INFLUENCE OF VIRAL AND HOST CHARACTERISTICS ON CLINICAL OUTCOME OF HEPATITIS B

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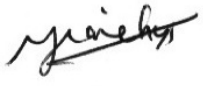
Witness's Signature  Date 25-6-2013

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Abstract (English version)

Hepatitis B is a global health problem, affecting >2 billion people worldwide. Chronicity develops in >90% of neonates infected by hepatitis B virus (HBV). After several decades of the infection, about a-quarter-to-half may progress to liver cirrhosis and hepatocellular carcinoma (HCC). In contrast, more than 90% of people infected during adulthood may clear the infection, resulting in acute self-limiting hepatitis. Both virus and host may influence clinical outcome of the infection. Basal core promoter (BCP; A1762T/G1764A) and precore stop codon (G1896A) mutations have been commonly associated with cirrhosis and HCC, whereas wild-types of these mutations with acute hepatitis. These associations have been inconsistent; the disease progression may have been affected by other non-viral factors. Host iron status could be one of the important factors; iron overload increases risk of cirrhosis and HCC. It has rarely been considered in chronic hepatitis B studies. The present study is divided into two sections. The aim of the first section is to investigate the influence of viral genomic variations and host iron markers (serum iron and serum ferritin) on progression to cirrhosis and HCC, whereas the second section to identify potential viral genomic variations associated with chronicity based on *in silico* observation. Overlapping polymerase chain reaction (PCR) was applied to amplify viral genomic fragments. The fragments were then sequenced and assembled. Sera were also sent to clinical laboratory for testing of the iron markers. To search for candidate nucleotides associated with cirrhosis, comparative sequence analysis was performed in 20 cirrhotic cases and 20 controls, whereas for HCC, the analysis was done in 21 HCC cases and 24 controls. The most potential viral marker was then applied in larger chronic hepatitis B populations, including 216 controls, 78 cirrhosis and 39 HCC cases. Binary logistic regression analysis showed that older age, cigarette smoking, family history of cirrhosis/HCC, HBV precore wild-type, serum iron and serum ferritin were associated independently

with HCC, whereas older age, male gender, Malay ethnicity, precore wild-type, serum iron and serum alanine aminotransferase (ALT) with cirrhosis. To search for candidate nucleotides associated with chronicity, comparative sequence analysis was conducted in 177 acute cases and 1,149 chronic cases. Binary logistic regression coupled with Bonferroni-correction identified four novel viral variants (G1171, T1785, A1786 and T3112) independently associated with acute hepatitis. These variants are located in enhancer-I-X-promoter and S promoter regions, mutations in which result in reduced viral replication and release. In conclusions, older age, precore wild-type and serum iron markers may increase the risk of progression to cirrhosis and HCC, but not to NAFLD. Cigarette smoking, male gender, Malay ethnicity and high serum ALT should also be considered. The present study has also identified novel variants (G1171, T1785, A1786 and T3112) highly specific for acute self-limited infection. These putatively replication-defective variants may be responsible for lower rate of chronicity in some cases of HBV infection. Further *in vitro* and *in vivo* investigations are required to confirm this hypothesis.

Abstrak (Malay version)

Hepatitis B adalah masalah kesihatan global yang menjejaskan lebih 2 ribu juta orang di seluruh dunia. Jangkitan hepatitis B kronik berlaku pada lebih daripada >90% bayi baru lahir yang dijangkiti oleh virus hepatitis B (HBV). Selepas jangkitan selama beberapa dekad, lebih kurang satu perempat hingga 40 peratus boleh mengalami komplikasi serius seperti sirosis hati dan kanser sel hati (HCC). Sebaliknya, lebih daripada 90% daripada orang yang dijangkiti semasa dewasa boleh mengawal jangkitan mereka yang terhad kepada hepatitis akut. Faktor-faktor melibatkan HBV and pesakit sendiri boleh mempengaruhi akibat jangkitan. Mutasi "basal core promoter" (BCP; A1762T/G1764A) dan "precore stop codon" (G1896A) telah sering dikaitkan dengan sirosis dan HCC, sedangkan nukleotida-nukleotida jenis liar tersebut sering berhubungkait dengan hepatitis akut. Hubungkait-hubungkait ini adalah tidak konsisten, perkembangan penyakit mungkin telah dipengaruhi oleh faktor-faktor yang bukan berkaitan dengan virus. Status zat besi pesakit boleh menjadi salah satu faktor penting; lebihan beban zat besi meningkatkan risiko sirosis dan HCC. Akan tetapi, ia jarang dipertimbangkan dalam kajian penyakit kronik hepatitis B. Kajian ini dibahagikan kepada dua bahagian. Tujuan bahagian pertama adalah untuk menyiasat pengaruh variasi virus genomik dan penanda zat besi pesakit (besi serum dan ferritin serum) pada perkembangan sirosis dan HCC, manakala bahagian kedua adalah untuk mengenalpasti variasi genomik virus berpotensi yang boleh dikaitkan dengan jangkitan kronik berdasarkan pemerhatian *in silico*. Tindakbalas rantaian polimerase (PCR) secara bertindih telah digunakan untuk mengamplikasi fragmen genomik virus. Fragmen tersebut kemudiannya disusun dan dipasang. Sera juga telah dihantar ke makmal klinikal untuk ujian-ujian penanda besi. Untuk mencari nukleotida calon virus yang dikaitkan dengan sirosis, analisis urutan genetik virus secara perbandingan telah dilaksanakan dalam 20 kes sirosis dan 20 kawalan, manakala bagi HCC, analisis telah dilakukan dalam 21 kes HCC dan 24

kawalan. Penanda virus yang paling berpotensi untuk mengakibatkan sirosis dan HCC kemudiannya diaplikasikan dalam populasi kronik hepatitis B yang lebih besar, termasuk 216 kawalan, 78 sirosis dan 39 kes HCC. Analisis regresi logistik secara perduaan menunjukkan bahawa umur yang lebih tua, merokok, sejarah keluarga sirosis/HCC, HBV precore jenis liar ("precore wild type"), besi serum dan ferritin serum dikaitkan secara bebas dengan HCC, sedangkan usia yang lebih tua, jantina lelaki, etnik Melayu, precore liar-jenis, besi serum dan serum alanine aminotransferase (ALT) dengan sirosis. Untuk mencari calon-calon nukleotida virus yang dikaitkan dengan jangkitan kronik, analisis urutan genetik virus secara perbandingan telah dijalankan pada 177 kes akut dan 1149 kes kronik. Regresi logistik secara perduaan disertakan dengan pembetulan Bonferroni mendapati bahawa empat varian virus novel (G1171, T1785, A1786 dan T3112) dikaitkan secara bebas dengan hepatitis akut. Varian-varian ini terletak dalam lingkungan "enhancer I-X promoter" dan "S promoter", kawasan-kawasan mutasi yang mengurangkan kadar replikasi and pelepasan virus. Kesimpulannya, usia yang lebih tua, precore jenis liar dan penanda zat besi serum yang tinggi boleh meningkatkan risiko perkembangan bagi sirosis dan HCC, tetapi tidak bagi NAFLD. Merokok, jantina lelaki, etnik Melayu dan ALT serum yang tinggi juga perlu dipertimbangkan. Kajian ini juga telah mengenal pasti varian novel (G1171, T1785, A1786 dan T3112) yang sangat khusus untuk jangkitan akut terhad sendiri. Varian replikasi-cacat tersebut mungkin bertanggungjawab bagi jangkitan kronik pada kadar yang lebih rendah dalam kes-kes jangkitan HBV. Penyiasatan *in vitro* dan *in vivo* yang selanjutnya diperlukan untuk mengesahkan pertemuan yang baru ini.

Acknowledgement

In the age of modern science and technology, information accumulates in ever more intimidating quantities. Disconnected facts and impenetrable problems give away to rational explanations, and simplicity emerges from chaos.

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Last, but not the least, I express my highest appreciation to my parents that have been my safe boat, supporting me spiritually and financially in an unlimited way.

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List of Symbols and Abbreviations

Abbreviation	Full name
95%CI	95% confidence interval
aa	amino acid
AASLD	American Association for the Study of Liver Diseases
AFP	alpha-fetoprotein
ALT	alanine aminotransferase
anti-HBc	hepatitis B core antibody
anti-HBs	hepatitis B surface antibody
AOR	adjusted odds ratio
AST	aspartate aminotransferase
BCP	basal core promoter
C	core
CURS	core upper regulatory sequence
dNTP	deoxynucleotide
dsDNA	double-stranded deoxyribonucleic acid
EASL	European Association for the Study of the Liver
EnhI	enhancer I
EnhII	enhancer II
EtBR	ethidium bromide
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B early antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBV-REVEAL	Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus
HBx	hepatitis B X protein
HCV	hepatitis C virus
kb	kilobase
LHBsAg	large hepatitis B surface antigen
MHBsAg	middle hepatitis B surface antigen
NRE	negative regulatory element
nt	nucleotide

Abbreviation	Full name
OR	odds ratio
P	polymerase
PCR	polymerase chain reaction
PNALT	persistently normal alanine aminotransferase
pre-C	precore
ROC	receiver operating characteristic
S	surface
SHBsAg	small hepatitis B surface antigen
SNP	single nucleotide polymorphism
TBE	Tris-base-Borate-EDTA
ULN	upper limit of normal
UMMC	University Malaya Medical Centre
ε	encapsidation signal

Chapter 1 General Introduction

1.1 Epidemiology and clinical manifestations of hepatitis B

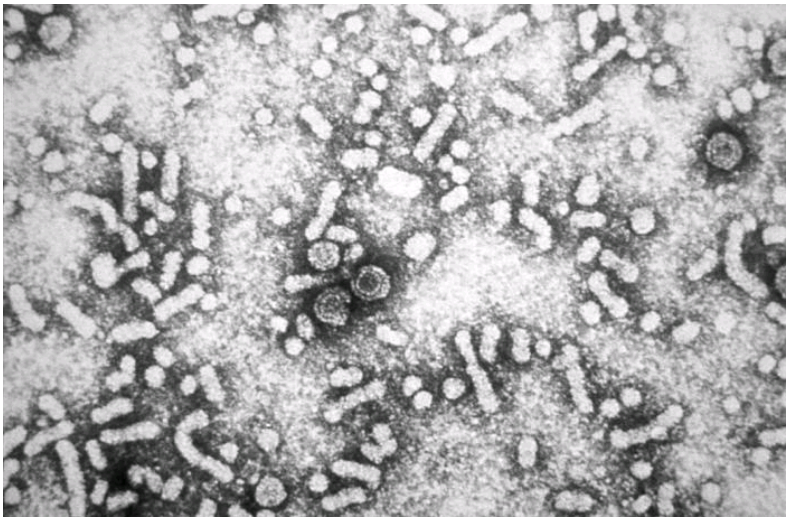
Hepatitis B virus (HBV) is a major cause of liver disease globally. More than 2 billion people world-wide have been infected by HBV, of whom 350 million develop chronic hepatitis and one million of them decease every year [Maddrey, 2000] and over 4 million acute cases [WHO, 2002]. About 75% of them are Asian [Merican et al., 2000; Arauz-Ruiz et al., 2002]. HBV is usually transmitted through body fluids, such as blood and semen, and rarely through saliva [WHO, 2002]. In Asia, HBV infection is highly endemic and predominantly acquired at birth or in early childhood [André, 2000]. The early infection results in persistency in more than 90% of children [Michel and Tiollais, 2010]. Malaysia has intermediate endemicity of HBV infection with a prevalence of 4.7% among the general population [Lim et al., 2003]. Prevalence of HBV infection was higher among Chinese and Malays than that in Indians [Qua and Goh, 2011]. Symptomless infection is common in children. The main symptoms of acute infection may include jaundice, dark urine, malaise, low-grade fever, nausea, anorexia and pale stool. The chronic infection often leads to severe complications.

Hepatitis B can manifest as acute hepatitis, fulminant hepatitis, chronic hepatitis, fatty liver, cirrhosis and hepatocellular carcinoma (HCC). Acute hepatitis B is mostly self-limiting, being less than 1% cases progress to fulminant hepatitis [Han, 2009]; the latter is associated with high mortality. One million people die per year from chronic complications, including cirrhosis and HCC [WHO, 2002]. The age at infection is the major factor determining chronicity [Juszczyk, 2000]. Risk of chronicity varies inversely with age. Chronic hepatitis B develops in about 90% of infected newborns, 29-40% of children and 5-10% of adults [Juszczyk, 2000]. Cirrhosis occurs in 15-40% of those with the chronic infection [Liaw et al., 1988]. Of these, 25% progress to HCC [Beasley et al., 1981]. Patients with cirrhosis are characterised by the formation of

nodules and scarring on liver tissue, whereas those with HCC suffer overgrowth and neoplastic transformation of the liver tissue. HCC is ranked at fifth in all cancers globally. HBV accounts for 60-80% of the liver cancer [Lopez, 2005]. In Taiwan, about 75% of HCC patients will die within 3 years [Changchien et al., 2008]. HBV DNA level, also called viral load, is a surrogate marker for viral replication. It has been shown to be one of the important predictive factors for progression to cirrhosis and HCC [Chen et al., 2006a; Iloeje et al., 2006]. Male gender, Asian or African ethnicity, longer duration of infection, family history of HCC, heavy alcohol drinking, tobacco smoking, aflatoxin exposure, obesity and diabetes mellitus are among the risk factors for HCC [Elgouhari et al., 2008; McMohan, 2009; Tanaka et al., 2011]. Risk factors for cirrhosis are similar to that of HCC, except for no ethnic difference and no family history of HCC [Elgouhari et al., 2008].

1.2 The virus

HBV is a partially double-stranded DNA (dsDNA) virus classified under the Family *Hepadnaviridae*. In 1970, the virion was first described as a 42 nm spheres under electron microscopy [Dane et al., 1970], as shown in Figure 1.1. The viral dsDNA is kept within a protein coat and surrounded by a lipid envelope [Seeger and Mason, 2009]. The DNA virus mainly replicates in liver. It uses a reverse-transcription and an RNA intermediate for replication. HBV has a high replication rate and the replication strategy is error-prone.



HBV virions

Figure 1.1 : Electron microscopic structure of hepatitis B virus.

1.2.1 Viral genome

The HBV genome has a size of about 3.2 kb. It is circular and highly compact. It consists of four overlapping open reading frames (ORFs), namely C (core), P (polymerase), S (surface) and X (X protein) (Figure 1.2). The C ORF can be divided into two overlapping domains, precore and core. Precore (pre-C) domain produces hepatitis B e antigen (HBeAg, a truncated, secretory precore protein) whereas core domain expresses hepatitis B core antigen (HBcAg, a subunit of virion protein coat). HBcAg is the building block for virion protein coat. Meanwhile, S ORF is divided into three overlapping domains, pre-S1, pre-S2 and S; each of the domains is responsible for

production of large (LHBsAg), middle (MHBsAg) and small hepatitis B surface antigen (SHBsAg) respectively. The surface antigens, mainly SHBsAg, are transmembrane proteins embedded within lipid envelope [Heermann et al., 1984]. P ORF is divided into four domains – terminal protein (aa1-182), spacer (aa183-348), reverse transcriptase-polymerase (aa349-691) and RNaseH (aa692-845) [Zoulim, 2004]. P ORF is involved in viral replication and antigen syntheses. X ORF produces X protein (HBx), known to have some gene trans-activating properties, and has been essential for viral replication [Keasler et al., 2009].

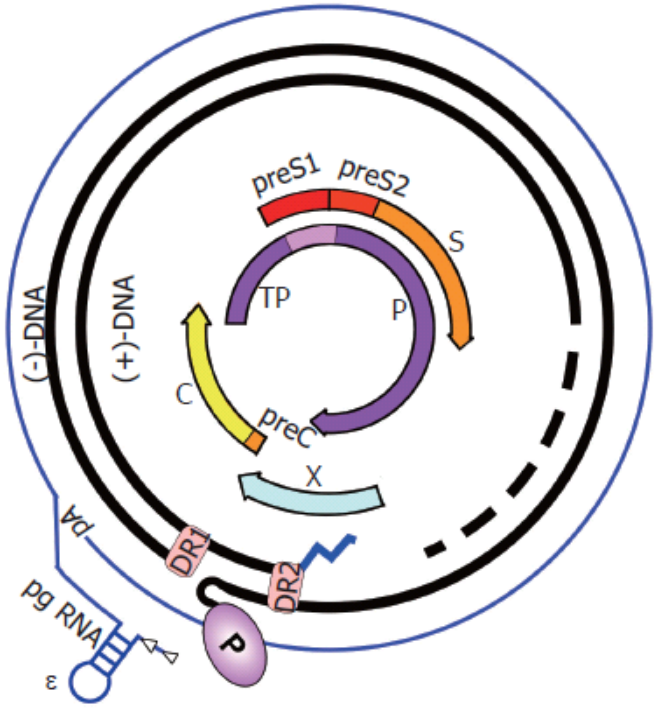


Figure 1.2 : HBV genome organisation [Beck and Nassal, 2007].

Viral regulatory sequences, such as enhancer I (EnhI), enhancer II (Enh II), negative regulatory element (NRE), core upper regulatory sequence (CURS), basal core promoter (BCP), S1 promoter, S2 promoter and X promoter, may also affect viral replication and antigen syntheses. For example, enhancer I mutation may decrease viral replication [Bock et al., 2000] and S2 promoter mutation may down-regulate HBsAg

production [Sengupta et al., 2007]. The nucleotide (nt) position of various coding and some of the regulatory regions are listed in Table 1.1.

Table 1.1 : Coding and regulatory regions in a HBV genome of 3215 nt.

Region	Typical genotype B/C genome (nt)	
	Start	End
Coding		
Pre-C	1814	1900
C	1901	2452
Pre-S1	2848	3204
Pre-S2	3211	154
S	155	835
X	1374	1838
P	2307	1623
Regulatory		
EnhI	1043	1235
EnhI-X promoter	950	1350
NRE	1611	1634
EnhII	1644	1666
CURS	1643	1741
BCP	1742	1849
ε	1847	1907
S1 promoter (pre-S1 promoter)	2710	2800
S2 promoter (S promoter)	3045	3180

BCP, basal core promoter; C, core; CURS, core upper regulatory sequence; ε, encapsidation signal; EnhI, enhancer I; EnhII, enhancer II; nt, nucleotide; NRE, negative regulatory element; P, polymerase; S, surface.
References: Ori et al., 1998; Norder et al., 1994; Moriyama, 1997; Kurbanov et al., 2005; Tanaka et al., 2006; Xu et al., 2006; Panjaworayan et al., 2007; Cui et al., 2010; Lin et al., 2012.

1.2.2 Viral genotype

HBV genotype was classified based on more than 8% genomic divergence [Okamoto et al., 1988]. Later, it was re-defined as 7.5% [Kramvis et al., 2008]. Currently, nine genotypes has been identified, namely genotypes A-I [Okamoto et al., 1988; Norder et al., 1994; Arauz-Ruiz et al., 2002; Kato et al., 2002; Olinger et al., 2008] and one provisional genotype J [Tatematsu et al., 2009]. Sub-genotyping was defined as between 4-7.5% genomic divergence [Kramvis et al., 2008]. However, this sub-genotyping definition has not been followed strictly by all researchers; some published sub-genotypes were more geographical-specific rather than based on the divergence rule [Pourkarim et al., 2010]. Besides these, a number of HBV recombinants have also been

found, for example, genotype B/C [Sugauchi et al., 2003] and C/D [Cui et al., 2002] recombinants.

1.2.3 Life cycle

Life cycle of HBV begins with the viral attachment on liver cell membrane via envelope protein; both pre-S1and HBsAg proteins could be involved in the viral entry [Le Duff et al., 2009]. The lipid envelope of the virus then fuses with the cell membrane, and subsequently the viral protein coat is released into the cytoplasm. The protein coat posseses nuclear localization signal located at C-terminal of HBcAg. This brings the virus to nuclear membrane. The viral relaxed circular DNA (RC-DNA) is then released into the nucleoplasm via the nuclear pore. The RC-DNA is then repaired to form covalently closed circular DNA (cccDNA) [Seeger and Mason, 2000]. The cccDNA, acting like mini-chromosome, start transcribing viral pregenomic RNA (pgRNA) and various viral mRNAs by host DNA-dependent RNA polymerase II (shown in Figure 1.3). The viral mRNAs encode various viral proteins described earlier, inclusive of viral reverse transcriptase-polymerase and so on, whereas pgRNA is packaged into a viral protein coat via an encapsidation signal (ε; shown in Figure 1.4) and subsequently converted into viral negative DNA strand by the viral reverse transcriptase. The complementary positive DNA strand is then synthesized by viral DNA polymerase [Beck and Nassal, 2007]. However, the synthesis halts half-way due to limited space in the protein coat, resulting in formation of a partially double-stranded DNA. The newly packaged virion can either be be transported out of plasma membrane for infecting other hepatocytes [Gerelsaikhan et al., 1996] or be re—transported into the nucleus for perpetuating replication [Tuttleman et al., 1986].

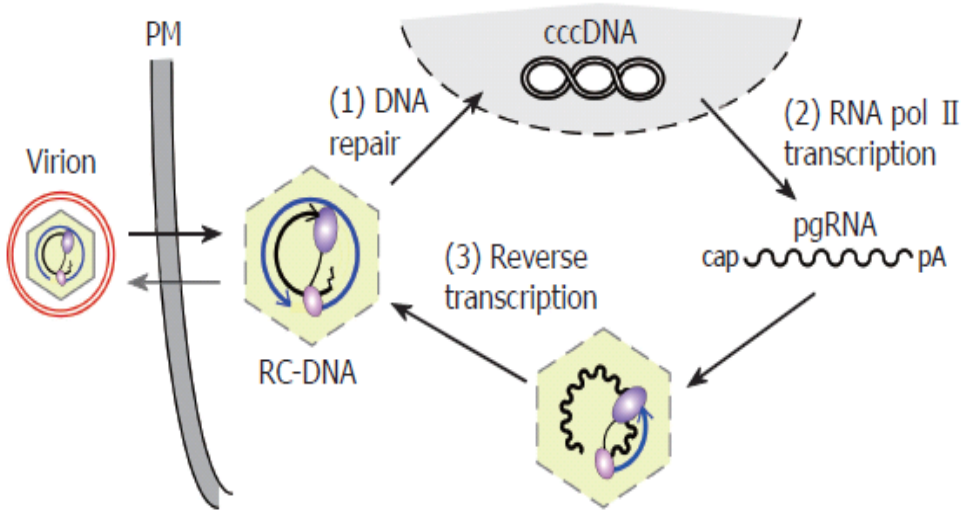


Figure 1.3 : Life cycle of HBV in a hepatocyte [Beck and Nassal, 2007].
cccDNA, covalently closed circular DNA; RC-DNA, relaxed circular DNA.

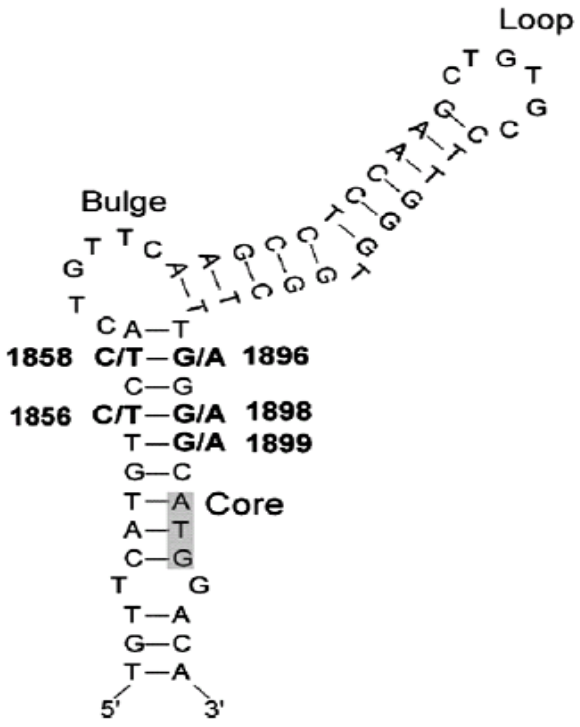


Figure 1.4 : ε loop structure of pgRNA [Sugauchi et al., 2004]. The stable Watson-Crick base pairing in the encapsidation signal sequence is required for efficient viral replication. For example, the pairing between C1858 and G1896 is a characteristic of genotype A [Li et al., 1993].
ε, encapsidation signal; pgRNA, pregenomic RNA.

1.3 Viral markers in various clinical manifestations

1.3.1 Viral protein markers

Hepatitis B virus was first identified in an HBV-infected Australian aborigine as the Australian antigen [Blumberg et al., 1967], now known as hepatitis B surface antigen (HBsAg). Acute hepatitis B occurs in HBV-infected individuals who clear HBsAg within 6 months whereas chronic hepatitis B is said to occur in those remaining HBsAg positive for more than 6 months [WHO, 2002].

In the early phase of HBV infection, hepatitis B early antigen (HBeAg) is usually positive, indicating active replication of the virus [Cheng et al., 2012]. However, the inverse is not true. HBeAg shares antigenic epitopes with HBcAg. HBcAg is targeted by immune system, which could lead to either viral clearance or necrosis of hepatocytes. HBeAg was thought to induce immune tolerance against HBV, more specifically, HBcAg [Milich et al., 1990]. Having said so, in a HBV-transgenic mouse model study, it was demonstrated that HBeAg was the major antigen inducing liver injury with respect to HBcAg [Frelin et al., 2009]. Persistent HBeAg positivity has been linked with HCC [Yang et al., 2002]. Late HBeAg seroconversion, especially at elder age, confers higher risk for development of cirrhosis [Lai and Yuen, 2007a]. Early HBeAg seroconversion usually confers a favourable clinical outcome [Lin and Kao, 2008].

Acute HBV infection is associated with a high titre of IgM hepatitis B core antibody (IgM anti-HBc). Those having resolved infection are expected to be negative for HBV DNA and HBsAg together with presence of hepatitis B surface antibody (anti-HBs) and IgG anti-HBc [Towell and Cowie, 2012].

1.3.2 Viral genetic markers

Viral genotype, T1762/A1764 (a common BCP double mutation) and A1896 (a common precore stop codon mutation) are the three most commonly studied viral markers in various clinical manifestations. In Asia, there is a greater association of HBV genotype C infection than genotype B, whereas in North America and Western Europe, incidence of HCC in genotype D infection is higher than that in genotype A [El-Serag, 2012]. The BCP mutation is known to suppress HBeAg expression [Buckwold et al., 1996] whereas the precore mutation to abolish the antigen synthesis [Buti et al., 2005]. The association of these markers with various clinical manifestations, however, has been largely controversial.

(a) Cirrhosis

About 80% of HCC patients have underlying cirrhosis. Patients with cirrhosis have higher predisposition to HCC. T1762/A1764 was more frequently encountered in cirrhosis cases compared to non-cirrhotic carriers while A1896 was more in asymptomatic carriers [Song et al., 2006]. Some researchers have proposed a complex algorithm of viral mutations to predict development of cirrhosis [Chen et al., 2007].

(b) Hepatocellular carcinoma

Extensive studies have been done to determine viral markers associated with HCC. A meta-analysis study reported that pre-S mutations, T1653, V1753 and T1762/A1764 were associated with an increased risk of developing HCC; relationship between A1896 with HCC was inconsistent [Liu et al., 2009]. Yet, some other studies reported no association between T1762/A1764 and HCC [Guo et al., 2008].

(c) Acute and chronic hepatitis

Viral markers highly specific for acute infection (non-fulminant hepatitis) of HBV could predict those who are likely to have a self-limiting infection, whereas those for chronic infection of the virus could predict a persistent infection. HBV genotype A has been shown to be associated with acute hepatitis whereas genotype C with chronic hepatitis [Kobayashi et al., 2004]. Individuals with T1762/A1764 and A1896 were reported to be more likely to develop acute hepatitis [Kobayashi et al., 2004; Liu et al., 2010] but not in the other [Chu et al., 1996].

1.4 Biochemical markers in various clinical manifestations

1.4.1 Serum aminotransferase

Aminotransferase is an enzyme involving conversion of amino acids. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are two of the enzymes found in cytoplasm and mitochondria of various tissues. Both of them are involved in energy production.

ALT is an enzyme found abundantly in the cytosol of liver tissue, but to lesser extent in kidney and other organs. ALT activity in liver is about 3000 times that of serum activity [Kim et al., 2008]. Measurement of the enzyme activity in serum has been regarded as a reliable and sensitive marker of active liver disease. A high level of serum ALT activity is commonly indicated in hepatocellular injury [Prati et al., 2002]. Furthermore, the elevation has been strongly associated with HCC [Ishiguro et al., 2009]. However, chronic hepatitis B patients with persistently normal ALT may have significant hepatic inflammation and fibrosis [Lai et al., 2007a].

Measurement of serum ALT is unlikely to be reliable for the prediction of risk of cirrhosis and HCC because they may fluctuate during the course of chronic HBV infection. Serum AST is sometimes combined with ALT as a ratio to reflect liver impairment, such as cirrhosis [Gianini et al., 2003]. Yet, Lin et al. (2008) did not find

such relationship. In addition, both tests may be elevated in response to certain drug use [Sokolove et al., 2010] and systemic inflammation [Al-Maini, et al., 2000; Curtis et al., 2010].

1.4.2 Serum alpha-fetoprotein

Alpha-fetoprotein (AFP) is a glycoprotein normally produced in the fetal yolk sac and liver during embryonic life. Serum AFP increases in markedly in hepatoblastoma (during childhood), HCC and certain germ cell cancers. Liver injury and increased cellular regeneration may increase serum AFP because patients with chronic active disease have higher serum AFP levels than the quiescent ones [Collazos et al., 2002]. According to a Malaysia report, about 70% of serum AFP >20 ng/ml was demonstrable in HCC [Yap and Peh, 1991]. About 44% of cirrhotic patients with persistently high serum AFP (>50 ng/ml) developed HCC after 2-10 year [Imaeda and Doi, 1992]. Serum AFP may fluctuate during the course of the disease (Figure 1.5). Serum AFP rises in acute hepatitis (12%), chronic hepatitis (34%), chronic severe hepatitis (67%) and cirrhosis (58%) [She et al., 2003]. Elevated serum AFP was detected in 58% of those with family history of HCC, higher than those without [She et al., 2003]. In HCV studies, serum AFP at cutoff of 16 ng/ml may have sensitivity ranged from 60-80% and specificity from 70-90% [Wright et al., 2007]. Even for diagnosis only (not to say early diagnosis), the sensitivity may go as low as 30% as in other HCC studies [Block et al., 2005; Marrero et al., 2005]. Based on these performance characteristics, serum AFP may not be a suitable marker for HCC; a better predictive marker for HCC and cirrhosis is required.

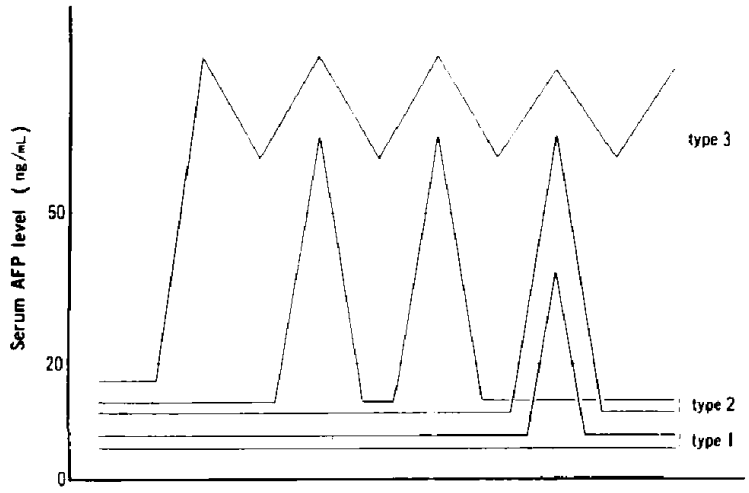


Figure 1.5 : Possible fluctuating patterns of serum AFP in cirrhotic patients. About 16% of type 1, 23% of type 2 and 33% of type 3 groups developed HCC after 2-10 years [Imaeda and Doi, 1992].

1.4.3 Serum iron and serum ferritin

Iron may present as free iron, transferring-bound and albumin-bound form in serum. Ferritin is the main storage molecule for iron and mitigates the oxidative process caused by free iron [Theil, 2003]. Serum iron and serum ferritin are two markers usually used to assess body iron status. Liver is a major iron storage site. Stainable liver iron has been correlated positively with serum iron and serum ferritin in chronic hepatitis C [Fernández-Rodriguez et al., 2004]. Serum ferritin has been shown to be higher in patients with cirrhosis compared to those without cirrhosis [Büyükaşik et al., 2011]. Serum ferritin may predict severe hepatic fibrosis and hepatic liver iron deposition according to a HCV study [Metwally et al., 2004]. No HBV study has conducted such study before. High serum ferritin is a manifestation of haemochromatosis, an iron overload disease in which affected individuals are prone to HCC [Kowdley, 2004].

1.5 Molecular techniques

Two molecular techniques were employed in this study, namely hemi-nested polymerase chain reaction (PCR) and chain termination sequencing.

1.5.1 Hemi-nested polymerase chain reaction

PCR is a molecular technique using heat-stable DNA polymerase to amplify DNA template exponentially. It usually involves using a pair of primers, a forward and reverse primer, for the amplification. A forward primer binds on negative-sense DNA and the reverse one binds on positive-sense DNA during the reaction. In nested PCR, two primer sets were used – outer primer set and inner primer set. Amplification starts with the outer set followed by the inner one. For hemi-nested PCR, one of the inner primers, either the forward or reverse, is similar with the respective outer primer. An example is shown in Figure 1.6. The amplification can be done simultaneously or separately (two-step).

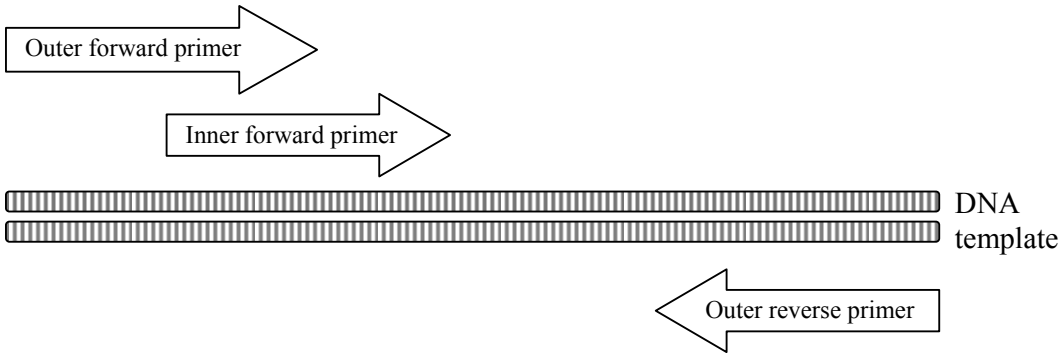


Figure 1.6 : An example of hemi-nested PCR. Two outer primers and one inner primer are used for amplification of a DNA template.

1.5.2 Dye-terminator cycle sequencing

DNA sequencing is a molecular technique used for determining the order of the nucleotide bases. Chain termination method is a PCR-based DNA sequencing method involving deoxynucleotide triphosphate (dNTPs) for elongation of a DNA strand and

dideoxynucleotide triphosphate (ddNTPs) to terminate elongation of the DNA strand. Dye-terminator cycle sequencing uses the method to decode a DNA template (See Figure 1.7). In this technique, the ddNTPs are labelled with four different fluorescent dyes, corresponding to four different bases present naturally in DNA – Adenine (A), Thymine (T), Guanine (G) and Cytosine (C) triphosphate. After a number of elongation and termination processes, the DNA template electrophoreses through a capillary, and the DNA sequence can be traced by an electronic chromatogram.

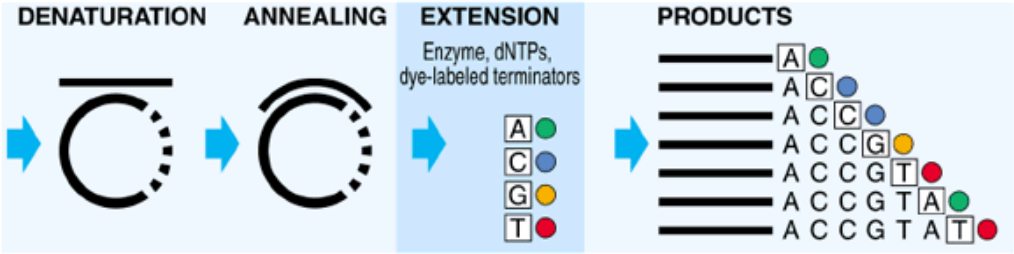


Figure 1.7 : Dye-terminator sequencing is a PCR-based sequencing method uses fluorescent dye-labelled terminators, ddNTPs.
Repeated elongation of DNA and termination cycle results in different lengths of DNA fragments. The DNA fragments are then separated through a capillary. This separation enables the reading of the DNA template sequence in order. ddNTPs, dideoxynucleoside triphosphate; PCR, polymerase chain reaction.
(Source: http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/dna-sequencing-fragment-analysis/overview-of-dna-sequencing/sequencing-chemistries.html)

1.6 Objectives of the present study

Cirrhosis and HCC take decades to develop. However, early detection is not often achieved due to the lack of reliable markers [Wright et al., 2007]. In addition, the markers may not be readily applicable in clinical settings and most are less manipulative, therefore have limited usefulness for preventing and improving the severe liver diseases. Meanwhile, viral marker for predicting chronicity has not been convincing despite several studies being conducted, mainly due to their limited sample size and the mainly focus on BCP and precore mutations. Therefore, the main objective of this study is to determine viral and/or host characteristics potentially predictive for various clinical outcomes. The specific objectives of the study are:

1. To identify candidate viral marker(s) of hepatitis B associated with cirrhosis and HCC.
2. To study the polymorphism of the viral marker(s) in chronic hepatitis B individuals.
3. To evaluate host iron marker(s) associated with cirrhosis and HCC.
4. To identify independent risk factors for prediction of clinical complications.
5. To identify candidate viral marker(s) of hepatitis B associated with chronicity of HBV infection.

Objectives 1-4 were achieved with biochemical and molecular approaches, whereas objective 5 with an *in silico* approach.

Chapter 2 Methodology

This study was divided into two sections. The first section was to determine the potential serum viral and host markers in association with cirrhosis and HCC. The second section was to determine the potential viral genomic mutations associated with chronicity based on *in silico* observation. Investigation on host factors was not possible on the latter.

2.1 Identifying markers for chronic complications

The present study intended to evaluate the potentials of viral and iron markers responsible for chronic complications, that is, liver cirrhosis and HCC.

2.1.1 Study design

This was a case–control study involving Malaysian subjects with chronic hepatitis B attending Medical Clinics in the University Malaya Medical Centre (UMMC), Kuala Lumpur. Approval for the study was obtained from the UMMC Medical Ethics Committee acting by the ethical standards of the Declaration of Helsinki. Between October 2007 and April 2010, all consecutive HBsAg positive patients attending the hepatitis clinic were invited to participate in the study. After obtaining informed consent, potential participants were interviewed with a preset questionnaire to obtain information on basic demographics, and personal and family history of illnesses. About 5 ml of blood were then drawn into a plain tube from each of them.

A grand total of 376 participants were recruited. These participants were divided into 4 groups, namely controls (N = 216), cirrhosis (N = 78) and HCC (N = 39) cases. The diagnosis of HCC was based on typical features of HCC on dynamic radiological imaging, and liver histology or alpha-fetoprotein (AFP) > 200 ng/mL. The diagnosis of cirrhosis was based on liver histology or at least two of the following clinical features:

ultrasonographic and radiologic evidence of a nodular or shrunken liver, splenomegaly, ascites, presence of varices, or platelet counts of <100,000/ml. Those who had other possible causes of cirrhosis and HCC, including autoimmune hepatitis, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alpha-1 anti-trypsin deficiency and other types of diagnosed cancer, were excluded. General controls were defined as those who were without significant liver diseases, whereas stringent controls were defined with additional inclusion criteria: (i) had a family history of HBV infection, (ii) were at least 50 years old, and (iii) were treatment-naïve. Additional exclusion criteria for all groups included a history of chronic alcoholism, chronic hepatitis C co-infection and other possible non-hepatic causes of systemic inflammation.

To save cost and time, only those HBV genomes from stringent controls, cirrhosis and HCC cases were sequenced and compared to search for potential viral markers predictive of cirrhosis and HCC. Only sera collected from these selected participants tested positive for the presence of HBV DNA by polymerase chain reaction (PCR) were subjected to whole genome sequencing. Only participants, from whom all HBV DNA fragments could be amplified successfully and sequenced fully, were included in the final analysis. At the time of our first publication on viral markers for cirrhosis [Chook et al., 2011], HBV genomes for 20 stringent controls and 20 cirrhosis cases were sequenced successfully and compared. About 10 months later, HBV genomes for 21 cases of HCC were sequenced successfully. Because it has taken longer time for the collection of HCC cases, the sample size of HBV genomic sequences for stringent controls sequenced successfully has also increased to 24 cases accordingly for comparison. Only viral marker(s) with the highest accuracy for identification of cirrhosis and HCC cases were further evaluated in a larger population of chronic

hepatitis B (N = 376) mentioned earlier (Figure 2.1). For the larger population screening, those with either past or recent antiviral treatment were included.

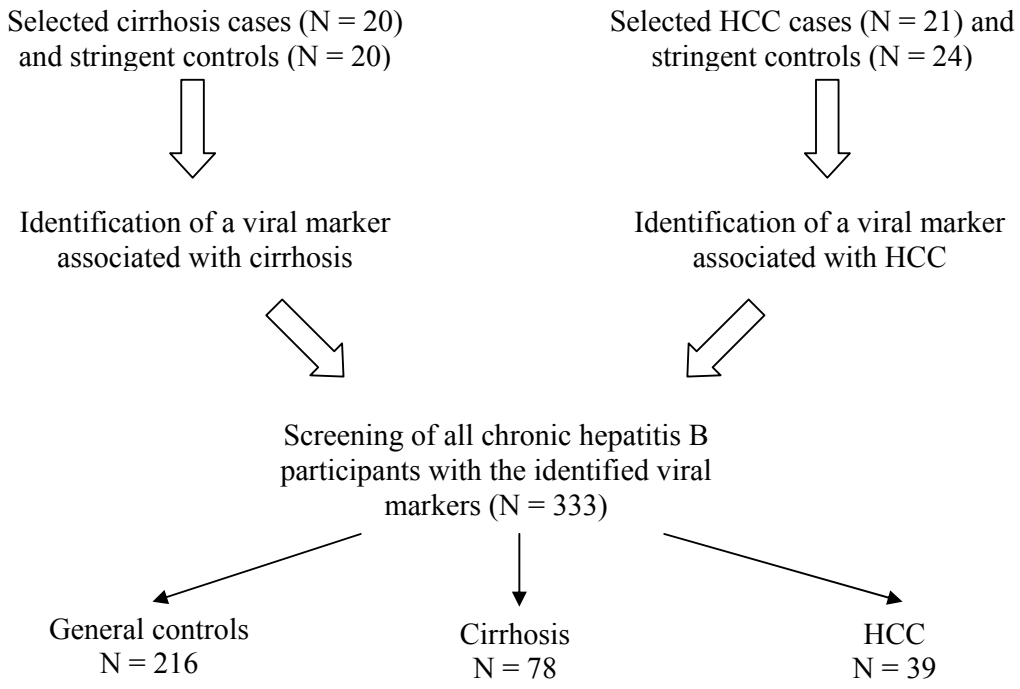


Figure 2.1 : Workflow encompassing study design from comparative sequence analysis of selected participants (to identify specific viral markers for cirrhosis and HCC) to screening of all chronic hepatitis B participants with the identified viral markers, which were potentially predictive of cirrhosis and HCC. General controls (inclusive of stringent controls) were all liver ultrasound normal chronic hepatitis B participants.

2.1.2 Routine laboratory assay

In searching for potential predictive markers for cirrhosis and HCC, all selected stringent controls, cirrhosis and HCC cases were tested for serum ALT (Siemens Dimension®, Deerfield, IL, USA), serum alpha-fetoprotein (AFP; Siemens Dimension®), serum iron, serum ferritin (Roche Diagnostics, Pleasaton, CA, USA), HBsAg positivity and HBeAg positivity (Architect, Abbott Laboratories, Dublin, Ireland). Viral load was determined using Roche COBAS Taqman HBV Test (Roche Molecular Diagnostics, Branchburg, NJ, USA). Those laboratory assays determined to be predictive for development of cirrhosis and HCC were further applied in all the chronic hepatitis B participants. According to UMMC laboratory guidelines, the upper limit of normal (ULN) for serum ALT, AFP, iron and ferritin were set at 65 IU/L, 6.7 IU/mL, 27.00 μmol/L and 291.0 μg/L respectively.

2.1.3 Viral DNA extraction

All sera collected were stored at -80ºC until use. Serum HBV DNA was extracted using Qiagen QIAamp DNA mini kit (Qiagen, Germany). It is a solid phase extraction using spin column. The extraction was done according to manufacturer's instruction. The extraction process basically involved using a mildly acidic guanidine compound to dissolve the viral lipid envelope and to denature protein together with a protease to digest the viral protein. The mixture was then passed through a spin column by microcentrifugation. The guanidine compound already in the mixture would simultaneously enhance the binding of viral DNA to the column during the pass-through. The column was then washed with a provided Qiagen wash buffer twice to remove the impurities that might inhibit PCR. The viral DNA captured in the column was then eluted out using a Qiagen elution buffer. The detailed procedure can be referred to the manufacturer's instruction manual.

2.1.4 Hemi-nested polymerase chain reaction optimisation

PCR amplification was performed using Qiagen HotStarTaq (Qiagen) with the six overlapping primer sets listed in Table 2.1. These primers were designed using Oligo Analyzer version 1.0.2 [Kuulasmaa, 2002] to collectively cover the entire genome of the virus with three hemi-nested PCR assays (using outer primer sets, namely 1441F-2839R, 251F-1797R, and 2365F-275R), generating a total of six fragments. The melting temperatures and qualities of these primers are shown in Table 2.2.

Table 2.1 : List of primer sets used in the amplification of HBV genome.

Primer name[a]	5'-Oligonucleotide-3'	Nucleotide position (Region covered)
Set 1		(Surface/X region)
251F	GAY TCG TGG TGG ACT TCT C	251–269
1262R	GTT CCG CRG TRT GGA TCG	1,245–1,262
Set 2		(Surface/X region)
596F	ACY TGT ATT CCC ATC CCA TC	596-615
1797R	CCA ATT TAT GCC TAC AGC CT	1,797-1,778
Sequencing Primer		
1709R	GGA GTR NGC CTC AAG GTC G	1,709-1,691
Set 3		(X/Core region)
1441F	CTG AAT CCY GCG GAC GA	1,441-1,457
Inner Primer		
1583F	ACT TCG CYT CAC CTC TGC A	1,583-1,601
2393R	KGC GAG GYG AGR GAG TTC	2,393-2,376
Set 4		(Core/Surface region)
1865F	CAM GCC TCY RAG CTG TGC	1,865-1,882
2839R	TST TGT TCC CAA GWA TAT GG	2,839-2,820
Set 5		(Core/Surface region)
2365F	CCY TAG AAG AAG AAC TCC CTC	2,365-2,385
275R	AAA YTG AGA GAA GTC CAC CAC	275-255
Set 6		(Surface region)
2826F	TCY TGG GAA CAA GAG CTA CA	2,826-2,845
649R	AGA AAC GGR CTG AGG CC	649-633
Sequencing Primer		
617R	AYG ATG GGA YGG GAA TAC A	617-599

HBV, hepatitis B virus.

[a]Suffix -F indicates forward primer; suffix -R indicates reverse primer. Sequencing primer was used for repeated sequencing whenever sequencing failure was encountered for the particular primer set.

Table 2.2 : Properties of six primer sets used for HBV genomic amplification.

Primer pair[a]	Predicted Tm, °C[b]	Self- and pair-annealing, kcal/mol[c]	Loop, kcal/mol[d]
(a) First round of PCR			
251F-1797R	58.0, 59.1	None; -2.16; -1.81	None, none
Second round of PCR			
251F-1262R	58.0, 58.4	None, -1.41; -1.43	None, -1.31
596f-1797R	59.7, 59.1	None, -2.16; -2.65	None, none
(b) First round of PCR			
1441F-2839R	64.5, 56.7	-13.30, -1.81; -1.43	-1.31, -1.69
Second round of PCR			
1583F-2393R	58.4, 62.8	-3.84, none; -3.03	None, none
1865F-2839R	63.3, 56.7	-3.13, -1.81; -0.69	-1.41, -1.69
(c) First round of PCR			
2365F-649R	57.3, 61.3	-0.95, -6.07; -3.03	None, -2.88
Second round of PCR			
2365F-275R	57.3, 58.2	-0.95, -2.16; -0.33	None, none
2826F-649R	59.6, 61.3	-3.86, -6.07; -1.53	-3.74, -2.88

HBV, hepatitis B virus; PCR, polymerase chain reaction; Tm, melting temperature.
[a]Suffix -F indicates forward primer; suffix -R indicates reverse primer.
[b]Prediction based on Nearest Neighbour (NN) method.
[c]The first two are the scores of self-annealing free energy for forward and reverse primers respectively. The third score is for pair-annealing free energy for both primers. The more negative the score, the higher the probability for the annealing to occur. Note that none indicates no secondary structure would be formed.
[d]The first score is the free energy of loop formation for forward primer whereas the second score is that for reverse primer. The more negative the score, the higher the probability for the formation to occur. Note that none indicates no secondary structure would be formed.

First round of the nested PCR assays were carried out in 50 µl PCR mixture volumes consisting of 5 µl of 10x KCl buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 10 µM of each primer, 2 µl of 10mM dNTPs, 0.2 µl of 5U/µl Qiagen HotStarTaq polymerase and 5 µl of a HBV DNA concentration of about 10^4 copies virus per ml. Second round of the nested PCR assays were carried out in 50 µl PCR mixture volumes consisting of 5 µl 10x KCl buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 10 µM of each primer, 2 µl of 10mM dNTPs, 0.2 µl of 5U/µl Qiagen HotStarTaq polymerase and 1 µl of first round PCR-amplified products. Annealing temperature of the PCR assays were optimised using gradient temperature setting in a MyCycler thermal cycler (Biorad, Hercules, CA, USA). The thermal profile used in both rounds of hemi-nested PCR are as follows:

Initial denaturing at 95ºC for 15 minutes; 40 cycles in 3 steps: 94ºC for 60 seconds, gradient annealing temperature of 55-64ºC for 90 seconds, 72ºC for 60 seconds; final extension at 72ºC for 10 minutes. The calculated annealing temperatures for the gradient setting were 55.0, 55.6, 56.7, 58.3, 60.4, 62.2, 63.3 and 64.0ºC. After the PCR amplification, the amplified PCR product was run under an agarose gel electrophoresis system (Easy-Cast^{TM} Electrophoresis System, Pittsburgh, PA, USA).

For casting of gel, exactly 0.9 g of LE agarose (Seakem®, Rockland, ME) was topped up with 60 ml of 1x Tris-base-Borate-EDTA (TBE) buffer in a 200 ml Schott bottle. Next, the mixture was cooked in a microwave. After the cooking, the gel solution was let to cool down to around 60-70ºC (hotness bearable by handhold). Approximately 3 µl of ethidium bromide (EtBr) was added into the bottle and the bottle was shook to ensure even mixing. The gel solution was then poured onto a gel-casting tray with a 14-teeth comb and left to solidify for at least 30 minutes. Finally, the amplified product was visualised under UV transillumination (Fotodyne®, Hartland, WI, USA). Gel picture was taken using a digital camera (Sony Cybershot DSC-P10, Japan). The correct size of a respective fragment was compared with a 100 bp ladder (i-Lad3, i-DNA, Singapore) or a 100 bp ladder plus (i-Lad4, i-DNA, Singapore) where appropriate. Amplified fragments with single band size were sent for sequencing.

2.1.5 Viral genomic sequencing

Before proceeding to sequencing, the amplified fragments were purified using QIAquick® PCR Purification kit (Qiagen). The purified products were then sent to a commercial laboratory (First BASE Laboratories Pte. Ltd., Selangor, Malaysia) for bidirectional sequencing using ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). All 65 complete genomes of HBV were submitted to GenBank. Accession numbers for stringent controls were HM011465–HM011484 and JQ027310-

JQ027313, cirrhosis cases were HM011485–HM011504 and HCC cases were JQ027314-JQ027334.

2.1.6 Complete HBV genome sequence analysis to identify candidate viral markers

HBV genotype was determined by applying HBV full genome in Viral Genotyping Tool available free (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) on National Center for Biotechnology Information (NCBI) website. Sequence alignment was done using SECentral Align Plus version 4 (Scientific and Educational software, US). Nucleotide numbering was referenced to that reported by Okamoto et al. [1988]. Whole genomic sequence analysis of HBV was compared for differences between the cirrhosis and stringent control groups, and between the HCC and stringent control groups. Mononucleotide variations were screened across the HBV whole genomes. The comparison was done by using matemathical, logical and string functions in Microsoft Excel 2007 to calculate the frequency of candidate nucleotides responsible for cirrhosis and HCC. Candidate mononucleotide(s) that achieved the minimum power of 80% for the identification of cirrhosis and HCC were selected for codon analysis. Single codons that gave the highest accuracy were used subsequently to screen all the chronic hepatitis B sera collected to verify the relationship of the candidate marker(s) with cirrhosis and HCC.

2.1.7 Screening of candidate viral markers associated with cirrhosis and HCC

Based on the previous whole genome sequence analysis, the most potential candidate viral marker was found to fall within the precore region. A hemi-nested PCR assay was performed to amplify the region; three primers were designed: a forward primer (1776F), an inner reverse primer (2325R) and an outer reverse primer (2820R). Properties and quality of the primers are listed in Table 2.3 and 2.4. The hemi-nested

PCR amplification was conducted in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) instead of Biorad MyCycler. Optimisation of annealing temperature from 50ºC to 60ºC with a step of 2ºC was carried out. The thermal profile used in both rounds of hemi-nested PCR are as follows: initial denaturing at 95ºC for 15 minutes; 40 cycles in 3 steps: 94ºC for 60 seconds, gradient annealing temperature of 55-64ºC for 90 seconds, 72ºC for 60 seconds; final extension at 72ºC for 10 minutes. The calculated annealing temperatures for the gradient setting were 55.0, 55.6, 56.7, 58.3, 60.4, 62.2, 63.3 and 64.0ºC. The first round of PCR generated a typical fragment size of 1045 nt and the second round 550 nt. The amplified products identified positive under agarose gel electrophoresis were then purified and sent for sequencing as previously described. Precore start codon and stop codon mutations were identified from the targeted sequencing. A precore wild-type is defined as a viral strain without both precore start codon and stop codon mutations, whereas a precore mutant is defined as that with either precore start codon or precore stop codon mutation.

Table 2.3 : Properties of primers targeting precore region.

Primer name[a]	**5'-Oligonucleotide-3'**	**Nucleotide position**	**Predicted Tm, ºC**
1776F	GAGRCTGHASKYAYAAATTG	1776-1795	55.5
2820R (outer)	TSTTGTTCCCAAGWATATGG	2801-2820	56.6
2325R (inner)	RTKGAYARGATAGGGGCA	2308-2325	57.3

Nt, nucleotide; Tm, melting temperature.
[a]Suffix -F indicates forward primer; suffix -R indicates reverse primer.

Table 2.4 : Secondary structure prediction of primers targeting precore region.

Primer pair[a]	Self- and pair-annealing, kcal/mol[b]	Loop, kcal/mol[c]
First round of PCR		
1776F-2820R	-7.04, -2.29; -2.03	None, -1.69
Second round of PCR		
1776F-2325R	-7.04, none; -2.77	None, none

HBV, hepatitis B virus; PCR, polymerase chain reaction; Tm, melting temperature.
[a]Suffix -F indicates forward primer; suffix -R indicates reverse primer.
[b]The first two are the scores of self-annealing free energy for forward and reverse primers respectively. The third score is for pair-annealing free energy for both primers. The more negative the score, the higher the probability for the annealing to occur. Note that none indicates no secondary structure would be formed.
[c]The first score is the free energy of loop formation for forward primer whereas the second score is that for reverse primer. The more negative the score, the higher the probability for the formation to occur. Note that none indicates no secondary structure would be formed.

2.2 Identification of markers for chronicity

2.2.1 Retrieval of HBV genomes

HBV genomes related to acute and chronic hepatitis B were identified by performing advanced search in National Centre for Biotechnology Information (NCBI) Nucleotide Database on 20 November 2011. The algorithm used to search HBV complete genomes was ‘("Hepatitis B virus" AND *complete genome* NOT chromosome) NOT Duck NOT Ross NOT Woodchuck NOT “Snow goose” NOT Heron NOT "Ground squirrel" NOT Sheldgoose NOT Stork NOT "Woolly monkey" NOT Orangutan’; only HBV genome sizes of 2,800-3,400nt were included. Under this search, 3,832 HBV genomes were successfully retrieved. The search was further refined by including those with acute or chronic hepatitis B only, and excluding gibbon, chimpanzee, other primate species and those with acute-on-chronic hepatitis, fulminant hepatitis or unknown liver conditions. Other exclusion criteria were age less than 12 years, non-serum/non-plasma/non-blood tissue, HIV co-infection and immune suppression. Finally, a total of 1,326 HBV genomes were obtained, being 177 from acute hepatitis and 1,149 from chronic hepatitis. Their accession numbers are listed in Appendix B and C respectively.

2.2.2 Complete HBV genome sequence analysis

HBV genotype was determined by Viral Genotyping Tool [Rozanov et al., 2004] available free (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) on National Center for Biotechnology Information (NCBI) website. Sequence alignment was carried out using SECentral Align Plus version 4. Nucleotide numbering was according to that reported by Okamoto et al. [1988]. Whole genomic differences of HBV between the acute and chronic hepatitis groups were compared. The comparison was done based on alignment by using matemathical, logical and string functions in Microsoft Excel 2007 to calculate the frequency of candidate nucleotides responsible for chronicity.

2.3 Statistical analysis

Statistical analyses were performed using the Statistical Program for Social Sciences (PASW Statistics 18 for windows, SPSS, Inc., Chicago, IL) and OpenEpi version 2.3 [Dean et al., 2009]. Categorical data for a 2x2 table, such as gender and viral mutations, were compared by Chi-square exact test. Continuous data were compared by Mann-Whitney U test; median and range were calculated. Odds ratio (OR), 95% confidence interval (CI) and Phi coefficient were calculated. Odds ratio of 1.0-1.5 indicates trivial positive association, >1.5-3.5 low positive association, >3.5-9.0 moderate positive association, >9.0 high positive association. Odds ratio of 0.7-1.0 indicates trivial negative association, 0.3-<0.7 low negative association, 0.1-<0.3 moderate negative association and 0.0-<0.1 high negative association. Phi coefficient was used to measure the strength of association (< 0.2 = small effect; 0.2–0.5 = moderate effect; > 0.5 = large effect); a positive sign for the coefficient indicates direct association, whereas a negative sign indicates inverse association. Where necessary, cutoff of continuous data was determined from receiver operating curve (ROC) analysis. Correlation between two

continuous variables was determined using scatter-plot and Spearman's rho correlation test. Binary logistic regression analysis was performed to examine the independent association between different variables and dichotomous outcome, specifically to estimate the independent risk of cirrhosis and HCC associated with precore mutations and serum iron markers after adjusted with basic demographic factors and ALT; forward and backward stepwise 'Conditional' (Bayesian) methods were applied using $P_{entry} < 0.05$ and $P_{removal} < 0.05$ respectively to obtain adjusted odds ratios (AORs) of independent predictors for risk of developing cirrhosis and HCC and their 95% CIs. Two-tailed P values of < 0.05 were considered to be statistically significant, unless otherwise specified.

Sensitivity was calculated by dividing the number of cases with marker over the total number of all cases, whereas specificity was calculated by dividing the number of controls without the marker over the total number of controls. Accuracy was calculated by dividing the total number of cases with marker and controls without marker over the grand total number of all cases and controls. In the study of cirrhosis and HCC, the cases were either cirrhosis or HCC, whereas the controls were stringent controls. The OpenEpi version 2.3 was used to calculate the minimum accuracy required for cirrhosis and HCC study to achieve at least 80% statistical power. For the comparison between acute and chronic hepatitis B, the cases were those with acute hepatitis, whereas the referents were those with chronic hepatitis. Binary logistic regression analysis was performed to examine the independent association between chronicity and viral genomic mutations; 'Enter' method was applied using $P_{entry} < 0.05$ and $P_{removal} < 0.05$ respectively to validate the independent predictors for chronicity. Chronic cases were set as 0 and acute cases as 1. Simple sampling method was chosen and bootstrapping sample number was set at 1,000. The viral mutations were included progressively based on accuracy in a descending order until it reached a P value of more than 0.05 in the

multivariate analysis. The significant candidate nucleotides were then tested for the best combination based on improvement in accuracy. The selection of candidate nucleotides was further refined with the statistical significance level re-calculated by Bonferroni correction in order to reduce false positivity [Pearson and Manolio, 2008]. Taking an usual two-tailed P value as 0.05 and number of single nucleotide polymorphisms (SNPs) present in a typical HBV genome as 3,139 (typical size of HBV genome is 3,215 nt; the SNP numbers were derived from 3,832 HBV complete genomes retrieved from GenBank), the two-tailed P value threshold was thus adjusted to 1.593×10^{-05} (= 0.05 divided by 3139).

Chapter 3 Hemi-nested PCR Optimisation

3.1 Overlapping PCR for HBV genomic sequencing

Six overlapping primer sets were designed to cover completely the HBV genome. Hemi-nested PCRs were performed in two steps, 40 cycles for the first round (using outer primer sets) and 40 cycles for the second round (using inner primer sets).

3.1.1 Optimisation of annealing temperature for outer primer sets

All primer sets were optimised with annealing temperature ranging from 55-64ºC. The gel pictures of the first round of hemi-nested PCRs are given in Figure 3.1. All primers seemed to be working well around 60ºC. Therefore, the annealing temperatures of all outer primer sets were synchronised to 60.4ºC.

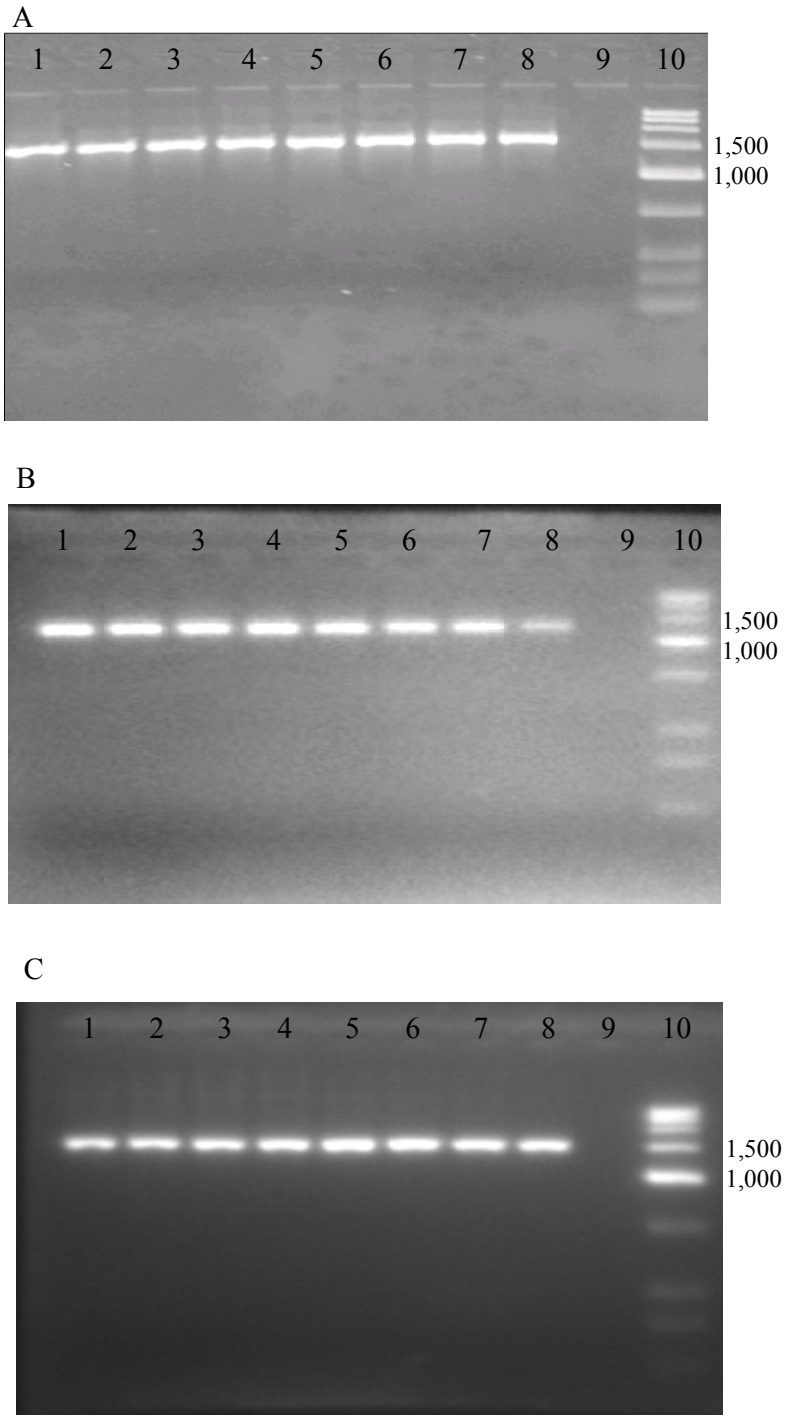


Figure 3.1 : Agarose gel (1.5%, w/v) pictures of first round hemi-nested PCR with gradient annealing temperature optimisation for primer sets (A) 251F-1797R (fragment size 1,547 bp), (B) 1441F-2839R (fragment size 1,399 bp) and (C) 2365F-649R (fragment size 1,500 bp).
Lane 1-9: 55.0, 55.6, 56.7, 58.3, 60.4, 62.2, 63.3, 64.0ºC and reagent control.
Lane 10: i-DNA 100 bp plus ladder (100, 200, 300, 600, 1000, 1500, 2000, 2500 and 3000 bp).

3.1.2 Optimisation of annealing temperature for inner primer sets

All primer sets were optimised with annealing temperature ranging from 55-64ºC. The gel pictures of the second round of hemi-nested PCRs are given in Figure 3.2. Most primers seemed to be working well around 60ºC. The annealing temperatures of all primer sets, except for 1865F-2839R, were synchronised to 60.4ºC; the annealing temperature of the unsynchronised primer set was determined to be 58.3ºC.

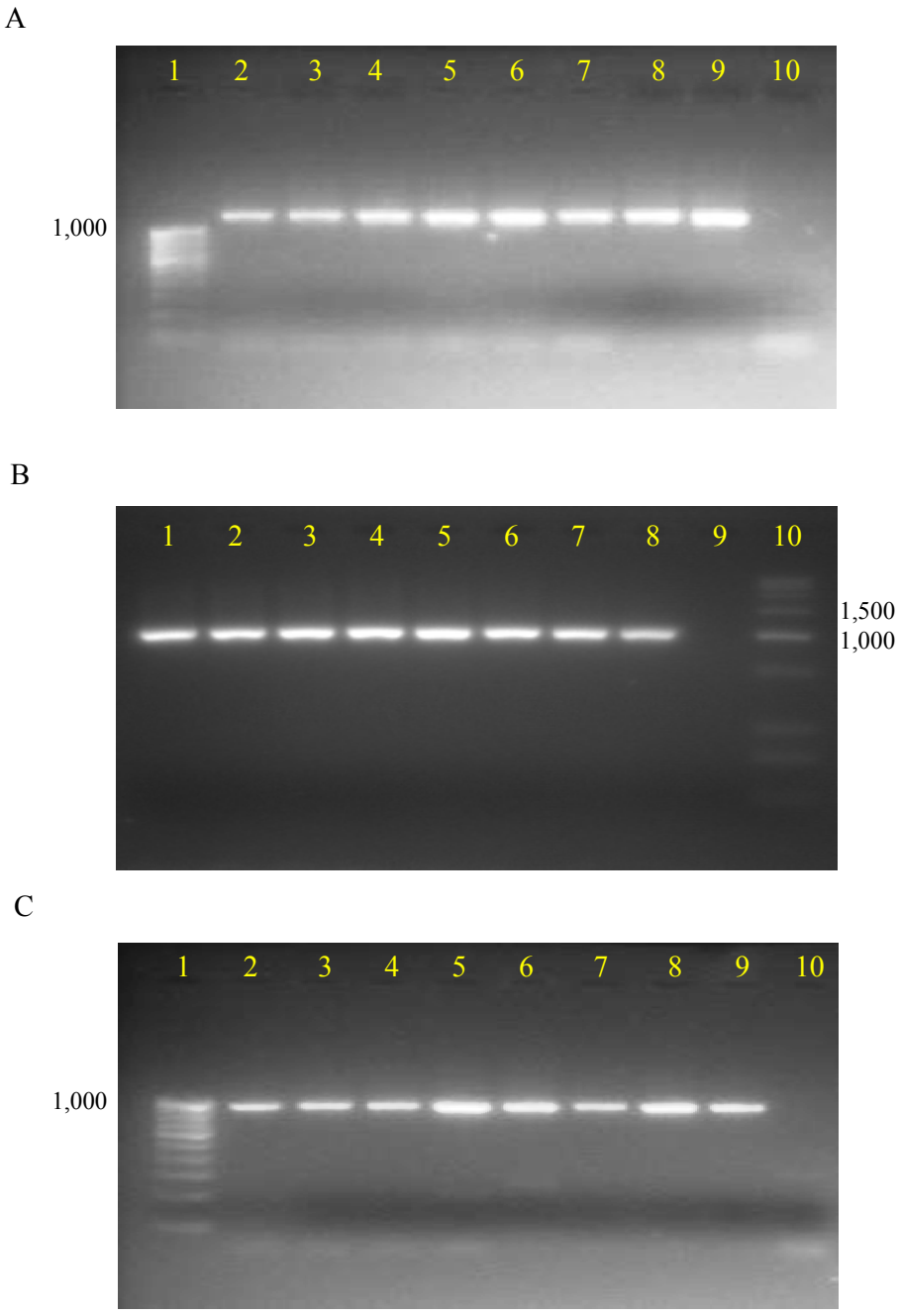
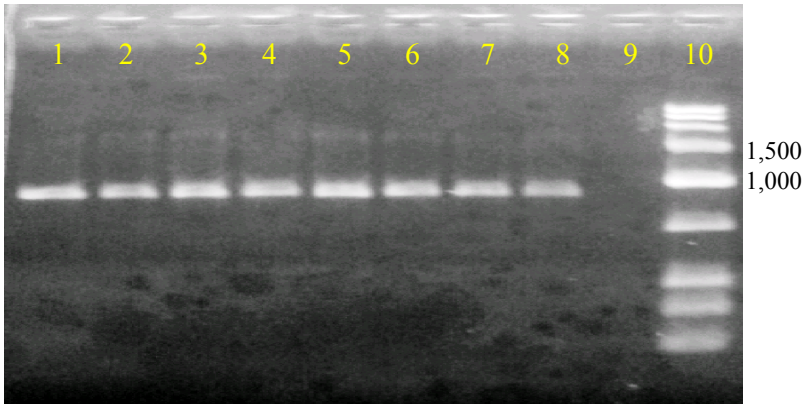
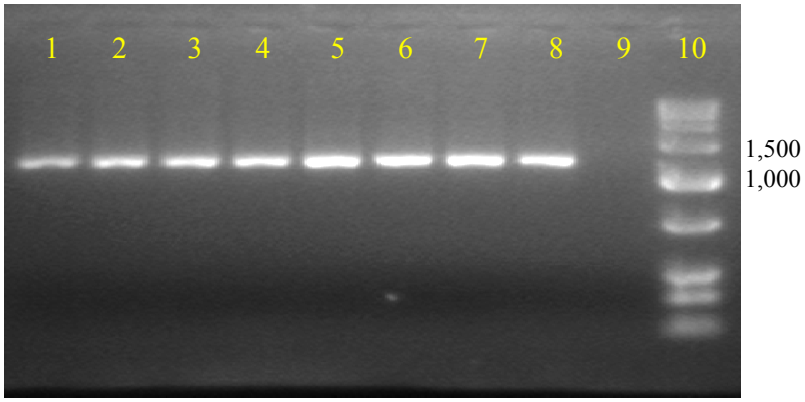


Figure 3.2 : Agarose gel (1.5%, w/v) pictures of first round hemi-nested PCR with gradient annealing temperature optimisation for primer sets (A) 596F-1797R (fragment size 1,202 bp), (B) 251F-1262R (fragment size 1,012 bp), (C) 1865F-2839R (fragment size 975 bp), (D) 1583F-2393R (fragment size 811 bp), (E) 2365F-275R (fragment size 1,126 bp) and (F) 2829F-649R (fragment size 1,036 bp).
Lane 1-9: 55.0, 55.6, 56.7, 58.3, 60.4, 62.2, 63.3, 64.0ºC and reagent control.
Lane 10: i-DNA 100 bp plus ladder (100, 200, 300, 600, 1,000, 1,500, 2,000, 2,500 and 3,000 bp).

D



E



F

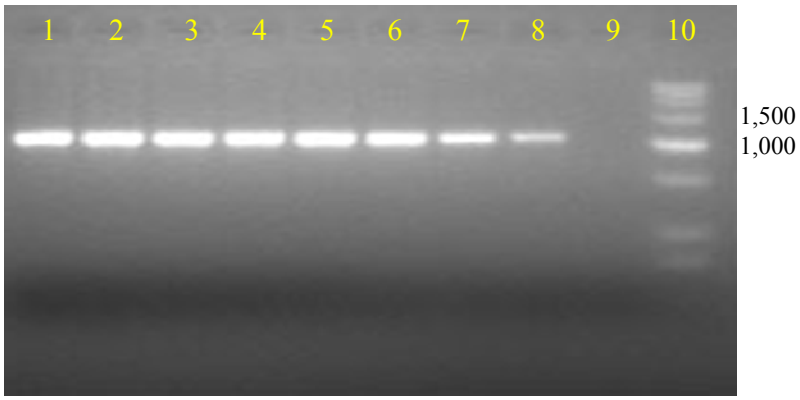


Figure 3.2, continued.

3.2 Hemi-nested PCR for targeted precore fragment sequencing

Two primer sets targeting precore region were optimised with annealing temperature ranging from 50-60ºC. The gel pictures of the first and second rounds of hemi-nested PCR are given in Figure 3.3. The optimal annealing temperature was determined to be 54ºC for both rounds of hemi-nested PCR.

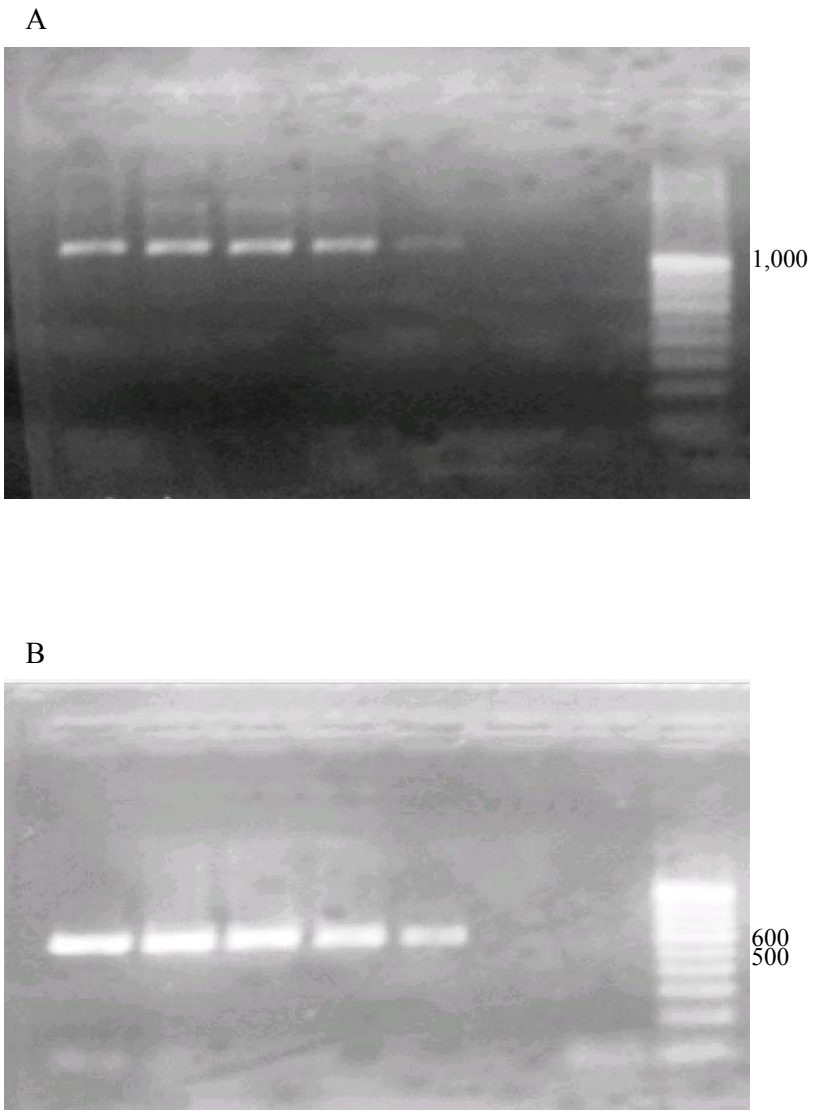


Figure 3.3 : Agarose gel (1.5%, w/v) pictures of first and second round hemi-nested PCR with annealing temperature optimisation for primer sets (A) 1776F-2820R (fragment size 1,045 bp) and (B) 1776F-2325R (fragment size 550 bp).
Lane 1-9: 50, 52, 54, 56, 58, 60ºC and reagent control.
Lane 10: i-DNA 100 bp plus ladder (100, 200, 300, 600, 1,000, 1,500, 2,000, 2,500, 3,000 bp).

Chapter 4 In Search of Markers for HBV-related Cirrhosis and HCC

4.1 Literature review

Chronic HBV infection is a serious public health problem because of its worldwide distribution and severe complications. Cirrhosis and HCC are the most severe complications particularly prevalent in Asia, where HBV-infected individuals usually acquire infection at birth or during early childhood. Viral markers, such as HBeAg positivity and viral load, and host markers, such as smoking and serum ferritin, are among the risk factors for cirrhosis and HCC. Cirrhosis per se is a predisposing factor for HCC [Muroyama et al., 2006]. Regardless of disease etiology, about 80% of HCC patients have underlying cirrhosis. In those infected by HBV only, more than 90% may have cirrhosis [Yang et al., 2011]. The annual incidence of HBV-related HCC in cirrhotic patients is about 1-15% [Sanyal et al., 2010]. The focus on predictive markers of the complications is usually on either viral or host markers alone. Often, the use of either viral or host markers alone, is not adequate to predict cirrhosis and HCC with high accuracy, suggesting that the HBV-related complications could be multi-factorial in origin.

4.1.1 Viral Markers

(a) HBeAg

Positivity for HBeAg usually indicates high viral replication. HBV-infected hepatocytes produce various viral antigens that can provoke host immune attack. When viral replication is high, more hepatocytes are expected to be infected. Subsequently these would provoke host immune attack on more liver tissues, resulting in a higher degree of liver damage. The interplay between the virus and the host immune response in long term leads to repeated hepatocyte injury and regeneration, and ultimately culminates in cirrhosis and HCC. In fact, chronic hepatic necro-inflammatory process has been

proposed as one of the mechanisms for HBV-related cirrhosis and HCC. In short, HBeAg might play a role in the development of liver complications.

Published reports, however, have given conflicting evidence on the link between HBeAg and cirrhosis. A Taiwanese study estimated the incidence of cirrhosis in HBeAg-positive chronic hepatitis B patients to be 2-5 per 100 person-years [Liaw et al., 1988]. In the same year, an Italian study demonstrated a much higher incidence of cirrhosis in those who were HBeAg-negative, 8-9 per 100 person-years [Fattovich et al., 1988]. Again in France, the proportion of cirrhosis in HBeAg-negative patients was higher than that in the HBeAg-positive ones [Zarski et al., 2006]. However, another study did lend support to the hypothesis of HBeAg positivity being linked to cirrhosis. The study showed that the hazard ratio of cirrhosis was more than 17 for HBeAg-seroconverted patients after age of 40 years compared to those before 30 years of age [Chen et al., 2010]. Overall, it seemed that HBeAg-negative patients, contrary to expectation, might have a higher risk for cirrhosis. Alternatively, the duration of exposure to HBeAg, but not age at visit, may influence the progression to cirrhosis. Hence, an age-adjusted or cohort analysis is required to further elucidate the link of HBeAg positivity with cirrhosis.

A large-scale cohort study involving more than 10,000 men found that the age-adjusted incidence rate of HCC among HBeAg-positive cases was at least 3 times higher than that among HBeAg-negative cases [Yang et al., 2002]. A more recent case-control study from India also showed the positive association of HBeAg with HCC [Asim et al., 2010]. The hazard ratio of HCC for HBeAg-seroconverted patients after age of 40 years compared to those before age of 30 years was more than 5 [Chen et al., 2010]. It seemed that a longer period of exposure to HBeAg might confer a higher risk of developing HCC. As in the case of cirrhosis, however, another study reported that earlier HBeAg seroconversion did not reduce such a risk [Yuen et al., 2003]. Other

studies showing that more than 80% of the HBV-cirrhotic patients have negative serum HBeAg at the time of diagnosis of HCC [Xu et al., 2009] and lower prevalence of HBeAg in cirrhosis and HCC [Liaw et al., 1984; Lin et al., 2007] underscore the inconsistent relationship between HBeAg and risk of HCC. The usefulness of HBeAg as a predictor for complications of hepatitis B is further hampered by the fact that individuals carrying viral mutants capable of abrogating HBeAg expression could also be having high viral replication that predisposes an individual to the development of cirrhosis and HCC.

(b) HBV DNA

Viral load of HBV, also called HBV DNA, is currently the most important marker of viral replication. Active viral replication is one of the risk factors associated with progression to severe complications [Fung and Lok, 2005]. Chronic hepatitis B patients with high HBV DNA level (higher than 4 log copies/ml or 2,000 IU/ml) and active liver disease are recommended for antiviral treatment as they are at risk for progression to cirrhosis and HCC [Keeffe et al., 2008, Liaw et al., 2008].

HBV DNA fluctuates during the course of chronic infection of HBV (shown in Figure 4.1). As such, a point estimate of HBV DNA level would be less accurate for predicting the development of cirrhosis and HCC. This is exemplified by the observation that the HBV DNA in children is usually high; yet, children are not at risk of the liver complications. It takes several decades for HBV-related cirrhosis and HCC to occur [Block et al., 2003]. Fluctuating or persistently high HBV DNA patterns are important in determining the risk [Harris et al., 2003; Kwon et al., 2010]. Many studies have been conducted to assess the capability of the HBV DNA marker to predict the development of liver diseases, especially HCC. The non-exhaustive lists of case-control and cohort studies on cirrhosis and HCC are summarised in Table 4.1 and 4.2

respectively. Most studies supported that chronic hepatitis B individuals with viral load ≥ 10,000 copies virus/mL were at higher risk for cirrhosis and HCC. A cohort study from Hong Kong indicated that a trough viral load more than 6.31×10^4 copies/mL was predictive for the development of HCC (trough viral load was defined as the lowest level of HBV DNA during follow-up) [Chan et al., 2009]. In addition, viral loads more than 10,000 copies/mL (>2,000 IU/mL) have a higher rate of HCC recurrence after liver resection [Hung et al., 2008], radiofrequency ablation [Goto et al., 2011] and liver transplantation [Li et al., 2011]. A large-scale case-control study from China showed that high viral load was a risk factor for HCC, but not for cirrhosis [Yin et al., 2011]. In contrast, some studies showed no association of a higher viral load (≥100,000 copies/mL) with risk of cirrhosis [Tsai et al., 2009] and HCC [Fattovich et al., 2002; Liu et al., 2006]. Another study found that 15% of HCC patients have low viral load, <1,000 copies/mL [Fung et al., 2007]. Chronic hepatitis B individuals with low viral load can still progress to the severe liver complications [Kim et al., 2010; Mendy et al., 2010]. Overall, elevated viral load is associated with a higher risk for progression to cirrhosis and HCC, but those with low viral load are also at risk. Multiple points or years of viral load estimation before diagnosis of cirrhosis and HCC, instead of single point determinations, are probably better for the prediction of liver complications.

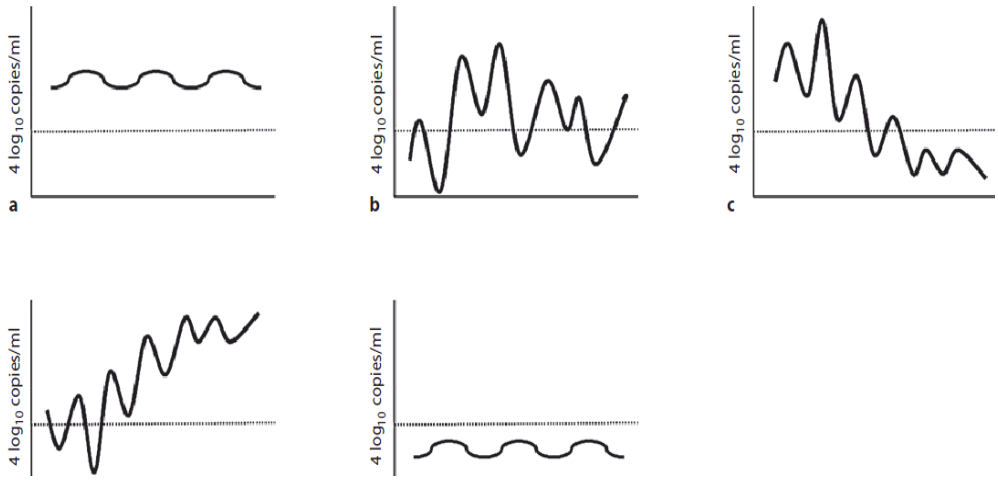


Figure 4.1 : Possible trends of HBV DNA levels.
a.) a persistently high pattern; b.) a fluctuating pattern; c.) a decreasing pattern; d.) an increasing pattern; e.) a persistently low pattern. Reference: Kwon et al. (2010). The value of 4 $\log_{10}$ copies/mL is equivalent to 10,000 copies/mL.

Table 4.1 : Serum HBV DNA levels in case-control and cohort studies of cirrhosis.

Author (year)	Sample size (Cirrhosis, control[a])	HBV DNA level (copies/mL)	OR/RR/HR
Case-control			
Yuan et al. (2005)	79, 158	10,000-99,999	1.5
		100,000-999,999	3.1
Cohort			
Chen et al. (2006b)	367, 1,316	1,600-99,999	1.5
		≥100,000	2.7
Iloeje et al. (2006)	365, 3,217	≥10,000	2.5
		≥100,000	5.9
Mendy et al. (2010)	53, 60	>10,000	17.3

HR, hazard ratio; OR, odds ratio; RR, relative risk.
Note: all results are significant ($P < 0.05$).
[a]Control indicates non-cirrhosis and non-HCC control.

Table 4.2 : Serum HBV DNA levels in case-control and cohort studies of HCC.

Author (year)	Sample size (HCC, control[a])	HBV DNA level (copies/mL)	OR/RR/HR
Case-control			
Yu et al. (2005)	154, 316	17,000-79,400	2.5
		≥81,200-794,000	2.4
Liu et al. (2006)	44, 45	≥100,000	2.6
Tong et al. (2007)	101, 67	>100,000	80.8
Liu et al. (2008)	170, 276	10,000-99,999	2.8
		100,000-999,999	48.4
Mendy et al. (2010)	126, 60	>10,000	38.8
Yin et al. (2011)	846, 190	≥10,000	4.5
Cohort			
Ohata et al. (2004)	21, 52	≥1000,000	3.1
Chen et al. (2006a)	164, 3489	10,000-99,999	2.3
		100,000-999,999	6.6
Yang et al. (2008)	153, 2,609	10,000-99,999	1.8
		100,000-999,999	3.4

HR, hazard ratio; OR, odds ratio; RR, relative risk.
Note: all results are significant ($P < 0.05$).
[a]Control indicates non-cirrhosis and non-HCC control.

(C) Viral genotype and mutations

Studies in the possible influence of viral genotype, BCP and precore mutations in HBV-related diseases have mushroomed over the past decade. Newly observed viral mutations, including X gene mutations and pre-S deletions, have emerged in the past few years. Viral genetics are less prone to fluctuation compared to viral load and other liver function parameters [Chu et al., 2012]. Hence, they may be more suitable for the determination of potential predictors for chronic liver complications in case-control studies compared to other parameters.

Viral genotype C is frequently associated with development of cirrhosis and HCC when compared to genotype B [Yin et al., 2011]. Nonetheless, some studies showed inconsistent findings [Lin et al., 2007; Yuan et al., 2007]. Viral genotypes A and D are less common in Malaysia. A study from India demonstrated no association between genotypes A and D with HCC [Asim et al., 2010]. Certain sub-genotypes, for example, sub-genotype C2, have been claimed to have higher disease-inducing capacity than C1 [Tanaka et al., 2006]. Viral genotyping and sub-genotyping are difficult to

apply in the clinical setting because for an accurate classification of the viral strains, complete genomic sequencing is required; this approach is labour-intensive and not cost-effective. The analysis of single or a few nucleotides is more practical. The genotyping method is further complicated by viral genetic recombination. For example, a HBV C/B recombinant, GQ377594 [Xu et al., 2010], when classified according to the genotype definitions [Okamoto et al., 1988; Kramvis et al., 2008], can be either genotype B or C. In fact, the problems of inadequate differentiation of HBV strains according to the conventional definition of genotyping have been brought up recently [Zhou et al., 2012]. Obviously, a new standard for classifying such viral sequences is warranted.

BCP is known to be the binding site for some liver transcription factors and may be responsible for differential viral gene regulation [Zheng et al., 2004]. V1753, T1762, A1764, T1766, A1768 and G1799 are some of the BCP mutations. As listed in Table 4.3-4.6, the BCP double mutations (T1762/A1764) have been intensively reported in various clinical association studies. However, no *in vitro* study has yet provided any evidence concerning the hepatocarcinogenic effects of the BCP mutations [Pollicino et al., 2011]. The mutations suppress HBeAg expression and additional BCP mutations, such as those at nucleotide position 1753 and 1766, could affect rate of viral replication [Jammeh et al., 2008]. Taken that HBeAg is one of the major antigen inducing liver injury [Frelin et al., 2009], it would be expected that BCP mutations imposed lesser extent of immune-mediated liver injury as a result of reduced HBeAg expression. Surprisingly, most studies supported a positive association of T1762/A1764 with cirrhosis and HCC. Also, quite a number found no association. However, in a large sample-sized cohort study, the BCP mutations did show a strong negative association with HCC [Yang et al., 2008]. Although associated significantly with cirrhosis and HCC, the BCP mutations can still be either less sensitive or less specific (<70%) for

prediction of progression to cirrhosis and HCC [Wang et al., 2007; Mendy et al., 2008; Utama et al., 2009; Cho et al., 2011; Kao et al., 2012]. Collectively, these association data point to the inconsistent biological relevance of T1762/A1764 mutations affecting disease pathogenesis.

A1896 is a common precore stop codon mutation interrupted in the middle of precore ORF; A1897 is the less common stop codon mutation. These mutations totally abrogate HBeAg expression [Jammeh et al., 2008]. Because HBeAg is harmful to hepatocytes [Frelin et al., 2009], one would expect that the termination of HBeAg expression by A1896 was probably hepatoprotective. This is indeed in agreement with two huge population age-adjusted studies [Tanaka et al., 2006], showing strong negative association of the mutation with HCC. Intriguingly, much evidence argues against the association of A1896 with cirrhosis and HCC (listed in Table 4.3 and 4.5 respectively). Even more suprisingly, some found positive association [Song et al., 2006; Kao et al., 2012].

Pre-S may be divided into pre-S1 and pre-S2, which are responsible for production of LHBsAg and MHBsAg respectively. Pre-S mutations may include deletions at the 3' terminus of pre-S1 region, at the 5' terminus of pre-S2 region and at the pre-S2 start codon, and point mutations at the pre-S2 start codon [Yeung et al., 2011]. Both pre-S1 and pre-S2 deletions could cause overproduction and accumulation of the envelope proteins in endoplasmic reticulum (ER), resulting in ER stress that may promote oxidative DNA damage and genomic instability [Fan et al., 2001; Hsieh et al., 2004]. This would indeed induce more liver damage and hepatocarcinogenesis. In addition, pre-S2 mutations may induce formation of ground glass hepatocytes, an indication of liver injury [Wang et al., 2003]. In addition, truncated pre-S2 sequence might either act as a gene transactivator integrated into the human genome [Hildt and Hofschneider, 1998; Wang et al., 2005] or induce uncontrolled cell cycle progression

via degradation of cyclin-dependent kinase inhibitors [Hsieh et al., 2007]. Clinical association of pre-S deletions with cirrhosis and HCC was inconsistent (see Table 4.4 and 4.6). Further, positions and lengths of pre-S deletions were variable, making interpretation and evaluation difficult. Besides the deletions, pre-S mutations like C2964, C3116 and pre-S2 start codon mutations have rarely been reported.

Less well-reported markers, like BCP mutations (such as V1753 and T1766/A1768), precore mutations (such as T1858 and A1899), core mutations (such as G1914) and X mutations (such as T1653 and A1689), have also gained some attention recently. The underlying mechanisms of these mutations in hepatocellular necrosis and carcinogenesis have been largely unknown. The risks of these mutations for cirrhosis and HCC are listed in Table 4.4 and 4.6 respectively.

Table 4.3 : Common HBV mutations in studies of cirrhosis.

Author (year)	Sample size Cirrhosis, control[a]	OR/RR/HR	*P* value
T1762/A1764			
Positive association			
Lin et al. (2005)	30, 50	5.0	0.005
Chen et al. (2006b)	46, 18	38.9	<0.050
Song et al. (2006)	40, 40	4.8	<0.001
Chen et al. (2007)	28, 113	3.3	0.005
Wang et al. (2007)	32, 132	2.6	0.030
Yuan et al. (2007)	14, 17	8.8	0.016
Mendy et al. (2008)	43, 21	3.6	0.040
Utama et al. (2009)	62, 61	6.0	<0.001
Cho et al. (2011)	65, 60	3.5	0.004
Yin et al. (2011)	188, 235 (CHB)/846 (ASC)	2.9	0.001
No association			
Preikschat et al. (2002)	14, 24	0.2	0.354
Tsai et al. (2009)	13, 38	1.2	1.000
Malik et al. (2012)	30, 116	1.7	0.267
A1896			
Positive association			
Chen et al. (2006b)	46, 18	3.2	<0.050
Song et al. (2006)	40, 40	6.8	<0.001
No association			
Lin et al. (2005)	31, 57	0.9	1.000
Chen et al. (2007)	28, 113	0.8	0.570
Wang et al. (2007)	32, 132	0.6	0.450
Mendy et al. (2008)	43, 21	2.3	0.200
Tsai et al. (2009)	13, 38	0.9	1.000
Malik et al. (2012)	30, 116	1.4	0.590
V1753, S1753 or C1753[b]			
Positive association			
Chen et al. (2007)	28, 113	2.9	0.009
Utama et al. (2009)	62, 61	4.0	0.001
No association			
Song et al. (2006)	40, 40	0.3	0.060
Wang et al. (2007)	32, 132	2.1	0.272
Malik et al. (2012)	30, 116	2.1	0.154

ASC, asymptomatic hepatitis B surface antigen carrier; BCP, basal core promoter; CHB, chronic hepatitis B; HR, hazard ratio; NA, not available; OR, odds ratio; RR, relative risk.

[a]Control indicates non-cirrhotic non-HCC control.

[b]Nucleotide V = A, C or G; S = C or G. All studies used V1753, except for Malik et al. (2012) that used S1753 and Guo et al. (2008) that used C1753.

Table 4.4 : Rarely reported HBV mutations in studies of cirrhosis.

Author (year)	Sample size Cirrhosis, control[a]	OR/RR/HR	*P* value
M1386			
Cho et al. (2011)	65, 60	4.2	0.004
T1653			
Wang et al. (2007)	32, 132	2.1	0.962
Cho et al. (2011)	65, 60	3.0	0.028
A1726/Y1727[b]			
Utama et al. (2009)	62, 61	0.2	0.001
T1764/G1766			
Malik et al. (2012)	30, 116	0.59	0.528
T1766/A1768			
Chen et al. (2007)	28, 113	6.5	<0.001
A1768			
Yin et al. (2011)	188, 235 (CHB)/846 (ASC)	3.1	0.009
G1799			
Chen et al. (2007)	28, 113	0.4	0.009
A1846			
Yin et al. (2011)	188, 235 (CHB)/846 (ASC)	1.9	0.029
T1858			
Wang et al. (2007)	32, 132	1.1	0.980
A1899			
Song et al. (2006)	40, 40	9.8	0.029
Wang et al. (2007)	32, 132	2.6	0.287
Malik et al. (2012)	30, 116	2.3	0.078
C2964			
Yin et al. (2010)	119, 603	10.0	<0.001
C3116			
Yin et al. (2010)	119, 603	3.4	0.005
Pre-S deletions			
Chen et al. (2006b)	46, 18	0.3	1.000
Chen et al. (2007)	28, 113	3.1	0.003

BCP, basal core promoter; HR, hazard ratio; NA, not available; OR, odds ratio; RR, relative risk.
[a]Control indicates non-cirrhotic non-HCC control.
[b]Nucleotide Y = C or T.

Table 4.5 : Common HBV mutations in studies of HCC.

Author (year)	Sample size HCC, control[a]	OR/RR/HR	*P* value
T1762/A1764			
Positive association			
Nakashima et al. (2004)	36, 38	58.7	<0.001
Lin et al. (2005)	25, 50	7.3	0.002
Chen et al. (2006b)	50, 46	7.2	<0.001
Liu et al. (2006)	44, 45	4.5	0.003
Tanaka et al. (2006)	180, 148	3.3	<0.001
Tong et al. (2007)	101, 67	12.9	<0.001
Wang et al. (2007)	47, 132	11.4	<0.001
Yuan et al. (2007)	8, 17	25.7	0.009
Chen et al. (2008)	80, 160	2.8	0.002
Mendy et al. (2008)	114, 21	3.4	0.024
Yang et al. (2008)	153, 2609	1.7	0.013
Kim et al. (2009)	135, 135	4.7	<0.001
Utama et al. (2009)	48, 61	4.8	<0.001
Asim et al. (2010)	150, 136	6.9	0.001
Cho et al. (2011)	69, 60	9.2	<0.001
Jang et al. (2012) – T1762	75, 75	11.7	0.034
Kao et al. (2012)	112, 56	6.3	<0.001
Malik et al. (2012)	30, 116	3.9	0.002
No association			
Muroyama et al. (2006)	39, 36	0.8	0.782
Livingston et al. (2007)	45, 43	1.3	0.670
Shinkai et al. (2007)	80, 80	2.9	>0.050
Elkady et al. (2008)	23, 25	1.1	1.000
Guo et al. (2008)	58, 71	0.7	0.448
Jang et al. (2012) – A1764	75, 75	NA	0.430

All are case-control studies, except for Muroyama et al. (2006) and Yang et al. (2008).

BCP, basal core promoter; HCC, hepatocellular carcinoma; HR, hazard ratio; NA, not available; OR, odds ratio; RR, relative risk.

[a]Control indicates non-cirrhotic non-HCC control.

[b]Nucleotide V = A, C or G; S = C or G. All studies used V1753, except for Malik et al. (2012) that used S1753 and Guo et al. (2008) that used C1753.

Table 4.5, continued.

Author (year)	Sample size HCC, control[a]	OR/RR/HR	*P* value
A1896			
Positive association			
Chen et al. (2006b)	50, 46	3.7	0.007
Tong et al. (2007)	101, 67	2.0	0.040
Kao et al. (2012)	112, 56	2.4	0.013
Malik et al. (2012)	30, 116	2.7	0.030
No association			
Park et al. (1999)	58, 16	2.1	0.473
Nakashima et al. (2004)	36, 38	0.3	0.073
Lin et al. (2005)	25, 50	0.8	0.860
Liu et al. (2006)	44, 45	1.9	0.260
Muroyama et al. (2006)	39, 36	1.6	0.358
Livingston et al. (2007)	45, 43	0.6	0.408
Shinkai et al. (2007)	80, 80	0.7	>0.050
Wang et al. (2007)	47, 132	0.7	0.336
Yuan et al. (2007)	8, 17	1.2	1.000
Chen et al. (2008)	80, 160	1.4	0.353
Elkady et al. (2008)	23, 25	2.0	0.444
Mendy et al. (2008)	119, 21	0.6	0.492
Kim et al. (2009)	135, 135	1.0	0.889
Asim et al. (2010)	150, 136	1.8	>0.050
Jang et al. (2012)	75, 75	NA	0.438
Negative association			
Tanaka et al. (2006)	180, 148	0.5	0.009
Yang et al. (2008)	153, 2,609	0.3	<0.001
V1753, S1753 or C1753[b]			
Positive association			
Tanaka et al. (2006)	180, 148	2.5	<0.001
Shinkai et al. (2007)	80, 80	8.0	<0.001
Wang et al. (2007)	47, 132	10.4	<0.001
Chen et al. (2008)	80, 160	2.1	0.041
Asim et al. (2010)	150, 136	3.7	0.041
Cho et al. (2011)	69, 60	7.8	<0.001
Jang et al. (2012)	75, 75	5.3	<0.001
Malik et al. (2012)	30, 116	3.7	0.005
No association			
Guo et al. (2008)	58, 71	1.9	0.108
Kim et al. (2009)	135, 135	0.6	0.106

All are case-control studies, except for Muroyama et al. (2006) and Yang et al. (2008).

BCP, basal core promoter; HCC, hepatocellular carcinoma; HR, hazard ratio; NA, not available; OR, odds ratio; RR, relative risk.

[a]Control indicates non-cirrhotic non-HCC control.

[b]Nucleotide V = A, C or G; S = C or G. All studies used V1753, except for Malik et al. (2012) that used S1753 and Guo et al. (2008) that used C1753.

Table 4.6 : Rarely reported HBV mutations in studies of HCC.

Author (year)	Sample size HCC, control[a]	OR/RR/HR	*P* value
A7			
Yin et al. (2010)	231, 603	7.2	<0.001
C53			
Yin et al. (2010)	231, 603	2.2	0.022
M1386			
Cho et al. (2011)	69, 60	3.9	0.002
T1485			
Muroyama et al. (2006)	91, 87	4.9	0.001
T1504/T1505			
Asim et al. (2010)	150, 136	7.6	0.012
A1613/T1653			
Tatsukawa et al. (2011)	40, 52	7.2	0.016
T1653			
Tanaka et al. (2006)	180, 148	2.2	0.001
Shinkai et al. (2007)	80, 80	4.4	0.006
Wang et al. (2007)	47, 132	15.4	<0.001
Guo et al. (2008)	58, 71	1.6	0.323
Kim et al. (2009)	135, 135	4.3	0.037
Asim et al. (2010)	150, 136	3.0	>0.050
Cho et al. (2011)	69, 60	4.4	<0.001
Jang et al. (2012)	75, 75	4.2	0.001
A1689			
Kim et al. (2009)	135, 135	3.1	0.026
A1726/Y1727[b]			
Utama et al. (2009)	48, 61	0.5	0.104
T1764/G1766			
Elkady et al. (2008)	23, 25	1.7	0.615
Guo et al. (2008)	58, 71	1.2	1.000
Malik et al. (2012)	30, 116	2.2	0.119
T1846			
Chen et al. (2008)	80, 160	2.0	0.018
Jang et al. (2012)	75, 75	6.5	<0.001
T1858			
Wang et al. (2007)	47, 132	3.0	0.004
Kim et al. (2009)	135, 135	0.8	0.463

All are case-control studies, except for Muroyama et al. (2006), Yang et al. (2008) and Yin et al. (2010).
aa, amino acid; BCP, basal core promoter; HCC, hepatocellular carcinoma; HR, hazard ratio; NA, not available; OR, odds ratio; RR, relative risk.
[a]Control indicates non-cirrhotic non-HCC control.
[b]Nucleotide Y = C or T.

Table 4.6, continued.

Author (year)	Sample size HCC, control[a]	OR/RR/HR	*P* value
A1899			
Wang et al. (2007)	47, 132	0.2	0.006
Chen et al. (2008)	80, 160	2.2	0.017
Asim et al. (2010)	150, 136	1.3	>0.050
Jang et al. (2012)	75, 75	NA	0.690
Malik et al. (2012)	30, 116	1.5	0.435
G1914			
Asim et al. (2010)	150, 136	8.2	0.002
Malik et al. (2012)	30, 116	15.0	<0.001
C2189			
Zhu et al. (2010)	103, 103	4.0	0.003
W2203			
Zhu et al. (2010)	103, 103	9.7	0.035
A2964			
Yin et al. (2010)	231, 603	25.7	<0.001
T3116			
Yin et al. (2010)	231, 603	3.8	<0.001
Pre-S deletions			
Chen et al. (2006b)	46, 50	NA	1.000
Lin et al. (2007)	64, 202	3.7	0.007
Chen et al. (2008)	80, 160	2.2	0.021
Yin et al., (2010) – Pre-S1	231, 603	7.3	<0.001
Yeung et al. (2011)	69, 69	5.1	0.012
Pre-S2 aa1-6 deletion			
Kao et al. (2012)	112, 56	136.4	0.039
Pre-S2 start codon mutation			
Yin et al. (2010)	231, 603	5.3	<0.001

All are case-control studies, except for Muroyama et al. (2006), Yang et al. (2008) and Yin et al. (2010).
aa, amino acid; BCP, basal core promoter; HCC, hepatocellular carcinoma; HR, hazard ratio; NA, not available; OR, odds ratio; RR, relative risk.
[a]Control indicates non-cirrhotic non-HCC control.
[b]Nucleotide Y = C or T.

4.1.2 Host Markers

(a) Age, gender and ethnicity

It is generally accepted that as a person gets older, the person is at a higher risk for cancer. This is also true in HBV-related HCC. The annual incidence of HCC in HBV-infected Asians was reported to be 0.5%, increasing to 2-fold at age of 70 years [Bruix and Sherman, 2005]. As the Asian population usually acquired HBV infection at birth or during early childhood, it is plausible that most of them are exposing to HBV antigens for a long period, and thus have a much higher risk of progression to HCC. Knowing that HBV antigens cause immune-mediated liver injury [Frelin et al., 2009], it is conceivable that long-term exposure to such antigens may induce significant liver damage (possibly lead to cirrhosis) and continual regeneration of hepatic cells. As a result of the rapid cellular renewal, accumulation of cellular replication errors may occur, and subsequently would lead to HCC. In reality, it is not feasible to obtain data on duration of infection. Most, if not all, studies reported age at visit [Cho et al., 2011; Kao et al., 2012]. Hence, the patients' family history of chronic HBV infection could be the best indication of childhood infection. There is little doubt that individuals of older age are more likely to develop cirrhosis [Fung and Lok, 2005; Zarski et al., 2006] and HCC [Yin et al., 2011]. A non-exhaustive list of publications on age in relation to cirrhosis and HCC is shown in Table 4.7. The evidence for the linking of older age with cirrhosis and HCC is strong. Most studies have shown that chronic HBV individuals developed cirrhosis and HCC at an average age of 45 years and above.

Table 4.7 : Age in studies of HBV-related cirrhosis and HCC.

Author (year)	Disease, control[a]		*P* value
	Sample size	Mean/Median age or age cutoff (OR)	
Disease: cirrhosis			
Song et al. (2006)	40, 40	54.2, 34.7	<0.001
Yin et al. (2010)	119, 603	51.6, 30.0	<0.001
Cho et al. (2011)	65, 60	51.6, 35.6	<0.001
Yin et al. (2011)	188, 235 (CHB)/846 (ASC)	50.9, 40.3/29.2 (1.1)	<0.001
Disease: HCC			
Liu et al. (2006)	44, 45	46.2, 44.8	0.590
Tanaka et al. (2006)	180, 148	≥50, <50 (2.9)	<0.001
Tong et al. (2007)	101, 67	53.3, 45.4	0.002
Yin et al. (2010)	231, 603	49.9, 30.0	<0.001
Cho et al. (2011)	69, 60	55.8, 35.6	<0.001
Wang et al. (2007)	47, 132	≥50, <50 (14.3)	<0.001
Chen et al. (2008)	80, 160	50.7, 48.9	0.210
Asim et al. (2010)	150, 136	>45, ≤45 (4.0)	0.001
Kao et al. (2012)	112, 56	46.3, 42.9	0.385

ASC, asymptomatic hepatitis B surface antigen carrier; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; OR, odds ratio.
[a]Sub-heading of this table indicated (in **bold**) whether the disease was cirrhosis or HCC. Control indicates non-cirrhotic non-HCC control.

Males tend to develop HCC at a younger age [Gonzalez and Keefee, 2012]. In most publications, a predominance of cirrhosis and HCC in male has been described, with male-to-female ratios ranging from 1.8:1.0 to 9.2:1.0 (shown in Table 4.8). In Peninsular Malaysia, age-standardised incidences of liver cancer in males and females were 7.2 and 2.7 per 100,000 persons respectively [National Cancer Registry, 2006]. Other Southeast Asian countries like Indonesia and Vietnam have the ratio of about 4:1 [El-Serag, 2012]. The male predominance is strengthened by the clinical observations of the more rapid progression to cirrhosis in males than females; hence cirrhosis that leads to HCC development is usually considered to be the disease of men and postmenopausal women [Shimizu, 2007]. The striking gender disparity findings have initiated *in vitro* and *in vivo* studies from time to time to explore the importance of sex hormones in HCC. Androgen has been long known to induce or at least promote hepatocarcinogenesis [De Maria et al., 2002], but some studies showed no correlation of histopathologic types of HCC with androgen receptor expression [Nagasue et al., 1989].

Hepatic androgen receptor increases the HBV viral titer by enhancing the viral RNA transcription through direct binding to the androgen response element near the viral core promoter [Wu et al., 2010]. Meanwhile, estrogen could be either protective [Wang et al., 2011; Zhang et al., 2012] or carcinogenic [Farinati et al., 2002; Trauner and Halilbasic, 2011], or having no effect at all [Manesis et al., 1995; Nagasue et al., 2009] in the liver. In addition, a recent study found that estrogen may repress transcription of HBV genes via interaction with a hepatocyte nuclear factor [Wang et al., 2012]. This may account for the lower viral load, and hence lower incidence of cirrhosis and HCC in HBV-infected females compared to males. The evidence on sex hormones related progression to cirrhosis and HCC is inconsistent. Other 'sex-dependent' factors like menstrual blood loss and smoking habit should be taken into consideration as well. The effects of these factors in chronic hepatitis B diseases will be described in later sections.

Table 4.8 : Male gender in studies of HBV-related cirrhosis and HCC.

Author (year)	Sample size Disease, control[a]	OR	*P* value
Disease: cirrhosis			
Song et al. (2006)	40, 40	6.0	<0.001
Yin et al. (2010)	119, 603	1.8	0.008
Cho et al. (2011)	65, 60	2.3	0.064
Yin et al. (2011)	188, 235 (CHB)/846 (ASC)	1.9	0.044
Disease: HCC			
Liu et al. (2006)	44, 45	4.4	0.020
Tanaka et al. (2006)	180, 148	2.3	0.014
Wang et al. (2007)	47, 132	0.8	0.810
Tong et al. (2007)	101, 67	6.5	<0.001
Asim et al. (2010)	150, 136	3.3	0.001
Yin et al. (2010)	231, 603	2.5	<0.001
Cho et al. (2011)	69, 60	0.6	0.270
Kao et al. (2012)	112, 56	9.2	<0.001

ASC, asymptomatic hepatitis B surface antigen carrier; CHB, chronic hepatitis B; OR, odds ratio.
[a]Sub-heading of this table indicated (in **bold**) whether the disease was cirrhosis or HCC. Control indicates non-cirrhotic non-HCC control.

Ethnicity may represent the genetic constituent of an individual. According to a Centre for Disease Control (CDC) report in HCC cases between 2001-2006, the incidences of HCC for Asian, Caucasian, African and America Indian/Alaska Native

populations were 7.8, 2.6, 4.2 and 3.2 per 100,000 persons respectively. The age-adjusted incidence of HCC has been reported to be 18–35 per 100,000 in Asian men and 3–10 per 100,000 in European men [Fung and Lok, 2005]. Asian countries like China, Korea and Thailand have higher prevalence of HBV infection (2.6-12% versus 0.5-1.0%) and higher incidence of HCC (11.4-47.1% versus 1.4-4.2%) than Western countries like USA, UK, Canada and Germany. It is possible that each ethnic group may have a unique genetic trait that would affect the progression of liver diseases. For example, certain human leukocyte antigen (HLA) genes like HLA-A, HLA-B, HLA-DP and HLA-DRB are ethnic-dependent [Easteal et al., 1989; Miao et al., 2007]. Genetic polymorphisms in such gene regions have been reported to be associated with either persistence [Ramezani et al., 2008; Gao et al., 2011] or clearance of HBV infection [Ramezani et al., 2008; Cho et al., 2008]. Those who are likely to have longer duration of infection would have higher risk of cirrhosis and HCC and vice versa. Another example is that Caucasians seems to have a higher frequency of the mutated HFE gene that predisposes them to develop haemochromatosis, an iron overload disease that would progress to cirrhosis and HCC [McLaren and Gordeuk, 2009]. However, Asians generally have higher body iron status than Caucasians [Harris et al., 2007]. In addition, each ethnic group may have dietary and living habits that would impact on their disease pathogenesis. For example, in some parts of China, the peanut and corn consumed were often contaminated by fungal toxin due to the humid weather [Yu, 1992], whereas the East African Bantu population consumes food highly contaminated with iron metal [Senba et al., 1989]. In addition, people in some Asian countries, like India, Sri Lanka, Taiwan, Malaysia and Myanmar, practice chewing betel quid which increases liver cancer risk [Wen et al., 2010]. Therefore, certain ethnic groups are prone to the development of cirrhosis and HCC. In general, Asians are more likely to develop liver

complications than other ethnic groups, largely due to the higher prevalence of HBV infection compared to the West.

The annual incidence of cirrhosis and HCC in Malaysia is 2-10% and 1-6% respectively [Merican et al., 2000]. The Chinese have the highest incidence of HCC among all ethnic groups. In UMMC, the prevalence of cirrhosis was highest in HBV infection (46.1%), followed by HCV infection (18.5%), cryptogenic (15.4%), alcoholic (12.6%) and autoimmune (2.0%); HBV-related cirrhosis was predominant in Malay (47.9%) and Chinese (58.8%) compared to Indian (5.6%) [Qua and Goh, 2011]. Unfortunately, data on the association of Malaysian ethnic groups (especially Malay and Chinese) with HBV-related cirrhosis and HCC are lacking in the aforementioned study.

(b) Cigarette/Tobacco smoking

About 22% of the world's populations are smokers [WHO, 2011]. Cigarette smoke contains over 4000 chemicals that not only predispose smokers to lung cancer, but also other cancers as well [American Lung Association, 2010]. Nicotine, carbon monoxide, heavy metals (lead, arsenic, and cadmium) and polycyclic aromatic hydrocarbons are among the carcinogens found in cigarette smoke [National Toxicology Program, 2005]. The liver is the primary site for detoxification of such compounds. Chronic exposure to the compounds induces hepatic oxidative stress to the liver [Altamirano and Bataller, 2010], resulting in alterations in liver histology [Canales et al., 2012; Wong et al., 2012]. Initial evidence arose from two retrospective studies proposing that cigarette smoking may increase prevalence and severity of alcoholic [Klatsky and Armstrong, 1992] and HBV-related cirrhosis [Yu et al., 1997]. It has also been associated with progression to advanced liver fibrosis in fatty liver patients [Zein et al., 2012]. Nicotine, in particular, induces fibrogenic changes in human liver [Soeda et al., 2012]. In fact, cigarette smoking has been long reported to increase the risk of developing HCC;

smokers had about 2.1 times higher risk than non-smokers [Yu et al., 1991]. Many studies supported the risk of HCC in smoking with relative risk ranging from 1.5 to 9.6 [Zhu et al., 2007; Di Costanzo et al., 2008; Altamirano and Bataller, 2010]. A HBV cohort study observed no increased risk of HCC in cigarette smokers [Yang et al., 2008], but another two large sample-sized cross-sectional studies demonstrated about 5 times higher likelihood of HCC developing in smokers compared to non-smokers [Chen et al., 2005a] and showed prospectively higher risk of mortality from HCC in HBV-infected men only [Jee et al., 2004]. Smoking may also cause early onset of HCC [Wan et al., 2011]. Many HBV mutation studies have not included cigarette smoking as part of their risk evaluation in cirrhosis and HCC [To name a few: Yin et al., 2010; Cho et al., 2011; Kao et al., 2012]. It may be necessary to include cigarette or tobacco smoking to be included in future viral mutational studies as part of the confounding factors in chronic HBV infection.

(c) Family history of cirrhosis/HCC

The influence of genetic inheritance, lifestyles and dietary behaviours from parents to children may alter the clinical course of chronic HBV infection. Genetic variations in host genes, such as cytokine and DNA repair genes were shown to contribute to susceptibility of HBV-related HCC [Chen et al., 2005a]. Those having family members who practice smoking or consume contaminated food may also have higher risk of cirrhosis and HCC overall as discussed in previous sections. In addition, specific HBV strains capable of inducing cirrhosis or HCC, if they ever exist, may also be acquired through parental transmission. In fact, family history of HCC has been included in Asian and American clinical guidelines as an important risk factor for surveillance of HCC [Gonzalez and Keeffe, 2012]. Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (HBV-REVEAL) study involving

23,820 chronic hepatitis B participants found that even those with no alcohol consumption possessed 45% and 72% 10-year risk of getting cirrhosis and HCC respectively [Chen and Yang, 2011]. In a US study, those with first degree family history of liver cancer in either HBV or HCV infection had over 60-fold higher risk for HCC than those without [Hassan et al., 2009], whereas a study from Taiwan, involving 4471 HBsAg positive participants, reported a higher odds ratio of suffering from cirrhosis if the individuals had family history of HCC [Yu et al., 2002]. In contrast, another Taiwan study showed no association of familial HCC history between chronic HBV carriers and non-cirrhotic HCC patients [Liu et al., 2006]. According to the latest meta-analysis, based on nine case-control and four cohort studies, for a total of approximately 3,600 HCC cases, the combination of family history of liver cancer and hepatitis B/C virus infection is associated with an over 70-fold elevated HCC risk [Turati et al., 2012]. In addition, those with strong family history of HCC have been associated with early onset of HCC [Wan et al., 2011; Park et al., 2012a].

(d) Serum ALT

Clinically, serum ALT has been used as a marker to assess disease activity in the liver. Serum ALT has been known to fluctuate during the course of chronic HBV infection; HBeAg-negative patients, during immune clearance and viral reactivation, frequently present fluctuating patterns in ALT levels (given in Figure 4.2) [Hadziyannis and Papatheodoridis, 2006].

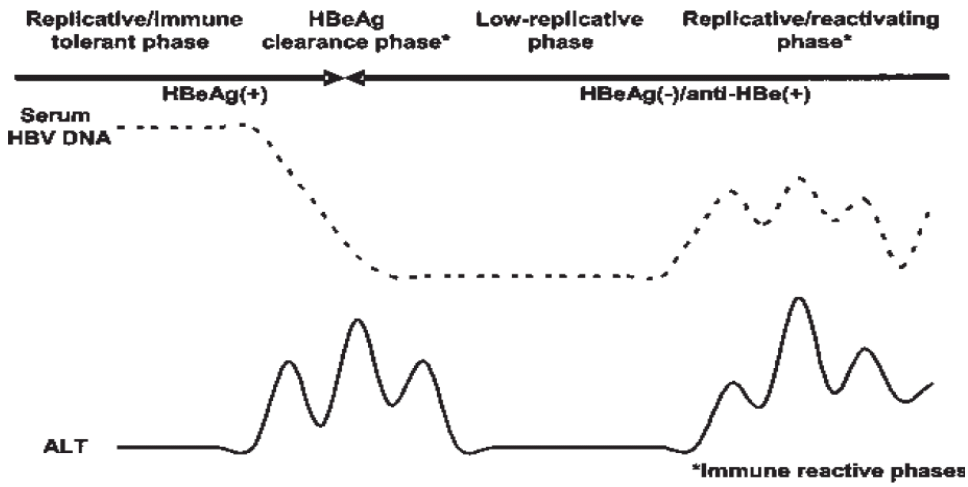


Figure 4.2 : Phases of chronic HBV infection. ALT is fluctuating during HBeAg clearance and viral reactivating phases [Hadziyannis and Papatheodoridis, 2006].
ALT, alanine aminotransferase; HBV, hepatitis B virus.

In a Taiwan HBV-REVEAL study, serum HBV DNA and ALT levels at both study entry and regular follow-up examinations were associated significantly with the risk of cirrhosis and HCC showing a dose-response relationship [Chen and Yang, 2011]. The current guidelines of the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) recommend initiation of antiviral treatment when ALT is elevated [Keeffe et al., 2008; EASL, 2009]. However, the reports on the relationship of serum ALT with cirrhosis and HCC were highly variable (Listed in Table 4.9). Surprisingly, quite a number of studies showed negative association of ALT with HCC. This may be due to the fact that lower serum ALT levels occur at a later stage of severe liver diseases as well as older age [Huo et al., 2006]. A recent large population histology study demonstrated that an elevated ALT did not accurately predict liver fibrosis in HBeAg-positive and –negative individuals [Seto et al., 2012]. Chronic hepatitis B patients with persistently normal ALT (PNALT) but high viral load were still at high risk of HCC [Nakazawa et al., 2011]. HBeAg-negative patients with ALT more than 0.5 times ULN were still at high

risk of cirrhosis [Wong et al., 2008]. Significant fibrosis and inflammation of liver was detected in 37% of patients with PNALT and thus the investigators suggested that a liver biopsy should be performed in patients older than 40 who had high normal ALT [Lai et al., 2007b]. Significant liver fibrosis was also found in 18% of patients with normal ALT in another study [Göbel et al., 2011]. A large cohort Chinese study showed an increased risk of liver complications in those with ALT of 0.5x-2.0x ULN but decreased risk in those of ALT >6x ULN [Yuen et al., 2005]. In brief, clinical application of high ALT level for the prediction of cirrhosis and HCC is difficult because the associations have been inconsistent.

Table 4.9 : ALT in studies of HBV-related cirrhosis and HCC.

Author (year)	Disease, control[a]		*P* value
	Sample size	Mean/Median ALT[b] or ALT cutoff (OR)	
Disease: cirrhosis			
Positive association			
Yin et al. (2010)	119, 603	≥45, <45 (7.6)	<0.001
Yin et al. (2011)	188, 235 (CHB)/846 (ASC)	≥45, <45 (2.9)	<0.001
Negative association			
Cho et al. (2011)	65, 60	89.9, 267.9	<0.001
Disease: HCC			
Positive association			
Tong et al. (2007)	101, 67	92.9, 19.9	<0.001
Asim et al. (2010)	150, 136	70.2, 51.8	0.001
Negative association			
Wang et al. (2007)	47, 132	63.9, 201.7	<0.001
Chen et al. (2008)	80, 160	84.2, 180.9	0.068
Cho et al. (2011)	69, 60	106.9, 267.9	<0.001
Jang et al. (2012)	75, 75	36.0, 45.0	0.028
No association			
Tanaka et al. (2006)	180, 148	≥50, <50 (1.8)	>0.050
Yin et al. (2010)	231, 603	≥45, <45 (NA)	>0.050

ALT, alanine aminotransferase; ASC, asymptomatic hepatitis B surface antigen carrier; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; NA, not available; OR, odds ratio.
[a]Sub-heading of this table indicated (in **bold**) whether the disease was cirrhosis or HCC. Control indicates non-cirrhotic non-HCC control.
[b]All ALT values were measured in U/L.

(e) Serum iron and serum ferritin

As mentioned in the previous section, male predominance in liver complications has been linked with sex hormonal difference, but this difference is not necessarily the only explanation. Dietary iron overload has been reported to be a risk factor for development of HCC [Mandishona et al., 1998]. Males are at higher risk of iron overload than females because women suffer monthly menstrual blood loss and men do not have a normal physiologic mechanism of significant iron excretion. In fact, iron excess has been associated with cirrhosis [Morrison et al., 2003; Clark et al., 2010] and HCC [Hellerbrand et al., 2003] because it accelerates hepatocyte proliferation [An et al., 2012] and induces hepatic DNA damage [Tanaka et al., 2008].

Body iron status is routinely assessed by serum iron and serum ferritin. The current most accurate method for quantification of iron in the liver is Magnetic Resonance Imaging (MRI) [Alústiza Echeverría et al., 2012]. However, this method is still undergoing universal standardisation. In a Poland chronic liver disease study, serum iron and serum ferritin levels exceeded the normal limits in 49% and 71% of liver complication cases respectively [Sikorska et al., 2003]. In a non-alcoholic-steatohepatitis-related cirrhosis study, iron deposits were more frequent in HCC patients than in controls [Sorrentino et al., 2009]. Another HCV-related study also showed a similar finding of iron deposits [Chapoutot et al., 2000]. The mean serum ferritin of cirrhosis cases (161-366 ng/mL for Child-Pugh stage A-C) was higher than that in hepatitis cases (68 ng/mL) and controls (80 ng/mL) but mean serum iron was lower in cirrhosis cases (72-89 μg/dL) than in hepatitis cases (120 μg/dL) and controls (110 μg/dL) [Büyükaşik et al., 2011].

The risk of HCC in iron overload patients is about 20-fold higher than in the general population [Dragani, 2010]. In a rat model study, deprivation of liver iron using an iron chelator suppresses the growth of HCC tissue through triggering cell cycle arrest

and apoptosis [Ba et al., 2011]. An earlier HBV study detected liver ferritin and stainable liver iron in more than 75% and 65% of 40 HCC patients respectively [Zhou et al., 1987]. HCC is almost always associated with iron overload and chronic viral infection in Myanmar; however, only 8 HBV-related HCC cases were studied – a rather small sample size [Win et al., 2000]. Since then, attention to iron has been fading in the field of HBV-related liver complications; much attention has been given to viral mutations solely. The importance of mild-to-moderate iron overload has been neglected in the disease progression of chronic hepatitis B. Perhaps, both viral mutations and iron status have roles to play in the pathogenesis of HBV-related cirrhosis and HCC. Hence, the main aim of the present study is to examine the influence of HBV mutations and serum iron markers in cirrhosis and HCC.

4.2 Identification of viral markers for cirrhosis

A total of 40 HBVs from the serum of 20 cirrhosis cases and 20 controls managed in the UMMC were successfully amplified, sequenced and assembled. These complete genome data were used for analysis. The selection of controls in this section was based on stringent control criteria mentioned previously in Chapter 2. Clinical and virological data of the participants are given in Table 4.10. There was no significant difference between cirrhosis cases and controls in age, gender, ethnicity, ALT, HBeAg positivity, viral load and viral genotype. By conducting comparative sequence analysis using SECentral Align Plus, six candidate nucleotides associated with cirrhosis were identified to have power more than 80%: one in the core region (G1896), two in the surface region (A87 and C2964), two in the polymerase region (A1359 and T2753) and one in the X region (C1799). Their corresponding codons are shown in Table 4.11. Three out of the six candidate nucleotides were in concordance with previous reports, one being associated positively (C2964) and two negatively (G1799 and A1896), as

shown in Table 4.12. Codon TGG (nt 1,895–1,897) gave the highest accuracy of 77.5% (31/40) followed by AAC (nt 86–88), AAA (nt 1,357–1,359), GTC (nt 1,797–1,799), CAT (nt 2,751–2,753), and RAC (nt 2,962–2,964), all with 75% accuracy (Table 4.11). A combination of codons ATG (nt 1,814–1,816) and TGG (nt 1,895–1,897) gave an even better accuracy of 80%. In contrast, accuracy was not improved by the combination of the other five codons in the surface, polymerase and X genes with their respective start codons. Hence, precore wild-type was the best viral marker for the identification of cirrhosis.

Table 4.10 : Comparison of clinical and virological characteristics between cirrhosis and control groups.

Characteristics	**Cirrhosis (n = 20)**	**Control (n = 20)**	***P* value[d]**
Age of visit, median year (range)	57 (35-78)	57.5 (50-70)	0.787
Gender (Male:Female)	10:10	5:15	0.191
Ethnicity (C:M:O)[a]	13:5:2	19:1:0	0.083
ALT	52.5 (22-140)	44 (32-130)	0.194
HBeAg positive:negative	5:15	1:19	0.182
Viral load[b]	17:3	12:8	0.155
Genotype C:B:A[c]	10:9:1	5:15:0	0.191

ALT, alanine aminotransferase; NA, not applicable;
[a]C, Chinese; M, Malay; O, Other ethnic groups; Other ethnic groups were not included for the calculation of statistical significance.
[b]Viral load >2,000 IU/ml: ≤2,000 IU/ml, which is equivalent to >10,000 copies/ml: ≤10,000 copies/ml.
[c]Including recombinants B/C grouped as genotype B and C/A grouped as genotype C; genotype A was not included in the calculation of statistical significance. Five B/C recombinants were found in control participants whereas 2 were in cases of cirrhosis. There was one C/A recombinant in each group.
[d]Mann-Whitney U test for continuous data and Chi-square exact test for categorical data.

Table 4.11 : Accuracy of specific codons for the identification of cirrhosis.

Codon (nucleotide position)	Gene	Cirrhosis n = 20 (%Sensitivity)	Control n = 20 (%Specificity)	Accuracy[a] (%)
TGG (nt 1895-1897)	*Core*	13 (65%)	2 (90%)	77.5
AAC (nt 86-88)	*Surface*	17 (85%)	7 (65%)	75.0
AAA (nt 1357-1359)	*Polymerase*	16 (80%)	6 (70%)	75.0
GTC (nt 1797-1799)	*X*	16 (80%)	6 (70%)	75.0
CAT (nt 2751-2753)	*Polymerase*	17 (85%)	7 (65%)	75.0
RAC (nt 2962-2964)	*Surface*	17 (85%)	7 (65%)	75.0
ATG (nt 1814-1816) … TGG (nt 1895-1897)	*Core*	13 (65%)	1 (95%)	80.0

[a]%Accuracy = (20 x %sensitivity + 20 x %specificity) / (20 + 20)

Table 4.12 : Association of reported HBV nucleotides with cirrhosis.

Nucleotide(s)[a]	Cirrhosis[d] n = 20 (%Sensitivity)	Control[d] n = 20 (%Specificity)	OR (95% CI, Phi)	*P* value[e]
M1386	15 (75)	19 (95)	0.16 (0.02-1.50, -0.280)	0.182
T1653	8 (40)	2 (90)	6.00 (1.08-33.27, 0.346)	0.065
A1726/Y1727	0 (100)	2 (90)	0.45 (0.038-5.392, -0.101)	0.481
C1753	5 (25)	4 (80)	1.33 (0.30-5.93, 0.060)	1.000
V1753	7 (35)	4 (80)	2.15 (0.52-9.00, 0.168)	0.480
T1762/A1764	13 (65)	6 (70)	4.33 (1.15-16.32, 0.350)	0.056
T1764/G1766	0 (0)	0 (100)	1.00 (0.058-17.12, 0.000)	1.000
C1766	2 (10)	1 (95)	2.11 (0.18-25.35, 0.095)	1.000
T1766/ A1768	1 (5)	0 (100)	1.1 (0.06-18.05, 0.006)	1.000
A1768	1 (5)	0 (100)	1.1 (0.06-18.05, 0.006)	1.000
G1799	4 (20)	14 (30)	0.11 (0.03-0.46, -0.503)	0.004
A1846	11 (55)	11 (45)	1.00 (0.288-3.476, 0.000)	1.000
T1858	14 (70)	19 (5)	0.12 (0.01-1.14, -0.329)	0.091
A1899	8 (40)	5 (75)	2.00 (0.52-7.72, 0.160)	0.501
C2964	17 (85)	7 (65)	10.52 (2.27-48.76, 0.510)	0.003
C3116	19 (95)	20 (0)	0.95 (0.06-16.29, -0.006)	1.000
Pre-S deletions[b]	1 (5)	1 (95)	1.00 (0.06-17.18, 0.000)	1.000
G1896	13 (65)	3 (85)	10.52 (2.27-48.76, 0.510)	0.003
G1896/G1897	13 (65)	2 (90)	16.71 (2.98-93.89, 0.568)	0.001
A1814/T1815[c]/ G1896/G1897	13 (65)	1 (95)	35.29 (3.87-321.93, 0.629)	<0.001

CI, confidence interval; HBV, hepatitis B virus; NA, not applicable; OR, odds ratio.
[a]M = A or C; V = A, C or G.
[b]Inclusive of pre-S1 and pre-S2 regions.
[c]These are the first 2 nucleotides of the precore start codon; the third nucleotide, G, is conserved in all participants.
[d]Value of zero was adjusted to 0.5 for calculation of odds ratio.
[e]Chi-square exact test.

4.3 Identification of viral markers for HCC

Complete HBV genomes from 45 chronic hepatitis B participants consisting of 21 HCC cases and 24 controls were included for analysis. The control in this section was based on stringent selection criteria mentioned previously in Chapter 2. Clinical and virological data of the participants are given in Table 4.13. Male gender, ALT, HBeAg positivity and viral genotype were associated significantly with HCC. There was no significant difference between cirrhosis cases and controls in age, ethnicity and viral load. Nucleotides G1896 and C1347 gave the highest accuracy (75.6%) for the identification of HCC, followed by T966, T1500 and C1799, each with an accuracy of 73.3%. Their corresponding codons are shown in Table 4.14. Two out of the five candidate nucleotides were in concordance with previous reports; these nucleotides were located at nucleotide position 1799 and 1896 respectively, as shown in Table 4.15. The corresponding codons were TGG (nt 1895-1897) with the highest accuracy of 77.8%, GTC (nt 1345-1347) with 75.6% accuracy, followed by GTC (nt 1797-1799), TCT (nt 1500-1502) and TMG (nt 966-968), all with 73.3% accuracy (Table 4.14). A combination of the TGG precore codon with its ATG start codon, designated as the precore wild-type (A1814/T1815/G1816 … T1895/G1896/G1897), gave an even better accuracy of 82.2%. In contrast, accuracy was not improved by the combination of the other four codons in the polymerase and X genes with their respective start codons. As in cirrhosis, precore wild-type was again the best viral marker for identification of HCC.

Table 4.13 : Comparison of clinical and virological characteristics between HCC and controls.

Characteristics	HCC (n = 21)	Control (n = 24)	*P* value[c]
Age of visit, median yr (range)	56 (30-74)	58 (50-69)	0.166
Gender (Male:Female)	17:4	6:18	<0.001
Ethnicity (Chinese:Malay)	15:6	22:2	0.121
ALT, median IU/L (range)	73 (28-305)	44 (26-130)	<0.001
HBeAg positive:negative	7:14	1:23	0.017
Viral load[a]	16:5	13:11	0.212
Genotype C:B[b]	13:8	6:18	0.017

ALT, alanine transaminase; HBeAg, hepatitis B early antigen; HCC, hepatocellular carcinoma.
[a] Viral load >2,000 IU/ml: ≤2,000 IU/ml, which is equivalent to >10,000 copies/ml: ≤10,000 copies/ml.
[b]Control: 6 B/C, 1 C/A, 1 C/B; HCC: 5 B/C, 3 C/A/D, 2 C/A, 1 C/A/B.
[c]Mann-Whitney U test for continuous data and Chi-square exact test for categorical data.

Table 4.14 : Accuracy of specific codons for the identification of HCC.

Codon (nucleotide position)	Gene	HCC, n = 21 (%sensitivity)	Control, n = 24 (%specificity)	Accuracy[a] (%)
TGG (nt 1895-1897)	*Core*	14 (66.7)	3 (87.5)	77.8
GTC (nt1345-1347)	*Polymerase*	17 (81.0)	7 (70.8)	75.6
TMG (nt 966-968)	*Polymerase*	15 (71.4)	6 (75.0)	73.3
TCT (nt 1500-1502)	*X*	16 (76.2)	7 (70.8)	73.3
GTC (nt 1797-1799)	*X*	16 (76.2)	7 (70.8)	73.3
ATG (nt 1814-1816) … TGG (nt 1895-1897)	*Core*	14 (66.7)	1 (4.2)	82.2

HCC, hepatocellular carcinoma
[a]%Accuracy = (21 x %sensitivity + 24 x %specificity)/(21+24)

Table 4.15 : Association of reported HBV nucleotides with HCC.

Nucleotide[a]	HCC[c] n = 21 (%Sensitivity)	Control[c] n = 24 (%Specificity)	OR (95% CI, Phi)	*P* value[d]
A7	3 (14)	3 (88)	1.17 (0.21-6.51, 0.026)	1.000
C53	4 (19)	4 (83)	1.18 (0.26-5.43, 0.031)	1.000
M1386	18 (86)	22 (8)	0.55 (0.08-3.63, -0.094)	0.652
T1485	4 (19)	0 (100)	5.64 (0.58-55.08, 0.241)	0.163
T1504/T1505	4 (19)	10 (58)	0.33 (0.09-1.28, -0.244)	0.121
A1613/T1653	3 (14)	0 (100)	4.00 (0.38-41.70, 0.182)	0.318
T1653	8 (38)	3 (88)	4.31 (0.97-19.24, 0.297)	0.081
A1689	21 (100)	24 (0)	0.88 (0.05-14.87, -0.013)	1.000
A1726/Y1727	0 (0)	2 (92)	0.52 (0.04-6.22, -0.077)	1.000
V1753	7 (33)	7 (71)	1.214 (0.343-4.298, 0.045)	1.000
T1762	14 (67)	8 (67)	4.000 (1.155-13.855, 0.333)	0.038
A1764	14 (67)	8 (67)	4.000 (1.155-13.855, 0.333)	0.038
T1762/A1764	14 (67)	6 (75)	8.000 (2.237-28.605, 0.470)	0.001
T1764/G1766	0 (0)	0 (100)	1.14 (0.07-19.42, 0.013)	1.000
T1846	11 (52)	12 (50)	1.10 (0.34-3.55, 0.024)	1.000
T1858	15 (71)	23 (4)	0.109 (0.012-0.995, -0.336)	0.039
A1899	6 (29)	7 (71)	0.97 (0.27-3.54, -0.007)	1.000
G1914	0 (0)	0 (100)	1.14 (0.07-19.42, 0.013)	1.000
C2189	6 (29)	6 (75)	1.20 (0.32-4.51, 0.040)	1.000
W2203	0 (0)	2 (92)	0.52 (0.04-6.22, -0.077)	1.000
A2964	5 (24)	15 (63)	0.19 (0.05-0.69, -0.388)	0.016
T3116	0 (0)	0 (100)	1.14 (0.07-19.42, 0.013)	1.000
Pre-S deletions[b]	4 (19)	1 (96)	5.41 (0.55-52.87, 0.236)	0.169
Pre-S2 aa1-6 deletion	2 (10)	0 (100)	2.53 (0.21-30.01, 0.111)	0.585
Pre-S2 start codon mutation	7 (33)	2 (92)	4.42 (0.82-23.79, 0.254)	0.140
G1896	14 (67)	4 (17)	10.000 (2.542-40.778, 0.509)	0.001
G1896/G1897	14 (67)	3 (13)	14.000 (3.086-63.510, 0.557)	<0.001
A1814/T1815/ G1816 … T1895/G1896/ G1897	14 (67)	1 (4)	46.000 (5.107-414.363, 0.661)	<0.001

aa, amino acid; CI, confidence interval; HCC, hepatocellular carcinoma; OR, odds ratio.
[a]M = A or C; V = A, C or G; W = A or T.
[b]Inclusive of pre-S1 and pre-S2 regions.
[c]Value of zero was adjusted to 0.5 for calculation of odds ratio.
[d]Chi-square exact test.

4.4 Clinical and virological characteristics in chronic hepatitis B participants

Due to the high accuracy of precore wild-type (A1814/T1815/G1816 … T1895/G1896/G1897) for the identification of cirrhosis and HCC cases, the precore marker was further used to screen for larger chronic hepatitis B populations at the UMMC. A total of 333 chronic hepatitis B participants were recruited, including 216 general controls (without clinical evidence of cirrhosis or HCC), 78 with cirrhosis and 39 with HCC. The clinical and virological data of these chronic hepatitis B participants

in HCC and cirrhosis were given in Table 4.16 and 4.17 respectively (Raw data are tabulated in Appendix A). Older age and male predominance was found in HCC and cirrhosis cases when compared to general controls. Ethnicity did not seem to be important in determining clinical outcome. In contrast, smoking increased risk of HCC but not cirrhosis when compared to general controls. Family history of cirrhosis/HCC was not a significant risk factor for all groups. ALT was significantly higher in all diseased groups with respect to general controls. AFP was significantly high in cirrhosis and HCC. The HCC participants have relatively higher frequency of HBeAg positivity than that of other groups. Overall, by comparing with the general control group, serum iron was somewhat significantly lower in HCC but higher in cirrhosis, whereas serum ferritin was significantly higher in all diseased groups.

Table 4.16 : Comparison of clinical and virological characteristics in HCC.

Characteristics[a]	**General control N = 216**	**HCC N = 39**	***P* value**[d]
Age, year	50 (18-78)	57 (30-74)	<0.001
Gender (male:female)	93:123	29:10	<0.001
Ethnicity (C:M:O)[b]	171:41:4	26:11:2	0.149
Smoker:Non-smoker	21:195	16:23	<0.001
Family history of HBV infection (Y:N:U)[c]	139:49:28	23:7:9	0.249
Family history of cirrhosis/HCC (Y:N:U)[c]	60:137:19	14:18:7	0.081
ALT, IU/L	42 (10-403)	73 (26-305)	<0.001
AFP, IU/mL	2.0 (<0.5-32.0)	379.0 (2.0-546300.0)	<0.001
HBeAg positive:negative	45:171	15:24	0.023
Precore wild-type:mutant:mixed	64:138:14	21:15:3	0.012
Serum iron	17.30 (1.70-44.68)	14.30 (2.31-49.10)	0.011
Serum ferritin	146.0 (3.8-1192.0)	422.2 (42.0-3383.0)	<0.001

ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.
[a]Continuous variables were expressed in median (range).
[b]C, Chinese; M, Malay; O, other ethnicity.
[c]Y, yes; N, no; U, unknown.
[d]Mann-Whitney U test for continuous data and Chi-square exact test for categorical data.

Table 4.17 : Comparison of clinical and virological characteristics in cirrhosis.

Characteristics[a]	General control N = 216	Cirrhosis N = 78	*P* value[d]
Age, year	50 (18-78)	57 (32-80)	<0.001
Gender (male:female)	93:123	48:30	0.006
Ethnicity (C:M:O)[b]	171:41:4	59:16:3	0.562
Smoker:Non-smoker	21:195	13:65	0.100
Family history of HBV infection (Y:N:U)[c]	139:49:28	44:23:11	0.435
Family history of cirrhosis/HCC (Y:N:U)[c]	60:137:19	22:48:8	0.918
ALT, IU/L	42 (10-403)	51 (22-1090)	<0.001
AFP, IU/mL	2.0 (<0.5-32.0)	4.0 (<0.5-69.0)	<0.001
HBeAg positive:negative	45:171	23:55	0.158
Precore wild-type:mutant:mixed	64:138:14	33:40:5	0.124
Serum iron (μmol/L)	17.30 (1.70-44.68)	20.35 (4.93-48.22)	<0.001
Serum ferritin (μg/L)	146.0 (3.8-1192.0)	184.6 (8.9-1842.0)	0.040

ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.
[a]Continuous variables were expressed in median (range).
[b]C, Chinese; M, Malay; O, other ethnicity.
[c]Y, yes; N, no; U, unknown.
[d]Mann-Whitney U test for continuous data and Chi-square exact test for categorical data.

4.5 Prevalence of precore mutations in chronic hepatitis B participants

Generally, 35.4% precore wild-type, 58.0% precore mutations and 6.6% both were detected in this study. Precore mutations were further divided into precore start codon and stop codon mutations. Precore start codon mutation was rarely present, being 7.8%, inclusive of mixed infection. CTG, TTG and ACG were the predominant precore start codon mutations (See Table 4.18). Precore stop codon mutation was the major precore mutation (See Table 4.19). TAG was the predominant precore stop codon mutation (54.7%) whereas the minor mutation was TGA (0.6%).

Table 4.18 : Frequency of precore start codon variants (nt 1814-1816).

nt 1814-1816[a]	Frequency N = 333	Percentage
Wild-type		
ATG	307	92.2
Mutant		
CTG	5	1.5
TTG	5	1.5
ACG	3	0.9
AAG	1	0.3
AGG	1	0.3
ATA	1	0.3
ATT	1	0.3
Mixed[b]		
AYG	5	1.5
MTG	2	0.6
AYS	1	0.3
WTG	1	0.3

nt, nucleotide;
[a]Mixed indicates mixed infection of multiple HBV precore strains.
[b]Y = C or T; M = A or C; W = A or T.

Table 4.19 : Frequency of precore stop codon variants (nt 1895-1897).

Nt 1895-1897[a]	Frequency N = 333	Percentage
Wild-type		
TGG	130	39.0
Mutant		
TAG	180	54.1
TGA	2	0.6
Mixed[b]		
TRG	20	6.0
TGR	1	0.3

nt, nucleotide.
[a]Mixed indicates mixed infection of multiple HBV precore strains.
[b]R= A or G

4.5.1 Precore mutations of stringent control and diseased groups

To recap, stringent control was applied based on family history of HBV infection, age of above 49 years and normal ultrasound findings. A total of 111 stringent control participants were available for analysis. The distribution of stringent control, NAFLD, cirrhosis and HCC is depicted in Figure 4.3. Stringent control has at least 3-fold lower frequency of precore wild-type compared to precore mutant, whereas the HCC group has at least 1.5-fold higher frequency of precore wild-type. Both cirrhosis and NAFLD

groups have ratios of precore wild-type-to-mutant near to one. Based on odds ratio and Phi value, precore wild-type has the strongest relationship with HCC (OR = 5.50, 95% CI 2.51-12.05; Phi = 0.365), followed by cirrhosis (OR = 3.27, 95% CI 1.74-6.13; Phi = 0.274).

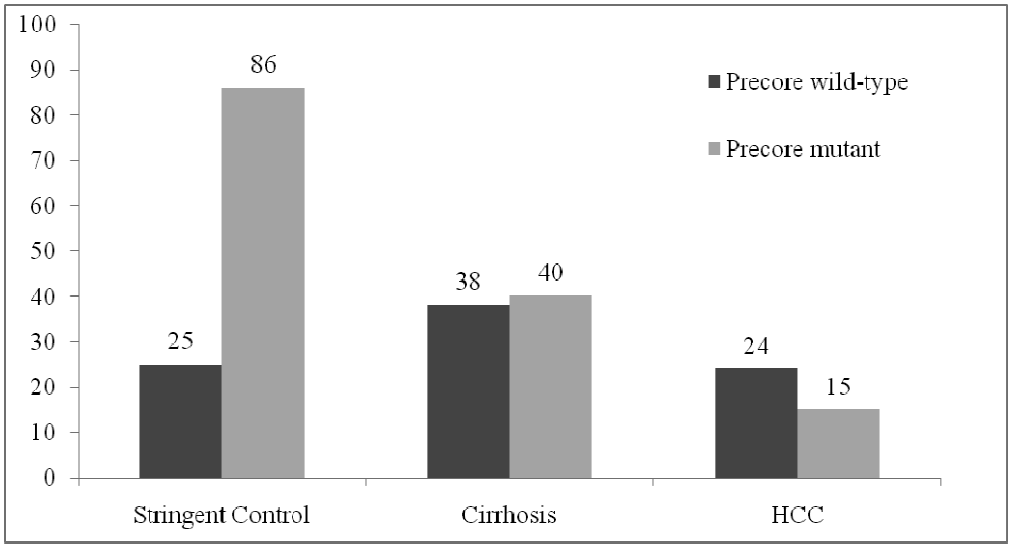
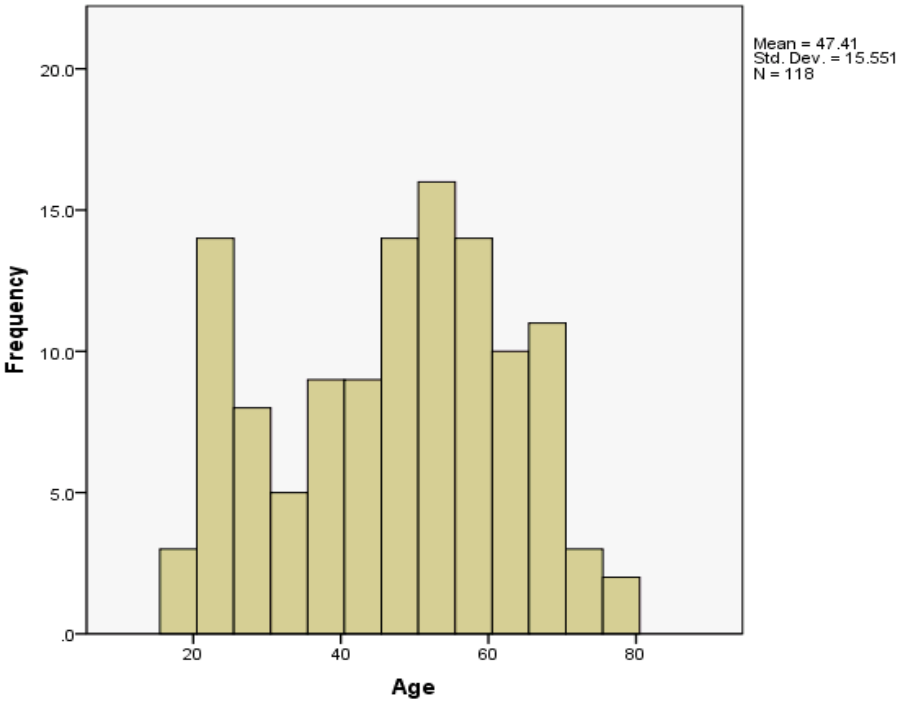


Figure 4.3 : HBV precore in stringent control, cirrhosis and HCC groups.
HCC versus stringent control, $P < 0.001$; cirrhosis versus stringent control, $P < 0.001$.
HCC, hepatocellular carcinoma.

4.5.2 Age-dependency of precore mutations

Mean ages of precore wild-type and mutant were 47.4 and 52.9 years respectively. Participants with precore mutations were significantly older that those with precore wild-type ($P = 0.001$; Mann-Whitney U test). Distribution of age in precore wild-type and mutant are shown in Figure 4.4.

(A) Precore wild-type



(B) Precore mutant

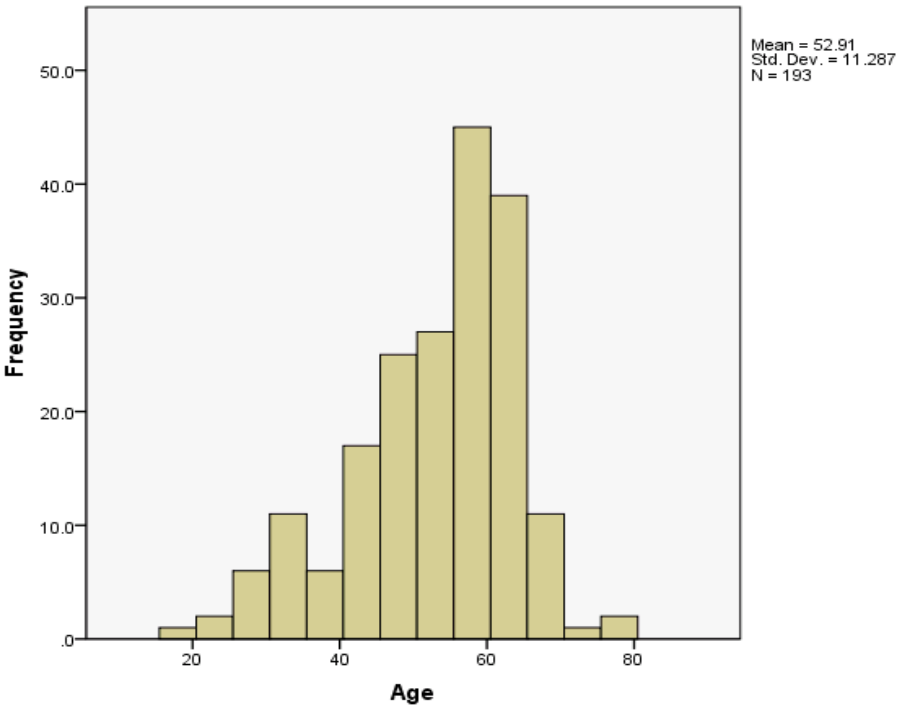


Figure 4.4 : Histogram of age in (A) precore wild-type and (B) precore mutant.
The distributions of both (A) and (B) were near to normal with the curve slightly skewed to left. The mean age of precore mutant was higher than precore wild-type (P = 0.001).

4.5.3 Precore mutations and HBeAg positivity

The Chi-square exact test was performed to examine the effects of precore mutations (inclusive of start and stop codon only) on HBeAg expression (given in Table 4.20). There was a significant difference in the distribution of precore mutations in HBeAg positivity ($P < 0.001$). Precore wild-type was more or less equal in HBeAg-positive and –negative chronic hepatitis B participants, whereas precore mutations were almost completely absent in the HBeAg-positive participants. These findings supported the facts that (1) the precore mutations abrogate HBeAg expression [Buti et al., 2005], and (2) expression of HBeAg can be affected by other mutations, such as BCP mutations [Jammeh et al., 2008].

Table 4.20 : Comparison of precore wild-type and mutant with HBeAg positivity

Precore[a]	HBeAg (%)	
	Positive	Negative
Wild-type, N = 118	51 (43.2)	67 (56.8)
Mutant, N = 193	5 (2.6)	188 (97.4)
Mixed, N = 22	11 (50.0)	11 (50.0)

HBeAg, hepatitis B e antigen.
Note: Chi-square exact test, $P < 0.001$.
[a]Precore wild-type means ATG (nt 1814-1816) … TGG (nt 1895-1897); Mixed indicates mixed infection of precore wild-type and mutant.

4.5.4 Familial transmission of precore mutations

Those with family history of HCC may be at higher risk of cirrhosis and HCC. Precore wild-type appeared to be the most likely the HBV hepatocarcinogenic strain in this study. However, whether the carcinogenic strain of HBV found is ‘inheritable’ has not been shown. This is important because it could explain why those with family history of HCC were at higher risk of severe liver complications. This study investigated the familial transmission of HBV precore strains in first degree relatives. Three families

were available for analysis. The precore data are given in Table 4.21. From family1 and family2, the data indicated that precore wild-type was likely to be 'inheritable' from the mother; four out of 5 siblings (80%) were infected with the same viral strain from their mothers. Yet, the quasispecies nature of HBV infection was observed in the mothers of family2 and family3. In family2, the two siblings were infected by precore wild-type and mutant respectively. The siblings in family3 were infected by HBV of two different precore mutations, one being stop codon mutation and another one start codon mutation. The transmission of precore variants among family members was heterogeneous. This showed that the viral strain was possibly, but not absolutely, 'inheritable'.

Table 4.21 : Familial transmission of HBV precore variants.

Label	Precore[a]		Age, year	Gender[b]	Relationship
	Start codon	Stop codon			
Family1					
HB471	ATG	TGG	30	M	Son
HB472	ATG	TGG	59	F	Mother
HB473	ATG	TGG	25	F	Daughter
HB525	ATG	TGG	36	M	Son
Family2					
HB483	ATG	TGR	51	F	Mother
HB484	AYG	TAG	30	M	Son
HB485	ATG	TGG	26	F	Daughter
Family3					
HB213	ATG	TAG	65	M	Brother
HB506	ATA	TGG	61	F	Sister

[a]R = A or G; Y = C or T.
[b]M indicates male, F indicates female.

4.6 Serum iron markers in chronic hepatitis B participants

Median values of serum iron and serum ferritin are shown in Table 4.16 and 4.17 respectively in the previous section. This section compared body iron status of the stringent control group with various diseased groups, using ULNs of serum iron and serum ferritin. A 2x2 cross-tabulation of Chi-square test was performed. When comparing with the stringent control, high serum iron level showed a significant relationship with the cirrhosis group (OR = 3.80, 95% CI 1.73-8.38; Phi = 0.251; *P* = 0.001) but not with other diseased groups (*P* > 0.05; shown in Table 4.5). For the same comparison, high serum ferritin showed a significant relationship with HCC group (OR = 6.47, 95% CI 2.92-14.35; Phi = 0.397) but not with other diseased groups (see Figure 4.6).

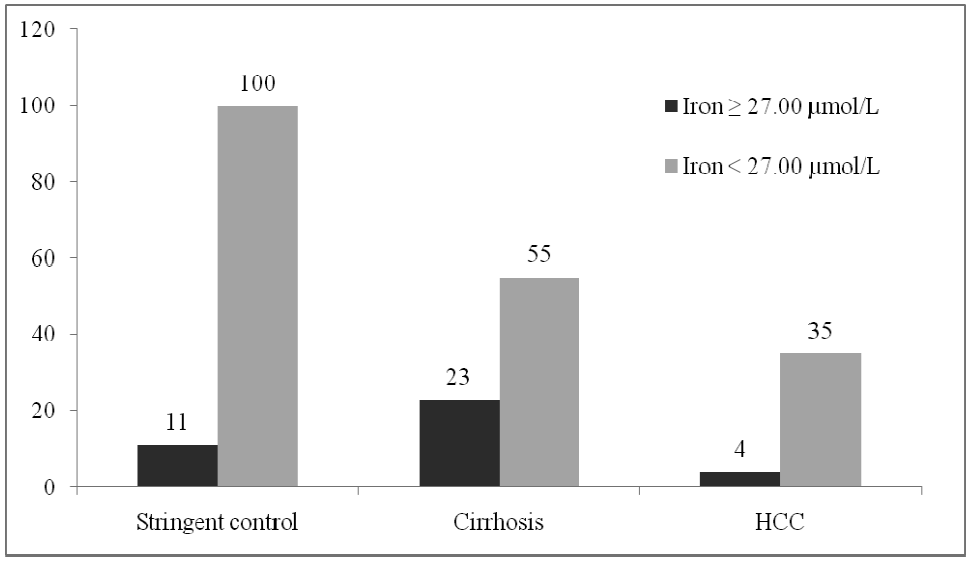


Figure 4.5 : Serum iron in stringent control, cirrhosis and HCC groups.
HCC versus stringent control, *P* = 1.000; cirrhosis versus stringent control, *P* = 0.001.
HCC, hepatocellular carcinoma; ULN, upper limit of normal.

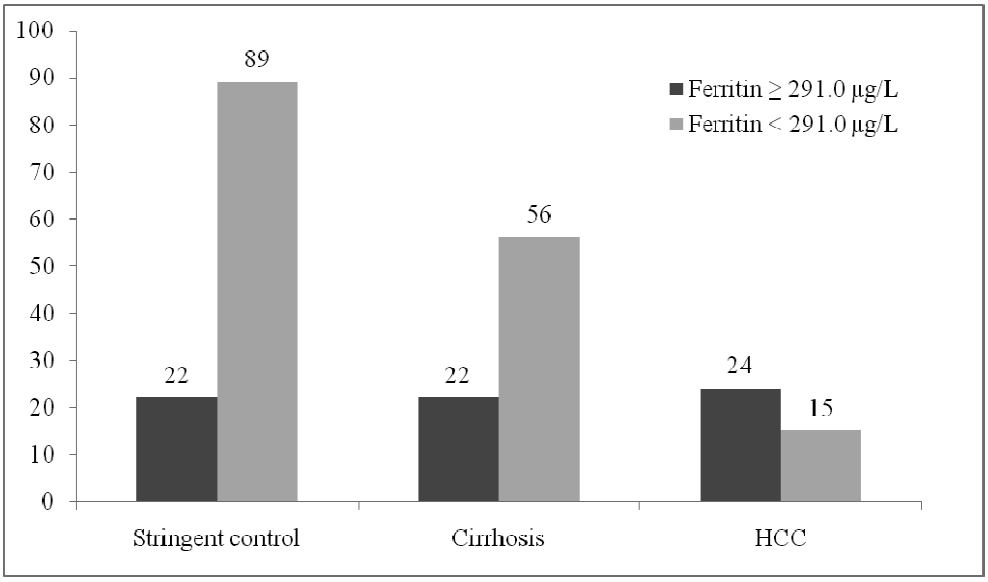


Figure 4.6 : Serum ferritin in stringent control, cirrhosis and HCC groups.
HCC versus stringent control, $P < 0.001$; cirrhosis versus stringent control, $P = 0.221$.
HCC, hepatocellular carcinoma; ULN, upper limit of normal.

4.6.1 Receiver operating characteristic (ROC) curve analysis

Clinical characteristics, such as age, serum ALT, serum iron and serum ferritin, were included for ROC curve analyses. The quality of a potentially predictive clinical parameter was judged by area under curve (AUC); a higher AUC value indicates the better a marker for identification of a disease.

(a) HCC

This section has included 39 HCC cases and 216 general controls for ROC curve analysis. Serum AFP, which is part of the diagnosis for HCC, was included for reference only. As shown in Figure 4.7, serum ferritin has the highest AUC value for identification of HCC (0.806; $P < 0.001$) when compared with serum ALT (0.771; $P < 0.001$), age (0.689; $P < 0.001$) and serum iron (-0.373; $P = 0.011$). Except for serum iron, all were positive markers for HCC. At the cutoff of 274.5 µg/L, serum ferritin has the highest accuracy for identification of HCC; the sensitivity was 71.8% and the

specificity was 81.0%. This cutoff is slightly lower than that used as the upper limit of normal reference range in UMMC (ULN = 291.0 μg/L). Meanwhile, serum ALT at an optimum cutoff of 54.5 U/L produced sensitivity and specificity of 71.8% and 77.3% respectively. This cutoff is much lower than that used as the upper limit of normal reference range by UMMC (ULN = 65 U/L). Age at cutoff of 56.5 years was most potentially predictive for HCC with sensitivity of 56.4% and specificity of 71.3%. Serum iron, which was determined to be a negative marker for HCC, had sensitivity of 55.1% and specificity of 66.7% at the optimum cutoff of 16.54 μmol/L. As most of the HCC participants had serum iron level under ULN of 27 μmol/L, the interpretation for this cutoff would be that those over 16.54 μmol/L but lower than 27.00 μmol/L had a decreased risk of HCC, whereas those below 16.54 μmol/L had an increased risk of HCC. For comparison with logistic regression model later (which was highly specific for HCC), the sensitivity was recalculated with respect to about 97% specificity. The data are given in Table 4.22. When adjusted to such high specificity, the sensitivity of all serum markers was less than 31%.

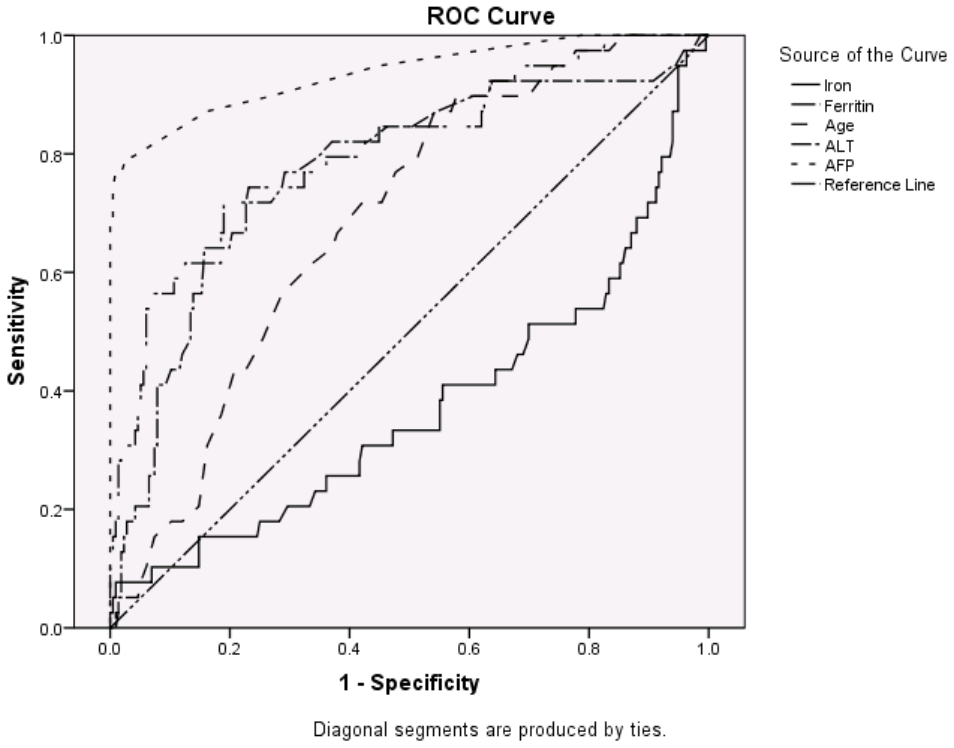


Figure 4.7 : ROC curves of age, serum ALT, serum AFP, serum iron, serum ferritin for identification of HCC.
AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic.

Table 4.22 : Sensitivity of age and serum markers for identification of HCC.

Characteristics	%Sensitivity	%Specificity
Age	5.1	97.2
Serum ALT	17.9	96.8
Serum iron	7.7	96.8
Serum ferritin	30.8	96.8

ALT, alanine aminotransferase; HCC, hepatocellular carcinoma.

(b) Cirrhosis

This section has included 78 cirrhotic cases and 216 general controls for ROC curve analysis. Serum AFP was included as a reference only. As shown in Figure 4.8, age has the highest AUC value for detecting cirrhosis (0.680; $P < 0.001$) when compared with serum iron (0.645; $P < 0.001$), serum ALT (0.644; $P < 0.001$) and serum ferritin (0.578; $P = 0.040$). Using the cutoff of 56.5 years, age had the highest accuracy for identification of cirrhosis; the sensitivity was 56.4% and the specificity was 71.3%. At cutoff of 22.82 μmol/L, serum iron had the highest potential for predicting cirrhosis with sensitivity of 43.6% and specificity of 81.9%. Serum ALT at an optimum cutoff of 53.5 U/L produced sensitivity of 46.2% and specificity of 75.5%. Lastly, serum ferritin at best cutoff of 198.9 μg/L had sensitivity of 47.4% and specificity of 65.3%. For comparison with logistic regression model later (which was highly specific for cirrhosis), the sensitivity was recalculated with respect to about 92% specificity. The data are given in Table 4.23. When adjusted to such high specificity, the sensitivity of all serum markers was less than 29%.

Table 4.23 : Sensitivity of age and serum markers for identification of cirrhosis.

Characteristics	%Sensitivity	%Specificity
Age	12.8	92.6
Serum ALT	6.4	92.1
Serum iron	28.2	92.1
Serum ferritin	16.7	92.1

ALT, alanine aminotransferase.

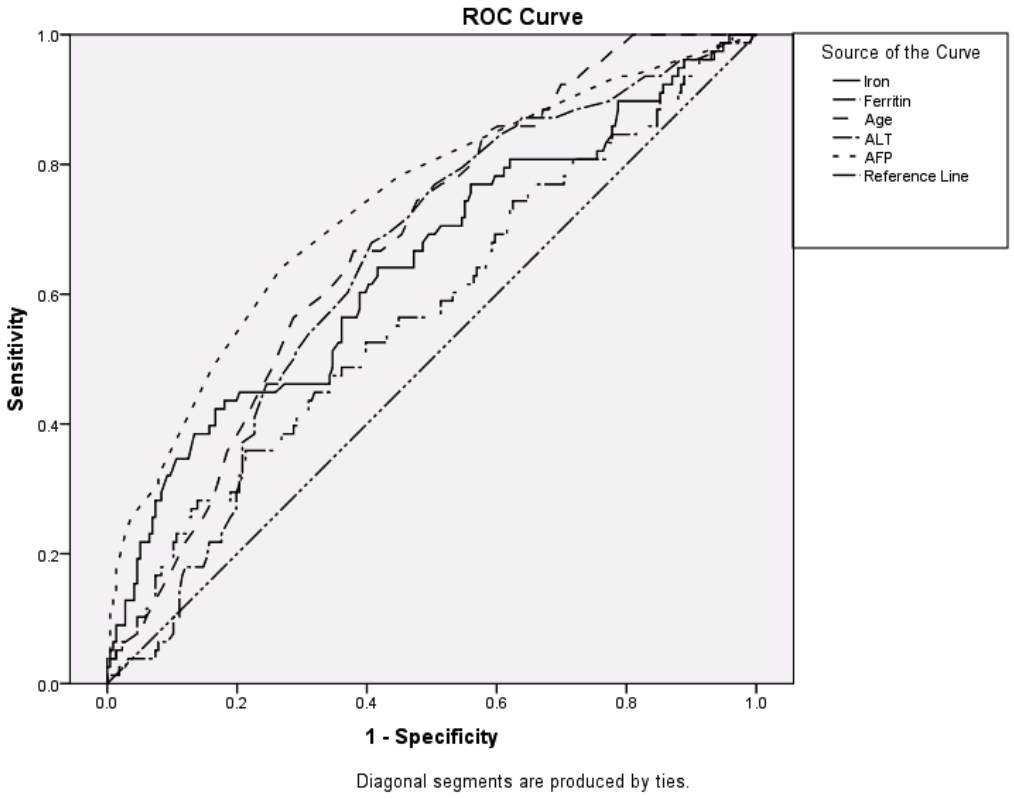
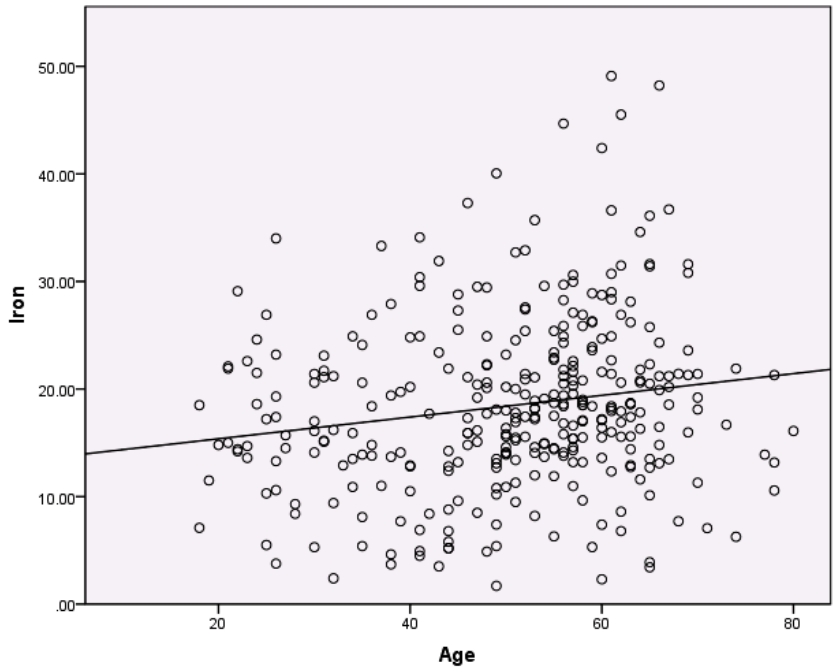


Figure 4.8 : ROC curves of age, serum ALT, serum AFP, serum iron, serum ferritin for identification of cirrhosis.
AFP, alpha-fetoprotein; ALT, alanine aminotransferase; ROC, receiver operating characteristic.

4.6.2 Correlation with age and ALT

Scatter-plots of serum iron and ferritin with age and serum ALT are given in Figure 4.9 and 4.10. Age was weakly associated with serum ferritin (rho = 0.343; $P < 0.001$), and serum iron (rho = 0.176; $P = 0.001$). Serum ALT was weakly associated with serum ferritin (rho = 0.341; $P < 0.001$) and trivially with serum iron was observed (rho = 0.139; P = 0.011).

(A)



(B)

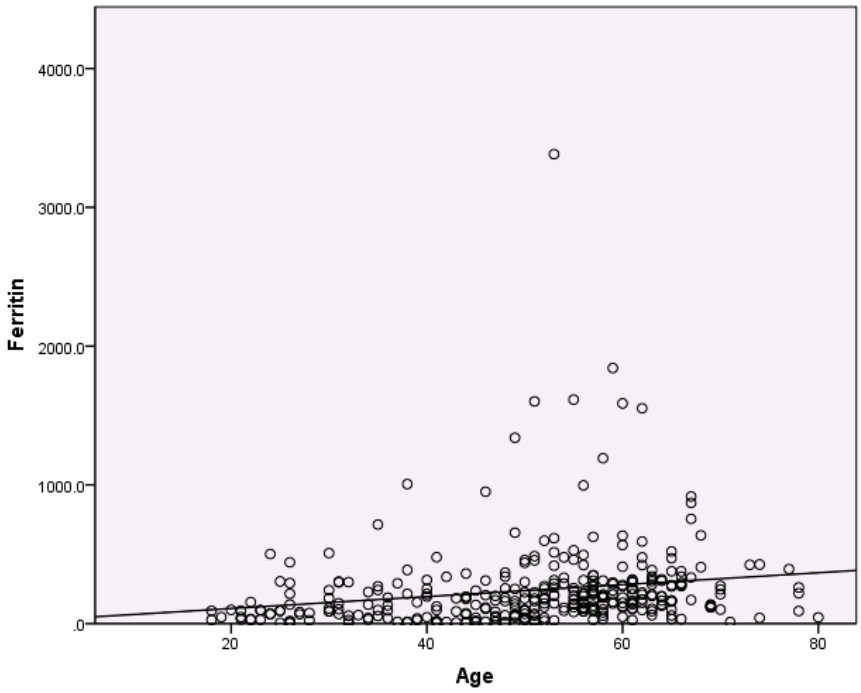
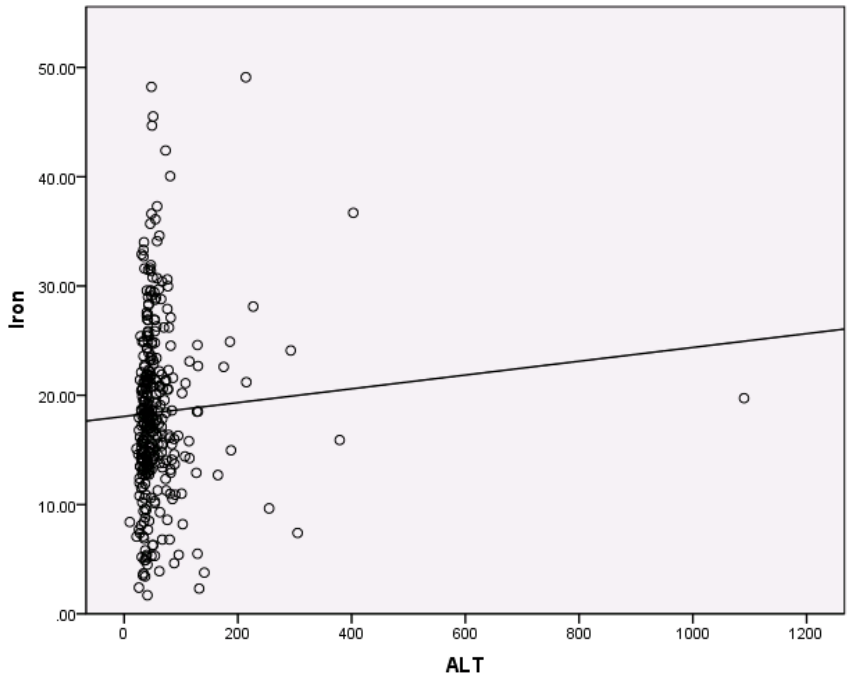


Figure 4.9 : Scatter-plot of (A) serum iron and (B) serum ferritin with age.

(A)



(B)

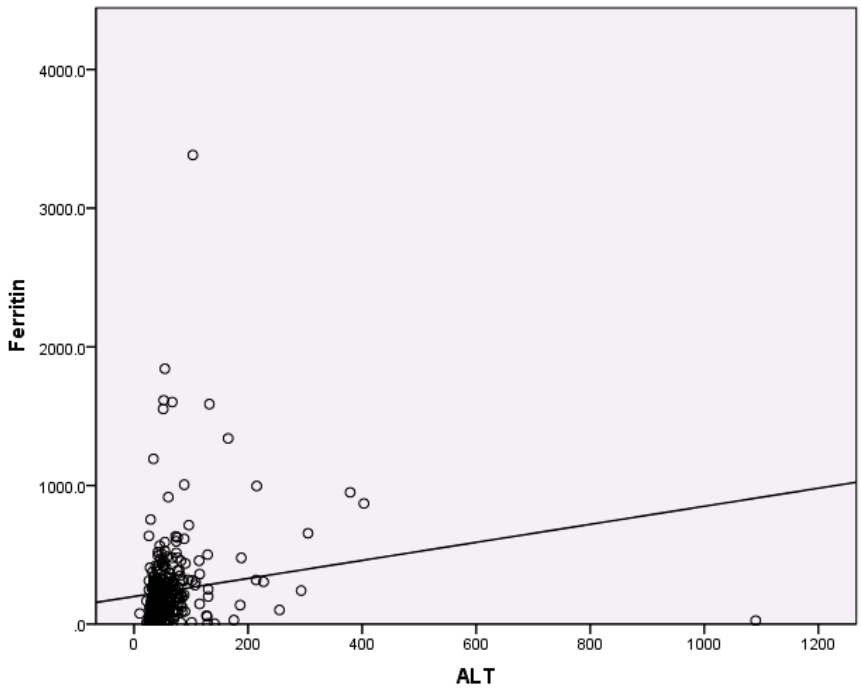


Figure 4.10 : Scatter-plot of (A) serum iron and (B) serum ferritin with ALT. ALT, alanine aminotransferase.

4.6.3 Association with gender, ethnicity and smoking

Serum iron of male patients (19.25 μmol/L; range 2.31-49.10 μmol/L) was significantly higher than that of female patients (15.90 μmol/L; range 1.70-44.68 μmol/L; $P < 0.001$). Similar significant result was also found in serum ferritin, with male having 238.6 μg/L (range 10.2-3383.0 μg/L) and female 102.2 μg/L (range 3.8-1601.8 μg/L; $P < 0.001$). Chinese ethnicity (18.20 μmol/L; range 1.70-49.10 μmol/L), was significantly associated with higher serum iron than non-Chinese (predominantly Malay; 15.05 μmol/L; range 2.31-37.29 μmol/L; $P = 0.008$). Similarly, Chinese had significantly higher serum ferritin level (187.9 μg/L, range 6.4-3383.0 μg/L) compared to non-Chinese 112.4 μg/L (range 3.8-1587.0 μg/L) ($P = 0.008$). Smokers (264.6 μg/L; range 25.7-3383 μg/L) had higher serum ferritin than non-smokers (155.2 μg/L; range 3.8-1842 μg/L; $P < 0.001$). This difference was not observed with serum iron ($P = 0.099$).

4.7 Potential independent predictors for cirrhosis and HCC

To investigate further whether the precore wild-type and serum iron markers were independently associated with cirrhosis and HCC, forward stepwise conditional binary logistic regression analyses were performed by comparing HCC (N = 39) and cirrhosis (N = 78) groups with general control group (N = 216) respectively. All clinical and virological factors were taken into consideration for the modeling. In initial steps of the modeling, only relevant factors were included step-by-step for analysis. At classification cutoff of 0.5, the accuracy, sensitivity and specificity of this prediction model for HCC were 89.0%, 46.2% and 96.8% respectively and those for cirrhosis were 76.5%, 33.3% and 92.1% respectively. The predictive values of each factor for HCC and cirrhosis are shown in Table 4.24 and 4.25 respectively. Backward stepwise modeling was also performed and the same results were generated. In initial steps of the modeling, irrelevant factors were excluded step-by-step for analysis.

According to the regression model, precore wild-type, high serum ferritin, older age, smoker and positive family history of cirrhosis/HCC were associated independently with HCC. Intriguingly, serum iron was an independent negative predictive factor for HCC, whereas precore wild-type, older age, male gender, non-Chinese ethnicity and high serum ALT were associated independently with cirrhosis. In contrast to the HCC model, serum iron was a positive predictive factor of cirrhosis. Unlike HCC, high serum ferritin, cigarette smoking and positive family history of cirrhosis/HCC did not seem to be a risk factor of cirrhosis. Precore wild-type and older age were the most robust predictive factor for cirrhosis and HCC.

Table 4.24 : Logistic regression analysis of clinical and virological variables with independent predictive value for HCC in 39 HCC participants and 216 general controls.

Factor	B score	AOR (95% CI)	*P* value
Age, year	0.076		0.001
1		1.08 (1.03-1.13)	
10		2.14 (1.37-3.36)	
18 (Maturation)		3.95 (1.76-8.87)	
20		4.60 (1.87-11.30)	
30		9.87 (2.56-38.00)	
40		21.17 (3.51-127.74)	
50		44.70 (4.83-429.45)	
Smoker	1.387		0.012
No		1.00 (referent)	
Yes		4.00 (1.36-11.75)	
Family history of cirrhosis/HCC			0.011
No		1.00 (referent)	
Yes	1.567	4.79 (1.66-13.82)	0.004
Unknown	1.360	3.90 (0.85-17.76)	0.079
Precore wild-type	1.437		0.002
Absence		1.00 (referent)	
Presence		4.21 (1.68-10.57)	
Serum iron (μmol/L)	-0.089		0.005
1.00		0.92 (0.86-0.97)	
10.00		0.41 (0.22-0.77)	
20.00		0.17 (0.05-0.59)	
27.00 (ULN)		0.09 (0.02-0.49)	
30.00		0.07 (0.01-0.45)	
40.00		0.03 (0.00-0.34)	
50.00		0.01 (0.00-0.26)	
Serum ferritin (μg/L)	0.005		<0.001
1.0		1.00 (1.00-1.01)	
50.0		1.27 (1.15-1.40)	
100.0		1.61 (1.32-1.96)	
150.0		2.03 (1.51-2.74)	
200.0		2.58 (1.73-3.83)	
250.0		3.27 (1.99-5.36)	
291.0 (ULN)		3.97 (2.23-7.07)	
300.0		4.14 (2.28-7.51)	
400.0		6.64 (3.00-14.70)	
500.0		10.67 (3.95-28.77)	

ALT, alanine aminotransferase; AOR, adjusted odds ratio; CI, confidence interval; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; ULN, upper limit of normal.
Note that gender, ethnicity, family history of HBV infection, ALT and HBeAg were not the independent predictive factors for HCC according to the multivariate analysis.

Table 4.25 : Logistic regression analysis of clinical and virological variables with independent predictive value for cirrhosis in 78 cirrhosis participants and 216 general controls.

Factor	B score	AOR (95% CI)	*P* value
Age, year	0.081		<0.001
1		1.08 (1.05-1.12)	
10		2.25 (1.68-3.00)	
18 (Maturation)		4.30 (2.55-7.24)	
20		5.05 (2.83-9.02)	
30		11.36 (4.76-27.11)	
40		25.53 (8.00-81.43)	
50		57.39 (13.46-244.62)	
Gender	0.618		0.045
Female		1.00 (referent)	
Male		1.85 (1.01-3.40)	
Ethnicity	1.208		0.002
Chinese		1.00 (referent)	
Non-Chinese[a]		3.35 (1.54-7.28)	
Precore wild-type	1.211		<0.001
Absence		1.00 (referent)	
Presence		3.36 (1.78-6.34)	
Serum iron (μmol/L)	0.067		0.001
1.00		1.07 (1.03-1.11)	
10.00		1.96 (1.31-2.94)	
20.00		3.85 (1.71-8.63)	
27.00 (ULN)		6.16 (2.07-18.36)	
30.00		7.54 (2.24-25.37)	
40.00		14.80 (2.94-74.53)	
50.00		29.02 (3.84-218.99)	
Serum ALT (U/L)	0.004		0.039
1.0		1.00 (1.00-1.01)	
10.0		1.04 (1.00-1.08)	
20.0		1.08 (1.00-1.16)	
30.0		1.12 (1.01-1.25)	
40.0		1.17 (1.01-1.35)	
50.0		1.21 (1.01-1.45)	
60.0		1.26 (1.01-1.57)	
65.0 (ULN)		1.28 (1.01-1.63)	
130.0 (2x ULN)		1.65 (1.03-2.64)	
325.0 (5x ULN)		3.48 (1.07-11.36)	

ALT, alanine aminotransferase; AOR, adjusted odds ratio; CI, confidence interval; ULN, upper limit of normal.

[a]For non-Chinese, majority were Malays (89%); about 11% were Indian and indigenous populations.

4.8 Discussion

Despite strong epidemiological link of HBV with cirrhosis and HCC [Beasley et al., 1981], no consistent viral mutation was found to be adequate for predicting the development of these liver diseases. Hence, the continued search for other potential markers that might enhance the use of viral mutations for this purpose. In this study, precore wild-type and serum ferritin were associated closely with HCC whereas precore wild-type and serum iron with cirrhosis in chronic hepatitis B participants. Age was an important factor in determining the clinical outcome. Other factors like cigarette smoking and family history of HCC/cirrhosis would predispose chronic hepatitis B individuals to HCC, whereas male gender, non-Chinese ethnicity and high serum ALT to cirrhosis. Viral load at the time of diagnosis was not associated significantly with cirrhosis and HCC in this case-control study. The serial viral load data were not available. It has been reported that the mean viral load at the time of diagnosis of HCC could be lower than before the development of HCC [Yu et al., 2005]. Therefore, single measurement of viral load may not be useful to predict risk of HCC.

In the study design, special attention was given to the selection of stringent controls. It takes decades for the development of HCC in chronic hepatitis B patients, and patients often present with cirrhosis and HCC after the age of 40-50 years [refer Table 4.7]. Therefore, one criterion for the selection of stringent controls was age of at least 50 years. Secondly, only participants with a family history were included, suggesting that the infection occurred at an early age (and hence, long duration of infection). The binary logistic regression analysis showed that chronic hepatitis B Malaysians already had a high risk (OR > 9.0) of developing cirrhosis and HCC by 30 years of age. However, the current international standard of age for HCC screening in chronic hepatitis B is above 40-50 years. Perhaps, the age cutoff for HCC screening in

Malaysia should be revised to be lower than 40 years so as not to miss out those who might be at high risk for cirrhosis and HCC.

Precore mutations totally abrogate HBeAg expression. Precore mutations can be divided into start codon mutation (at nucleotide position 1814-1816) or newly introduced stop codon mutations (at nucleotide position 1895-1897). Precore start codon mutation was rare, being 7.8% in this study. Precore stop codon mutation was the most common, being 54.1% in TAG form and 0.6% in TGA form. On the other hand, precore wild-type may express HBeAg. Precore wild-type was associated with HBeAg positivity in this study. The ability of HBV antigens to induce long-term immune-mediated liver injury has been proposed as one of the possible mechanisms of inducing cirrhosis and HCC. Several lines of evidence pointed out that precore wild-type may have higher disease-inducing capacity than precore mutant: (i) HBeAg positivity was associated with HCC [Yang et al., 2002]; (ii) transgenic mice with HBeAg positive phenotype produced more liver injury than those without [Frelin et al., 2009]; and (iii) individuals with delayed HBeAg seroconversion presented with more severe liver pathology [Lin and Kao, 2008; Chan et al., 2009; Chen et al., 2010]. In this study, HBeAg was not associated with cirrhosis and HCC. This could be due to low levels of HBeAg expression that were not detected by the diagnostic assay used. Such low level of expression is probably due to BCP mutation rather than precore mutation. More importantly, the low undetectable HBeAg level might still be able to induce liver injury. It was also observed that precore wild-type had a co-linearity relationship with HBeAg and was predominant over HBeAg in the logistic regression analysis. Owing to this, HBeAg was excluded as a predictive factor during the stepwise logistic regression analysis process. This observation also indicated that precore wild-type was the better predictor of cirrhosis and HCC than HBeAg.

BCP double mutation (T1762/A1764) may reduce HBeAg expression, but not terminate HBeAg expression. One less known finding is that other BCP mutations like V1753 (coupled with T1762/A1764) and T1766/A1768 may actually increase HBeAg expression [Jammeh et al., 2008]. T1762/A1764 has been commonly reported in clinical association studies, especially in HCC. The inconsistent association of the BCP mutation with cirrhosis and HCC might be partly due to the capability of BCP mutations to either up- or down-regulate HBeAg expression. This study did lend support to the relationship of T1762/A1764 with HCC, but not with cirrhosis. On the other hand, another BCP mutation, G1799, was associated with cirrhosis and HCC in this study. Precore wild-type, A1814/T1815/G1816 … T1895/G1896/G1897, appeared to be the top and consistent viral marker related to cirrhosis and HCC in this study. As reported by two large sample size, age-adjusted studies [Tanaka et al., 2006; Yang et al., 2008], precore wild-type was a much stronger predictor of liver complications than BCP mutation. However, many other previous investigations reported otherwise [Tsai et al., 2009; Kao et al., 2012; Malik et al., 2012]. Owing to such inconsistent associations of precore wild-type with cirrhosis and HCC, a recent large sample size study excluded the precore wild-type in their clinical investigation [Yin et al., 2011]. The inconsistency could be because most, if not all, of the studies reporting discordant results have not controlled or adjusted for age in the selection of controls or in the analysis. It is generally accepted that older age is linked to higher occurrence of cirrhosis and HCC, as was observed in this study. In addition, the presence of precore wild-type was age-dependent as also shown in this study, where patients harbouring the precore wild-type were 5 years younger than those with the precore mutation. Perhaps, age-adjustment is required to show the association between precore wild-type with cirrhosis and HCC.

Besides precore wild-type and BCP mutations, pre-S mutation may also affect the pathogenesis of HBV infection. Accumulation of pre-S1/S2 antigens may induce

oxidative stress and DNA damage in hepatocytes, predisposing the infected persons to cirrhosis and HCC [Hsieh et al., 2004]. A pre-S variation previously related to cirrhosis and HCC were also shown in this study. The pre-S variations were located at nucleotide position 2964. In this study, C2964 was associated positively (OR = 10.5) with cirrhosis in agreement with a previous study (Yin et al., 2010), whereas A2964 was associated negatively (OR = 0.19) with HCC in contrast to the previous study. However, the sequence change from A2964 to C2964 at pre-S1 codon 39 does not cause any amino acid change; both encode for proline. The likely explanation is that the synonymous change might affect gene expression level via codon usage bias during protein translation. However, further studies are warranted to prove such a hypothesis.

Dietary intake of iron has been overlooked in studies on HBV-related liver diseases. Hepatic iron accumulation has been well reported in HCV-related liver damage [Hézode et al., 1999; Thorborn et al., 2002]. Recently, hepatic iron overload was found to be common in chronic hepatitis B, especially in males [Sebastiani et al., 2012]. Experimentally, excess iron deposition in liver is hepatotoxic and may worsen liver injury. Firstly, iron may facilitate viral replication [Kakizaki et al., 2000; Theurl et al., 2004; Park et al., 2012b]. Secondly, iron catalyses free hydroxyl radical formation via biochemical processes like lipid peroxidation, leading to progressive liver damage and thus increased risk of cirrhosis and HCC. Iron deposition in chronic hepatitis B may also be simply the result of iron released from damaged hepatocytes – a vicious cycle of iron accumulation. In addition, interferon treatment, an immune-booster, was not effective in chronic hepatitis B individuals with elevated serum ferritin levels [Bayraktar et al., 1998]. This is also true for interferon/ribavirin treatment in chronic hepatitis C [Fujita et al., 2007]. High serum ferritin might have somehow cause failure of the immune response to clear the virus. This stresses the important role of body iron status in immune control/clearance of the virus. High viral load has been known to

increase the risk of developing cirrhosis and HCC. By impeding the viral replication through iron reduction procedures like phlebotomy and dietary iron restriction, it may reduce the risk of progression to such complications. Therefore, one of the aims of this study was to address the clinical significance of serum iron markers in the course of chronic hepatitis B.

Moderately elevated serum iron markers were detected in cirrhotic and HCC participants in this study, a finding consistent with the belief that mild-to-moderate iron overload may be adequate to aggravate HBV-related liver damage. Serum ferritin correlates well with hepatic iron deposition in chronic hepatitis B [Sebastiani et al., 2012]. To more accurately assess effects of the iron input on HBV-related disease outcomes, serum iron and serum ferritin tests were carried out. From ROC analysis, serum ferritin level of ≥274.5 μg/L most accurately correlated with HCC and serum iron level of ≥22.82 μmol/L with cirrhosis. The ferritin level was comparable to the ULN of serum ferritin (291.0 μg/L) used in the UMMC but the serum iron level was much lower than the 27.0 μmol/L ULN in UMMC. According to logistic regression analysis, high serum ferritin was associated independently with HCC and high serum iron with cirrhosis. Chronic hepatitis B participants with serum ferritin level near ULN had a moderate risk of HCC (AOR > 3.5), whereas those with serum iron level as low as 20 μmol/L had a similar risk of cirrhosis. The high serum ferritin in HCC was unlikely to be due to liver inflammation because its relationship with serum ALT was rather weak. In addition, it was observed that serum ferritin surpassing the ULN only accounted for about 60% of the HCC participants, leaving 40% with normal level of serum ferritin. Further logistic regression ‘enter’ method adjusted for age and ALT was also conducted and the analysis showed that serum ferritin was independently associated with HCC regardless of the serum ALT level (Not shown in results). Serum iron was not correlated with serum ALT, indicating the high serum iron is probably not from liver

inflammation. The observation that elevated serum ferritin associated with HCC also explained the high male-to-female ratio (= 1.6) in HCC as Asian men generally have higher serum ferritin than Asian women [Harris et al., 2007]. High serum ferritin was not universally found in participants with cirrhosis because some of them could have encountered blood loss from liver complications prior to the time of diagnosis, and therefore serum ferritin could have dropped by the time the blood sample was collected for testing. Another interesting result was that low serum iron was independently associated with HCC. This might be due to the high demand of iron for supporting cellular proliferation in HCC tissues, making less iron available in the serum. Serum ferritin increased significantly with age, indicating that the serum marker could be accumulative during lifetime. However, such relationship was weak with serum iron. Overall, these observations highlight the possible role of serum iron markers affecting the disease outcome of chronic HBV infection.

It is well known that cigarette contains thousands of carcinogenic compounds that could increase the risk of various cancers. The logistic regression analysis showed that cigarette smoking was independently associated with HCC, but not male gender. Cigarette smoking could have diminished the contribution of male gender to risk of HCC in the multivariate analysis because male gender was associated significantly with cigarette smoking in univariate analysis ($P < 0.001$, Chi-square exact test). Smokers were four times more likely to develop HCC than non-smokers. However, cigarette smoking was not related to cirrhosis in this study. In the present study, men were twice more likely to have cirrhosis than women as have been shown in other studies (refer Table 4.8). In logistic regression analysis, non-Chinese ethnicity was a significant independent predictive factor for cirrhosis. This may be partly because non-Chinese ethnicity, mainly Malays, was associated significantly with cigarette smoking ($P = 0.007$, Chi-square exact test).

Chronic hepatitis B individuals with strong family history of HCC are prone to develop cirrhosis and HCC [Hassan et al., 2009; Chen and Yang, 2011]. This is supported by the evidence provided in this study that those with family history of cirrhosis/HCC were about 5 times more likely to develop HCC than those without, provided in this study. However, this was not true in cirrhosis. Genetic inheritance of HCC-susceptibility genes, fungal poisoning, dietary habits and misuse of hepatotoxic medicines could have accounted for the familial aggregation of HCC [Yu et al., 2000]. This study added new knowledge that precore wild-type could have accounted for the familial aggregation of HCC. Given that precore wild-type was associated with HBeAg positivity and HCC in this study plus an estimation of 31%–85% of the babies born to HBeAg-positive mothers becoming infected by the virus [Beasley et al., 1977], 'inheritance' of precore wild-type was highly possible and probably accounted for the familial aggregation of HCC. According to data on familial transmission in this study, both precore wild-type and mutant could be transmitted fairly vertically. In brief, family history of cirrhosis/HCC is a risk factor for progression to HCC, but not cirrhosis. Host and environmental factors might dilute the effect of familial predisposition on the progression to cirrhosis.

Point estimate of serum ALT is not always an accurate predictor for severe liver diseases. Although a dose-response relationship of serum ALT with risks of HCC and cirrhosis was observed in univariate analyses, elevated serum ALT was associated independently with cirrhosis only in multivariate analyses. Having said that, elevated serum ALT was probably the least important risk factor for cirrhosis because a chronic hepatitis B person with serum ALT raised to 2x ULN has merely a weak association with cirrhosis (OR = 1.65). In addition, by the time serum ALT is high, the liver damage has already occurred. A much earlier serum marker is preferred.

4.9 Conclusion

The present study showed that older age, cigarette smoking, family history of cirrhosis/HCC, HBV precore wild-type, serum iron and serum ferritin were associated independently with HCC, whereas older age, male gender, Malay ethnicity, precore wild-type, serum iron and serum ALT were associated independently with cirrhosis. This study suggested that those with precore wild-type or high body iron status should be given special attention because they are probably at a much higher risk of progression to cirrhosis and HCC. They should be carefully assessed for suitability of specific hepatitis B treatments which may lower the risk for developing the liver complications.

4.10 Limitations

Firstly, as bidirectional sequencing instead of clonal sequencing was done, the presence of quasispecies or certain deletion mutants may be missed. Secondly, the rise of serum free iron in liver cirrhosis might be due to the reduced synthesis of serum transferrin in the cirrhotic liver. Certain infections and systemic inflammatory diseases may increase serum ferritin level. The possibility of elevated serum ferritin being due to systemic inflammatory diseases cannot be excluded in this study due to the lack of relevant clinical information in the participants. However, systemic inflammatory diseases are rare. Lastly, serum transferrin saturation test has not been done to confirm the true elevation of serum ferritin partly because many of the participants were not fasting at the time of blood collection.

Chapter 5 In Search of HBV Markers for Chronicity

5.1 Literature review

Acute HBV infection is defined as a sudden elevation of serum ALT and increased titre of anti-HBc IgM [Lok et al., 1985; Chen et al., 2006c]. Recovery from the acute infection is marked by disappearance of HBsAg and presence of anti-HBs, together with normalisation of liver function tests [Krugman et al., 1979; Hoofnagle, 1981]; this is so-called acute self-limited hepatitis. However, viremia may still be detected in some of the patients [Akahane et al., 2002]. The majority of acute infections are self-limiting [Juszczyk, 2000]. Less than 10% of children and 30-50% of adults with acute HBV infection will have icteric disease [WHO, 2002]. The acute infection may progress to either chronicity (where HBsAg persists) in <5% of adults or fulminant hepatitis (where massive hepatic necrosis occurs) in about 0.5% of patients [Heathcote J et al., 2003; Han, 2009].

Both host immunity and viral characteristics may influence chronicity of HBV infection. Age at acquiring infection determines whether an acute infection will progress to chronic carriage. Babies and children of early age with under-developed immunity often fail to resolve the viral infection. Chronicity develops in 25-50% of children infected at 1-5 years of age but <5% in older children and adults [WHO, 2002]. The age-related chronicity reflects the importance of host immune control. In addition, human leukocyte antigen (HLA)-DP genes have been associated with immune clearance and chronicity. HLA-DP genes are class II HLA genes that encode proteins expressed on the surface of antigen-presenting cells and thereby play a critical role in inducing clonal expansion of viral antigen-specific CD4+ T-helper lymphocytes. According to a latest meta-analysis on genome-wide association study (GWAS), HLA-DPA1 rs3077 and HLA-DPB1 rs9277542 SNPs were associated with protective effects against persistency and clearance of HBV infection in East Asian populations [Nishida et al.,

2012]. The associations were further strengthened by the finding of decreased expression of HLA-DPA1 and HLA-DPB1 mRNAs in chronically HBV-infected persons [O'Brien et al., 2011].

HBeAg is a truncated protein product of HBV precore protein; it is secreted out from viral-infected hepatocytes into bloodstream [Ahn et al., 2003]. Most, if not all, acute hepatitis B patients are serum HBeAg-positive [WHO, 2002]. HBeAg is one of the immune evasion strategies of HBV. HBeAg may act as a tolerogen, protecting HBV-infected hepatocytes from host immune attack and thereby fostering persistent infection [Chen et al., 2005b]. This is supported by the evidence that HBeAg may disrupt innate immune signaling, thereby compromising host capability to clear the virus [Bauer et al., 2011]. The viral antigen properties have given the virus an opportunity to avoid the immune clearance. There were very few clinical reports on HBV mutations associated with chronicity. Ogawa and colleagues (2002) found the predominance of precore wild-type (92%) and BCP wild-type (76%) among patients with acute hepatitis B. In fact, BCP (A1762/G1764) and precore (G1896) wild-types were the two most common HBV variants reported to be associated positively with acute hepatitis, in other words, lower rate of chronicity (given in Table 5.1); however, one study reported negative association of BCP with acute hepatitis [Liu et al., 2010] and another reported no association of precore wild-type with acute hepatitis [Chu et al., 1996]. Contrary to expectation, these reports implied that HBeAg did not provide an advantage to persistency of HBV infection in host.

Table 5.1 : Common HBV variants associated with acute and chronic hepatitis.

Author (year)	Acute, chronic	OR	*P* value
A1762/G1764			
Kobayashi et al. (2004)	43, 203	13.53	<0.001
Kusumoto et al. (2008)	36, 36[a]	23.80	<0.001
Liu et al. (2010)	182, 325	0.317	<0.001
G1896			
Chu et al. (1996)	12, 60	0.43	0.280
Kobayashi et al. (2004)	43, 203	15.19	<0.001
Kusumoto et al. (2008)	36, 36[a]	17.16	<0.001
Liu et al. (2010)	182, 325	4.35	<0.001

HBV, hepatitis B virus; OR, odds ratio.
[a]All the chronic cases in the study were acute-on-chronic hepatitis B.

Most of the acute hepatitis studies have focused only on precore and BCP regions. Further, these associations have been inconsistent. The importance of other viral genes contributing to chronicity might have been overlooked. Therefore, the present study intended to investigate whether certain viral genetic variations were associated with either higher or lower rate of chronicity in HBV-infected patients based on large-scale *in silico* observation.

5.2 *In silico* observation of viral markers associated with chronicity

Sequence data from 1,326 HBV complete genomes comprising 177 acute hepatitis and 1,149 chronic hepatitis cases were successfully identified from NCBI website. About 75% of the acute cases were self-limited (HBsAg clearance was not indicated in the rest), whereas chronic cases encompassed a wide range of clinical conditions, such as chronic hepatitis, cirrhosis and HCC. The majority were from East Asian countries with acute cases from China (74.01%), Belgium (18.64%), Japan (3.39%) and others, and chronic cases from China (46.91%), Japan (14.10%), South Korea (5.92%), Taiwan (2.52%), Belgium (2.44%) and others. Both acute and chronic cases were more or less geographical similar; about 77% of acute and 72% of chronic cases were from East Asian countries. The distribution of HBV genotypes in acute and chronic cases is given

in Table 5.2. Genotype A were prevalent in acute cases, genotypes B, E-G and I in chronic cases and genotype C almost equally in both acute and chronic cases.

Table 5.2 : Genotype distribution in 177 acute and 1,149 chronic hepatitis B viral genomes retrieved from NCBI.

Genotype	Acute (%)	Chronic (%)
A	35 (19.77)	59 (5.13)
B	30 (16.95)	262 (22.80)
C	102 (57.63)	637 (55.44)
D	8 (4.52)	85 (7.40)
E	0 (0.00)	17 (1.48)
F	0 (0.00)	49 (4.26)
G	0 (0.00)	11 (0.96)
H	1 (0.56)	8 (0.70)
I	1 (0.56)	20 (1.74)
J	0 (0.00)	1 (0.09)

NCBI: National Center for Biotechnology Information.

Seventeen HBV nucleotides were identified to be potentially distinguishable between acute and chronic cases. Internal validation was carried out by performing binary logistic regression analysis bootstrapped 1,000 times to select the nucleotides independently associated with chronicity, as given in Table 5.3. Nine candidate nucleotides were found to be associated with lower rate of chronicity but none with higher rate of chronicity. The one with the highest accuracy was A1786, followed in descending order by G1171, T1785, T3112, C504, C2398, G2669, A382 and A2341. These nucleotides were then one-by-one combined in the descending order of accuracy to see if there were degradations in accuracy. The combination of A1786/ G1171/ T1785/ T3112/ A382/ A2341 improved the accuracy from 88.39% to 90.72%; C504, C2398 and G2669 were excluded as they degraded the accuracy when combined with the others. Bonferroni correction was further applied in order to reduce false positives resulting from the analysis of large sample size data; as a result, two nucleotides, A382 and A2341, were excluded (given in Table 5.4), leaving A1786/ G1171/ T1785/ T3112 which gave a high accuracy (90.1%) for predicting lower rate of chronicity (Table 5.3).

Table 5.3 : Candidate nucleotides associated significantly with acute and chronic hepatitis using logistic regression analysis set for 1,000 bootstrapped samples. The selection was carried out based on a descending order of accuracy. The significant candidate nucleotides were then combined progressively based on the order of accuracy again. The candidate nucleotides that showed degraded accuracy in combination were excluded from further assessment.

Nucleotide	Acute n = 177 (%sensitivity)	Chronic n = 1149 (%specificity)	%Accuracy[a]	*P* value
A1786	26 (14.69)	3 (99.74)	88.39	0.001
G1171	18 (10.17)	2 (99.83)	87.86	0.001
T1785	13 (7.34)	0 (100.00)	87.59	0.001
T3112	32 (18.08)	20 (98.26)	87.56	0.001
C504	18 (10.17)	7 (99.39)	87.48	0.024
C801	12 (6.78)	2 (99.83)	87.41	0.708
C2398	32 (18.08)	23 (98.00)	87.33	0.003
T1499	21 (11.86)	15 (98.69)	87.10	0.082
G2669	34 (19.21)	28 (97.56)	87.10	0.001
A382	5 (2.82)	0 (100.00)	87.03	0.001
A2215	31 (17.51)	26 (97.74)	87.03	0.073
A2341	5 (2.82)	0 (100.00)	87.03	0.001
C2659	31 (17.51)	26 (97.74)	87.03	0.364
G1784	4 (2.26)	0 (100.00)	86.95	0.848
G2239	31 (17.75)	27 (97.65)	86.95	0.670
A2245	33 (18.64)	29 (97.48)	86.95	0.118
A2662	32 (18.08)	28 (97.56)	86.95	0.354
Combinations of significant nucleotides				
A1786 or G1171	40 (22.60)	5 (99.56)	89.29	<0.001
A1786 or G1171 or T1785	40 (22.60)	5 (99.56)	89.29	<0.001
A1786 or G1171 or T1785 or T3112	70 (39.55)	25 (97.82)	90.05	<0.001
A1786 or G1171 or T1785 or T3112 or C504	73(41.24)	31 (97.30)	89.82	<0.001
Excluded C504[b]				
A1786 or G1171 or T1785 or T3112 or C2398	71 (40.11)	31 (97.30)	89.67	<0.001
Excluded C2398[b]				
A1786 or G1171 or T1785 or T3112 or G2669	72 (40.68)	50 (95.65)	88.31	<0.001
Excluded G2669[b]				
A1786 or G1171 or T1785 or T3112 or A382	75 (42.37)	25 (97.82)	90.42	<0.001
A1786 or G1171 or T1785 or T3112 or A382 or A2341[c]	79 (44.63)	25 (97.82)	90.72	<0.001

[a]% Accuracy = (177 x %sensitivity + 1149 x %specificity)/(177 + 1149). Accuracy of random guess was calculated to be (1149/1327 x 100% =) 86.58%. This was used as accuracy cutoff.
[b]C504, C2398 and G2669 were excluded as they decreased the overall accuracy when combined.
[c]This was the best combination where it gave the highest accuracy. Hence, they were included for next step analysis.

Table 5.4 : Refined selection of candidate nucleotides independently associated with acute and chronic hepatitis B based on a Bonferroni-corrected *P* value to reduce false positivity.

Nucleotide	OR (95% CI)[a]	Phi	*P* value[b]
A1786	65.78 (19.67-219.93)	0.335	8.663E-21
G1171	64.93 (14.92-282.44)	0.279	1.236E-14
T1785	91.08 (11.84-700.82)	0.242	3.536E-11
T3112	12.46 (6.94-22.36)	0.286	9.461E-17
A382[c]	*33.40 (3.88-287.61)*	*0.139*	*2.150E-04*
A2341[c]	*33.40 (3.88-287.61)*	*0.139*	*2.150E-04*

CI, confidence interval; HBV, hepatitis B virus; NA, not applicable; OR, odds ratio.
[a]Value of zero was adjusted to 0.5 for calculation of odds ratio.
[b]Chi-square exact test; *P* value of 1.593E-05 is equivalent to 1.593×10^{-05}.
[c]A382 and A2341 were excluded because their *P* values exceeded the threshold *P* value, 1.593E-05; the threshold *P* value was determined based on 3139 SNPs in 3832 complete genomes of HBV [Pearson and Manolio et al., 2008].

5.3 Discussion

More than 70% of circulating HBVs are from Asia. From all complete genomes of HBV retrieved in the present study, about 70-80% were from East Asian countries; this is true for acute and chronic cases. From this *in silico* observation, viral genotype A was prevalent in acute cases, in accordance with other studies [Kobayashi et al., 2004; Suzuki et al., 2005]; however, genotype C was present equally in both acute and chronic cases, contrasting with the aforementioned studies. Under rigorous selection procedure, four candidate nucleotides, namely A1786, G1171, T1785 and T3112, were associated strongly with acute hepatitis (mostly self-limited), suggesting the existence of viral strains potentially responsible for a lower rate of chronicity in HBV-infected individuals.

Several lines of evidence indicated that HBx is essential for initiating and maintaining viral replication and antigen expression after infection [Lucifora et al., 2011]. Firstly, HBx increases deoxyribonucleotide synthesis which is essential for optimal activity of viral polymerase [Cohen et al., 2010]. Secondly, HBx is required for

establishment of chronicity in a woodchuck model [Zoulim et al., 1994]. Furthermore, HBx restores replicative activity of X-defective HBV strains *in vitro* and *in vivo* [Keasler et al., 2009] and blocking HBx activity inhibits viral replication [Carmona et al., 2009; Xie et al., 2012]. Small interfering RNA targeting HBx inhibits the expression of HBsAg, HBeAg and the replication of HBV DNA [Xie et al., 2011]. T1785 and A1786 are located near the 3' end of X gene (corresponding to C-terminal of HBx), which is the region critical for stability and function of HBx; truncated C-terminal HBx was not able to stimulate viral replication in a woodchuck and mouse model [Lizzano et al., 2011; Luo et al., 2012]. HBV possessing both T1785 and A1786 variants form a stop signal (TAG) of translation at codon 138 of X gene. If translated, this would produce a C-terminal truncated HBx protein that is non-functional for viral replication, similar to that reported in the aforementioned study. The substitution of A1786 with G1786 causes switching of arginine to lysine. An arginine-rich domain in a protein may work as a nuclear localisation signal, whereas a lysine-rich domain may function as a nuclear export signal [Demart et al., 2003; Michaud et al., 2008]. The switch may favour the cytoplasmic-over-nuclear distribution of HBx, as evidenced in the HBV core protein [Garcia et al., 2009]. Truncation and cytoplasmic localisation of HBx could lead to inefficient viral replication, reducing viral capability to compete with host vigourous immune clearance, thereby lowering rate of chronicity.

G1171 falls within the viral EnhI-X promoter complex (nt 950-1,350) [Guo et al., 1991]. EnhI mutation has been known to reduce viral replication [Bock et al., 2000], whereas X promoter, located upstream of X gene, may affect transcription of X mRNA which is required for HBx production. Deletion of EnhI greatly reduces the activity of X promoter, indicating that EnhI may be required for maximal activity of X promoter; deletion of nt 970-1,116 partially inactivates X promoter, but deletion of nt 964-1,217 completely abolishes X promoter activity [Treinin and Laub, 1987]. G1171 is located in

the sequence region of the latter, which is critical for regulating the viral X promoter activity. Therefore, G1171 variant may down-regulate expression of HBx, resulting in lower rate of chronicity.

T3112 is located within the polymerase region, overlapping with pre-S1 and S promoter. Pre-S1 (nt 2848-3204) and S promoter (nt 3045-3180) share a large overlapping region. In fact, most S promoter mutations coincide with pre-S1 mutations but the inverse is not true. T3112 is unlikely to have an impact on the polymerase gene regulation because it falls within the spacer region which is non-functional [Bock et al., 1997]. T3112 switches proline to serine in the pre-S1 region. The amino acid change is probably not causing any secondary protein structure alteration as both proline and serine are frequently encountered in loops and turns structures. T3112 is also positioned near CCAAT motif (nt 3,137-3,141) within the S2 promoter region which regulates MHBsAg and SHBsAg (also called HBsAg) production [Bock et al., 1999]. S promoter deletion variants may lead to predominant expression of pre-S1 mRNA over pre-S2/S mRNA, and intracellular accumulation of surface proteins, especially the LHBsAg [Xu and Yen, 1996; Melegari et al., 1997; Lin et al., 2012]. Several S2 promoter mutations at 5' and 3' terminus of CCAAT-box, like C3007, A3008, C3039, G3103, T3105, C3126 and A3127, have also been reported to affect surface protein expression [Kimbi et al., 2004]. Retention of LHBsAg within hepatocytes may induce endoplasmic reticulum stress, producing oxidative damage that may cause genomic instability or self-destruction of hepatocytes, and thus lead to less suitable or fewer factories for virion production [Hsieh et al., 2004]. S promoter mutations may also inhibit virion secretion [Melegari et al., 1997]; however, two other studies did not support such findings [Xu and Yen, 1996; Bock et al., 1997]. T3112 could be another novel S2 promoter variant potentially capable of reducing MHBsAg and SHBsAg secretion and over-producing LHBsAg that may inhibit virion production. If this holds true, it would confer a negative

impact on viral immune-escape strategy and viral replication. Host immune defense may eventually wipe off the inferior virus strain, resulting in acute self-limited infection. Further experimental studies are needed to confirm this hypothesis.

5.4 Conclusion

The present study discovered several novel viral variants that might predispose a HBV-infected individual to a lower rate of chronicity. They are located within X gene, EnhI-X promoter complex and S promoter region. They are highly specific but insufficiently sensitive, indicating the possible involvement of other factors in the development of acute hepatitis. As this is an *in silico* observation study, further *in vitro* and *in vivo* experiments are required to decipher the clinical importance of these specific viral variants and their effects on viral replication and release.

5.5 Limitations

1. Rigour classification of hepatitis cases is not possible in this *in silico* observation study as many of the publications sourced did not provide sufficient details for the definition of acute hepatitis.
2. The imbalanced sample size could lead to false high accuracy. To resolve this, accuracy cutoff of random guess was adjusted from 50% to >85%.
3. The influence of HBV quasispecies on the development of chronicity could not be investigated as most of the HBV sequences published were not cloned sequences.

Appendix A: Raw Data of Cirrhosis and HCC Study

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB003	ATG	TGG	6.90	18.1	41	0	0	0	0	0	0	35	2.0	N
HB006	ATG	TGG	21.50	67.5	24	1	0	0	1	0	1	40	2.0	N
HB007	ATG	TAG	13.20	11.1	45	0	0	0	1	1	0	82	0.5	N
HB008	ATG	TGG	10.90	227.8	34	0	0	0	0	0	1	38	4.0	N
HB009	ATG	TAG	23.40	88.7	43	1	1	0	1	1	0	47	5.0	C
HB015	ATG	TAG	14.40	201.3	55	1	0	0	0	0	0	51	0.5	N
HB021	ATG	TAG	19.30	215.8	26	1	1	1	2	2	0	57	5.0	N
HB022	ATG	TGG	14.10	89.3	30	0	2	0	0	0	1	84	2.0	N
HB028	ATG	TAG	16.90	174.7	62	1	0	1	0	0	0	39	2.0	N
HB031	ATG	TAG	12.40	66.4	44	0	0	0	0	0	0	27	4.0	N
HB033	ATG	TAG	20.20	332.9	67	1	0	0	0	0	0	40	2.0	C
HB034	ATG	TGG	15.60	300.4	50	1	0	0	1	1	1	68	5.0	C
HB038	ATG	TAG	19.00	310.8	58	0	0	0	0	0	1	42	10.0	C
HB039	ATG	TAG	21.90	40.9	74	1	0	1	0	0	0	40	0.5	N
HB043	ATG	TAG	31.90	182.8	43	1	1	1	1	1	0	47	4.0	C
HB044	ATG	TGG	22.10	31.3	21	0	0	0	1	1	1	30	3.0	N
HB045	TTG	TGG	34.10	124.2	41	1	1	0	1	0	0	58	4.0	C
HB047	ATG	TGG	30.60	625.0	57	0	0	0	0	1	1	76	3.0	N
HB049	ATG	TGG	24.90	137.5	34	1	0	1	1	0	1	186	3.0	C
HB053	ATG	TGG	24.31	280.6	66	0	0	0	2	0	0	41	3.0	H
HB056	ATG	TGG	23.20	70.8	50	1	0	0	1	1	1	51	2.0	N
HB057	ATG	TGG	10.57	218.6	78	0	0	0	0	0	0	36	2.0	C
HB059	ATG	TGG	21.10	115.5	46	1	0	1	1	1	0	41	3.0	N
HB060	ATG	TGG	8.40	77.3	28	0	0	0	0	0	1	10	4.0	N
HB062	ATG	TAG	13.50	41.4	34	0	0	0	1	0	0	28	4.0	N
HB063	ATG	TAG	22.30	267.6	65	1	0	0	2	0	0	43	2.0	N
HB066	ATG	TAG	3.90	158.7	65	1	1	0	0	0	0	62	2.0	N
HB073	ATG	TGG	11.90	118.7	55	0	0	0	1	1	0	37	3.0	N
HB075	ATG	TRG	14.80	292.4	66	0	0	0	0	0	0	34	2.0	N
HB077	ATG	TGG	14.60	28.9	57	1	0	0	1	0	0	31	2.0	N
HB079	AYG	TGG	20.20	314.9	40	1	0	0	1	0	1	102	3.0	N
HB082	ATG	TAG	13.70	13.4	38	0	0	0	1	0	0	40	2.0	N
HB083	ATG	TGG	24.80	251.0	40	1	0	0	1	0	0	54	3.0	N
HB084	ATG	TAG	23.10	146.4	31	1	0	0	1	0	0	115	2.0	N
HB088	ATG	TRG	13.90	101.3	50	0	0	0	1	1	1	34	2.0	N
HB091	ATG	TGG	5.50	5.8	25	0	1	0	0	0	1	129	6.0	N
HB093	ATG	TAG	15.90	30.0	34	0	1	0	2	2	0	35	0.5	N
HB095	ATG	TAG	20.50	100.4	65	0	0	0	2	2	0	32	4.0	C
HB099	ATG	TAG	15.90	950.8	46	1	0	0	0	0	0	379	5.0	N

Pc start, precore start codon variant; Pc stop, precore stop codon variant; eth, ethnicity; smo, smoker; fhx, family history of infection; fhcc, family history of HCC; eAg, HBeAg; alt, alanine aminotransferase; afp, alpha-fetoprotein; cond, disease condition.

Sex: 0 = female, 1 = male; eth: 0 = Chinese, 1 = Malay, 2 = others; smo: 0 = non-smoker, 1 = smoker; fhx: 0 = no, 1 = yes, 2 = unknown; fhcc: 0 = no, 1 = yes, 2 = unknown; eAg: 0 = negative, 1 = positive; cond: N = control without significant liver disease, C = cirrhosis and H = HCC.

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB103	ATG	TGG	21.30	90.7	78	1	2	0	0	0	1	72	5.0	N
HB104	ATG	TAG	21.80	197.9	56	0	0	0	1	0	0	48	0.5	N
HB106	ATG	TGG	14.80	99.1	20	0	1	0	0	0	1	43	2.0	N
HB107	ATG	TRG	9.30	24.6	28	0	0	0	1	0	0	63	5.0	N
HB109	ATG	TGG	14.50	528.2	55	1	0	0	0	0	0	54	3.0	N
HB110	TTG	TAG	5.80	10.2	44	0	1	0	1	0	0	37	2.0	N
HB114	ATG	TAG	17.30	162.4	51	1	0	0	0	0	0	41	3.0	N
HB118	ATG	TAG	13.17	260.7	78	1	0	0	0	0	0	48	4.0	C
HB129	ATG	TGG	36.11	278.1	65	1	0	1	0	0	0	55	15.0	H
HB131	ATG	TAG	10.30	93.6	25	0	0	0	1	1	0	54	0.5	N
HB133	ATG	TAG	20.20	43.1	50	0	1	0	0	1	0	35	8.0	N
HB134	ACG	TGG	17.00	210.7	58	0	0	0	1	0	0	45	2.0	N
HB136	ATG	TAG	29.60	98.4	41	1	0	0	1	0	0	47	0.5	N
HB137	ATG	TAG	15.47	109.8	51	0	0	0	1	1	0	49	3.0	N
HB139	ATG	TAG	17.40	441.7	26	1	0	1	0	0	0	48	0.5	N
HB141	ATG	TGG	21.90	193.1	44	0	0	0	1	0	0	42	0.5	N
HB142	ATG	TGG	24.90	60.1	48	0	0	0	1	2	0	42	2.0	C
HB143	ATG	TGG	15.70	67.7	27	0	0	0	0	0	0	30	6.0	N
HB145	ATG	TAG	20.80	198.8	64	1	1	1	1	0	0	51	2.0	N
HB146	ATG	TGG	14.50	83.4	27	1	0	0	1	0	1	60	0.5	N
HB148	ATG	TAG	10.80	118.4	49	0	1	0	1	1	0	27	2.0	N
HB151	ATG	TGG	16.68	423.7	73	1	0	0	1	0	0	51	8.0	C
HB152	CTG	TGG	18.90	146.4	53	0	0	0	1	1	0	36	0.5	N
HB155	ATG	TRG	11.00	13.0	37	0	1	0	0	0	1	101	5.0	N
HB156	ATG	TAG	14.00	213.3	34	1	0	0	1	0	0	86	4.0	F
HB158	ATG	TGG	12.80	46.1	40	0	0	0	1	0	0	32	4.0	N
HB166	ATG	TGG	14.70	97.8	23	1	0	0	0	0	1	46	3.0	N
HB171	ATG	TAG	30.40	478.4	41	1	1	0	0	0	0	67	7.0	C
HB172	ATG	TAG	14.88	288.8	54	0	0	0	1	0	0	44	3.0	N
HB173	ATG	TRG	3.77	4.6	26	0	1	0	0	0	1	141	3.0	N
HB174	ATG	TAG	23.20	137.3	26	1	1	1	1	0	0	46	5.0	N
HB177	ATG	TAG	15.60	198.6	63	0	0	0	0	0	0	48	2.0	N
HB178	ATG	TGG	7.06	11.3	71	0	1	0	0	0	1	22	3.0	C
HB181	ATG	TGG	17.42	120.9	52	0	0	1	1	1	1	46	3.0	C
HB182	ATG	TGG	26.90	199.4	58	0	0	0	2	0	1	53	4.0	N
HB185	ATG	TAG	18.40	119.0	56	0	1	0	1	1	0	37	0.5	N
HB187	ATG	TAG	14.10	153.3	39	1	1	1	2	0	0	30	3.0	N
HB188	ATG	TGG	17.50	237.3	55	1	0	0	1	0	0	34	8.0	N
HB192	ATG	TAG	20.79	279.1	57	0	0	0	1	1	0	47	2.0	N
HB193	ATG	TAG	20.61	165.7	64	1	0	0	1	0	0	32	2.0	N

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB197	ATG	TAG	13.50	377.3	65	0	0	0	1	1	0	41	4.0	C
HB198	ATG	TAG	15.40	238.6	57	1	0	0	0	0	0	67	0.5	N
HB199	ATG	TRG	16.20	57.0	32	1	1	0	0	0	0	59	3.0	N
HB200	ATG	TRG	13.80	38.8	36	0	1	0	1	0	0	70	3.0	N
HB201	ATG	TGG	14.10	94.1	50	0	0	0	2	0	0	41	4.0	N
HB202	ATG	TGG	11.50	46.9	19	0	0	0	1	0	1	30	2.0	N
HB205	ATG	TAG	13.10	270.7	66	0	0	0	0	0	0	43	2.0	N
HB206	ATG	TGG	23.90	210.7	59	1	0	0	1	2	1	43	2.0	C
HB209	ATG	TAG	13.90	55.5	35	0	0	0	1	1	0	41	0.5	N
HB212	ATG	TAG	13.50	48.3	49	0	1	0	2	0	0	34	3.0	N
HB213	ATG	TAG	12.70	165.5	65	1	0	0	1	0	0	37	2.0	N
HB215	ATG	TAG	18.09	249.8	49	0	0	0	1	1	0	49	5.0	N
HB217	ATG	TAG	22.68	251.8	61	0	0	0	1	2	0	130	4.0	N
HB219	ATG	TAG	18.20	204.5	53	1	0	0	0	0	0	36	2.0	N
HB220	ATG	TAG	21.70	69.7	31	1	1	0	0	0	0	37	2.0	N
HB221	ATG	TAG	14.40	28.0	22	0	1	0	1	0	0	39	0.5	N
HB223	ATG	TRG	17.80	486.1	51	1	0	0	1	1	0	58	5.0	C
HB225	ATG	TAG	36.70	870.0	67	1	0	1	2	0	0	403	3.0	N
HB228	ATG	TAG	31.62	167.0	65	0	0	0	0	0	0	46	10.0	C
HB229	ATG	TAG	15.20	297.4	31	1	1	0	1	2	0	45	3.0	N
HB231	ATG	TRG	18.40	187.8	36	1	0	0	1	0	0	74	3.0	N
HB232	ATG	TGG	3.43	59.4	65	1	0	0	2	0	0	37	4.0	N
HB233	ATG	TGG	14.20	155.2	22	1	1	0	2	0	0	52	3.0	N
HB234	ATG	TGG	13.30	18.4	26	0	1	0	1	0	1	39	0.5	N
HB239	ATG	TAG	21.80	129.0	64	0	0	0	0	0	0	46	2.0	N
HB240	ATG	TAG	24.90	90.4	56	0	1	0	1	0	0	35	6.0	N
HB241	ATG	TAG	8.40	12.4	42	0	0	0	1	0	0	35	15.0	N
HB242	ATG	TAG	19.40	86.8	56	0	1	0	1	1	0	35	2.0	N
HB243	ATG	TAG	22.60	345.1	57	0	0	0	0	0	0	36	2.0	N
HB246	ATG	TAG	15.51	125.4	60	0	0	0	1	2	0	84	4.0	N
HB247	ATG	TAG	17.00	241.1	30	1	0	1	0	2	0	48	6.0	N
HB248	ATG	TAG	24.90	110.9	61	0	1	0	0	0	0	49	5.0	C
HB250	ATG	TGG	18.61	320.3	63	0	0	0	2	0	1	84	4.0	N
HB251	ATG	TGA	15.97	122.8	69	0	0	0	1	0	0	40	5.0	N
HB253	ATG	TGG	13.60	94.8	23	1	0	0	1	0	1	44	2.0	N
HB254	AAG	TGG	15.10	167.8	47	1	0	0	1	0	0	22	6.0	N
HB256	ATG	TAG	24.90	13.0	41	0	0	0	1	1	0	32	4.0	N
HB257	AYG	TAG	15.24	225.5	51	1	0	0	1	1	0	48	4.0	N
HB258	ATG	TRG	21.10	302.8	31	1	1	1	1	1	1	108	3.0	N
HB259	ATG	TGG	24.60	501.6	24	1	1	0	1	1	1	129	3.0	N
HB260	ATG	TGG	8.80	76.0	44	1	1	1	2	0	1	37	6.0	N
HB264	ATG	TGG	4.93	15.1	41	0	0	0	0	0	1	38	3.0	C
HB266	ATG	TAG	18.40	298.4	61	0	0	0	1	1	0	43	2.0	N
HB267	ATG	TAG	20.10	338.7	48	1	1	1	1	1	0	57	3.0	N

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB268	ATG	TGG	29.10	33.2	22	0	1	0	0	0	1	48	2.0	N
HB269	ATG	TAG	15.10	103.8	31	0	1	0	0	0	0	30	2.0	N
HB271	ATG	TAG	19.20	246.1	70	0	0	0	0	0	0	41	3.0	N
HB273	ATG	TGG	33.30	290.2	37	1	0	0	1	1	1	34	2.0	N
HB281	CTG	TGG	15.56	42.0	52	0	0	0	1	0	0	35	3.0	N
HB284	ATG	TAG	31.48	223.8	62	1	0	0	1	1	0	42	4.0	N
HB285	ATG	TAG	13.79	106.2	56	0	1	0	0	0	0	63	6.0	C
HB286	ATG	TGG	20.59	269.3	35	1	1	0	1	0	1	77	3.0	C
HB287	ATG	TRG	12.90	213.5	40	1	0	0	1	0	1	82	2.0	N
HB289	ATG	TAG	26.20	385.8	63	1	0	1	2	0	0	79	4.0	C
HB291	MTG	TRG	27.40	252.1	52	1	0	0	1	0	0	42	2.0	N
HB294	ATG	TAG	30.71	305.3	61	1	2	0	0	0	1	58	4.0	C
HB298	ATG	TGG	37.29	106.9	46	1	1	0	0	0	1	58	5.0	C
HB299	ATG	TAG	21.59	309.6	57	1	0	0	1	0	0	86	407.0	H
HB301	ATG	TGG	34.60	193.5	64	1	0	0	0	0	0	62	22.0	C
HB302	ATG	TAG	16.60	100.2	57	0	0	0	2	0	0	48	0.5	N
HB304	ATG	TAG	19.73	26.4	39	0	0	0	1	1	0	1,090	3.0	C
HB310	ATG	TGG	4.63	1,006.0	38	1	1	0	0	0	1	88	39,235.0	H
HB312	ATG	TAG	13.60	117.0	60	1	2	0	1	1	0	39	3.0	C
HB315	ATG	TGG	2.31	1,587.0	60	1	1	1	1	0	0	132	71.0	H
HB316	ATG	TGG	21.20	338.4	66	1	0	1	1	0	0	34	3.0	N
HB317	ATG	TAG	15.80	152.5	56	0	0	0	1	0	0	34	2.0	N
HB318	ATG	TRG	15.50	34.3	58	1	0	0	1	1	0	60	0.5	C
HB319	ATG	TAG	17.10	187.9	58	0	0	0	1	1	0	38	0.5	N
HB322	ATG	TAG	5.40	161.9	49	0	0	0	1	1	0	40	2.0	H
HB326	ATG	TAG	9.50	25.6	51	0	0	0	1	0	0	39	2.0	N
HB328	ATG	TGG	19.10	330.8	54	1	0	0	0	0	0	64	69.0	C
HB332	ATG	TGG	20.00	57.8	51	0	0	0	1	1	1	40	6.0	C
HB333	ATG	TAG	20.30	264.4	57	1	0	0	1	0	0	63	19.0	C
HB335	ATG	TAG	17.30	310.9	46	1	0	1	1	1	0	35	2.0	N
HB336	MTG	TAG	18.80	1,192.0	58	1	0	0	0	0	0	34	5.0	N
HB337	ATG	TGG	21.30	117.4	69	0	0	0	1	1	1	37	2.0	N
HB338	ATG	TAG	17.70	337.4	42	1	0	0	1	1	0	57	5.0	N
HB339	ATG	TAG	13.20	80.7	58	1	1	0	0	0	1	45	2.0	C
HB341	ATG	TAG	21.40	25.7	61	1	0	1	1	0	1	55	4.0	C
HB343	ATG	TRG	14.80	132.2	36	1	2	1	2	0	1	66	0.5	N
HB345	TTG	TAG	17.10	189.2	60	1	0	0	0	0	0	66	32.0	N
HB347	CTG	TGG	28.34	103.4	61	1	0	0	0	0	0	43	4.0	C
HB348	ATG	TAG	14.30	168.4	57	0	0	0	1	1	0	30	3.0	H
HB349	ATG	TGG	5.20	9.7	44	0	0	0	1	0	1	39	2.0	N
HB350	ATG	TGG	22.20	368.1	48	1	0	0	1	0	0	62	0.5	N
HB363	ATG	TAG	24.68	150.2	60	1	0	0	1	0	0	49	2.0	N

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB365	ATG	TGG	28.26	422.2	56	1	0	1	0	0	1	43	2.0	H
HB370	ATG	TAG	12.75	209.7	63	0	0	0	1	0	0	44	2.0	N
HB371	ATG	TAG	28.80	238.5	45	1	0	0	2	0	0	65	0.5	N
HB374	TTG	TGG	5.31	1,842.0	59	1	0	0	1	0	0	54	2.0	C
HB378	ATG	TGG	28.90	238.2	59	1	0	1	1	0	1	56	5.0	C
HB380	ATG	TGG	49.10	318.6	61	1	0	1	1	1	1	214	8.0	H
HB381	ATG	TGG	32.70	181.5	51	0	0	0	1	1	0	34	0.5	C
HB383	ATG	TAG	16.40	409.7	60	1	0	1	1	0	0	56	4.0	H
HB385	ATG	TAG	13.70	91.8	54	0	0	0	1	0	0	89	4.0	N
HB386	ATG	TAG	27.58	18.3	52	0	0	0	1	0	0	40	3.0	C
HB388	ATG	TAG	6.30	460.8	55	1	0	0	1	0	0	51	2,980.0	H
HB390	ATG	TAG	19.90	376.2	66	1	0	0	0	0	0	32	2.0	C
HB391	ATG	TGG	22.30	186.4	48	1	0	1	1	0	0	78	47.0	C
HB392	ATG	TGG	22.90	1,614.7	55	1	0	0	1	0	1	52	14.0	C
HB393	ATG	TGG	17.20	304.1	25	1	0	0	0	0	0	40	2.0	N
HB397	ATG	TGG	7.70	37.8	39	0	1	0	1	2	1	42	0.5	N
HB400	ATG	TGG	29.58	113.9	54	0	1	0	2	0	0	40	48.0	C
HB403	ATG	TAG	20.41	12.9	47	0	1	0	1	1	0	30	0.5	C
HB404	ATG	TGG	6.80	209.3	62	1	0	0	1	0	0	67	5.0	H
HB405	ATG	TAG	17.40	340.5	63	1	2	0	2	2	0	61	31.0	C
HB406	ATG	TAG	9.40	25.3	32	0	1	0	2	0	0	34	4.0	C
HB409	ATG	TGG	5.20	10.2	44	1	0	0	1	2	1	31	2.0	N
HB410	ATG	TGG	23.41	256.2	55	0	0	0	1	2	1	57	3.0	C
HB413	ATG	TAG	17.83	308.2	64	1	0	1	2	2	0	67	5.0	C
HB415	ACG	TAG	20.50	155.4	56	0	0	0	1	0	0	77	5.0	N
HB419	ATG	TGG	27.90	386.5	38	1	0	0	1	0	0	76	5.0	C
HB420	ATG	TGG	29.98	219.8	57	1	0	0	1	0	0	77	11.0	C
HB423	AYS	TGG	18.70	143.0	55	0	0	0	1	1	0	42	5.0	C
HB424	ATG	TAG	21.09	201.8	53	0	0	0	1	2	0	47	4.0	N
HB425	ATG	TAG	28.12	306.2	63	1	0	0	1	0	0	227	3.0	N
HB428	ATG	TGG	18.60	61.0	63	0	0	0	1	1	1	49	9.0	C
HB430	ATG	TRG	28.74	269.0	60	1	0	0	1	1	1	54	14.0	C
HB433	ATG	TGG	19.55	91.7	57	1	1	0	0	0	1	71	28.0	H
HB434	ATG	TGG	15.38	129.4	57	0	0	0	1	1	0	44	3.0	C
HB438	ATG	TAG	18.19	139.6	61	1	0	0	1	1	0	35	2.0	N
HB439	ATG	TGA	17.20	260.9	53	1	0	0	2	0	0	31	5.0	N
HB444	ATG	TGG	16.15	42.0	47	0	1	0	1	1	1	28	228.0	H
HB445	ATG	TAG	26.30	297.2	59	0	0	0	1	0	0	57	3.0	N
HB448	ATG	TAG	10.98	350.4	57	0	0	0	1	1	0	81	8.0	C
HB450	ATG	TAG	14.10	144.6	56	0	0	0	0	0	0	33	2.0	N
HB451	ATG	TAG	14.06	181.2	50	1	1	0	1	1	0	50	2.0	N
HB452	ATG	TAG	18.20	313.2	53	1	0	0	1	0	0	35	2.0	N
HB454	ATG	TAG	25.86	495.3	56	1	0	0	1	1	0	42	6.0	C
HB455	ATG	TAG	15.56	150.8	62	0	0	0	1	1	0	33	2.0	N

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB456	ATG	TAG	29.00	150.6	61	1	0	1	1	0	0	42	2.0	N
HB461	ATG	TAG	19.40	215.2	38	1	0	0	2	2	0	53	0.5	N
HB463	ATG	TAG	4.89	3.8	48	0	1	0	1	0	0	36	3.0	N
HB464	ATG	TAG	12.90	61.9	33	0	1	0	2	2	0	127	0.5	N
HB465	ATG	TAG	8.10	97.7	35	0	0	0	1	1	0	30	2.0	N
HB467	ATG	TAG	29.70	159.3	56	0	0	0	1	0	0	61	2.0	C
HB468	ATG	TGG	14.06	513.7	53	1	0	1	1	1	0	74	7.0	N
HB469	ATG	TAG	16.10	45.4	80	0	0	0	0	0	0	35	5.0	C
HB470	ATG	TAG	18.01	225.8	50	1	0	0	1	0	0	40	2.0	N
HB471	ATG	TGG	21.40	109.2	30	1	0	0	1	1	1	40	2.0	N
HB472	ATG	TGG	23.60	158.1	59	0	0	0	0	1	1	48	2.0	N
HB473	ATG	TGG	26.90	91.2	25	0	0	0	1	1	1	42	0.5	N
HB475	ATG	TGG	29.50	85.8	47	0	0	0	1	0	1	46	3.0	C
HB477	ATG	TAG	19.50	270.6	52	1	0	0	2	0	0	34	2.0	N
HB478	ATG	TAG	31.60	131.3	69	0	0	0	1	1	0	36	3.0	N
HB484	AYG	TAG	20.60	89.1	30	1	0	0	1	1	0	68	13.0	N
HB485	ATG	TGG	10.60	14.2	26	0	0	0	1	1	1	47	4.0	N
HB487	ATG	TGG	25.40	138.1	55	1	0	0	1	1	0	40	0.5	N
HB488	ATG	TAG	17.70	162.2	48	1	0	0	1	0	0	43	2.0	N
HB490	ATG	TAG	32.90	186.8	52	1	0	1	1	0	0	31	3.0	N
HB492	ATG	TGG	11.29	8.9	51	0	0	0	1	1	0	59	2.0	C
HB496	ATG	TAG	17.40	417.7	53	1	0	0	2	0	0	53	2.0	N
HB501	ATG	TAG	20.60	9.6	48	0	0	0	1	1	0	41	3.0	N
HB502	ATG	TAG	42.40	634.1	60	0	0	0	2	2	1	73	17.0	H
HB503	ATG	TGG	14.97	478.1	54	1	0	0	1	1	0	188	270.0	H
HB505	ATG	TAG	20.90	175.5	52	1	0	0	1	0	0	63	2.0	N
HB506	ATA	TGG	17.96	248.6	61	0	0	0	1	0	0	26	3.0	N
HB507	ATG	TAG	22.73	172.2	55	1	0	0	1	0	0	36	2.0	N
HB508	ATG	TAG	24.30	219.4	56	0	0	0	1	0	0	40	2.0	N
HB510	ATG	TGG	40.05	140.4	49	1	0	0	0	0	0	81	0.5	C
HB511	ATG	TGG	3.51	7.4	43	0	0	0	1	1	0	33	0.5	N
HB513	ATG	TAG	18.50	27.4	18	0	0	0	1	1	0	45	0.5	N
HB519	ATG	TAG	18.40	143.9	59	1	0	0	1	0	0	40	4.0	N
HB521	ATG	TAG	23.59	137.3	69	0	0	0	1	1	0	34	0.5	N
HB522	ATG	TGG	16.48	276.4	66	1	0	0	1	1	1	67	23,249.0	H
HB523	ATG	TGR	13.90	393.5	77	0	0	0	0	0	0	40	5.0	N
HB524	ATG	TAG	35.70	289.0	53	1	0	0	1	0	0	46	0.5	N
HB526	ATG	TGG	16.38	94.0	50	1	0	0	1	1	1	78	6.0	C
HB528	ATG	TAG	7.39	49.3	60	0	0	0	1	0	0	28	2.0	N
HB531	ATG	TAG	25.76	520.4	65	1	0	0	1	0	0	42	5.0	N
HB532	ATG	TAG	45.51	1,552.0	62	1	0	1	0	0	0	51	2.0	C
HB534	ATG	TGG	18.55	916.1	67	1	0	1	0	0	1	60	8,541.0	H
HB535	ATG	TAG	11.60	138.2	64	0	1	0	1	0	0	33	0.5	N

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB536	AYG	TAG	18.10	272.5	70	0	0	0	1	0	0	32	0.5	N
HB537	ATG	TGG	6.26	426.1	74	1	0	1	2	2	0	50	5.0	H
HB540	ATG	TGG	24.54	456.4	51	1	1	0	1	1	1	82	6.0	H
HB541	ATG	TAG	44.68	110.1	56	0	0	0	1	1	0	49	6.0	N
HB547	ATG	TAG	22.14	112.1	57	0	0	0	1	1	0	44	4.0	N
HB548	ATG	TAG	17.92	99.0	62	0	0	1	1	1	0	47	133.0	H
HB551	ATG	TRG	14.60	615.9	53	1	0	1	2	0	1	88	34,451.5	H
HB556	ATG	TGG	7.71	636.5	68	1	1	0	2	0	1	26	6,290.7	H
HB559	ATG	TGG	11.29	100.9	70	1	1	1	2	2	0	74	546,300.0	H
HB560	ATG	TGG	12.34	251.0	61	1	1	1	1	2	1	73	3,062.0	H
HB562	ATG	TAG	25.86	138.5	58	0	0	0	1	1	0	54	2.0	N
HB565	ATG	TAG	16.30	320.2	64	1	0	0	2	2	0	95	5.0	C
HB567	ATG	TAG	16.92	70.6	51	0	0	0	1	0	0	43	1.0	N
HB568	ACG	TGG	4.50	6.5	41	0	0	0	2	0	0	41	9.0	N
HB571	ATG	TAG	21.59	566.8	60	1	0	1	1	1	0	45	2.0	N
HB572	ATG	TRG	26.90	591.8	62	1	0	1	1	0	1	54	17.0	C
HB574	ATG	TAG	10.12	33.7	65	1	1	1	2	0	0	54	5.0	C
HB576	ATG	TAG	10.20	62.3	49	0	0	0	1	0	0	32	0.5	N
HB581	ATG	TAG	48.22	33.0	66	1	0	1	0	0	0	48	7.0	C
HB582	ATG	TAG	36.61	162.9	61	1	0	0	2	0	0	48	5.5	C
HB586	ATG	TGG	13.47	754.4	67	1	0	0	2	0	0	29	20.0	C
HB587	ATG	TAG	29.44	253.8	48	1	0	0	1	1	0	53	5.0	N
HB588	ATG	TAG	18.51	199.0	58	0	0	0	1	0	0	130	17.0	C
HB589	TTG	TAG	27.10	211.3	57	1	0	0	1	0	0	82	4.0	C
HB594	ATG	TAG	21.41	406.9	68	0	0	0	2	0	0	28	4.0	N
HB597	ATG	TAG	14.24	361.2	44	0	1	0	2	2	0	115	6.0	N
HB600	ATG	TGG	15.80	457.9	50	0	0	0	0	0	0	114	3.0	N
HB601	ATG	TAG	20.80	193.4	58	1	1	1	1	2	0	63	3.0	N
HB605	ATG	TAG	11.98	23.5	53	0	0	0	1	1	0	27	5.0	N
HB608	ATG	TGG	16.80	313.1	56	0	2	0	1	2	0	26	2.0	N
HB613	ATG	TGG	9.65	102.2	58	0	0	0	1	1	1	255	355.0	H
HB617	ATG	TGG	22.60	29.3	23	0	0	0	1	0	0	175	1.0	N
HB620	ATG	TAG	15.90	14.4	46	0	0	0	1	1	0	33	2.0	N
HB622	ATG	TAG	26.90	95.3	36	1	1	0	2	2	0	41	2.0	C
HB624	ATG	TGG	18.60	71.0	24	0	0	0	0	0	0	31	0.5	N
HB626	ATG	TAG	9.60	135.0	45	0	1	0	0	0	0	37	2.0	N
HB628	ATG	TAG	21.20	299.4	32	1	0	0	1	0	0	42	0.5	N
HB630	ATG	TGG	16.10	183.9	30	1	1	0	1	0	1	80	3.0	N
HB642	ATG	TAG	27.30	42.5	45	1	0	0	2	2	0	40	2.0	N
HB643	ATG	TGG	2.40	6.4	32	0	0	0	2	2	1	26	2.0	N
HB652	ATG	TGG	6.80	192.3	44	1	0	0	1	1	1	80	0.5	N
HB655	ATG	TAG	8.50	54.4	47	0	1	0	1	1	0	44	5.0	N

Appendix A, continued

Label	Pc start	Pc stop	iron	ferritin	age	sex	eth	smo	fhx	fhcc	eAg	alt	afp	cond
HB662	ATG	TGG	21.90	47.6	21	0	0	0	0	0	1	66	4.0	N
HB665	ATG	TAG	13.10	63.2	49	0	0	0	1	1	0	32	0.5	N
HB668	ATG	TAG	3.70	8.6	38	0	0	0	2	2	0	34	1.0	N
HB669	ATG	TAG	18.70	107.8	63	0	0	0	1	0	0	30	3.0	N
HB676	ATG	TGG	21.20	171.1	67	0	0	0	0	0	0	36	0.5	N
HB681	ATG	TAG	12.80	177.1	44	1	0	0	1	2	0	40	0.5	N
HB693	ATG	TRG	24.10	242.2	35	1	0	0	2	0	1	293	5.0	N
HB695	ATG	TAG	21.40	597.8	52	1	0	0	2	2	0	74	6.0	N
HB707	ATG	TGG	25.50	21.1	45	0	0	0	1	0	1	43	3.0	N
HB708	ATG	TGG	26.20	285.5	59	1	0	0	1	0	1	70	7.0	N
HB709	ATG	TAG	14.80	205.5	46	0	0	0	1	0	0	32	2.0	N
HB714	ATG	TGG	15.00	93.1	21	0	1	0	1	1	1	51	2.0	N
HB716	CTG	TGG	17.20	278.5	60	1	0	0	0	0	0	45	0.5	C
HB718	ATG	TAG	12.92	89.8	63	0	0	0	0	0	0	41	2.0	N
HB720	ATG	TAG	19.50	83.6	55	0	0	0	1	0	0	37	1.0	N
HB724	ATG	TAG	1.70	7.2	49	0	0	0	1	1	0	41	0.5	N
HB725	ATG	TAG	5.30	508.2	30	0	1	0	1	1	0	48	534,637.0	H
HB728	ATG	TAG	21.40	211.7	70	0	0	0	0	0	0	42	0.5	N
HB734	ATG	TGG	34.00	292.5	26	1	0	0	0	0	0	35	2.0	N
HB740	ATG	TGG	7.10	92.5	18	1	0	0	1	0	1	34	0.5	N
HB743	AGG	TGG	13.40	1,601.8	51	0	0	0	1	2	0	67	3,052.0	H
HB749	ATG	TRG	10.90	438.4	50	1	0	1	1	1	1	90	2,574.0	H
HB753	ATG	TAG	8.20	3,383.0	53	1	0	1	0	0	0	103	4,720.1	H
HB756	ATT	TGG	18.50	56.5	58	0	0	0	1	1	0	128	778.0	H
HB762	ATG	TAG	14.40	281.4	63	1	0	0	2	2	0	107	577.0	H
HB763	ATG	TGG	30.80	118.2	69	0	0	0	1	1	1	50	5.0	C
HB777	ATG	TAG	31.40	471.5	65	0	0	0	1	1	0	47	3.0	N
HB780	ATG	TGG	19.20	199.9	47	1	1	1	0	0	1	45	8.0	C
HB781	ATG	TAG	12.70	1,340.0	49	1	0	0	2	2	0	165	54.5	H
HB785	ATG	TGG	10.50	195.2	40	1	0	0	1	1	0	85	28,534.1	H
HB794	ATG	TAG	13.20	236.4	57	1	0	0	1	0	0	34	4.0	C
HB795	ATG	TGG	16.00	211.5	61	0	0	0	1	1	0	88	2.0	C
HB798	ATG	TAG	18.60	211.0	56	0	0	0	1	1	0	37	4.0	N
HB802	ATG	TGG	25.40	145.5	52	0	0	0	1	1	0	29	2.0	N
HB804	ATG	TRG	21.20	996.1	56	1	1	0	1	0	0	215	2,333.0	H
HB805	ATG	TAG	14.60	25.8	50	0	0	0	1	0	0	25	4.0	N
HB806	ATG	TGG	5.40	713.8	35	1	0	1	1	0	0	96	227,935.0	H
HB807	ATG	TAG	8.60	477.6	62	1	2	1	2	0	0	76	379.0	H
HB808	ATG	TAG	7.40	655.9	49	1	0	0	0	0	0	305	131.5	H
HB809	ATG	TGG	20.60	422.0	62	1	1	1	1	1	1	38	1,730.0	H

Appendix B: Accession Numbers for Acute Hepatitis B

AB078032.1	GQ377519.1	GQ377569.1	GQ377619.1
AB116266.1	GQ377520.1	GQ377570.1	GQ377620.1
AB120308.1	GQ377521.1	GQ377571.1	GQ377621.1
AB231908.1	GQ377522.1	GQ377572.1	GQ377622.1
AB266536.1	GQ377523.1	GQ377573.1	GQ377623.1
AB298720.1	GQ377524.1	GQ377574.1	GQ377624.1
AY233274.1	GQ377525.1	GQ377575.1	GQ377625.1
AY233279.1	GQ377526.1	GQ377576.1	GQ377626.1
AY233283.1	GQ377527.1	GQ377577.1	GQ377627.1
AY233287.1	GQ377528.1	GQ377578.1	GQ377628.1
AY233292.1	GQ377529.1	GQ377579.1	GQ377629.1
DQ991753.2	GQ377530.1	GQ377580.1	GQ377630.1
EU859898.1	GQ377531.1	GQ377581.1	GQ377631.1
EU859899.1	GQ377532.1	GQ377582.1	GQ377632.1
EU859900.1	GQ377533.1	GQ377583.1	GQ377633.1
EU859901.1	GQ377534.1	GQ377584.1	GQ377634.1
EU859902.1	GQ377535.1	GQ377585.1	GQ377635.1
EU859903.1	GQ377536.1	GQ377586.1	GQ377636.1
EU859904.1	GQ377537.1	GQ377587.1	GQ377637.1
EU859905.1	GQ377538.1	GQ377588.1	GQ377638.1
EU859906.1	GQ377539.1	GQ377589.1	GQ377639.1
EU859907.1	GQ377540.1	GQ377590.1	GQ377640.1
EU859908.1	GQ377541.1	GQ377591.1	GQ377641.1
EU859909.1	GQ377542.1	GQ377592.1	GQ377642.1
EU859910.1	GQ377543.1	GQ377593.1	GQ377643.1
EU859911.1	GQ377544.1	GQ377594.1	GQ377644.1
EU859912.1	GQ377545.1	GQ377595.1	HPBADRM
EU859913.1	GQ377546.1	GQ377596.1	
EU859914.1	GQ377547.1	GQ377597.1	
EU859915.1	GQ377548.1	GQ377598.1	
EU859916.1	GQ377549.1	GQ377599.1	
EU859917.1	GQ377550.1	GQ377600.1	
EU859918.1	GQ377551.1	GQ377601.1	
EU859919.1	GQ377552.1	GQ377602.1	
EU859920.1	GQ377553.1	GQ377603.1	
EU859921.1	GQ377554.1	GQ377604.1	
EU859922.1	GQ377555.1	GQ377605.1	
EU859923.1	GQ377556.1	GQ377606.1	
EU859924.1	GQ377557.1	GQ377607.1	
EU859925.1	GQ377558.1	GQ377608.1	
EU859926.1	GQ377559.1	GQ377609.1	
EU859927.1	GQ377560.1	GQ377610.1	
EU859928.1	GQ377561.1	GQ377611.1	
FJ349229.1	GQ377562.1	GQ377612.1	
FJ349232.1	GQ377563.1	GQ377613.1	
GQ377514.1	GQ377564.1	GQ377614.1	
GQ377515.1	GQ377565.1	GQ377615.1	
GQ377516.1	GQ377566.1	GQ377616.1	
GQ377517.1	GQ377567.1	GQ377617.1	
GQ377518.1	GQ377568.1	GQ377618.1	

Appendix C: Accession Numbers for Chronic Hepatitis B

AB048702.1	AB078033.2	AB116093.1	AB219426.1	AB367393.1
AB048703.1	AB090268.1	AB116094.1	AB219427.1	AB367394.1
AB048704.1	AB090269.1	AB117758.1	AB219428.1	AB367395.1
AB048705.1	AB090270.1	AB117759.1	AB219429.1	AB367396.1
AB056513.1	AB091255.1	AB119251.1	AB219430.1	AB367397.1
AB056514.1	AB091256.1	AB119252.1	AB219529.1	AB367398.1
AB056515.1	AB106884.1	AB119253.1	AB219530.1	AB367399.1
AB056516.1	AB106885.1	AB119254.1	AB219531.1	AB367400.1
AB073821.1	AB109475.1	AB119255.1	AB219532.1	AB367401.1
AB073822.1	AB109476.1	AB119256.1	AB219533.1	AB367402.1
AB073823.1	AB109477.1	AB176642.1	AB219534.1	AB367403.1
AB073824.1	AB109478.1	AB176643.1	AB241109.1	AB367404.1
AB073825.1	AB109479.1	AB188241.1	AB241110.1	AB367405.1
AB073826.1	AB110075.1	AB188242.1	AB241111.1	AB367406.1
AB073827.1	AB111112.1	AB188243.2	AB241112.1	AB367407.1
AB073828.1	AB111113.1	AB188244.1	AB241113.1	AB367408.1
AB073829.1	AB111114.1	AB188245.2	AB241114.1	AB367409.1
AB073830.1	AB111115.1	AB195930.1	AB241115.1	AB367410.1
AB073831.1	AB111116.1	AB195931.1	AB241116.1	AB367411.1
AB073832.1	AB111117.1	AB195932.1	AB241117.1	AB367412.1
AB073833.1	AB111118.1	AB195933.1	AB246317.1	AB367413.1
AB073834.1	AB111119.1	AB195934.1	AB246335.1	AB367414.1
AB073835.1	AB111120.1	AB195935.1	AB246336.1	AB367415.1
AB073836.1	AB111121.1	AB195936.1	AB246337.1	AB367416.1
AB073837.1	AB111122.1	AB195937.1	AB246338.1	AB367417.1
AB073838.1	AB111123.1	AB195938.1	AB246339.1	AB367418.1
AB073839.1	AB111124.1	AB195939.1	AB246340.1	AB367419.1
AB073840.1	AB111125.1	AB195940.1	AB246341.1	AB367420.1
AB073841.1	AB113875.1	AB195941.1	AB246342.1	AB367421.1
AB073842.1	AB113876.1	AB195942.1	AB246343.1	AB367422.1
AB073843.1	AB113877.1	AB195943.1	AB246344.1	AB367423.1
AB073844.1	AB113878.1	AB195944.1	AB246345.1	AB367424.1
AB073845.1	AB115551.1	AB195945.1	AB246346.1	AB367425.1
AB073846.1	AB116076.1	AB195946.1	AB246347.1	AB367426.1
AB073847.1	AB116077.1	AB195947.1	AB246348.1	AB367427.1
AB073848.1	AB116078.1	AB195948.1	AB247916.1	AB367428.1
AB073849.1	AB116079.1	AB195949.1	AB250109.1	AB367429.1
AB073850.1	AB116080.1	AB195950.1	AB288026.1	AB367430.1
AB073851.1	AB116081.1	AB195951.1	AB298362.1	AB367431.1
AB073852.1	AB116082.1	AB195952.1	AB353764.1	AB367432.1
AB073853.1	AB116083.1	AB195953.1	AB365445.1	AB367433.1
AB073854.1	AB116084.1	AB195954.1	AB365446.1	AB367434.1
AB073855.1	AB116085.1	AB195955.1	AB365447.1	AB367435.1
AB073856.1	AB116086.1	AB195956.1	AB365448.1	AB485808.1
AB073857.1	AB116087.1	AB195957.1	AB365449.1	AB485809.1
AB073858.1	AB116088.1	AB202071.1	AB365450.1	AB485810.1
AB074047.1	AB116089.1	AB202072.1	AB365451.1	AB486012.1
AB074755.1	AB116090.1	AB205010.1	AB365452.1	AB493827.1
AB074756.1	AB116091.1	AB210819.1	AB365453.1	AB493828.1
AB076678.1	AB116092.1	AB210820.1	AB367392.1	AB493829.1

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AB493830.1	AF223965.1	AY206386.1	AY233289.1	AY739674.1
AB493831.1	AF297619.1	AY206387.1	AY233290.1	AY739675.1
AB493832.1	AF297620.1	AY206388.1	AY233291.1	AY741794.1
AB493833.1	AJ309371.1	AY206389.1	AY233293.1	AY741795.1
AB493834.1	AJ344115.1	AY206390.1	AY233294.1	AY741796.1
AB493835.1	AJ344116.1	AY206391.1	AY233295.1	AY741797.1
AB493836.1	AJ344117.1	AY206392.1	AY233296.1	AY741798.1
AB493837.1	AY033072.1	AY206393.1	AY247030.1	AY796030.1
AB493838.1	AY033073.1	AY217355.1	AY247031.1	AY796031.1
AB493839.1	AY057947.1	AY217356.1	AY247032.1	AY796032.1
AB493840.1	AY057948.1	AY217357.1	AY293309.1	AY800249.1
AB493841.1	AY090452.1	AY217358.1	AY373428.1	AY817509.1
AB493842.1	AY090453.1	AY217359.1	AY373429.1	AY817510.1
AB493843.1	AY090454.1	AY217360.1	AY373430.1	AY817511.1
AB493844.1	AY090455.1	AY217361.1	AY373431.1	AY817512.1
AB493845.1	AY090456.1	AY217362.1	AY373432.1	AY817513.1
AB493846.1	AY090457.1	AY217363.1	AY596102.1	AY817514.1
AB493847.1	AY090458.1	AY217364.1	AY596103.1	AY817515.1
AB493848.1	AY090459.1	AY217365.1	AY596104.1	AY935700.1
AB549213.1	AY090460.1	AY217366.1	AY596105.1	DQ078791.1
AF043593.1	AY090461.1	AY217367.1	AY596106.1	DQ089756.1
AF043594.1	AY167089.1	AY217368.1	AY596107.1	DQ089757.1
AF068756.1	AY167090.1	AY217369.1	AY596108.1	DQ089758.1
AF121239.1	AY167091.1	AY217370.1	AY596109.1	DQ089759.1
AF121240.1	AY167092.1	AY217371.1	AY596110.1	DQ089760.1
AF121241.1	AY167093.1	AY217372.1	AY596111.1	DQ089761.1
AF121242.1	AY167094.1	AY217373.1	AY596112.1	DQ089762.1
AF121243.1	AY167095.1	AY217374.1	AY641558.1	DQ089763.1
AF121244.1	AY167096.1	AY217375.1	AY641559.1	DQ089764.1
AF121245.1	AY167097.1	AY217376.1	AY641560.1	DQ089765.1
AF121246.1	AY167098.1	AY217377.1	AY641561.1	DQ089766.1
AF121247.1	AY167099.1	AY217378.1	AY641562.1	DQ089767.1
AF121248.1	AY167100.1	AY220697.1	AY641563.1	DQ089768.1
AF121249.1	AY167101.1	AY220698.1	AY721605.1	DQ089769.1
AF121250.1	AY167102.1	AY220699.1	AY721606.1	DQ089770.1
AF121251.1	AY179734.1	AY220700.1	AY721607.1	DQ089771.1
AF160501.1	AY179735.1	AY220701.1	AY721608.1	DQ089772.1
AF182802.1	AY206373.1	AY220702.1	AY721609.1	DQ089773.1
AF182803.1	AY206374.1	AY220703.1	AY721610.1	DQ089774.1
AF223954.1	AY206375.1	AY220704.1	AY721611.1	DQ089775.1
AF223955.1	AY206376.1	AY233275.1	AY721612.1	DQ089776.1
AF223956.1	AY206377.1	AY233276.1	AY738139.1	DQ089777.1
AF223957.1	AY206378.1	AY233277.1	AY738140.1	DQ089778.1
AF223958.1	AY206379.1	AY233278.1	AY738141.1	DQ089779.1
AF223959.1	AY206380.1	AY233280.1	AY738142.1	DQ089780.1
AF223960.1	AY206381.1	AY233281.1	AY738143.1	DQ089781.1
AF223961.1	AY206382.1	AY233282.1	AY738144.1	DQ089782.1
AF223962.1	AY206383.1	AY233285.1	AY738145.1	DQ089783.1
AF223963.1	AY206384.1	AY233286.1	AY738146.1	DQ089784.1
AF223964.1	AY206385.1	AY233288.1	AY738147.1	DQ089785.1

Appendix C, continued

DQ089786.1	DQ478895.1	EU579441.1	FJ032354.1	FJ386590.1
DQ089787.1	DQ478896.1	EU579442.1	FJ032355.1	FJ386591.1
DQ089788.1	DQ478897.1	EU579443.1	FJ032356.1	FJ386592.1
DQ089789.1	DQ478898.1	EU589335.1	FJ032357.1	FJ386593.1
DQ089790.1	DQ478899.1	EU589336.1	FJ032358.1	FJ386594.1
DQ089791.1	DQ478900.1	EU589337.1	FJ032359.1	FJ386595.1
DQ089792.1	DQ478901.1	EU589338.1	FJ032360.1	FJ386596.1
DQ089793.1	DQ536410.1	EU589339.1	FJ032361.1	FJ386597.1
DQ089794.1	DQ536412.1	EU589340.1	FJ349205.1	FJ386598.1
DQ089795.1	DQ776247.2	EU589341.1	FJ349206.1	FJ386599.1
DQ089796.1	DQ890381.1	EU589342.1	FJ349207.1	FJ386600.1
DQ089797.1	DQ995801.1	EU589343.1	FJ349208.1	FJ386601.1
DQ089798.1	DQ995802.1	EU589344.1	FJ349209.1	FJ386602.1
DQ089799.1	DQ995803.1	EU589345.1	FJ349210.1	FJ386603.1
DQ089800.1	DQ995804.1	EU589346.1	FJ349211.1	FJ386604.1
DQ089801.1	EF137802.1	EU595030.1	FJ349212.1	FJ386605.1
DQ089802.1	EF137803.1	EU595031.1	FJ349213.1	FJ386606.1
DQ089803.1	EF464097.1	EU796066.1	FJ349214.1	FJ386607.1
DQ089804.1	EF464098.1	EU796067.1	FJ349216.1	FJ386608.1
DQ377158.1	EF464099.1	EU796068.1	FJ349217.1	FJ386609.1
DQ463787.1	EF473971.1	EU796069.1	FJ349218.1	FJ386610.1
DQ463788.1	EF473972.1	EU796070.1	FJ349219.1	FJ386611.1
DQ463789.1	EF473973.1	EU796071.1	FJ349220.1	FJ386612.1
DQ463790.1	EF473974.1	EU796072.1	FJ349221.1	FJ386613.1
DQ463791.1	EF473975.1	EU833889.1	FJ349222.1	FJ386614.1
DQ463792.1	EF473976.1	EU833890.1	FJ349223.1	FJ386615.1
DQ463793.1	EF473977.1	EU833891.1	FJ349225.1	FJ386616.1
DQ463794.1	EU305540.1	FJ032331.1	FJ349227.1	FJ386617.1
DQ463795.1	EU305541.1	FJ032332.1	FJ349234.1	FJ386618.1
DQ463796.1	EU305542.1	FJ032333.1	FJ349235.1	FJ386619.1
DQ463797.1	EU305543.1	FJ032334.1	FJ349236.1	FJ386620.1
DQ463798.1	EU305544.1	FJ032335.1	FJ349240.1	FJ386621.1
DQ463799.1	EU305545.1	FJ032336.1	FJ356715.1	FJ386622.1
DQ463800.1	EU305547.1	FJ032337.1	FJ356716.1	FJ386623.1
DQ463801.1	EU305548.1	FJ032338.1	FJ386574.1	FJ386624.1
DQ463802.1	EU366116.2	FJ032339.1	FJ386575.1	FJ386625.1
DQ478881.1	EU366118.2	FJ032340.1	FJ386576.1	FJ386626.1
DQ478882.1	EU366129.2	FJ032341.1	FJ386577.1	FJ386627.1
DQ478883.1	EU366132.2	FJ032342.1	FJ386578.1	FJ386628.1
DQ478884.1	EU366133.2	FJ032343.1	FJ386579.1	FJ386629.1
DQ478885.1	EU410079.1	FJ032344.1	FJ386580.1	FJ386630.1
DQ478886.1	EU410080.1	FJ032345.1	FJ386581.1	FJ386631.1
DQ478887.1	EU410081.1	FJ032346.1	FJ386582.1	FJ386632.1
DQ478888.1	EU410082.1	FJ032347.1	FJ386583.1	FJ386633.1
DQ478889.1	EU547558.1	FJ032348.1	FJ386584.1	FJ386634.1
DQ478890.1	EU547559.1	FJ032349.1	FJ386585.1	FJ386635.1
DQ478891.1	EU547560.1	FJ032350.1	FJ386586.1	FJ386636.1
DQ478892.1	EU547561.1	FJ032351.1	FJ386587.1	FJ386637.1
DQ478893.1	EU547562.1	FJ032352.1	FJ386588.1	FJ386638.1
DQ478894.1	EU547563.1	FJ032353.1	FJ386589.1	FJ386639.1

Appendix C, continued

FJ386640.1	FJ518810.1	FJ562264.1	FJ562314.1	FJ787452.1
FJ386641.1	FJ518811.1	FJ562265.1	FJ562315.1	FJ787453.1
FJ386642.1	FJ518812.1	FJ562266.1	FJ562316.1	FJ787454.1
FJ386643.1	FJ518813.1	FJ562267.1	FJ562317.1	FJ787455.1
FJ386644.1	FJ562218.1	FJ562268.1	FJ562318.1	FJ787456.1
FJ386645.1	FJ562219.1	FJ562269.1	FJ562319.1	FJ787457.1
FJ386646.1	FJ562220.1	FJ562270.1	FJ562320.1	FJ787458.1
FJ386647.1	FJ562221.1	FJ562271.1	FJ562321.1	FJ787459.1
FJ386648.1	FJ562222.1	FJ562272.1	FJ562322.1	FJ787460.1
FJ386649.1	FJ562223.1	FJ562273.1	FJ562323.1	FJ787461.1
FJ386650.1	FJ562224.1	FJ562274.1	FJ562324.1	FJ787462.1
FJ386651.1	FJ562225.1	FJ562275.1	FJ562325.1	FJ787463.1
FJ386652.1	FJ562226.1	FJ562276.1	FJ562326.1	FJ787464.1
FJ386653.1	FJ562227.1	FJ562277.1	FJ562327.1	FJ787465.1
FJ386654.1	FJ562228.1	FJ562278.1	FJ562328.1	FJ787466.1
FJ386655.1	FJ562229.1	FJ562279.1	FJ562329.1	FJ787467.1
FJ386656.1	FJ562230.1	FJ562280.1	FJ562330.1	FJ787468.1
FJ386657.1	FJ562231.1	FJ562281.1	FJ562331.1	FJ787469.1
FJ386658.1	FJ562232.1	FJ562282.1	FJ562332.1	FJ787470.1
FJ386659.1	FJ562233.1	FJ562283.1	FJ562333.1	FJ787471.1
FJ386660.1	FJ562234.1	FJ562284.1	FJ562334.1	FJ787472.1
FJ386661.1	FJ562235.1	FJ562285.1	FJ562335.1	FJ787473.1
FJ386662.1	FJ562236.1	FJ562286.1	FJ562336.1	FJ787474.1
FJ386663.1	FJ562237.1	FJ562287.1	FJ562337.1	FJ787475.1
FJ386664.1	FJ562238.1	FJ562288.1	FJ562338.1	FJ787476.1
FJ386665.1	FJ562239.1	FJ562289.1	FJ562339.1	FJ787477.1
FJ386666.1	FJ562240.1	FJ562290.1	FJ562340.1	FJ787478.1
FJ386667.1	FJ562241.1	FJ562291.1	FJ589065.1	FJ787479.1
FJ386668.1	FJ562242.1	FJ562292.1	FJ589067.1	FJ787480.1
FJ386669.1	FJ562243.1	FJ562293.1	FJ657519.1	FJ787481.1
FJ386670.1	FJ562244.1	FJ562294.1	FJ657522.1	FJ787482.1
FJ386671.1	FJ562245.1	FJ562295.1	FJ657525.1	FJ787483.1
FJ386672.1	FJ562246.1	FJ562296.1	FJ657528.1	FJ787484.1
FJ386673.1	FJ562247.1	FJ562297.1	FJ657529.1	FJ787485.1
FJ386674.1	FJ562248.1	FJ562298.1	FJ787436.1	FJ787486.1
FJ386675.1	FJ562249.1	FJ562299.1	FJ787437.1	FJ787487.1
FJ386676.1	FJ562250.1	FJ562300.1	FJ787438.1	FJ787488.1
FJ386677.1	FJ562251.1	FJ562301.1	FJ787439.1	FJ787489.1
FJ386678.1	FJ562252.1	FJ562302.1	FJ787440.1	FJ787490.1
FJ386679.1	FJ562253.1	FJ562303.1	FJ787441.1	FJ882610.1
FJ386680.1	FJ562254.1	FJ562304.1	FJ787442.1	FJ882611.1
FJ386681.1	FJ562255.1	FJ562305.1	FJ787443.1	FJ882612.1
FJ386682.1	FJ562256.1	FJ562306.1	FJ787444.1	FJ882613.1
FJ386683.1	FJ562257.1	FJ562307.1	FJ787445.1	FJ882614.1
FJ386684.1	FJ562258.1	FJ562308.1	FJ787446.1	FJ882615.1
FJ386685.1	FJ562259.1	FJ562309.1	FJ787447.1	FJ882616.1
FJ386686.1	FJ562260.1	FJ562310.1	FJ787448.1	FJ882617.1
FJ386687.1	FJ562261.1	FJ562311.1	FJ787449.1	FJ882618.1
FJ386688.1	FJ562262.1	FJ562312.1	FJ787450.1	GQ227692.1
FJ386689.1	FJ562263.1	FJ562313.1	FJ787451.1	GQ227693.1

Appendix C, continued

GQ227694.1	GQ475346.1	HM011497.1
GQ227695.1	GQ475347.1	HM011498.1
GQ227696.1	GQ475348.1	HM011499.1
GQ227697.1	GQ475349.1	HM011500.1
GQ259588.1	GQ475350.1	HM011501.1
GQ331046.1	GQ475351.1	HM011502.1
GQ331047.1	GQ475352.1	HM011503.1
GQ331048.1	GQ475353.1	HM011504.1
GQ372968.1	GQ475354.1	HM585187.1
GQ475305.1	GQ475355.1	HM585188.1
GQ475306.1	GQ475356.1	HM585189.1
GQ475307.1	GQ475357.1	HM585190.1
GQ475308.1	GQ872210.1	HM585191.1
GQ475309.1	GU357842.1	HM585192.1
GQ475310.1	GU357843.1	HM585193.1
GQ475311.1	GU357844.1	HM585194.1
GQ475312.1	GU357845.1	HM585195.1
GQ475313.1	GU357846.1	HM585196.1
GQ475314.1	HM011465.1	HM585197.1
GQ475315.1	HM011466.1	HM585198.1
GQ475316.1	HM011467.1	HM585199.1
GQ475317.1	HM011468.1	HM585200.1
GQ475318.1	HM011469.1	HM590471.1
GQ475319.1	HM011470.1	HM590472.1
GQ475320.1	HM011471.1	HM590473.1
GQ475321.1	HM011472.1	HM590474.1
GQ475322.1	HM011473.1	HM622135.1
GQ475323.1	HM011474.1	HM627320.1
GQ475324.1	HM011475.1	HPBADW1
GQ475325.1	HM011476.1	HPBADW2
GQ475326.1	HM011477.1	HPBADW3
GQ475327.1	HM011478.1	HQ231877.1
GQ475328.1	HM011479.1	HQ231878.1
GQ475329.1	HM011480.1	HQ231879.1
GQ475330.1	HM011481.1	HQ231880.1
GQ475331.1	HM011482.1	HQ231881.1
GQ475332.1	HM011483.1	HQ231882.1
GQ475333.1	HM011484.1	HQ231883.1
GQ475334.1	HM011485.1	HQ231884.1
GQ475335.1	HM011486.1	HQ231885.1
GQ475336.1	HM011487.1	HQ236014.1
GQ475337.1	HM011488.1	HQ378247.1
GQ475338.1	HM011489.1	HQ622095.1
GQ475339.1	HM011490.1	NC_003977.1
GQ475340.1	HM011491.1	X59795.1
GQ475341.1	HM011492.1	Y18855.1
GQ475342.1	HM011493.1	Y18856.1
GQ475343.1	HM011494.1	Y18857.1
GQ475344.1	HM011495.1	Y18858.1
GQ475345.1	HM011496.1	

Scholarly Contributions

List of publications

Chook JB, Ngeow YF, Ng KP, Tiang YP, Khang TF, Mohamed R (2013). Comparative analysis of viral genomes from acute and chronic hepatitis B reveals novel variants associated with lower rate of chronicity. *J Med Virol* **85**:419-424. [ISI-cited publication]

Chook JB, Ngeow YF, Yap SF, Tan TC, Mohamed R (2011). Combined use of wild-type HBV precore and high serum iron marker as a potential tool for the prediction of cirrhosis in chronic hepatitis B infection. *J Med Virol* **83**:594-601. [ISI-cited publication]

List of conference presentations

Rosmawati M, Chook JB, Yap SF, Tan TC, Ngeow YF. Hepatitis B precore wild-type together with serum ferritin for the better prediction of hepatocellular carcinoma. BIT's 2nd Annual Congress of Biomarkers. Oral presentation at Beijing, China, 7-9 November 2011.

Chook JB, Ngeow YF, Yap SF, Tan TC, Mohamed R. Novel combination biomarker in hepatitis B-related cirrhosis. The 21st conference of the Asian Pacific Association for the Study of Liver (APASL). Poster presentation at Bangkok, Thailand, 17-20 February 2011.

Chook JB, Ngeow YF, Tan TC, Yap SF, Mohamed R. Better prediction of the development of liver cirrhosis in chronic hepatitis B infection using both HBV genetic and serum iron biomarkers. Oral presentation at 46th Malaysian Society of Parasitology and Tropical Medicine (MSPTM) Annual Scientific Conference: Infectious Disease: From Epidemic to Pandemic in Grand Season Hotel, Kuala Lumpur, Malaysia on 24-25 March 2010.

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