AUTOPHAGY GENES (*ATG16L1* AND *IRGM*) VARIANTS AND HEPATITIS B VIRUS INFECTION SUSCEPTIBILITY

Thesis submitted in fulfillment for the requirement of the Degree of

Doctor of Philosophy

By

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DECLARATION

I hereby declare that the work contained in the thesis entitled **"Autophagy genes (*ATG16L1* and *IRGM*) variants and Hepatitis B Virus infection susceptibility"**, submitted at Jaypee University of Information Technology, Waknaghat, India, has been done by me under the supervision of Dr. Harish Changotra. The work has not been submitted to any other organization for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

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CERTIFICATE

This is to certify that the thesis entitled, **"Autophagy genes (*ATG16L1* and *IRGM*) variants and Hepatitis B Virus infection susceptibility"** which is being submitted by **Ambika Sharma (Enrolment No. 136557)** in fulfillment for the award of the **Degree of Philosophy in Biotechnology by Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by her under my supervision. This work has not been submitted partially or wholly to any other Institute or University for the award of this or any other degree or diploma.

Supervisor **Dated**

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DEDICATED TO

MY

GRANDPARENTS

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"Kind words can be short and easy to speak, but their echoes are truly endless"

(Mother Teresa)

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Ambika Sharma

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LIST OF ABBREVIATIONS

%	Percentage
μg	Microgram
μl	Microlitre
μM	Micromolar
^{o}C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
AASLD	American Association for the Study of Liver Diseases
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
APASL	Asian Pacific Association for the Study of the Liver
A-RFLP	Artificial-RFLP
ATP	Adenosine 5'-triphosphate
ATGs	Autophagy-related genes
ATG16L1	Autophagy related 16 like 1
CASL	Canadian Association for the Study of the Liver
CHB	Chronic hepatitis B
CD	Crohn's disease
CMA	Chaperone-mediated autophagy
cccDNA	Covalently closed circular DNA
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DRs	Direct repeats
dNTPs	2'-deoxynucleotide 5'-triphosphate
EASL	European Association for the Study of the Liver
EDTA	Ethylenediaminetetraacetic acid
Enh1	Enhancer1

ER	Endoplasmic reticulum
EtBr	Ethidium bromide
g	Gram
GWAS	Genome-wide association studies
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HbcAg	Hepatitis B core antigen
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
IRGM	Immunity-related GTPase
kb	Kilobase
LC	Liver cirrhosis
LD	Linkage disequilibrium
LFT	Liver function test
M	Molarity
mg	Milligram
ml	Milliliter
mM	Millimolar
MHC	Major histocompatibility complex
mRNA	messenger RNA
N	Normality
NAs	Nucleos(t)ide analogues
Na+	Sodium
NaOH	Sodium hydroxide
NEB	New England Biolabs
NLS	Nuclear localization signal
nm	Nanometer
ng	Nanogram
NTCP	Sodium taurocholate cotransporting polypeptide

OR	Odds ratio
ORF	Open Reading Frame
PCD	Programmed cell death
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
pH	Pouvoir hydrogen
qPCR	Quantitative PCR
RBC	Red blood cell
rcDNA	relaxed circular DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RPM	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SHBs	Small surface protein
SNPs	Single Nucleotide Polymorphisms
T-ARMS	Tetra-primer amplification refractory mutation system
Taq	Thermus aquaticus
TE	Tris-EDTA
TMEM59	Transmembrane protein 59
TNF-α	Tumour necrosis factor-alpha
U	Unit
UC	Ulcerative colitis
ULK1	Unc-51 Like Autophagy Activating Kinase 1
UV	Ultraviolet
WBC	White blood cell
WHO	World Health Organization

ABSTRACT

Hepatitis B virus infection is a severe liver inflammation and is the main cause of morbidity and mortality in developing countries. Along with the environmental factors and virus-related factors, host genetic factors also play an imperative role in HBV pathogenesis and disease outcome. Host genetic factors are known as an important factor in patient's susceptibility to HBV infection. Recently, a large number of studies reported the connection of many viral infections with the autophagy process. Moreover, HBV also enhances the autophagy process, without promoting protein degradation and use the process for its own replication. Single Nucleotide Polymorphisms (SNPs) in the autophagy genes are strongly associated with many diseases such as Crohn's disease, asthma, cancer, Paget disease of bones, and various neurodegenerative diseases. Understanding of molecular mechanism of autophagy pathway and its modulation could be helpful in the management of liver diseases including HBV infection. *IRGM* and *ATG16L1* are two important genes of autophagy pathway, which helps in the initiation and autophagosome formation. Various association studies documented the role of SNPs in these genes with many diseases. However, no association studies have been conducted to see the role of SNPs of *IRGM* and *ATG16L1* genes with HBV infection. In this study, we have genotyped three promoter region SNPs (rs4958842, rs4958843 and rs4958846) of *IRGM* gene and three intronic (rs2241879, rs13005285, and rs7587633) and one exonic (rs2241880) SNP of *ATG16L1* gene in 551 HBV infected and 247 healthy control individuals. HBV infected individuals belong to different clinical categories such as asymptomatic, acute, chronic hepatitis B and liver cirrhosis. HBV DNA levels and plasma AST and ALT levels were also correlated with different genotypes of the SNPs. In case of *IRGM* SNPs, we found the protective association of the SNP rs4958842 and rs4958846 with HBV infection and rs4958843 was associated with HBV infection susceptibility. We found the association of *ATG16L1* SNPs rs2241880, rs2241879, and rs13005285 with HBV infection susceptibility and rs7587633 was associated with protection from HBV infection. In conclusion, we have identified *IRGM* and *ATG16L1* genetic variants as a genetic susceptibility factor in HBV infection. These SNPs could be used as biomarkers to see the HBV infection susceptibility in our population and further the influence of these SNPs on the treatment response of HBV infected patients could be studied.

CHAPTER-1

INTRODUCTION

Hepatitis is a liver infection caused by the hepatitis B virus and it represents a serious public health problem globally with significant morbidity and mortality [1]. Jaundice, abdominal pain, liver enlargement, muscles or joint pain, loss of appetite and fever, characterize it. Hepatitis B virus (HBV) transmission occurs from direct contact with infected blood, parenterally via infected blood transfusions, intravenous drug abuse, vertically from an infected mother to child, horizontally (*e.g.* between children in a household) and via sexual routes [2]. The HBV can survive outside the body for at least 7 days and during this period it is capable of causing infection. The average incubation period of HBV is 75 days and can vary from 30-180 days [3]. According to World Health Organization estimate, worldwide around 257 million people are living with chronic HBV infection [3]. HBV leads to a wide range of liver disease from acute to chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [4]. Individuals with chronic HBV infection are more prone to develop liver cirrhosis (LC) and hepatocellular carcinoma (HCC) and HBV is the primary risk factor for the development of HCC [3].

India has one-fifth of the World's population and carries the second largest burden of HBV [5]. According to the WHO estimates, in India, around 40 million people are chronic carriers of HBV [6]. Among 26 million infants born every year in India, approximately 1 million develop chronic HBV infection [7]. In India each year, around one million people die from HBV induced liver diseases, which equates to about two HBV-related deaths per minute. Moreover, due to the wide geographical variations, different socioeconomic status, religion and cultures the estimated prevalence of HBsAg in the tribal population of India, is 3.1% and in non-tribal population, it is 11.85% [8]. Based on the prevalence of HBsAg, different areas of the world are classified as having low (<2%), intermediate (2-7%) and high (≥8%) HBV endemicity [9]. Countries which have high endemicity include South-East Asia, China, most of Africa, most of Pacific Islands, the Amazon basin and parts of the Middle East, countries with intermediate endemicity include South Asia, Eastern and Southern Europe, Russia and Central and South America and low

endemicity areas include United States, Western Europe, and Australia. India falls in South Asia has been grouped as a country with intermediate to high HBV endemicity [9].

The HBV is an enveloped partially double-stranded DNA virus that belongs to the hepatotropic DNA virus family (*hepadnaviridae*). It has the smallest genome of 3.2 kb among all DNA viruses [10]. The infectious HBV virion (Dane particles) has a double-shelled spherical structure which is 42 nm in diameter and consists of a lipid envelope containing HBsAg that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg) [4]. Nucleocapsid contains partially double-stranded DNA with two asymmetric strands. The plus strand of the HBV DNA is incomplete on the other hand minus strand or negative strand is complete but contains a nick at a unique site [11]. A negative strand of the DNA is not covalently closed due to the presence of viral polymerase on the 5' end of the strand [4]. With the help of electron microscopy, two types of particles were observed in the serum of infected individuals 42 nm to 47 nm Dane particles which determine the infectivity of the individual and 20 nm spherical subviral particles that do not contain the viral capsid [11, 12]. The Dane particles may range from as low as 10^2 particles mL^{-1} of serum in the occult and asymptomatic infections to more than 10^8 particles mL^{-1} during the active replicative phase of infection [11]. At the 5′ ends of both the strands short repeat sequence and direct repeats (DRs) of around 11 nucleotides are present which play a vital role in priming viral replication [13]. Vaccination programmes for HBV effectively decreased the burden of the infection all over the world, but it is still a major public health concern and various improvements are required in order to reduce the burden of this deadly disease [14]. Environmental, host genetics, immune responses, virus genotypes and viral load are the factors, which influence the susceptibility and progression of this disease [15, 16].

Various studies showed that HBV induces the autophagy process and use it for its own replication [17]. Autophagy is an important cellular process that helps in degradation of dead cell organelles, unwanted proteins, in the elimination of various intracellular pathogens and protects cells from various stress conditions [18]. In the autophagy process, unwanted cell material is enclosed by isolating membrane or phagophore that form double-membrane vesicle called as autophagosome, which delivers the cytoplasmic contents to the lysosome [19]. Autophagosome formation is an essential step and various autophagy-related genes (ATGs) regulate this step. Till now in the yeast 40 autophagy-related proteins have been identified, among them, 18 proteins are

involved in autophagosome formation [20, 21]. A study showed that small surface protein (SHBs) of HBV enhances the autophagy process without promoting degradation of proteins via lysosome [22]. Other study demonstrated that HBV induces both autophagy and autophagic degradation [23]. Tang et al in their study demonstrated that HBx protein increases the autophagy process by up-regulating the expression of beclin-1[24]. HBx protein also inhibits the autophagic degradation by impairing lysosomal acidification that ultimately affects lysosomal degradative capacity [25]. In a study conducted by Tian and colleagues lower levels of HBV DNA, HBsAg and HBeAg were found in the liver-specific Atg5 knockout mice, which shows that autophagy inhibition leads to a reduction in HBV replication [19]. Alteration in autophagy pathway genes and proteins has been well documented in the implications of various diseases such as neurodegenerative diseases, cardiovascular disorders, cancer, aging and other inflammatory diseases. Modulation in autophagy process has already shown better results in the treatment of various diseases such as cancers, Alzheimer's and Parkinson's diseases etc. [26]. A number of polymorphisms in various genes such as *MHC* class I and class II loci, cytokines, chemokines and vitamin D receptor have been shown to play role in HBV persistence and disease outcomes. Various polymorphisms in genes *Th17, HLA-DP*, *IL-28B* and *eIF-2a* are found to be associated with sustained responses to pegylated interferon's treatment [27-29]. Identification of various polymorphisms of the genes those directly or indirectly play role in the HBV infection, treatment and vaccination would be helpful in developing more effective treatments regimens and preventive measures [30]. Various polymorphisms of many genes play role in the pathogenesis of HBV infection but no such polymorphisms in autophagy-related genes have been studied so far to see their association with HBV infection and outcome. HBV virus uses the autophagy process to enhance its replication, hence indicating the possibility of targeting the pathway for the treatment of HBV infection [31].

A recent study demonstrated the increased expression of *ATG16L1* gene (an autophagy gene) in HepG2 and HepG2.215 cell lines (cell lines infected with HBV genome) in comparison with THLE-2 cell line (normal liver cell line) suggesting its role in HBV infection [32]. *ATG16L1* protein makes a complex with the ATG12–ATG5 conjugate that helps in the recruitment of LC3 to the developing phagophore [33]. The ATG12–ATG5/ATG16L1 complex is a ~800-kDa protein that dissociates from the completed autophagosome [34]. Defects in *ATG16L1* gene affect

the formation of autophagosome, degradation of long-lived proteins and clearance of bacteria [35]. Atg16l1 protein mainly consists of three domains, N-terminal domain where Atg5 binds, a central coiled-coil domain that helps in self-oligomerization and WD repeats C domain [34]. WD domain provides docking stage for an amino acid motif that present in the intracellular region of TMEM59 (transmembrane protein) and other proteins that lead to increase in the autophagy process (xenophagy) by the activation of LC3 lipidation [36]. Over-expression of *ATG16L1* was found to be associated with various diseases such as oral squamous cell carcinoma disease, Crohn's disease and colorectal cancer [32]. *ATG16L1* gene polymorphisms found to be associated with various diseases such as Crohn's disease, inflammatory bowel disease, psoriasis vulgaris, palmoplantar pustulosis, a Paget disease of bones and gastric cancer[37-43]. Another important autophagy gene, IRGM helps in the initiation of autophagy pathway and it is the most commonly targeted protein by RNA viruses [44]. *IRGM* interacts with various other autophagy pathway genes such as *ATG5*, *ATG10*, *MAP1CL3C* and *SH3GLB1*, *ULK1* and *BECN1* which regulates earlier steps of the pathway, suggesting its role in nucleation or elongation step of autophagy [45, 46]. These proteins along-with *IRGM* regulate the initial steps of the autophagosome formation and viral infection modulates this interaction, which in turn modulate the autophagy process [45]. Role of polymorphisms of *IRGM* gene has already been well documented in the pathogenesis of many diseases such as inflammatory bowel disease, tuberculosis, and many others [47-52]. Defects in the autophagy pathway have been implicated in various human disease, hence it can be a viable target for drug discovery and to develop more effective therapeutic approaches for various diseases [53, 54]. Therefore, in this study, we have targeted polymorphisms of two important autophagy genes *ATG16L1* and *IRGM* which are extensively studied in the implications of various diseases, and we have genotyped these SNPs in 550 HBV infected patients and 247 healthy control subjects to find out their association with HBV susceptibility.

RATIONALE OF THE STUDY

Various polymorphisms of Interferon-*λ4, HLA-DPA1, HLA-DPB1, NTCP and IL-28B* genes are found to be associated with HBV infection and treatment response to the drugs. Hepatitis B virus uses the autophagy process for its own replication. Increase in the autophagy process also leads to the increase in the levels of HBV DNA and inhibition of autophagy lead to a decrease in HBV

DNA levels. This pathway can be targeted to develop more effective therapeutic approaches and to treat HBV infected individuals. In this study, we have targeted two important autophagy genes *ATG16L1* and *IRGM*, which help in initial steps in autophagy process, and any disruption or defects in these genes modulate the autophagy process. We explored four polymorphisms of ATG16L1 gene (rs2241880, rs2241879, rs13005285 and rs7587633) and three promoter region polymorphisms (rs4958842, rs4958843, and rs4958846) of *IRGM* gene. If studied SNPs found to be associated with HBV infection, after studying them in a large cohort and after their validation they can be used as prognostic markers in HBV infection.

Keeping in view the research gaps, the current study was undertaken with the following objectives

Objective 1: To develop A-RFLP methods for genotyping promoter SNPs (rs4958842, rs4958843, rs4958846) of *IRGM*.

Objective 2: To genotype promoter SNPs in healthy controls and HBV infected patients and find their role in HBV susceptibility.

Objective 3: To develop A-RFLP (for rs13005285 and rs7587633) and RFLP (for rs2241879) methods for genotyping intronic and exonic SNPs of *ATG16L1*.

Objective 4: To genotype *ATG16L1* SNPs in healthy controls and HBV infected patients and to look for their genetic association in HBV susceptibility.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Hepatitis B virus infection

Hepatitis B caused by hepatitis B virus (HBV) represents a serious public health problem globally. Jaundice, abdominal pain, liver enlargement, muscles or joint pain, loss of appetite and fever, characterizes it. Hepatitis B virus transmission occurs vertically from an infected mother to child, horizontally (*e.g.* between children in a household), sexually and parenterally via blood transfusions, intravenous drug abuse, etc. [2]. HBV can survive outside the body for at least 7 days and during this period; it is capable of causing infection. The average incubation period of HBV is 75 days but can vary from 30-180 days [3].

2.1.1 Epidemiology

According to World Health Organization estimates, worldwide 2 billion people are living with HBV infections and more than 257 million people (around 3.4% of total population) are chronic carriers. HBV causes chronic infection and puts people at high risk of death from cirrhosis and liver cancer. Out of 257 million people, those are infected with HBV; 2.7 million people are co-infected with HIV [3]. Each year around one million people die from HBV induced liver diseases, which equates to about two HBV infections related deaths per minute. Different areas of the world, on the prevalence of HBsAg, are classified as having low (<2%; United States, Western Europe, and Australia), intermediate (2-7%; South Asia, Eastern and Southern Europe, Russia and Central and South America) and high (≥8%; China, South-East Asia, the majority of Africa, the majority of Pacific Islands, the part of Amazon and some areas of the Middle East) HBV endemicity [9]. Effective vaccines are available for HBV infection; still, it is a major public health problem in many developing countries. Worldwide incidence of CHB infection is mentioned in Figure 2.1.

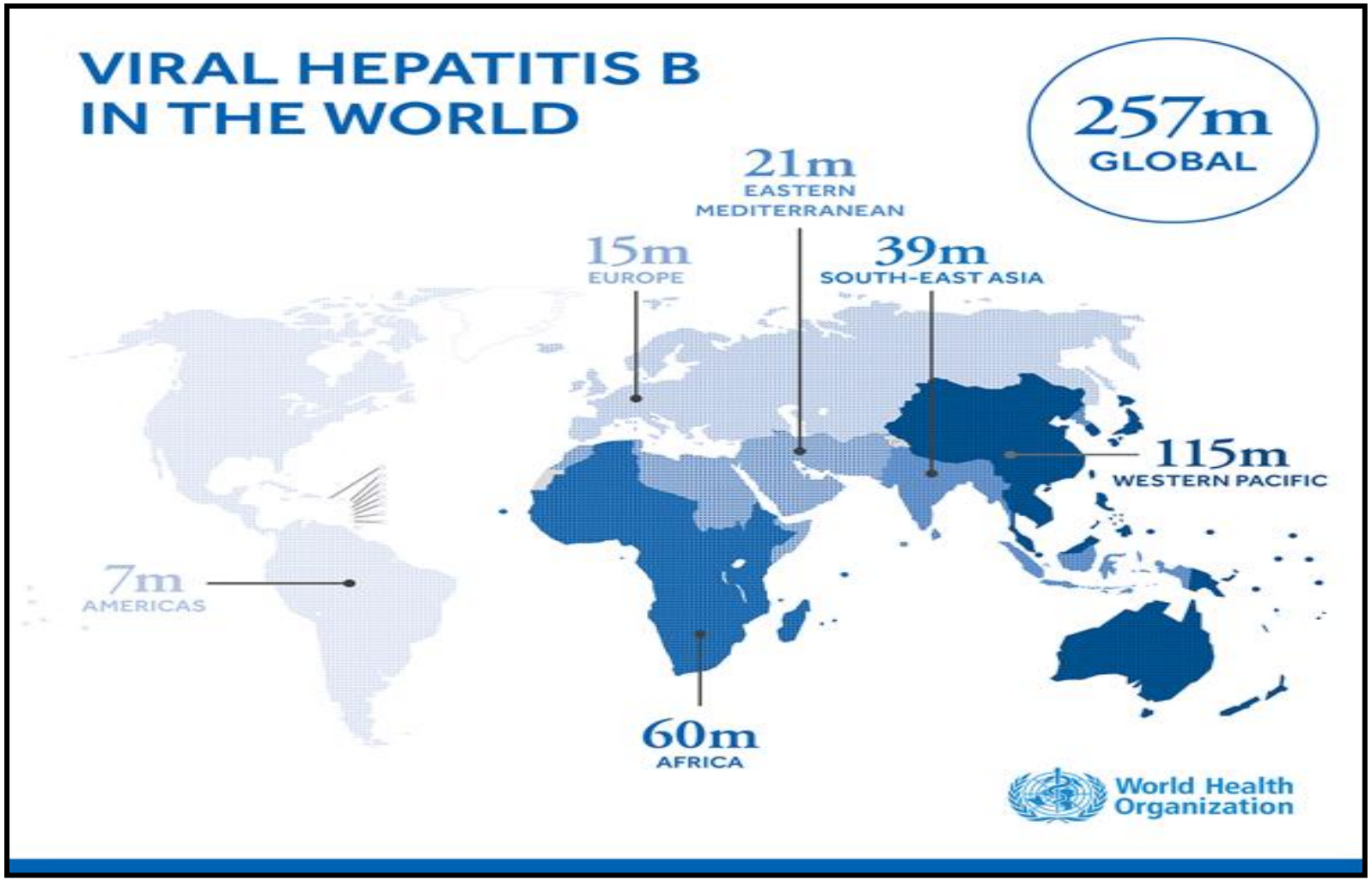

Figure 2.1: Worldwide occurrence of CHB infection (WHO) [3].

2.1.2 Prevalence of HBV infection in India

Since India accounts for one-fifth of the World's population and it carries the second largest burden of HBV infection [5]. The prevalence of HBsAg was reported up to 3-4.2% and around 40 million HBV carriers are present in India [55]. In India every year around 26 million infants are born and approximately 1 million infants have the risk of developing chronic HBV infection [7] and around 100,000 die every year from the infection [56]. Moreover, due to the presence of wide geographical variations, different socioeconomic status, religion and cultures the estimated prevalence of HBsAg in the tribal population of India, is 3.1% and in non-tribal population, it is 11.85% [8]. In India horizontal transmission (saliva or open wounds) is the main route of HBV transmission mainly in the childhood [5]. In India, 1.1 million people are intravenous drug users and 2.7-10.8% of them were reported positive for HBsAg [5].

2.1.3 Etiology

HBV is one of the smallest viruses and the infection caused by the virus is the most common cause of contagious liver diseases [11, 57]. HBV replicates via reverse transcription and infects the hepatocytes in a non-cytopathic manner [58]. In the serum of infected person, three types of viral particles of HBsAg are observed, two viral particles are small in size and spherical in shape with 20 nm diameter and one viral particle is of filamentous shape with a diameter of 22 nm [4]. Chronic infection, cirrhosis of liver, and hepatocellular carcinoma are the clinical outcomes of HBV infection [11]. HBV infection is the most common chronic infection worldwide and it accounts for 50% cases of HCC [59]**.** On the basis of capability of host immune response to clear the infection, it causes either acute infection (less than six months) or chronic and life-long infection [58]. Various environmental factors (alcohol and aflatoxin), viral related factors (HBV genotypes, HBV DNA levels, co-infection with other viruses), host-related factors (age, sex, immunological and genetic factors) influence the outcome of HBV infection [60]. Studies demonstrated that mutations rate in HBV is 10 times greater than other DNA viruses and mutations may occur naturally or due to the pressure from antiviral therapies [61].

2.1.4 HBV Structure and genome organization

The HBV is an enveloped DNA virus that belongs to the hepatotropic DNA virus family (*hepadnaviridae*) and it has the smallest genome of 3.2 kb among all DNA viruses [10]. The

infectious HBV virion (Dane particles) has a double-shelled spherical structure of diameter 42 nm and consists of a lipid envelope containing hepatitis B surface antigen (HBsAg) that surrounds an inner nucleocapsid that contain hepatitis B core antigen (HBcAg) [4]. The schematic representation of the structure of HBV is shown in Figure 2.2. Nucleocapsid contains partially double-stranded DNA with two asymmetric strands. The plus strand of the HBV DNA is incomplete, on the other hand, minus strand or negative strand is complete but contains a nick at a unique site [11]. The negative strand of the DNA is not covalently closed due to the presence of viral polymerase on the 5' end of the strand [4]. With the help of electron microscopy, two types of particles were observed in the serum of infected individuals that ranges from 42 nm to 47 nm Dane particles which determines the infectivity of the individual and 20 nm spherical subviral particles that lack the viral capsid [11, 12]. The Dane particles ranges between as low as 10^2 particles mL^{-1} of serum in the occult and asymptomatic infections to more than 10^8 particles mL^{-1} during the active replicative phase of infection [11]. The organization of HBV genome is shown in Figure 2.3. The HBV genome mainly contains four promoters, two enhancers (Enh1 and Enh2) and two direct repeats (DR1 and DR2) [62]. Enhancers and promoters of the HBV are embedded within the ORFs. At the 5′ ends of both the strands short repeat sequence and direct repeats (DRs) of around 11 nucleotides are present which play a vital role in priming viral replication [13]. The HBV genome contains four partially overlapping Open Reading Frames (ORFs) encoding genes: (1) precore/core gene, coding for the nucleocapsid protein and for the secreted, non-structural, pre-core protein, (HBeAg) (2) the polymerase gene coding for the reverse transcriptase, RNase H and the terminal protein domains (3) PreS1/L, PreS2/M and Surface/S-gene coding for the three envelope proteins (4) X gene, coding for the regulatory X-protein [62-64].

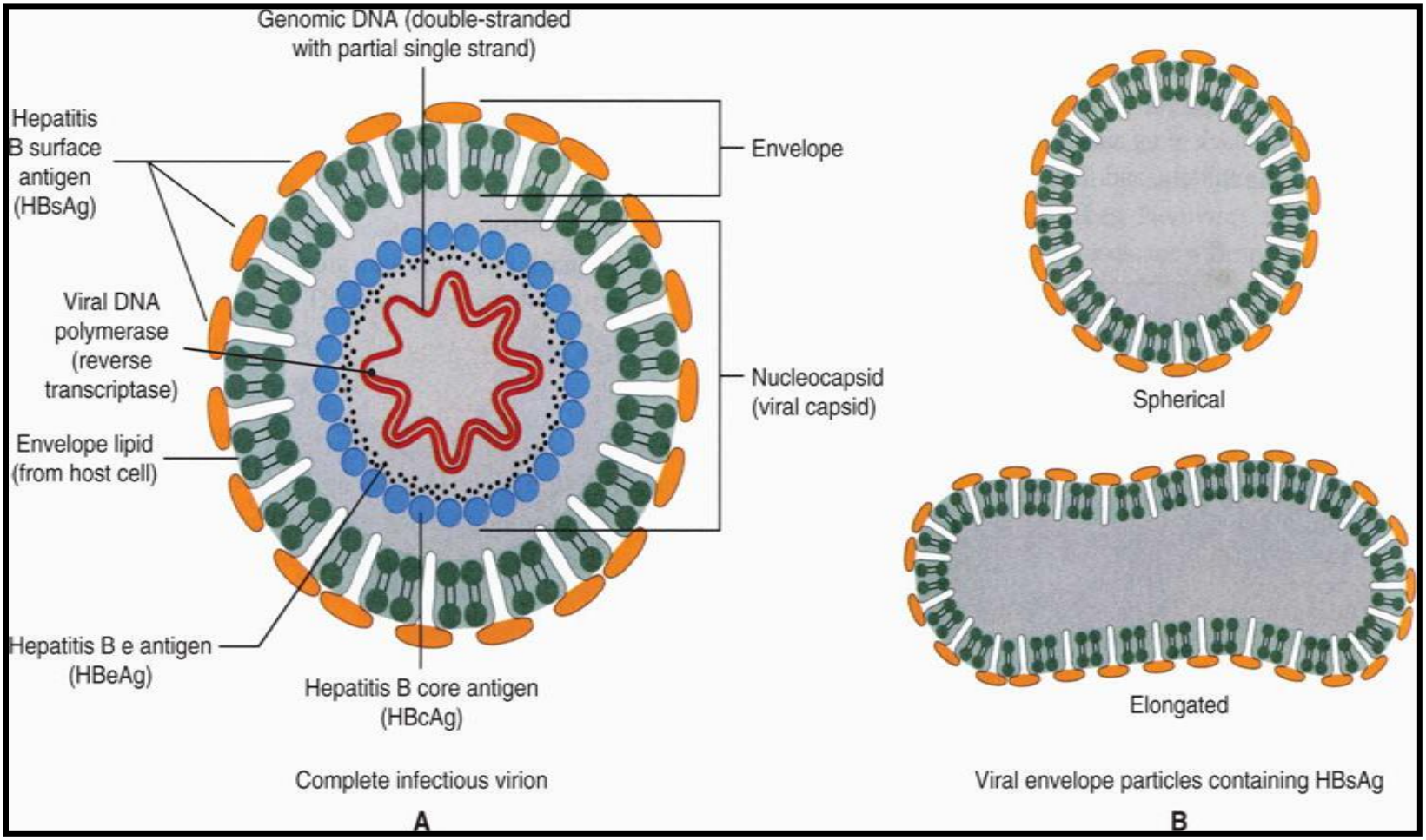

Figure 2.2: Schematic representation of HBV virus (A). The 42 nm "Dane particle" (B) The 22 nm filamentous and circular forms of HBsAg or protein coat [65].

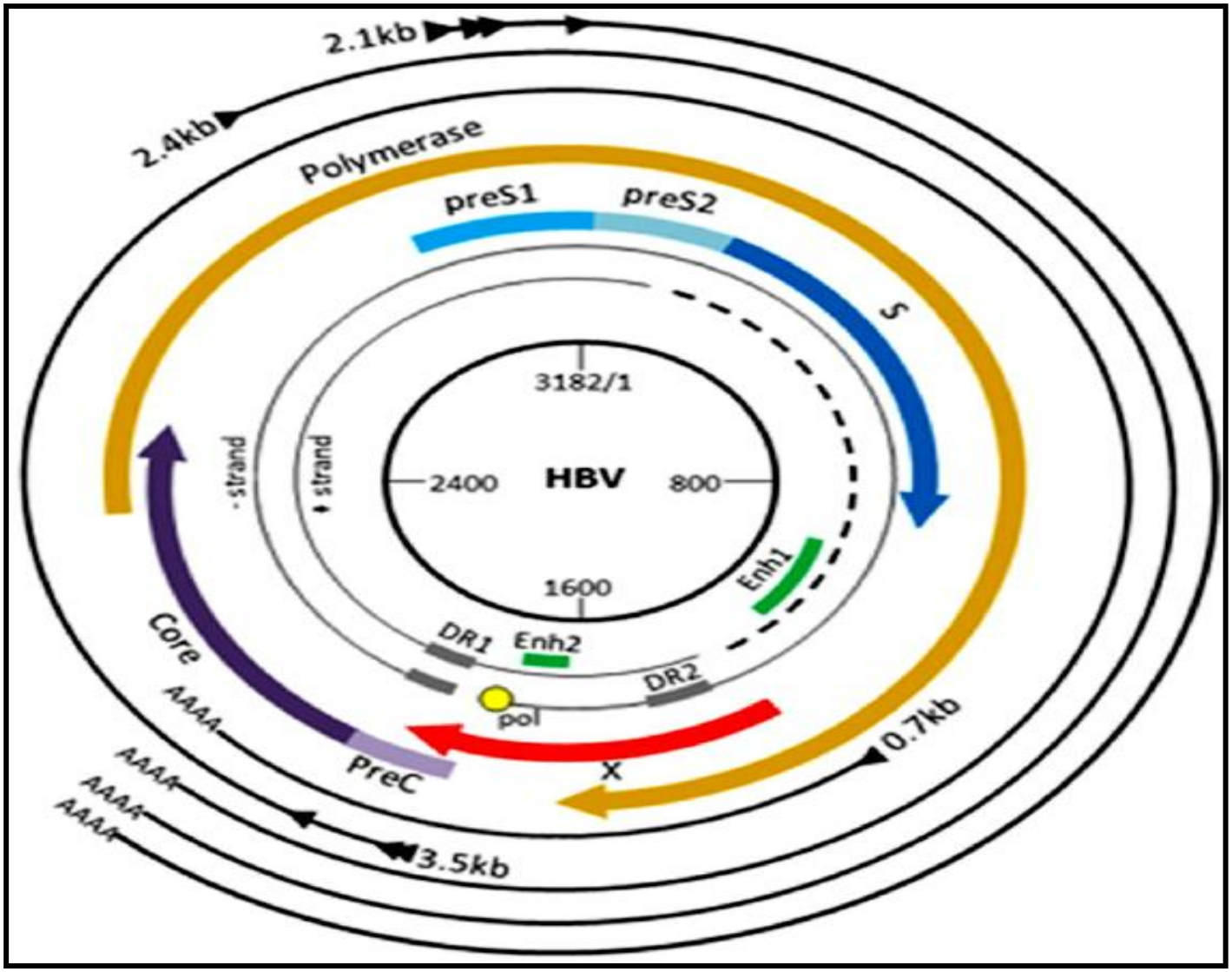

Figure 2.3: Diagram, showing the organization of HBV genome [62].

2.1.5 Lifecycle of HBV

HBV is one of the few known non-retroviral viruses that replicate via reverse transcription process. The life cycle of virus starts with entry into a host cell, followed by transport of its genetic material to the nucleus, conversion into cccDNA and ends with transcription, translation and virus assembly [66]. Representation of the complete life cycle of the HBV is shown in Figure 2.4 [67].

2.1.5.1 Interaction and Entry of HBV

The interaction between HBV and it host is initiated by the reversible binding between hepatocyte-specific preS1 that are present on the outer membrane of mature Dane particles and heparan sulfate proteoglycans on hepatocytes [68]. Little was known about the HBV receptor that helps in the internalization of the virus, recently Yan and colleagues identified a hepatocyte-specific receptor namely sodium taurocholate cotransporting polypeptide (NTCP) predominantly expressed in the liver [69]. The virus may enter into the host cells by both, endocytosis and direct fusion of the viral envelope with the plasma membrane [70].

2.1.5.2 Release and translocation of nucleocapsid to the nucleus

After release into the cytoplasm, the relaxed circular DNA (rcDNA) containing nucleocapsid is transported to the nucleus for further modifications via nuclear pore complex (NPC) [64, 71]. Cellular microtubules assist in the transportation of capsid by their interaction with the cytoplasm [72]. Transportation of the capsid through NPC is mediated by the interaction of the nuclear localization signal (NLS) that is present on the C terminal of capsid protein with the two nuclear import receptors, α, and β importins.

2.1.5.3 Formation of covalently closed circular DNA (cccDNA)

After the release of nucleocapsid into the nucleus, the incomplete plus-strand of the partially dsDNA (rcDNA) of the virus is completed by viral polymerase [73]. Covalently closed circular DNA (cccDNA) formed after the covalent ligation of both DNA strands and each infected cell found to contain 1-50 cccDNA molecules [64]. Throughout the lifespan of the hepatocyte, the

viral cccDNA can persist in the nucleus of the hepatocytes and its elimination is still a therapeutic challenge [72].

2.1.5.4 Transcription

Viral cccDNA serves as a template for the synthesis of RNA. All the viral RNA species (pre-core mRNA, pregenomic mRNA, mRNA for L, M and S proteins and mRNA for x protein) are transcribed from the cccDNA using the cellular transcriptional machinery. Various host transcription factors such as CREB, STAT1, STAT2, etc, chromatin-modifying enzymes, hepatocytes nuclear factors and viral factors (core and regulatory X protein) regulate the process of transcription [68, 72] All the transcription regulators, regulate the viral gene expression by interacting with the viral promoters of four ORF regions [74]. Pre-genomic RNA (PgRNA) has a ε-stem-loop proximal to the 5′-end where polymerase binds and triggers the capsid formation [75]. Initially, three nucleotides (5´– GAA – 3´) are synthesized by utilizing the bulge region of ε as the template and a tyrosine residue in the amino-terminal domain of the polymerase molecule as a primer [76].

2.1.5.5 Translation of pgRNA

In the cytoplasm, pgRNA is translated into the core protein, the viral polymerase, and the sgRNA into the regulatory X-protein and the three envelope proteins (L, M and S envelope proteins) [64].

2.1.5.6 Nucleocapsid assembly and envelopment

Assembly of the nucleocapsid occurs in the cytosol and results in the packaging of a 3.5 kb pgRNA molecule together with various viral and cellular factors [77]. Pregenomic RNA (pgRNA) and polymerase are encapsidated within core particles where the viral DNA is synthesis (rcDNA) initiated by the reverse transcription activity of the polymerase, following synthesis of a negative strand of DNA and degradation of RNA template by RNase H [75]. Three co-terminal proteins S, M and L make the viral envelope and span the membrane several times. These viral envelope proteins not only release from infected cells but also released as subviral lipoprotein particles of diameter 2 nm without capsid [77]. HBV surface proteins envelop the DNA containing nucleocapsid in the pre-Golgi compartment of the endoplasmic reticulum and are secreted as complete Dane particles via the vesicular transport pathways into the blood. 5´ end of

a negative strand of the newly formed rcDNA is bound to the viral polymerase. Newly formed rcDNA has two fates; first, it may travel to the nucleus for further amplification to cccDNA to maintain the pool of cccDNA, secondly, it can move towards endoplasmic reticulum for its further envelopment with glycoproteins that resides in the ER membrane [78].

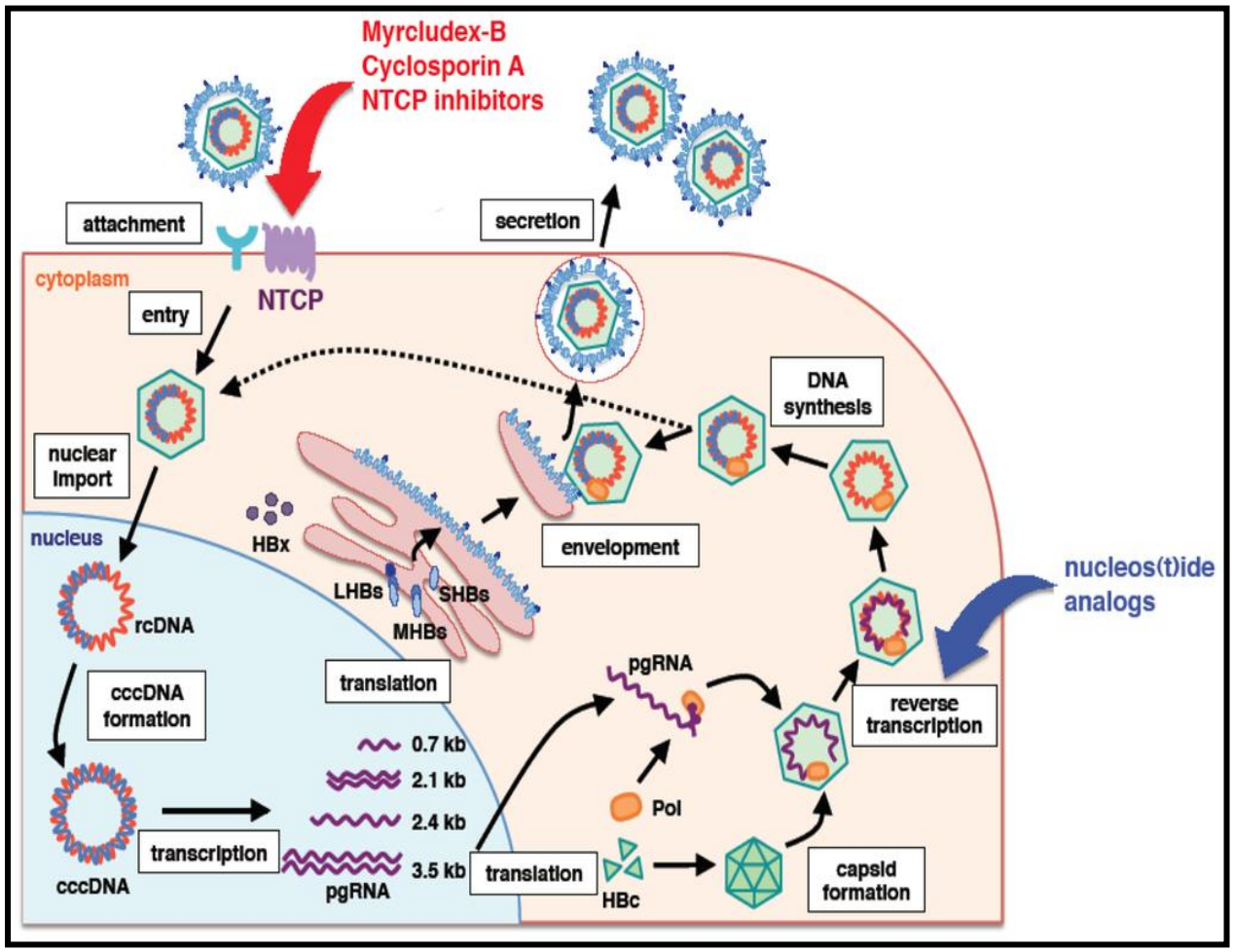

Figure 2.4: Representation of different stages involved in the lifecycle of HBV [67].

2.1.6 Distribution of different genotypes and subgenotypes of HBV and their importance

Viral polymerase of HBV lacks proofreading activity and due to this reason, it has evolved into various genotypes [79]. HBV has mainly classified into eight (A-H) well-known genotypes on the basis of genome divergence but two more genotypes (I, J) have also been identified and each genotype has distinct geographical distribution [80]. Among all the reported genotypes, genotype A is present in sub-Saharan Africa, Northern Europe, and Western Africa. Genotypes B and C are common in Asia; genotype C is primarily observed in Southeast Asia; genotype D is dominant in Africa, Europe, Mediterranean countries, and India; genotype G is reported in France, Germany, and the United States; and genotype H is commonly encountered in Central and South America [80]. Newly found genotype I have recently been reported in Laos and Vietnam and J genotype has been identified in the Ryukyu Islands in Japan [80]. Out of eight genotypes of HBV, A-D, F, H and I has been further divided into 35 sub-genotypes and they all differ in genome length, the size of ORFs and the proteins translated [81]. Among different genotypes of HBV >8% difference in nucleotides was observed and for subgenotypes, 4%-8% difference was reported [80]. Various studies showed that different genotypes of HBV found to be associated with various clinical outcomes and treatment efficacy in patients with the infection [82]. In India, the most important characteristics of HBV epidemiology are the dominance of genotype D and its nine sub-genotypes (D1-D9) [80].

2.1.7 Clinical manifestation of HBV infection

Chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) are the main clinical outcomes of HBV infection. Acute HBV infection can be either symptomatic or asymptomatic and it provides life-long immunity if immune system completely clear the virus [61]. More than 90% of infants, who are not immunized, if get infected develop a chronic infection. Most of the older children and adults recover from the infection whereas, 5%–10% are not able to clear the infection and develop chronic infection [4]. Some individuals with chronic infection have mild liver diseases, but 15%-25% develop an active disease that ultimately leads to liver cirrhosis and hepatocellular carcinoma (HCC). Worldwide HBV infection is the major risk factor for the development of HCC as HBV endemic regions carry 85% of the disease burden [83]. The representation of clinical outcomes of the HBV infection is shown in Figure 2.5

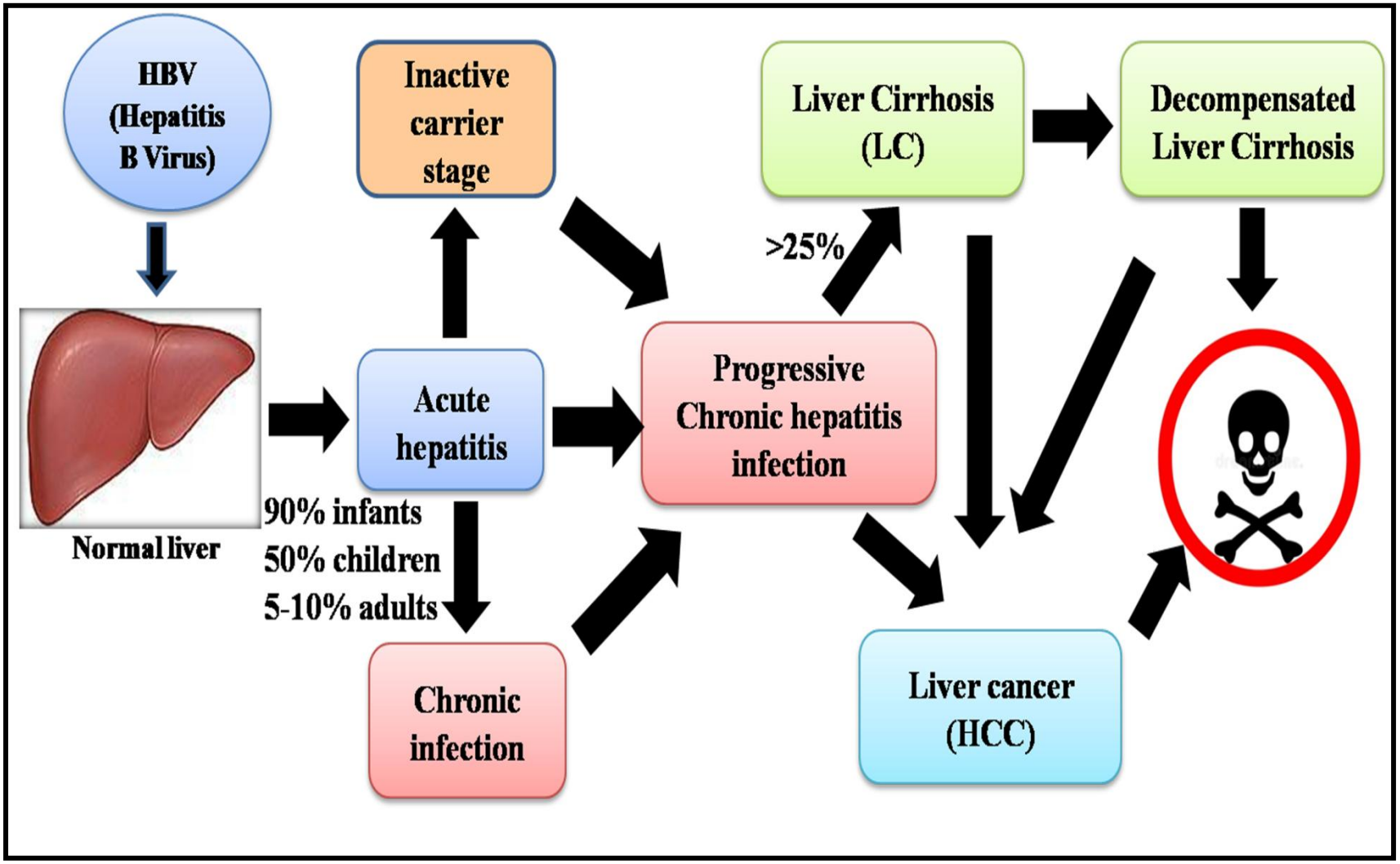

Figure 2.5: Schematic representation of the clinical outcomes of the HBV infection

Based on host and virus interaction HBV infection is divided into three phases [84].

(1) Immune tolerant phase: In this phase, after acute HBV infection some patients may remain HBeAg +ve, normal ALT levels, high HBV DNA levels (usually > 2×10^6-2×10^7 IU/mL) with minimum histological changes in the liver. This phase can last for many years in children who acquire infection in perinatal period

(2) Immune clearance phase: This phase is characterized by HBeAg (+ve) CHB and increase in ALT and HBV DNA levels. Hepatic decompensation is also observed in this phase of HBV infection.

(3) Immune control phase: Low HBV DNA levels, normal ALT levels with mild or no liver injury characterizes this phase. This stage may last for some years or even stays for a lifetime. Some patients in this stage resolve the infection and develop HBeAb but some may reactivate chronic HBV infection.

(4) HBeAg negative CHB: Patients in this phase of CHB infection have a higher rate of fibrosis than HBeAg positive CHB patients. Elevated ALT and HBV DNA levels characterize this phase.

2.1.8 Diagnosis of HBV infection

The diagnosis of various stages of HBV infection is based on the identification of various clinical, biochemical, histological and serological parameters [4]. In the serum of HBV, infected patients, viral antigens and their respective antibodies are present that help in the diagnosis of the exact clinical stage of the infection. Various serological and virological markers used for the diagnosis of HBV infection are shown in Table 2.1. The serological pattern in acute and chronic HBV infection is shown in Figure 2.6 [4].

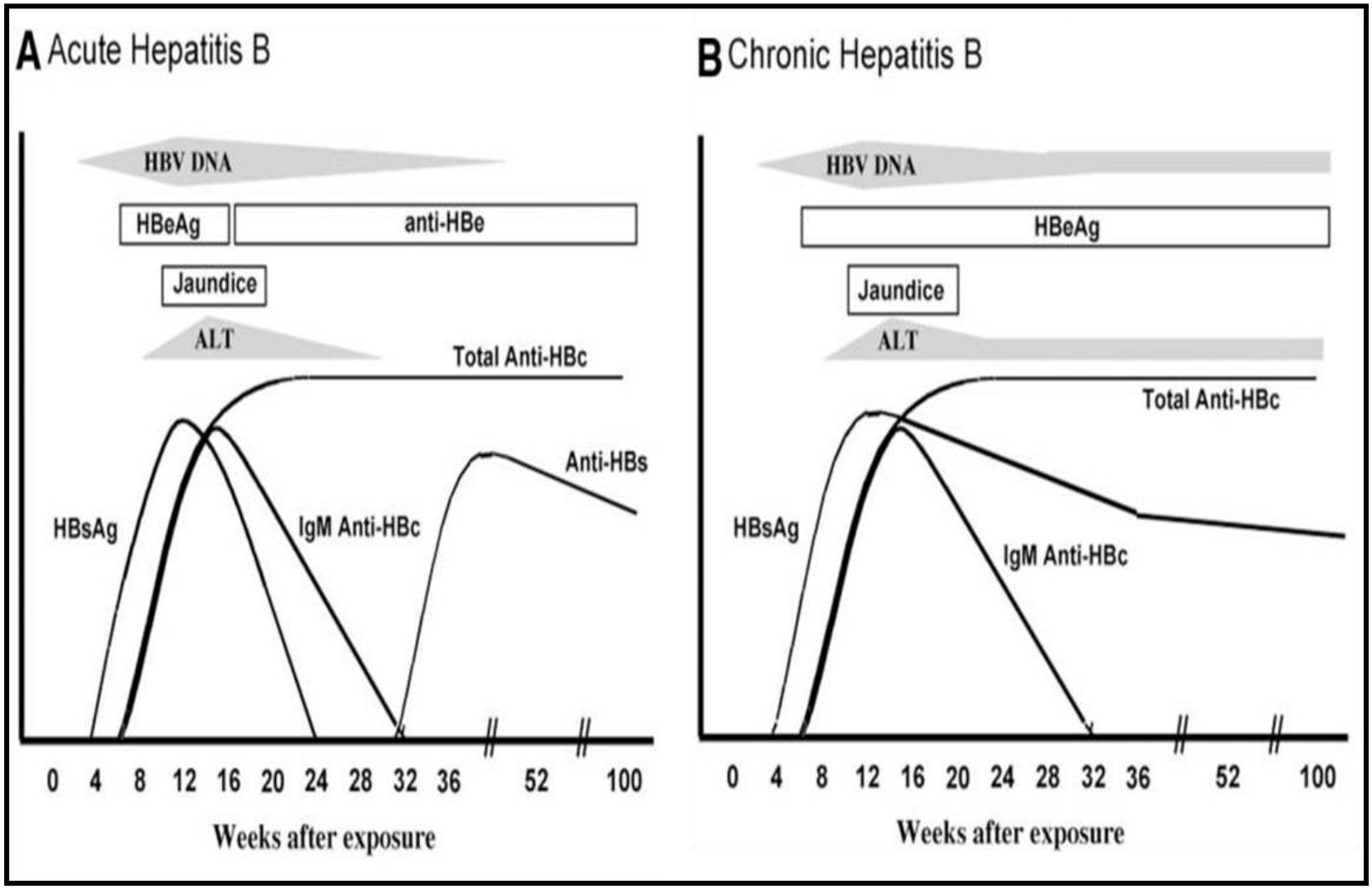

Figure 2.6 Serological profile of acute and chronic HBV infected patients [4].

Table 2.1: Serological and Virological markers used for the identification of stage of HBV infection

Serological markers	Consequences
HBsAg	Acute and Chronic HBV infection
HBeAg	High level of HBV DNA and infectivity, marker for treatment response
HBV DNA	Level of HBV DNA and primary marker for treatment response
Anti-HBc (IgM)	Acute HBV infection could be seen in CHB infection
Anti-HBc (IgG)	Recovered or Chronic HBV infection
Anti-HBs	Recovered HBV infection and also a marker of HBV vaccination
Anti-HBe	Low-level HBV replication and infectivity
Anti-HBc (IgG) and anti-HBs	Past HBV infection, could lose anti-HBs
Anti-HBc (IgG) and HBsAg	Chronic HBV infection

2.1.9 Treatment and preventive approaches used for HBV infection

Patients with different stages of HBV infection and different age groups require different treatment approaches to eliminate the infection. The main goal of the HBV treatment is to prevent the progression of the disease in the patients [85]. Various organizations like World Health Organization (WHO), American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), the Asian Pacific Association for the Study of the Liver (APASL), the Canadian Association for the Study of the Liver (CASL), the National Institute for Health and Clinical Excellence (NICE) proposed various guidelines for the treatment of HBV infection [85].

2.1.9.1 Antivirals

Chronic HBV infection cannot be cured but its progression to LC and HCC can be controlled with the help of antivirals as they suppress viral DNA replication [86]. Currently, two main therapeutic approaches used for the treatment of chronic infection are pegylated-interferon (IFN)-α and nucleos(t)ide analogues (NAs), like lamivudine, adefovir, telbivudine, entecavir and tenofovir [87].

2.1.9.2 Immunization with vaccines

The HBV vaccine helps to prevent the progression of chronic HBV infection to LC and HCC [88]. Plasma-derived vaccines were the first available vaccines but since 1986, recombinant HBV vaccines are being used and it has been proven as a safest and effective vaccine [86]. There are four recombinant vaccines available for HBV, namely, Engerix-B, Recombivax HB, Pediarix and twinrix [89]. Immunization against HBV is the best way to protect ourselves from the infection.

2.2 Genetic factors in HBV infection

Various environmental factors (alcohol and aflatoxin), viral factors (HBV genotypes, HBV DNA levels, co-infection with other viruses), host-related factors (age, sex, immunological and genetic factors) influence the outcome of HBV infection [60]. Host-related genetic factors are not much studied for their association with the progression and outcome of the HBV infection. However, many host gene variants such as *HLA* I and II, various cytokines and chemokines influence the susceptibility to HBV infection [60]. One of the important host factors which are associated with HBV infection and its outcome is *HLA* (Human leukocyte antigen) system. Role of various polymorphisms in the *HLA* region or nearby the *HLA* region is well documented in the association with persistent HBV infection, spontaneous clearance of HBV infection, seroconversion, and progression of CHB into liver cirrhosis and hepatocellular carcinoma [90]. Variants of *HLA* genes were also found to be associated with treatment efficacy towards interferon's, nucleot(s)ides analogues and HBV vaccines [91]. Variants of *IL-4* gene found to be associated with increased risk of CHB in Chinese males [92]. *IFN-γ* gene variant (+879) was shown to be associated with the development of chronic

HBV infection [93]. Levels of an important cytokine, *TNF-α* was also found to be elevated in HBV infected patients [30]. Studies conducted in German and Chinese population demonstrated that a promoter region variant, -238A/G of *TNF-α* is significantly associated with chronic HBV infection susceptibility and its persistence [30, 94]. Another promoter region polymorphism of the *TNF-α* gene (-308A) was shown to be associated with HBV infection progression in Chinese Han population [95]. A study conducted by Robek et al reported that *IL-28B* or *IFN-λ-3* inhibit

the replication of HBV in hepatocytes cell lines and can be used as a new target for the treatment of the disease [96]. Various polymorphisms of *IL-28B* gene (rs12979860, rs12980275 and 8099917) have been shown to be associated with sustained viral response and in the spontaneous clearance of chronic HBV infection [97]. *IL-28B* polymorphism rs12979860 play an important role in the development of long-term chronic HBV infection [98]. Another study demonstrated the association of *IL-28B* polymorphisms (rs12979860 and rs8099917) with rapid viral response to pegylated interferon therapy in HCV patients infected with genotype 3 [99]. Besides *HLA*, cytokine and chemokines polymorphisms, various polymorphisms of other genes such as *APOBEC3G, Caveolin-1, Vitamin D receptor, TLR, LTBR, TNFAIP3* and many others are also shown to be associated with HBV infection [100-106]**.**

2.3 Autophagy

The concept of autophagy emerged during the 1960s when scientists observed that cells degrade their own contents by forming vesicle-like structures and transport it to the lysosome for further degradation.

2.3.1 What is autophagy?

Autophagy is a highly conserved biological process that helps in the degradation of dead organelles in the cell, long live proteins and other unwanted cell content with the help of lysosome and helps in maintaining cellular homeostasis. The pathway begins with the sequestration of dead cell organelles and cytoplasmic content via a double- membrane termed as phagophore which can be derived from several cellular compartments such as endoplasmic reticulum, Golgi complex, mitochondria, or Endoplasmic reticulum-mitochondria associated membranes as well as the plasma membrane [107]. The autophagy pathway is an important component of defense against many viral infections and pathogen degradation [108].

Autophagy performs various other functions such as cellular differentiation, housekeeping, growth control, cell defense, tissue remodeling, and acclimatization [109]. During starvation or in nutrient-deprived conditions autophagy transfer, damaged cellular content for degradation and promotes recycling of nutrients [110, 111].

2.3.2 Types of autophagy

In higher eukaryotes, mainly three types of autophagy process have been described; macroautophagy, microautophagy, and chaperone-mediated autophagy; all degrade cytosolic contents via lysosome with a common mode of degradation but with different mechanism [112-114]. The pictorial representation of the different types of autophagy is shown in Figure 2.7.

2.3.2.1 Microautophagy

Microautophagy is the direct engulfment of the cytoplasmic constituents by the lysosome [115]. In this process, lysosomal membrane invaginates and differentiates into an autophagic tube that encloses degraded cytoplasmic constituents [116]. Vps1p a member of dynamin-related GTPase family helps in the regulation of invagination process [117]. Microautophagic invaginations are normally lipid-rich and transferred into the lumen for degradation and they regulate lipid to protein ratio on the lysosomal surface [118]. Microautophagy is a constitutive process but starvation conditions and rapamycin can also induce it [116].

2.3.2.2 Macroautophagy

It is another type of autophagic process begins with the formation of double-membrane vesicle called an autophagosome. Macroautophagy consists of various molecular processes such as the formation of autophagosome, nucleation, elongation, maturation, and degradation of autophagosome [119]. Autophagosome enveloped degraded proteins, intracellular pathogens and other targeted cytosolic constituents fuse with the lysosome (autolysosome) for further degradation [120]. After degradation, products are recycled to the cytosol to generate energy, to protect cell under stress conditions and to maintain cellular homeostasis [121, 122]. Microautophagy and macroautophagy are further of two types, selective and nonselective and are best characterized in yeast [123]. Nonselective autophagy is used under starvation conditions for processing of bulk cytoplasm, whereas selective autophagy use set of machinery and various specific components for targeting damaged cell organelles, pathogens etc [120].

2.3.2.3 Chaperone-mediated autophagy

The third type of autophagic process is known as chaperone-mediated autophagy in which translocation of targeted proteins across the lysosomal membrane occurs with the help of heat shock protein-70 (chaperone protein). Targeted proteins and chaperone proteins are recognized by the lysosomal membrane receptor, lysosomal-associated membrane protein 2A (LAMP-2A) that further leads to their unfolding and degradation [124]. Proteins that undergo degradation by chaperone-mediated autophagy are selected individually through a recognition motif present in the amino acid sequences of CMA substrate that allow the removal of specific proteins without disturbing non-targeted neighboring proteins making CMA an efficient process [125]. Only specific proteins that contain a unique recognition motif undergo degradation by chaperone-mediated autophagy (CMA), making it an efficient system of degradation of damaged proteins [125]. The molecular basis of autophagy was first connected to the human diseases by the Beth Levine's laboratory through the identification of *BECN1/VPS30/ ATG6* as a tumor suppressor gene [126].

2.3.3 Steps of autophagy process

1. Induction and nucleation with the formation of an isolation membrane (also called phagophore).
2. Atg5–Atg12 conjugation, their interaction with Atg16l1 and multimerization of Atg5–Atg12- Atg16l1 complex at the phagophore
3. Processing of LC3 and its insertion into the phagophore membrane
4. Capturing of targets for degradation
5. Fusion of the autophagosome and the lysosome, the digestion of the autophagosome content and the release of the digested components back to the cytosol [127].

Phagophore membrane originated from the endoplasmic reticulum, Golgi apparatus and from the outer membrane of mitochondria. In the yeast, the pre-autophagosomal structure (PAS) that contains Atg9 is the precursor of phagophore formation and no such structure is still described in mammals [128]. Two ubiquitin-like conjugation systems help in the elongation and maturation of autophagosome. Initially, Atg5 make conjugate with Atg12 in the presence of Atg7 (E1 like ubiquitin) and Atg10 (E2 like ubiquitin system) [128]. Atg5-Atg12 make a complex with Atg16l1

and the Atg5-Atg12-Atg16l1 complex dimerize itself in the outer membrane of phagophore and helps in the formation of the autophagosome [128]. LC-II is also essential for the closure of autophagosome and it is present in the outer membrane and in the lumen of the autophagosome [129]. Until now in the yeast approximately 40 autophagy-related proteins have been identified, among them, 18 proteins are involved in autophagosome formation and most of them have mammalian homologs [21, 129]. For cargo selection, LC3-II binds to the various adaptor proteins like p62, Nbr-1 and Alfy and their associated ubiquitinated protein. After that autophagosome fuses with the vesicle and the vesicle fuses with the lysosome, which starts the degradation of selected cargo, and associated adaptor proteins [21].

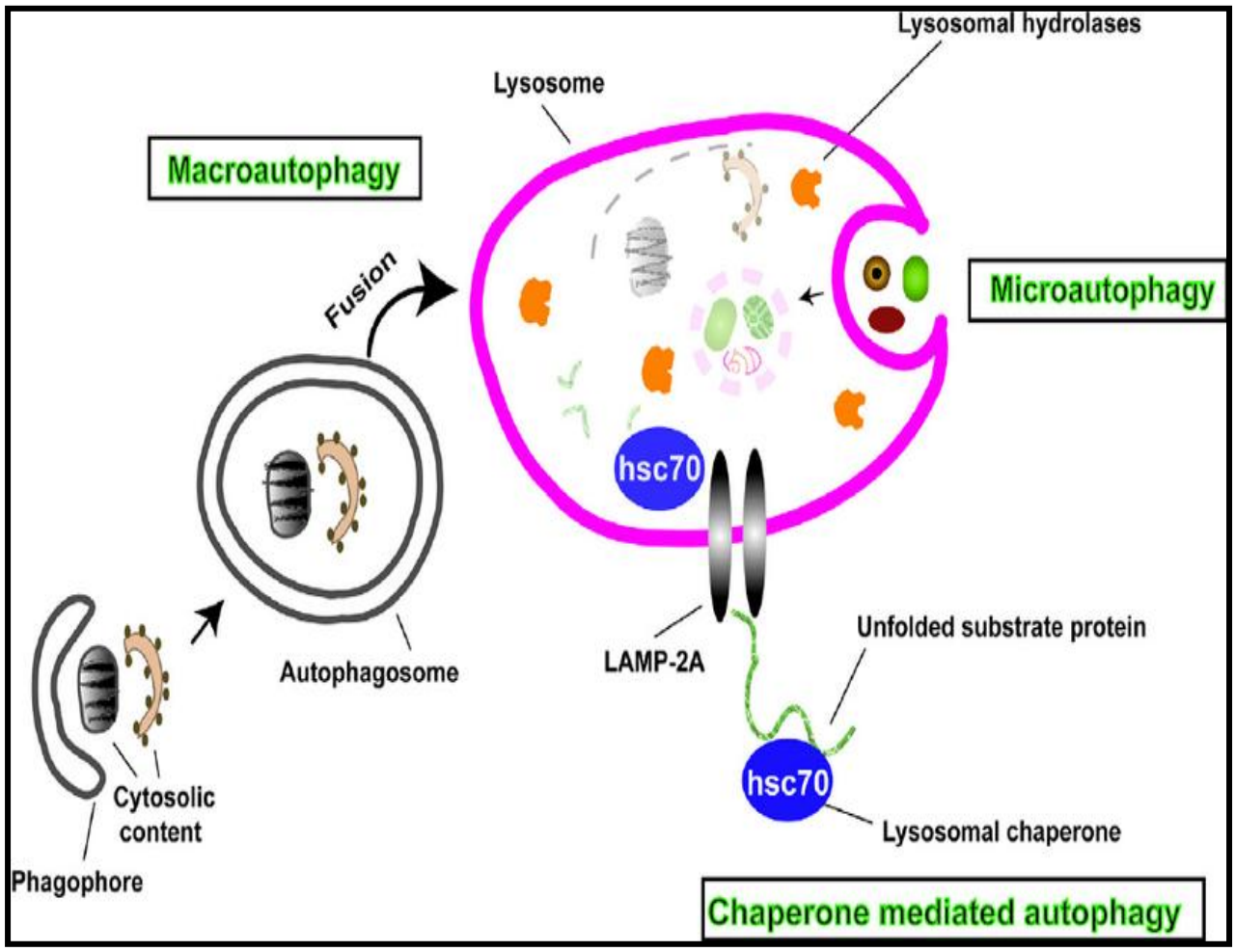

Figure 2.7: Diagram showing types of autophagy and various steps involved in the pathway [130].

2.4 Autophagy genes and their wide role in various diseases

Autophagy is a multistep process which helps in maintaining cellular homeostasis and defects in various steps of the pathway lead to the implications of a wide range of diseases such as neurodegenerative diseases (Alzheimer's, Parkinson's), various cancers, liver diseases, cardiovascular diseases and many immune-related disorders [131]. Liang and colleagues reported that monoallelic loss of Beclin-1, that helps in the initiation of autophagy, leads to cancer and it was the first connection that shows the relationship between autophagy and cancer [126]. Figure 2.8 shows that defects in the autophagy genes that are involved in the initiation and autophagosome formation lead to several diseases. During the past few years, researchers are paying attention in exploring the relationship of autophagy and viruses [108]. Autophagy-related genes (ATGs) play the antiviral role that helps in the elimination of viruses but various studies show that viruses evade the autophagy process and use the pathway for their own replication [108]. Studies have shown that autophagy pathway could be modulated for the development of potential therapeutic targets for a wide range of diseases [132].

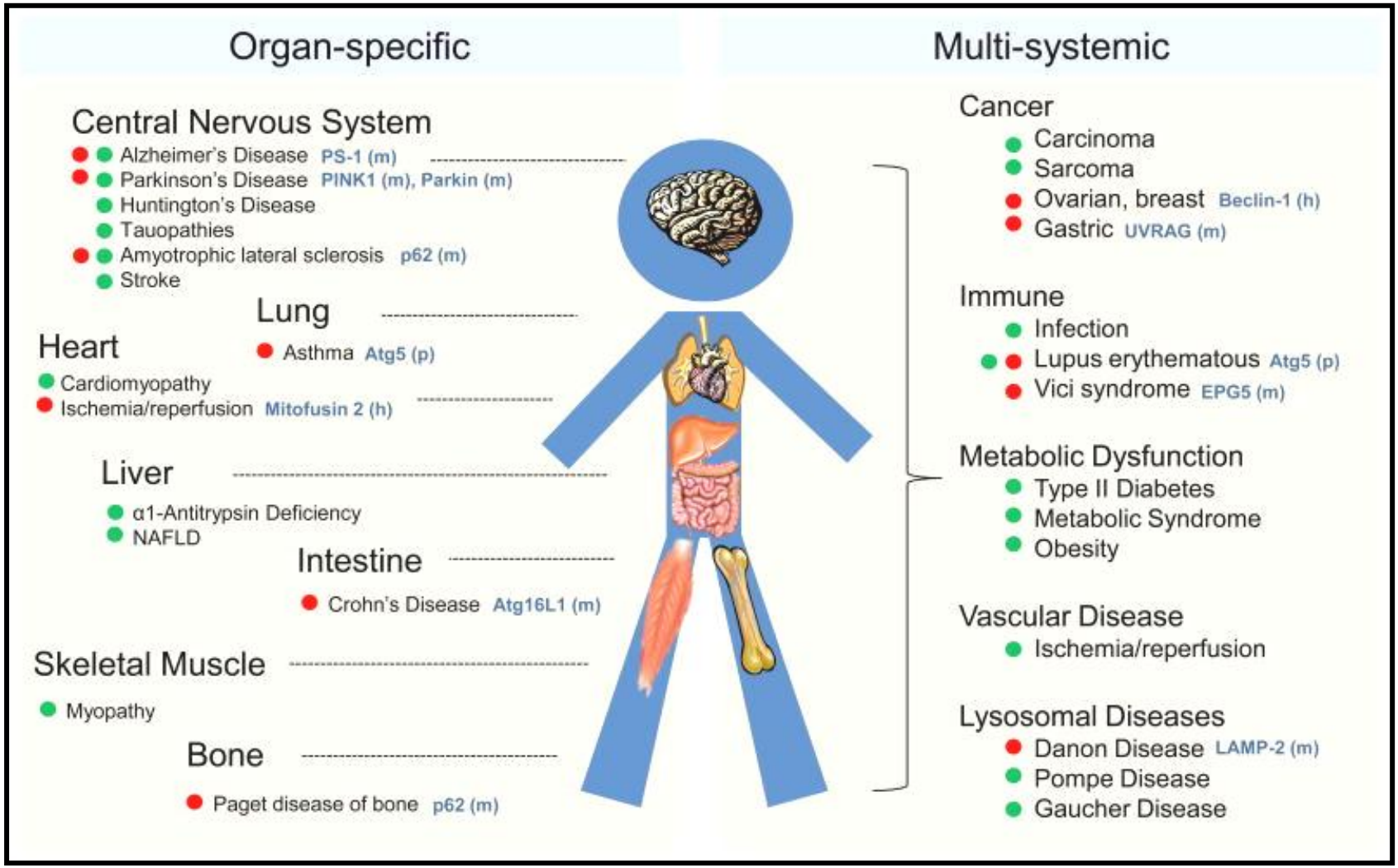

Figure 2.8: Various diseases resulted from defects in autophagy genes [131].

2.5 HBV and autophagy

The relationship of autophagy process with various viruses has been well-studied e.g. enteroviruses (poliovirus and coxsackievirus) [133]. Studies have shown that infection with these viruses results in the formation of double-membrane vesicles (autophagosome) which serves as the site for the replication of these viruses [134, 135]. Many other viruses such as dengue virus, human parainfluenza virus type 3, influenza A virus, HIV use different mechanisms to utilize this pathway for their benefit [133]. However, the exact mechanism how HBV uses this process is still controversial. A study conducted by Dona and colleagues reported that HBV helps in the enhancement of autophagy response without increasing the protein degradation by this pathway, hence use the autophagy process for its own replication [31]. They demonstrated that this pathway could be targeted for the treatment of HBV infected patients [31]. In another study conducted by Tian and colleagues, liver-specific knockout of Atg5 gene was found to inhibit autophagy process in transgenic mice and hence HBV replication [136]. Small surface proteins (SHBs) of HBV were also reported to induce the autophagy process by interacting with an autophagy protein LC3 [22].

It has been reported that suppression of autophagy resulted in reduced expression of HBV polymerase and HBcAg, but exerts no effect on expression of HBsAg [23]. Moreover, another study reported that HBx protein of the virus activates the autophagy process, but inhibits lysosomal-associated degradation that leads to the consequences of HBV associated hepatocellular carcinoma (HCC) [25]. Two other studies demonstrated that HBx protein activates the autophagy process by up-regulating the BECN1 gene expression or by activating class III phosphatidylinositol 3- kinase [24, 31]. A study reported that autophagy machinery is also required for the envelopment of HBV but not helpful in the virus release [22]. Studies reported that HBV uses the autophagy process for its envelopment and replication by inhibiting the autophagy degradation pathway. A study showed that *ATG16L1* (autophagy gene) gene might have role in HBV infection as the mRNA level of the gene was found significantly increased in the HepG2 and HepG2.2.15 cells lines [137]. Another important autophagy gene, (*IRGM)* is involved in autophagy-mediated immunity against bacteria and many viruses seem to have developed strategies to influence autophagy through the selective targeting of Irgm protein [138].

ATG16L1 and *IRGM* play important roles in autophagy process and might play role in HBV pathogenesis.

2.6 Immunity-related GTPase M (*IRGM*) gene

Immunity-related GTPases (IRGs) belongs to p47 GTPase family and are one of the earliest known resistance system against intracellular pathogens like bacteria and protozoa [139]. Immunity-related GTPases (IRGs) are induced by IFN-γ family of proteins and various proteins of this system are found to accumulate and activate at the vacuolar membrane of many pathogens like *Toxoplasma gondii, Chlamydia trachomatis* and *Encephalitozoon cuniculi* and helps in their destruction [140, 141]. In mouse, 23 IRGs are divided into 5 different subfamilies: *IRGA, IRGB, IRGC, IRGD, and IRGM* based on homology across the GTP-binding domain [142]. Only two copies of IRGs, *IRGC* (chromosome 19) and *IRGM* (chromosome 5) are present in humans, out of them *IRGC* is not involved in immunity but *IRGM* gene plays a role in autophagy-targeted destruction pathogens like *Mycobacterium tuberculosis* and *Salmonella typhimurium* [139, 142]. Based on the lysine (canonical) and methionine (non-canonical) containing GI motifs in the conserved GTPase domain, IRGs are divided into GKS and GMS IRGs [143]. *IRGM* subfamily possesses non-canonical GMS sequence of GTP-binding compared to other subfamilies, which have canonical GKS sequence [144]. *IRGM* is a 55.878 kb gene located at chromosome 5q33.1 and encodes a GTP binding protein of 181 amino acids, that helps in autophagy regulation in response to intracellular pathogens and plays an important role in innate immunity [106]. *IRGM* gene comprises 5 exons, first long and coding exon is followed by 4 shorter exons that extend 50 kb downstream from the first coding exon [145]. Five different splice isoforms of *IRGM* (a-e) are present in humans, which differ with each other in the C-terminal end [138]. Unlike others IRGs gene, the promoter region of *IRGM* gene has lost its interferon's response elements; hence its function cannot be induced by IFN-gamma [145]. Insertion of Endogenous Retrovirus elements (ERV9) in the *IRGM* gene during the evolution and is responsible for the rebirth of the gene and it serves as a functional promoter of the *IRGM* gene [146].

2.6.1 *IRGM* and autophagy

Irgm helps in the autophagy process by interacting with proteins of autophagy pathway such as Atg5, Atg10, MAP1CL3C and SH3GLB1 that plays role in the initial step of autophagosome formation [45]. Recent studies have shown that Irgm also interacts with two other autophagy-related proteins Ulk1 and Beclin-1 that helps in the formation of autophagosome initiation complex and plays an essential role in innate immunity [46]. Figure 2.9 shows a schematic representation of the *IRGM* gene involved in the autophagy process**.** Mouse ortholog of the *IRGM*, *IRGM1 was* the first gene demonstrating the role of autophagy in immune responses against intracellular microorganisms [147]. Singh et al in a study reported that with the help of mitochondrial lipid (cardiolipin), Irgm translocates to mitochondria and regulates the autophagy process by affecting mitochondrial fission [148]. *IRGM* gene also induces autophagy process by converting LC-1 to LC-II in the human macrophages and decreases *Mycobacterium tuberculosis* load and provides resistance against the pathogen [149].

2.6.2 *IRGM* gene polymorphisms and diseases

Number of studies demonstrates the role of many polymorphisms of *IRGM* gene in various diseases including leprosy, sepsis, gastric cancer, language impairment, Crohn's disease and tuberculosis [47-52]. Single Nucleotide Polymorphisms (SNPs), the most common genetic variations in the human genome, have been shown to be involved in various functions such as phenotypes, protein structure stability, control of gene expression etc. and have been shown to associate with many diseases [150]. Polymorphisms in *IRGM* gene regulate the expression of many cytokines including IL-4, IL-6, IFN-γ, IL-1β and play an autophagy-mediated antimicrobial role [47, 151].

2.6.2.1 *IRGM* and Crohn's disease

IRGM gene polymorphisms that are associated with tuberculosis and Crohn's disease are one of the earliest known examples demonstrating the role of autophagy in human diseases [152]. McCarroll and colleagues in their study reported a 20 kb deletion polymorphism upstream of the IRGM gene in strong linkage disequilibrium with the SNPs that are strongly associated with the Crohn's disease [153]. Wellcome Trust Case Control Consortium (WTCCC) also discovered

many SNPs near the *IRGM* gene that are associated with the pathogenesis of Crohn's disease [154, 155]. Prescott and colleagues reported that small indel polymorphism in the promoter region and 5'UTR region of the *IRGM* gene are associated with Crohn's disease susceptibility [156]. Reduced expression of *IRGM* gene was also observed in the lymphocytes of Crohn's disease patients [156].

Glas et al in a Genome-Wide Association Study (GWAS) analyzed six IRGM SNPs and reported a 20 kb deletion polymorphism (13371189) upstream of IRGM gene as a candidate SNPs, which was found to be associated with Crohn's disease in the German population [157]. In another study, three SNPs, rs1000113, rs9637876 and rs13361189 of *IRGM* gene were found significantly associated with Crohn's disease in an Indian population [158]. Exonic region polymorphism of *IRGM* gene, rs10065172 was found significantly associated with increased susceptibility to Crohn's disease [159].

2.6.2.2 *IRGM* and tuberculosis

Various studies demonstrated the role of many polymorphisms of IRGM gene in the susceptibility of tuberculosis. Singh et al induced the autophagy in the U937 cell lines (macrophage cell lines) by treating them with Human IFN-g (hIFN-g) and then silence the *IRGM* expression with the help of siRNA [149]. Silencing of *IRGM* resulted in decreased levels of LC3-II as in comparison to controls group, which shows that in the human macrophages *IRGM* plays an essential role in the completion of autophagy process [149]. Che et al sequenced the 1.7 kb promoter region of the *IRGM* gene and they analyzed three SNPs (1208 A/G (rs4958842), −1161 C/T (rs4958843), and −947 C/T (rs4958846) in tuberculosis patients and in healthy controls among Chinese population [160].

They reported that polymorphisms 1208 A/G (rs4958842) was associated with decreased susceptibility to tuberculosis and no association was observed for other two polymorphisms [160]. In another replicating study conducted in Iranian population, variants −1161 C/T and −947 C/T were found to be associated with decreased susceptibility to tuberculosis infection [161]. These promoter region polymorphisms were again studied in Chinese Hubei Han population and polymorphism −947 C/T was again found to be associated with decreased susceptibility to tuberculosis [51]. Two haplotypes, ACC and ACT of these polymorphisms were

also found to be associated with decreased and increased susceptibility to pulmonary tuberculosis and in addition, the ACT haplotype reduced the luciferase activity of *IRGM* promoter and hence decreased the expression of *IRGM* gene in tuberculosis patients [51]. A study in African population reported that the promoter region polymorphism of *IRGM* gene (-261T) when present in homozygous TT condition, might enhance the *IRGM* gene expression which ultimately increases the autophagy process and hence degradation of the translocated bacteria [162]**.**

Another polymorphic allele of the exonic region of *IRGM* gene, (rs10065172 T) was found to be associated with decreased susceptibility of tuberculosis in Asian population [163]. In another replication study in African Americans, rs10065172 C/T genotype was associated with decreased *IRGM* expression in lymphoblastoid cell line and makes individuals more susceptible to tuberculosis infection [164]. Two polymorphisms of the *IRGM* gene, rs4958842 and rs13361189 were found to be associated with increased risk of leprosy [47]. The study demonstrated that *Mycobacterium leprae* infected individuals with rs13361189 CC genotype expressed significantly higher levels of cytokines (INF-γ and IL-4) than those individuals with TT genotype. *IRGM* gene also provides autophagy-dependent antibacterial response against *Salmonella thyphymurium* and adherent-invasive *Escherichia coli* [165, 166].

2.6.2.3 *IRGM* and viral diseases

Roles of various polymorphisms of *IRGM* gene have been well documented in the bacterial diseases, but their role in viral diseases still needs attention. By generation of yeast two-hybrid system maps and with the help of bioinformatics analysis, Gregoire et al reported the interaction of 44 human autophagy-associated protein with around 83 proteins of several RNA viruses [45]. Irgm protein found to interact with other various proteins of autophagy pathway that includes Atg5, Atg10, MAP1CL3C and SH3GLB1 and LC3 [45, 138]. Irgm protein reported to interact with 12 viral proteins of five different classes of RNA viruses Chikungunya virus (ChikV), Mumps virus (MuV), Hepatitis C virus (HCV), Measles virus (MeV), and HIV-1 and except for MuV virus all other viruses reported to manipulate the autophagy process for their own replication [138].

A recent study reported that in response to HCV infection, *IRGM* regulates the, fragmentation of Golgi membranes and autophagy process that ultimately enhance the replication of the virus

[167]. Infection with various viruses modulates the autophagy process by using different mechanisms and use the process to promote their replication and this pathway could be targeted to combat the viral infections [145].

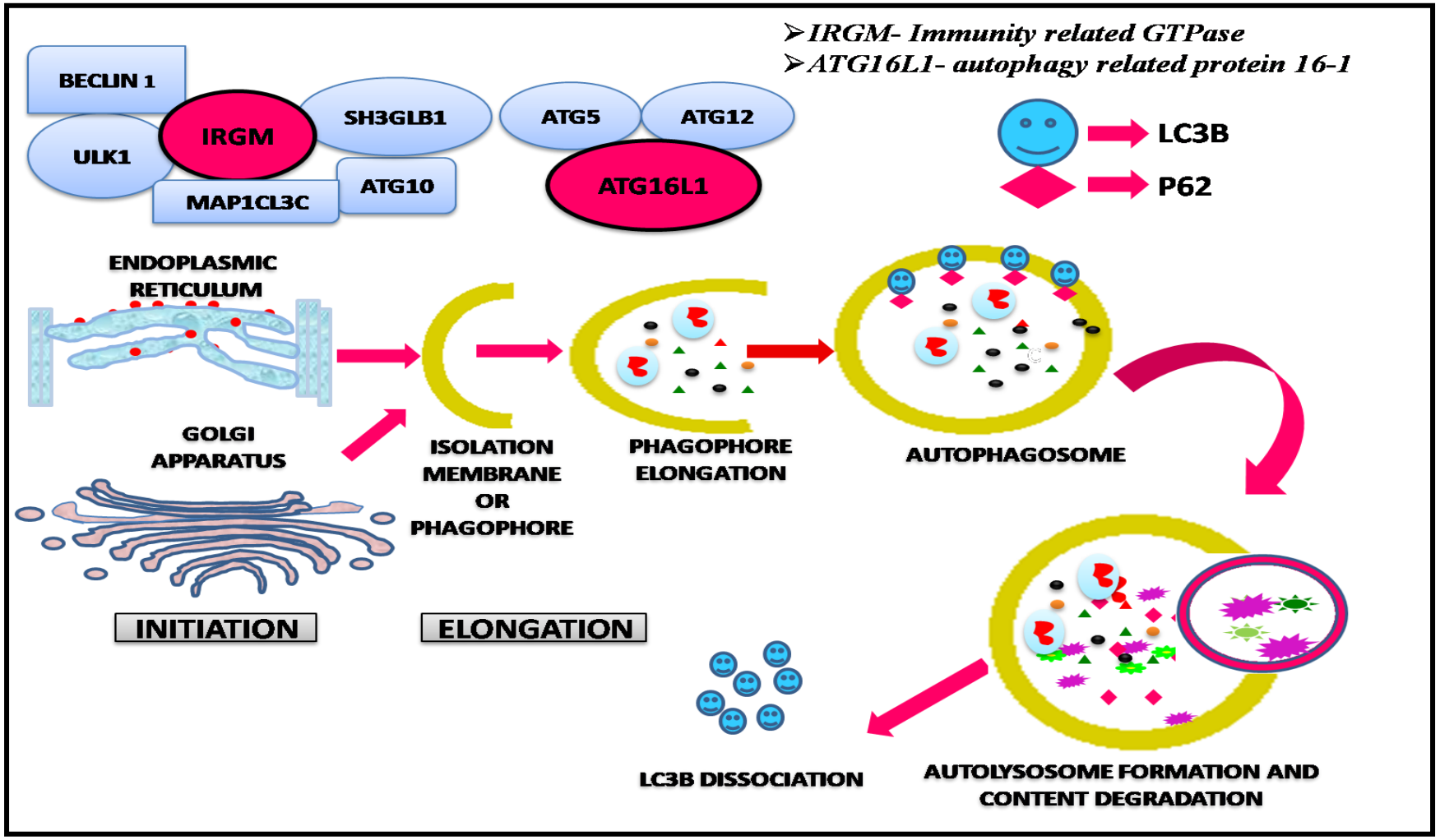

Figure 2.9: Schematic representation of the macroautophagy-showing role of *IRGM* and *ATG16L1* in initiation and elongation processes of autophagy.

2.7 Autophagy-related 16 like 1 (*ATG16L1)* gene

ATG16L1 gene is mapped to human chromosome 2q37.1 and encodes a protein of 607 aa [168]. *ATG16L1* is an 85.624 kb gene and comprises of 19 exons [169]. *ATG16L1* gene consists of 18 transcript variants that encode different isoforms of the protein [170]. *ATG16L1*-203 transcript of the gene codes for the 607 aa protein [170]. Out of many essential genes which are involved in autophagocytosis, *ATG16L1* is an important gene that targets and helps in the destruction of proteins that are derived from pathogen during innate immune response [171]. During nutrient-deprived conditions, *ATG16L1* controls the production of IL-1β, which is induced by toll-like receptor by downregulating p62 levels and helps in clearance of intracellular pathogens [172, 173].

2.7.1 *ATG16L1*, autophagy, and various human diseases/disorders

The protein encoded by *ATG16L1* gene is an important component of a large protein complex that is crucial for autophagy process [34]. Atg16l1 makes a 350 kDa multimeric complex with Atg5 and Atg12 and the Atg16l1 complex functions as E3 like enzyme of ubiquitin system [174-176]. Atg16l1 protein of mammals contains N terminal domain that binds to ATG5, C terminal domain of WD repeats and a coiled-coil domain [177]. On the other hand yeast, Atg16l1 protein contains only a coiled-coil domain and Atg5 binding N terminal domain [178]. WD domain at carboxyl terminal of Atg16l1 helps in recruitment and binding of the Atg16l1 complex with ubiquitin and direct binding occurs with FIP200 [179]. Yeast Atg16l1 protein only contains an N-terminal domain that binds to Atg5 and a coiled-coil domain hence it is much smaller than mammalian Atg16l1 protein [178].

Figure 2.9 shows the schematic representation of the *ATG16L1* gene involved in the autophagy process. Studies reported that WD domain provides docking stage for an amino acid motif that present in the intracellular region of TMEM59 (transmembrane protein) and other proteins that leads to increase in autophagy process (xenophagy) by the activation of LC3 lipidation [36]. The *ATG16L1* complex localizes to the isolation membrane and on completion of autophagosome, formation, complex gets dissociated from the membrane [180]. Studies on various populations reported the role of many polymorphisms of *ATG16L1* gene in various diseases such as psoriasis, palmoplantar pustulosis and Inflammatory bowel diseases (IBD) mainly Crohn's disease (CD)

[40, 41, 181-183]. Defects in the autophagy process in the neurons lead to various neurodegenerative disorders such as Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease and many others [184]. Studies reported a large number of autophagic vesicles in the brain of patients with neurodegenerative disorders [185]. A recent study on rat model showed that Atg16l1 interact with protein Kif21a (Kinesin Family Member 21A) and this interaction helps in the transportation of autophagic vesicles in neurons of rat [185]. Knockdown of *ATG16L1* in the rats showed decreased levels of autophagic vesicles mainly autolysosome in the neurons of the rats [185].

Defects or dysregulations in the *ATG16L1* gene leads to various consequences such as imperfect antigen presentation, elevated levels of inflammatory cytokines, poor bacterial clearance and abnormal function of the Paneth cells of the small intestine [186]. It has been reported that knockdown of *ATG16L1* gene interrupts the destruction of *Salmonella typhimurium* via autophagy pathway, which is responsible for inflammatory bowel disease [187]. The coding region polymorphism rs2241880 (T300A) of the *ATG16L1* gene has been considered an important variant which is associated with IBD [188]. The polymorphism rs2241880 (A/G) presents at the C terminal domain of WD repeats and encode for a Threonine to Alanine substitution (T300A). Elevated expression of the *ATG16L1* gene has been reported in the intestinal epithelial cells and in various immune cells such as $CD4^+$, $CD8^{+,}$ and $CD19^+$ primary human T cells [188, 189]. Moreover, another study reported a reduction in antibacterial autophagy in the human epithelial cell lines, that express mutant CD associated protein Alanine as compared to cell lines that express wild protein Threonine [190].

It has been reported that nsSNP variant T300A increase the pathogen infection by enhancing the cleavage of Atg16l1 protein and thereby resulting in a decrease in autophagy that leads to metabolic stress [191]. In Caucasian population, the polymorphism T300A also reported to increase the infection risk of bacterium *Helicobacter pylori* (H. pylori) which cause gastric cancer [192]. In gastric cancer patients, T300A polymorphism of the *ATG16L1* gene found to be associated with lower risk of cancer and mRNA levels were found significantly higher in the patients with gastric cancer [43]. In the colorectal cancer patients with the mutant alanine protein (T300A), elevated levels of type I interferon's (IFN-I) and MxA protein was reported suggesting the role of cytokines in the disease [193]. *ATG16L1* gene is an important gene of autophagy

process and defects or mutations in this gene are found to be associated with a number of diseases but the role of *ATG16L1* gene in viral diseases still need to be explored. Autophagy pathway has been documented as an important pathway in the wide range of liver diseases such as chronic liver injury, liver adenoma, viral hepatitis, alcoholic and fatty liver diseases [194]. A recent study showed that Atg5-Atg12-Atg16l1 complex by interacting with Rab33 (GTPase helps in autophagosome formation) helps in the HBV capsid formation and its release [195].

In another study, Tantithavorn et al determined and compared the expression of *ATG16L1* in various cell lines such as HepG2 (human hepatoma cell lines), HepG2.2.15 (HBV genome transfected human hepatoma cell lines), THLE-2 cell lines (primary normal liver cells) and HCC patients liver tissues [137]. Elevated expression of *ATG16L1* mRNA in HepG2 and HepG2.2.15 in comparison to THLE-2 cell lines was reported and in addition, up-regulation of levels of *ATG16L1* mRNA was observed in HepG2.2.15 in comparison to HepG2 cells showing the role of *ATG16L1* gene in HBV infection [137].

2.8 Single Nucleotides Polymorphisms (SNPs)

In any two individuals, 99.9% of DNA sequences are same, 0.1% of the genome contains sequence variations, and these variations are called as Single nucleotide polymorphisms (SNPs) or SNIPs [196]. SNPs are defined as a type of polymorphism with the variation of only single nucleotide base [197]. Most of the SNPs are biallelic only less than 0.1% of SNPs are triallelic and thus they are easy to study [198]. SNPs are found to be associated with diversity in the population, susceptibility to various diseases, individual's response to medicines [196]. Around 10 million SNPs are present throughout the human genome, which shows that one SNP occurs after 300 nucleotide bases [197].

2.8.1 Applications of SNPs studies

2.8.1.1 Pharmacogenomics studies

In pharmacogenomics, genetic variations of the individuals are used for the studying of drug response (efficacy and toxicity), as it has been well documented that due to the presence of SNPs in the genes (drug metabolizing enzymes, drug targets etc) different individuals respond

differently towards various drugs [199, 200]. Every year thousands of people die due to the adverse effect of drugs and to enhance the efficacy of drugs and to decrease their adverse effects we need to study the relationship of genetic variants (SNPs) with individual's drug response [200]. In pharmacogenomics two approached are used for mapping of SNPs [201].

2.8.1.1.1 Candidate gene approach

Candidate gene approach uses the already established knowledge of the disease pathogenesis to identify genes that are related to the disease. SNPs that are present in the selected genes were then genotyped in case and control group to look for their association with the particular disease. By using this approach lots of candidate genes variants that are associated with drug response are identified such as thiopurine methyltransferase (TPMT), 5-lipoxygenase; ALOX5 etc. Various candidate genes that are associated with HBV infection are *IFNG* [93], *TNF* [93], *ESR1* [202], *VDR* [203], *MBP* [204], *CTLA4* [205] and *HLA* [206]. This approach is helpful in the developing of personalized medicines and more effective diagnostics and treatment approaches [207].

2.8.1.1.2. Linkage disequilibrium (LD) mapping

Linkage disequilibrium is defined as the non-random association between pairs of linked markers that are present on different loci [208]. Groups of genes or alleles that are inherited together are called haplotypes and the region of the human genome that consists of haplotypes is associated with the phenotype of the diseases [209]. Throughout the genome, LD helps us to understand the population history, the breeding system, and geographic subdivision pattern. In each genomic region, LD mapping helps us to understand the evolutionary history of the genes that are associated with inherited diseases and also the evolution of the linked genes [208].

2.8.1.2. Risk profiling or diagnostics

After the identification of SNPs or their haplotypes that are associated with any disease, the information generated from the data can be used to develop prognostic and diagnostic tools [210]. Two important goals of genetic risk prediction are (1) to quantify disease risk in early pathogenesis and to opting any preventive measure (2) it provides better diagnostics to individuals if established diagnostics methods are not well informative [210].

2.8.1.3. Forensic investigation

SNPs are considered as efficient markers for forensic investigation in some special cases; such as a mass disaster or in the case when DNA is fragmented substantially [211]. SNPs possess unique characteristics such as amenability to automation and DNA sample of around 60-80 bp can also be detected. Based on forensic analyses SNPs can be divided into four categories: (1) Identity-testing SNP (2) Lineage informative SNPs (3) Ancestry informative SNPs (4) Phenotype informative SNPs [211].

CHAPTER-3

MATERIALS AND METHODS

3.1 Study population

The present work was designed to ascertain the role of *IRGM* and *ATG16L1* genes in the susceptibility to hepatitis B virus infection. To achieve the power of study >0.8% 551 HBV infected subjects and 247 healthy individuals were recruited. Blood samples from HBV infected as well as healthy controls were also collected from the same geographic area. HBV infected samples of four different categories were collected from Department of Hepatology Out Patient Department (OPD) at the PGIMER Chandigarh during the period 2013-2016. The diagnosis of hepatitis B infection was based on various serological and biochemical parameters that included AST and ALT levels, presence of HBsAg and HBeAg. Patients under 18 years of age, having co-infection with any another virus such as HCV and HIV or any other kind of liver diseases were excluded from the study**.** Table 3.1 listed the criteria for inclusion and exclusion of HBV infected patients and healthy controls. Informed consents were taken from both infected individuals and healthy controls participating in the study. During sample collection, care was taken to avoid any healthy control that has personal or family history of hepatitis B. This study was performed in Department of Biotechnology and Bioinformatics (BT & BI) of Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh and the ethical committee of the Jaypee University of Information Technology, Solan approved this study.

Table 3.1: Inclusion and Exclusion criteria for the selection of HBV infected and healthy control individuals

	Inclusion criteria	**Exclusion criteria**
HBV infected patients	• HBsAg positive • Elevated levels of HBV DNA • Detection of scarring of liver • Ascites detection with ultrasound • Resident of North India	• HCV positive individuals • HIV positive individuals • Patients under 18 year of age • Pregnant women • Any other type of liver disease
Healthy Controls	• No personal or family history of HBV infection • Resident of North India • Age and sex matched to that of HBV patients	• Personal or family history of HBV infection • Individuals under 18 years of age • Pregnant women

3.2 Sample collection, transportation, and processing

Blood samples were taken with the help of sterile and disposable syringes of ml. Two types of vacutainers (with or without EDTA coating) were used for the collection of the venous blood. All the collected samples were transported to the laboratory under cold chain. Serum was separated from the blood in the plain vacutainers (without EDTA) and used for testing biochemical parameters and HBV DNA isolation. Processing of infected blood was performed carefully in the Biosafety Cabinet Class II (Telstar, BioII Advance).

3.2.1 Assessment of biochemical parameter

Serum of infected individuals was used for the identification of biochemical parameters such as Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT), IgM and IgG levels. AST and ALT levels were measured by using Erba Mannheim diagnostic kit. IgM immunoglobulins in the serum of acute patients were measured by using Bioneovan ELISA kit and the levels of IgG in chronic individuals were measured using Autobio ELISA kit.

3.2.2 DNA isolation

DNA was isolated from the blood samples collected in EDTA coated vials by using Inorganic method given by Miller et al. with minor modifications [212].

3.2.2.1 Protocol for isolation of genomic DNA from blood

- In a 2 ml micro-centrifuge tube, 400 µl of a blood sample taken and 1200 µl of RBC lysis buffer was also added and the tube was kept on a rocker at room temperature (RT) with slow shaking. The bright red color of the blood indicates lysis of the RBCs.
- After lysis, blood was centrifuged at 5,000 rpm for 2 minutes to obtain a white pellet of WBC.
- The supernatant was discarded and WBC pellet was resuspended in 300 µl TE buffer (pH 8.0).

- After vortexing, the above mixture 22 µl of 10% SDS solution was added and the mixture was incubated at 56ºC for 30 minutes.
- After incubation, 160 µl of 7.5 M ammonium acetate was added to the above mixture and mixed vigorously for about 1 minute on a vortex machine.
- The mixture was centrifuged at 13,000 rpm, for 15 minutes at room temperature.
- The supernatant was transferred to a fresh microcentrifuge tube, and to this, twice the volume of chilled ethanol (95%) was added. DNA was precipitated by gently moving the micro-centrifuge tube upside down a few times.
- To obtain the DNA pellet, precipitated DNA was centrifuged at 13,000 rpm for 10 minutes.
- The supernatant was then discarded and the pellet was washed with 150 µl of 70 % ethanol.
- The micro-centrifuge tube containing DNA has centrifuged again at 13,000 rpm for 10 minutes. The pellet was then air-dried at RT for about 10-15 minutes.
- DNA pellet obtained was dissolved in 60 µl of TE buffer (pH 7.5) by incubating it at 65º C for 10 minutes.
- The dissolved DNA was initially stored at 4ºC and finally stored at -20ºC for further use.

3.2.3 Analysis of quality and quantity of DNA

Quantity and quality of isolated DNA were checked by spectrophotometric analysis and agarose gel electrophoresis**.**

3.2.3.1 Spectrophotometric analysis

Multiskan GO 1.00.40 (µDrop Plate, Thermo Scientific) was used for the quantification of DNA in micro-liter volumes. The nitrogenous bases in the nucleotides (DNA) have a maximum absorption at about 260 nm (Lambert-Beer's equation).

3.2.3.1.1 Procedure

- The µDrop Plate was thoroughly wiped out with the help of distilled water.
- The low-volume measurement area consists of two quartz slides, the top clear quartz slide, and the bottom partially Teflon-coated quartz slide.

- The bottom slide contains 16 sample positions, arranged in a 2 x 8 matrix, onto which we pipetted out the samples.
- TE buffer (pH-7.5) was used a blank.
- The Optical density (OD) of TE buffer was subtracted from the Optical density (OD) of DNA for both 260 nm and 280 nm, which represented only the absorbance of DNA.
- Amount of DNA can be calculated by using the formula: DNA concentration (µg/ml) = Abs260 x 50 µg/ml.
- The 260/280 ratio gives an estimate of the protein contamination of the sample.
- For a good quality sample, the value should be between 1.8 and 2.0.
- A value smaller than 1.8 indicates the presence of proteins and a value higher than 2.0 indicates probable contamination, such as phenols.

3.2.3.2 Agarose gel electrophoresis (Sambrook and Russell, 2001)

The quality of DNA was also measured with the help of agarose gel electrophoresis

3.2.3.2.1 Procedure

- Agarose el of concentration 0.8% was used in the method. We have dissolved 0.4 grams of agarose in 50 ml of 1X TAE buffer and then gently heated in a microwave oven.
- When the agarose gel cooled down then we added ethidium bromide (final concentration = 0.5 µg/ml) to it and mixed gently.
- Agarose gel was poured into a gel caster and allowed to solidify at room temperature.
- Five µl of genomic DNA was mixed with one µl of gel loading dye and the sample was loaded onto the gel and electrophoresis was done in 1X TAE buffer initially at 50 volts then at 100 volts.
- Bromophenol dye front was monitored and run was stopped when dye front reached near another side of the gel.

3.2.4 Photo documentation

- After the completion of electrophoresis, the gel was removed from the tank and placed on the UV-transilluminator of Gel documentation system (Bio-Rad).

3.3 Genotyping of Single Nucleotide Polymorphisms

Single nucleotide polymorphisms of two genes *ATG16L1* (rs2241880, rs2241879, rs13005285 and rs7587633) and *IRGM* (rs4958842, rs4958843, and rs4958846) included in the study were genotyped by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. We can perform PCR-RFLP if either natural restriction enzyme site containing polymorphic site present or by creating artificial restriction site. When natural restriction site for any enzyme is not present, so to perform PCR-RFLP, mismatches can be introduced in the primers accordingly for the creation of artificial restriction site for the available restriction enzyme. Artificial restriction site based PCR-RFLP is called artificial restriction length polymorphism (A-RFLP) method.

3.4 Strategy for the creation of restriction site

In order to develop A-RFLP methods, we have designed forward primers manually in such a way that nucleotide mismatch/s towards 5´ end resulted in the creation of restriction enzyme sites after PCR amplification. The reverse primer was also designed manually and various parameters of both the primers were analyzed by using IDT OligoAnalyzer tool [213]. These primers bind adjacent to the polymorphic site and the polymorphic nucleotides present in this site are added by the polymerase after amplification. To differentiate digested products proficiently a tail of 39 bp of A and C nucleotides was added that resulted in increase in the smallest DNA fragment. Figure 3.1 shows the schematic representation for the creation of an artificial restriction site [214].

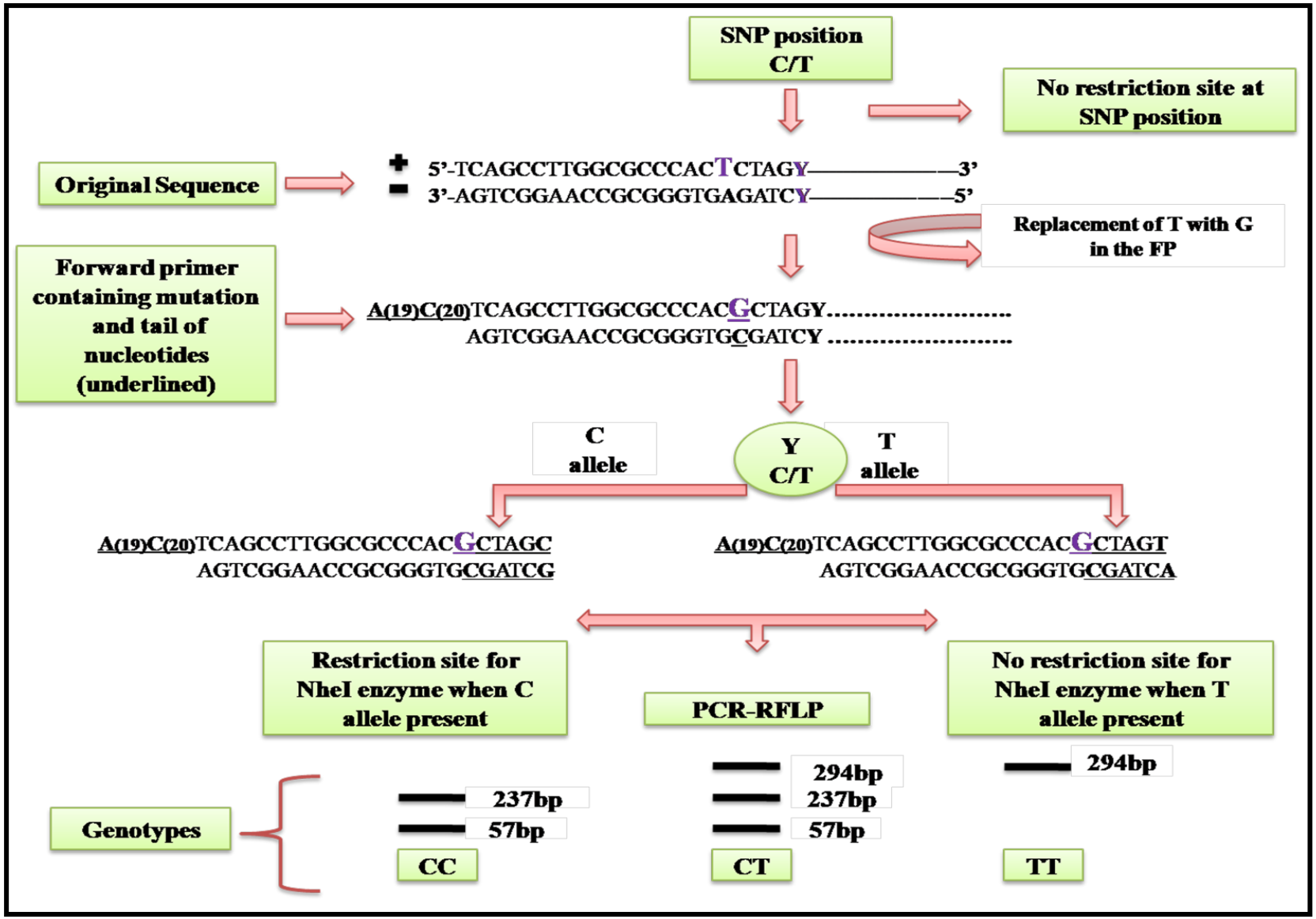

Figure 3.1: Schematic diagram for creation of artificial restriction site for development of A-RFLP [214].

3.5 Genotyping of three promoter region polymorphisms rs4958842 (G/A), rs4958843 (T/C) and rs4958846 (T/C) of *IRGM* gene

The three-promoter region polymorphisms (rs4958842, rs4958843, and rs4958846) do not have any restriction enzyme digestion site, therefore, we developed A-RFLP methods for genotyping these polymorphisms. Primer sequences and restriction enzymes used to perform the PCR-RFLP have been shown in Table 3.2**.** A tail (of nucleotides A and C) of approximately 39 bp was also added in the forward primer to differentiate restriction enzyme digested products. Amplification with these primers results in the introduction of restriction sites of enzymes *Nru*I (for rs4958842 A/G when A is present), *Nhe*I (for –rs4958843 C/T when C is present) and *Sal*I (for rs4958846 C/T when C is present).

3.5.1 Optimization of PCR amplification conditions and sequencing of samples

We have tried various parameters for optimization of PCR amplification conditions for the three SNPs and these parameters included gradients of NEB Taq DNA polymerase, Tm, and primers concentration. We have tried temperature gradient range from 58ºC to 68ºC for rs4958842, 59ºC to 69ºC for rs4958843 and 58ºC to 68ºC for rs4958846. Briefly, genomic DNA for all three SNPs was amplified in 25 µl reaction mixture with a different temperature range, Taq DNA polymerase (New England Biolabs) concentration varied from 0.5U-1U, primers concentration varied from 0.08 µM -0.28 µM for each primer, 0.2 mM of dNTPs and 40 ng of genomic DNA. The PCR cycling parameters were: Initial denaturation at 95ºC for 3 minutes, followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at different temperatures 58ºC - 68ºC (rs4958842), 59ºC - 69ºC (rs4958843) and 58ºC - 68ºC (rs4958846) for 40 seconds, extension at 68ºC for 30 seconds followed by final extension at 68ºC for 7 minutes. PCR products were then checked on 2% “EtBr” stained agarose gel by using agarose gel electrophoresis.

After amplification initially ten samples were send for sequencing for the respective SNP to get the control genotypes. For sequencing PCR reaction was performed in 30 µl of reaction volume and after PCR completion, we have loaded whole of the PCR product on the gel and allowed the electrophoresis to be complete at 100 Volts. After the completion of the run, with the help of

UV-transilluminator, we cut the desired band with a sterile blade and processed the DNA for its purification from the agarose gel. After purification DNA samples were send for sequencing.

3.5.2 Optimization of RFLP conditions

Control samples that we got after sequencing, were further used for the optimization of RFLP conditions. We have optimized the units of restriction enzymes to perform A-RFLP. Units of restriction enzymes were varied from 0.5-2U for each SNP. Amplified products then subjected to digestion with respective restriction enzymes. All the three SNPs rs4958842, rs4958843, and rs4958846 were genotyped by using *Nru*I, *Nhe*I, and *Sal*I restriction enzymes, respectively. Ten µl PCR products were digested in 15 µl of the reaction mixture with varying units of restriction enzymes at 37ºC. Digested products were then resolved on 3.5% agarose gel and visualized by ethidium bromide staining under UV light.

Table 3.2: Primer sequences for amplification of target region for *IRGM* polymorphisms, nucleotides mismatches introduced in these, recognition sites and restriction enzymes used

Polymorphisms	Forward Primer sequence with tail of A and C nucleotides at 5'end[a]	Reverse Primer sequence	Recognition site	Restriction Enzyme
rs4958842 (−1208 A/G)	***A*****(19)*****C*****(20)**AGCATGCTGGCAGCCCTCGC***G***	GGGCCGGATGGCTGCTCTGA	TCGCGA*	*Nru*I
rs4958843 (−1161 C/T)	***A*****(19)*****C*****(20)**TCAGCCTTGGCGCCCAC***G***CTAG	CCCTCACTGCCAGGGGCCAT	GCTAGC*	*Nhe*I
rs4958846 (−947 C/T)	***A*****(19)*****C*****(20)**AGAGCAGCCATCCGGC***GT***CGA	TGCACTCTTCAGCCCTTGGGC	GTCGAC*	*Sal*I

3.6 Genotyping of *ATG16L1* gene polymorphisms

PCR-RFLP and A-RFLP methods were employed for genotyping of rs2241880, rs2241879, rs13005285 and rs7587633 polymorphisms. Polymorphism rs2241880 was genotyped applying PCR-RFLP method given by Csongei et al. rs2241879 was also genotyped by using the PCR-RFLP method. For other two intronic region polymorphisms (rs13005285 and rs7587633), we have designed forward primers with mismatches and SNPs were genotyped using A-RFLP methods. Primer pairs used for the amplification of all the four SNPs with the respective restriction enzymes are mentioned in Table 3.3.

3.6.1 Genotyping of rs2241880 (A/G) and rs2241879 (G/A) polymorphisms

3.6.1.1 Optimization of PCR amplification conditions

PCR amplification was done by using specific primer pairs and gradient PCR was carried out to select the optimum temperature for PCR amplification. The SNP rs2241880 was genotyped by applying PCR-RFLP method given by Csongei et al [38]. As natural restriction site was present at the SNP position for rs2241879, we have designed primers by using blast tool and further various parameters of the primers were checked by using IDT OligoAnalyzer tool [213, 215]. Genomic DNA (40ng) was amplified in a 20 µl reaction mixture containing 0.5U i-Taq DNA polymerase (iNtRON Biotechnology) 0.2 mM of dNTPs and 0.2 µM of each primer. Initially, genomic DNA was denatured at 94ºC for 3 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at different temperature range 53ºC-57ºC (rs2241880) and 48ºC-58ºC (rs2241879) for 40 seconds and extension at 72ºC for 30 seconds, and a final extension at 72ºC for 7 minutes in a thermal cycler. After the final extension amplified PCR products were removed and further analyzed on 2% ethidium bromide gel by using agarose gel electrophoresis.

3.6.1.2 Optimization of RFLP conditions

To perform restriction fragment length polymorphism, 10 µl of amplified PCR products were incubated with different units of restriction enzymes varied from 0.5-2U for both the SNPs and samples were incubated overnight at 37ºC for rs2241880 and 65ºC for rs2241879. *Sfa*NI and *Tsp*45I restriction enzymes were used for rs2241880 and rs2241879 SNPs, respectively. After the restriction digestion, digested PCR products were resolved on ethidium bromide stained agarose gel for visualization of DNA bands.

Table 3.3: Primer sequences used for amplification of target region for *ATG16L1* polymorphisms, nucleotide mismatches introduced in these, recognition sites of enzymes and restriction enzymes used

Polymorphisms	Forward Primer sequence	Reverse Primer sequence	Recognition Site	Restriction Enzymes
rs2241880 (A/G)	CTCTGTCACCATATCAAGCGTGG	TCTAGAAGGACAGGCTATCAACAGA	GCATC	*Sfa*NI
rs2241879 (G/A)	TTTGCCCCATCCCTCAT	ATTTCTTAGGAGACGCTCTG	GTSAC	*Tsp*45I
rs13005285 (G/T)	***A(19)C(20)***AGCCCAGTGGAGCAGAGAAA**G**CT	CCTCCTACAGGCATTCATTCACCTC	AAGCTT	*Hin*dIII
rs7587633 (C/T)	***A(19)C(20)***TAGGCTTGTGATGTTACTCAG**A**A**T**T	CCAGAGATGACAATCAAGATAAGGAC	GAATTC	*Eco*RI

3.6.2 Genotyping of intronic SNPs rs13005285 (G/T) and rs7587633 (C/T)

3.6.2.1 Development of A-RFLP methods

To genotype intronic SNPs rs13005285 and rs7587633 by applying PCR-RFLP, we have developed Artificial-RFLP method as no restriction enzyme site was present at the polymorphic position. The strategy of incorporating mismatches for the creation of restriction site has been already discussed in section 3.4 and shown in Fig 3.1. For the development of A-RFLP method, we designed forward primer with mismatches towards 5´-end so that amplified product should have restriction enzyme site for a *Hin*dIII and *Eco*RI restriction enzymes for SNPs rs13005285 and rs7587633, respectively.

3.6.2.2 Optimization of PCR amplification conditions

Gradient PCR was performed for the selection of optimum temperature to avoid the nonspecific amplification. Briefly, genomic DNA for the SNPs rs13005285 and rs7587633 was amplified in 20 µl reaction mixture containing 0.5U i-Taq DNA polymerase (iNtRON Biotechnology), 0.2 mM of dNTPs and 0.25 mM of each primer. Initially, genomic DNA was denatured at 94ºC for 3 minutes followed by followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at different temperature range from 55ºC-59ºC (rs13005285) and 55ºC-65ºC (7587633) for 40 seconds and extension at 72ºC for 30 seconds, and a final extension at 72ºC for 7 minutes in a thermal cycler. Amplified PCR products were analyzed on 2% ethidium bromide-stained agarose gel.

3.6.2.3 Optimization of A-RFLP conditions

10 µl of amplified PCR products were digested with restriction enzymes varied between 0.5-2U for both the SNPs and samples were incubated at 37ºC. *Hin*dIII and *Eco*RI were used for rs13005285 and rs7587633, respectively. After the restriction digestion, digested PCR products were separated on 3.5% ethidium bromide-stained agarose gel for visualization of DNA bands.

3.7 HBV DNA isolation and quantification

Serum HBV DNA levels vary significantly in various stages of HBV infection that helps in characterizing these stages and also shown to be associated with the disease progression. It has been reported that higher viral load is related to increased risk of liver cancer and hepatocellular carcinoma [216]. We were interested to correlate HBV DNA levels with the various genotypes of polymorphisms. HBV DNA from serum samples of the patients was isolated using QIAamp DNA Blood Mini Kit (QIAGEN) and the dried pellet was stored at −20°C until further use. 300 bp of HBV DNA was amplified and cloned into a pGEMT-easy vector for construction of standard curve. HBV DNA quantification was done with quantitative Real-Time PCR (using primers; forward: 5´-CCT GGY TAT CGY TGG ATG TGT-3´ and reverse: 5´-GGA CAV ACG KGC AAC ATA CCT T-3´. These primers amplify 116 bp DNA fragment) protocol given by Roberta et al.

3.8 Statistical analysis

Relative odds of the occurrence of the outcome of any disease or disorder in the presence of any kind of exposure can be measured by using Odds ratios and confidence interval (CI) signifies the precision of the odds ratios [217]. Odds ratios also compares the magnitude of any outcome in relation to exposure, OR=1 signifies that exposure does not affect odds of outcome, OR>1 signifies that exposure is associated with higher odds of outcome and lastly OR<1 shows that exposure is associated with lower odds of outcome [217]. Odds Ratios (ORs) and confidence intervals (CI) for all the alleles and genotypes were obtained by logistic regression using Review Manager5.3 software. Studied SNPs were tested for Hardy-Weinberg equilibrium in the control population. Student's t-test was used to compare continuous variables while Pearson's chi-squared analysis was used to compare categorical variables. Values are expressed as a mean ± standard deviation.

Association of the SNP with HBV infection was tested using different genetic models (Allelic model, dominant model, co-dominant model and homozygous model) in five types of patients groups (HBV vs. control, Asymptomatic vs. control, Acute vs. control, CHB vs. control and Liver cirrhosis vs. control). An association was considered significant if a p-value of < 0.05 was attained. A 2-tailed p-value <0.05 was considered statistically significant. Comparisons between

the genotypes, enzyme levels (AST, ALT) and HBV viral load levels were performed using the Student's t-test. Haplotype analysis in the *ATG16L1* and *IRGM* gene was done by SHEsis software [218]. After the haplotype analysis, the haplotypes with frequency lower than 3% were excluded.

CHAPTER-4

RESULTS

4.1 Demographic characteristics of HBV infected patients and healthy control subjects enrolled in the study

A total of 551 HBV infected subjects (220 Female, mean age 35.65±12.58 and 331 male, mean age 38.49±14.81) and 247 healthy individuals (108 Female, mean age 37.26±11.79 and 139 male, mean age 35.24±13.76) were recruited for the study. Blood samples of HBV infected patients were collected from the Hepatology, Out Patient Department (OPD) at the Postgraduate Institute of Medical Education & Research (PGIMER) Chandigarh, India, from 2013 to 2016. The diagnosis of HBV infection was based on various biochemical and serological parameters (AST and ALT levels, HBsAg, HBeAg and HBV DNA levels). On the basis of clinical diagnosis report of the patients, they were classified into different HBV infection stages that include (1) asymptomatic carrier, (2) acute, (3) chronic and (4) cirrhosis. Asymptomatic HBV carriers were HBsAg positive for a period more than six months but carry normal AST and ALT levels.

The immune system of the individuals makes antibodies against HBV infection and is able to get rid of the infection within six months. This short-range infectivity is called acute hepatitis B. Patients belonging to the acute group were IgM positive with increased levels of AST and ALT enzymes. Chronic group of HBV patients was positive for HBsAg for six or more than six months, with elevated levels of enzymes AST and ALT and HBV DNA more than 10^5 copies /ml with no evidence of cirrhosis. Chronic group patients were positive for IgG and IgM immunoglobulins. Cirrhosis group patients were HBsAg positive with elevated AST and ALT levels, HBV DNA more than 10^5 copies/ml, scarring of liver and ultrasound also detect ascites development.

Patients having co-infection with other viruses such as HCV, HDV, and HIV or any other kind of liver disease were excluded from the study. All the healthy control subjects were matched with the infected individuals for their age, gender, and geographical location. Clinical and demographic parameters of HBV infected individuals and healthy controls are listed in Table 4.1

Table 4.1 Demographic and clinical characteristics of all subjects included in the study

Variables	Total HBV infected Individuals (n=551)	HBV Categories				Control (247)
		Asymptomatic (n=111)	Acute (n=104)	CHB (286)	Cirrhosis (50)	
Female count	225(41%)	41 (37%)	44 (42%)	123 (43%)	17 (34%)	129 (52.2%)
Male count	326 (59%)	70 (63)	60 (58)	163 (57%)	33 (66%)	118 (47.8%)
Age (Yrs) Mean±SD	37.02±13.69	35.48±12.31	38.60±13.26	36.89±14.06	44.96±12.96	36.32±10.09
HBsAg	+ve	+ve	+ve	+ve	+ve	-ve
ALT (IU/L)	89±86.4	49.9±71.6	85±56.9	89.2±86.5	88±81	12±2.8
AST (IU/L)	87.9±72.9	59±43.6	67.6±49	87.9±73.02	87.4±71.2	16±3.2

4.2 Quality assessment and quantification of DNA

Genomic DNA of all the collected samples (patients and healthy control) was isolated applying inorganic salting out method [212]. Quality and quantity of DNA was checked on 0.8% agarose gel stained with EtBr and by spectrophotometric analysis, respectively. The average concentration of isolated DNA in the samples was observed 520ng/µl. The 260/280 ratio for all the DNA samples was in the range of 1.75-1.79. The representative image of the genomic DNA of different samples is shown in Figure 4.1.

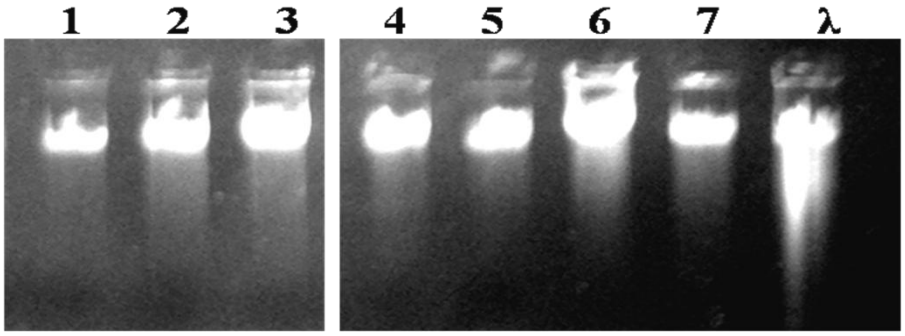

Figure 4.1 Quantification and quality analysis of DNA on 0.8% agarose gel. λ DNA concentration= 300 ng/µl and different genomic DNA samples are shown in Lane 1-7.

4.3. Genotyping of *IRGM* promoter region polymorphisms, rs4958842 (G/A), rs4958843 (T/C) and rs4958846 (T/C) by applying newly developed Artificial-Restriction Fragment Length Polymorphism (A-RFLP) methods

4.3.1 Genotyping of rs4958842 (G/A)

Briefly, after performing gradient PCR we obtained a single band of size 318 bp at temperature 66ºC. Figure 4.2 represents the representative gel image of gradient PCR. We have initially amplified 10 samples with the specific primers and amplification was checked on 2% EtBr stained agarose gel. Amplified samples were sequenced to get the control samples of known genotypes (GG, AG and AA). These controls were used to optimize the A-RFLP conditions (given in section 3.5.2 of materials and methods). Optimized reaction components along with PCR and A-RFLP reaction conditions are shown in Table 4.2.

DNA samples were amplified, digested with *Nru*I (New England Biolabs) restriction enzyme and checked on 3.5% agarose gel. Sequencing results were concordant with the A-RFLP results. A representative agarose gel picture along with chromatogram for sequencing results showing genotypes of rs4958842 SNP in the studied population is shown in Figure 4.3.[214] M represents the 100 bp DNA marker. An amplicon of 318 bp size corresponds to GG genotype; 260 bp and 58 bp bands correspond to AA genotype; and banding pattern of 318 bp, 260 bp, and 58 bp represents AG genotype. The SNP rs4958842 was genotyped in 551 HBV infected individuals and 247 healthy control subjects applying these optimized conditions of A-RFLP method [214].

Table 4.2: Optimized reaction components and amplification conditions to perform A-RFLP for genotyping *IRGM* polymorphisms

Optimized components and conditions	rs4958842 (G/A)	rs4958843 (T/C)	rs4958846 (T/C)
PCR REACTION COMPONENTS			
Genomic DNA (ng)	40	40	40
Forward primer (µM)	0.2	0.2	0.2
Reverse primer (µM)	0.2	0.2	0.2
dNTPs (mM) (New England Biolabs)	0.2	0.2	0.2
Taq DNA polymerase (U) (New England Biolabs)	0.625	0.625	0.625
Total reaction Volume (µl)	25	25	25
PCR AMPLIFICATION CONDITIONS			
Initial denaturation	95°C-3´	95°C-3´	95°C-3´
Denaturation	95°C-30´´ ×35 cycles	95°C-30´´ ×35 cycles	95°C-30´´ ×35 cycles
Annealing	**66°C-40´´**	**59°C-40´´**	**65°C-40´´**
Extension	68°C-30´´	68°C-30´´	68°C-30´´
Final extension	68°C-7´	68°C-7´	68°C-7´
ARTIFICIAL-RFLP			
Amplified DNA (µl)	10	10	10
Restriction Enzyme (IU)	1	1	1
Buffer (10X)	1X	1X	1X
Total reaction volume (µl)	15	15	15

4.3.1.1 Distribution of genotypic and allelic frequencies of SNP rs4958842 (G/A)

Genotypic and allelic frequencies of the SNP in the genotyped samples were analyzed using Review Manager 5.3. Distribution of genotypic and allelic frequencies of the SNP in 551 HBV infected samples of different categories and 247 healthy control samples are shown in Table 4.3. On comparing genotypic frequencies in both the studied groups, we found that HBV infected group had a maximum percentage of wild genotype GG (63%) and healthy controls had a maximum percentage of GA genotype (51%) as shown in Table 4.3. To see whether any genotypic and allelic difference exists between HBV infection categories they were compared with healthy controls and the percentage of genotype GG was again found to be higher in all the categories as compared to healthy controls. Percentage of wild allele G was higher in HBV infected individuals in totality and also in different categories along with healthy controls shown in Table 4.3.

Association of the SNP with HBV infection was tested using different genetic models (allelic model, dominant model, co-dominant model and homozygous model) in five analysis groups, HBV vs. Control, Asymptomatic vs. Control, Acute vs. Control, CHB vs. Control and Liver cirrhosis vs. Control. The promoter region SNP rs4958842 (G/A) was found to be associated with protection from HBV infection. On comparing frequencies of total HBV infected individuals with healthy controls, we found that A allele was statistically significantly associated with protection from HBV infection in allelic (Odds Ratio (OR) =0.61, 95%CI=0.48-0.78, p=0.0001), co-dominant (OR=0.52, 95%CI=0.38-0.71, p<0.0001) and dominant (OR=0.51, 95% CI=0.38-0.70, p<0.0001) genetic models. On comparing different categories of HBV infection, we found a protective association of the SNP in CHB group in allelic, co-dominant and dominant models. We did not find any association of the SNP rs4958842 in asymptomatic, acute and cirrhosis groups. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value under different genetic models have been shown in Table 4.3.

4.3.1.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs4958842 (G/A)

HBV DNA was quantified as mentioned earlier applying Real-Time PCR and the data from patients were segregated according to three genotypes i.e. GG, GA, and AA. Table 4.4 shows the

average levels of HBV DNA, AST and ALT levels in the patients of different genotypes GG, GA, and AA. On further analysis, we did not find any difference in HBV DNA levels in various infection stages and in any of the genetic models tested. Furthermore, we have compared biochemical parameters (AST and ALT) in different genotypes and did not observe any significant difference in the mean AST levels in different genotypes. In context to ALT levels, we observed significant association only in cirrhosis category in the homozygous model (GG vs. GA, p=0.009). No association of ALT levels was observed in other categories.

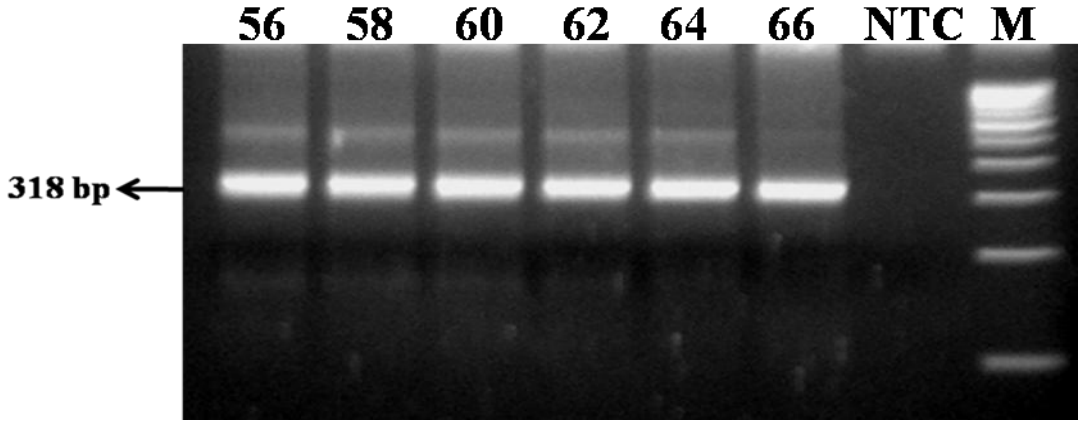

Figure 4.2: Representative agarose gel image of gradient PCR (gradient temperature in increasing order from 56°C-66°C). M represents 100 bp marker and NTC (Non-template control) represents the negative control.

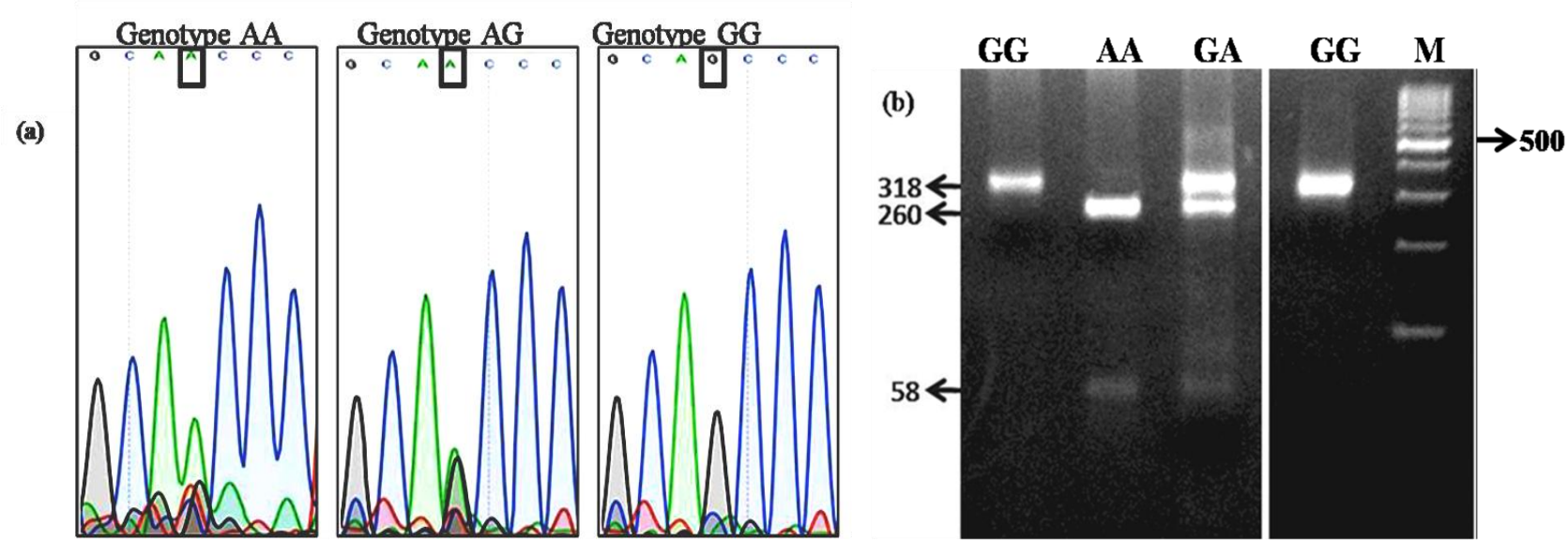

Figure 4.3 (a) Representative chromatogram of sequenced samples showing three genotypes GG, AA and GA (b) representative A-RFLP agarose gel electrophoresis picture for rs4958842 [214].

Table 4.3 Genotypes and alleles frequencies distribution of promoter polymorphism rs4958842 in HBV infected patients and healthy controls.

Polymorphism rs4958842 G/A MAF(A)=0.2037/1020	HBV (n/N[%]) (N=551)	Asymptomatic Carriers (n/N[%]) (N=111)	Acute Carriers (n/N[%]) (N=104)	CHB (n/N[%]) (N=286)	Cirrhosis (n/N[%]) (N=50)	Control (n/N[%]) (N=247)	HWE p-value
GG	348 (63)	62 (56)	58 (56)	200 (70)	28 (56)	116 (47)	
GA	197 (36)	48 (43)	44 (42)	83 (29)	22 (44)	126 (51)	
AA	6 (1)	1 (1)	2 (2)	3 (1)	0 (0)	5 (2)	-
G	893 (81)	172 (77.5)	160 (77)	483 (84.5)	78 (78)	358 (72.5)	
A	209 (19)	50 (22.5)	48 (23)	89 (15.5)	22 (22)	136 (27.5)	
HBV vs. Control	**Odds Ratio (95%CI)**			**p-value**			
GG	1^{Ref}						
GA	0.52 (0.38 to 0.71)			**< 0.0001***			
AA	0.40 (0.12 to 1.34)			0.14			
GA+AA	0.51 (0.38 to 0.70)			**< 0.0001***			
G	1^{Ref}						
A	0.61 (0.48 to 0.78)			**0.0001***			
Asymptomatic vs. Control							
GG	1^{Ref}						
GA	0.71 (0.45 to 1.12)			0.14			
AA	0.37 (0.04 to 3.27)			0.37			
GA+AA	0.69 (0.44 to 1.09)			0.12			
G	1^{Ref}						
A	0.76 (0.52 to 1.10)			0.15			
Acute vs. Control							
GG	1^{Ref}						
GA	0.69 (0.43 to 1.11)			0.13			
AA	0.80 (0.15 to 4.24)			0.79			
GA+AA	0.70 (0.44 to 1.11)			0.13			
G	1^{Ref}						
A	0.78 (0.54 to 1.15)			0.22			
CHB vs. Control							
GG	1^{Ref}						
GA	0.38 (0.26 to 0.54)			**< 0.0001***			
AA	0.34 (0.08 to 1.48)			0.15			
GA+AA	0.38 (0.26 to 0.54)			**< 0.0001***			
G	1^{Ref}						
A	0.48 (0.35 to 0.65)			**< 0.0001***			
Cirrhosis vs. Control							
GG	1^{Ref}						
GA	0.72 (0.39 to 1.33)			0.30			
AA	0.37 (0.02 to 6.91)			0.50			
GA+AA	0.69 (0.37 to 1.28)			0.24			
G	1^{Ref}						
A	0.74 (0.44 to 1.23)			0.25			

*** p-value <0.05 was considered significant and shown in bold**

Table 4.4 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs4958842 G/A in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	GG[62]	83.23±86.29		54±38		51±89	
[111]	GA[48]	81.31±53.92	0.88	64±58	0.27	51±63	1.00
	AA[1]	28	NA	44±19	NA	57±0	NA
	GA +AA[49]	78±53	0.72	58±39	0.58	56±62	0.73
	GA +GG[110]	82±74	NA	58±48	NA	NA	NA
Acute	GG[58]	1384.018±1292		73±59		93±68	
[104]	GA[44]	1449.488±1442	0.81	59±39	0.17	73±42	0.09
	AA[2]	2785±2828	0.15	80±12	0.86	94±6	0.98
	GA +AA[46]	NA	0.64	60±38	0.19	74±41	0.19
	GA +GG[102]	1411±1350	0.16	67±48	0.70	84±59	0.81
Chronic	GG[200]	1672550±2495100		105±86		108±105	
[286]	GA[83]	2421504±5704788	0.25	103±78	0.85	103±70	0.69
	AA[3]	31867±7207	0.47	38±7	0.17	25±7	0.17
	GA +AA[86]	NA	0.19	100±77	0.64	99±70	0.46
	GA +GG[283]	2183030±4920743	0.45	105±84	0.16	107±97	0.47
Cirrhosis	GG[28]	768678675±13480225705		146±46		126±71	
[50]	GA[22]	798825033±1948984531	0.42	195±94	**0.01***	187±88	**0.009***
	AA[0]	-	NA	NA	NA	NA	NA
	GA +AA[22]	NA	NA	NA	NA	NA	NA
	GA +GG[50]	NA	NA	NA	NA	NA	NA

*** p-value <0.05 was considered significant and shown in bold**

4.3.2 Genotyping of rs4958843 (T/C)

Gradient PCR was done and we obtained a single band of size 294 bp at temperature 59ºC shown in Figure 4.4. Initially, 10 samples have been amplified with the specific primers and amplified samples were sequenced to get the control samples of known genotypes (CC, TC, and TT). These controls were used to optimize the A-RFLP conditions (given in 3.5.2 section of materials and methods). Optimized PCR and RFLP reaction components and conditions for the SNP rs4958843 are shown in Table 4.2. DNA samples were amplified, digested with *Nhe*I (New England Biolabs) restriction enzyme and checked on 3.5% agarose gel. Sequencing results were concordant with the A-RFLP results.

A representative agarose gel picture along with chromatogram for sequencing results showing genotypes of rs4958843 in the studied population is shown in Figure 4.5 [219]. M represents the 100 bp DNA marker and an amplicon of 294 bp size corresponds to TT genotype; 237 bp and 57 bp corresponding to CC genotype; and banding pattern of 294 bp, 237 bp, and 57 bp represents TC genotype. Further, polymorphism rs4958843 was genotyped in 551 HBV infected individuals and 247 healthy control subjects by applying these optimized conditions of A-RFLP method. [219]**.**

4.3.2.1 Distribution of genotypic and allelic frequencies of SNP rs4958843 (T/C)

Distribution of genotypic and allelic frequencies of the SNP in 551 HBV samples and 247 healthy control samples are shown in Table 4.5. This SNP was in HWE equilibrium in the control population (p=0.3258). On comparing genotypic frequencies in both the studied groups, we found that both the groups (HBV infected and healthy controls) had a maximum percentage of TT genotype; 59%, and 62%, respectively. Further, genotypic and allelic frequencies of different HBV infection categories were compared with healthy controls and the percentage of TT genotype and T allele was found higher in all the categories along with healthy controls, shown in Table 4.5. Both the groups had almost equal percentage of T allele i.e. 79% in infected group and 79.4% in healthy control group.

We did not find any association of rs4958843 (T/C) with HBV infection in the allelic model (OR =1.03, 95% CI=0.79-1.33, p=0.82). On analyzing by using various genetic models, we again did

not find any association of the SNP. On comparing different stages of HBV infection, we found significant association of the SNP in the susceptibility of CHB group in co-dominant (OR =1.52, 95% CI=1.07-2.16, p=0.01) and dominant (OR =1.41, 95% CI=1.00-2.00, p=0.04) genetic models. No association of the SNP rs4958843 was observed in asymptomatic, acute and cirrhosis groups. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value under different genetic models have been shown in Table 4.5.

4.3.2.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs4958843 (T/C)

Mean levels of HBV DNA, AST, and ALT along with standard deviation (STDEV) are shown in Table 4.6. We found a statistically significant difference in DNA levels on comparing homozygous mutant genotype with heterozygous genotype (TT vs. TC, p=0.0003) and in a combination of heterozygous and homozygous mutant genotypes (TT vs. TC+CC, p=0.0003). No significant difference was observed in the mean values of HBV DNA levels in all the HBV infected categories when they were correlated with different genotypes of the SNP. Furthermore, genotypes were correlated with biochemical parameters (AST and ALT) by using various genetic models.

No significant differences in the mean AST levels of different genotypes were observed in any of the HBV categories and in any of the genetic model tested. On comparing ALT levels significant difference in the mean ALT levels was observed in the chronic category when we compared homozygous wild genotype with homozygous mutant and heterozygous genotype (TT vs. TC+CC, p=0.02). We did not observe any significant difference in mean ALT levels in other HBV categories.

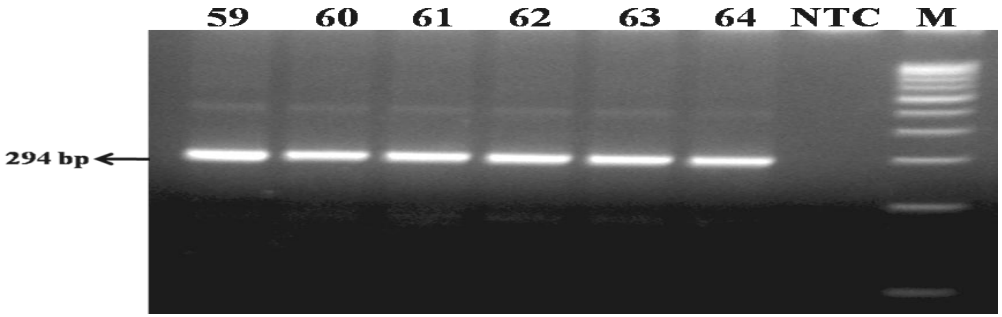

Figure 4.4: **Representative agarose gel image of gradient PCR (temperature in increasing order from 59°C-64°C). M represents 100 bp DNA marker and NTC (Non-template control) represents the negative control**

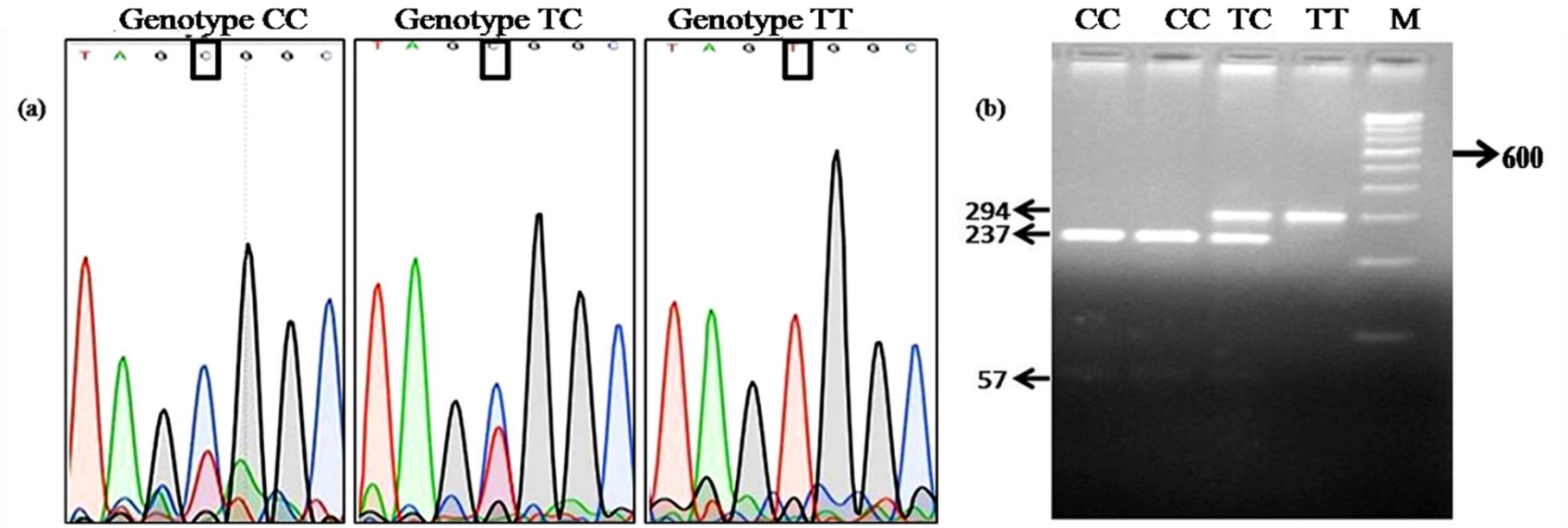

Figure 4.5 (a) Representative chromatogram of sequenced samples showing three genotypes CC, TC, and TT (b) representative A-RFLP agarose gel electrophoresis picture for rs4958843 [219].

Table 4.5 Genotypes and alleles frequencies distribution of promoter polymorphism rs4958843 in HBV infected patients and healthy controls individuals

Polymorphism rs4958843 (T/C) MAF(C)=0.3033/1519	HBV (n/N[%]) (N=551)	Asymptomatic Carriers (n/N[%]) (N=111)	Acute Carriers (n/N[%]) (N=104)	CHB (n/N[%]) (N=286)	Cirrhosis (n/N[%]) (N=50)	Control (n/N[%]) (N=247)	HWE p-value
TT	324 (59)	73 (66)	72 (69)	153 (53)	26 (52)	153 (62)	0.3258
TC	221 (40)	35 (31)	31 (30)	131 (46)	24 (48)	86 (35)	
CC	6 (1)	3 (3)	1 (1)	2 (1)	0 (0)	8 (3)	
T	869 (79)	181 (81.5)	175 (84)	437 (76.4)	76 (76)	392 (79.4)	
C	233 (21)	41 (18.5)	33 (16)	135 (23.6)	24 (24)	102 (20.6)	
HBV vs. Control	**Odds Ratio (95%CI)**			**p-value**			
TT	1^{Ref}						
TC	1.21 (0.89 to 1.66)			0.23			
CC	0.35 (0.12 to 1.04)			0.06			
TC+CC	1.14 (0.84 to 1.55)			0.40			
T	1^{Ref}						
C	1.03(0.79 to 1.33)			0.82			
Asymptomatic vs. Control							
TT	1^{Ref}						
TC	0.85 (0.52 to 1.38)			0.51			
CC	0.78 (0.20 to 3.04)			0.72			
TC+CC	0.84 (0.53 to 1.35)			0.48			
T	1^{Ref}						
C	0.87(0.58 to 1.30)			0.50			
Acute vs. Control							
TT	1^{Ref}						
TC	0.76 (0.46 to 1.25)			0.29			
CC	0.26 (0.03 to 2.16)			0.21			
TC+CC	0.72 (0.44 to 1.18)			0.19			
T	1^{Ref}						
C	0.72 (0.47 to 1.11)			0.14			
CHB vs. Control							
TT	1^{Ref}						
TC	1.52 (1.07 to 2.16)			**0.01***			
CC	0.25 (0.05 to 1.19)			0.08			
TC+CC	1.41 (1.00 to 2.00)			**0.04***			
T	1^{Ref}						
C	1.18 (0.88 to 1.58)			0.24			
Cirrhosis vs. Control							
TT	1^{Ref}						
TC	1.64 (0.88 to 3.03)			0.11			
CC	0.34 (0.01 to 6.08)			0.46			
TC+CC	1.50 (0.81 to 2.76)			0.19			
T	1^{Ref}						
C	1.21(0.73 to 2.01)			0.45			

*** p-value <0.05 was considered significant and shown in bold**

Table 4.6 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs4958843 T/C in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	TT[73]	84.13±67.97		58±46		45±57	
[111]	TC[35]	70.53±61.33	0.29	57±54	0.92	65±111	0.21
	CC[3]	93.66± 27.13	0.81	72±13	0.60	45±11	1.00
	TC+CC[38]	72±59	0.35	58±52	1.00	63±108	0.25
	CT+TT[108]	79±65	0.71	58±48	0.61	51±79	0.89
Acute	TT[72]	1599±1294		66±54		85±61	
[104]	TC[31]	715.7±219.2	**0.0003***	71±46	0.65	86±53	0.93
	CC[1]	854	NA	32±0	NA	37±0	NA
	TC+CC[32]	720±217	**0.0003***	70±46	0.71	84±53	0.93
	TC+TT[103]	NA	NA	NA	NA	NA	NA
Chronic	TT[153]	2023877±3983369		99±53		95±74	
[286]	CT[131]	3087416±6580516	0.09	113±111	0.16	112±119	0.14
	CC[2]	53325±4896	0.48	52±38	0.21	81±59	0.79
	TC+CC[133]	3021457±6522989	0.11	112±110	0.19	121±118	**0.02***
	TC+TT[284]	2490796±5295186	0.51	105±84	0.37	107±97	0.70
Cirrhosis	TT[26]	278626852±332654124		165±47		147±83	
[50]	TC[24]	663622777±1903935005	0.31	169±98	0.85	158±87	0.64
	CC[0]	-	NA	-	NA	-	NA
	TC+CC[24]	NA	NA	NA	NA	NA	NA
	TC+TT [50]	NA	NA	NA	NA	NA	NA

***p-value <0.05 was considered significant and shown in bold**

4.3.3 Genotyping of rs4958846 (T/C)

Gradient PCR was performed by using specific primers for the SNP and we obtained a single band of size 296 bp at temperature 65ºC as shown in Figure 4.6. Initially, 10 samples have been amplified and the samples were sequenced to get the control samples of known genotypes (CT and TT). These controls were used to optimize the A-RFLP conditions (given in 3.5.2 section of materials and methods). Optimized reaction components along with PCR and A-RFLP reaction conditions for the SNP rs4958846 are shown in Table 4.2. DNA samples were amplified, digested with *Sal*I (New England Biolabs) restriction enzyme and checked on 3.5% agarose gel. Sequencing results were concordant with the A-RFLP results. A representative agarose gel picture along with chromatogram for sequencing results showing genotypes of rs4958846 SNP in the studied population is shown in Figure 4.7 [214]. M represents the 50 bp DNA marker and an amplicon of 296 bp size corresponds to TT genotype, 240 bp and 56 bp corresponding to CC genotype and banding pattern of 296 bp, 240 bp, and 56 bp represents CT genotype. The SNP rs4958846 was genotyped in 551 HBV infected individuals and 247 healthy control subjects applying these optimized conditions of A-RFLP method [214].

4.3.3.1 Distribution of genotypic and allelic frequencies of SNP rs4958846 (T/C)

Distribution of genotypic and allelic frequencies of the SNP in HBV samples and healthy control samples are shown in Table 4.7. This SNP was in HWE equilibrium in the control population (p=0.088). On comparing genotypic frequencies, we found that HBV infected patients and healthy controls had a maximum percentage of TC genotype; 49%, and 52%, respectively. The percentage of genotype TC genotype was again found higher in asymptomatic (69%) and acute (50%) groups but in the chronic and cirrhosis categories, TT genotype had a maximum percentage (53% and 52%). Percentage of T allele was found higher in HBV infected individuals in totality and also in different categories along with healthy controls shown in Table 4.7. We did not find any association of the SNP rs4958846 (T/C) with HBV infection susceptibility or in protection (OR =1.03, 95% CI=0.79-1.33, p=0.82). On analyzing the data by using different genetic models, we did not find any association of the SNP. We found significant association of the SNP in the susceptibility of CHB group in co-dominant (OR=1.52, 95% CI=1.07-2.16, p=0.01) and dominant (OR =1.41, 95% CI=1.00-2.00, p=0.04) models. We did not find any

association of the SNP in asymptomatic, acute and cirrhosis groups. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value under different genetic models have been shown in Table 4.7.

4.3.2.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs4958846 (T/C)

Mean levels of HBV DNA, AST, and ALT along with standard deviation (STDEV) are shown in Table 4.8. On comparing genotypes of the SNP with mean HBV DNA levels in all the categories we did not observe any statistically significant difference in the HBV DNA levels. Furthermore, genotypes were correlated with biochemical parameters (AST and ALT) by using various genetic models. Mean difference in the AST levels was observed in asymptomatic and chronic categories. A significant difference was observed in the AST levels in homozygous wild and homozygous mutant genotypes (TT vs. CC, p=0.05) in the asymptomatic category. In the chronic category, a significant difference was observed in the AST levels in homozygous wild and homozygous mutant genotypes (TT vs. CC, p=0.003; CT+TT vs. CC, p=0.003). No significant difference was observed in the mean ALT levels of different genotypes of the SNPs under any genetic model.

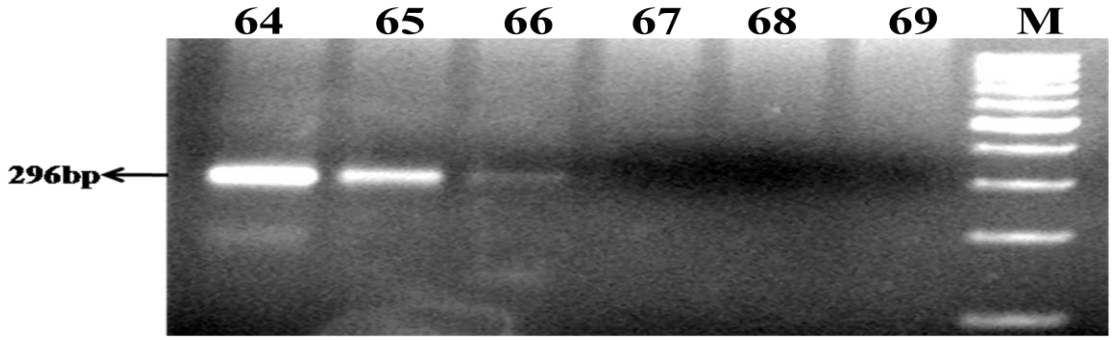

Figure 4.6: Representative agarose gel image of gradient PCR (temperature in increasing order from 64°C-69°C). M represents 100 bp DNA marker.

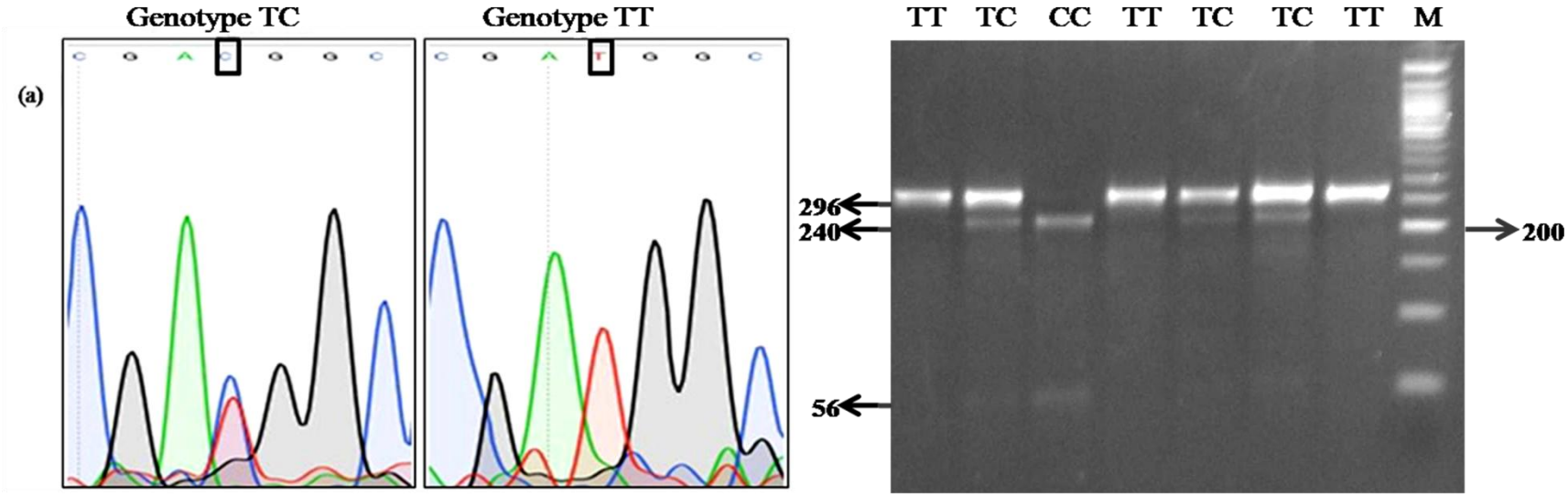

Figure 4.7 (a) Representative chromatogram of sequenced samples showing genotypes CT and TT (b) representative A-RFLP agarose gel electrophoresis picture for rs4958846 [214].

Table 4.7 Genotypes and alleles frequencies distribution of promoter polymorphism rs4958846 in HBV patients and healthy controls individuals

Polymorphism rs4958846 (T/C) MAF(C)=0.3722/1864	HBV (n/N[%]) (N=551)	Asymptomatic Carriers (n/N[%]) (N=111)	Acute Carriers (n/N[%]) (N=104)	CHB (n/N[%]) (N=286)	Cirrhosis (n/N[%]) (N=50)	Control (n/N[%]) (N=247)	HWE p-value
TT	247 (45)	26 (23.5)	45 (43)	150 (52.5)	26 (52)	91 (37)	0.088
TC	272 (49)	76 (68.5)	52 (50)	123 (43)	24 (48)	128 (52)	
CC	32 (6)	9 (8)	7 (7)	13 (4.5)	0 (0)	28 (11)	
T	766 (69.5)	128 (57.7)	142 (68.3)	423 (74)	76 (76)	310 (62.8)	
C	336 (30.5)	94 (42.3)	66 (31.7)	149 (26)	24 (24)	184 (37.2)	
HBV vs. Control	**Odds Ratio (95%CI)**			**p-value**			
TT	1^{Ref}						
TC	0.78 (0.57 to 1.08)			0.13			
CC	0.42 (0.24 to 0.74)			**0.0003***			
TC+CC	0.72 (0.53 to 0.98)			**0.04***			
T	1^{Ref}						
C	0.74 (0.59 to 0.92)			**0.007***			
Asymptomatic vs. Control							
TT	1^{Ref}						
TC	2.07 (1.23 to 3.49)			**0.005***			
CC	1.12 (0.47 to 2.68)			0.79			
TC+CC	1.90 (1.14 to 3.17)			**0.01***			
T	1^{Ref}						
C	1.23 (0.89 to 1.70)			0.19			
Acute vs. Control							
TT	1^{Ref}						
TC	0.82 (0.50 to 1.32)			0.42			
CC	0.50 (0.20 to 1.24)			0.13			
TC+CC	0.76 (0.47 to 1.21)			0.25			
T	1^{Ref}						
C	0.78 (0.55 to 1.10)			0.16			
CHB vs. Control							
TT	1^{Ref}						
TC	0.58 (0.40 to 0.83)			**0.0032***			
CC	0.28 (0.13 to 0.57)			**0.0004***			
TC+CC	0.52 (0.37 to 0.74)			**0.0003***			
T	1^{Ref}						
C	0.59 (0.45 to 0.77)			**0.0001***			
Cirrhosis vs. Control							
TT	1^{Ref}						
TC	0.65 (0.35 to 1.21)			0.18			
CC	0.06 (0.003 to 1.02)			0.06			
TC+CC	0.53 (0.29 to 0.99)			0.04			
T	1^{Ref}						
C	0.53 (0.32 to 0.87)			**0.01***			

*** p-value <0.05 was considered significant and shown in bold**

Table 4.8 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs4958846 T/C in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	TT[26]	96.21±99.1		48±20		62±125	
[111]	CT[76]	87.26±78.26	0.60	61±55	0.24	47±58	0.41
	CC[9]	32±9.53	0.06	67±35	**0.05***	53±58	0.83
	CT+CC[85]	83±76	0.48	61±53	0.22	48±57	0.42
	CT+TT[102]	89±83	0.04	57±49	0.55	51±80	0.94
Acute	TT[45]	1596±1450		65±37		79±33	
[104]	CT[52]	1513±1428	0.77	70±63	0.64	88±77	0.46
	CC[7]	1946±1814	0.56	61±33	0.79	92±31	0.33
	CT+CC[59]	1565±1467	0.91	69±60	0.69	89±73	0.39
	CT+TT[97]	1550±1430	0.48	68±52	0.72	84± 60	0.71
Chronic	TT[150]	2592641±41721406		98±33		98±42	
[286]	CT[123]	2487305±6526763	0.97	97±60	0.86	103±86	0.53
	CC[13]	632775±900357	0.86	143±149	**0.003***	126±121	0.06
	CT+CC[136]	2354838±6276858.08	0.94	103±78	0.47	106±91	0.33
	CT+TT[273]	2543929±5363683	0.20	97±47	**0.003***	100±66	0.18
Cirrhosis	TT[26]	572847208±1808674621		162±38		140±89	
[50]	CT[24]	3758132578±1.4528E+10	0.27	172±97	0.62	163±79	0.34
	CC[0]	NA	NA	-	NA	-	NA
	CT+CC[24]	NA	NA	NA	NA	NA	NA
	CT+TT[50]	NA	N A	NA	NA	NA	NA

***p-value <0.05 was considered significant and shown in bold**

4.4. Genotyping of *ATG16L1* polymorphisms rs2241880 (A/G), rs2241879 (G/A), rs13005285 (G/T) and rs7587633 (C/T)

4.4.1 Genotyping of rs2241880 (T300A, A/G)

Genotyping of the SNP rs2241880 was done in the 551 HBV infected individuals and 247 healthy control subjects by using the method given by Csongei et al. with minor modifications [38]. Optimized PCR and RFLP reaction conditions are shown in Table 4.9. We got a single band of size 270 bp at the temperature 55ºC that was used for the further amplification. Figure 4.8 shows the representative gel image of gradient PCR. Amplification was checked; samples were digested with *Sfa*NI (New England Biolabs) restriction enzyme and checked on 2.5% agarose gel.

A representative agarose gel picture showing three genotypes (AG, GG, and AA) in the studied population is shown in Figure 4.9. M represents the 100 bp DNA marker. An amplicon of 282 bp size corresponds to AA genotype, 172 bp, and 110 bp corresponds to GG genotype and banding pattern of 282 bp, 172 bp and 110 bp represents AG genotype. The SNP rs2241880 was genotyped in 551 HBV infected individuals and 247 healthy control subjects applying these optimized conditions.

4.4.1.1 Distribution of genotypic and allelic frequencies of rs2241880 (A/G)

Distribution of genotypic and allelic frequencies of the SNP in 551 HBV samples of different categories and 247 healthy control samples are shown in Table 4.10. On comparing genotypic frequencies in the HBV infected and healthy control subjects, we found maximum percentage of AG genotype i.e. 48%, and 61%, respectively. The percentage of genotype AG was found higher in all the categories of HBV infection. Percentage of mutant allele G (53%) was found higher in HBV infected individuals in totality in comparison to the percentage of the mutant allele in (46%) healthy control group. When we observed percentage of mutant allele G in different categories, higher percentage of this allele in asymptomatic, chronic and cirrhosis group but in acute group percentage of the mutant allele was found less.

The SNP rs2241880 (A/G) was found to be associated in HBV infection susceptibility in allelic (OR =1.31, 95% CI=1.06-1.63, p=0.01) and homozygous (OR =1.96, 95% CI=1.22-3.16,

p=0.005) genetic models. On comparing different categories we found a significant association of the mutant allele G in the susceptibility of asymptomatic and CHB group in allelic and homozygous genetic models. In the acute category of infection, we found a protective association in the co-dominant model. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value in different genetic models have been shown in Table 4.10.

4.4.2.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs2241880 (A/G)

HBV DNA was quantified as mentioned earlier applying Real-Time PCR and the data from patients were segregated according to three genotypes (GG, AG and AA). Table 4.11 shows the mean levels of HBV DNA in the patients of different genotypes. We found statistically significant different DNA levels in the genetic models (GG+AG vs. AA) and (GG vs. AG+AA) in the asymptomatic patients. In the patients at the acute stage of HBV infection, no difference in viral load was observed in any of the genetic models, while in the patients with chronic infection, we observed the statistically significant difference in the viral load in all the genetic models. In liver cirrhosis patients, there was a significant difference in DNA levels was observed in all models except for (GG vs. AA).

Furthermore, genotypes were correlated with biochemical parameters (AST and ALT) by using genotype combinations. Significant differences in mean ALT levels were observed in genotypes (AA vs. AG +GG, p=0.03, and AA vs. AG, p=0.007). No significant difference was observed in AST and ALT levels in different genotypes in other HBV infection categories.

Table 4.9: Optimized reaction components and amplification conditions to perform PCR-RFLP and A-RFLP for genotyping of *ATG16L1* polymorphisms

Optimized components and conditions	rs2241880 (A/G)	rs2241879 (G/A)	rs13005285 (G/T)	rs7587633 (C/T)
PCR REACTION COMPONENTS				
Genomic DNA (ng)	40	40	40	40
Forward primer (μM)	0.2	0.2	0.2	0.2
Reverse primer (μM)	0.2	0.2	0.2	0.2
dNTPs (mM) New England Biolabs)	0.2	0.2	0.2	0.2
i-Taq DNA polymerase (U) (iNtRON Biotechnology)	0.5	0.5	0.5	0.5
Total reaction Volume (μl)	20	20	20	20
PCR AMPLIFICATION CONDITIONS				
Initial denaturation	94°C-3´	94°C-3´	94°C-3´	94°C-3´
Denaturation	94°C-30´´ ×35 cycles	94°C-30´´ ×35 cycles	94°C-30´´ ×35 cycles	94°C-30´´ ×35 cycles
Annealing	**55°C-40´´**	**50°C-40´´**	**59°C-40´´**	**57°C-40´´**
Extension	72°C-30´´	72°C-30´´	72°C-30´´	72°C-30´´
Final extension	72°C-7´	72°C-7´	72°C-7´	72°C-7´
PCR-RFLP & A-RFLP				
Amplified DNA (μl)	10	10	10	10
Restriction Enzyme (IU)	0.5	0.5	1	1
Buffer (10X)	1X	1X	1X	1X
Total reaction volume (μl)	15	15	15	15

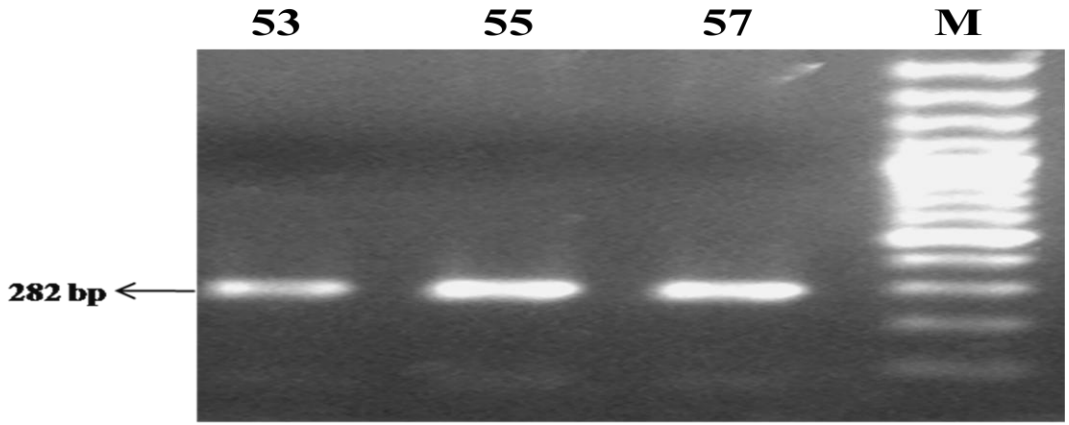

Figure 4.8: Representative agarose gel image of gradient PCR Lane 1 to lane 3 represents the gradient temperature in increasing order from 53°C-57°C. M represents 100 bp DNA marker.

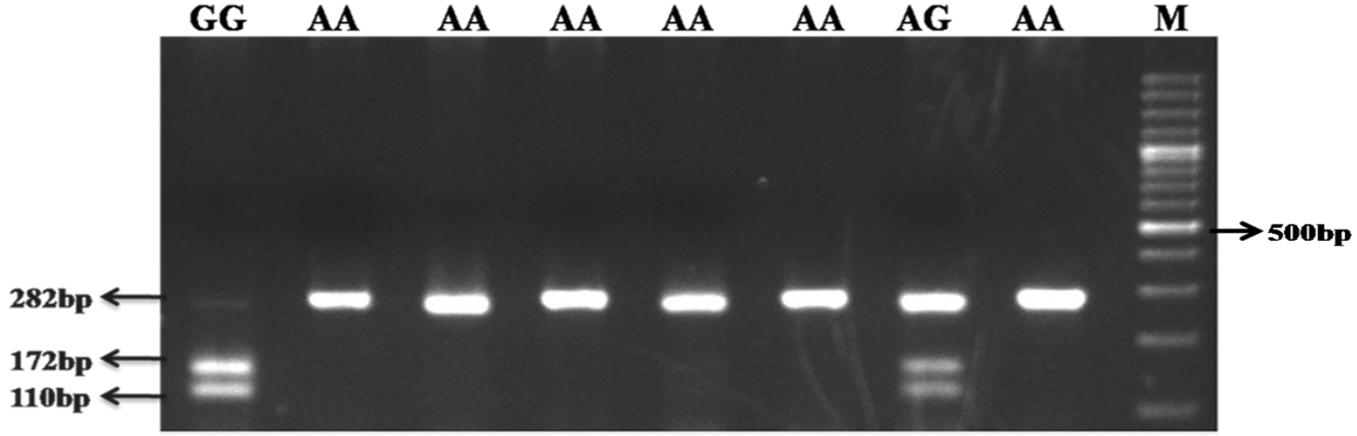

Figure 4.9: Representative agarose gel electrophoresis showing genotyping of SNP rs2241880 by applying PCR-RFLP method. Lane 1 represents GG genotype, Lane 2-6 and 8 represents AA genotype, Lane 7 represents AG genotype. M represents 100 bp marker.

Table 4.10 Genotypes and allele frequencies distribution of polymorphism rs2241880 (A/G) in HBV patients and healthy controls individuals

Polymorphism **rs2241880 A/G MAF(G)=0.3960/1983**	**HBV (n/N[%]) (N=551)**	**Asymptomatic Carriers (n/N[%]) (N=111)**	**Acute Carriers (n/N[%]) (N=104)**	**CHB (n/N[%]) (N=286)**	**Cirrhosis (n/N[%]) (N=50)**	**Control (n/N[%]) (N=247)**	**HWE p-value**
AA	125 (23)	26 (23.4)	33 (33.9)	55 (19.2)	11 (22)	57 (23.1)	0.0003
AG	266 (48)	44 (39.7)	47 (41.5)	153 (53.5)	22 (44)	151 (61.1)	
GG	160 (29)	41(36.9)	24 (24.6)	78 (27.3)	17 (34)	39 (15.8)	
A	516 (47)	96 (43.2)	113 (54.3)	263 (46)	44 (44)	265 (53.6)	
G	586 (53)	126 (56.8)	95 (45.7)	309 (54)	56 (56)	229 (46.4)	
HBV vs. Control	**Odds Ratio (95%CI)**			**p-value**			
AA	1^{Ref}			-			
AG	0.82 (0.56 to 1.19)			0.29			
GG	1.96 (1.22 to 3.16)			**0.005***			
AG+GG	1.02 (0.72-1.46)			0.90			
A	1^{Ref}			-			
G	1.31 (1.06 to 1.63)			**0.01***			
Asymptomatic vs. Control							
AA	1^{Ref}			-			
AG	0.64 (0.36 to 1.13)			0.13			
GG	2.30 (1.22 to 4.36)			**0.01***			
AG+GG	0.98 (0.58 to 1.67)			0.94			
A	1^{Ref}			-			
G	1.52 (1.10 to 2.09)			**0.01***			
Acute vs. Control							
AA	1^{Ref}			-			
AG	0.54 (0.31 to 0.92)			**0.02***			
GG	1.06 (0.55 to 2.06)			0.86			
AG+GG	0.64 (0.39 to 0.07)			0.09			
A	1^{Ref}			-			
G	0.97 (0.70 to 1.35)			0.8683			
CHB vs. Control							
AA	1^{Ref}			-			
AG	1.05 (0.68 to 1.62)			0.83			
GG	2.07 (1.22 to 3.53)			**0.0075***			
AG+GG	1.26 (0.83 to 1.91)			0.28			
A	1^{Ref}			-			
G	1.36 (1.07 to 1.73)			**0.01***			
Cirrhosis vs. Control							
AA	1^{Ref}			-			
AG	0.76 (0.34 to 1.66)			0.48			
GG	2.26 (0.95 to 5.34)			0.0636			
AG+GG	1.06 (0.51 to 2.21)			0.86			
A	1^{Ref}			-			
G	1.47 (0.95 to 2.27)			0.08			

*** p-value <0.05 was considered significant and shown in bold**

Table 4.11 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs2241880 A/G in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	AA[26]	254±86		57±51		70±136	
[111]	AG[44]	223±107	0.21	57±41	1.00	47±57	0.32
	GG[41]	258±118	0.88	61±53	0.76	44±38	0.25
	AG +GG[85]	114±113	**< 0.0001***	59±47	0.85	45±49	0.15
	AG+AA[70]	110±100	**< 0.0001***	57±45	0.67	55±94	0.47
Acute	AA[125]	1807±1502		66±30		80±38	
[104]	AG[266]	1318±1280	0.11	63±40	0.72	86±51	0.24
	GG[160]	1524±1536	0.48	80±85	0.38	88±91	0.35
	AG +GG[426]	1388±1364	0.16	68±59	0.85	87±66	0.25
	AG+AA[391]	1503±1385	0.94	64±36	0.18	83±46	0.39
Chronic	AA[55]	9878565±3045121		107±82		102±68	
[286]	AG[153]	7856767±2892934	**< 0.0001***	109±106	0.89	117±133	0.42
	GG[78]	12366547±4075401	**< 0.0001***	96±31	0.28	95±31	0.42
	AG +GG[231]	2269652.85±3434466.72	**< 0.0001***	104±85	0.81	108±105	0.68
	AG+AA[208]	1938555.96±2983638.46	**< 0.0001***	108±98	0.28	111± 113	0.21
Cirrhosis	AA[11]	100000000±157870747		157±33		101±60	
[50]	AG[22]	654987897±273958805	**< 0.0001***	161±47	0.80	178±79	**0.007***
	GG[17]	86789876±118307083	0.80	181±113	0.50	140±90	0.21
	AG +GG[39]	265110292.7± 256507123	**0.04***	169±80	0.63	162±85	**0.03***
	AG+AA[33]	291640047.3±262131707	**0.004***	160±43	0.34	158±81	0.47

*** p-value <0.05 was considered significant and shown in bold**

4.4.2 Genotyping of rs2241879 (G/A)

Gradient PCR was performed by using specific primers for the SNP and we got a single band of 366 bp without any non-specific amplification at 50ºC. Optimized PCR and RFLP reaction components and conditions for the SNP rs2241879 are shown in Table 4.9. Representative image of the gradient PCR is shown in Figure 4.10. Isolated DNA from 551 HBV infected individuals and 247 healthy control subjects were amplified, digested with *Tsp*45I (New England Biolabs) restriction enzyme and checked on 2.5% agarose gel. A representative agarose gel picture showing three genotypes (AG, GG, and AA) is shown in Figure 4.11. M represents the 100 bp marker and an amplicon of 366 bp represents AA genotype, 211 bp, and 155 bp band size represent GG genotype and banding pattern of 366 bp, 211 bp and 155 bp represents GA genotype. The SNP rs2241879 was genotyped in 551 HBV infected individuals and 247 healthy control subjects applying these optimized conditions of PCR-RFLP.

4.4.2.1 Distribution of genotypic and allelic frequencies of rs2241879 (G/A)

Distribution of genotypic and allelic frequencies of the SNP in all the samples is shown in Table 4.12. The SNP was in the control population was in accordance to HWE (p=0.0719). On comparing genotypic frequencies in HBV infected and healthy control subjects we found maximum percentage of heterozygous GA genotype i.e. 48%, and 55%, respectively. The percentage of heterozygous genotype GA was again found higher in asymptomatic, acute and chronic categories but in cirrhosis category, homozygous mutant genotype AA had a maximum percentage. Both the studied groups (HBV and healthy controls) had a maximum percentage of mutant allele A i.e. 66% and 57%, respectively. When we observed percentage of mutant allele A in different HBV infection stages, we found a higher percentage of the mutant allele in all the categories. SNP rs2241879 (G/A) was found to be associated with HBV infection susceptibility in allelic (OR =1.57, 95% CI=1.26-1.95, p<0.0001) and homozygous (OR =12.58, 95% CI=1.59-4.18, p=0.0001) genetic models.

On comparing different categories of HBV infection we found a significant association of the mutant allele A in the susceptibility of chronic and cirrhosis infection in allelic, dominant and homozygous models. We did not find any association of the SNP in the asymptomatic and acute

category in any of the genetic models. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value under different genetic models have been shown in Table 4.12.

4.4.2.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs2241879 (G/A)

Table 4.13 shows the mean levels of HBV DNA in the patients of different genotypes, GG, GA, and AA. As the SNP rs2241879 was associated with HBV infection risk but we did not observe higher viral load in mutant genotype AA. We found a statistically significant difference in DNA levels in the genetic models (GG vs. GA+AA), (GG vs. AA) and (GG vs. GA) in the asymptomatic patients. In the patients with acute stage of HBV infection, no difference in viral load was observed in any of the genetic models, while in the patients with chronic and cirrhosis we observed the statistically significant difference in viral load in the same genetic models as observed in the asymptomatic category. No significant difference in the mean AST and ALT levels of different genotypes was observed in other HBV categories in any of the genetic models.

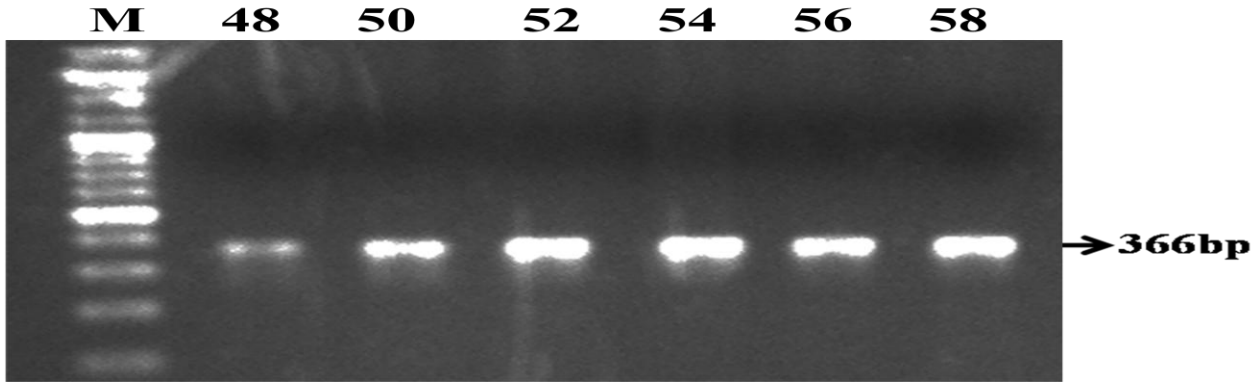

Figure 4.10: **Representative agarose gel image of gradient PCR (temperature in increasing order from 48°C-58°C). M represents 100 bp DNA marker.**

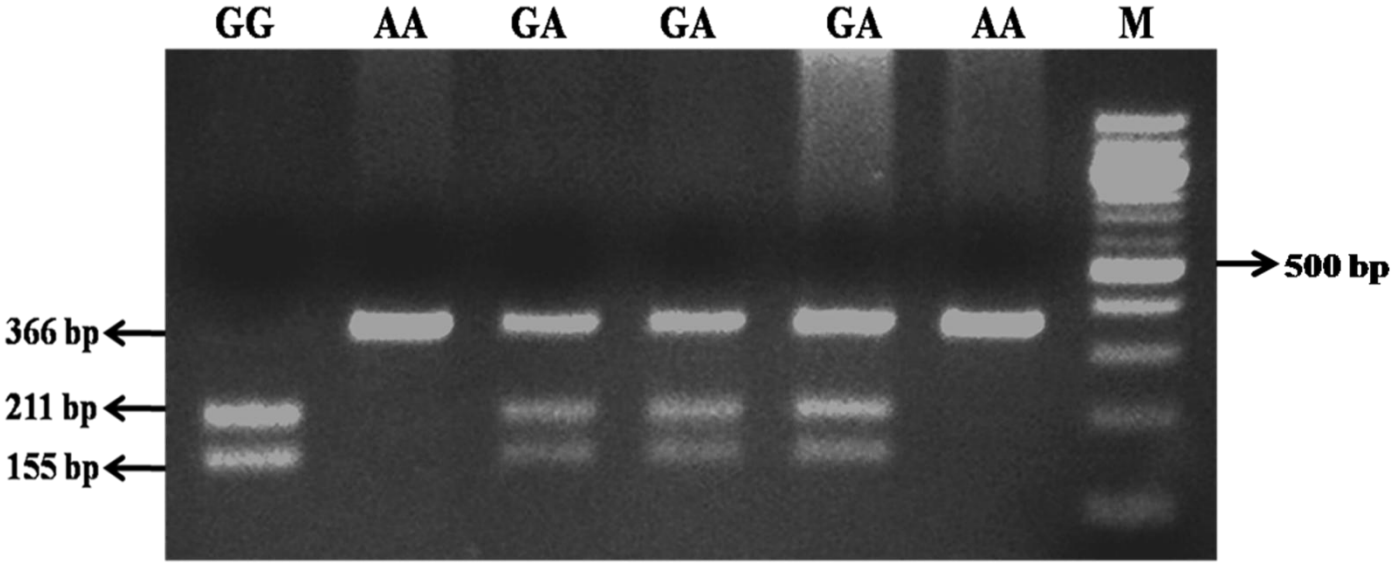

Figure 4.11: Representative agarose gel electrophoresis showing genotyping of SNP rs2241879 by applying PCR-RFLP method. Lane 1 represents GG genotype, Lane 2 and 6 represents AA genotype, Lane 3-5 represents GA genotype. M represents 100 bp DNA marker.

Table 4.12 Genotypes and allele frequencies distribution of polymorphism rs2241879 (G/A) in HBV infected patients and healthy controls individuals

Polymorphism rs2241879 G/A MAF(A)=0.3946/1976	HBV (n/N[%]) (N=551)	Asymptomatic Carriers (n/N[%]) (N=111)	Acute Carriers (n/N[%]) (N=104)	CHB (n/N[%]) (N=286)	Cirrhosis (n/N[%]) (N=50)	Control (n/N[%]) (N=247)	HWE p-value
GG	55 (10)	15 (13.5)	11 (10.6)	27 (9.4)	2 (4)	42 (17)	0.0719
GA	263 (47.7)	54 (48.6)	57 (54.8)	135 (47.2)	17 (34)	136 (55)	
AA	233 (42.3)	42 (37.9)	36 (34.6)	124 (43.4)	31 (62)	69 (28)	
G	373 (33.8)	84 (37.8)	79 (38)	189 (33)	21 (21)	220 (44.5)	
A	729 (66.2)	138 (62.2)	129 (62)	383 (67)	79 (79)	274 (56.5)	
	Odds Ratio (95%CI)			**p-value**			
HBV vs. Control							
GG	1^{Ref}			-			
GA	1.48 (0.94 to 2.32)			0.09			
AA	2.58 (1.59 to 4.18)			**0.0001***			
GA+AA	1.10 (0.71 to 1.71)			0.66			
G	1^{Ref}			-			
A	1.57 (1.26 to 1.95)			**< 0.0001***			
Asymptomatic vs. Control							
GG	1^{Ref}						
GA	1.11 (0.57 to 2.17)			0.75			
AA	1.70 (0.84 to 3.44)			0.14			
GA+AA	1.31 (0.69 to 2.48)			0.40			
G	1^{Ref}						
A	1.31 (0.95 to 1.82)			0.09			
Acute vs. Control							
GG	1^{Ref}						
GA	1.60 (0.76 to 3.33)			0.20			
AA	1.99 (0.91 to 4.33)			0.08			
GA+AA	1.73 (0.85 to 3.51)			0.12			
G	1^{Ref}						
A	1.31 (0.94 to 1.83)			0.10			
CHB vs. Control							
GG	1^{Ref}						
GA	1.54 (0.90 to 2.64)			0.11			
AA	2.79 (1.58 to 4.92)			**0.0004***			
GA+AA	1.96 (1.17 to 3.29)			**0.01***			
G	1^{Ref}						
A	1.62 (1.27 to 2.09)			**0.0001***			
Cirrhosis vs. Control							
GG	1^{Ref}						
GA	2.62 (0.58 to 11.82)			0.20			
AA	9.43 (2.15 to 41.46)			**0.0030**			
GA+AA	4.92 (1.15 to 21.02)			**0.03**			
G	1^{Ref}						
A	3.02 (1.80 to 5.04)			**< 0.0001**			

* **p value <0.05 was considered significant and shown in bold**

Table 4.13 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs2241879 G/A in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	GG[15]	238±140		52±15		33±21	
[111]	GA[54]	96±91	**< 0.0001***	55±46	0.80	51±91	0.45
	AA[42]	125±135	**0.007***	64±57	0.42	58±73	0.19
	GA+AA[96]	108±113	**0.0001***	59±51	0.59	54±83	0.19
	GA+GG[69]	107±105	0.43	55±41	0.33	47±82	0.47
Acute	GG[11]	1171±1069		54±28		71±31	
[104]	GA[57]	827±484	0.09	71±64	0.39	93±72	0.25
	AA[36]	952±715	0.43	65±29	0.27	76±37	0.64
	GA+AA[93]	876±583	0.15	69±53	0.35	86±61	0.64
	GA+GG[68]	928±711	0.87	69±60	0.70	89±67	0.35
Chronic	GG[27]	32984±34327		95±32		102±34	
[286]	GA[135]	524083±1071398	**0.01***	108±108	0.53	105±113	0.89
	AA[124]	487928±927487	**0.01***	102±53	0.51	109±85	0.67
	GA+AA[259]	508085±1006041	**0.01***	105±87	0.55	109±85	0.67
	GA+GG[162]	481379±1032533	0.95	106±101	0.68	104±105	0.66
Cirrhosis	GG[2]	512171158±78779334		123±2		123±2	
[50]	GA[17]	93496871±40721023	**< 0.0001***	183±78	0.30	161±106	0.62
	AA[31]	103513422±111363666	**< 0.0001***	161±73	0.47	149±74	0.62
	GA+AA[48]	98004318±78234998	**< 0.0001***	168±74	0.39	153±85	0.62
	GA+GG[19]	157908299±163153736	0.16	174±75	0.54	155±99	0.80

*** p-value <0.05 was considered significant and shown in bold**

4.4.3 Genotyping of rs13005285 (G/T)

To genotype the SNP rs13005285 by applying PCR-RFLP, natural restriction site was not present for any available restriction enzyme at the SNP position. Strategy for the development of A-RFLP is described under section 3.6.2 in materials and methods**.** We have successfully optimized A-RFLP reaction conditions for the SNP and further genotyping of the SNP was done by applying newly developed method. Optimized PCR-RFLP reaction components and conditions are shown in Table 4.9.

Briefly**,** gradient PCR was performed by using specific primers and we have chosen 59 º C temperatures as we got a single band of 269 bp without any nonspecific amplification at that temperature. Representative image of the gradient PCR is shown in Figure 4.12. DNA was amplified; samples were digested with *Hin*dIII (New England Biolabs) restriction enzyme and checked on 3.5% agarose gel. A representative agarose gel picture showing three genotypes, (GG, GT, and TT) is shown in Figure 4.13. M represents the 100 bp DNA marker and an amplicon of 269 bp represents GG genotype, 219 and 50 bp band size represent TT genotype and banding pattern of 269 bp, 219 bp, and 50 bp represents GT genotype. The SNP rs13005285 was genotyped in 551 HBV infected individuals and 247 healthy control subjects applying these optimized conditions of A-RFLP method.

4.4.3.1 Distribution of genotypic and allelic frequencies of rs13005285 (G/T)

Distribution of genotypic and allelic frequencies of the SNP in HBV infected samples and healthy control samples have been shown in Table 4.14. This SNP in the control population was in accordance to HWE (p=0.3536). On comparing genotypic frequencies in both the studied groups, we found that both the groups had a maximum percentage of homozygous wild genotype GG, 49%, and 58% respectively. The percentage of homozygous wild genotype GG was found higher in chronic and cirrhosis categories but in asymptomatic category, both GG and GT genotypes were found at equal frequencies. In acute category, heterozygous GT genotype had a maximum percentage.

Both the studied groups (HBV and healthy controls) had a maximum percentage of wild allele G i.e. 70% and 78%, respectively. When we observed percentage of allele G in different HBV

infection categories we found a higher percentage of the allele in all the categories. The SNP rs13005285 (G/T) was found to be associated with HBV infection susceptibility in allelic (OR =1.39, 95% CI=1.09-1.77, p=0.008), dominant (Odds ratio (OR) =1.42, 95% CI=1.05-1.93, p=0.021) and homozygous (OR =2.26, 95% CI=1.13-4.50, p=0.019) genetic models. On comparing different categories we found a significant association of the SNP in the susceptibility of asymptomatic and acute categories in allelic, dominant, co-dominant and homozygous models and in cirrhosis category, we found the association of the SNP only in the homozygous model. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value in different genetic models have been shown in Table 4.14.

4.4.3.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs13005285 (G/T)

Mean levels of HBV DNA, AST, and ALT along-with STDEV among different genotypes (GT, TT, and GG) of the SNP are shown in Table 4.15. We found a statistical significant difference in the HBV DNA levels when we compared homozygous mutant genotype with homozygous wild and heterozygous genotype (GG vs. TT and GT+GG vs. TT) in the asymptomatic and chronic category.

No difference in a mean viral load of different genotypes was observed in acute and cirrhosis category. No significant difference in the mean AST levels of different genotypes was observed in HBV categories under any genetic model. A significant difference in the homozygous mutant, homozygous wild genotype was observed in ALT levels in the only asymptomatic category (GG vs. TT, p=0.03; GT+GG vs. TT, p=0.001).

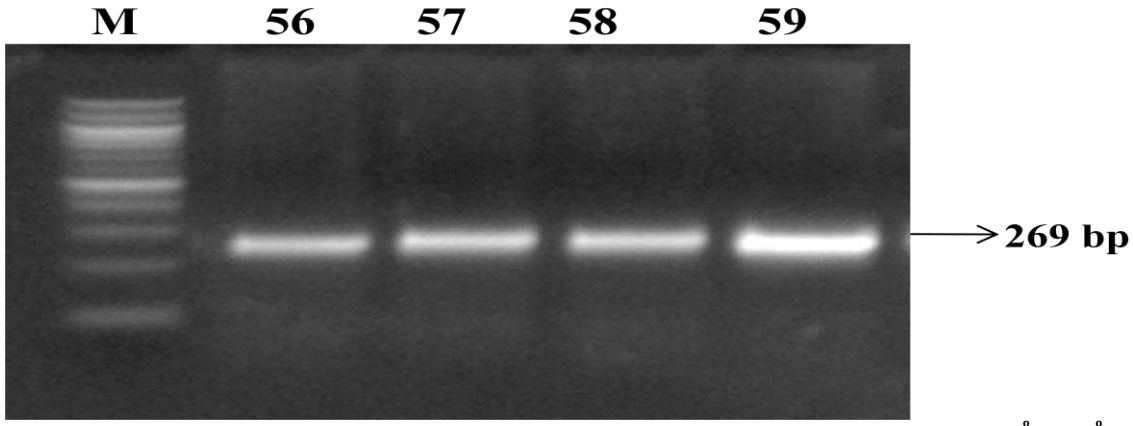

Figure 4.12: **Representative agarose gel image of gradient PCR (temperature in increasing order from 56°C-59°C) M represents 100 bp DNA marker.**

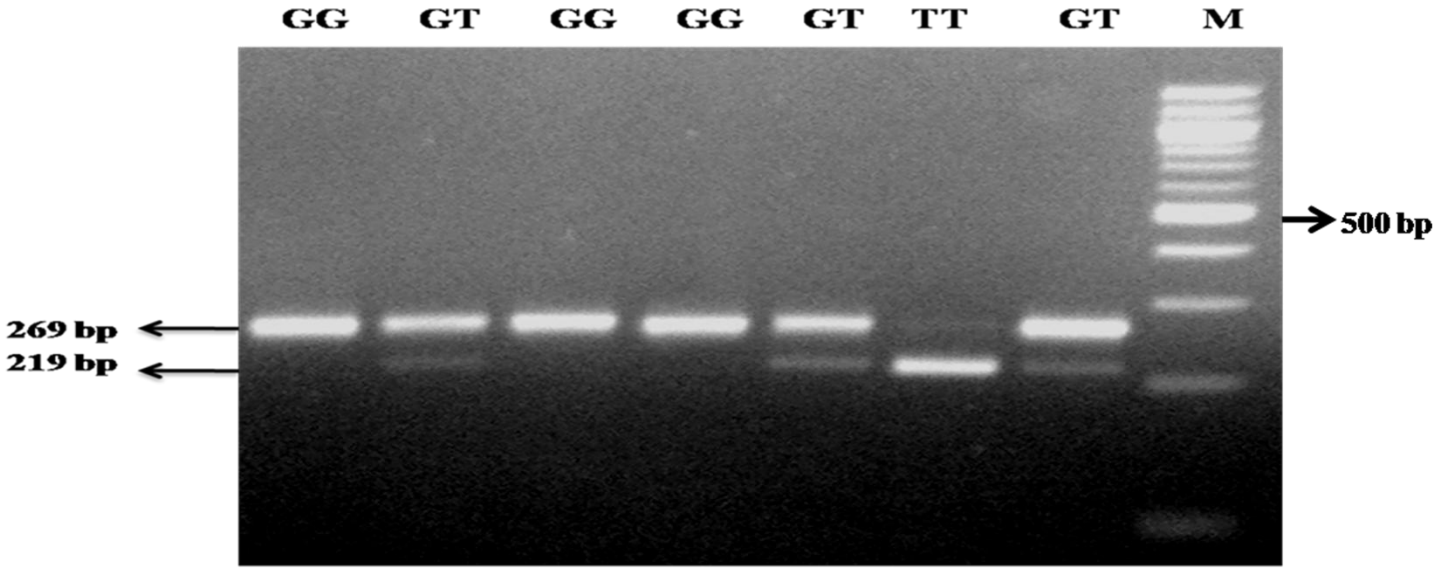

Figure 4.13: Representative agarose gel electrophoresis showing genotyping of SNP rs13005285 (G/T) by applying A-RFLP method. Lane 1, 3 and 4 represents GG genotype, Lane 2 and 5 represents GT genotype, Lane 6 represents TT genotype and M represents 100 bp DNA marker.

Table 4.14 Genotypes and allele frequencies distribution of polymorphism rs13005285 (G/T) in HBV patients and healthy controls individuals

Polymorphism rs13005285 G/T MAF(T)=0.4962/2485	HBV (n/N[%]) (N=551)	Asymptomatic Carriers (n/N[%]) (N=111)	Acute Carriers (n/N[%]) (N=104)	CHB (n/N[%]) (N=286)	Cirrhosis (n/N[%]) (N=50)	Control (n/N[%]) (N=247)	HWE p-value
GG	268 (48.6)	47 (42.3)	44 (42.3)	151 (52.8)	26 (52)	142 (57.5)	0.3536
GT	236 (42.8)	46 (41.5)	52 (50)	120 (42)	18 (36)	94 (38)	
TT	47 (8.6	18 (16.2)	8 (7.7)	15 (5.2)	6 (12)	11 (4.5)	
G	772 (70)	140 (63)	140 (67.3)	422 (73.8)	70 (70)	378 (76.5)	
T	330 (30)	82 (37)	68 (32.7)	150 (26.2)	30 (30)	116 (23.5)	
	Odds Ratio (95%CI)			**p-value**			
HBV vs. Control							
GG	1^{Ref}			-			
GT	1.33 (0.97 to 1.82)			0.07			
TT	2.26 (1.13 to 4.50)			**0.019***			
GT+TT	1.42 (1.05 to 1.93)			**0.021***			
G	1^{Ref}			-			
T	1.39 (1.09 to 1.77)			**0.0080***			
Asymptomatic vs. Control							
GG	1^{Ref}						
GT	1.47 (0.91 to 2.39)			0.11			
TT	4.94 (2.17 to 11.21)			**0.0001***			
GT+TT	1.84 (1.17 to 2.89)			**0.0083***			
G	1^{Ref}						
T	1.90 (1.35 to 2.68)			**0.0002***			
Acute vs. Control							
GG	1^{Ref}						
GT	1.78 (1.10 to 2.88)			**0.017***			
TT	2.34 (0.88 to 6.20)			0.085			
GT+TT	1.84 (1.15 to 2.90)			**0.0097***			
G	1^{Ref}						
T	1.58 (1.10 to 2.26)			**0.0116***			
CHB vs. Control							
GG	1^{Ref}						
GT	1.20 (0.84 to 1.71)			0.311			
TT	1.28 (0.56 to 2.88)			0.54			
GT+TT	1.20 (0.85 to 1.70)			0.277			
G	1^{Ref}						
T	1.15 (0.87 to 1.53)			0.3024			
Cirrhosis vs. Control							
GG	1^{Ref}						
GT	1.04 (0.54 to 2.01)			0.89			
TT	2.97 (1.01 to 8.76)			**0.04***			
GT+TT	1.24 (0.67 to 2.29)			0.47			
G	1^{Ref}						
T	1.39 (0.86 to 2.24)			0.16			

*** p value <0.05 was considered significant and shown in bold**

Table 4.15 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs13005285 G/T in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	GG[47]	145±129		63±59		46±57	
[111]	GT[46]	124±101	0.38	51±22	0.19	36±19	0.26
	TT[18]	42±27	**0.001***	61±63	0.90	104±162	**0.03***
	GT+TT[64]	109±97	0.09	54±38	0.33	55±91	0.55
	GT+GG[93]	135±116	**0.001***	57±45	0.74	41±43	**0.0015***
Acute	GG[44]	1708±1410		65±30		81±49	
[104]	GT[52]	1469±1392	0.40	69±70	0.72	88±69	0.57
	TT[8]	1470±1856	0.67	73±24	0.47	77±38	0.82
	GT+TT[60]	1469±1420	0.39	69±62	0.69	87±65	0.60
	GT+GG[96]	1580±1398	0.83	67±53	0.75	85±60	0.71
Chronic	GG[151]	1571729±2977623		98±49		103±78	
[286]	GT[120]	3084404±7265018	**0.02***	110±101	0.20	109±113	0.60
	TT[15]	923766±2292713	0.41	118±155	0.25	118±126	0.50
	GT+TT[135]	2863429±6945310	**0.03***	111±108	0.18	110±114	0.54
	GT+GG[271]	2283046±5467853	0.34	104±78	0.52	106±95	0.64
Cirrhosis	GG[26]	266689610±343526373		157±43		134±51	
[50]	GT[18]	228249072±261146315	0.69	180±96	0.28	167±103	0.16
	TT[6]	256625770±282616420	0.94	123±2	0.06	133±7	0.96
	GT+TT[24]	231587507±254467166	0.62	174±93	0.40	159±90	0.22
	GT+GG[44]	246806573±298783173	0.93	168±74	0.14	147±77	0.75

*** p-value <0.05 was considered significant and shown in bold**

4.4.4 Genotyping of rs7587633 (C/T)

Genotyping of the SNP rs7587633 (C/T) was done by using newly developed A-RFLP method described under section 3.6.2 in material and methods. Optimized PCR-RFLP reaction components and conditions are shown in Table 4.9. Briefly, gradient PCR was performed and we obtained a single 467 bp band at 57°C which was selected for the further amplification. Representative image of the gradient PCR is shown in Figure 4.14. DNA was amplified and samples were digested with *Eco*RI (New England Biolabs) restriction enzyme and were checked on 3.5% agarose gel. A representative agarose gel picture showing three genotypes, (CC, CT, and TT) are shown in Figure 4.15. After restriction digestion, we have got amplicon of 467 bp that represents homozygous TT genotype, 416 bp, and 51 bp amplicons represent homozygous CC genotype and banding pattern of 467 bp, 416 bp and 51 bp represents heterozygous CT genotype.

4.4.4.1 Distribution of genotypic and allelic frequencies of rs7587633 (C/T)

Distribution of genotypic and allelic frequencies of the SNP in both the studied groups is shown in Table 4.16. The genotypic frequencies of this SNP in the control population was in accordance to HWE (p=0.22). On comparing genotypic frequencies in HBV infected and healthy subjects, we found that both the groups had a maximum percentage of homozygous wild genotype CC i.e. 69% and 65%, respectively. The percentage of homozygous wild genotype CC was found higher in all the HBV infection categories. HBV infected group and healthy controls had a maximum percentage of wild allele C i.e. 81% and 80%, respectively. When we observed percentage of allele C in different categories we again found a higher percentage of the allele in all the HBV infection categories.

On data analysis, we did not find any association of the SNP rs7587633 (C/T) with HBV infection susceptibility or in protection in any genetic model. On comparing different categories, we found the significant protective association of the SNP in the asymptomatic category in dominant (OR =0.54, 95% CI=0.32-0.91, p=0.021), and co-dominant (OR =0.41, 95% CI=0.22-0.74, p=0.0037) genetic models. Genotypic and allelic frequencies of all the studied groups along with ORs and p-value under different genetic models have been shown in

Table 4.16. The SNP rs7587633 was genotyped in 551 HBV infected individuals and 247 healthy control subjects applying these optimized conditions of A-RFLP method.

4.4.4.2 Comparison of HBV DNA levels, AST and ALT with different genotypes of the SNP rs7587633 (C/T)

Mean levels of HBV DNA, AST, and ALT along-with STDEV among different genotypes (CT, TT, CC) of the SNP are shown in Table 4.17. We found a statistically significant difference in the HBV DNA levels in chronic and cirrhosis categories under different genetic models. Higher HBV DNA levels were observed in homozygous mutant genotype as compared to homozygous wild genotype (CC vs. TT, p=0.0001; CT+CC vs. TT, p=0.0001). No significant difference in the mean AST and ALT levels of different genotypes was observed in HBV categories in any genetic model.

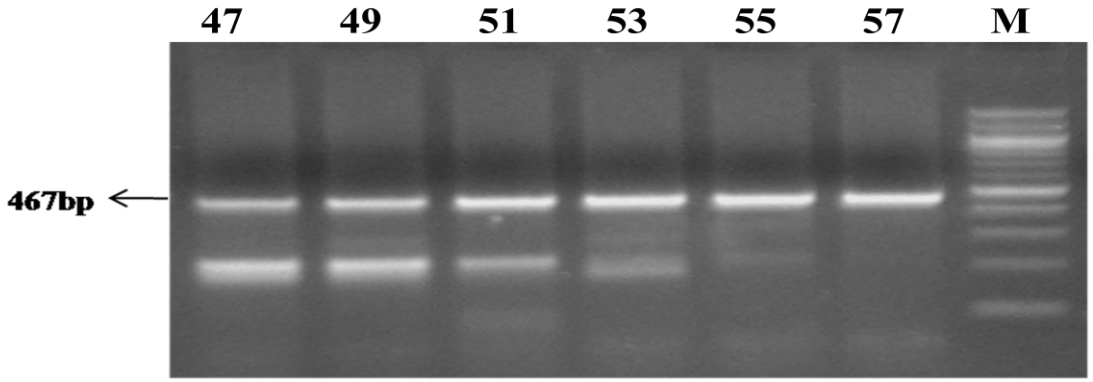

Figure 4.14: **Representative agarose gel image of gradient PCR Lane 1 to lane 6 represents the gradient temperature in increasing order from 58°C-68°C. M represents 100 bp DNA marker and NTC (Non-template control) represents the negative control.**

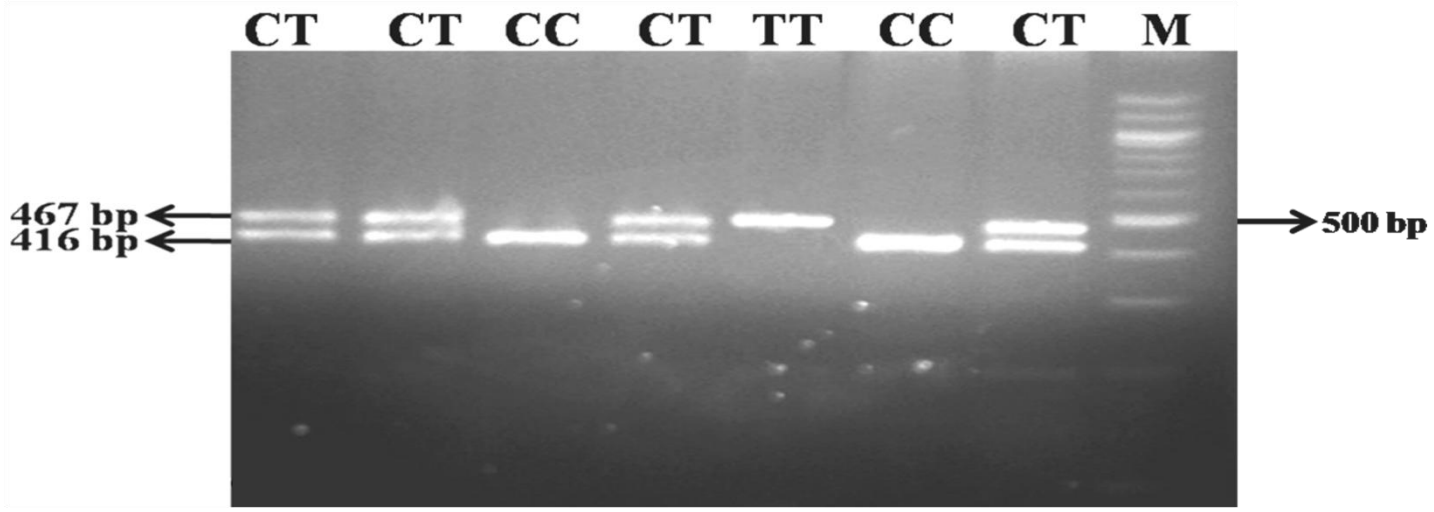

Figure 4.15: Representative agarose gel electrophoresis showing genotyping of SNP rs7587633 (C/T) by applying A-RFLP method. Lane 1, 2, 4 and 7 represents CT genotype, Lane 3 and 6 represents CC genotype, Lane 5 represents TT genotype and M represents 100 bp DNA marker.

Table 4.16 Genotypes and allele frequencies distribution of polymorphism rs7587633 (C/T) in HBV patients and healthy controls individuals

Polymorphism rs7587633 (C/T) MAF(T)=0.3470/1738	HBV (n/N[%]) (N=551)	Asymptomatic Carriers (n/N[%]) (N=111)	Acute Carriers (n/N[%]) (N=104)	CHB (n/N[%]) (N=286)	Cirrhosis (n/N[%]) (N=50)	Control (n/N[%]) (N=247)	HWE p-value
CC	381 (69)	86 (77.5)	72 (69)	185 (64.7)	38 (76)	161 (65.2)	0.22
CT	133 (24)	16 (14.4)	25 (24)	83 (29)	9 (18)	73 (29.6)	
TT	37 (7)	9 (8.1)	7 (7)	18 (6.3)	3 (6)	13 (5.2)	
C	895 (81.2)	188 (84.7)	169 (81.2)	453 (79.2)	85 (85)	395 (80)	
T	207 (18.8)	34 (15.3)	39 (18.8)	119 (20.8)	15 (15)	99 (20)	
HBV vs. Control	**Odds Ratio (95%CI)**			**p value**			
CC	1^{Ref}			-			
CT	0.76 (0.54 to 1.08)			0.13			
TT	1.20 (0.62 to 2.32)			0.58			
CT+TT	0.83 (0.60 to 1.14)			0.26			
C	1^{Ref}			-			
T	0.92 (0.70 to 1.20)			0.55			
Asymptomatic vs. Control							
CC	1^{Ref}			-			
CT	0.41 (0.22 to 0.74)			**0.0037***			
TT	1.29 (0.53 to 3.15)			0.56			
CT+TT	0.54 (0.32 to 0.91)			**0.021***			
C	1^{Ref}			-			
T	0.72 (0.47 to 1.10)			0.133			
Acute vs. Control							
CC	1^{Ref}			-			
CT	0.76 (0.44 to 1.30)			0.32			
TT	1.20 (0.46 to 3.14)			0.70			
CT+TT	0.83 (0.50 to 1.36)			0.46			
C	1^{Ref}			-			
T	0.92 (0.60 to 1.39)			0.69			
CHB vs. Control							
CC	1^{Ref}			-			
CT	0.98 (0.67 to 1.44)			0.95			
TT	1.20 (0.57 to 2.53)			0.62			
CT+TT	1.02 (0.71 to 1.46)			0.90			
C	1^{Ref}			-			
T	1.04 (0.77 to 1.41)			0.75			
Cirrhosis vs. Control							
CC	1^{Ref}						
CT	0.41 (0.22 to 0.74)			**0.0037***			
TT	1.29 (0.53 to 3.15)			0.56			
CT+TT	0.54 (0.32 to 0.91)			**0.021 ***			
C	1^{Ref}			-			
T	0.70 (0.38 to 1.27)			0.2450			

*** p-value <0.05 was considered significant and shown in bold**

Table 4.17 Comparison of HBV DNA, AST and ALT levels with different genotypes of rs7587633 C/T in HBV infected patients

HBV infection category No. of patients [N]	Genotypes[N] Genetic Models	HBV DNA (Copies /ml, mean ± SD)	p-value	AST (U/L) [Mean±SD]	p-value	ALT (U/L) [Mean±SD]	p-value
Asymptomatic	CC[86]	128±118		128±118		58±47	
[111]	CT[16]	76±68	0.09	76±68	0.09	63±64	0.71
	TT[9]	97±28	0.43	97±28	0.43	48±23	0.53
	CT+TT[25]	83±57	0.06	83±57	0.06	57±53	0.92
	CT+CC[102]	120±113	0.54	120±113	0.54	59±49	0.50
Acute	CC[72]	1654±1775		66±34		66±34	
[104]	CT[25]	1307±1358	0.37	79±85	0.28	79±85	0.28
	TT[7]	733±123	0.17	45±29	0.12	45±29	0.11
	CT+TT[32]	1168±1203	0.16	72±77	0.58	72±77	0.58
	CT+CC[97]	1565±1447	0.13	69±52	0.23	69±52	0.23
Chronic	CC[185]	2076667±3009531		104±63		104±63	
[286]	CT[83]	1743201±3390372	0.42	103±121	0.92	103±121	0.92
	TT[18]	45676578±16891293	**<0.0001***	113±31	0.55	113±31	0.55
	CT+TT[101]	3086751±7581322	0.11	105±109	0.92	105±109	0.92
	CT+CC[268]	1959273±3144244	**<0.0001***	104±87	0.66	104±87	0.66
Cirrhosis	CC[38]	249817758±254182376		175±80		175±80	
[50]	CT[9]	63683615±9812543	**0.03***	135±52	0.16	135±52	0.16
	TT[3]	23717828149±33288515793	**<0.0001***	154±26	0.65	154±26	0.65
	CT+TT[12]	7948398459±20418399044	**0.02***	282±453	0.16	282±453	0.16
	CT+CC[47]	216970556±240964439	**<0.0001***	175±80	0.65	175±80	0.65

*** p value <0.05 was considered significant and shown in bold**

4.5 Haplotype and linkage disequilibrium analysis in the *IRGM* and *ATG16L1* SNPs

4.5.1 Haplotype and linkage disequilibrium analysis in the *IRGM* SNPs

As mentioned in the section material and methods, haplotype analysis in the *IRGM* gene was done by SHEsis software. Haplotypes of three loci of *IRGM* gene, rs4958842, rs4958843 and rs4958846 and their frequencies in case and control group (HBV infected and healthy group) are shown in Table 4.18. We got seven haplotypes and we have excluded the haplotypes with a frequency lower than 3% from the study. Among seven predicted haplotypes only three haplotypes ATT, GTC and GTT were found to be associated with HBV infection susceptibility. The haplotypes ATT (OR=0.479, 95%CI=0.334- 0.688, p=0.001) and GTC (OR=0.686, 95%CI=0.511-0.921, p=0.01) a role of as a protective factor against hepatitis B infection whereas haplotype GTT appeared as a risk factor against hepatitis B infection (OR=2.01, 95% CI= (1.55-2.60), p=<0.0001). On linkage disequilibrium analysis we did not find any linkage between the three loci of *IRGM* gene (Figure 4.16).

4.5.2 Haplotype and linkage disequilibrium analysis in the *ATG16L1* SNP

Haplotypes of four loci of *ATG16L1* gene, rs2241880, rs2241879, rs13005285 and rs7587633 and their frequencies in HBV infected and healthy control group (case and control group) are shown in Table 4.19. Twelve haplotypes were predicted on analysis and haplotypes with frequencies lower than 3% were excluded from the study. Out of total twelve predicted haplotypes, only three haplotypes i.e. GGGC, AGTC, AATC were found to be associated with HBV infection susceptibility.

The haplotypes GGGC **(**OR=0.497, 95% CI=0.325-0.759), AGTC (OR=0.374, 95% CI=0.234-0.596, p=0.001) and GTC (OR=0.686, 95% CI=0.511-0.921, p=0.01) were appeared as protective factor against hepatitis B infection whereas haplotype GTT appeared as a risk factor against hepatitis B infection (OR=2.01,95% CI=(1.55-2.60), p=<0.0001). On linkage disequilibrium analysis we did not find any linkage between the four loci of *ATG16L1* gene (Figure 4.16).

Table 4.18. Haplotype frequencies of rs4958842, rs4958843 and rs4958846 in *IRGM* gene in both cases and control groups

Haplotypes (rs4958842-rs4958843-rs4958846)	Case (freq)	Control(freq)	χ2	Fisher's p	OR (95%CI)
A C C	15.25(0.019)	14.26(0.035)	2.887	0.089379	0.535 (0.257-1.113)
A T C	60.31(0.076)	27.99(0.070)	0.174	0.676432	1.104 (0.693-1.759)
A T T	**69.20(0.087)**	**66.99(0.167)**	**16.480**	**4.99e-005**	**0.479 (0.334-0.688)**
G C C	31.65(0.040)	23.54(0.059)	2.069	0.150411	0.671 (0.388-1.160)
G C T	106.86(0.135)	51.44(0.128)	0.123	0.725536	1.066 (0.746-1.523)
G T C	**138.79(0.175)**	**95.20(0.237)**	**6.336**	**0.011861**	**0.686 (0.511-0.921)**
G T T	**358.69(0.453)**	**117.81(0.293)**	**28.983**	**7.64e-008**	**2.016 (1.558-2.607)**

Table 4.19. Haplotype frequencies of rs2241880, rs2241879, rs13005285 and rs7587633 in *ATG16L1* gene in both cases and control groups.

Haplotypes	Case (freq)	Control (freq)	$\chi 2$	Fisher's p	OR (95%CI)
G G G C	**47.33(0.061)**	**47.11(0.118)**	**10.806**	**0.001019**	**0.497 (0.325-0.759)**
G G T C	36.10(0.047)	14.76(0.037)	0.707	0.400535	1.303 (0.702-2.418)
G A G C	172.75(0.223)	76.31(0.191)	2.116	0.145814	1.252 (0.925-1.695)
G A G T	34.47(0.045)	25.18(0.063)	1.661	0.197539	0.707 (0.416-1.201)
G A T C	51.37(0.066)	19.16(0.048)	1.795	0.180385	1.444 (0.841-2.477)
A G G C	91.20(0.118)	42.96(0.107)	0.415	0.519717	1.135 (0.772-1.669)
A G G T	20.47(0.026)	18.32(0.046)	2.885	0.089448	0.576 (0.303-1.096)
A G T C	**33.76(0.044)**	**44.18(0.110)**	**18.247**	**1.98e-005**	**0.374 (0.234-0.596)**
A G T T	23.74(0.031)	14.23(0.036)	0.153	0.695341	0.875 (0.448-1.708)
A G G C	119.52(0.154)	54.75(0.137)	0.870	0.351061	1.179 (0.834-1.668)
A A G T	29.07(0.038)	22.19(0.055)	1.833	0.175839	0.677 0.384-1.194)
A A T C	**70.98(0.092)**	**5.77(0.014)**	**26.558**	**2.66e-007**	**7.071 (2.998-16.676)**

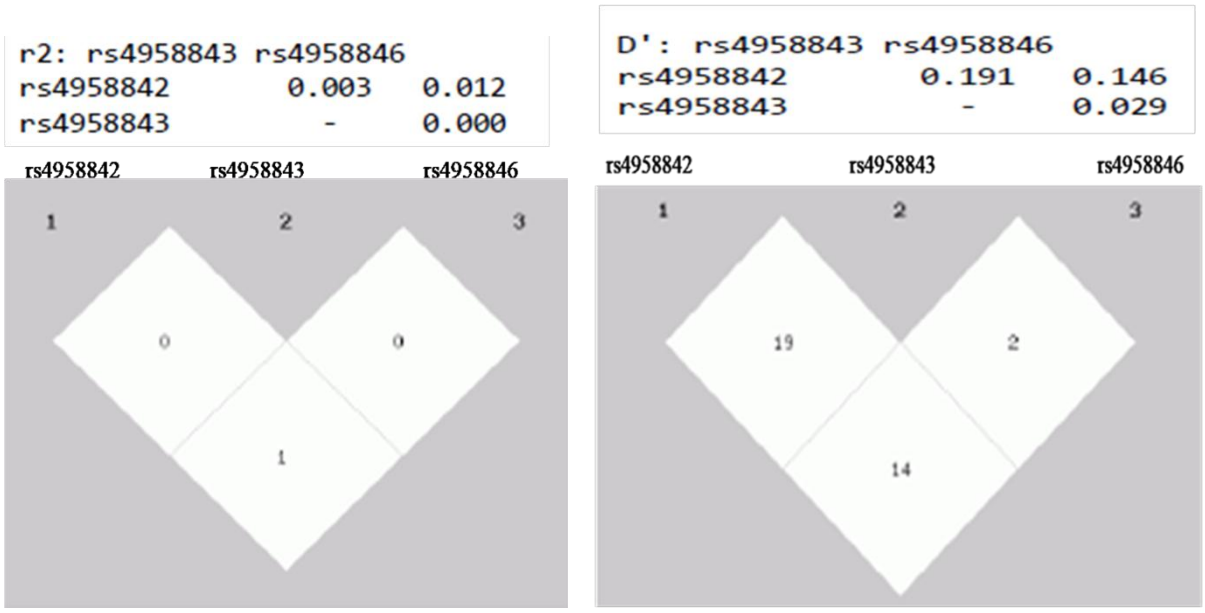

Figure 4.16: Linkage disequilibrium in between three SNPs (rs4958842, rs4958843 and rs4958846) of *IRGM* gene among cases and controls

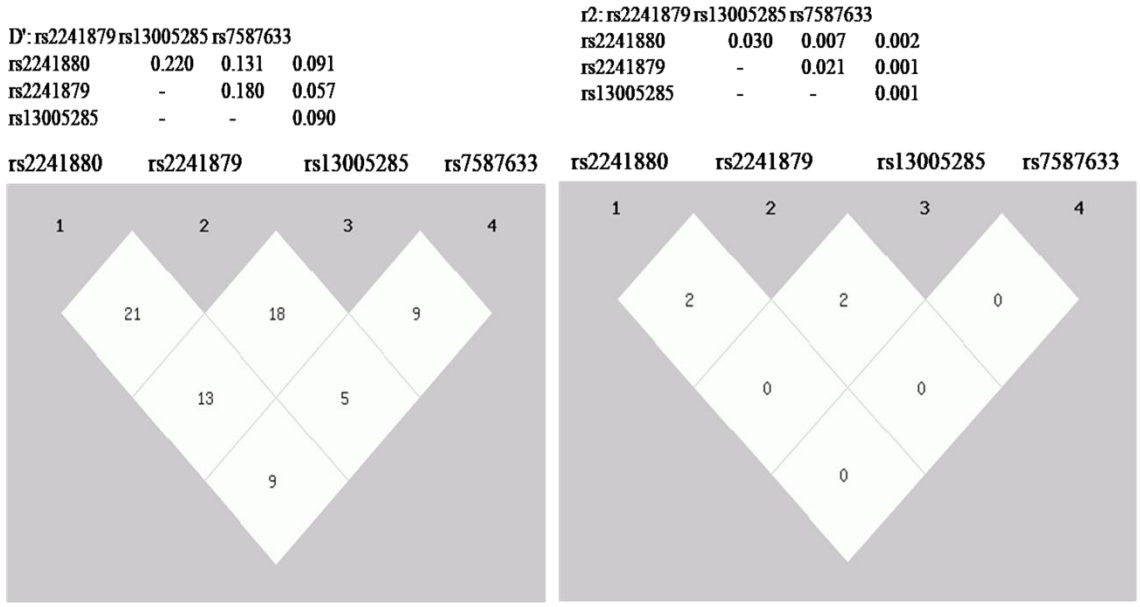

Figure 4.17: Linkage disequilibrium in between four SNPs (rs13005285, rs7587633, rs2241879 and rs2241880) SNPs of *ATG16L1* gene among cases and controls

CHAPTER-5

DISCUSSION

Although Hepatitis B is a vaccine-preventable disease but still worldwide there are around 257 million people are infected with this virus [214]. Out of total infants who acquire HBV infection during birth from their mothers, 90% of them become chronically infected and the children who get infected at an age between 1-5 years also develop chronic infection. [220]. This number is small in adults and only 5% of the adults progress to chronic infection. With the increase in age, the risk of chronic hepatitis B infection decreases [220]. In 2015, worldwide 8,87,000 people died due to the complication of HBV infection such as LC and HCC [3]. HBV infection is considered as a second main cause of liver cancer (HCC) related mortality worldwide [221]. Various environmental, virus related and host-related factors play role in the pathogenesis of HBV infection [60]. One important host related factor is Single Nucleotide Polymorphisms (SNPs). Various studies have documented the role of SNPs with HBV infection susceptibility. Polymorphisms of various genes such as members of the innate immune system, cytokines genes and many other genes have been found to be associated with the pathogenesis of HBV infection [30, 222, 223]. Various studies reported that HBV uses or modulate the autophagy process in different ways for its own replication [23, 25]. It has been reported that Atg5-Atg12-Atg16l1 complex helps in the HBV capsid formation and its release, hence showing the role of this complex in HBV infection [195].

ATG16L1 and *IRGM* genes are two important genes of the autophagy process. *IRGM* helps in the initiation process of autophagy [145] and *ATG16L1* helps in the autophagosome elongation [191]. Polymorphisms in these autophagy genes have been well documented in various diseases [145, 176, 224, 225]. No case-control association studies have been done so far to see the role of *IRGM* and *ATG16L1* genes SNPs in HBV infection susceptibility. The aim of the present study was to look for the polymorphisms in two important autophagy genes *IRGM* and *ATG16L1* which could be associated with HBV infection.

5.1. Newly developed A-RFLP methods for genotyping of rs4958842 (G/A), rs4958843 (T/C) and rs4958846 (T/C) polymorphisms of *IRGM* gene

The promoter region polymorphisms of *IRGM* gene studied in various populations have been genotyped by various high throughput methods such as using direct sequencing, ligation detection reaction, and T-ARMS [51, 160, 161]. The genotyping of the SNP by using various high throughput methods require sophisticated and pricey instruments. So, we have developed A-RFLP methods for the genotyping of these SNPs [219, 226] These newly developed A-RFLP methods are cost-effective, does not require expensive instrumentation and can be used in small-scale laboratories. To genotype these SNPs using the PCR-RFLP method, restriction site of any available restriction enzymes was not present at SNP position. So we have created mismatches in the forward primers to create restriction sites of enzymes *Nru*I, *Nhe*I and *Sal*I for the SNPs rs4958842, rs4958843 and rs4958846 respectively. Genotypes were also confirmed by sequencing and A-RFLP results and sequencing results were concordant.

5.1.1 *IRGM* promoter SNPs rs4958842 (A/G) and rs4958846 (C/T) are associated with HBV protection while rs4958843 (C/T) is associated with increased HBV infection susceptibility

The selected promoter region polymorphisms rs4958842, rs4958843 and rs4958846 were genotyped by in 551 HBV infected and 247 healthy control samples to see their role in HBV infection susceptibility. Statistical analysis was done to see the difference in allelic and genotypic frequencies in HBV infected and healthy control group. Moreover, HBV DNA levels and plasma AST and ALT levels were also compared with different genotypes of the SNPs. Association of the SNPs with HBV infection susceptibility was assessed by using various genetic models such as allelic, homozygous, dominant and co-dominant models.

The polymorphism rs4958842 (-1208 A/G) was found to play protective role in HBV infection in totality and also in the CHB group in the allelic, dominant and co-dominant models (Table 4.3). Different genotypes of the SNP rs4958842 were correlated with mean levels of HBV DNA and we did not find any significant difference among them. SNP rs4958842 (G/A) is associated with the protection of HBV (CHB group), and we also observed low levels of HBV DNA of mutant genotype AA in CHB group. A significant difference was observed in the levels of AST (GA; p=0.01) and ALT (GA; 0.009) only in cirrhosis group of patients (Table 4.4).

For the polymorphism rs4958843 (-1161 C/T) we did not find any association with HBV infection susceptibility in totality in any genetic model used but significant association was observed in susceptibility to CHB infection in dominant and co-dominant genetic models (Table 4.5). On comparing genotype of the SNP rs4958843 with HBV DNA levels we found the statistically significant difference between these only in the acute category (TC; p=0.003, TC+CC; p=0.003), and in case of ALT levels, a significant difference was observed only in CHB group (TC+CC; p=0.02). No difference was observed between AST levels when compared with genotypes of the SNP rs4958843 (Table 4.6). The SNP rs4958843 is associated with HBV susceptibility (CHB group) but we did not find any correlation between higher levels of HBV DNA with mutant genotype CC.

SNP rs4958846 (-947 C/T) was also found to be significantly associated with protection from HBV infection in totality in allelic, dominant and homozygous genetic models and in CHB infection in allelic, dominant, co-dominant and homozygous genetic models and in cirrhosis category only in the allelic model (Table 4.7). For SNP rs4958846 no significant difference was observed in the mean HBV DNA and ALT levels of different genotypes among all HBV infection categories. A significant difference was observed in the mean levels of AST of different genotypes in asymptomatic (CC; p=0.05) and CHB (CC; p=0.003, CT+TT; p=0.003) group (Table 4.8).

Numbers of case-control association studies have been conducted to see the association of various SNPs of the *IRGM* gene in a wide range of diseases such as IBD (CD and UC) [157, 227, 228], tuberculosis [51, 160, 161] and leprosy [47]. Che et al sequenced the 1.7 kb promoter region of the *IRGM* gene and identified 29 SNPs, out of which 11 SNPs were novel [160]. Three promoter region SNPs (rs4958842 (-1208 A/G), rs4958843 (-1161 C/T) and rs4958846 (-947 C/T) were selected and genotyped in 216 TB patients 275 and healthy control subjects to see their association in TB susceptibility.

SNP rs4958842 (-1208 A/G) was found to be associated with decreased susceptibility to TB infection [160]. However, the role of three promoter region polymorphisms (rs4958842, rs4958843, rs4958846) of the *IRGM* gene has been documented by very few studies only in tuberculosis [51, 161] and leprosy [47]. To validate the role of these three promoter region polymorphisms in a wide range of disease pathogenesis, these polymorphisms need to be studied

in different diseases in different populations. We have compared our study results with the previous studies published on these SNPs and we observed that our results were consistent with the study conducted by Chen et al in Chinese population where polymorphism -1208A/G was found associated with decreased susceptibility to TB infection [160]. In contrast to our results, Bahari et al reported no association of the SNP −1208 A/G with TB in Iranian population and SNPs rs4958843 (-1161 C/T) and rs4958846 (-947 C/T) found to be associated with decreased susceptibility to TB infection [161]. Another study on Chinese population again reported no association of the SNP −1208 A/G with leprosy [47]. In another study conducted by Yuan et al in Chinese Hubei Han population, no significant association of the SNPs −1208 A/G and (-1161 C/T) was observed, but SNP (-947 C/T) was found to be associated with decreased susceptibility of HBV infection. In a study, Maura et al compared HBV DNA and plasma AST and ALT levels with the genotypes of SNPs rs1800450, rs1800451, and rs5030737 of MBL gene and reported that wild AA genotype is associated with these parameters [229]. Another study also reported a significant difference in HBV DNA and plasma ALT levels of different genotypes of the SNPs present in the HBV core gene [230].

5.1.2 Haplotype analysis identifies GTT as risk factor for HBV infection and ATT and GTC plays a protective role in North Indian population

Haplotypes analysis is considered more powerful than individual SNP analysis particularly when there is weak linkage disequilibria exist between SNPs [231]. As discussed above, *IRGM* gene promoter region polymorphisms have been well documented in various diseases. The association of haplotypes of these three SNPs. rs4958842, rs4958843, and rs4958846 has already been reported in association with TB infection [51]. All the SNPs (rs4958842, rs4958843, and rs4958846) were in linkage disequilibrium. Yuan et al have identified six haplotypes, out of them ACC haplotype found to be associated with decreased susceptibility and ACT haplotype found to be associated with increased susceptibility to TB infection [51]. Furthermore, ACT haplotype shown to reduce the luciferase activity of *IRGM* promoter and decreased expression of *IRGM* was reported in TB patients [51]. In another study, haplotype ACC was again found to be associated with decreased susceptibility and GCC haplotype associated with risk to TB infection [160]. We have carried out haplotype and linkage disequilibrium (LD) analysis of these four SNPs, and we have got seven total haplotypes among cases and controls. Out of seven

haplotypes, only three haplotypes found to be associated with HBV infection ($p<0.05$). In contrast to previous studies, ATT and GTC haplotypes were found associated with HBV protection and GTT haplotype was associated with increased susceptibility to HBV infection in our population. Moreover, on LD analysis we did not find any linkage disequilibrium between the three SNPs.

5.2 *ATG16L1* gene polymorphisms

Jochen Hampe and colleagues performed genome-wide association study and found that many non-synonymous SNPs of *ATG16L1* carries virtually all the disease risk exerted by the *ATG16L1* locus [188]. They have identified the *ATG16L1* as a candidate gene responsible for Crohn's disease [188]. The present study includes four extensively studied SNPs, rs2241880 (A/G), rs2241879 (G/A), rs13005285 (G/T) and rs7587633(C/T) that span a region of 36 kb within *ATG16L1* gene. These four SNPs are found to be associated with various diseases such as Crohn's disease, inflammatory bowel disease, psoriasis vulgaris, palmoplantar pustulosis, Paget disease of bones and gastric cancer [37-43]. SNPs of *ATG16L1* gene found to be associated with various diseases including bacterial diseases but their role in viral diseases still need to be explored. We have genotyped these four SNPs in 551 HBV infected individuals and 247 healthy control subjects.

5.2.1 nsSNP rs2241880 (A/G) and intronic SNP rs2241879 (G/A) are associated with increased HBV infection susceptibility

Out of four *ATG16L1* SNPs included in the study, the only coding variant is rs2241880 (T300A).The substitution in rs2241880 from T300A enhances the cleavage of *ATG16L1* protein by caspase-3 and caspase-7 mediated pathway during stress conditions and starvation and destabilization of *ATG16L1* protein lead to the reduction in autophagy. Reduced autophagy in results leads to less bacterial killing and elevated production of interleukin-1b [191, 232]. The nsSNP rs2241880 has already been studied in various diseases such as Crohn's disease and ulcerative colitis (IBD) in different population [233-236], gastric cancer [43], Paget Disease of Bone [42], Buruli ulcer [237], palmoplantar pustulosis [41], Psoriasis Vulgaris [40], colorectal cancer [193] and rheumatoid arthritis [238]. First GWAS identified rs2241880 SNP as a risk allele for Crohn's disease and after that various replication studies among European population

supported the GWAS [239]. Meta-analysis study conducted by Zhang et al have shown that G allele of the SNP rs2241880 in co-dominant genetic model found to be associated with Crohn's disease in Caucasians population, but not in Asian population [233]. Another study, conducted by Sadabad et al showed that in the CD patients risk allele G is associated with the pathogens of families, Enterobacteriaceae, Bacteroidaceae, and Fusobacteriaceae, whereas the patients with wild allele A inhabit beneficial bacteria in their intestinal mucosal membrane [235]. In another study on Romanian population homozygous mutant GG and AG genotype was found to be associated with lower risk of gastric cancer and in the dominant genetic model strongest association was observed [43]. In the Spanish population, mutant allele G was found to be associated with increased risk of Paget disease of bones (PDB) [42]. Capela et al reported the protective role of G allele in the Buruli cancer patients [237]. Higher frequency distribution of rs2241880 G allele was observed in patients with palmoplantar pustulosis disease in Estonian population [41].

In comparison with previous studies on SNP rs2241880, in our studied population also the frequency of a mutant allele (rs2241880, A/G) was found higher in HBV patients (53%) as compared to healthy control group (46%) (Table 4.10). We observed a significant association of the mutant allele G in the increased susceptibility to HBV infection. Furthermore, in the homozygous genetic model, we again observed a significant association of the allele in HBV infection susceptibility. In asymptomatic and chronic categories of HBV infection, we again observed a significant association of the mutant allele in HBV infection risk in the allelic and homozygous model. We observed a statistically significant difference in the mean levels of HBV DNA according to genotypes of the SNP in asymptomatic (AG+GG; p=< 0.0001, AG+AA; p=< 0.0001), chronic (p=< 0.0001) and cirrhosis category (AG; p=< 0.0001, AG+GG; p=0.04, AG+AA; p=0.004) (Table 4.11). On comparing biochemical parameters AST and ALT with different genotypes of the SNP we did not find any association with AST levels but for ALT we found significant difference in the levels according to genotypes only in cirrhosis category (AG; p=0.007, AG+GG; p=0.03) (Table 4.11)

The intronic region variant rs2241879 (G/A) has already been well studied in various diseases such as palmoplantar pustulosis [41], psoriasis vulgaris [40], Crohn's disease [37]. In German population mutant allele, A of the SNPs rs2241879 (G/A) was found to be associated with

protection with Crohn's disease [37]. Moreover, in the patients with palmoplantar pustulosis disease (chronic inflammatory skin disease), higher frequency of the mutant allele was observed [41]. Additionally, the mutant allele was identified in lower frequency among the patients with Psoriasis vulgaris and the variant found to be associated with protection with the disease in only co-dominant model [40]. In our studied population frequency of mutant allele A (rs2241879 (G/A) was found higher in both, HBV patients (66%) and healthy control group individuals (56%) (Table 4.12). We observed a significant association of the mutant allele A in the increased susceptibility to HBV infection.

Furthermore, in the dominant genetic model, we again observed a significant association of the mutant allele A in HBV infection susceptibility. On category wise analysis we did not observe any association of the SNP in asymptomatic and acute categories but in chronic and cirrhosis categories significant association was observed with HBV infection risk in allelic, dominant and homozygous genetic models (Table 4.12). Increase in the Odds ratios was observed from chronic to cirrhosis category, shows that the SNP might play role in HBV progression. Significant difference was observed in the means levels of HBV DNA according to genotypes in asymptomatic (GA; p=< 0.0001, AA; p=0.007, GA+AA; p=0.0001), chronic (GA; p=0.01, = AA; p=0.01, GA+AA; p=0.01), and in cirrhosis categories (GA; p=< 0.0001, AA; p=< 0.0001, GA+AA; p=< 0.0001). On comparing biochemical parameters AST and ALT with different genotypes of the SNP we did not find any association with these parameters (Table 4.13). Both the SNPs rs2241880 and rs2241879 predispose the individuals of North Indian Population to HBV risks.

5.2.2 Intronic SNP rs13005285 (G/T) is associated with HBV infection susceptibility and rs7587633 (C/T) is associated with HBV protection

Another, two intronic region variants that we genotyped in our study are rs13005285 (G/T) and rs7587633 (C/T). Both the variants have already been studied in only two diseases, palmoplantar pustulosis [41], psoriasis Vulgaris [40]. Both the intronic region variants were genotyped by using SNPlex Genotyping System. To genotype these intronic region variants by applying PCR-RFLP, a restriction site for any restriction enzymes was not present at the SNPs position. So in order to perform genotyping by applying PCR-RFLP, we have created restriction sites for the enzymes *Hin*dIII and *Eco*RI. Further genotyping was done with the help of newly developed A-

RFLP methods. In the patients with palmoplantar pustulosis, lower frequency of mutant allele present in both the cases and controls in both the SNPs (rs13005285 G/T) and rs7587633 (C/T) [41]. Moreover, in another study again low frequency of mutant allele observed for both the SNPs in the patients with Psoriasis vulgaris [40]. Similar to the results of previous studies, in our studied population frequency of mutant allele T (rs13005285 (G/T) was found lower in both, HBV patients (30%) and healthy control group individuals (23%) as compared to wild allele G. We observed significant association of the mutant allele T in the increased susceptibility of HBV infection (allelic model). Furthermore, in dominant and homozygous genetic models we again observed a significant association of the mutant allele T in HBV infection susceptibility. On category wise analysis we observed a significant association in asymptomatic, acute and cirrhosis categories with HBV infection risk in allelic, dominant and homozygous genetic models (Table 4.14). We did not observe any significant increase in the OR, which shows that SNP might not play any role in HBV progression. A significant difference was observed in the mean levels of HBV DNA in asymptomatic (TT; p=0.001, GT+GG; p=0.001) and chronic categories (GT; p=0.02, GT+TT; p=003) and ALT levels only in the asymptomatic category (TT; p=0.03, GT+GG; p=0.0015). No difference was observed in the mean levels of AST according to different genotypes (Table 4.15).

In our studied population frequency of mutant allele T (rs7587633 (C/T) was found lower in both, HBV patients (19%) and healthy control group individuals (20%) as compared to wild allele C. We did not find any significant association of the SNP in the susceptibility of HBV infection. Furthermore, on category wise analysis we observed a significant protective association of the SNP in asymptomatic and cirrhosis categories in dominant and co-dominant models (Table 4.16). A significant difference was observed in the mean levels of HBV DNA according to genotypes in chronic (TT; p=<0.0001, CT+CC=<0.0001) and cirrhosis categories. On comparing biochemical parameters AST and ALT with different genotypes of the SNP we did not find any association with these parameters (Table 4.17).

5.2.3 Haplotype analysis identifies AATC as risk factor for HBV infection and GGGC and AGTC plays a protective role in North Indian population

As discussed above, rs2241880, rs2241879, rs13005285 and rs7587633 variants of the *ATG16L1* gene have been well documented in various diseases. There are only two studies conducted by

Douroudis et al, which included all the four SNPs. Haplotypes analysis was conducted in both the studies. We did not find any linkage disequilibrium between these SNPs. Four haplotypes were observed in the palmoplantar pustulosis patients and control group i.e. AGGC, AGTC, AGTT, and GAGC. In the patients with palmoplantar pustulosis, GAGC haplotype was found higher as compared to control group subjects [41]. Douroudis et al genotyped six SNPs in Psoriasis Vulgaris patients and significant linkage disequilibrium was exist in the *ATG16L1* SNPs (D´> 0.95) [40]. AGGT haplotype was found to be associated with decreased susceptibility to Psoriasis Vulgaris.

We have carried out haplotype and linkage disequilibrium (LD) analysis of these four SNPs, and we have got total twelve haplotypes among cases and controls. Out of twelve haplotypes, only three haplotypes found to be associated with HBV infection ($p<0.05$). In our population, two haplotypes GGGC and AGTC were found to be associated with decreased HBV infection risk and haplotype AATC was found to be associated with increased HBV infection risk. The frequency of AGTC haplotype was found lower in HBV group and AATC haplotype was found higher in HBV group as compared to control group. The equivalent frequency of GGGC haplotype was found in both the groups.

CHAPTER-6

CONCLUSION AND FUTURE PROSPECTS

It has been well documented in various studies that SNPs play an imperative role in the pathogenesis of a broad range of diseases such as various neurodegenerative disorders, Inflammatory Bowel Disease, various cancers, viral diseases such as hepatitis B, hepatitis C, and many others. Instead of availability of effective vaccination against HBV, the burden of hepatitis B is still very high, mainly in developing countries. Environmental and virus-related factors that play role in the pathogenesis of HBV infection are well studied, but host-related factors or host genetics needs to be explored in order to develop effective preventive measures or therapeutic approaches to combat this deadly disease. Many studies elucidated the role of polymorphisms in various genes such as cytokines, MHC class I and MHC class II and many others with HBV infection. Recently, it has been shown that HBV uses the important process of the immune system i.e. autophagy for its own benefit that helps in its own replication. Moreover, studies have also demonstrated that inhibition of this pathway results in inhibition of HBV replication and this pathway could be targeted for antiviral treatment and better management of patients infected with this virus. Elevated mRNA expression levels of *ATG5* and *ATG12* were reported in HepG2.2.15 cell lines as compared to HepG2 cell lines. Higher levels of autophagy have been observed in HBV infected patients. There are various ways by which HBV replicates by using the autophagy process, which helps in maintaining cellular homeostasis. The expression level of an autophagy gene (*ATG16L1*) was found to be higher in HepG2.2.15 cell lines providing the evidence of the role of autophagy in HBV infection. *IRGM*, another autophagy gene is the common target of various viruses and play important role in the pathogenesis of viral diseases. In this study we have analyzed SNPs which are present in the intronic and promoter regions of the *ATG16L1* and *IRGM* gene. It has been well documented that if SNPs or genetic variants are present in the coding region of the gene, promoter region, intronic region and miRA binding sites they might alter the gene function. Intronic region SNPs may affect alternating splicing of the mRNA and hence genes function. So, we have hypothesized in our study that SNPs of autophagy genes might play role in the pathogenesis of HBV infection. In this study, we have genotyped SNPs of two important autophagy genes, *IRGM* and *ATG16L1* in 551 HBV infected and 247

healthy control patients from North Indian Population. Three promoter region SNPs (rs4958842, rs4958843, and rs4958846) of *IRGM* gene and four intronic (rs2241879, rs13005285 and rs7587633) and exonic (rs2241880) SNPs of *ATG16L1* gene which have already been found to be associated with various diseases were included in this study. We have developed A-RFLP methods to genotype three SNPs (rs4958842, rs4958843, and rs4958846) of *IRGM* gene and two SNPs (rs13005285 and rs7587633) of *ATG16L1* gene. These five SNPs were further genotyped by using newly developed A-RFLP methods.

In case of *IRGM* SNPs, we found the protective association of the SNP rs4958842 (G/A) with HBV infection. The other SNP rs4958843 (T/C) was found to be associated with HBV infection risk only in chronically (CHB) infected patients. The SNP rs4958846 (T/C) was also found to be associated with protection from HBV infection when compared with all HBV infected individuals as a group, in CHB and cirrhosis categories of patients.

rs2241880, T300A (G/A) is the only nsSNP of the *ATG16L1* gene that was included in the study. The SNP rs2241880 (A/G) is responsible for the change of an amino acid Threonine with Alanine in the protein at AA 300. The SNP has been implicated as a risk factor in Crohn's disease. In our population, the SNP rs2241880 was found to be associated with HBV infection risk. The other three SNPs of ATG16L1 gene are present in the intronic region of the gene. The SNP rs2241879 (G/A) was found to be associated with an increase in HBV infection risk. The SNP rs13005285 (G/T) also predisposes individuals to HBV infection risk. The intronic region SNP rs7587633 (C/T) of ATG16L1 gene was found to be associated with decreased susceptibility to HBV infection risk. In nutshell, the study highlights the importance of autophagy genes SNPs in the pathogenesis of HBV infection. We have successfully genotyped seven SNPs of *IRGM* and *ATG16L1* genes in a sample of North Indian Population. Some SNPs predispose individuals of the studied population to HBV infection and some helps in protection from HBV. We found an association of all the SNPs in HBV infection susceptibility. Further studies on genotyping of these SNPs in different population and in more number of samples are needed to explicate the role of these SNPs in HBV infection. After validating the role of these SNPs in a large cohort and if found associated with HBV, can be used as biomarkers for the management of this dreadful disease in our population.

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Reagents and buffers: All the chemical reagents and buffers used in the study were prepared in the nuclease free water

Red Blood cell (RBC) lysis buffer		
Ingredients	**Volume (ml)**	**Final Concentration**
Tris (8.0)	10	1 M
EDTA	2	0.5 M
NH_4Cl (pH-8.0)	125	1 M
Final volume was made 1000 ml with distilled water		

Ammonium Chloride (NH_4Cl)		
Ingredients	**Amount (g)**	**Final Concentration**
Ammonium Chloride (NH_4Cl)	5.35	1 M
5.35 grams of NH_4Cl dissolved in 80 ml of distilled water and final volume was made up to 100 ml.		

Agarose gel (2%)		
Ingredients	**Amount (g)**	**Final Concentration**
Agarose	2	2%
Dissolve 2 grams agarose in 100 ml of 1X TAE buffer, dissolved completely by heating in microwave oven.		

Tris (hydroxymethyl) amino methane-chloride (Tris-Cl)		
Ingredients	**Amount (g)**	**Final Concentration**
Tris base	12.11	1 M
pH was set to 8.0 by using 1N HCL. Added autoclaved distilled water to make up the final volume to 100 ml.		

Di-sodium ethylene diamine tetra acetate (Na_2EDTA)		
Ingredients	**Amount (g)**	**Final Concentration**
EDTA	18.61	0.5 M
18.61 grams of EDTA was dissolved in 50 ml of distilled water and placed over magnetic stirrer. 10 M NaOH was added to the above mixture. Allowed the salt to dissolve completely. Final volume was made up to 100 ml and pH was set at 8.0.		

Tris EDTA (TE) buffer (pH-8.0)		
Ingredients	**Volume (ml)**	**Final Concentration**
Tris Cl	10	1 M
EDTA	2	0.5 M
Final volume was made 1000 ml with distilled water.		

Tris EDTA (TE) buffer (pH-7.0)		
Ingredients	**Volume (ml)**	**Final Concentration**
Tris Cl (pH-7.3)	10	1 M
EDTA	2	0.5 M
Final volume was made 1000 ml with distilled water		

Sodium dodecyl sulphate (SDS) (10%)		
Ingredients	**Amount (g)**	**Final Concentration**
SDS	10	10%
Final volume was made 100 ml with distilled water		

Ammonium acetate (7.5 M)		
Ingredients	**Amount (g)**	**Final Concentration**
Ammonium acetate	28.9	7.5 M
28.9 grams of the salt was dissolved in 20 ml of distilled water and final volume was made 50 ml with distilled water		

Ethyl alcohol (70%)		
Ingredients	**Volume (ml)**	**Final Concentration**
Dehydrated ethyl alcohol	70	70%
70 ml of Dehydrated ethyl alcohol was mixed in 30 ml of autoclaved distilled water to obtain final volume of 100 ml		

Tris-acetic acid-EDTA (TAE) buffer (50X)		
Ingredients	**Amount (g)**	**Final Concentration**
Tris base	242	50X
Dissolved in 500 ml of distilled autoclaved water and 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH-8.0) was added. Final volume was made 1000 ml.		

Ethidium Bromide (EtBr) (10mg/ml)		
Ingredients	**Amount (mg)**	**Final Concentration**
Ethidium Bromide	50	10mg/ml
Added 5 ml of distilled autoclaved water and completely dissolved by heating in microwave oven.		

LIST OF PUBLICATIONS

[1] Sharma A and Changotra H (2017) Novel artificial restriction fragment length polymorphism methods for genotyping IRGM promoter polymorphisms. *Inflammatory Bowel Disease* 23(10): E52–E53 **(Impact Factor: 4.6; SCOPUS).**

[2] Sharma A and Changotra H (2017) Mutagenic primer based PCR-RFLP assay for genotyping IRGM gene promoter variant rs4958843 (C/T). *Journal of Clinical Laboratory Analysis*. doi: 10.1002/jcla.22346. **(Impact Factor: 1.52; SCOPUS).**

CONFERENCE ABSTRACTS

[1] **Sharma A,** Duseja A and Changotra H (2018) Association of *IRGM* gene promoter polymorphisms with HBV infection. 43rd Annual Meeting of Indian Society of Human Genetics on Population and Medical Genomics, Centre for Cellular and Molecular Biology, Hyderabad, India, 12-14 March, 2018.

[2] Sharma A, Duseja A, Changotra H (2017) Association of *IRGM* promoter polymorphisms with hepatitis B virus infection in North Indian population. 42nd Annual Meeting of the Indian Society of Human Genetics & International Symposium on Trends in Human Genetic Research & Management, Indian Institute of Sciences, Bangalore, 2-4 March, 2017.

[3] Sharma A, Duseja A and Changotra H (2017) Association of *IRGM* gene variant rs4958842 with Hepatitis B infection in North Indian population. 20th Punjab Science Congress, IET Bhaddal, Ropar, Punjab, 7-9 February 2017.

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