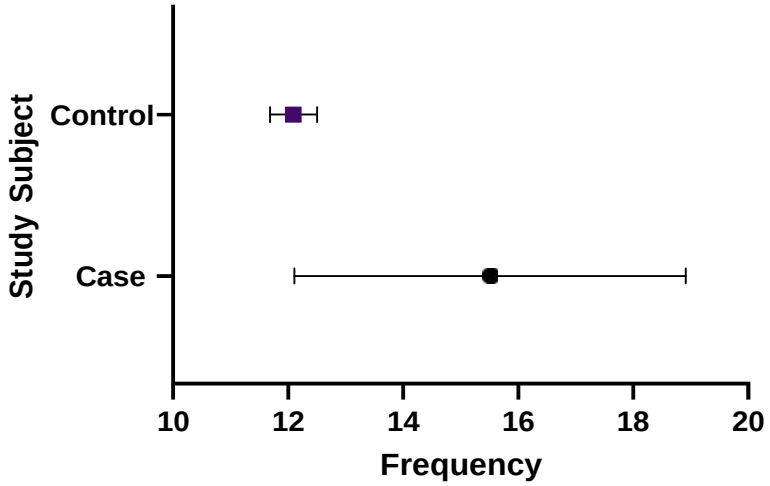


Figure 3.12: Frequency of plasma prothrombin time (PT) level between liver cancer and healthy subjects

Discussion and Conclusion

4 Discussion and Conclusion

4.1 Discussion

In this study, GC of exon 4, an SNP (single nucleotide polymorphism) in the TP53 gene, has been investigated for its probable activity in the progression of HCC patients. The outcome of the current study indicated a significant association between the p53 codon 72 polymorphism and perceptivity to HCC in a Bangladeshi population GC: OR= 0.17 and 95% CI, 0.09 –0.31; CC: OR= 0.11 and 95% CI, 0.06 –0.23; where p-vale was <0.0001 in each case. The TP53- CC72 variant bearing chance was OR = 0.26 and 95% CI, 0.14-0.47, P <0.0001 and $\chi 2$ = 0.0004. Among the liver cancer patients, the repetition for the GG, GC, and CC genotypes were 20.17%, 49.58%, and 30.25%, respectively, whereas, in the healthy people, these frequencies were 63.33%, 26.00%, and 10.67%, respectively ($\chi 2$ = 11.79). In the liver cancer patients the allelic frequencies (TP53-GG72 variant, 0.45; TP53-CC72 variant, 0.55) were significantly distinct from the controls (TP53-GG72 variant, 0.67; TP53-CC72 variant, 0.24). These results support previous data (Zhuet al., Afifiet al., Kang et al.) [163-166]. This indicates an association between the TP53GG72GC polymorphism and raise the risk for HCC.

This study had found the GG72CC was significantly higher in both male and female patients (OR= 0.09, 95%CI= 0.04-0.23, p<0.0001; OR=0.15, 95% CI=0.04-0.63, $p=0$.156 respectively) compared with GG genotype of control. But there was no significant association in the frequency of GC in females, whereas, in male patients, a significant association was found (OR= 0.12, 95% CI= 0.06- 0.24, p<0.0001) in the TP53 gene at codon 72 genotype in Bangladeshi cohort. No previous studies have correlated TP53 genotypes and hepatocellular carcinoma risk with gender.

Analyses were performed with smoking status between case and control and found GC: OR=0.29; 95% CI, 0.05-1.60 and CC: OR= 0.31; 95% CI, 0.23-2.37 where p-value was 0.3193 and0.6026 respectively in smoker but GC: OR=0.16; 95% CI, 0.07-0.39 and CC:OR= 0.08; 95%CI, 0.03-0.20 where p-value was significant (P<0.0001) in nonsmoker. Alcohol consumption was insignificant among this study subject. It may be living status and social or

religious value in this cohort, or it may also be a factor of sample size. However, those factors may increase the risk for HCC. Still, there was no correlation found in TP53 codon 72polymorphismandthe same study was informed by Zhang et al [167]; they did not find any association between the TP53 codon 72 polymorphism and risk of HCC in Guangxi, China. Still, Mu et al. [168] found a correlation between the TP53 codon 72polymorphism and perceptivity to HCC in Shandong, China. Zhu et al. [169] did not observe any correlation between TP53 codon72 polymorphism and the risk of HCC, which data concerned my results.

Genotyping variant frequency was analyzed with a family history of liver cancer. Still, no association was found among the study subjects, and no previous studies correlated TP53 SNPs and the risk of hepatocellular carcinoma.

Furthermore, TP53 codon 72 genotype also was analyzed with HBV and HCV infection, but no significant association was found in this study subjects. HBV infection (GG/GC): OR=1.74; 95% CI, 0.55-5.29 and (GG/CC): OR=2.7, 95% CI, 0.77-8.43 where p-value was 0.5658 and 0.1499 respectively. In HCV infection (GG/GC): OR= 0.59; 95% CI, 0.05-3.93 and (GG/CC): OR=0.36; 95% CI, 0.03-2.47 and p-value was >0.05 in each genotype. However, the present observation was associated with results reported by Zhang et al [167], Jiajing Cai et al. [170], while Mu et al, [168] noticed a correlation between the TP53 codon polymorphism perceptivity to HCC. Zhu et al. [168] didn't observe any correlation between TP53 codon 72 polymorphism and risk of HCC. Still, they proved that the pro allele increased the risk of HCC in negative hepatitis B virus subjects and extended an interdepended effect on the risk of HCC. Ezzikouri et al. [171] reported that TP53 codon 72 polymorphism codon 72 polymorphism granted a significantly increased riskof HCC in a Moroccan population. Sümbül et al. [172] recommended the GG/CC (Pro/Pro) homozygote may be a genetic susceptivity factor for HCC in the Turkish population, especially in males and HBV-infected patients. Yoon et al. [173] suggested that the pro allele was linked with an elevated risk of HCC in hepatitis B virus (HBV)-infected patients in a Korean population. In addition, Di Vuolo et al. [174] suggested that this polymorphism was not an effective risk factor for developing HCC in Italian patients. This dissimilarity among the studies because of the topographical location of the study subjects, the sample size, analytical methods, confounding factors, and genotyping assays.

In this study, genetic polymorphism at codon249 in exon 7 was analyzed, and found the frequency distribution of genotype inpatient and controls among 119 patients of hepatocellular carcinoma; the homozygous wild type was 48.74%, the heterozygous mutant was 42.86 %, the homozygous mutant was 8.40% compared to control. Heterozygous mutant (GT) of TP53 at249 positions were significantly associated with liver cancer risk when compared with the controls (OR=0.19; 95% CI=0.10 – 0.35; p<0.0001), whereas no association was found with homozygous mutant variants (TT) (OR=0.43; 95% CI=0.18-1.17; *p*=0.124) when GG was considered as a reference group. The TP53-TT249 variant carriage risk was OR = 0.21and 95% CI, 0.11-0.41, *P* <0.0001 and $\chi 2 = 0.67$. Among the HCC patients, the frequencies for the GG, GT, and TT genotypes were 48.74%, 42.86%, and 8.40%, respectively, whereas, in the healthy people, these frequencies were 80.67%, 13.33%, and 6.00%, respectively ($\chi 2 = 23.68$). The allelic frequencies in the HCC patients (TP53-GG249 variant, 0.70; TP53- TT249 variant, 0.30) were significantly different from those in the controls (TP53-GG249 variant, 0.87; TP53-TT249 variant, 0.13). This result indicated that GT was associated significantly with hepatocellular carcinoma in the Bangladeshi population. At the same time, TT did not find an association with comparing GG in this cohort. These results agreed with another study done by Mona et al. in Egypt [175], who noted that in the control group, 20 subjects had no pointmutation at codon 249 of exon 7, while in the HCC group, out of 30 patients, 4 patients (13.3%) showed a point mutation at codon 249 of exon 7. TP53 gene generally exhibits mutational hotspots of codon 249 in HCC, *i.e.*, particular G to T mutation commonly occurs in the third baseof codon 249. It has also been reported that this unique mutation is involved in the differentiation,carcinogenesis, and metastasis of HCC [176-181].

From this study gender-wise genotype was analyzed and found genotype of GT and TT were significantly higher in a male patient (OR=0.14, 95% CI =0.07-0.029, p<0.0001; OR=0.23,95% CI= 0.06-0.08, p=0.049respectively) compared with GG genotype of control. There was no significant association in the frequency in females. No previous studies have correlated TP53 genotypes at codon 249 and hepatocellular carcinoma risk with gender.

Similarly, TP53 gene codon 249 genotype was analyzed with smoking status, family cancer history, and alcohol consumption history, but there was no associated risk with HCC. No correlated data had been found in the previous study.

In this study, 87 patients were HBV infected, and 09 patients were HCV infected. HBV and HCV infection correlation was also analyzed for TP53 codon 249 in exon 7, but no significant association was found in this study subjects. HBV infection (GG/GT): OR= 0.79; 95% CI, 0.35-1.89 and (GG/TT): OR= 1.46, 95% CI, 0.43-5.85 where p-value was 0.6631and 0.7194respectively. In HCV infection (GG/GT): OR=0.28; 95% CI, 0.84-18.03 and (GG/TT): OR=0.41; 95% CI, 0.05-6.40 and p-value was >0.05 in each genotype. There is no association found in the Bangladeshi cohort. But Hesham Gamal et al. [182] reported that HBV and HCV were associated with mutation codon 249 in exon 7. Genotype also was analyzed according to gender with HBV and HCV infection, but there was no significant association.

4.1 Conclusion

This case-control study confirmed that the TP53 GC and CC genotypes at codon 72 in exon 4 were significantly linked with HCC in the Bangladeshi population. This study also observed TP53 GT genotype at codon 249 in exon 7 was significantly related to the risk of developing hepatocellular carcinoma in the Bangladeshi cohort. The result of our study was not following some of the study results mentioned above for different populations might be due to ethnic and racial differences.

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Appendix

Definition of Major terms

Polymorphism: Polymorphism occurs whenever the generation of a species have two or more distinctly different morphs or variants, usually known as alternative phenotypes.

Genotype: A cell's genotype, and hence an organism's or individual's genetic composition, defines one of its features (phenotype). In 1903, the word was invented by Wilhelm Johannsen, a Danish botanist, plant physiologist, and geneticist.

Phenotype: A phenotype is a collection of an organism's observable qualities, such as its appearance, maturation, biological or physiological features, behavior patterns, and behavior outcomes. A phenotype seems to be the result of the expression about an organism's genetic code, its genotype, and even the impact of environmental factors and their interactions.

Allele: A variant form of a gene is known as an allele. Diverse alleles sometimes can generate differing phenotypic features, such as different coloring.

Mutation: A mutation occurs when the nucleotide sequence of an organism's genome, virus genome, extrachromosomal DNA, or other genetic components is permanently altered.

SNP: A single-nucleotide polymorphism (SNP) is an alteration in a single nucleotide that occurs at a specific location in the genome, and each variation is present to some degree throughout a population (e.g. > 1%)

PCR: Polymerase chain reaction (PCR) is a molecular biology technique for amplifying a single copy or only few clones of a DNA segment over multiple orders of magnitude, resulting in thousands to millions of copies of a specific DNA sequence.

RFLP: Restriction fragment length polymorphism, or RFLP, is a molecular biology technique that takes use of variations in homologous DNA sequences. It refers to the variation in homologous DNA molecules from various positions of restriction enzyme sites, as well as a related scientific methodology for illustrating these segments.

DNA: Deoxyribonucleic acid (DNA) is a thread-like chain of nucleotides that contains the genetic code information including all living beings and many viruses' formation, differentiation, operation, and multiplication.

Gel Electrophoresis: Gel electrophoresis is a method that uses duration and charge to segregate and analyse macromolecules (DNA, RNA, and proteins) and its fragments. It's utilized in clinical chemistry to split proteins via charge and/or length (IEF agarose, that is nearly duration variable), as well as in biochemistry and molecular biology to isolate a combined populace of DNA and RNA fragments by using period to estimate the dimensions of DNA and RNA fragments or to split proteins via rate.

PCR-CTPP: A new PCR method, PCR-CTPP (polymerase chain response with confronting -pair primers) turned into invented to genotype a pretty large variety of samples in a value-powerful and time-saving manner. On this method, allele-precise DNA products are amplified by way of applying as it should be designed -pair primers (4 primers) into an ordinary PCR tube.

Liver Cancer: A tumor in which the most cancers starts all through maturity in cells inside the liver. Additionally referred to as hepatocellular carcinoma or hepatoma. Primary liver cancer isn't like cancer that has metastasized (unfold) from every other vicinity in the body to the liver.

TP53: A gene that produces a protein that is positioned inside the nucleus of cells and is worried in cellular department and demise. Most cancers cells may grow and spread within the body as a result of mutations (changes) inside the TP53 gene. Many types of most cancers, as well as a genetic disorder termed Li-Fraumeni syndrome, have shown these modifications. The TP53 gene belongs to the tumor suppressor gene family. Also referred to as the tumor protein p53 gene and the p53 gene.

UV Transilluminator: In molecular biology labs, UV-trans illuminators are also used to visualize DNA (or RNA) which has been separated by electrophoresis on an agarose gel. The agarose gel is coloured with a fluorescent dye which binds to nucleic acid throughout or quickly during electrophoresis. When the colored gel is exposed to UVB light, the DNA/dye fluoresces and becomes visible.

Agarose: Agarose is a polymer derived primarily from red seaweed. It is a linear polymer composed of the agarobiose repeating unit, which is a disaccharide composed of D-galactose and 3,6-anhydro-L-galactopyranose. Agarose is extensively employed in molecular biology for electrophoresis separation of big molecules, particularly DNA.

Centrivap Concentrator: Entrivap benchtop vacuum concentrators are designed to unexpectedly pay attention a couple of small samples using centrifugal pressure vacuum and warmth.

Questionnaires

ID # Date:

Thesis Title:

Study of p53 gene polymorphism and risk of hepatocellular carcinoma: in a Bangladeshi

cohortPatient ID: Contact Number: Age: Sex:

Name of Patient:

1. How long you are suffering from liver diseases?

o < 6 months o > 6 months

2. Have any person who suffered or was suffering in liver disease in your family?

o Yes o No o Don't know

If yes,

- o Father
- o Mother
- o Brother
- o Sister

3. What is your occupation?

o Farmer o Housewife o Job o Day Laborer

o Factory Worker o Street Hawker o Driver

4. Have you any surgical history?

o Yes o No

If yes then –

- o General Surgery
- o Caesarean Section
- o Dental Surgery

5. Have you blood transfusion history?

- o Yes
- o No

6. Are you take alcohol or was taken?

- o Yes
- o No

7. Have you taken any steroidal medicine?

- o Yes
- o No

8. Are you smoker?

- o Yes
- o No
- o Smoking habit was but left now

9. Do you take a lot of pain killers?

- o Yes
- o No
- o Don't know

10. Have you taken a lot of antibiotic drugs?

- o Yes
- o No
- o Don't know

11. How long you suffered in jaundice? (H/O Jaundice)

- o < 6 months
- o > 6 months

12. Medical history:

a). Histological report of liver:

b). Grade &Stage of Cancer: (The Barcelona clinic liver cancer (BCLC) staging classification)

- stage 0 (very early stage)
- stage A (early stage)
- stage B (intermediate stage)
- stage C (advanced stage)
- stage D (end-stage disease)

c). Duration of Cancer:

d). Biochemical Test

i). S. Albumin:

ii). S. ALT level:

iii). S.ALP:

iv). Prothrombin Time:

vi). Alpha feto Protein level:

e). Virus conformation Test

i). HBSAg:

ii). Anti-HCV:

গবেষণা কার্যক্রমে অংশগ্রহণের সম্মতিপত্র

Title of Study:

Study of TP*53* gene polymorphism and risk of hepatocellular carcinoma: in a Bangladeshi cohort

Principal investigator: Md. Abdur Rahman, M.Phil Researcher, Department of Biochemistry & Molecular Biology, Dhaka University, Dhaka.

১. **এই গবেষণা সম্পর্কে কি সাধারণ তথ্য জানা উচিৎ?**

আপনাকে এই গবেষণায় অংশগ্রহণ করতে বলা হচ্ছে। আপনি এই গবেষণায় স্বেচ্ছায় অংশগ্রহণ করতেও পারেন বা নাও করতে পারেন অথবা আপনি গবেষণা থেকে যে কোন সময়ে যে কোন কারণে চলেও যেতে পারেন। এই গবেষণার কিছু ঝুকি আছে। এই গবেষণার বিস্তারিত নিম্নে বর্ণনা করা হলো। এটা গুরুত্বপূর্ণ যে আপনি নিম্নে উল্লেখিত তথ্যসমূহ জেনে ও বুঝে এই গবেষণায় অংশগ্রহণের সিদ্ধান্ত নিতে পারবেন। আপনাকে এই সম্মতিপত্রের একটি কপি দেয়া হবে। আপনি এই গবেষণাকারী এবং তাকে সাহায্যকারী সদস্যদের নাম জানতে পারবেন এবং এই গবেষণা সম্পর্কে যে কোন সময়ে যে কোন তথ্য জানতে পারবেন।

২. **এই গবেষণার উদ্দেশ্য কি?**

এই গবেষণার উদ্দেশ্য হলো TP*53* gene polymorphism and risk of hepatocellular carcinoma: in a Bangladeshi cohort কে পরীক্ষা করে দেখা। আমরা আপনাকে এই গবেষণায় অংশগ্রহণ করার জন্য বলছি কারণ আপনি একজন লিভার ক্যান্সার আক্রন্ত রোগী। আমরা আপনার TP53 Gene Polymorphism নির্ণয় করব। যখন গবেষণা শেষ হবে এই গবেষণা ব্যাখ্যা করতে সাহায্য করবে কেন এবং কোন Polymorphic Gene liver Cancer এর জন্য দায়ী এবং ভবিষৎতে এ রোগের চিকিৎসা করতে সাহায্য করবে।

৩. **এই গবেষণায় অংশগ্রহণ না করার কোন কারণ আছে কি?**

আপনি এই গবেষণায় অংশগ্রণ নাও করতে পারেন যদি আপনি আপনার নাড়ীর রক্ত দিতে এবং গবেষণার সঙ্গে সংশিষ্ট প্রশ্ন পত্রের উত্তর দিতে অনিচ্ছুক হন।

৪. **কত লোক এই গবেষণায় অন্তর্ভূক্ত হবে?**

এই গবেষণায় অংশগ্রহণকারীগণ বাংলাদেশ থেকে নিযুক্ত হবেন। যদি আপনি অংশগ্রহণ করার সিদ্ধান্ত নেন, আপনি হবেন এই গবেষণায় সম্ভাব্য ২৭০ জন অংশগ্রহণকারীর ১ জন।

৫. গবেষণায়অংশ গ্রহণ করলে কি হবে?

সম্মতি পত্র স্বাক্ষর করার পর ১টি প্রশ্নপত্র পূরণ করা হবে ও আপনার আর্থণ্ডসামাজিক অবস্থা চিকিৎসার ইতিবৃত্ত, সাম্প্রতিক চিকিৎসা এবং ধুমপান সম্পর্কে। তা ছাড়া আপনার শিরা থেকে ১টি সূচের সাহায্যে ৬ মিলি মিটার রক্তের নমুণা সংগ্রহ করা হবে।

৬. গবেষণা থেকে সম্ভাব্য কি সুবিধা পেতে পারেন?

এই গবেষণায় অংশগ্রহণে আপনি সরাসরি কোন সুবিধা পাবেন না। কিন্তু এই গবেষণার ফলাফল থেকে TP53 Gene Polymorphism সঙ্গে বাংলাদেশী Liver Cancer রোগীর সংশিষ্টতা সম্পর্কে জানা যাবে।

৭. গবেষণায় অংশগ্রহণের সম্ভাব্য ঝুঁকি/অসুবিধা কি?

রক্ত নেওয়ার সময় সামান্য অসুবিধা হতে পারে। অভিজ্ঞ নার্স রক্ত সংগ্রহ করবেন। রক্ত নেয়ার সময় শিরা ফুটা করার কারণে সামান্য ব্যাথা অনুভব করতে পারেন। কদাচিৎ শিরায় সূঁচ ফোটানোর পর ঐ জায়গা সামান্য কালচে হতে পারে। আপনি সাময়িক অচেতনতা অনুভব করতে পারেন। আপনাকে নিশ্চয়তা দেওয়া হবে যে, আপনি যদি কোন রকম স্বাস্থ্য ঝুকির মধ্যে হন তবে সকল প্রকার জরুরী স্বাস্থ্য সেবা প্রদান করা হবে। সংক্রমন প্রতিরোধের জন্য জীবানুমুক্ত পদ্ধতি ব্যবহার করা হবে।

সকল তথ্য পরীক্ষা নিরীক্ষা ও পর্যালোচনার জন্য কোডেড নমূণা বাবহার করে গবেষণায় অংশগ্রহণকারীর গোপণীয়তা রক্ষা করা হবে। সাক্ষাৎকার গ্রহণের সময় প্রত্যেক অংশগ্রহণকারীর এই কোড নির্ধারণ করা হবে। এই নমুনা এই প্রতিষ্ঠান বা অন্য কোন প্রতিষ্ঠানের কোন গবেষকের সাথে শেয়ার করা হতে পারে। এই গবেষণা এক সাথে অনেক জায়গায় করা হতে পারে। আপনার বাক্তিগত পরিচয়ের তথ্য অন্য গবেষকের নিকট দেয়া হবে না।

৮. যদি আপনি এই গবেষণায় অংশগ্রহণ করতে না চান তাহলে অন্য কি উপায় আছে?

আপনি এই গবেষণায় অংশগ্রহণ নাও করতে পারেন। অংশগ্রহণ না করার জন্য আপনার চিকিৎসার কোন প্রকার অসুবিধা হবে না এই নিশ্চয়তা দেওয়া হবে।

৯. কিভাবে আপনার গোপনীয়তা রক্ষা করা হবে?

আপনার সকল ব্যক্তিগত তথ্য ঢাকা বিশ্ববিদ্যালয়ের গবেষণা অফিসের গোপন জায়গায় রাখা হবে। আপনার নাম একটি কোড প্রতিস্থাপন করে আপনার কাছ থেকে নেওয়া সকল নমুনা গবেষণা কাজে ব্যবহার করা হবে। আপনার নামসহ ব্যক্তিগত তথ্য কোডে দিয়ে একটি নির্দিষ্ট কাগজের ফাইল তৈরী করে তথ্য সমূহ কম্পিউটারে এবং ফাইলটি কেবিনেটে তালাবদ্ধ করে রাখা হবে।

আপনার নাম গবেষণা দলের সদস্য অথবা বাংলাদেশের বাহিরে কারও নিকট বলা ও দেয়া হবে না। এই গবেষণায় অংশগ্রহণকারীদের কোন ব্যাক্তিগত তথ্য নির্দিষ্টভাবে কোন প্রতিবেদন বা প্রকাশনায় ব্যবহার করা হবে না। যদিও গবেষণার সকল কাগজপত্র গোপন রাখার সকল উদ্দ্যোগ নেয়া হবে তবে অনেক সময় সরকার অথবা রাষ্ট্র

আইনের প্রয়োজন অনুযায়ী ব্যক্তিগত তথ্যসহ গবেষণার কাগজপত্র প্রকাশ করা হতে পারে। এটা খুবই অস্বাভাবিক, যদি কখনও প্রকাশের প্রয়োজন হয় তবে এই গবেষণা কাজে অংশগ্রহণকৃত প্রতিষ্ঠান অনুমোদিত আইনের মাধ্যমে গোপণীয়তা রক্ষার পদক্ষেপ নিবে।

১০. যদি গবেষণা শেষ হওযার পূর্বে আপনার অংশগ্রহণ বন্ধ করেন তাহলে কি হবে?

আপনি যেকোন সময়ে গবেষণা থেকে আপনার নাম প্রত্যাহার করে চলে যেতে পারেন। যেকোন সময়ে গবেষণা থেকে বাদ দেওয়ার অধিকার গবেষকের আছে। যেমন, আপনি যদি অনাকাঙ্খিত প্রতিক্রিয়া করেন অথবা গবেষণার নির্দেশনা অনুসরণ করতে ব্যর্থ হন অথবা পুরো গবেষণা কাজ বন্ধ হয়ে যায়।

১১. গবেষণায় অংশগ্রহণের সুবিধাদিঃ

এই গবেষণায় সার্বিক অংশগ্রহণের জন্য আপনাকে ক্ষতিপূরণ হিসাবে কোন পারশ্রমিক/সম্মানী দেওয়া হবে না ।

১২. এই গবেষণায় অংশগ্রহণের জন্য কোন খরচ দিতে হবে কিনা?

এই গবেষণায় অংশগ্রহণের জন্য আপনাকে খরচ দিতে হবে না। এই গবেষণায় অংশগ্রহণের সকল পরীক্ষা নিরীক্ষা বিনা খরচে করা হবে। একমাত্র আপনার খরচ হলো আপনার সাক্ষাৎকারের জন্য সময় ব্যয় করতে হবে।

১৩. এই গবেষণা সম্পর্কে যদি কোন প্রশ্ন থাকে?

এই গবেষণা সম্পর্কে যে কোন প্রশ্ন এবং উত্তর পাবার অধিকার আপনার আছে। এই গবেষণা সম্পর্কে যে কোন তথ্য জানার জন্য যোগাযোগ করবেনঃ অধ্যাপক ডঃ ইয়ারুল কবীর, প্রাণরসায়ন ও অণুপ্রাণ বিজ্ঞান বিভাগ, ঢাকা বিশ্ববিদ্যালয়, ঢাকা-১০০০। অথবা, অধ্যাপক ডাঃ মামুন আল মাহতাব, লিভার বিভাগ, বঙ্গবন্ধু শেখ মুজিব মেডিকেল বিশ্ববিদ্যালয়, ঢাকা-১০০০।

অংশগ্রহণকারীর অঙ্গীকারনামাঃ

আমি উপরে উল্লেখিত সকল তথ্য পড়েছি। এ সম্বন্ধে আমার যে সকল প্রশ্ন ছিল সব জিজ্ঞেস করেছি। আমি নিজের ইচ্ছায় এই গবেষণায় অংশগ্রহণ করছি।

গবেষণায় অংশগ্রহণকারী স্বাক্ষর (বা বৃদ্ধাগুলীর ছাপ) **সম্মতি গ্রহণকারীর স্বাক্ষরঃ**

তারিখঃ **তারিখঃ**

স্বাক্ষীর স্বাক্ষরঃ

তারিখঃ