MOLECULAR ASSOCIATION STUDIES IN PREDICTING RISK OF ADVANCED LIVER DISEASE IN HBV INFECTED PATIENTS

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ABSTRACT

Hepatitis B virus infection (HBV) is a serious public health problem and a risk factor for liver cirrhosis and hepatocellular carcinoma (HCC). Globally it affects more than 2 billion individuals and over 600,000 persons die annually due to the consequence of the disease. In this study, we aim to investigate association between genetic polymorphisms of various candidate genes, of microRNAs expression as well as of HLA-DQ gene and risk of progression of HBV infection to cirrhosis / HCC in a Malaysian population. A total of 526 subjects were enrolled in this study. The participants were made up of 423 chronic HBV patients without cirrhosis/HCC and 103 chronic HBV patients with cirrhosis /HCC. Genotyping of 19 SNPs from 10 genes was carried out using Sequenom MassARRAY® platform. The microRNA profiling was performed using Affymetrix GeneChip® miRNA 3.0 Array. The HLA typing of HLA-DQA1 and HLA–DQB1 was done using the LAB Type PCR- sequence specific oligonucleotide (PCR-SSO) probes technique and Luminex profiling system. Data was analyzed using SPSS version 16.0 statistical software. The microarray results were analyzed using Expression console software, Transcriptome Analysis Console (TAC) and the Ingenuity Pathway Analysis (IPA) software. The raw data from Luminex were analyzed by HLA Fusion software. The result of genetic association study showed that rs12304647 of microRNA-196A2 has a protective effect from progression to cirrhosis/HCC (OR=0.37, 95% CI=0.15 - 0.89, p=0.027). The results of miRNA profiling revealed that six miRNAs were up-regulated (miRNA-935, miRNA-342-5p, miRNA- 339, miRNA- 4508, miRNA- 3615 and miRNA-3200-5p) while two down-regulated (miRNA-182 and miRNA-4485) between the HBV with and without cirrhosis/HCC groups (fold change ≥ 2 or ≤ -2, p-value ≤ 0.05). The results of HLA typing revealed the presence of five HLA-DQA1 alleles (-DQA1*01, -DQA1*02, -DQA1*03, -DQA1*05 and -DQA1*06) and five HLA-DQB1 alleles (-DQB1*02, -DQB1*03, -DQB1*04, -DQB1*05 and -DQB1*06). Among all patients in this study, 66% of HBV

patients with cirrhosis / HCC and 62% of HBV patients without cirrhosis / HCC are carries of HLA-DQA1*01 (p value= 0.908, OR= 0.95, CI= 0.41 - 2.10). Moreover, 73% of HBV patients with cirrhosis / HCC and 60% of HBV patients without cirrhosis / HCC are carries of HLA-DQB1*03 (p value= 0.320, OR= 1.56, CI= 0.65 - 3.72).

In summary, this study showed that there is a significant association between microRNA-196A2 rs12304647 gene and reduced risk of progression to cirrhosis/HCC in patients with chronic HBV infection. In addition, the eight identified microRNAs may have a significant clinical value in diagnosis of progression of disease in HBV infected patients. However, no significant association was found between the identified HLA-DQ alleles and progression of HBV to cirrhosis and HCC.

ABSTRAK

Jangkitan virus Hepatitis B (HBV) adalah masalah kesihatan awam yang serius dan punca yang paling penting bagi sirosis hati dan karsinoma hepatoselular (HCC). Pada peringkat global, ia memberi kesan kepada lebih daripada 2 bilion individu dan lebih daripada 600,000 orang maut setiap tahun akibat daripada penyakit ini. Dalam kajian ini, objektif kami ialah untuk menyiasat perkaitan antara polimorfisme genetik pelbagai gen calon, antara microRNAs serta antara gen HLA- DQ dan risiko perkembangan jangkitan HBV kepada sirosis / HCC di kalangan penduduk Malaysia. Seramai 526 orang subjek terlibat dalam kajian ini. Para subjek terdiri daripada 423 orang pesakit HBV kronik tanpa sirosis / HCC dan 103 orang pesakit HBV kronik yang mengalami sirosis / HCC. Pencirian genotaip bagi 19 polimorfisme nukleotid tunggal (SNP) daripada 10 gen telah dijalankan dengan menggunakan platform SEQUENOM MassARRAY®. Profil microRNA dilakukan dengan menggunakan Affymetrix GeneChip® miRNA 3.0 Array. Pencirian HLA bagi HLA- DQA1 dan HLA-DQB1 telah dilakukan dengan menggunakan Jenis PCR- urutan oligonucleotide tertentu (PCR- SSO) dan pemprofilan sistem Luminex. Penganalisan data dilakukan dengan menggunakan perisian statistik SPSS versi 16.0. Keputusan microarray dianalisis dengan menggunakan perisian konsol Expression , Transcriptome Analisis Console (TAC) dan perisian Ingenuity Pathway Analisis (IPA). Data mentah dari Luminex dianalisis dengan menggunakan perisian HLA Fusion. Hasil kajian menunjukkan bahawa rs12304647 daripada microRNA - 196A2 mempunyai kesan perlindungan daripada perkembangan kepada sirosis / HCC (OR=0.37, 95% CI=0.15 - 0.89, p=0.027). Keputusan profil miRNA mendedahkan bahawa enam miRNA telah dikawal selia menaik (miRNA-935, miRNA-342-5p, miRNA- 339, miRNA- 4508, miRNA- 3615 and miRNA-3200-5p) manakala dua ke dikawal selia menurun (miRNA-182 and miRNA-4485) apabila perbandingan dibuat antara kumpulan HBV dengan dan HBV tanpa sirosis / HCC (fold change ≥ 2 or ≤ -2, p-value ≤ 0.05). Keputusan jenis HLA

mendedahkan kehadhiran lima alel bagi HLA- DQA1 (-DQA1*01, -DQA1*02, -DQA1*03, -DQA1*05 and -DQA1*06) dan lima alel bagi HLA- DQB1 (-DQB1*02, -DQB1*03, -DQB1*04, -DQB1*05 and -DQB1*06). Kami mendapati bahawa HLA-DQA1*01 dan HLA-DQB1*03 adalah alel yang paling biasa terdapat di kalangan pesakit HBV manakala kekerapan alel tersebut dalam HBV dengan sirosis / HCC dibandingkan dengan HBV tanpa sirosis / kumpulan HCC ialah 66% lawan 62% bagi HLA-DQA1*01 and 73% lawan 60% bagi HLA-DQB1*03. Kesimpulannya, kajian ini menunjukkan bahawa terdapat hubungan yang signifikan antara gen microRNA-196A2 rs12304647 dan penurunan risiko bagi perkembangan kepada sirosis / HCC pada pesakit yang dijangkitkan HBV kronik. Di samping itu, lapan microRNA dikenal pasti mungkin mempunyai nilai klinikal penting dalam diagnosis perkembangan penyakit dalam HBV pesakit yang dijangkiti . Walau bagaimanapun, tiada hubungan yang signifikan yang dapat dikenal pasti antara alel HLA-DQ dan perkembangan HBV kepada sirosis dan HCC.

Dedication

I dedicate this thesis to

My mother (Rest in Peace) and father,

My brothers Behrouz and Behzad

And

To GOD almighty

For his infinite blessings

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Sincerely,

Behnaz Riazalhosseini 2016

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LIST OF SYMBOLS AND ABBREVIATIONS

% : Percentage

± : Plus minus

µg : microgram

µL : microliter

CHB : Chronic hepatitis B

LC : Liver cirrhosis

MiRNA : microRNA

HCC : Hepatocellular carcinoma

HBcAg : Hepatitis B core antigen

HBsAg : Hepatitis B surface antigen

HBeAg : Hepatitis B e antigen

HBV : Hepatitis B virus

HCV : Hepatitis C virus

HLA : Human Leucocyte Antigen

SNP : Single nucleotide polymorphism

WHO : World Health Organization

IL-6 : Interleukin-16

IL28-B : Interleukin-28-B

STAT4 : Signal Transducer and Activator of Transcription 4

CXCR1 : Chemokine (C-X-C Motif) receptor 1

FOXP3 : Forkhead Box P3

miR146A : microRNA146A

miR196A2 : microRNA196A2

RAD52 : RAD52 Homolog, DNA Repair Protein

ATF6 : Activating Transcription Factor 6

TEP1 : Telomerase-Associated Protein 1

GRIN2A : Glutamate receptor ionotropic N-methyl D-aspartate 2A

VEGFA : Vascular endothelial growth factor alpha

KIF1B : Kinesin family member 1B

IGF1R : Insulin-like growth factor 1 receptor

DLC1 : Deleted in Liver Cancer 1

TNF-α : Tumour necrosis factor-alpha

GWAS : Genome wide association study

TSG : Tumour suppressor gene

IL-8 : Interleukin-8

UPR : Unfolded protein response

EDTA : Ethylene diamine tetra acetic acid

UMMC : University Malaya Medical Centre

HWE : Hardy-Weinberg Equilibrium

SPSS : Statistical Package for the Social Sciences

OR : Odds ratio

CI : Confidence intervals

LD : Linkage disequilibrium

ALT : Alanine aminotransferase

AST : Aspartate aminotransferase

TAC : Transcriptome Analysis Console

IPA : Ingenuity Pathway Analysis

TGFβ : Transforming growth factor beta

VEGF : Vascular endothelial growth factor

MTSS1 : Metastasis suppressor 1

FASN : Fatty acid synthase

HMGCR : 3-hydroxyl-3-methylglutaryl CoA reductase

SREBP : Sterol regulatory element- binding protein

CEBPA : CCAAT enhancer binding protein alpha

HLA : Human Leucocyte Antigen

MHC : Major Histocompatibility Complex

APC : Antigens presenting cells

NK : Natural Killer cells

PCR : Polymerase chain reaction amplification

SSO : Sequence specific oligonucleotide

RISC : RNA-induced silencing complex

SMAD4 : SMAD family member 4

CHAPTER 1: GENERAL INTRODUCTION

1.1 General introduction

Hepatitis B virus (HBV) infection is a serious public health problem affecting approximately 2 billion people worldwide (Liaw & Chu, 2009; Zhang et al., 2015). HBV is the most significant cause of chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (He, Zhao, Zhang, & Lin, 2006; Pratedrat et al., 2015). According to the World Health Organization (WHO), about 240 million individuals are chronically infected with the HBV virus and around 780,000 deaths per year is reported due to the consequences of HBV infection including LC and HCC (World Health Organization, 2015).

HBV is a potentially life-threatening infection that can be transferred through contact with the body fluids or blood of an infected individual (World Health Organization, 2015). Although it can be prevented by available vaccine (World Health Organization, 2015), however, mother to child transmission is still the main important mechanism of infection especially in countries with a high incidence of HBV infection (Gentile & Borgia, 2014). The high endemic area of HBV infection and HBV-associated liver disease are mostly in Southeast Asia (including Malaysia) and Africa, with a prevalence of approximately 8% of population (Kao & Chen; Zeng, 2014). Even with current advances in management and treatment of HBV, patients with LC and HCC still have a poor prognosis. For patients with HBV- related HCC, there is a five year survival rate of 5% in developing countries due to the lack of effective treatment in most patients (Peng., 2014). To date, HBV infection remains a serious public problem due to its complex aetiology and variable epidemiology between different populations (Akkiz, Bayram, Bekar, Akgollu, & Ulger, 2011; Kar, 2014; Kou et al., 2014). Although environmental factors such as alcohol drinking and smoking result in susceptibility to progression of

HBV to cirrhosis and HCC, the results from ethnic differences, familial and twin studies have indicated the significant role of genetic factors in determining the consequence of HBV infection (Feitelson, 1995; Frodsham, 2005; Mbarek et al., 2011; Ohbayashi, Okochi, & Mayumi; Zhong et al., 2012). Over the past years, molecular genetic studies has led to the identification of some polymorphic genes which are involved in HBV infection outcomes. However, more recently advances in technology and discovery of circulatory microRNA have enabled researchers to find possible non-invasive biomarkers for prognostic of HBV-related liver disease (Xie., 2014). Lately, it has shown that polymorphisms in Human Leucocyte Antigen (HLA) effect on immune response may lead to susceptibility or resistance to HBV infection as well as progression of HBV disease (Hennig & Hall, 2012).

Collective evidence have reported a link between some candidate genes and CHB progression in different populations such as Japanese, Chinese, Turkish and Egyptian (Akkiz, Bayram, Bekar, Akgollu, & Uskudar, 2011; Kamatani et al., 2009; Othman, Aref, Mohamed, & Ibrahim, 2013; Wu et al., 2013). In addition, many studies indicated differentially expressed miRNA between HBV infection and HBV associated cirrhosis and HCC (Li., 2010; Tsai et al., 2009; Zhou et al., 2011). MicroRNAs can regulate gene expression at the post-transcriptional level and play an important role in regulating epigenetic machinery (Xie, Zhang, Liu, Zeng, & Wu, 2014). Considering the significant roles of miRNAs in various crucial biological processes, it is clear that dysregulation of miRNAs expression is implicated in a variety of human diseases such as HBV infection and HBV associated cirrhosis and HCC (Liu, Yeh, & Chen, 2011). Furthermore, several studies have shown the link between specific HLA alleles with disease progression in HBV infected individuals (Al-Qahtani . 2014; Doganay et al., 2014; Lin et al., 2010). It have been reported that HLA gene contributes to the host response against HBV infection and individuals with different HLA type may be different in predisposition or resistance

to outcomes of HBV infection (Jiang, Wang, Liu, & Liu, 2003). Earlier studies indicated a relationship between HLA class II alleles and susceptibility or persistent of HBV infection (Liu & Cheng, 2007; Singh, Kaul, Kaul, & Khan, 2007), however, the results were inconsistent between the different populations.

Considering the important role of genetic polymorphism, miRNA function and HLA molecules in the consequences of HBV infection, we aim to investigate the impact of single nucleotide polymorphism (SNP), miRNA profiling and HLA allele between HBV infected patients with and without cirrhosis and HCC with the purpose of finding biomarkers for diagnosis and treatment strategies for prevention of HBV disease progression in Malaysia.

This study contains three different sections investigating possible biomarkers of HBV associated cirrhosis and HCC at the levels of (a) genomics (b) HLA alleles and (c) microRNAs. Utilizing the biomarkers for differentiating HBV disease stages could help physicians in early diagnosis and appropriate management to be taken. Information from the present study may be utilized to improve the management of those HBV patients who are at higher risk to progress to LC and HCC.

In the following chapters of the thesis, the different scopes of the work representing studies on the (a) genomics (b) HLA alleles and (c) microRNAs aspect of the work, will be in separate chapters, each complete with literature review, justification, specific objectives, methodology, results, discussion and conclusion. This is to be followed by a General Discussion and Conclusion at the end.

1.2 Literature review

1.2.1 Definition of hepatitis

Hepatitis is a general term referring to inflammation of the liver (World Health Organization, 2016). It is usually caused by viral infection but there are other possible causes of hepatitis such as alcohol and certain drugs (Figure 1.1) (World Health Organization, 2016). Viral hepatitis refers to infections caused by viruses and the most common viral infection of the liver are hepatitis A, B, C, D and E (World Health Organization, 2016). Among these, hepatitis B, C and D are more dangerous due to the mode of transmission by infected blood or blood products, invasive medical procedures using contaminated equipment and for HBV transmission from mother to baby at birth as well as by sexual contact (Table 1.1). However, hepatitis A and E can be transmitted by contaminated water or food (World Health Organization, 2016). Hepatitis B and C together are the most common cause of liver cirrhosis and HCC in the world (World Health Organization, 2016). In the present study we focus only on hepatitis B infection because of high prevalence of this infectious disease in the Southeast Asian region including Malaysia (Scotto, Martinelli, Di Tullio, & Fazio, 2010).

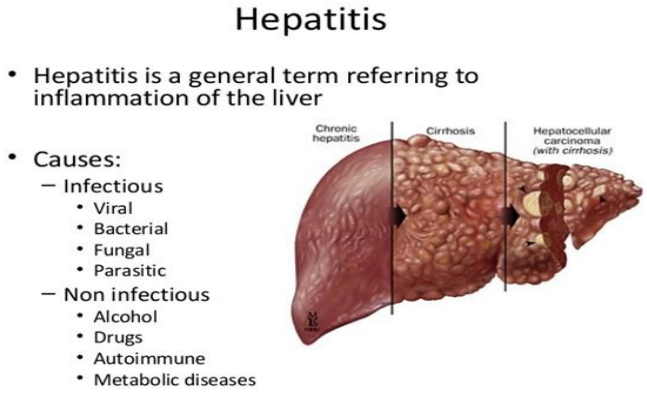


Figure 1.1: Inflammation of the liver

Available at http://www.slideshare.net/drashokrattan/laboratory-diagnosis-of-viral-hepatitis-b-c

Table 1.1: Groups of people at high risk of HBV infection

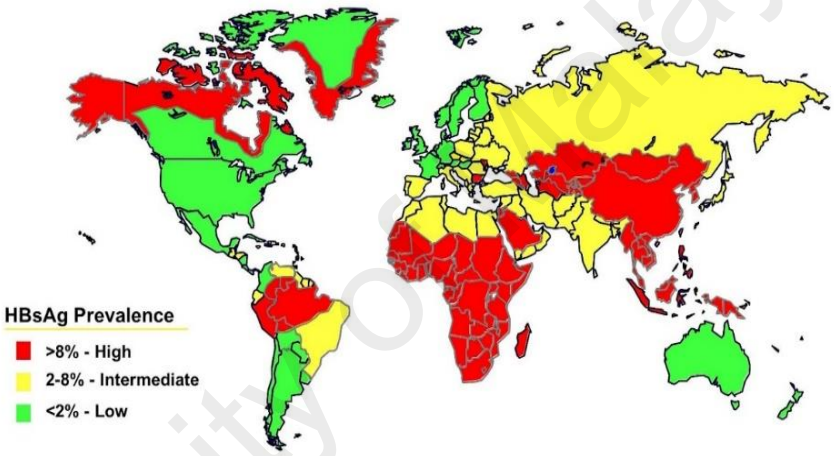
People at increased risk for HBV infection
Infants born to HBV-infected mothers
Intravenous drug users
Hemodialysis patients
Household contacts of HBV-infected persons
Persons with a history of sexually transmitted disease
Health care workers
Sexual partners of HBV-infected persons
Immigrants and children of immigrants from hyper-endemic areas

1.2.2 Epidemiology of HBV infection

Hepatitis B is a viral infection that attacks the liver and can lead to both acute and chronic liver diseases (Sarkar & Chakravarty, 2015; World Health Organization, 2015). In the acute phase, which is during the first six months after HBV infection, the immune system is able to fight the infection and clear the virus from the body. However, acute hepatitis B may develop to chronic HBV if the immune system cannot clear the virus and HBV infection persist in the body for more than 6 months (Ayub et al., 2013). The hepatitis B surface antigen (HBsAg) is the first serological marker in the serum that appears during the incubation period of HBV virus life cycle (World Health Organization, 2016).

Hepatitis B virus (HBV) infection is a public health problem affecting more than 2 billion people globally (Posuwan et al., 2014; Zhang et al., 2015). The incidence of HBV infection varies in different parts of the world (Figure 1.2), from more than 8% of the population in regions where the diseases is greatly endemic (Southeast Asia and Africa),

then in intermediate area with 2-7% of the population and low endemic area with less than 2% of the population (United State, Australia and Western Europe) (Hou, Liu, & Gu, 2005; Tunçbilek, 2014; Zeng, 2014). The incidence rate differs in many ways such as age at infection and predominant mode of transmission. For instance, in high endemic countries the predominant age at infection is perinatal and early childhood, however, the main mode of transmission is maternal-infant and unsterile medical equipment (Croagh & Lubel, 2014).



Source: Available at: http://uscis.gov/graphics/shared/aboutus/statistics/yearbook/2002.pdf. Accessed: July 8, 2005.

Figure 1.2 Geographical variation in hepatitis B prevalence

It has been reported that progression from acute to chronic infection occur in about 90% of perinatal infected patients, approximately 20% of infected HBV patients during childhood and less than 5% of adults infected (Croagh & Lubel, 2014).

1.2.3 Chronic HBV-associated cirrhosis and HCC

Chronic hepatitis B (CHB) is a major global health problem due to its worldwide distribution and its potential adverse liver disease such as liver cirrhosis and HCC (Peng, Chien, & Liaw, 2012). Following progression of CHB to liver cirrhosis, a large amount of healthy liver tissue is permanently replaced with scar tissue and eventually leads to inappropriate function of the liver. A non-functioning liver can give rise to serious complications such as bleeding in the digestive tract and increased fluid within the abdomen. People with cirrhosis are at higher risk of liver cancer as compared to those without cirrhosis. HBV can lead to hepatocellular carcinoma in the absence of cirrhosis, although the majority of HBV-associated HCC (70-90%) happen in patients with underling cirrhosis (Yang et al., 2011). Subsequent progression to HCC, cancerous cells spread of liver tissue and continue to multiply. As it grow, malignant tumor spread to other organs of the body that cause damage. Malignant cells may form secondary mass (metastasis) if they are carried in the bloodstream or lymph channels. The first symptoms of HCC are quite non-specific, however, as the cancer develops more specific symptoms appears (Table 1.2) (Sun & Sarna, 2008).

Table 1.2: Commons signs and symptoms of hepatocellular carcinoma.

commons signs and symptoms of HCC
Early stage
Nausea and vomiting Weight loss Fever
Developed stage
Right upper quadrant pain Jaundice Palpable liver mass Ascites Splenomegaly Asthenia Severe itching Peripheral edema

CHB infection results in approximately one-third of all liver cirrhosis and more than one-half of all HCC cases in the world (Perz, Armstrong, Farrington, Hutin, & Bell, 2006). Asia (specially Southeast-Asia) and Africa have the highest percentage of CHB infected patients with 75% of the world`s CHB population (Croagh & Lubel, 2014). Globally around one million deaths is reported each year due to complication of liver cirrhosis and HCC (Guan., 2011 ; Xu., 2008). The most common cause of cirrhosis is viral hepatitis (hepatitis B and C), however, it is varied depending on the area as in high endemic area of HBV infection (Southeast Asia and Africa), chronic hepatitis B is the major cause of cirrhosis while chronic hepatitis C and alcoholic liver disease are the most common cause of cirrhosis in the United State (Schuppan & Afdhal, 2008; Yang et al., 2011). HCC is known as a primary cancer of hepatocytes and majority of the HCC cases occur due to HBV infection (Ali et al., 2014). Globally, HCC is the sixth most common malignancy and the third leading cause of death because of its complex nature (Forner, Llovet, & Bruix, 2012). Annually, about 70% of HCC cases are associated with chronic hepatitis B infection in the world (Figure 1.3)(Yano et al., 2013).

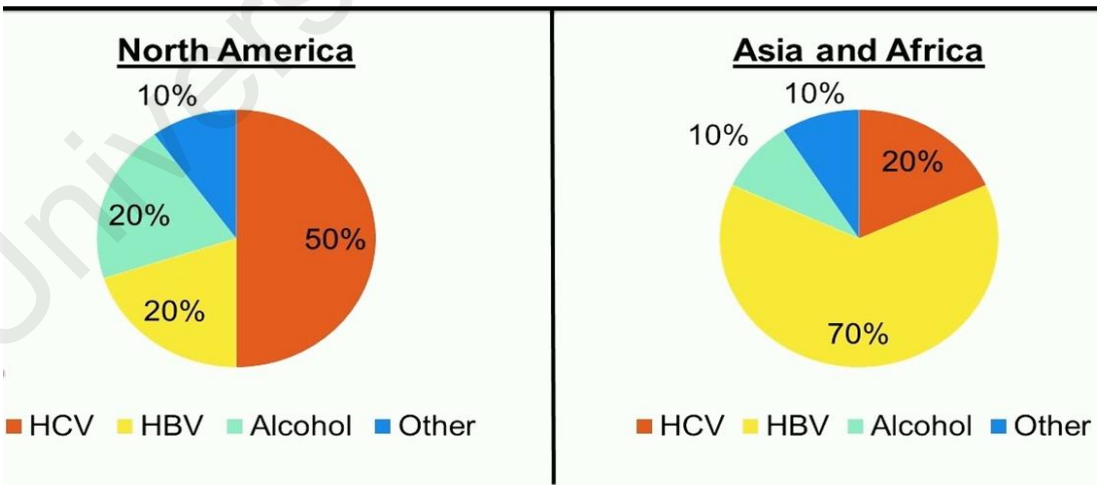


Figure 1.3: Percentage of the most important risk factors causing HCC

Adapted from Forner and Bruix (2012)

The natural history of CHB is determined by a complex interaction of host, viral and life style factors (Croagh & Lubel, 2014; Yim & Lok, 2006).

1.3 Etiological factors of CHB progression to cirrhosis and HCC

Risk factors to enhance possibility of cirrhosis and HCC among HBV infected patients can be grouped as (Figure 1.4);

- viral factors (genotype, level of HBV DNA , genomic mutation and co- infection with other virus),
- lifestyle (alcohol drinking, aflatoxin and cigarette smoking),
- Host characteristics (genetic and immunological background, ethnicity, gender and age).

  (Brunetto, Rodriguez, & Bonino, 1999; Chou et al., 2008; Ito et al., 2010; Kim, Lee, Hwang, Kook, & Kim, 2012; Park & Keeffe, 2004; Tunçbilek, 2014; Turati et al., 2012; Yang. et al., 2008).

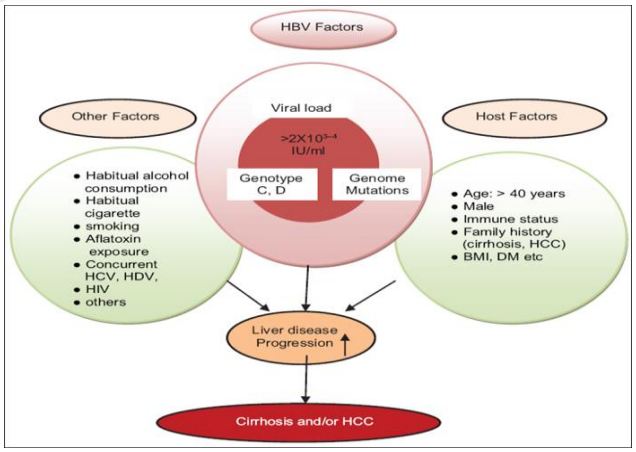


Figure 1.4: Factors interacting to affect the **C**HB progression to cirrhosis / HCC

Adapted from Hepatitis B Annual (2010). http://www.hepatitisbannual.org/currentissue.asp?sabs=y

1.3.1 Viral factors

Accumulating evidences indicated that HBV genotypes (A-H) play a significant role on the consequences of liver disease as well as response to treatment (Liu., 2005; Mayerat, Mantegani, & Frei, 1999; Scotto et al., 2010; Sumi et al., 2003). It has been identified that HBV genotypes B and C are prevalent in the Asian population while genotype C has a higher risk to increase the progression of HBV infection than genotype B (Kao, Chen, Lai, & Chen, 2002). Earlier study suggested that HBV genotypes are related to different features of clinical progression of liver disease in several ethnicities (Tong., 2014). For instance, in Asian ethnicity HBV genotype B is linked to less active disease and slower progression to cirrhosis as well as less risk of occurrence of HCC compared to HBV genotype C (El-Serag & Rudolph, 2007; Yang. et al., 2008). In contrast, in North America and western Europe HBV genotype D has a higher risk of triggering HCC compared to HBV genotype A (El-Serag, 2012; El-Serag & Rudolph, 2007). With regard to HBV genome mutation, existing studies have shown that the specific mutations of enhancer II / core promoter (A1762T/G1764A) and mutant of HBV virus pre-S deletions are associated with increased risk of HCC (Yeung et al., 2011; Yuen et al., 2008). The results of one study in Taiwanese men showed that HBV infected patients with higher levels of HBV DNA replication are in higher risk of progression to HCC. The study also showed that HBV patients with positive Hepatitis B e antigen (HBeAg) are associated with an increased risk of HCC occurrence (Yang et al., 2002). Evidence confirmed that HBeAg could prevent production of IL-6 through the suppression of nuclear factor kappa β gene expression. It has been identified that HBeAg might impair both innate and adaptive immune response with the purpose of promoting HBV progression. (Ayub et al., 2013).

The results of three independent meta-analyses in different populations have confirmed the increased risk of progression to HCC in HBV infected patients with co-infection of HCV (Cho et al., 2011; Donato, Boffetta, & Puoti, 1998; Shi, Zhu, Liu, & Xie, 2005).

1.3.2 Lifestyle

Epidemiological evidence showed that aflatoxin exposure may increase the carcinogenicity of HBV infection (Chen et al., 1996; Qian et al., 1994; Wang et al., 1996). It is believed that the presence of the 249^{ser} TP53 mutation is due to the mutagenic ability of aflatoxin that affects the *P53* gene to cause DNA damage (Szymanska et al., 2004). Earlier evidence reported that alcohol drinking and cigarette smoking are independently related to increased risk of mortality in HBV-linked HCC (Jee, Ohrr, Sull, & Samet, 2004). Furthermore, it has been confirmed that around 25% of HBV-associated HCC patients are attributed to smoking (Jee et al., 2004). Numerous mechanisms have been reported with regards to adverse effect of cigarette smoking on liver malignancy. Cigarette smoking increases the production of pro-inflammatory cytokines such as IL-6, IL-1 and TNF-alpha as well as increasing fibrogenic mediators (angiotensin II and TGFbeta1) and angiogenic factors such as VEGF-A (Bataller, 2006). The results of some studies indicated that alcohol consumption enhance the risk of liver cancer among HBV infected patients (Adami et al., 1992; Henry, Bosch, & Bowers, 2002; La Vecchia, Negri, Decarli, D'Avanzo, & Franceschi, 1988; Tanaka et al., 1992). Alcohol drinking stimulate carcinogenesis by preventing DNA methylation as well as by interacting with retinoid

metabolism. In addition, acetaldehyde which is the most toxic ethanol metabolite is known as a cancerous agent (Seitz & Stickel, 2007).

1.3.3 Host genetic factors

Evidences have shown that male gender, Asian or African race, family history of HCC and older age are risk factors to increase the possibility of chronic hepatitis B progression to cirrhosis and HCC (El-Serag, 2012; El-Serag & Rudolph, 2007; Turati et al., 2012; Yu et al., 2000).

It has been proven that females have significantly lower risk of cirrhosis and HCC than males (Hennig & Hall, 2012; Tangkijvanich, Mahachai, Suwangool, & Poovorawan, 2004). The possible reason could be the effect of sex hormones, as the result of one animal study confirmed that the hormonal effect might be related to testosterone`s capability in increasing the transforming growth factor alpha (TGF-α) associated with hepatocyte proliferation (Matsumoto, Takagi, & Mori, 2000). Results from familial studies revealed the influence of genetic factors on clinical consequences of HBV infection after contact with HBV virus (Carrilho et al., 2005; Hann, Kim, London, Whitford, & Blumberg, 1982; Lin et al., 1989). It has been determined that the risk of HBV progression to cirrhosis is approximately three times more in patients with a first degree family history of HCC compared with those without (Yu et al., 2002). Recently a study by Loomba *et al,* has reported that family history of HCC increases risk of HCC occurrence in HBV infected patients (Loomba et al., 2013). Another study confirmed that the combination of family history of liver cancer together with increased levels of HBV/HCV serum markers is correlated with an over 70-fold raised HCC risk (Turati et al., 2012). Studies of susceptibility to HBV infection in families and also between

different ethnic groups are a useful method to investigate the role of genetic factors in progression of HBV to cirrhosis / HCC.

Genetic factor involved in HBV progression to cirrhosis and HCC is the main focus of this study and play a significant role in HBV infection and disease progression. The results of numerous host genetic studies showed an association between candidate genes and susceptibility to resistance or progression to cirrhosis / HCC in chronic hepatitis B infected patients (Cong et al., 2014; Kou et al., 2014; Minmin et al., 2011; Peng et al., 2014; Qi et al., 2014; Suriapranata et al., 2010; Xu et al., 2013a). In fact, genetic variation which is alteration in the sequence of DNA amongst individuals can differentiate between people with higher or lower risk for getting certain disease (National Human Genome Research Institute, 2015). Single nucleonic polymorphism (SNP) is the most common type of genetic variation in the human genome and identification of SNPs that contribute to predisposition to human disease will provide effective diagnostic information that will help prevention and treatment of human disease (Suh & Vijg, 2005). Genetic polymorphisms in cytokine genes such as interleukin-16 (IL-16), IL-10 and tumour necrosis factor-α (TNF-α) have been shown to be associated with either resistance or predisposition to HBV-related HCC (Cheong et al., 2006; Li, Deng, et al., 2011). Moreover, genetic variation in non-cytokine genes such as osteopontin, estrogen receptor alpha and miRNAs have been shown to be linked to HBV associated liver diseases (Akkiz, Bayram, Bekar, Akgollu, & Ulger, 2011; Andrade Júnior & Andrade, 2004; Guo et al., 2011; Xie et al., 2007; Xu et al., 2013a; Z. Yan et al., 2012).

CHAPTER 2: THE ROLE OF SINGLE NUCLEOTIDE POLYMORPHISMS IN SUSCEPTIBILITY TO LIVER DISEASE PROGRESSION IN HBV INFECTED INDIVIDUALS

2.1 Background of study

Numerous studies have revealed the role of genetic factors in the consequence of HBV infection (Hennig & Hall, 2012; Minmin et al., 2011; Tunçbilek, 2014; Yano, Seo, Azuma, & Hayashi, 2013). Candidate genes involved in biological pathways play important roles in HBV infection outcomes. Single nucleotide polymorphisms in various candidate genes affect the expression of these genes resulting in different consequences of HBV infection. Increasing evidences suggest that polymorphisms in these genes account for the differences in occurrence of chronic hepatitis B progression among different ethnicities within the same geographical location (Al-Qahtani et al., 2012; Bao et al., 2015; Jiang et al., 2015; Kwak et al., 2012; Li, Li, & Wu, 2015b; Saxena, Chawla, Verma, & Kaur, 2014; Sopipong, Tangkijvanich, Payungporn, Posuwan, & Poovorawan, 2013; Zhang, Li, Yao, & Li, 2016). For instance, Tong *et al*, found that Asian family members have a significantly increased levels of HBV markers such as hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) in comparison with the non-Asian family members (Tong et al., 1979). In another study, Zhao *et al* revealed that the incidence of HBsAg in Mongoloid population is (19.1%) approximately two times more than Han Chinese population in the same region (Zhao et al., 2001). These studies have shown significant insights into the fact that different ethnicities in the same area have different outcomes of HBV infection. Malaysia has a multiethnic population which is composed of three different ethnic groups namely; Malay, Chinese and Indians with varying genetic background. Therefore the study of genetic predisposition to progression of disease in HBV infected patients in this population is vital. Thus, this study is aimed

to investigate the role of genetic factors in liver disease progression in HBV infected individuals in the Malaysian population.

Against this back ground, we selected 19 SNPs in 10 genes involved in HBV progression to cirrhosis and HCC. These candidate genes are involved in many pathways such as the immune system, DNA transcription pathways, apoptosis and tumour suppressor gene (Ayub, Ashfaq, & Haque, 2013; Chen et al., 2016; Lan, Chang, Wu, & Yuan, 2015; Z. Zhang, Zhang, Sun, Ma, & Chen, 2015). In addition, haplotype analysis of candidate SNPs will be performed for possible association with HBV progression to cirrhosis and HCC. Haplotype is a set of alleles of closely linked loci that are found in a particular position on a chromosome and tend to be inherited as a unit (Andrade Júnior & Andrade, 2004). In addition, haplotype can also refer to the inheritance of a group of SNPs, with variations at particular positions in the DNA sequence amongst individuals. Although particular SNPs can be indicated for certain genotype-phenotype association, however, haplotypes have been identified to show greater evidence for this association (Liu et al., 2015; Shin et al., 2003). The study of haplotype within genes can help researchers to identify patterns of genetic variation that are associated with the disease.

2.2 Candidate genes for association with HBV infection outcome

Numerous studies have reported the association between genetic polymorphism in various candidate genes with the HBV infection outcomes (Al-Qahtani et al., 2012; Du et al., 2006; Hohler et al., 1997; Kummee, Tangkijvanich, Poovorawan, & Hirankarn, 2007; Li, Jiang, et al., 2011; Ramezani et al., 2008; Thursz et al., 1995). Single nucleotide polymorphisms in different candidate genes involved in HBV progression to cirrhosis

and HCC have been studied in various populations worldwide such as Saudi Arabian, Chinese, Korean and Indian (Al-Qahtani et al., 2012; Almajhdi et al., 2013; Bao et al., 2015; Kim et al., 2014; Mathew, Abdel-Hafiz, Raza, Fatima, & Qadri, 2016; Pratedrat et al., 2015; Saxena et al., 2014; Xie, Xing, et al., 2014). For this study we selected a total of 19 SNPs from 10 genes that have been earlier identified to have association with HBV-linked cirrhosis / HCC in other populations.

2.2.1 Interleukin-6 (*IL-6*)

Interleukin-6 (*IL-6*) is a multifunctional cytokine with a vital role in regulation of immune response to infectious disease (Park, Lee, et al., 2003). *IL-6* play an important role in regulation of the immune reactions of many target cells such as hepatocytes (Zhao, Gao, Zhou, Pan, & Li, 2013). *IL-6* also plays crucial role in the control of balance of pro-inflammatory and anti-inflammatory pathways (Zhao et al., 2013). Early studies have shown that polymorphisms in the *IL-6* gene may influence symptoms in liver disease. It has been reported that single nucleotide polymorphisms in *IL-6* may contribute to progression of CHB disease to cirrhosis and HCC (Tang et al., 2013). Cussigh *et al*, reported that *IL-6* promoter polymorphisms affect the progress of HCV virus infection (Cussigh et al., 2011). In another study Kuo *et al* stated that *IL-6* was capable of suppressing the HBV replication and inhibiting the accumulation of HBV covalently closed circular DNA (cccDNA) in a human hepatoma cell line (Kuo et al., 2009). In contrast, other studies have shown that increased serum levels of *IL-6* were related to increased risk of HBV- linked cirrhosis/HCC, indicating that *IL-6* serum level might be used as a possible biomarker for prompt prediction of infected HBV patients who are at

risk of progression (Sheng et al., 2015; Wong et al., 2009). Thus it is essential to investigate the association between *IL-6* gene polymorphism and progression of HBV infection in our subjects.

2.2.2 Kinesin family member 1B (*KIF1B*)

It has been shown that over-expression of mitotic Kinesin-like protein2, a member of the kinesin family, leading to proliferation of hepatocyte and therefore resulting in tumour aggressiveness in HCC. Thus, members of the Kinesin family are appropriate candidates for genetic studies related to liver disease (Gasnereau et al., 2012). Recently, a genome wide association study (GWAS) revealed the relationship between the kinesin family member 1B (*KIF1B*) gene and HBV-associated HCC in the Chinese population (Zhang, Zhai, et al., 2010). The results of this study showed that rs17401966, rs12734551 and rs3748578 SNPs have strong association with fatal liver disease in HBV patients. However, Sawai *et al*, revealed no association between rs17401966 and HBV associated HCC in the Japanese, Hong Kong and Korean populations (Sawai et al., 2012). Moreover, Sopipong *et al*, reported that there is no relation between rs17401966 of *KIF1B* gene and HBV-linked HCC in Thai patients (Sopipong et al., 2013). In addition, there was no significant association of the *KIF1B* gene with HBV-associated HCC in Saudi Arabian ethnicity (Al-Qahtani et al., 2012). Due to vital role of the Kinesin family members in HBV-associated liver disease, it is necessary to investigate the role of *KIF1B* polymorphisms with HBV-related cirrhosis and HCC in the Malaysians.

2.2.3 MicroRNA-196A2

MiRNA genes have been shown to play vital roles in human cancer progression and metastasis (Esquela-Kerscher & Slack, 2006). Evidence has shown that some SNPs located in the regions of miRNA affect mRNA expression while some other miRNA play a principal role in modulating target mRNA (Fu, Tan, Hou, Hu, & Liu, 2012; Potenza et al., 2011). Genetic polymorphisms in miRNA genes have been implicated in various cancers such as lung cancer (Hu et al., 2008), breast cancer (Gao et al., 2011; Hu et al., 2009) , colorectal cancer (Zhu et al., 2012), gastric cancer (Peng et al., 2010) and HCC (Peng et al., 2014; Qi et al., 2010; Xu et al., 2013). It has been demonstrated that miRNAs play important roles in HBV progression to HCC by post-transcriptionally modifying the expression of human genes (Qi et al., 2010). Single nucleotide polymorphism in miRNA sequences may change the expression and maturation of miRNA and consequently increase the possibility of carcinogenesis (Wu et al., 2008). Studies have revealed that the *miRNA-196A2* is associated with HBV related liver diseases in Chinese population (Li, Li, Song, & Liu, 2010; Qi et al., 2010). Qi *et al,* showed the association between *miRNA196A2* polymorphism and predisposition to HBV related HCC in Chinese men (Qi et al., 2010). Li *et al,* revealed the link between *miRNA-196A2* rs11614913 genotype and size of tumor in cirrhosis-associated HCC Chinese patients (Li, Li, et al., 2010). A number of studies have shown that polymorphisms in *miRNA196A2* gene is associated with HBV- related HCC (Han et al., 2013; Kim et al., 2014; Li, Li, & Wu, 2015; Li, Li, et al., 2010; Qi et al., 2014). The association between the *miRNA-196A2* polymorphisms and progression of HBV infection varies with ethnicity, therefore, it is necessary to investigate the role of *miRNA-196A2* polymorphisms with HBV-related HCC in the Malaysians.

2.2.4 MicroRNA-146A

Existing studies have shown that polymorphism in miRNA gene might affect miRNA expression that could lead to decreased expression of mRNA (Duan, Pak, & Jin, 2007; Iwai & Naraba, 2005). It has been reported that *miRNA-146A* can suppress the sensitivity of HCC cell line to interferon alpha through SMAD4 gene (SMAD family member 4), indicating that this miRNA might be suitable potential therapeutic target in HCC patients on interferon based therapy (Tomokuni et al., 2011). Jiang *et al*, has identified that CC genotype of *miRNA-146A* rs2910164 polymorphism is associated with susceptibility for acute-on-chronic hepatitis B liver failure (Jiang et al., 2013), while the GG genotype is associated with higher expression of *miRNA-146A* and increased risk of HCC especially in male Asian population (Xu et al., 2008). Association of miRNA gene polymorphism with liver malignancy has been identified in many studies (Kwak et al., 2012; Qi et al., 2014; Zhang et al., 2013; Zhou, Dong, et al., 2014). For instance it has been reported that *miRNA-146A* is associated with liver diseases in the Chinese population (Wang et al., 2013). However, there is no information on the role of *miRNA-146A* polymorphism with HBV-associated liver disease in the Malaysian population. The association between the *miRNA-146A* polymorphisms and progression of HBV infection varies with ethnicity, therefore, it is necessary to investigate the role of *miRNA-146A* polymorphisms with HBV-related HCC in the Malaysian population.

2.2.5 Deleted in liver cancer 1 gene (*DLC1*)

The gene, Deleted in Liver Cancer 1 (*DLC1*), is mapped in chromosome 8p21-22 and was first isolated from human hepatocellular carcinoma cells (Yuan et al., 1998). The

DLC1 gene is found to be deleted in liver cancer and in many cancers including kidney, breast, colon, lung, prostate, uterus and stomach (Liao & Lo, 2008; Matsuyama et al., 2001). Evidence have shown that variants in *DLC1* gene are associated with HBV related HCC in both HCC cell line as well as in human HCC, as found by Xie *et al*, which showed that rs3816747 of the *DLC1* gene might have a predictive value for HCC predisposition in the Chinese population (Ng, Liang, Cao, & Lee, 2000; Wong, Lee, Ching, Jin, & Ng, 2003; Xie et al., 2015). *DLC1* is an important candidate gene due to its biological function such as promoter of DNA methylation, mRNA expression and genome deletion (Guan, Zhou, Soulitzis, Spandidos, & Popescu, 2006; Seng et al., 2007; Zhang et al., 2007). The *DLC1* is a potent tumour suppressor gene (TSG) and plays a critical role in the pathogenesis of HCC. Tumour suppressor gene act through the signalling pathways by protecting tumour initiation and development (Xue, Krasnitz, Lucito, Sordella, VanAelst, Cordon-Cardo, Singer, Kuehnel, Wigler, & Powers, 2008). Considering the important role of *DLC1* gene in HBV-associated HCC and the significant role of chromosome 8p in HCC malignancy, additional investigations are needed to understand the role of SNPs located on the 8p region with HBV-associated liver disease in different populations. Thus this study is aimed at evaluating the association between SNPs rs3739298, rs532841 and rs7821974 of the *DLC1* gene and the progression of HBV infection in the Malaysian population**.**

2.2.6 Vascular endothelial growth factor A (VEGFA)

Vascular endothelial growth factor A (VEGFA) is one of the most important mediators of angiogenesis and plays a vital role in carcinogenesis and development of

cancer through stimulating tumor growth (Wu et al., 2013). VEGFA activates various signaling pathways that stimulate endothelial cell growth, differentiation, migration and vascular permeability (Mann et al., 2007; Tammela et al., 2008). Genetic variations in the VEGFA gene have been implicated in susceptibility to numerous cancer such as ovarian cancer, breast cancer, bladder cancer and glioma (Balasubramanian et al., 2007; Garcia-Closas et al., 2007; Hefler et al., 2007; Li, Zhao, et al., 2011). Polymorphism in the VEGFA gene has been reported in many studies with regard to progression of HBV infection (Giacalone et al., 2011; He et al., 2010; Kong et al., 2007; Wu et al., 2009). The results of two studies which were conducted on HCC human tissues showed an increased expression of VEGFA mRNA and related protein in human hepatocellular carcinoma (Iavarone et al., 2007; Yao et al., 2005). In the present study, we investigated the role of the rs833061 of VEGFA gene that has been studied in relation to HBV progression to HCC in other populations. Considering the important role of VEGFA gene in initiation and development of cancer, we aim to study the association of VEGFA SNP rs833061 with risk of progression of HBV to cirrhosis and HCC in Malaysians.

2.2.7 CXC chemokine receptor 1 (CXCR1)

CXC chemokine receptor 1 (CXCR1) is a cell surface receptor for IL-8, a pro-inflammatory chemokine that has been involved in the metastasis and development of several malignancies such as breast, prostate, glioma and ovarian (Brat, Bellail, & Van Meir, 2005; Derin et al., 2007; Fulmer, 2010; Singh & Lokeshwar, 2009). It has been reported that CXCR1 regulate cell migration and alteration of their expression has been linked with poor prognosis of gastric cancers (Li, Wang, et al., 2014). Earlier study

showed that genetic variation in rs2234671 CXCR1 gene polymorphism is associated with susceptibility to HBV infection in Saudi Arabian patients, however they could not find any association between rs2234671 CXCR1 and HBV disease progression (Almajhdi et al., 2013). Thus in this study we aim to investigate the impact of CXCR1 rs2234671 gene polymorphism in relation to HBV progression to cirrhosis and HCC in the Malaysian population.

2.2.8 RAD52

Evidence has shown that *RAD52* is involved in DNA repair and play a dominant role in genetic recombination by promoting the annealing of complementary single-stranded DNA (Kim, Allen, Wagener, Shen, & Nickoloff, 2001; Shinohara & Ogawa, 1995). It has been demonstrated that *RAD52* plays a vital role in maintaining genomic stability and inhibit carcinogenesis (Li, Guo, et al., 2015; Timofeeva et al., 2012), Result of one *in vivo* study identified that knockdown of *RAD52* gene reduces cell growth in animal bronchial epithelial cells, however, overexpression of *RAD52* leads to increasing amount of cell proliferation (Lieberman et al., 2016). This study also showed that reduction of *RAD52* in mouse lung tumor cells changed the cell cycle distribution and increased DNA damage accumulation and this is related to increased tumor cell death. Evidence confirmed an association between rs7963551 SNP of *RAD52* and susceptibility to lung cancer risk in the Chinese population (Han et al., 2015). In another study using human liver tissue, it was shown that rs7963551 SNP of *RAD52* was associated with predisposition to HBV progression to HCC in the Chinese population (Li, Guo, et al., 2015). They also showed that presence of rs7963551 SNP was related to a significant

increase of *RAD52* mRNA expression. We aim to examine the rs7963551of the *RAD52* gene in relation to HBV progression to cirrhosis and HCC in the Malaysian population.

2.2.9 Telomerase- associated protein 1 (TEP1)

The Telomerase- associated protein 1 is a telomerase that adds a new telomere to the ends of chromosome and play a significant role in protection of telomeres from degradation (Jung et al., 2014). Previous study has reported that telomere dysfunction results in telomere-based chromosomal instability that lead to early stage of hepatocarcinogenesis (Farazi et al., 2003). Earlier studies have shown the association between SNPs of telomere maintenance genes with various cancer such as breast cancer, non-small cell lung cancer and endometrial cancer risk (Catarino et al., 2010; Prescott, McGrath, Lee, Buring, & De Vivo, 2010; Shen et al., 2010). It has been proven that functional genetic variations in telomere maintenance genes that possibly has effects on activity of telomeres and length of telomeres play a vital role in the progression of HCC (Jung et al., 2014). Recently Jung *et al* reported that rs1713449 variant of the *TEP1* gene was significantly associated with decreased survival in Korean CHB-linked HCC patients (Jung et al., 2014). However, little is known about the effect of *TEP1* genetic variations on risk of liver disease progression in other populations. Thus, considering the important role of *TEP1* gene in HCC tumorigenesis, we aimed to investigate the association between rs1713449 of the *TEP1* gene and the risk of HBV progression to cirrhosis and HCC in the Malaysian population.

2.2.10 Activating transcription factor 6 (ATF6)

Activating transcription factor 6 is an important transcriptional activator of unfolded protein response (UPR) which is involved in the regulation of gene expression of endoplasmic reticulum chaperones and apoptotic genes (Namba, Ishihara, Tanaka, Hoshino, & Mizushima, 2007; Wu et al., 2014). It has been proven that UPR plays a vital role in malignancy, thus UPR is more activated in cancer cells as compared to normal cells (Suh, Kim, Kim, Chung, & Song, 2012; Wu et al., 2014). Evidence showed that ATF6 is involved in several physiological processes such as apoptosis and cell differentiation that are crucial in tumorigenesis (So, de la Fuente, Walter, Shuman, & Bernales, 2009). Elevation of ATF6 mRNA was reported in hepatocarcinogenesis (Shuda et al., 2003). Recently a study has stated that rs2070150 of the ATF6 gene was significantly related to HBV associated HCC in the Chinese population. The study showed that polymorphism in ATF6 gene was related to predisposition to HCC by influencing ATF6 expression. Their functional study also showed that expression level of ATF6 was lower in HCC patients as compared to those without progression to HCC (Wu et al., 2014). However, no association was found between ATF6 (rs2070150) and risk of HCC malignancy in the Thai HBV patients (Makkoch et al., 2016). We aimed to investigate the impact of ATF6 rs2070150 gene polymorphism on the risk of HBV progression to cirrhosis and HCC in the Malaysian population.

2.2.11 Studies of genetic polymorphisms and risk of progression in chronic hepatitis B infection in various populations.

Table 2.1: List of HBV associated cirrhosis/HCC SNPs in different populations

Study population	Gene	SNP	HBV progression to cirrhosis	HBV progression to HCC	Reference
Chinese	KIF1B	rs3748578 rs12734551 rs17401966	---------	increased risk	(Zhang, et al., 2010)
Germany	KIF1B	rs17401966	---------	increased risk	(Casper, Grunhage, & Lammert, 2011)
Thai	KIF1B	rs17401966	no association	no association	(Sopipong et al., 2013)
Saudi Arabian	KIF1B	rs3748578 rs12734551 rs17401966	no association	no association	(Al-Qahtani et al., 2012)
Japanese, Hong Kong and Korean	KIF1B	rs17401966	---------	no association	(Sawai et al., 2012)
Chinese	miRNA-146A	rs2910164	---------	increased risk	(Wang et al., 2013)
Male Asian	miRNA-146A	rs2910164	--------	increased risk	(Xu et al., 2008)
Chinese	DLC1	rs7821974 rs532841 rs3739298	increased risk	increased risk	(Dong et al., 2009)
Chinese	DLC1	rs3816747	increased risk	increased risk	(Xie et al., 2015)
Korean	TEP1	rs1713449	--------	increased risk	(Jung et al., 2014)
Turkish	miRNA-196A2	rs11614913	increased risk	increased risk	(Akkiz, et al., 2011)
Chinese	miRNA-196A2	rs11614913	-------	increased risk	(Qi et al., 2014)
Chinese	miRNA-196A2	rs11614913	no association	no association	(Han et al., 2013)

Table 2.1 Continued

Korean	miRNA-196A2	rs12304647	protective effect	protective effect	(Kim et al., 2014)
Chinese	miRNA-196A2	rs11614913	increased risk	increased risk	(Li, Li, et al., 2010)
Caucasian	miRNA-196A2	rs11614913	--------	increased risk	(Xu et al., 2013)
Chinese	miRNA-196A2	rs12304647	---------	increased risk	(Li, Li, & Wu, 2015)
Chinese	ATF6	rs2070150	increased risk	increased risk	(Wu et al., 2014)
Thai	ATF6	rs2070150	---------	no association	(Makkoch et al., 2016)
Chinese	IL-6	rs1800796	increased risk	increased risk	(Tang et al., 2014)
Chinese	IL-6	rs2069837 rs17147230	---------	increased risk	(Zheng et al., 2015)
Korean	IL-6	rs2069837 rs1800796	no association	no association	(Park, Lee, et al., 2003)
Chinese	RAD52	rs7963551	--------	increased risk	(Li, Guo, et al., 2015)
Saudi Arabian	CXCR1	rs2234671	no association	no association	(Almajhdi et al., 2013)
Han Chinese	VEGFA	rs833061	-------	increased risk	(Wu et al., 2013)
Italy	VEGFA	rs833061	increased risk	increased risk	(Giacalone et al., 2011)

2.2.12 Specific objectives of the study

- To investigate the association between various candidate genes with progression to cirrhosis and /or HCC in HBV infected patients in Malaysia.
- To determine the association between single nucleotide polymorphisms (SNPs) with progression to cirrhosis and /or HCC in HBV infected patients in the Malaysian population.

- To investigate the association between haplotypes of variants of interest with progression to cirrhosis and/or HCC amongst chronic HBV infection in Malaysia.

2.2.13 Justification of the study

Chronic hepatitis B (CHB) has been a major public health problem due to its multifactorial nature. It is now clear that many etiological pathways are involved in the mechanism that results in progression of CHB to serious liver disease. Each of these pathways consists of different genes and some of these genes have been studied in different populations with the results showing ethnic differences. Thus there is a need for genetic studies of CHB in various geographical location, and it would be especially interesting if this were to be carried out in a multiethnic setting. Malaysia is a multiethnic country with three major ethnic groups, namely Malays, Chinese and Indians. Each of the ethnic groups consist of people from different genetic background, therefore providing a good setting to study ethnic differences in predisposition of CHB progression. The finding from this study involved genes represented by different pathways, thus the report from the present study will broaden our knowledge on the different pathways involved in progression of chronic hepatitis B. This may help to improve our understanding on the mechanisms leading to progression of CHB to cirrhosis and hepatocellular carcinoma in the Malaysian population. Prior information on the genetic profiles of subjects may help to predict those infected with CHB who are at higher risk to progress to cirrhosis and hepatocellular carcinoma for better management of high risk patients.

2.2.14 Hypothesis of the study

The hypothesis for this part of the study are:

The null hypotheses (H_0)

- There is no association between the identified candidate genes with progression to cirrhosis and /or HCC in HBV infected patients in Malaysia.
- There is no association between the identified SNPs with progression to cirrhosis and /or HCC in HBV infected patients in the Malaysian population.
- There is no association between identified haplotypes of variants with progression to cirrhosis and/or HCC amongst chronic HBV infected in Malaysia.

The research hypotheses (H_1):

- There is an association between investigated candidate genes with progression to cirrhosis and /or HCC in HBV infected patients in Malaysia.
- There is an association between investigated SNPs with progression to cirrhosis and /or HCC in HBV infected patients in the Malaysian population.
- There is an association between investigated haplotypes of SNPs with progression to cirrhosis and/or HCC amongst chronic HBV infected in Malaysia.

2.3 Material and methods

2.3.1 Materials

2.3.1.1 Blood collection

Alcohol swabs, 6 mL EDTA tubes, sterile plaster, disposable gloves.

2.3.1.2 DNA extraction

Mini-centrifuge machine, 1.5 mL micro centrifuge tubes (Axygen, Poland), GeneAll$^{@}$ Exgene^{TM} DNA purification kit (Dongnam-ro, Songpa-gu, Seoul, South Korea), pipette and pipette tips (Eppendorf).

2.3.1.3 Nanodrop

Nano-drop 2000c spectrophotometer (Thermo Fisher scientific, USA), 1.5mL micro-centrifuge tubes (Axygen, Poland), pipette and pipette tips (Eppendorf).

2.3.1.4 Sequenom mass array genotyping

Sequenom MassARRAY® platform (Sequenom, San Diego, CA), MassARRAY AssayDesign software package (V4.0) (Sequenom, San Diego, CA), various primers (Integrated DNA Technology, USA), SpectroClean Resin (Sequenom, San Diego, CA), MassARRAY Nanodispenser (Sequenom, San Diego, CA) and MassARRAY Analyzer Compact MALDI-TOF Mass Spectrometer (Sequenom, San Diego, CA).

2.3.1.5 Instrumentation

Refrigerated Centrifuge SIGMA 2-16 PK (Sartorius), Micro Centrifuge (THERMO), Micropipette (Eppendorf), Thermo Block TDB 120 (BIOSAN), Vortex (Thermolyne, USA), Nano-drop 2000c spectrophotometer (Thermo Fisher scientific, USA).

2.3.2 Methods

2.3.2.1 Subjects

This case-control study involved a total of 526 Malaysian subjects who were prospectively enrolled from July 2012 to December 2014. The participants were made up of 423 chronic HBV patients without either cirrhosis or HCC and 103 chronic HBV patients diagnosed with liver cirrhosis or with cirrhosis and HCC. Eligibility for participation was based on the following inclusion criteria; chronic hepatitis B without cirrhosis and HCC was determined by persistence of HBsAg for more than six months. Liver cirrhosis among chronic HBV-infected patients with cirrhosis were confirmed by liver biopsies and/or clinical, biochemical or radiological evidence of cirrhosis (Chook, Ngeow, Yap, Tan, & Mohamed, 2011). Diagnoses of HCC were based on liver histology and/or radiological evidence of liver mass with arterial hypervascularity and washout in the venous-delayed phase on dynamic imaging (Bruix & Sherman, 2011).

Exclusion criteria were as follows; other possible causes of liver disease such as hepatitis C infection, autoimmune hepatitis, hemochromatosis, Wilson's disease, primary biliary cirrhosis and alpha-1 anti-trypsin deficiency. The study protocol (MEC Ref. No: 938.42) was approved by the Medical Ethics Committee of the University of

Malaya Medical Centre (UMMC) and written informed consent was obtained from all participants.

2.3.2.2 Isolation of buffy coat

Two millilitres of venous blood were collected from patients into EDTA tubes to prevent blood clotting. After the samples were centrifuged at 2000 x g for 10 minutes, three layers were formed; the erythrocytes at the bottom layer, the buffy coat at the second layer and plasma at the upper layer. The buffy coat from each samples were collected and stored in 1.5mL micro centrifuge tubes and kept in the -80ºC freezer for subsequent DNA extraction.

2.3.2.3 DNA extraction from buffy coat

Genomic DNA was extracted from buffy coat using the GeneAll$^{@}$ Exgene^{TM} DNA purification kit (Dongnam-ro, Songpa-gu, Seoul, South Korea) according to the manufacture's procedure. Twenty µL of GeneAll protease enzyme was added into a 1.5mL micro centrifuge tube. A total of 200 µL concentrated buffy coat was then added into the micro centrifuge tube follow by addition of 200 µL of BL buffer. The mixture was thoroughly mixed by pulse vortex and then spun down briefly. The mixture was then incubated at 56ºC for 10 minutes in a thermos block heater. The sample was then shortly centrifuged to remove any droplets formed on the lid. A volume of 200 µL of absolute ethanol was added into the sample. Again the sample was mixed and spun down briefly to remove any drops from the lid. After that the mixture was transferred to GeneAll SV

column and was centrifuged at 6000 x g for 3 minutes. The filtrate was discarded and replaced with a new one. Next, a volume of 500 µL of BW buffer was added into the spin column and centrifuged at 6000 x g for 1 minute. After that the collection tube containing the filtrate was discarded and replaced with a new 2 mL collection tube. A volume of 600 µL of BW buffer was added to the spin column and centrifuged at 6000 x g for 1 minute. Then the collection tube containing the filtrate was discarded and replaced with a new collection tube. After that 700 µL of TW buffer was added and the mixture was centrifuged at 6000 x g for 1 minute. The filtrate in the collection tube was discarded, however, the collection tube was not changed. The spin column was centrifuged for one minute at full speed to entirely empty the column from buffer TW. The SV column was then placed in a new 1.5 mL micro centrifuge tube. A total volume of 200 µL of AE buffer was added into the column and then incubated for I minute at room temperature after that the column was centrifuged at full speed for I minute to yield the extracted DNA. Finally the spin column was discarded and the micro centrifuge tube containing the eluted DNA were kept in the -80ºC until used.

2.3.2.4 Determination of concentration and purity of DNA

Extracted DNA were tested for concentration and purity using NanoDrop spectrophotometer. Prior to sample measurement the NanoDrop was calibrated to zero using a blank (AE buffer) and after that DNA samples were measured. DNA concentration with reading of ≥1.8 for 260/280 and 260/230 absorbance ratios were considered to be of good quality DNA.

2.3.2.5 Selection of SNPs

Selection of SNPs was based on literatures involving candidate genes as well as meta-analysis from online database such as PubMed and NCBI.

2.3.2.6 Sequenom MassARRAY genotyping

The candidate SNPs were genotyped using Sequenom MassARRAY® platform (Sequenom, San Diego, CA). This assay is designed to detect sequence differences at the single nucleotide level. In brief, a volume of 1 µL of genomic DNA with average concentration between 10-20 ng/µL were used in each amplification reaction. After single base extraction, the reaction products were desalted with Spectro-Clean resin. A total of 10 nL of reaction products was loaded on to the SpectroCHIP using the Mass ARRAY Nano-dispenser and the mass was found with a Mass ARRAY Analyzer Compact MALDI-TOF mass spectrometer. The Mass ARRAY Typer 4.0 software was employed for analysis of data. Genotypes were assigned after cluster analysis using the default setting of Gaussian mixture model. Clusters were examined to yield a clear cluster separation with good signal to cut off noise. A blank and five duplicates were presented for quality control, and they were excluded if they had any of the following: Assay with >10% call rate in blank checks; >25% call rate within the blank control; < 99.5% concordance in duplicate checks and assay with <80% call rate in the same Spectro Chip.

2.4 Statistical analysis

Statistical analysis was performed using SPSS version 16.0. Data were presented as percentage or mean ± standard deviation. Hardy-Weinberg Equilibrium (HWE) for each SNP was tested using the chi-squared test. The genotype and allele frequencies for the candidate SNPs among the HBV infected with and without cirrhosis/HCC were calculated. The test for association between the SNPs in HBV-infected with and without cirrhosis/HCC was performed using logistic regression to calculate the odds ratio (OR) with 95% confidence intervals (CI). Multivariate logistic regression models were adjusted for confounding factors such as age and gender. Statistical significance was defined by $P<0.05$.

Construction of linkage disequilibrium (LD) blocks and haplotype analyses was performed using Haploview 4.2 software. The association of the haplotype distribution was compared between HBV-infected with and without progression by calculating the permutation P values generated using a chi squared statistics with one degree of freedom.

2.5 Results

2.5.1 Demographic characteristic of subject

A total of 526 chronic hepatitis B patients (284 males and 242 females) were recruited in this study. Table 2.2 shows the distribution of demographic features among HBV patients in Malaysia.

Table 2.2: Demographic characteristic of the study subject.

	HBV without cirrhosis / HCC (n= 423)	**HBV with cirrhosis / HCC (n= 103)**	**P**
Age [a]	52.63 ± 14.63	63.44±11.23	0.000
Ethnicity			0.003
Malay	116 (27.5%)	29 (28.2%)	
Chinese	295 (69.9%)	72 (69.9%)	
Indian	11(2.6%)	2 (1.9%)	
Gender			0.008
Male	218 (51.5%)	66 (64.1%)	
Female	205 (48.5%)	37 (35.9%)	

[a] Age presented as mean ± standard deviation.

2.5.2 Single nucleotide polymorphism of *IL-6* gene

2.5.2.1 Allele frequencies of *IL-6* SNPs

Table 2.3 shows the allelic distribution of *IL6* polymorphisms in case and control groups. There were no significant differences in the allele frequencies of all three SNPs between HBV-infected with and without cirrhosis/HCC.

Table 2.3: Allelic distributions for *IL-6* gene polymorphisms in HBV-infected with and without cirrhosis/HCC.

IL6 rs2069837	**HBV without cirrhosis/HCC (n= 423)**	**HBV with cirrhosis/HCC (n= 103)**	**OR [a] (95%Cl)**	**p [b]**
A	716 (85%)	169 (83%)	1.16 (0.75 - 1.79)	0.506
G	130 (15%)	35 (17%)		
IL6 rs1800796				
G	219 (26%)	64 (31%)	0.77 (0.55 - 1.08)	0.130
C	627 (74%)	142 (69%)		
IL6 rs2066992				
G	224 (26%)	64 (31%)	0.80 (0.57 - 1.14)	0.185
T	622 (74%)	142 (69%)		

[a]: OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b:] p value were determined using Chi square test.

2.5.2.2 Genotype association and modeling of *IL-6* SNPs

Table 2.4 shows the genotype association and modeling of *IL6* polymorphisms in case and control groups. There were no significant differences in genotype frequencies of all three SNPs between HBV-infected with and without cirrhosis/HCC.

Table 2.4: Genotypic association and modeling of *IL-6* gene polymorphisms in HBV-infected with and without cirrhosis/HCC.

IL6 rs2069837	**HBV without cirrhosis/HCC (n= 423)**	**HBV with cirrhosis/HCC (n= 103)**	OR [a] (95%Cl)	p [b]
AA	299 (70.7%)	67 (65.7%)	1	-
AG	118 (27.9%)	35 (34.3%)	1.34 (0.82-2.19)	0.248
GG	6 (1.4%)	0	-	NA [d]
AA vs AG + GG			1.27 (0.78-2.07)	0.342
AA + AG vs GG			-	NA
HWE [c] p	0.136	0.036		
IL6 rs1800796				
GG	28 (6.6%)	8 (7.8%)	1	-
GC	163 (38.5%)	48 (46.6%)	1.15 (0.47-2.84)	0.759
CC	232 (54.9%)	47 (45.6%)	0.77 (0.32-1.90)	0.570
GG vs GC+CC			0.93 (0.39-2.21)	0.860
GG + GC vs CC			0.68 (0.43 -1.08)	0.103
HWE p	0.930	0.371		
IL6 rs2066992				
GG	31 (7.3%)	8 (7.8%)	1	-
GT	162 (38.3%)	48 (46.6%)	1.34 (0.55-3.27)	0.520
TT	230 (54.4%)	47 (45.6%)	0.89 (0.37-2.17)	0.805
GG vs GT+TT			1.07 (0.46-2.53)	0.870
GG + GT vs TT			0.67 (0.44-1.10)	0.125
HWE p	0.736	0.371		

[a]: OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b:] p value were determined using Chi square test.

[c:] Hardy-Weinberg Equilibrium.

[d:] Not Applicable

2.5.2.3 Haplotype analysis of *IL-6* SNPs

Table 2.5 shows the overall population haplotype block frequency. The haplotype combination, namely CT and GG, was made between *IL6* rs1800796 and *IL6* rs2066992. However, no association was seen between these polymorphism and progression of HBV infection.

Table 2.5: *IL-6* haplotypes and their frequencies between CHB with and without cirrhosis and HCC.

	Frequency				
Haplotype	**All**	**Case**	**Control**	**Pearson's p [a]**	**Chi Square**
group I vs group II					
CT	0.724	142.00(0.689)	619.98(0.733)	0.129	2.302
GG	0.266	64.00(0.311)	215.98(0.255)	0.129	2.302

[a]: Pearson's p value were determined using Chi square test.

All frequency <0.03 was ignored in analysis

Group I= Control: CHB patients without cirrhosis/HCC

Group II: Case: CHB patients with cirrhosis/HCC

Figure 2.1 shows the LD pattern of the *IL-6 gene.* The LD between *IL-6* rs1800796 and *IL6* rs2066992 was high (D'= 0.99).

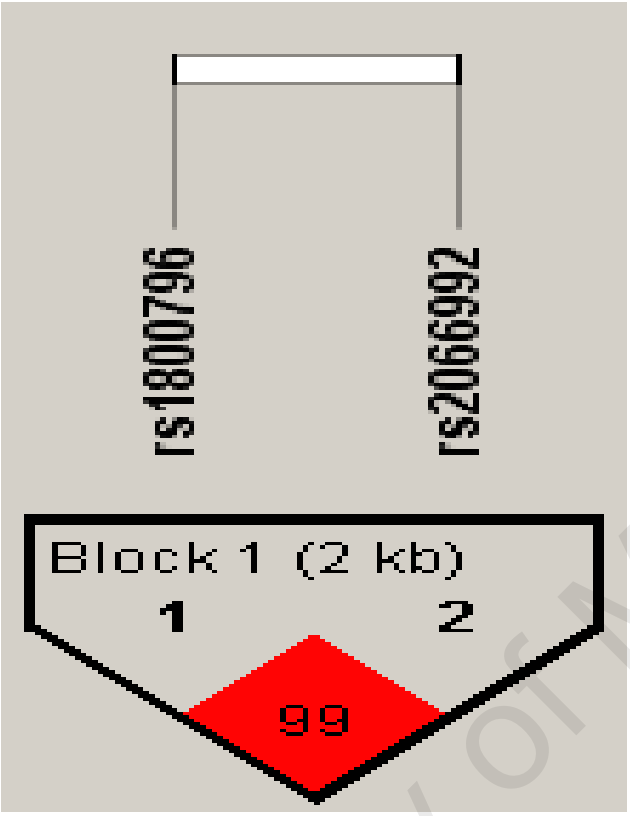


Figure 2.1: Linkage disequilibrium analysis of two *IL-6* SNPs

2.5.3 Single nucleotide polymorphism of *KIF1B* gene

2.5.3.1 Allele frequencies of *KIF1B* SNPs

Table 2.6 shows the allelic frequency distribution of *KIF1B* polymorphism among chronic HBV without cirrhosis / HCC and chronic HBV with cirrhosis and HCC. There was no significant difference between allelic frequency of any of the *KIF1B* variants among the chronic HBV without cirrhosis / HCC and chronic HBV with cirrhosis and HCC.

Table 2.6: Allelic association between the *KIF1B* polymorphisms and HBV with and without cirrhosis/HCC.

Subjects	Allele frequency		OR(95% CI)	p
rs3748578	**G**	**A**		
CHB without cirrhosis/HCC	0.75 (n=631)	0.25 (n=215)	0.95 (0.65 - 1.38)	0.79
CHB with cirrhosis/HCC	0.75 (n=155)	0.25 (n=51)		
rs8019	**T**	**G**		
CHB without cirrhosis/HCC	0.70 (n=499)	0.30 (n=219)	0.97 (0.68 - 1.38)	0.88
CHB with cirrhosis/HCC	0.70 (n=120)	0.30 (n=52)		
rs12734551	**T**	**G**		
CHB without cirrhosis/HCC	0.78 (n=660)	0.22 (n=186)	0.78 (0.50 - 1.20)	0.26
CHB with cirrhosis/HCC	0.80 (n=166)	0.20 (n=40)		
rs17401966	**A**	**G**		
CHB without cirrhosis/HCC	0.78 (n=660)	0.22 (n=186)	0.78 (0.50 - 1.20)	0.26
CHB with cirrhosis/HCC	0.80 (n=166)	0.20 (n=40)		
rs17401924	**A**	**G**		
CHB without cirrhosis/HCC	0.78 (n=660)	0.22 (n=186)	0.79 (0.51 - 1.22)	0.29
CHB with cirrhosis/HCC	0.80 (n=164)	0.20 (n=40)		

OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

p value was determined using Chi square test.

2.5.3.2 Genotype association and modeling of *KIF1B* SNPs

Table 2.7 shows the genotype association and modeling of *KIF1B* polymorphisms in chronic HBV with and without cirrhosis/HCC. However, there were no significant variation in genotype frequencies of selected SNPs between case and control groups.

Table 2.7: Genotype association and modeling of *KIF1B* polymorphisms in chronic HBV with and without cirrhosis/HCC.

KIF1B rs3748578	**CHB without cirrhosis / HCC (n= 423)**	**CHB with cirrhosis / HCC (n= 103)**	**OR [a](95%Cl)**	**p [b]**
GG	233 (55.1%)	60 (58.2%)	1	-
GA	165 (39.0%)	35 (34.0%)	0.77 (0.48 - 1.27)	0.31
AA	25 (5.9%)	8 (7.8%)	1.29 (0.53 - 3.15)	0.57
GG + GA vs. AA			0.70 (0.29 - 1.67)	0.42
GG vs. GA + AA			0.84 (0.53 - 1.34)	0.46
HWE [c] p	0.055	0. 370		
KIF1B rs8019				
TT	189 (52.7%)	46 (53.5%)	1	-
TG	121 (33.7%)	28 (32.5%)	0.94 (0.54 - 1.64)	0.83
GG	49 (13.6%)	12 (14.0%)	0.97 (0.46 - 2.05)	0.94
TT+TG vs. GG			0.99 (0.50 - 1.97)	0.97
TT vs. TG+ GG			0.95 (0.57 - 1.56)	0.84
HWE p	0.0001	0.034		
KIF1B rs12734551				
TT	245 (57.9%)	66 (64.1%)	1	-
TG	170 (40.2%)	34 (33.0%)	0.68 (0.42 - 1.11)	0.12
GG	8 (1.9%)	3 (2.9%)	1.39 (0.32 - 6.02)	0.66
TT+TG vs. GG			0.62 (0.15 - 2.66)	0.52
TT vs. TG + GG			0.71 (0.44 - 1.14)	0.15
HWE p	0.0004	0.58		

Table 2.7 continue:

KIF1B rs17401966	CHB without cirrhosis / HCC (n= 423)	CHB with cirrhosis/ HCC (n= 103)	OR [a] (95%Cl)	p [b]
AA	245 (57.9%)	66 (64.1%)	1	-
AG	170 (40.2%)	34 (33.0%)	0.68 (0.42 - 1.11)	0.12
GG	8 (1.9%)	3 (2.9%)	1.39 (0.32 - 6.02)	0.66
AA+AG vs. GG			0.62 (0.15 - 2.66)	0.52
AA vs. AG +GG			0.71 (0.44 - 1.14)	0.15
HWE [c] p	0.0004	0.586		
KIF1B rs17401924				
AA	245 (57.9%)	65 (63.8%)	1	-
AG	170 (40.2%)	34 (33.3%)	0.69 (0.43 - 1.13)	0.14
GG	8 (1.9%)	3 (2.9%)	1.42 (0.33 - 6.16)	0.64
AA+AG vs. GG			0.61 (0.14 - 2.63)	0.51
AA vs. AG +GG			0.72 (0.45 - 1.16)	0.18
HWE p	0.0004	0.561		

[a] OR and 95% CI were calculated using logistic regression, adjusted for gender and age

[b] p value was determined using Chi square test.

[c] Hardy-Weinberg Equilibrium.

2.5.4 Single nucleotide polymorphism of *miRNA-196A2* gene

2.5.4.1 Allele frequencies of *miRNA-196A2* gene

Table 2.8 shows the allelic distributions for *miRNA-196A2* gene polymorphisms in HBV-infected with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.8: Allelic distributions for *miRNA-196A2* gene polymorphisms in HBV-infected with and without cirrhosis/HCC.

	Allele	**frequency**	**OR [a] (95% CI)**	**p [b]**
MIR196A2 rs12304647	**A**	**C**		
CHB without cirrhosis / HCC	0.77 (n=654)	0.22 (n=192)	1.12 (0.78 -1.59)	0.53
CHB with cirrhosis / HCC	0.75 (n=155)	0.24 (n=51)		
MIR196A2 rs11614913	**C**	**T**		
CHB without cirrhosis /HCC	0.48 (n=409)	0.52 (n=437)	0.95 (0.71-1.28)	0.76
CHB with cirrhosis / HCC	0.49 (n=102)	0.51 (n=104)		

[a] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b]: p value were determined using Chi square test.

2.5.4.2 Genotype association and modeling of *miRNA-196A2* gene

Table 2.9 shows that the rs12304647 SNP of *MIR196A2* in recessive model was significantly associated with decreased risk of cirrhosis/HCC. Under recessive models there were no significant differences in genotype frequencies of *MIR196A2* rs11614913 SNPs between HBV patients without cirrhosis/HCC versus HBV patients diagnosed with liver cirrhosis or cirrhosis and HCC respectively.

Table 2.9: Genotypic association and modeling of *miRNA196A2* rs12304647 polymorphisms in HBV-infected with and without cirrhosis/HCC.

MIR196A2 rs12304647	CHB without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [a](95%Cl)	p [b]
AA	250 (59.1%)	61 (59.2%)	1	-
AC	154 (36.4%)	33 (32.1%)	0.87 (0.55-1.40)	0.580
CC	19 (4.5%)	9 (8.7%)	1.94 (0.83- 4.50)	0.120
AA+AC vs. CC			0.37(0.15 - 0.89)	**0.027**
AA vs AC +CC			1.13 (0.76 -1.67)	0.547
HWE [c] p	0.439	0.155		
MIR196A2 rs11614913				
CC	103 (24.3%)	27 (26.2%)	1	-
CT	203 (48.0%)	48 (46.6%)	0.90 (0.53-1.52)	0.701
TT	117 (27.7%)	28 (27.2%)	0.91 (0.50- 1.64)	0.760
CC+ CT vs. TT			1.21 (0.72 -2.02)	0.474
CC vs CT + TT			0.89 (0.37 -1.15)	0.796
HWE p	0.421	0.492		

[a]: OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b:] p value was determined using Chi square test.

[c:] Hardy-Weinberg Equilibrium.

2.5.4.3 Haplotype analysis of *miRNA-196A2* SNPs

Table 2.10 shows the overall population haplotype block frequency. The haplotype block consists of three haplotypes namely AT, AC and CC. None of the miRNA-196A2 gene haplotypes was significantly associated with cirrhosis/HCC.

Table 2.10: Haplotype analysis for *miRNA-196A2* gene polymorphism between HBV with and without cirrhosis/HCC.

	Frequency				
Haplotype	**All**	**Case [a]**	**Control [b]**	**P Value [c]**	**Chi Square**
Block 1					
AT	0.482	0.476	0.485	0.6493	0.207
AC	0.266	0.286	0.255	0.0939	2.806
CC	0.252	0.238	0.260	0.2374	1.396

[a] HBV with cirrhosis / HCC

[b] HBV without cirrhosis / HCC

[c] P Value < 0.05 is considered significant

Figure 2.2 shows the LD pattern of the *miRNA-196a2* gene. A complete LD was seen at rs12304647 SNP with rs11614913 (D'=1.0).

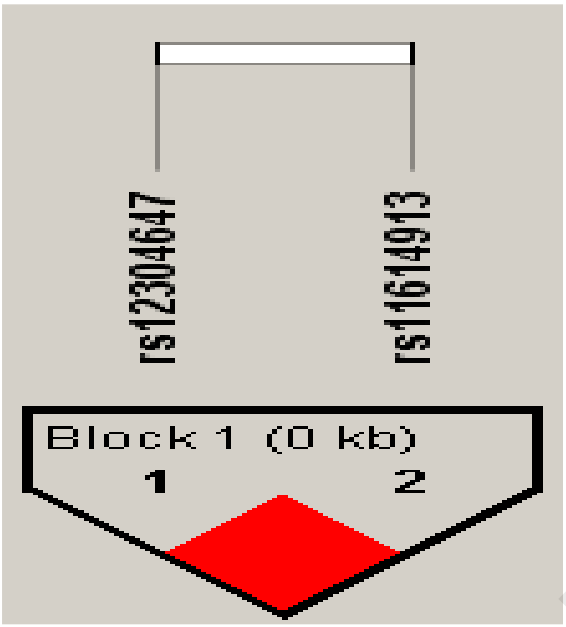


Figure 2.2: Linkage disequilibrium analysis of two *miRNA-196a2* SNPs

2.5.5 Single nucleotide polymorphism of *miRNA146a*

2.5.5.1 Allele frequency of *miRNA146a*

Table 2.11 shows that there was no significant difference in allele frequency of *miRNA146a* rs2910164 SNP between HBV patients without cirrhosis/HCC versus HBV patients with cirrhosis / HCC.

Table 2.11: Allelic distributions for *miRNA-196A2* gene polymorphisms in HBV-infected with and without cirrhosis/HCC.

	Allele	**frequency**	**OR[a] (95% CI)**	**p[b]**
MiRNA146a rs2910164	**C**	**G**		
CHB without cirrhosis / HCC	0.62 (n=524)	0.38 (n=322)	1.03 (0.76- 0.40)	0.84
CHB with cirrhosis / HCC	0.61 (n=126)	0.39 (n=80)		

[a] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b] p value were determined using Chi square test.

2.5.5.2 Genotype association and modeling of *miRNA146a*

Table 2.12 shows the genotype association and modeling of *miRNA146a* polymorphisms in chronic HBV with and without cirrhosis/HCC. However, there was no significant variation in genotype frequency of rs2910164 between case and control groups.

Table 2.12: Genotype frequency and modeling of *miRNA146a* rs2910164 polymorphisms in chronic HBV with and without cirrhosis / HCC.

miRNA146a rs2910164	chronic HBV without cirrhosis / HCC (n= 423)	chronic HBV with cirrhosis / HCC (n= 103)	OR [a] (95%Cl)	p [b]
CC	167 (39.5%)	40 (38.8%)	1	-
CG	190 (44.9%)	46 (44.7%)	1.01 (0.63- 1.62)	0.960
GG	66 (15.6%)	17 (16.5%)	1.07 (0.57- 2.02)	0.821
CC+ CG vs GG			0.91(0.49- 1.68)	0.756
CC vs CG + GG			0.97 (0.67 - 1.40)	0.863
HWE [c] p	0.330	0.543		

[a] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b] p value was determined using Chi square test.

[c] Hardy-Weinberg Equilibrium.

2.5.6 Single nucleotide polymorphism of *DLC1*gene

2.5.6.1 Allele frequencies of *DLC1*gene

Table 2.13 shows the allelic distributions for *DLC1* gene polymorphisms in HBV-infected with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.13: Allelic association between three SNPs of *DLC1* gene and HBV related liver disease in the Malaysian patients.

SNP	Allele	frequency	OR [b] (95% CI)	p[c]
rs3739298	**G**	**T**		
CHB [a] without cirrhosis / HCC	n=322 (39.9%)	n=486 (60.1%)	1.01 (0.72 -1.42)	0.956
CHB with cirrhosis / HCC	n=80 (42.5%)	n=108 (57.5%)		
rs532841	**G**	**A**		
CHB without cirrhosis / HCC	n=54 (63.9%)	n=305 (36.1%)	0.99 (0.71-1.39)	0.988
CHB with cirrhosis / HCC	n=127 (61.7%)	n=79 (38.3%)		
rs7821974	**T**	**C**		
CHB without cirrhosis / HCC	n=457 (54.1%)	n=389 (45.9%)	0.95 (0.71-1.29)	0.741
CHB with cirrhosis / HCC	n=111 (53.9%)	n=95 (46.1%)		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

2.5.6.2 Genotype association and modeling of *DLC1*gene

Table 2.14 shows the genotype association and modeling of *DLC1* polymorphisms in chronic HBV with and without cirrhosis/HCC. However, there were no significant variances in genotype frequencies of selected SNPs between case and control groups.

Table 2.14: Genotype association and modeling between three SNPs of *DLC1* polymorphisms in HBV related liver disease in the Malaysian subjects.

	CHB cirrhosis / HCC (n= 423)	**CHB cirrhosis / HCC (n= 103)**	**OR (95%Cl) [a]**	**p [b]**
DLC1 rs3739298				
GG	72 (17.8%)	12 (12.8%)	1	-
GT	178 (44.1%)	56 (59.6%)	2.16 (1.05 - 4.46)	0.066
TT	154 (38.1%)	26 (27.7%)	1.30 (0.59 - 2.84)	0.512
GG+GT vs. TT			0.71 (0.43 - 1.21)	0.211
GG vs GT +TT			0.73 (0.36 - 1.44)	0.361
HWE [c] p	0.103	0.034		
DLC1 rs532841				
GG	175 (41.4%)	35 (34.0%)	1	-
GA	191 (45.2%)	57 (55.3%)	1.43 (0.87 - 2.36)	0.153
AA	57 (13.5%)	11 (10.7%)	0.74 (0.34 - 1.63)	0.459
GG+ GA vs AA			0.60 (0.29 - 1.25)	0.172
GG vs GA+ AA			0.71 (0.49- 1.03)	0.086
HWE p	0.67	0.084		
DLC1 rs7821974				
TT	132 (31.2%)	30 (29.1%)	1	-
TC	193 (45.6%)	51 (49.5%)	1.21 (0.71- 2.08)	0.477
CC	98 (23.2%)	22 (21.4%)	0.87 (0.46 -1.66)	0.673
TT+ TC vs CC			0.77 (0.44 - 1.34)	0.359
TT vs TC+ CC			1.03 (0.98- 1.21)	0.375
HWE p	0.094	0.97		

[a] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b] P value were determined using Chi square test.

[c] Hardy-Weinberg Equilibrium.

2.5.7 Single nucleotide polymorphism of *VEGFA* gene

2.5.7.1 Allele frequencies of *VEGFA* gene

Table 2.15 shows the allelic distribution for *VEGFA* gene polymorphism in HBV with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.15: Allelic association between rs833061 of *VEGFA* gene and HBV related liver disease in the Malaysian subjects.

VEGFA rs833061	CHB [a] without cirrhosis/HCC (n= 423)	CHB with cirrhosis/HCC (n= 103)	OR (95%CI) [b]	p [c]
C	248 (29%)	57 (28%)	1.12 (0.78 - 1.61)	0.548
T	598 (71%)	149 (72%)		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

2.5.7.2 Genotype association and modeling of *VEGFA* gene

Table 2.16 shows the genotype association and modeling of *VEGFA* polymorphisms in chronic HBV with and without cirrhosis/HCC. However, there were no significant variation in genotype frequency of rs833061 between case and control groups.

Table 2.16: Genotype frequency and modeling of *VEGFA* rs833061 polymorphism in chronic HBV with and without cirrhosis / HCC.

VEGFA rs833061	CHB without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [a] (95%Cl)	p [b]
CC	39 (9.2%)	5 (4.9%)	1	-
CT	170 (40.2%)	47 (45.6%)	2.25 (0.80 - 3.32)	0.125
TT	214 (50.6%)	51 (49.5%)	1.99 (0.71- 2.56)	0.188
CC+ CT vs TT			0.96 (0.63- 1.47)	0.845
CC vs CT+ TT			2.11 (0.77- 2.74)	0.145
HWE [c] p	0.53	0.16		

[a] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[b] p value were determined using Chi square test.

[c] Hardy-Weinberg Equilibrium.

2.5.8 Single nucleotide polymorphism of *CXCR1* gene

2.5.8.1 Allele frequency of *CXCR1* gene

Table 2.17 shows the allelic distribution for *CXCR1* gene polymorphism in HBV with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.17: Allelic association between rs2234671 of *CXCR1* gene and HBV related liver disease in the Malaysian subjects.

CXCR1 rs2234671	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [b] (95%Cl)	P [c]
G	786 (93%)	185 (90%)	1.53 (0.89 - 2.61)	0.125
C	60 (7%)	21 (10%)		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

2.5.8.2 Genotype association and modeling of *CXCR1*gene

Table 2.18 shows the genotype association and modeling of *CXCR1* polymorphism in chronic HBV with and without cirrhosis/HCC. However, there was no significant variation in genotype frequency of rs2910164 between case and control groups.

Table 2.18: Genotype frequency and modeling of *CXCR1* rs2234671 polymorphism in chronic HBV with and without cirrhosis / HCC.

CXCR1 rs2234671	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [b] (95%Cl)	P [c]
GG	364 (86.1%)	82 (79.6%)	1	-
GC	58 (13.7%)	21 (20.4%)	1.47 (0.85 - 2.64)	0.201
CC	1 (0.2%)	0 (0%)	-	NA [e]
GG+ GC vs CC			-	NA
GG vs GC+ CC			1.46 (0.81 -2.62)	0.208
HWE [d] p	0.41	0.25		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

[d] Hardy-Weinberg Equilibrium.

[e] Not applicable

2.5.9 Single nucleotide polymorphism of *RAD52* gene

2.5.9.1 Allele frequency of *RAD52* gene

Table 2.19 shows the allelic distribution for *RAD52* gene polymorphism in HBV with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.19: Allelic association between rs7963551 of *RAD52* gene and HBV related liver disease in the Malaysian subjects.

RAD52 rs7963551	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR (95%Cl) [b]	p [c]
G	699 (82.6%)	172 (84%)	1.53 (0.89 - 2.61)	0.125
A	147 (17.4%)	34 (16%)		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

2.5.9.2 Genotype association and modeling of *RAD52* gene

Table 2.20 shows the genotype association and modeling of *RAD52* polymorphism in chronic HBV with and without cirrhosis/HCC. However, there were no significant variation in genotype frequency of rs7963551 between case and control groups.

Table 2.20: Genotype frequency and modeling of *RAD52* rs7963551 polymorphism in chronic HBV with and without cirrhosis / HCC.

RAD52 rs7963551	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR (95%Cl) [b]	p [c]
GG	285 (67.4%)	71 (68.9%)	1	-
GA	129 (30.5%)	30 (29.1%)	0.72 (0.43 - 1.19)	0.205
AA	9 (2.1%)	2 (1.9%)	1.04 (0.21 - 2.07)	0.961
GG + GA vs AA			1.14 (0.24 - 3.53)	0.866
GG vs GA+ AA			0.74 (0.45 - 1.21)	0.228
HWE [d] p	0.201	0.564		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

[d] Hardy-Weinberg Equilibrium.

2.5.10 Single nucleotide polymorphism of *TEP1* gene

2.5.10.1 Allele frequency of *TEP1*gene

Table 2.21 shows the allelic distribution for *TEP1* gene polymorphism in HBV with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.21: Allelic association between rs1713449 of *TEP1* gene and HBV related liver disease in the Malaysian subjects.

TEP1 rs1713449	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [b](95%Cl)	p[c]
G	594 (70%)	142 (70%)	1.07 (0.76 - 1.52)	0.694
A	252 (30%)	64 (30%)		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

2.5.10.2 Genotype association and modeling of *TEP1* gene

Table 2.22 shows the genotype association and modeling of *TEP1* polymorphism in chronic HBV with and without cirrhosis/HCC. However, there were no significant variation in genotype frequency of rs1713449 between case and control groups.

Table 2.22: Genotype frequency and modeling of *TEP1* rs1713449 polymorphism in chronic HBV with and without cirrhosis / HCC.

TEP1 rs1713449	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [b](95%Cl)	p [c]
GG	213 (50.4%)	50 (48.5%)	1	-
GA	168 (39.7%)	42 (40.8%)	0.99 (0.62 - 1.61)	0.975
AA	42 (9.9%)	11 (10.7%)	1.26 (0.57 - 2.78)	0.565
GG+ GA vs AA			1.27 (0.59 - 2.71)	0.543
GG vs GA + AA			1.04 (0.66 -1.64)	0.872
HWE [d] p	0.298	0.626		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

[d] Hardy-Weinberg Equilibrium.

2.5.11 Single nucleotide polymorphism of *ATF6* gene

2.5.11.1 Allele frequency of *ATF6* gene

Table 2.23 shows the allelic distribution for *ATF6* gene polymorphism in HBV with and without cirrhosis/HCC. However, none of the alleles were significantly associated with progression of HBV to cirrhosis / HCC.

Table 2.23: Allelic association between rs2070150 of *ATF6* gene and HBV related liver disease in the Malaysian subjects.

ATF6 rs2070150	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [b](95%Cl)	p [c]
G	574 (68%)	136 (66%)	1.31 (0.82 - 1.08)	0.257
C	270 (32%)	70 (34%)		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

2.5.11.2 Genotype association and modeling of *TEP1* gene

Table 2.24 shows the genotype association and modeling of *ATF6* polymorphism in chronic HBV with and without cirrhosis/HCC. However, there were no significant variation in genotype frequency of rs1713449 between case and control groups.

Table 2.24: Genotype frequency and modeling of *ATF6* rs2070150 polymorphism in chronic HBV with and without cirrhosis / HCC.

ATF6 rs2070150	CHB [a] without cirrhosis / HCC (n= 423)	CHB with cirrhosis / HCC (n= 103)	OR [b] (95%Cl)	p [c]
GG	197 (46.7%)	41 (39.8%)	1	-
GC	180 (42.7%)	54 (52.4%)	1.40 (0.86 - 1.26)	0.171
CC	45 (10.7%)	8 (7.8%)	0.92 (0.39 - 2.17)	0.847
GG + GC vs CC			0.77 (0.34 - 1.75)	0.532
GG vs GC+ CC			1.31 (0.82 -1.07)	0.257
HWE [d] p	0.685	0.087		

[a] CHB: Chronic hepatitis B.

[b] OR and 95% CI were calculated using logistic regression, adjusted for gender and age.

[c] P value were determined using Chi square test.

[d] Hardy-Weinberg Equilibrium.

2.6 Discussion

2.6.1 Single nucleotide polymorphism

In this study, we found a significant association between *miRNA-196A2* (rs12304647) polymorphism with HBV progression to cirrhosis and HCC.

2.6.1.1 *miRNA-196A2*

We investigated two SNPs in the *miRNA-196A2* gene and rs12304647 variant was significantly related to HBV- associated HCC in Malaysian patients.

Our results showed that rs12304647 of *miRNA-196A2* gene has a protective effect against progression to cirrhosis/HCC (OR=0.37, 95% CI=0.15 - 0.89, p=0.027). However, there was no significant association with the second SNP (rs11614913) of *miRNA-196A2* with progression of the HBV infection.

The role of *miRNA-196A2* with HBV-related liver disease has been investigated in different populations (Akkiz, Bayram, Bekar, Akgollu, & Ulger, 2011; Kim et al., 2014; Kwak et al., 2012; Qi et al., 2014). The effect of *microRNA-196A2* gene polymorphism with liver malignancy has been inconsistent in many studies and this may be due to the differences in genetic makeup. Two studies have shown that the *miRNA-196A2* is associated with liver diseases in Chinese population. Qi *et al*, showed the association between *miRNA-196A2* polymorphism and predisposition to HBV related HCC in Chinese men (Qi et al., 2010) . Li *et al*, revealed the link between *miRNA-196A2* rs11614913 genotype and size of tumor in cirrhosis-associated HCC Chinese patients (Li, Li, et al., 2010). The result of a study in Turkish population revealed that the CC genotype of the *miRNA-196A2* rs11614913 polymorphism might be a genetic risk factor

for progression to HCC in HBV infected patients (Akkiz, Bayram, Bekar, Akgollu, & Ulger, 2011). Recently a meta-analysis provided strong evidence that polymorphism within *miRNA-196A2* rs11614913 is related to HCC risk in the Asian population (Chen et al., 2016a). However, result of another meta-analysis showed that miR-196A2 C>T was associated with decreased risk of HCC in Asian population with HBV infection (Wang, Zhang, et al., 2014).

In this study, the rs12304647 of *miRNA196A2* was found to be significantly protective against HBV progression to cirrhosis/HCC in Malaysians. Similarly, Kou *et al*, reported that *miRNA196-A2* polymorphism had a protective effect from development of the HBV infection to cirrhosis/ HCC. The study also found that there is no association between miR-499 A>G, miRNA-149 C>T or miR-146a G>C polymorphisms with HCC occurrence (Kou et al., 2014). Qi *et al*, have reported that miRNA196A2 C>T and miRNA 499 C>T improved the risk of HCC and different genotypes of the polymorphism in miRNA genes can influence the levels of biochemical markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in HCC patients (Qi et al., 2014). It has been found that *miRNA-196A2* polymorphism is significantly associated with predisposition to progression of HBV to HCC in male Chinese people (Qi et al., 2010) . In contrast, none of the *miRNA-196A2* haplotypes were significantly associated with progression to cirrhosis/HCC in this population. However, Kim *et al* had reported an association between CC genotype of *miRNA-196A2* rs12304647 and predisposition to HBV related HCC in Korean HBV-infected patients. The contrasting results between Kim *et al,* with that of the current study might be due to dissimilarity in contact with varied genotypes of HBV virus infection (Kim et al., 2014). Differences in patients' characteristic, for instance ethnicity, environmental factors and host genetic variations could clarify the inconsistency between this study and other conflicting reports. The main HBV genotype in the South Korean population as reported by Kim *et*

al is the C genotype (Sunbul, 2014), which is different from that of the Malaysian population with equal frequency of the B and C genotype in the Malay ethnic group while the frequency of the B genotype in the Chinese ethnic group is 80%.

It is notable that in the case of *miRNA-196A2* rs12304647, there are no differences in allele (C or A) and genotype frequencies (AA, AC and CC) between chronic hepatitis B with and without cirrhosis and HCC. However, the significant association of rs12304647 was seen under the recessive model but not in the additive or dominant models. This indicates that the association is model dependent in which two copies of allele C is required for protection (OR= 0.37, 95% CI=0.15 - 0.89, p=0.027) in pooled Malaysian population. In other words, CHB individuals who do not carry the CC genotypes might have higher risk of progression of HBV disease.

A complete LD was seen between rs12304647 and rs11614913 of *miRNA-196A2* (D`= 1, r^2 =1.0). In all subjects the haplotype AT was seen frequently compared to AC and CC.

To the best of our knowledge, this is the first study to investigate the association between *miRNA-196A2* rs12304647 and rs11614913 polymorphisms and HBV-related cirrhosis/ HCC occurrence in the Malaysian population.

2.6.1.2 Interleukin-6

Evidence has shown that host innate immunity plays a crucial role in controlling HBV infection and interleukin-6 (*IL-6*) is the main factor in regulation of innate immune response (Lu et al., 2014). Moreover, host genetic background and its interaction with

the HBV virus affect the consequence of long term infection (Tunçbilek, 2014). *IL-6* has been shown to be involved in HBV infection in both *in vitro* and *in vivo* research (Galun et al., 2000). Emerging evidence revealed that the *IL-6* polymorphisms are linked to risk of hepatitis B infection and to development of the disease to liver cirrhosis and HCC (Tang et al., 2014). The result of a study revealed that genetic variation in the promoter region of IL-6 - 572 may be related to HCC incidence in males (Liu et al., 2012).

In our study we did not find any significant dissimilarity in genotype/allelic frequencies of rs2069837, rs1800796 and rs2066992 SNPs between HBV patients with and without cirrhosis/HCC. Hence, these SNPs were not related to progression to liver disease in HBV infection in the Malaysian population. Similarly, Park *et al* reported that *IL-6* variants were not associated with HBV consequences (Park, Lee, et al., 2003b). In contrast, another finding in Europeans indicated that *IL-6* variant may play a role in the clinical progression of HBV infection (Zhao et al., 2013). This contradictory finding may possibly due to the different genetic pools in different ethnicities of study`s subjects.

We also examined the association of *IL-6* haplotypes with progression of HBV infection in the Malaysian population. All three SNPs were common variants including one functional polymorphism of *IL-6* promoter rs1800796 and two (rs2069837 and rs2066992) in intron 2 region. The haplotype combination, namely CT and GG, was made between *IL6* rs1800796 and *IL6* rs2066992. Although the *IL6* rs1800796 and *IL6* rs2066992 were in strong LD (D'=0.99), however, the haplotypes showed no association with progression of HBV infection.

In summary, we investigated the effect of interlukine-6 gene variants with relation to progression of HBV infection in Malaysian subjects. We found no significant differences in the allele and genotype frequencies of rs2069837, rs1800796 and rs2066992 SNPs between HBV patients with and without cirrhosis/HCC. Our results suggest that these

SNPs of *IL-6* are unlikely to contribute to the progression of HBV infection in the Malaysian population.

2.6.1.3 *KIF1B* gene

In the present study, we investigated the genetic association between five SNPs in the *KIF1B* gene, namely, rs3748578, rs8019, rs12734551, rs17401966 and rs17401924 and HBV-related liver disease in Malaysians. Due to SNP deviation from HWE, we could not examine the association of *KIF1B* haplotypes with progression of HBV infection in the Malaysian population.

A GWAS study discovered the association between the *KIF1B* rs17401966 and HBV related HCC in the Chinese population (Zhang et al., 2010). However, in our study none of the five SNPs in *KIF1B* gene were found to have any association with development of CHB to cirrhosis and HCC. Results of our study is in agreement with some other studies which concluded that there was no association between *KIF1B* polymorphism and progression of CHB to cirrhosis and HCC in the Japanese, Korean, Hong Kong and Saudi Arabian populations (Al-Qahtani et al., 2012; Sawai et al., 2012).

KIF1B gene belongs to the Kinesin family and two isoforms of this gene, *KIF1B*α and *KIF1B*β are responsible for transportation of mitochondria and synaptic vesicle ancestors, respectively (MacAskill & Kittler, 2010). It has been proposed that *KIF1B*α can inhibit energy expenses of cancer cells through modifying mitochondria transport which lead to inhibition of cancer growth (Schlisio et al., 2008). Similarly, *KIF1B*β can induce apoptosis which can result in prevention of malignant alteration and development

(Chen et al., 2003). Hence it appears that *KIF1B* may act as a tumor suppressor, however, the results of studies are conflicting. Strunze and colleagues demonstrated the involvement of the kinesin family in stimulating viral infection by increasing the nuclear envelope permeability, thereby assisting the entry of viral DNA into the nucleus (Strunze et al., 2011). In addition, it has revealed that increased expression of a member of the Kinesin family, miototic kinesin-like protein 2, leads to tumor aggressiveness in HCC (Al-Qahtani et al., 2012).

Considering the vital role of two isoforms (alpha and beta) of the *KIF1B* gene in tumor prevention and with inconsistency in result from the different studies, more studies on *KIF1B* variants are needed. In addition, effects of other factors such as alcohol drinking, smoking and aflatoxin exposure study should be considered. The HBV genotype can be another cause of differences in role of *KIF1B* in progression of HBV disease in different studies. The differences in frequency of HCC are most likely because of locality variant in contact with hepatitis virus infection and environmental pathogens (Kew, 2014). For instance, in China the main HBV genotypes are B and C with the high prevalence of genotype B in the south and genotype C in the north area. Similarly, genotypes B and C are dominant in Malaysia with varied frequency in three Malaysian ethnic. There is an equal frequency of genotypes B and C in Malays ethnicity but 80% frequency with genotypes B in the Chinese in Malaysia (Meldal, Bon, Prati, Ayob, & Allain, 2011).

In summary, this study showed no association between polymorphisms in the *KIF1B* gene and HBV-related cirrhosis and/or HCC in the Malaysian population.

2.6.1.4 *DLC1*

We investigated three SNPs rs3739298, rs532841 and rs7821974 of *DLC1*gene and none of the variants were associated with HBV progression to cirrhosis and HCC in Malaysian patients. The deleted in liver cancer 1 gene (*DLC1*) is a tumor suppressor gene. It has been revealed that the *DLC1* gene is one of the common deregulated genes in the cancer genome. Deficiency of *DLC1* expression was seen in 90% of HCC cell line as well as more than 40% of primary HCC in human (Zimonjic & Popescu, 2012). Currently, a genome wide sequencing analysis have discovered that a missense mutation of *DLC1* has been associated with some cancers (Liao, Shih, & Lo, 2008; Park, Durkin, Thorgeirsson, & Popescu, 2003). It has been found that *DLC1* gene is down-regulated in some solid tumors such as ovarian, lung, breast and liver cancers (Ullmannova & Popescu, 2006). Studies have indicated that down regulation of *DLC1* can be attributed to epigenetic and genetic mechanisms. The *DLC1* as a tumor suppressor gene can prevent cell proliferation and reduce aggressiveness of HCC cell lines (Wong et al., 2005). Evidence has shown that *DLC1* mRNA encode RhoGAP protein which usually catalyze the hydrolysis of GTP bound to small RhoGAP family of proteins. As a result, Rho proteins return to the inactive basal state. Thus, RhoGAP negatively control Rho-mediated cellular processes, such as cell cycle progression and gene expression (Moon & Zheng, 2003; Peck, Douglas, Wu, & Burbelo, 2002). Clinically it has been shown that overexpression of Rho proteins in many human cancers lead to increased aggressiveness of tumors (Horiuchi et al., 2003; Kamai et al., 2003). RhoGAP proteins are essential controllers of regular biological processes such as transcription, metastasis and tumorigenesis in many of the cancers (Ko et al., 2013). Research has identified that activity of RhoGAP plays the main role in the tumor suppressor functions of *DLC1* (Lahoz & Hall, 2008). In a mouse model, knockdown of *DLC1* was shown to collaborate with Myc (proto oncogene protein) to stimulate hepatocarcinogenesis (Xue, Krasnitz,

Lucito, Sordella, VanAelst, Cordon-Cardo, Singer, Kuehnel, Wigler, & Powers, 2008). Moreover, mouse hepatoma cells with silenced *DLC1* revealed increased active Rho protein levels which offers great evidence for the activation of Rho as a result of deregulated *DLC1* in tumorigenesis *in vivo*. Consequently, this indicates the role of *DLC1* as a tumor suppressor gene with negative regulatory function in tumor development through down regulation of Rho family proteins (Lahoz & Hall, 2008). The mutations in *DLC1* gene that decrease its expression result in decrease activity of the *DLC1* gene in preventing cancer progression through down regulation of Rho family proteins (Lahoz & Hall, 2008).

Considering that HCC is heterogeneous and complex; the identification of related predicting markers based on patients' genetic data may aid in therapeutic strategies, early diagnosis and management of the disease (Zimonjic & Popescu, 2012). Chromosome 8p contains a large number of tumor suppressor genes (TSGs) and has been known to have a divergent pattern of genomic imbalances in HCC (Zimonjic & Popescu, 2012). The *DLC1* is a potent TSG located in chromosome 8p and which plays a critical role in the pathogenesis of HCC. Tumor suppressor gene act through the signaling pathways by protecting tumor initiation and development (Xue, Krasnitz, Lucito, Sordella, VanAelst, Cordon-Cardo, Singer, Kuehnel, Wigler, Powers, et al., 2008). Hence, therapeutic intervention which can target signaling networks involved in *DLC1* functions could lead to potential treatment of HCC.

2.6.1.5 Other candidate genes

We did not find any association between other candidate genes (*VEGFA*, *CXCR1*, *RAD52*, *ATF6 and TEP1*) polymorphism and progression to cirrhosis/HCC in HBV infected patients in the Malaysian population. Compared to previous results, we propose that the differences in results is because of variabilities in genetic background in certain ethnicity.

2.7 Conclusion

In summary, our study showed a significant association between *miRNA-196A2* rs12304647 with progression of disease in chronic HBV. The significant result achieved after adjustment of confounding factors had indicated that genetic polymorphism in *miRNA-196A2* variant is a predictor of cirrhosis / HCC among CHB infected patients in Malaysia. In this study, we investigated SNPs in some genes leading to progression of CHB disease for better understanding of whether these genes are related to cirrhosis / HCC in CHB patients. This study also shows proof of ethnic differences in allele and genotype frequencies of different gene polymorphism in the Malaysian population. Malaysia is a multi-ethnic country containing three different genetic background, hence the result of this study can assist researchers and clinicians on how to identify and manage those infected HBV patients who are at high risk of progression.

CHAPTER 3: THE ROLE OF HUMAN LEUCOCYTE ANTIGEN IN PATIENT SUSCEPTIBILITY TO LIVER DISEASE PROGRESSION IN HBV INFECTED INDIVIDUALS

3.1 Introduction

3.1.1 Background of study

The Human Leucocyte Antigen (HLA) or the Major Histocompatibility Complex (MHC) in human, consist of more than 200 genes and located on chromosome 6p21 (Figure 3.1) (Li, Zhang, et al., 2014; Robinson et al., 2009; Shiina, Hosomichi, Inoko, & Kulski, 2009). It has been reported that HLA region encodes a number of molecules which play important roles in the immune system which are involved in defense against infections (Al-Qahtani et al., 2014; Gough & Simmonds, 2007).

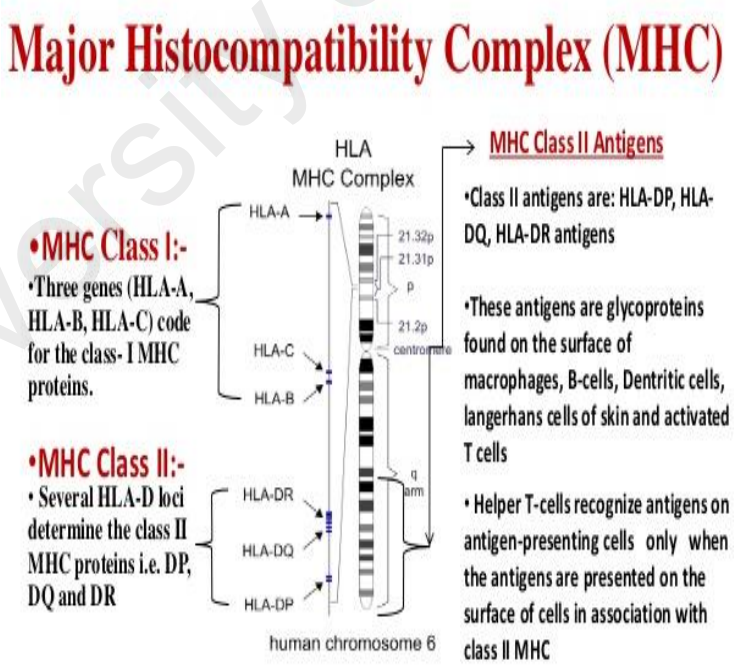


Figure 3.1: Human MHC resides on the arm of chromosome 6

Adapted from https://www.google.com/search

The HLA genes are classified into three clusters: class I, class II and class III (Mangalam, Taneja, & David, 2013). In human, HLA class I consist of three genes, namely, HLA-A, HLA-B and HLA-C which are expressed on the surface of all nucleated cells whereas the HLA class II are expressed entirely on the surface of antigens presenting cells (APC) such as B cells, macrophages and dendritic cells. HLA class II consist of HLA-DP, HLA-DQ and HLA-DR (Figure 3.1) (Tamori & Kawada, 2013; van Lith, McEwen-Smith, & Benham, 2010). The important role of HLA in the immune system is played by class I and class II in differentiation of self and non-self-antigen (Bardi et al., 2012; Blackwell, Jamieson, & Burgner, 2009).

The HLA system trigger immune response against pathogens with presenting antigens to $CD8^+$ and $CD4^+$ T cells by HLA class I and class II respectively (Horton et al., 2008; Singh et al., 2007). In general, interaction of HLA regulated immune system components such as T lymphocyte, B lymphocyte, Natural Killer cells (NK) and cytokines is the response of host immune in challenge to hepatitis infection (Tamori & Kawada, 2013). It is believed that an effective association of HLA molecules to the viral antigens led to an appropriate immune response against viral infection. Indeed, active presentation of antigens to $CD8^+$ T cells and $CD4^+$T cells by HLA class I and class II respectively is a critical role of immune system in facing to viral infection (Blackwell et al., 2009). Besides the important role of HLA in susceptibility to a variety of disease including infectious diseases it also plays a crucial role in organ transplant (Sheldon & Poulton, 2006).

According to the WHO Nomenclature Committee an HLA allele name has a specific structure (Figure 3.2) that may be composed of four, six or eight digits depend on its sequence (Marsh et al., 2010).

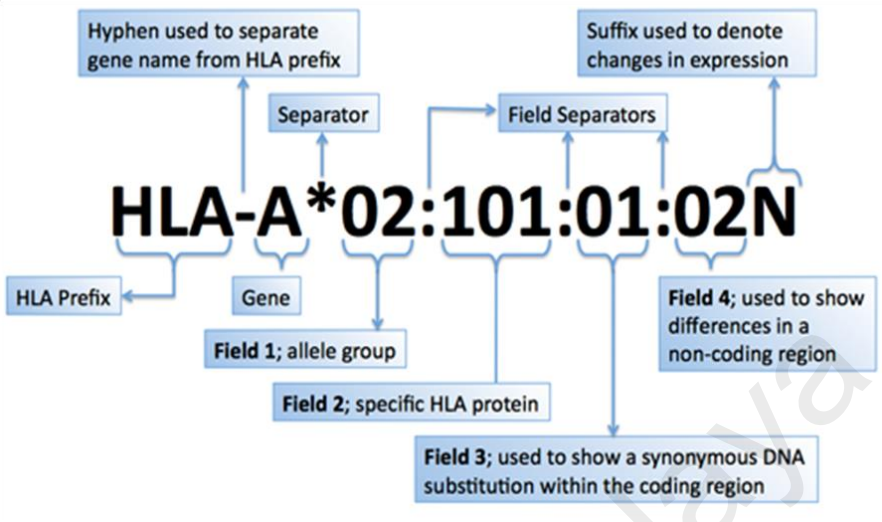


Figure 3.2: HLA Nomenclature

Adapted from http://hla.alleles.org/nomenclature/naming.html

3.2 Literature review

3.2.1 Overview of study

Numerous studies confirmed the role of HLA genes in determining consequences of the HBV infection, however, the results are inconsistent between different populations such as Turkish, Taiwanese, African American and Chinese (Doganay et al., 2014; Meng, Chen, Ma, & Liu, 2003; Thio et al., 1999; Wu et al., 2004) .

A significant association has been reported between HLA-DR7 and susceptibility to persistence of HBV infection in Qatar population while in another study HLA-DR7 was associated with recovery of HBV infection in Han Chinese population (Wu et al., 2004). Furthermore, in three separate studies, an association was seen between HLA-DR13 and

clearance of HBV infection in Gambian, Koreans and Europeans (Ahn et al., 2000; Hohler et al., 1997; Thursz et al., 1995). In addition, HLA-DR0406 was reported to be associated with recovery of HBV infection in Han Chinese (Wu et al., 2004).

Due to the role of HLA class II genes in HBV infection it is important to investigate the effect of these genes in defining progression of the HBV infection in Malaysia. Since previous studies showed ethnic-specific results, it will be interesting to explore the role of HLA class II genes in the multi-ethnic Malaysian population. Thus this part of the study aimed to investigate the role of HLA class II (particularly of HLA-DQ) with liver disease progression in HBV infected individuals in the Malaysian population with the purpose of identifying relation of HLA-DQ allele involved in immune response.

3.2.2 Role of HLA class II in immune system and infectious disease

HLA class II loci is the most variable region in the human genome and play a significant role in susceptibility to a variety of disease including infectious disease (Mangalam et al., 2013). HLA class II molecules are encoded by HLA-DP, HLA-DQ and HLA-DR loci and have approximately 70% similarity with each other. They play vital roles in adaptive immune system in defense against infection (Traherne, 2008; van Lith et al., 2010).

The HLA class II consist of two polypeptides chains alpha and beta (Mangalam et al., 2013; Reith, Siegrist, Durand, Barras, & Mach, 1994; van Lith et al., 2010). Each alpha and beta chain has 2 domains which alpha2 and beta2 regions are highly conserved while alpha1 and beta1 are highly polymorphic (Mangalam et al., 2013). As alpha1 and beta1

join to each other to form the antigen binding groove, high polymorphism in this region result in generation of new class II molecules with capability to identify new epitopes (Mangalam et al., 2013). The high polymorphism in this region contributes to the dissimilarities in susceptibility to disease among different ethnic groups. For HLA-DQ and HLA-DP both chains are polymorphic while for HLA-DR most variability comes from HLA-DR-beta (Alves, Souza, Meyer, Toralles, & Brites, 2006).

In order to have an effective immune response against an infectious agent, the HLA molecules should bond to the peptides derived from this pathogen and the range of the T cells must include clones that can be activated by this HLA-peptide association (Alves et al., 2006). Susceptibility to an infectious disease may be because of defectiveness in some stages of this system. It has been reported that the HLA alleles vary in different populations with different ethnic groups (Huang et al., 2016; Klein & Sato, 2000). Studies propose that the HLA class II alleles that confer defence against certain pathogens are dominant in region where they cause endemic disease (Carrington et al., 1999; Klein & Sato, 2000) and they are able to support an effective immune response to clear the infection (Apanius, Penn, Slev, Ruff, & Potts, 1997; Oliver & Piertney, 2012; Trowsdale, 2011).

3.2.3 List of HLA class II in HBV infection and HBV-associated cirrhosis/HCC in other publications.

Table 3.1: HLA class II in HBV infection and HBV-associated cirrhosis/HCC

Study population	HLA	Chronicity of HBV infection	Progression of HBV to cirrhosis/HCC	Reference
Chinese	DRB1*0301 DQA1*0501 DQB1*0301 DQA1*0301 DRB1*1101	risk factor risk factor risk factor protective factor protective factor		(Jiang et al, 2003)
Chinese	DRB1*06 DRB1*08 DRB1*16 DRB1*07	risk factor risk factor risk factor protective factor		(Han,.Yang,. Zheng, Tang, & Zhu, 2005)
Indian	DRB1*15 DRB1*11 DRB1*13	risk factor risk factor protective factor		(Amarapurpar, Patel, & Kankonkar, 2003)
African Americans	DQA1 *0501 DQB1 *0301	risk factor risk factor		(Thio et al, 1999)
Turkish	DR7 DR13 DQ3	risk factor risk factor risk factor		(Karan et al, 2002)
Chinese	DRB1*04 DQB1*02 DQB1*06		risk factor risk factor protective factor	(Pan et al, 2009)
Chinese	DRB1*1501 DQA1*0302 DQB1*0302		protective factor protective factor protective factor	(Donaldson et al,2001)
Egyptian	DRB1*04 DQB1*02 DQB1*06		risk factor risk factor protective factor	(El-Chennawi et al., 2008)

3.2.4 HLA-DQ and HBV infection

Numerous evidence have shown the association of HLA-DQ molecules with the consequences of HBV infection (Doganay et al., 2014; Jiang et al., 2003; Karan, Tascioglu, Ozturk, Palanduz, & Carin, 2002; Meng et al., 2003; Thio et al., 1999; Wu et al., 2006), however, the majority of these studies were focused on the clearance and persistence of HBV infection and there is only a few reports on the effect of HLA-DQ alleles with progression of HBV disease.

For instance, Doganay *et al*, reported that HLA-DQB1*03:01 were more frequent in patients with active HBV infection than in inactive patients in Turkish population (Doganay et al., 2014). Moreover, Karan *et al*, stated that HLA-DQ3 was associated with high risk of chronic hepatitis B infection in the Turkish population (Karan et al., 2002). Liu *et al*, identified that HLA-DQA1*0102 was associated with protection from chronic hepatitis B infection in the Han Chinese population (Liu & Cheng, 2007). In another study, it was shown that HLA-DQB1*0503 is related to earlier HBeAg seroconversion in Taiwanese children (Wu et al., 2006). It have been also stated that HLA-DQB1*0503 allele and HLA-DQB1*0303 allele are independently protective genetic factors to chronic HBV in Han Chinese population (Xi-Lin et al., 2006). A study in the Japanese population reported that HLA-DQA1*0102-HLA-DQB1*0604 and HLA-DQA1*0101-HLA-DQB1*0501 are protective haplotypes while HLA-DQA1*0102- HLA-DQB1*0303 and HLA-DQA1*0301- HLA-DQB1*0601 are risk haplotypes of persistent HBV infection (Mbarek et al., 2011).

3.2.5 HLA-DQ and HBV-associated cirrhosis / HCC

With regard to HBV progression to cirrhosis and HCC a few studies have been conducted so far. For instance, HLA-DQA1*0104 was found to be a protective allele from progression of CHB to liver cirrhosis in Han Chinese population (Liu & Cheng, 2007). A recent meta-analysis has investigated the association between HLA-DQB1 alleles and risk of HCC and had shown that HLA-DQB1*02 and HLA-DQB1*0502 are risk factors for HCC occurrence whereas HLA-DQB1*03 and HLA-DQB1*0602 have protective effect on incidence of HCC (Xin et al., 2011). A study in Egyptian patients was reported that HLA-DQB1*02 alleles might be a risk factor for the incidence of HCC while HLA-DQB1*06 might be a protective allele in occurrence of HCC (El-Chennawi et al., 2008).

3.2.6 Study objectives

- To investigate the association between HLA-DQ alleles with progression to cirrhosis and /or HCC in HBV infected patients in Malaysia.
- To investigate the association between haplotypes of HLA-DQA1 and HLA-DQB1 with progression to cirrhosis and/or HCC amongst chronic HBV infection in Malaysia.

3.2.7 Justification of the study

HLA molecules are reported to be ethnic specific causing differential outcomes of HBV infection in different population. Malaysia has a multi ethnic population, thus the

study of HLA in relation to progression of HBV infection is essential. Understanding the role of HLA in immune response in defense of HBV infection will lead to effective design of novel immune therapies.

3.2.8 Hypothesis of the study

The hypotheses for this study are:

The null hypotheses (H_0)

- There exist no association between HLA-DQ alleles with progression to cirrhosis and /or HCC in HBV infected patients in Malaysia.
- There exist no association between haplotypes of HLA-DQA1 and HLA-DQB1 with progression to cirrhosis and/or HCC amongst chronic HBV infection in Malaysia.

The research hypotheses (H_1)

- There is an association between HLA-DQ alleles with progression to cirrhosis and /or HCC in HBV infected patients in Malaysia.
- There is an association between haplotypes of HLA-DQA1 and HLA-DQB1 with progression to cirrhosis and/or HCC amongst chronic HBV infection in Malaysia.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Blood collection

Alcohol swabs, 6 mL EDTA tubes, sterile plaster, disposable gloves.

3.3.1.2 DNA extraction

Mini-centrifuge machine, 1.5mL micro-centrifuge tubes (Axygen, Poland), GeneAll$^{@}$ Exgene^{TM} DNA purification kit (Dongnam-ro, Songpa-gu, Seoul, South Korea), pipette and pipette tips (Eppendorf).

3.3.1.3 Nanodrop

Nano-drop 2000c spectrophotometer (Thermo Fisher scientific, USA), 1.5 mL micro-centrifuge tubes (Axygen, Poland), pipette and pipette tips (Eppendorf).

3.3.1.4 HLA-DQ genotyping

GeneAll$^{@}$ Exgene^{TM} DNA purification kit (Dongnam-ro, Songpa-gu, Seoul, South Korea), LAB Type polymerase chain reaction amplification (PCR) with sequence specific oligonucleotide (PCR-SSO) probes, HLA DQA1/DQB1 One Lambda kit (Canoga Park, California, USA).

3.3.1.5 Instrumentation

Polymerase chain reaction amplification (PCR) machine, Luminex profiling system (xMAP; Luminex, Austin, TX). Refrigerated centrifuge.

3.3.2 Methods

3.3.2.1 Subjects

A total of 121 HBV chronically infected patients (76 males and 45 females) were enrolled in this part of the study. Of them, 52 were diagnosed with chronic hepatitis B (CHB) without liver cirrhosis / HCC and 69 CHB with liver cirrhosis / HCC.

The inclusion and exclusion criteria for participants are the same as those for the subjects mentioned in Chapter 2.

3.3.2.2 HLA-DQA1 / -DQB1 genotyping

A low resolution (2-digit) genotyping was used to determine the HLA-DQA1 and HLA-DQB1 genotypes between CHB patients with and without cirrhosis/HCC. LAB Type polymerase chain reaction amplification (PCR) with sequence specific oligonucleotide (PCR-SSO) probes technique together with HLA DQA1/DQB1 One Lambda kit (Canoga Park, California, USA) and a Luminex profiling system (xMAP; Luminex, Austin, TX) were used for genotyping according to the manufacturer's protocol. The samples were used in final DNA concentration of 20 ng/µl and a good

quality with an absorbance of 260/280 <1.80 and >1.65. Briefly, the target DNA was amplified with biotinylated specific primers for the purpose of detection by R-Phycoerythrin conjugated Streptavidine (SAPE). The amplified DNA was then denatured, neutralized and hybridized with the oligonucleotide probes which are labelled with SAPE and finally detected by Luminex 200 system. The HLA alleles were recognised by HLA visual 1.0 software via referring to HLA typing pattern data for -DQA1 and -DQB1 provided by manufacturer.

3.4 Statistical analysis

Allele frequencies of HLA-DQA1 and -DQB1 were calculated with the direct counting method. A chi-square test was done to examine the deviation from Hardy-Weinberg equilibrium using SPSS version 16.0. Logistic regression was applied to conclude the association between HLA-DQA1 and -DQB1 with disease progression in CHB patients. The Linkage Disequilibrium of two locus haplotype was calculated using the SPSS version 16.0. Odd ratios (ORs) with 95% confidence intervals (CI) were calculated after adjustment for confounding factors such as sex and age. The data were presented as percentage or mean ± standard deviation. A p-value less than 0.05 was considered to be statistically significant. The significance levels for α was set according to the number of observed alleles at each locus. For HLA-DQA1 and -DQB1 alleles, the number of observed alleles was 5 for each of them. Thus, the significance levels for HLA-DQA1 and -DQB1 alleles were set at $\alpha=0.05/5$ after Bonferroni correction.

3.5 Results

Demographics of the 121 study subjects are presented in Table 3.2. All of the different ethnic groups were pooled and it included 44 Malays and 77 Chinese, five HLA-DQA1 alleles (-DQA1*01, -DQA1*02, -DQA1*03, -DQA1*05 and -DQA1*06) and five HLA-DQB1 alleles (-DQB1*02, -DQB1*03, -DQB1*04, -DQB1*05 and -DQB1*06) were observed. The HLA-DQA1 and HLA-DQB1 allele's frequencies and their associations with progression of HBV disease are presented in Table 3.3. Among all patients in this study, 66% of HBV patients with cirrhosis / HCC (n=46) and 62% of HBV patients without cirrhosis / HCC (n=32) are carriers of HLA-DQA1*01 (p value= 0.908, OR= 0.95, CI= 0.41 - 2.10). In addition, 73% of HBV patients with cirrhosis / HCC (n=50) and 60% of HBV patients without cirrhosis / HCC (n=31) are carriers of HLA-DQB1*03 (p value= 0.320, OR= 1.56, CI= 0.65 - 3.72). Furthermore, 17% of HBV patients with cirrhosis / HCC (n=12) and 8% of HBV patients without cirrhosis / HCC (n=4) are carriers of HLA-DQA1*02 (p value= 0.167, OR= 2.49, CI= 0.68 - 8.05). Whereas 4% of HBV patients with cirrhosis / HCC (n=3) and 12% of HBV patients without cirrhosis / HCC (n=6) were carries HLA-DQB1*04 (p value= 0.455, OR= 0.55, CI= 0.12 - 2.61). There were no significant association with progression of CHB and HLA alleles in Malaysian population. The DQA1-DQB1 haplotypes frequencies and their associations with progression of HBV disease are presented in Table 3.4. Twenty five HLADQA1-DQB1 haplotypes were identified between CHB patients with and without cirrhosis/HCC. In this study, 62% of HBV patients with cirrhosis/HCC (n=32) and 48% of HBV patients without cirrhosis/HCC (n=25) were carriers of haplotype DQA1*01-DQB1*05 (p value= 0.495, OR= 0.76, CI= 0.34 - 1.68). None of the haplotypes were significantly associated with progression of HBV disease.

Table 3.2: The characteristic of participants for HLA typing.

	HBV infected without LC/HCC (n= 52)	**HBV infected with LC/HCC (n= 69)**	**P**
Age	55.23 ± 10.76	65.84 ± 9.21	0.000
Ethnicity			0.239
Malay	22 (42.3%)	22 (31.8%)	
Chinese	30 (57.7%)	47 (68.2%)	
Indian	-	-	
Gender			0.313
Male	30 (57.7%)	46 (66.7%)	
Female	22 (42.3%)	23 (33.3%)	

LC: liver cirrhosis; HCC: hepatocellular Carcinoma

P <0.05 was considered significant

Table 3.3: The allele frequencies and association of HLA-DQA1/-DQB1 with disease progression in CHB patients with and without cirrhosis/HCC.

Allele	HBV infected without cirrhosis/HCC (n= 52)		HBV infected with cirrhosis/HCC (n= 69)		X^2	OR (95%)	p Value
DQA1	Count	%	Count	%			
01	32	62%	46	**66%**	0.560	0.95 (0.41 - 2.10)	0.908
02	4	8%	12	17%	0.119	2.49 (0.68- 8.05)	0.167
03	22	42%	24	35%	0.399	0.88 (0.39 - 2.01)	0.773
05	12	23%	20	29%	0.466	1.58 (0.64 - 3.87)	0.319
06	13	25%	24	35%	0.248	1.18 (0.49 - 2.85)	0.709
DQB1							
02	9	17%	21	30%	0.096	2.28 (0.87 - 6.01)	0.098
03	31	60%	50	**73%**	0.137	1.56 (0.65 - 3.72)	0.320
04	6	12%	3	4%	0.136	0.55 (0.12 - 2.61)	0.455
05	25	48%	31	45%	0.731	0.74 (0.33 - 1.63)	0.449
06	10	19%	18	26%	0.376	1.21 (0.47 - 3.05)	0.695
Serology							
DQ2	8	15%	21	30%	0.055	2.49 (091 -6.80)	0.076
DQ4	5	9%	2	3%	0.117	0.49 (0.08 -3.05)	0.441
DQ5	24	46%	30	44%	0.893	0.79 (0.36 - 1.75)	0.570
DQ6	10	19%	17	25%	0.376	1.21 (0.48 - 3.05)	0.690
DQ7	21	40%	33	48%	0.546	0.89 (0.39 - 1.99)	0.778
DQ8	9	17%	8	12%	0.371	0.87 (0.28 - 2.76)	0.821
DQ9	12	23%	13	19%	0.569	0.75 (0.27 - 2.01)	0.561

X^2: Pearson Chi Square p value and OR were calculated in presence versus absence of each allele. Association of HLA-DQA1 and –DQB1 alleles with HBV disease progression assessed by logistic regression analysis adjusted for gender and age.

Table 3.4: The associations of DQA1-DQB1 haplotypes with disease progression in CHB patients with and without cirrhosis/HCC.

Haplotype		HBV infected without cirrhosis/HCC (n= 52)		HBV infected with cirrhosis/HCC (n= 69)		(X^2)	OR (95%)	p value
HLA-DQA	HLA-DQB1	Count	%	Count	%			
01	02	4	7.7%	10	14.5%	0.247	1.70 (0.45 - 6.50)	0.437
01	03	13	25%	28	40.6%	0.073	1.38 (0.58 - 3.30)	0.469
01	04	2	3.8%	1	1.5%	0.401	0.37 (0.03 - 5.37)	0.464
01	05	25	48.1%	32	61.5%	0.853	0.76 (0.34 - 1.68)	0.495
01	06	10	19%	18	26%	0.376	1.21 (0.48 - 3.05)	0.695
02	02	4	7.7%	11	15.9%	0.173	2.02 (0.55 - 7.40)	0.289
02	03	1	1.9%	8	11.6%	0.045	4.64 (0.86 - 6.37)	0.066
02	04	0	0	1	1.5%	-	-	NA
02	05	2	3.8%	2	2.9%	0.773	0.503 (0.06 - 4.12)	0.522
02	06	0	0	1	1.5%	-	-	NA
03	02	3	5.8%	5	7.2%	0.746	1.69 (0.32 - 6.97)	0.536
03	03	19	36.5%	23	33.3%	0.714	1.05 (0.45 - 2.43)	0.916
03	04	6	11.5%	3	4.3%	0.136	0.55 (0.12 - 2.61)	0.455
03	05	3	5.8%	3	4.3%	0.721	0.75 (0.13 - 4.25)	0.745
03	06	1	1.9%	9	13%	-	-	NA
05	02	5	9.6%	11	15.9%	0.309	2.39 (0.69 - 6.21)	0.168
05	03	9	17.3%	12	17.4%	0.99	1.14 (0.41 -3.21)	0.804
05	04	2	3.8%	0	0	-	-	NA
05	05	4	7.7%	9	13%	0.347	1.81 (0.48 - 4.82)	0.384
05	06	4	7.7%	3	4.3%	0.435	0.53 (0.11 - 2.49)	0.419
06	02	1	1.9%	5	7.2%	0.182	5.30 (0.47 - 5.77)	0.177
06	03	13	25%	24	34.8%	0.248	1.18 (0.49 - 2.85)	0.709
06	04	2	3.8%	1	1.5%	0.401	0.53 (0.04 - 6.52)	0.619
06	05	5	9.6%	9	13%	0.559	0.81 (0.22 - 2.88)	0.738
06	06	0	0	2	2.9%	-	-	NA

X^2: Pearson Chi Square p value and OR were calculated in presence versus absence of each allele. Association of HLA-DQA1 and –DQB1 alleles with HBV disease progression assessed by logistic regression analysis adjusted for gender and age. NA: not available.

3.6 Discussion

In this study we found that 66% of HBV patients with cirrhosis / HCC and 62% of HBV patients without cirrhosis / HCC are carriers of HLA-DQA1*01. In addition, 73% of HBV patients with cirrhosis / HCC and 60% of HBV patients without cirrhosis / HCC are carries of HLA-DQB1*03. We could not find any association between HLA-DQA1 and HLA–DQB1 allele / haplotype and HBV disease progression in the Malaysian population.

Numerous evidence have shown the association of HLA-II molecules with inflammation, auto-immunity and infectious disease (Hong et al., 2005; Shiina et al., 2009; Yue et al., 2015), such as hepatitis B infection (Mbarek et al., 2011; Xi-Lin et al., 2006). HLA-DQ is greatly polymorphic particularly in exon 2 which codes for antigen binding sites. Thus, high variations in this region lead to identifying a number of alleles which are associated with consequences of HBV infection (Al-Qahtani et al., 2014; Mangalam et al., 2013). It has been confirmed that the presence of particular HLA class II alleles influence the effective immune response to infection (Oliver & Piertney, 2012; Trowsdale, 2011).

With respect to HBV infection, HLA system is known to influence the outcomes of disease. Several studies indicated the association of HLA–DQA1 and –DQB1 alleles with HBV infection outcomes in different populations. For instance, Jiang *et al*, have conducted a study in Chinese Han population among patients with chronic hepatitis B, acute hepatitis B and healthy controls subjects. Their results showed that HLA–DQB1*0301 and HLA–DQA1*0501 are significantly associated with predisposition to chronic hepatitis B while HLA–DQA1*0301 is closely associated with resistance to chronicity of HBV infection (Jiang et al., 2003) . In another study conducted in African Americans by Thio *et al*, have demonstrated that two HLA alleles, HLA–DQB1*0301

and HLA–DQA1*0501 as well as their corresponding haplotypes are associated with HBV persistence (Thio et al., 1999). A study by Mbarek *et al*, was conducted on CHB patients and non-HBV controls revealed that HLA-DQA1*0102–DQB1*0604 and HLA-DQA1*0101–DQB1*0501 are protective haplotypes while HLA-DQA1*0102–DQB1*0303 and HLA-DQA1*0301–DQB1*0601 are risk haplotypes in the Japanese population (Mbarek et al., 2011). Furthermore, two studies from China have revealed that the HLA-DQB1*03:03 and DQB1*05:03 are associated with HBV viral persistence (Han, Yang, Zheng, Tang, & Zhu, 2005; Xi-Lin et al., 2006). In addition, DQB1*05:01 allele is more common in chronic active HBV than inactive patients indicating that this allele is associated with chronic HBV persistent (Doganay et al., 2014). However, majority of these studies focused on clearance and persistence of HBV infection and there is only a few reports on effect of HLA-DQ alleles and HBV progression to cirrhosis / HCC.

To date, very little is known about association of HLA-DQ with susceptibility or resistance to progression of HBV disease in different population. With regards to HLA-DQA1 the only report is from China that HLA-DQA1*0104 has a protecting effect on progression of HBV infection to liver cirrhosis (Liu & Cheng, 2007). However, HLA-DQB1*02 and HLA-DQB1*06 were identified as a risk allele and protective allele in relation to HBV progression to HCC respectively (El-Chennawi et al., 2008).

This is the first study that demonstrated the distribution of HLA-DQA1 and -DQB1 alleles and haplotypes in HBV infected patients in the Malaysian population.

It is hoped that understanding the role of HLA-DQ in immune response in defense of HBV infection will lead to effective design of novel immune therapies. Given the inconclusive result of previous studies, further association studies with regard to

progression of HBV to cirrhosis and HCC and HLA-DQ alleles are required to provide strategies for the management and prevention of chronic HBV infection.

3.7 Conclusion

We did not find a positive association between HLA-DQ allele and their corresponding haplotype with HBV progression to cirrhosis and HCC in Malaysian.

CHAPTER 4: MICRORNA PROFILING IN PATIENT SUSCEPTIBILITY TO LIVER DISEASE PROGRESSION IN HBV INFECTED INDIVIDUALS

4.1 Introduction

4.1.1 Background of study

Transcriptome is the complete set of RNA (transcripts) encoded by the genome at a specific time. Understanding the transcriptome is necessary for interpreting the functional elements of the genome as well as for understanding the disease (Wang, Gerstein, & Snyder, 2009). Comparison of transcriptomes allows the identification of genes that are differentially expressed in response to the treatment or in distinct cell population.

MicroRNAs (miRNAs) are a class of evolutionarily conserved RNA molecules which are transcribed from the genome (George & Mittal, 2010). They regulate more than 30% of all human genes and mediate gene expression at the post transcriptionally levels in both human and plants (Wang & Xi, 2013). MiRNAs are involved in the control of many crucial biological process, such as cell growth, differentiation, development, tumorigenesis and apoptosis (Baskerville & Bartel, 2005; Carmell, Xuan, Zhang, & Hannon, 2002; Lee, Kim, Chung, Kim, & Dutta, 2005; Takamizawa et al., 2004). MiRNA control gene expression by binding to specific mRNAs and inhibit their translation into protein (Figure 4.1). They are capable to downregulate hundreds of genes at the same time, thus they can regulate entire transcriptional programs that determine important cellular activities (Behne & Copur, 2012).

MiRNA play a vital role in the development of the immune system. They are also involved in adaptive immune response by antigen presentation as well as innate

immunity through cytokine responses and regulation of Toll-like receptor signaling (Taganov, Boldin, Chang, & Baltimore, 2006).

It has been identified that miRNAs are involved in important biological factors such as hepatocyte differentiation (Coulouarn, Factor, Andersen, Durkin, & Thorgeirsson, 2009; Murakami et al., 2006), metastasis (Budhu et al., 2008; Wong et al., 2011), HBV or HCV infection (Ura et al., 2009), patient survival (Xiong et al., 2010; Zhang et al., 2010), and tumor recurrence (Ji et al., 2009). Thus this part of the study is aimed at investigating the differences in microRNAs expression between HBV-infected patients with and without cirrhosis and HCC with the purpose of identifying regulatory biomarkers for diagnosis of those HBV infected who are at higher risk to progress to cirrhosis and HCC. Specific microRNA can serve as biomarker for prevention and diagnosis of progression of HBV disease.

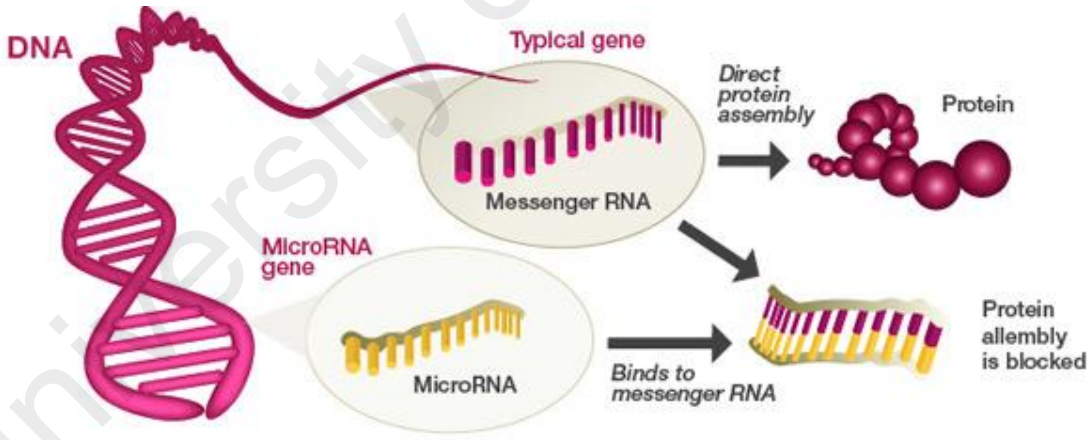


Figure 4.1: Role of MiRNA in controlling gene expression

Adapted from http://www.longevinex.com/articles/micrornas-aging-and-resveratrol/

4.2 Literature review

4.2.1 Overview of study

MicroRNAs are a class of endogenous noncoding RNAs with approximately 22 nucleotides in length (Srinivasan, Selvan, Archunan, Gulyas, & Padmanabhan, 2013). They play a vital role in the post transcriptional regulation of gene expression by repressing translation of target mRNAs (Ji et al., 2011). MicroRNAs are abundantly present in all human cells and are capable of suppressing many genes (Srinivasan et al., 2013). It has been proven that miRNAs act as potential diagnostic biomarkers and numerous aspects of miRNAs might provide novel ways of using these in disease diagnosis (Figure 4.2) (Paranjape, Slack, & Weidhaas, 2009).

Evidence have demonstrated the regulatory role of microRNAs in liver diseases such as viral hepatitis infection (Srinivasan et al., 2013; Sun, Lu, Wang, & Jin, 2013; van der Ree, de Bruijne, Kootstra, Jansen, & Reesink, 2014). In addition, differentially expressed miRNAs has been detected in many cancers such as prostate, kidney, breast and liver cancer (Catto et al., 2011; Corcoran, Friel, Duffy, Crown, & O'Driscoll, 2011; Jiang et al., 2008). Numerous studies have shown that miRNAs expression are significantly different between HCC and non-tumor tissue (Kutay et al., 2006; Ladeiro et al., 2008; Meng et al., 2007; Pineau et al., 2010). The result of other studies revealed that some serum miRNAs levels such as miRNA-122, miRNA-16, miRNA-25, miRNA- 375 miRNA-21, miRNA-223 and let-7f were expressed differentially between HBV patients with and without HCC (Qu, Zhang, Li, Afdhal, & Albitar, 2011; Xu et al., 2011). Another study by Qi *et al*, revealed that serum microRNA-122 may serve as a potential biomarker for detection of HCC in healthy individuals, however it cannot identify HCC in chronic HBV infected patients (Qi et al., 2011). Yuan *et al* tested 19 tumor/ non tumor liver tissue of HBV positive HCC patients to find the association of microRNA-148a in tumor-

genesis. Their results revealed that higher expression of miR-148a is associated with the elevation of tumorigenesis. They concluded that microRNA-148a may be used as a non-invasive diagnostic indicator and / or therapeutic target related to hepatocarcinogenesis (Yuan et al., 2012).

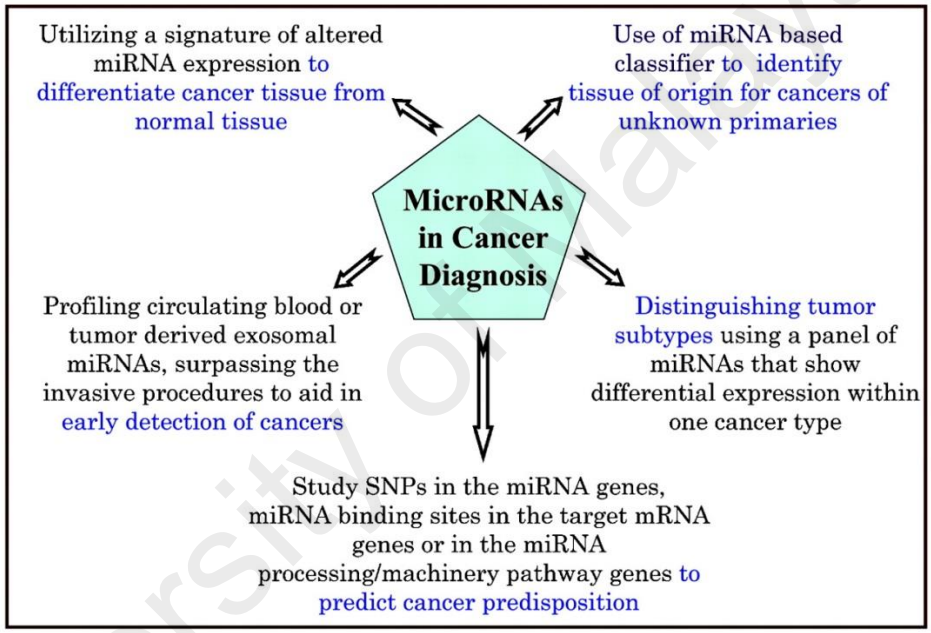


Figure 4.2: MicroRNA`s application in cancer diagnosis

Adapted from Paranjape and Slack (2009)

4.2.2 MicroRNA biogenesis pathway

MiRNA genes are transcribed as pri-miRNAs by RNA polymerase II in the nucleus. The long pri-miRNAs are then cleaved by the microprocessor which consist of Drosha enzyme into a precursor miRNA (pre-miRNA). The pre-miRNA is transferred to the cytoplasm via Exportin-5 where it will be cut by Dicer enzyme into miRNA-miRNA* duplex. The miRNA* is normally degraded, however, the mature miRNA is loaded into the RNA-induced silencing complex (RISC) where addition regulations will be carry out, depending on the level of complementarity between the miRNA and its target in the 3' untranslated region of the mRNA. In perfect complementarity, the mRNA will be cleaved by RISC and degraded while in case of defective complementarity, translation will be suppressed. (Figure 4.3) (Borel, Konstantinova, & Jansen, 2012).

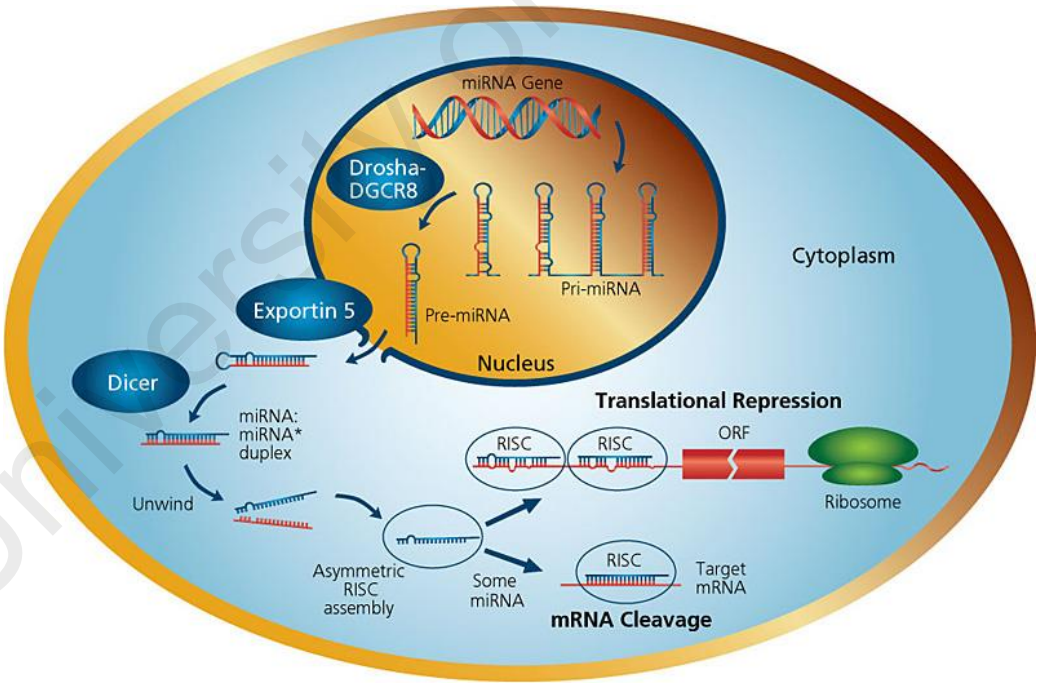


Figure 4.3: MicroRNA biogenesis pathway

Adapted from www.sigmaaldrich.com

4.2.3 MicroRNA biogenesis pathways in cancer

MicroRNAs are crucial regulators of gene expression and aberrant expression of individual miRNA are linked to human cancer. In addition, miRNA depletion caused by epigenetic and genetic variations in mechanisms of the miRNA biogenesis is oncogenic (Figure 4.4) (Lin & Gregory, 2015). Evidence showed that up-regulated miRNAs may act as oncogenes by down-regulation of tumor suppressor genes (TSG) while, the down-regulated miRNAs may function as TSGs by regulating genes involved in apoptosis (George & Mittal, 2010; Zhang, Pan, Cobb, & Anderson, 2007). All of these facts highlights the significance of miRNA deregulation in cancer.

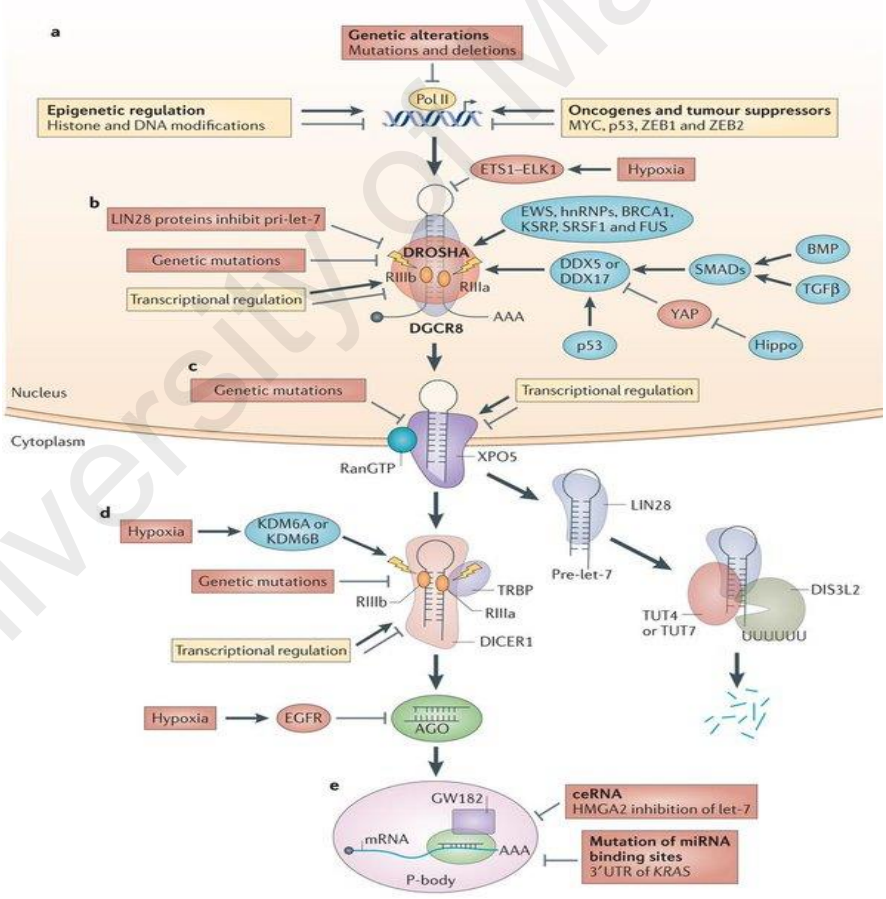


Figure 4.4: Overview of miRNA biogenesis pathways in cancer.

Adapted from NatureReviewsCancer (2015)

4.2.4 Aberrantly expressed microRNAs in HBV-associated cirrhosis/HCC in other studies.

Table 4.1 shows the identified aberrantly expressed microRNAs between HBV with and without progression to cirrhosis/HCC in other studies.

Table 4.1: Aberrantly expressed microRNAs in HBV-associated cirrhosis/HCC

microRNA	expression	source of microRNA	Reference
miR-16, miR-199a	Down Down	Serum	(Zhang, Yang, et al., 2010)
miR-191 miR-122	Up Down	Tissue	(Spaniel et al., 2013)
miR-122, miR-192, miR-21, miR-223, miR-26a miR-27a , miR-801	Up Up Up Down Up Down Up	Plasma	(Zhou et al., 2011)
miR-96	Up	tissue	(Ladeiro et al., 2008)
miR-375, miR-92a	Up	Serum	(Li, Hu, et al., 2010)
miR-125b-5p, miR-223-3p	Up Down	Plasma Plasma	(Giray et al., 2014)
miR-25, let-7f	Up Up	Serum Serum	(Li, Hu, et al., 2010)
miR-20a-5p miR-25-3p miR-30a-5p miR-92a-3p miR-132-3p miR-185-5p miR-320a miR-324-3p	Up	Plasma	(Wen et al., 2015)

Table 4.1: continue

microRNA	expression	source of microRNA	Reference
miR-152	Down	Tissue	(Dang et al., 2014)
miR-101	Down	Tissue	(Fu et al., 2013)
miR-152	Down	Tissue	(Huang, Wang, Guo, & Sun, 2010)
miR-22	Up	Tissue	(Jiang et al., 2011)
miR-18a	Up	Serum	(Li, Guo, Wang, Mao, & Gao, 2012)
miRNA-139	Down	Tissue	(Li, Yin, et al., 2014)
miR-24-3p	Up	Serum	(Meng, Wang, & Jia, 2014)
miR132	Down	Tissue	(Wei et al., 2013)
miR-122	Up	Serum	(Xing, Jiang, Huang, & Xu, 2014)
miR-885-5p	Up	Serum	(Gui et al., 2011)
miR-145, miR-199b, miR-224	Down Down Up	Tissue	(Gao et al., 2011)
miR-221	Up	Serum	(Zhang et al., 2011)

4.2.5 Objectives of study

- To determine microRNA profile in HBV samples.
- To investigate the association between microRNA expression in HBV samples with and without progression to cirrhosis/HCC.

4.2.6 Justification of the study

Disease-specific transcription regulators such as miRNA are ethnicity specific causing differential incidence of HBV outcomes between different populations. Malaysia is a multi-ethnic country, thus study of miRNA expression in relation to consequence of HBV infection would be of interest.

4.2.7 Hypothesis of the study

The hypotheses for this part of study are:

The null hypotheses (H_0)

- There exists no difference in miRNA profiles between HBV patients without progression compared to those with progression to cirrhosis and/or HCC.
- There is no association between miRNA expression and progression of HBV.

The research hypotheses (H_1)

- There exists difference in miRNA profiles between HBV patients without progression compared to those with progression to cirrhosis and/or HCC.
- There is an association between miRNA expression and progression of HBV.

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Blood collection

Tempus ™ Blood RNA Tubes (Applied Biosystems, Foster City, CA), alcohol swabs, cotton swabs, sterile plaster (hansplast) and disposable gloves.

4.3.1.2 RNA extraction

RNA extraction kit (NORGEN BIOTEK CORPORATION CANADA), RNase spray (Thermo scientific, US), absolute ethanol, 1.5 mL micro-centrifuge tubes (Axygen, Poland), pipettes and pipette tips (Eppendorf).

4.3.1.3 Nanodrop

Nano-drop 2000c spectrophotometer (Thermo Fisher scientific, USA), 1.5 mL micro-centrifuge tubes (Axygen, Poland), pipettes and pipette tips (Eppendorf).

4.3.1.4 Microarray experiment

FlashTaq Biotin HSR RNA Labelling kit (Affymetrix), Nuclease Free Water (Applied Biosystems), Affymetrix® Gene Chip® miRNA 3.0 Arrays, Phosphate buffer saline with PH=7.4 (Applied Biosystems), Optical adhesive covers (Applied Biosystems), 1mM Tris (Applied Biosystems), Eukaryotic Hybridization Control Kit and GeneChip miRNA 3.0

array, Laser Tough-Spots 3/8" diameter, GeneChip Hybridization Wash and stain kit (Affymetrix).

4.3.1.5 Instrumentation and statistical software

Affymetrix® Hybridization oven 645, Affymetrix® GeneChip® Command Console software, GeneChip® Fluidics station 450 Affymetrix), GeneChip® Scanner 3000 7G, Affymetrix Expression Console™ software, StepOne™ Real Time PCR system (Applied Biosystems).

4.3.2 Methods

4.3.2.1 Subjects

Twelve subjects from the University Malaya Medical Centre (UMMC) were recruited for this part of study. The participants were classified into three groups; the HBV patients with progression to cirrhosis and/or HCC (n=4), the HBV patients without progression (n=4) and healthy controls (n=4). Inclusion and exclusion criteria for participants are the same as subjects mentioned in chapter 2.

The expression profiles of microRNA in samples of participants in each group were detected using Affymetrix miRNA array platform.

4.3.2.2 Sample collection

Three millilitres of blood were collected from each individual in to a Tempus ^{TM} Blood RNA Tubes containing 6 mL of stabilizing reagent. Immediately after the tempus tube was filled, the blood was stabilized by shaking the contents of the tube vigorously for 10 seconds to ensure that the stabilizing reagent makes uniform contact with the sample. The samples were then kept in -80ºC until used.

4.3.2.3 Total RNA isolation

Total RNA, including microRNAs, was isolated from whole blood using the Preserved Blood RNA Purification Kit I according to the manufacture's protocol. Briefly, the whole contents of the tempus tube was emptied into a 15 mL conical tube. The final volume was adjusted to 12 mL by adding the Tempus blood RNA Tube diluent. The conical tube was then closed and mixed by vortexing vigorously for 30 seconds. After that the tubes were centrifuge at 4ºC at 4000 x g for 30 minutes. The supernatant was carefully discarded and the tubes then left reversed on paper towel for 2 minutes to dry excessive liquid. A volume of 600 µL of Lysis solution was added to the RNA pellet followed by vortexing of the tubes for a few seconds to re-suspend the pellet. After that 300 µL of absolute ethanol was added and vortexed briefly to mix then proceeded for step 2 for binding RNA to column. Assembled the column with collection tube and applied up to 600 µL of the lysate with the absolute ethanol onto the column followed by centrifuge for one minute at 4000 x g. Discarded the flow-through and reassembled the spin column with its collection tube. After that applied 400 µL of Wash Solution to the column and centrifuge for 1 minute. The washing step was repeated for the second and third time then the flow-through was discarded, the spin column was then reassembled

with its collection tube. The column was spun for two minutes with the intention of thoroughly drying the resin and the collection tube was then discarded. The column was placed into a new 1.7 mL Elution tube and 50 µL of Elution Solution was added to the column followed by centrifuging for 2 minutes at 200 x g, then for 1 minute at 14000 x g. The purified RNA was then kept in -80ºC freezer before use.

4.3.2.4 Determination of concentration and purity of RNA

The total RNA purity and concentration was quantified by the Nano-drop 2000c spectrophotometer (Thermo Fisher scientific, USA). The integrity of total RNA was determined by using an Agilent 2100 bio-analyser (Agilent, Palo Alto, CA) following the manufacturer's procedure. However, high quality RNA with RNA Integrity Number (RIN) ≥ 8 and high purity with an optical density of 1.8 – 2.0 for 260/280 ratio consider as intact RNA.

4.3.2.5 MicroRNA microarray profiling

Twelve samples were tested by the Affymetrix Gene Chip® miRNA 3.0 Arrays (each contains 2999 probe sets). The miRNA labelling was performed using FlashTaq Biotin HSR RNA Labelling kit, 10 rxn (P/N 901910). For the labelling step, a minimum of 130 ng of total RNA was used for poly (A) tailing. Briefly, nuclease free water was used to adjust the volume of RNA to 8 µL then add 2 µL RNA Spike Control Oligos. The ATP mixture was diluted in 1 mM Tris and the Poly (A) tailing master mix was prepared according to the Affymetrix protocol. A volume of 5 µL of master mix was added to the 10 µL RNA Spike Control Oligos, followed by incubation at 37ºC for 15 min. About 15

µL of tailed RNA was proceeded for ligation step by adding 4 µL 5X FlashTaq Biotin HSR ligation mixture followed by 2 µL of T4 DNA ligase to each sample then incubated at room temperature for 30 min. After stopping the reaction by adding 2.5 µL HSR stop solution, 23.5 µL of ligated sample was added. The Enzyme Linked Oligo Sorbent Assay (ELOSA) quality control was done prior to array hybridization according to the manufacturer's procedure. A volume of 21.5 µL biotin labelled sample was then used for hybridization on Affymetrix Gene Chip® miRNA 3.0 Arrays. After preparing the oven, 110.5 µL hybridization cocktail was added to the 21.5 µL biotin-labelled sample according to the manufacturers protocol and incubated at 99ºC for 5 min then 45ºC for 5 min. A total of 130 µL was finally inserted into the arrays, placed in the oven trays then transferred to the hybridization oven for incubation at 48ºC and 60 rpm for 18 hours. To proceed for washing and staining, the hybridization cocktail from each array was removed and transferred to a new tube and filled with array holding buffer. The washing and staining was then followed by placement of arrays in fluidics station 450. The arrays were then washed and stained with Buffer A, Buffer B, cocktail 1 and 2 followed by scanning using Affymetrix Command console software (version 1.3.1, Affymetrix). After scanning, the CEL files were extracted for additional analysis using Transcriptome Analysis Console (TAC) software and Ingenuity Pathway Analysis (IPA).

4.3.2.6 Quality control

The raw data obtained from Affymetrix were analyzed using Expression Console software for normalization and quality. The successful labelling and ligation was confirmed by the presence of background signal intensity higher than 1000 compared to the control oligo's 2, 23, 29, 31 and 36 RNA probe sets according to the manufacture protocol.

4.4 Statistical analysis

Expression analysis of microRNAs in samples were carried out using the Affymetrix Transcriptome Analysis Console software. TAC enabled the identification of differentially expressed microRNAs in different groups. It provides the fold changes of microRNAs which expressed differentially between HBV with and without cirrhosis/HCC groups. A false discovery rate of 20%, fold change of ≥ 2 for up-regulated miRNA and fold change ≤ -2 for down-regulated miRNA together with a ANOVA p value of less than 0.05 was considered to determine the most differentially expressed microRNA between HBV with and without cirrhosis and/or HCC.

4.5 Result

To identify differentially expressed miRNAs between the two groups, miRNA microarray was done using 12 samples which were matched in terms of age, gender (only males were chosen) and ethnicity (only the Chinese ethnic group was chosen). The demographics of the study subjects are shown in Table 3.1. Microarray data revealed a number of differentially expressed microRNAs between CHB with and without progression to cirrhosis/HCC as shown in Table 3.2. Eight detectable microRNAs with P value < 0.05 and fold expression change ≥ 2 or ≤ -2 were identified between the CHB with cirrhosis/HCC and CHB without cirrhosis/HCC groups.

4.5.1 Demographic characteristic of subject

Table 4.2 shows the demographic of HBV patients with and without progression to cirrhosis and/or HCC as well as healthy controls in Malaysia.

Table 4.2: Demographic of study subjects

	HBV infected without cirrhosis/HCC (n= 4)	**HBV infected with cirrhosis/HCC (n= 4)**	**Healthy control (n=4)**
Age (years) Median range	55 40– 73	60 45 – 76	49 40 – 55
Ethnicity (Chinese)	4 (100%)	4 (100%)	4 (100%)
Gender (Male)	4 (100%)	4 (100%)	4 (100%)
HBV DNA	+	+	-
HBs Ag [a]	+	+	-
HCV RNA	-	-	-

[a] Hepatitis B surface antigen

4.5.2 MiRNA microarray result

Table 4.3: Differentially expressed miRNA between CHB with and without cirrhosis and / or HCC.

Transcript Cluster ID	Fold Change [a]	ANOVA p-value [b]
hp_hsa-mir-935_st	2.83	0.030138
hsa-miR-342-5p_st	2.53	0.044610
hp_hsa-mir-339_st	2.27	0.039739
hp_hsa-mir-4508_st	2.27	0.041339
hsa-miR-3615_st	2.08	0.028634
hsa-miR-3200-5p_st	2.01	0.001203
hsa-miR-182_st	-2.03	0.019457
hp_hsa-mir-4485_st	-2.29	0.040389

[a] The results are shown as the mean fold change in miRNA expression of CHB with cirrhosis and/or HCC versus CHB without cirrhosis/HCC.

Fold expression change ≥ 2 or ≤ -2 were considered significant.

[b] p-value < 0.05 considered significant.

4.6 Discussion

It has been demonstrated that circulating miRNAs are ideal biomarkers in cancer as well as HBV associated liver disease due to their stability in the circulation (Ji et al., 2011; Li, Hu, et al., 2010; Qi et al., 2011). An earlier study has reported both oncogenic and tumor suppressor roles of miRNAs (Esquela-Kerscher & Slack, 2006).

In this study, the microarray result revealed eight differentially expressed microRNA between HBV patients with progression to cirrhosis/HCC and HBV patients without progression to cirrhosis/HCC. The miRNA-935, miRNA-342, miRNA- 339, miRNA-4508, miRNA-3615 and miRNA-3200 were ≥ 2 fold highly expressed in patients with progression compare to those without progression. Meanwhile miRNA-182 and miRNA-4485 were ≤ -2 fold less expressed in patients with HBV progression compare to those without progression (Table 3.2). These miRNAs might be involved in HBV progression but the mechanism of their action is still to be elucidated.

Aberrant expression of five miRNAs reported in this study (miRNA-3615, miRNA-342, miRNA-339, miRNA-182 and miRNA-4485) have been reported in relation to liver cirrhosis or HCC malignancy (Wojcicka et al., 2014) as well as some other cancers such as lung adenocarcinoma (Tian et al., 2016), melanoma (Weber et al., 2016), colorectal cancer (Ghanbari et al., 2015; Zhou et al., 2013) gastric cancer (Yan, Yu, et al., 2016b; Yang et al., 2014), intrahepatic cholangiocarcinoma (Plieskatt et al., 2014) , colon cancer (Yang et al., 2016) and breast cancer (Boo et al., 2016; Cittelly et al., 2010). Recently Wojcicka *et al*, employed next generation sequencing to investigate the expression of microRNA in tissue samples of patient with liver cirrhosis and hepatocellular carcinoma. Their results revealed up-regulation of miR-339, miR-3615, miR-342 in cirrhotic liver tissue compared to non- cirrhotic liver tissue (Wojcicka et al., 2014). This is similar to the results in this study as miR-339, miR-3615 and miR-342 were up-regulated in

samples of patients with progression to liver cirrhosis as compared to patients without progression. However, they also showed up-regulation of miR-182 in cirrhotic liver tissues compared to non-cirrhotic liver tissue which is in contrast to our results whereby this miR-182 was down-regulated in patients with progression. This slide differences in results might be due to the differences in experimental settings such as source of the miRNA (circulatory miRNA versus miRNA in HCC cell and tissues) as well as differences in etiology of liver disease (HBV-related cirrhosis/HCC versus HBV/HCV-related cirrhosis/HCC).

Interestingly, our finding also revealed three novel miRNAs associated with HCC malignancy (miRNA-935, miRNA-4508 and miRNA-3200). As far as we know these miRNAs have not been reported in relation to HBV- associated liver disease. However, they were previously reported as a non-invasive biomarker in gastric cancer and breast cancer (Boo et al., 2016; Yan, Yu, et al., 2016; Zhou, Wang, et al., 2014). Yan *et al* reported the down-regulation of miR-935 in gastric carcinoma tissue compare to normal tissue samples as well as in gastric cell line carcinoma than that of non- gastric cell (Yan, Yu, et al., 2016). In another study by Boo *et al* showed that up-regulation of miR-4508 is associated with breast cancer chemo- resistance and self-renewal ability (Boo et al., 2016). This suggests that the novel miRNAs identified in this study may serve as potential prognostic biomarker in HBV progression.

Aberrant expression of miRNAs is reported in a number of cancers and deregulated miRNAs have been confirmed to play vital role in starting and development of cancers by regulating the expression of numerous tumour suppressor genes or oncogenes. For instance, it has been proven that mir-342 act as a tumour suppressor gene, thus, its expression will increase during tumorigenesis (Yan, Cao, et al., 2016). It has also been identified that tumorigenesis depends on angiogenesis, a process of formation of new

blood vessels from an existing vasculature (Folkman, 1971). For this purpose, endothelial cells should be changed from a resting state to a rapid growth state. This change depends on some positive regulators of angiogenesis such as transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF) (Bergers & Hanahan, 2008). The change may also involve down-regulation of endogenous inhibitors of angiogenesis (Ribatti, 2009). The eventual result is the net balance between negative and positive regulators. It has been reported that miRNA-342 regulates angiogenesis probably through modulating VEGFR and endoglin (co-receptor of TGF-β receptor signalling pathway) mediated TGF-β signalling (Yan, Cao, et al., 2016). All of these finding suggest that miRNA-342 supressed angiogenesis. Earlier study have been confirmed that miRNA-182 is a prominent regulator of cancer associated processes, with supporting effect in angiogenesis and cell proliferation (Wei, Lei, & Hu, 2015). Wang *et al*, have conducted a study using normal and HCC liver tissue and reported that miRNA-182 stimulate metastasis of HCC by preventing the expression of Metastasis suppressor 1 (MTSS1) (Wang et al., 2012).

Recent evidence has shown the association between miRNAs and their targeted gene in various cancers disease. For instance, the impact of miRNA-342 on targeted genes including fatty acid synthase (FASN) and 3-hydroxyl-3-methylglutaryl CoA reductase (HMGCR) is reported in prostate cancer (Li et al., 2013). It has been demonstrated that miRNA-342 control lipogenesis and cholesterogenesis in prostate cancer cells by preventing Sterol regulatory element- binding protein (SREBP-1and 2) expression as well as down- regulation of FASN and HMGCR (Li et al., 2013). In another study, it has been verified that miRNA-182 is an important factor involved in HCC development and play a role as an upstream regulator of CEBPA (CCAAT enhancer binding protein alpha) in human (Wang, Ren, et al., 2014). They showed that miRNA-182 plays a vital role in CEBPA pathway by directly suppressing CEBPA and controls the proliferation of

tumour cells. The CEBPA is also reported in some cancer disease such as lung cancer and breast cancer (Wang, Ren, et al., 2014).

Very little is known about the aberrantly expressed microRNA and their target gene in HBV associated cirrhosis / HCC. To date, HBV miRNA pattern have been examined in many studies but with regard to progression of HBV infection there are limited studies which have mostly been done with liver tissues (*in vivo*) and HepG2 cells (*in vitro*) (Ladeiro et al., 2008; Pineau et al., 2010; Wong et al., 2008).

It should be noted that our study involved Chinese men only, thus this results needs to be replicated in females as well as in other ethnicities. In addition, our microarray results should be validated by another method such as quantitative RT-PCR. Considering both oncogenic and tumor suppressor role of miRNA in tumorigenesis of HCC more investigation using larger sample size is needed to provide additional evidence for these to be used as potential biomarkers for diagnosis and progression of the disease .

4.7 Conclusion

Results from this study suggests that the miRNA-935, miRNA-342, miRNA-339, miRNA-4508, miRNA-3615, miRNA-3200, miRNA-182 and miRNA-4485 can be used as a non-invasive marker to identify CHB patients who are at risk of progression to cirrhosis and HCC. Discovery of an effective and reliable tool for early diagnosis of cirrhosis and HCC would play an essential role in the management of patients with chronic HBV.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

5.1 General Discussion

A significant role of host genetic factors in determining susceptibility to HBV infection and progression of disease is now well established. Studies of candidate gene have identified many strong associations with outcomes of HBV infection. Moreover, it has been documented that variation in HLA class II molecules may have an effect on immune response thereby impacting on susceptibility or resistance to HBV infection as well as progression of HBV disease. In addition, evidence showed that profile expression of microRNAs is different from chronic HBV infection to cirrhosis and HCC.

With regards to HBV disease, most reported genetic studies have been conducted in Asia due to the high incidence rate of HBV infection. Considering the genetic variations between populations, there is a need for each country to investigate this association in their population to provide effective knowledge to prevent mortality.

Therefore, we investigate the impact of single nucleotide polymorphism (SNP), HLA allele and miRNA profiling between HBV infected patients with and without cirrhosis and HCC with the purpose of finding biomarkers for diagnosis and treatment strategies for prevention of HBV disease progression in Malaysia.

Prior information on the genetic profiles of patients may help to predict those infected with CHB who are at higher risk to progress to cirrhosis and HCC for better management of the disease.

However, this study has a few limitations: potential confounding factors (hepatic inflammation and fibrosis), as well as viral factors were not analyzed.

5.2 General Conclusion

Considering the variability between populations with regards to genetic association studies, miRNA profiles and HLA alleles on clinical outcomes of chronic HBV infections, this study has provided knowledge on the genetics, transcriptomics and HLA allele frequency of HBV progression to cirrhosis/HCC in the Malaysian population. One significant SNP and miRNAs profiles reported in the present study that was not found in other populations will offer additional information of relevant variants of progression of HBV infection specific to this population. This may assist physicians in identifying those infected HBV patients who are at higher risk to progress to cirrhosis/HCC.

To the best of our knowledge, this is the first study that is carried out in Malaysian population which provides wide information on association of genetic polymorphism, transcriptomics and HLA allele frequency of HBV disease progression.

5.3 Future Studies

For the significant results obtained in the present study, additional studies with larger samples size are required. Moreover, functional work is required with focus on pathways to better understand the details of the mechanisms involved in HBV progression to cirrhosis /HCC.

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SUPPLEMENTARY

LIST OF PUBLICATIONS

Published

1. Behnaz Riazalhosseini, Zahurin Mohamed, Yamunah Devi Apalasamy, Hooi Sian Eng, Rosmawati Mohamed. Association between microRNA-196A2 and microRNA-146A polymorphisms and progression to cirrhosis and hepatocellular carcinoma in patients with viral hepatitis B. Journal of Pharmacogenetics and Genomics. (Accepted). 2016 Feb; 26(2):74-9. doi:10.1097/FPC.0000000000000187.

Submitted

1. Behnaz Riazalhosseini, Rosmawati Mohamed, Yamunah Devi Apalasamy, Immaculate Mbongo Langmia, Zahurin Mohamed. Genetic variant in DLC1 gene is associated with increased risk of chronicity of disease in hepatitis B infection. (Journal of Molecular Carcinogenesis)

2. Behnaz Riazalhosseini, Rosmawati Mohamed, Yamunah Devi Apalasamy, Immaculate Mbongo Langmia, Zahurin Mohamed. Circulating microRNA as a marker for predicting liver disease progression in patients with chronic hepatitis B. (Pharmacology Journal)

3. Behnaz Riazalhosseini, Rosmawati Mohamed, Yamunah Devi Apalasamy, Hooi Sian Eng, Zahurin Mohamed. Prevalence of HLA-DQA1 / HLA-DQB1 alleles and haplotypes in patients with hepatitis B infection. (European Journal of Clinical Microbiology and Infection)

4. Behnaz Riazalhosseini, Rosmawati Mohamed, Yamunah Devi Apalasamy, Zahurin Mohamed. Interleukin-6 gene polymorphisms and association with resolution or progression of hepatitis B infection (Journal of HUMAN BIOLOGY).

Abstract in Conference

1. B. Riazalhosseini, R. Mohamed, Z. Mohamed. Association of microRNA196A2 gene polymorphisms with risk of chronic hepatitis B infection in Malaysian Population. Pharmacogenomics Symposium 2015, Next generation Pharmacogenomics (Poster presentation).

2. Riazalhosseini Behnaz. Mohamed Rosmawati. Mohamed Zahurin. MICRORNA PROFILING TO IDENTIFY CHRONIC HEPATITIS B INDIVIDUALS AT RISK OF LIVER DISEASE PROGRESSION. The Asian Pacific Association for the Study of the Liver (APASL) 2015. (Poster presentation).