ความสัมพันธ์ระหว่างรูปแบบความหลากหลายของยีน HLA-DRB1 และยีน TNF-alpha กับความเสี่ยงต่อการเกิดโรคและ/หรือการดำเนินโรคไวรัสตับอักเสบบีเรื้อรังในประชากรไทย

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ASSOCIATION BETWEEN POLYMORPHISMS OF HLA-DRB1 GENE AND TNF-ALPHA GENE WITH SUSCEPTIBILITY AND/OR DISEASE PROGRESSION OF CHRONIC HEPATITIS B INFECTION IN THAI POPULATION

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พิทยา คำมี : ความสัมพันธ์ระหว่างรูปแบบความหลากหลายของยีน HLA-DRB1 และยีน TNF- alpha กับความเสี่ยงต่อการเกิดโรคและ/หรือการดำเนินโรคไวรัสตับอักเสบบีเรื้อรังในประชากรไทย (ASSOCIATION BETWEEN POLYMORPHISMS OF HLA-DRB1 GENE AND TNF-ALPHA GENE WITH SUSCEPTIBILITY AND/OR DISEASE PROGRESSION OF CHRONIC HEPATITIS B INFECTION IN THAI POPULATION)
อ. ที่ปรึกษา: ผศ.พญ.ดร. ณัฏฐิยา หิรัญกาญจน์, อ. ที่ปรึกษาร่วม: รศ.พญ. ปรียาจิต เจริญวงศ์, 104 หน้า ISBN 974-17-3699-1

โรคไวรัสตับอักเสบบีเรื้อรัง เป็นสาเหตุสำคัญของโรคตับและมะเร็งตับ ผู้ที่ติดเชื้อไวรัสตับอักเสบบีแต่ละรายมีการดำเนินโรคที่แตกต่างกันซึ่งยังไม่ทราบสาเหตุแน่ชัด จากหลักฐานการศึกษาพบว่าปัจจัยทางพันธุกรรมมีส่วนเกี่ยวข้องกับโรคไวรัสตับอักเสบบีเรื้อรังในคู่ฝาแฝดในไต้หวัน และมีรายงานการศึกษากลุ่มยีนที่มีบทบาทสำคัญในระบบภูมิคุ้มกันมีความสัมพันธ์กับการเกิดโรคและการดำเนินโรคไวรัสตับอักเสบบีเรื้อรังในประชากรหลายกลุ่ม โดยพบว่าความหลากหลายของรูปแบบยีนดังกล่าวอาจมีผลต่อการแสดงออกของยีนทำให้การทำงานของยีนเกิดความหลากหลายด้วยซึ่งอาจมีผลทำให้เกิดการตอบสนองทางภูมิคุ้มกันและการดำเนินของโรคที่มีความแตกต่างกันในผู้ติดเชื้อ งานวิจัยนี้มีวัตถุประสงค์เพื่อทำการศึกษาความหลากของยีน HLA-DRB1 และ ยีน TNF-alpha ระหว่างผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีชนิดเรื้อรังเปรียบเทียบกับกลุ่มควบคุมซึ่งเป็นผู้ที่เคยได้รับเชื้อไวรัสตับอักเสบบีและสามารถกำจัดเชื้อได้ โดยใช้การศึกษาแบบ population-based case-control โดยรวบรวมผู้ป่วยโรคไวรัสตับอักเสบบีเรื้อรัง 150 คน (เป็นมะเร็งตับ 50 คน และไม่เป็นมะเร็งตับ 100 คน) และผู้ที่เคยได้รับเชื้อไวรัสตับอักเสบบีและสามารถกำจัดเชื้อได้ 100 คนซึ่งมีเชื้อสายและถิ่นกำเนิดเดียวกัน ใช้วิธี PCR-SSP และ PCR-RFLP หารูปแบบความหลากหลายในยีน HLA-DRB1 และยีน TNF-alpha ผลการศึกษาพบว่ารูปแบบของ HLA-DRB1*1301-2 ในผู้ที่เคยติดเชื้อแล้วหายจากโรคได้เองมากกว่าผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีชนิดเรื้อรังอย่างมีนัยสำคัญทางสถิติ (OR=0.04, 95%CI=0.00-0.26, p=0.0004) และพบ -863A allele ของยีน TNF-alpha ในผู้ที่ติดเชื้อไวรัสตับอักเสบบีเรื้อรังมากกว่าผู้ที่ติดเชื้อแล้วหายจากโรคได้เอง (OR= 1.63, 95%CI=1-2.65, p=0.0495) ซึ่งผลที่ได้นี้น่าสนใจที่ HLA-DRB1*1301-2 เป็นยีนที่สามารถตอบสนองทางภูมิคุ้มกันต่อเชื้อไวรัสตับอักเสบบีได้ดีและมีความสัมพันธ์กับการต้านทานโรคเช่นเดียวกับในประชากรหลายเชื้อชาตินอกจากนี้ยังพบว่า ยีน TNF-alpha มีความสัมพันธ์กับการดำเนินโรคไวรัสตับอักเสบบีเรื้อรัง จากผลการศึกษาพบว่ารูปแบบของ -863A allele และ -238A allele ของยีน TNF-alpha มีลักษณะการถ่ายทอดยีนแบบยีนเด่นในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีชนิดเรื้อรังชนิดที่มีมะเร็งตับมากว่าผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีชนิดเรื้อรังชนิดที่ไม่มีมะเร็งตับ (OR=2.17, 95%CI=1.03-4.59, p=0.0414 และ OR=3.69, 95%CI=0.94-15.42, p=0.046 ตามลำดับ) และจากการวิเคราะห์ haplotype ของยีน TNF-α พบว่า CGG/CGG haplotype มีความสัมพันธ์กับการต้านทานการดำเนินโรคไปเป็นมะเร็งตับในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีชนิดเรื้อรัง (OR=0.37, 95%CI=0.17-0.79, p=0.009) ซึ่งสอดคล้องกับความสัมพันธ์ของ -863A allele และ -238A allele ของยีน TNF-alpha และจากการศึกษาไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของยีน HLA-DR12 และความหลากหลายของรูปแบบยีน TNF-alpha ที่ตำแหน่ง –308(G/A) เมื่อเปรียบเทียบระหว่างกลุ่มผู้ป่วยกับกลุ่มควบคุม โดยสรุปคือรูปแบบของยีน HLA-DRB1*1301-2 มีความสัมพันธ์กับการต้านทานโรคไวรัสตับอักเสบบีเรื้อรัง นอกจากนี้ยังพบว่า -863A allele และ -238A allele ของยีน TNF-alpha สามารถใช้เป็นเครื่องหมายของยีนในการกำหนดความเสี่ยงต่อการเกิดโรคมะเร็งตับในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีชนิดเรื้อรังในประชากรไทย

สาขาวิชา จุลชีววิทยาทางการแพทย์ (สหสาขาวิชา) ลายมือชื่อนิสิต.....พิทยา คำมี.....
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

#4689120020 : MAJOR MEDICAL MICROBIOLOGY

KEYWORD : CHRONIC HEPATITIS B INFECTION / HLA-DRB1 GENE POLYMORPHISMS / TNF-ALPHA GENE POLYMORPHISMS

PITTAYA KUMMEE: ASSOCIATION BETWEEN POLYMORPHISMS OF HLA-DRB1 GENE WITH SUSCEPTIBILITY AND/OR DISEASE PROGRESSION OF CHRONIC HEPATITIS B INFECTION IN THAI POPULATION

THESIS ADVISOR: ASST.PROF.NATTIYA HIRANKARN, M.D., Ph.D. THESIS CO- ADVISOR: ASSOC. PREEYACHIT CHAREONWONGSE, M.D., 104 pp.

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Chronic hepatitis B virus (HBV) infection is caused of liver disease and liver cancer. However, the factor which determined the different outcome in an individual patient remains unclear. The evidence in twin studies supporting genetic factors was associated with HBV infection. In addition, considerable evidence suggests that host genetic factor play an important role in the pathogenesis and clinical outcome of the disease in several ethic groups. The aim of this study was to identify the polymorphisms of HLA-DRB1 gene and TNF-α gene and to determine the association with susceptibility and/or disease progression of chronic hepatitis B infection in Thai population. Population-based case-control study included 150 chronic HBV patients (100 patients without HCC and 50 patients with HCC) and 100 patients who transient from HBV infection to serve as control with similar ethic and geographic background. HLA-DRB1 gene and TNF-α gene polymorphisms were identified by PCR-sequence specific primer (SSP) and PCR-restriction fragment length polymorphism (RFLP), respectively. The result of this study demonstrated that HLA-DRB1*1301-2 phenotype in the transient HBV patients was significantly higher than chronic HBV patients (OR=0.04, 95%CI=0.00-0.26, p=0.0004) and -863A allele of TNF-alpha gene was increased in chronic HBV patients compared with transient HBV patients (OR= 1.63, 95%CI=1-2.65, p=0.0495). Interestingly, HLA-DRB1*1301-2 show a strong association with the clearance of HBV which similar to another study in several ethic groups. Moreover, we also found an association between TNF-α and the progression of chronic HBV infection. The present study showed that the frequencies of the -863A allele and -238A allele was significantly increased in chronic HBV patients with HCC compared with chronic HBV patients without HCC (OR=2.17, 95%CI=1.03-4.59, p=0.0414 and OR=3.69, 95%CI=0.94-15.42, p=0.046, respectively). The effect of the -863A allele and -238A allele were similar to autosomal dominant mode of inheritance. Haplotype analysis revealed that the homozygosity of the significantly most common haplotype (CGG/CGG) was a protective marker for HCC (OR=0.37, 95%CI=0.17-0.79, p=0.009) supporting the positive association of -863A and -238A genotype. No significant association in HLA-DR12 and TNF-α gene polymorphisms at position -308(G/A) were found between chronic HBV patients and recovered patients. In conclusion, our study showed that HLA-DRB1*1301-2 is a host factor that might be a protective allele in chronic hepatitis B infection. Furthermore, the -863A allele and -238A allele of TNF- α gene were identified as a genetic marker for hepatocellular carcinoma development in patient with chronic HBV infection in Thai population.

Field of Study Medical Microbiology (Inter-Department) Student' signature Pittaya Kummee

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ABBREVIATIONS

APCs	antigen-presenting cells
bp	base pair
CD	Cluster of Differentiation
95% CI	95% Confidence Interval
°C	degree Celsius
CTL	cytotoxic T lymphocyte
et al	et alii
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IFN	Interferon
kDa	Kilodalton
HBV	Hepatitis B virus
HBc	Hepatitis B core
HBs	Hepatitis B surface
HCC	Hepatocellular carcinoma
HLA	Human Leukocyte Antigen
µl	microliter
µg	microgram
ml	milliliter
mM	millimolar
MW	molecular weight
ng	nanogram
NK	Natural Killer
OR	Odd Ratio
PCR	Polymerase Chain Reaction

RFLP	restriction fragment-langth polymorphism
SSP	sequence specific primer
SDS	Sodium Dodecyl sulfate
SNP	Single Nucleotide Polymorphism
TGF	Transforming growth factor
TNF	Tumor necrosis factor
U	Unit
VNTR	variable numbers of tandem repeats

CHAPTER I

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a global public health problem with an estimated 350 million people chronically infected worldwide. In area where the prevalence is high, such as Southeast Asia, China and Africa, the major mode of infection in HBV-endemic areas is perinatal transmission (Lee.,1997). The outcome of HBV infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to cirrhosis or hepatocellular carcinoma. However, the factor which determine the outcome in an individual patient are unclear. They may be classified into three categories: environmental, viral and host genetic factor. The mechanisms underlying resolution of acute HBV infection or its progression to chronicity could not be explained completely by the virus itself and environmental factor. Thus, progress of HBV infection might be affect by host genetic susceptibility. The evidence in support of genetic factors arises from twin studies conducted in Taiwan, in these studies it was demonstrated that the degree of concordance for hepatitis B surface antigen (HBsAg) status was significantly higher in monozygotic twins than in dizygotic twins (Lin, Chen et al., 1989).

Chronic HBV infection is complex disease with no clear Mendelian pattern of inheritance. The outcome of disease is influenced by environmental factor. The influence of specific genes on the outcome of HBV infection has only been established using disease association studies. This is a simple technique of studying polymorphic genes between a group of subjects with the condition (cases) and a suitably selected control group. The choice of candidate genes for this method is strongly determined by the function (or putative function) of the gene and its possible role in the host immune response to HBV infection and disease progression. Since HBV is generally believed not to have a direct cytopathic effected hepatocyte, most available experimental evidence suggested that liver damage in hepatitis B is produces by the cellular immune response against viral antigens present in infected hepatocytes. Therefore, the genetic component of chronic HBV infection have been

speculated in immune response genes and many studies have reported of the association between HLA class II genes and cytokine genes with susceptibility of chronic HBV infection.

The HLA class II is a critical genetic factor that determines individuals variations of immune response. The fundamental role of class II molecules is to present peptide to interact with T cell receptor on $CD4^+$ T cell which is important in present self or non self peptide to T cell and positive and negative selection in T cell development. Polymorphisms at these loci influence immune responses and may explain the variation in outcome and progression of HBV infection. There are many reports of the significant associations between HLA-DRB1 and chronic HBV infection, studies demonstrated independently that HLA-DR13 is involved in the elimination of HBV. In Gambia, the HLA allele DRB*1302 was associated with protection against persistent HBV infection in both children and adults (Thursz, Kwiatkowski et al., 1995). Hohler et al have confirmed that DRB*1302 and DRB*1301 alleles conferred resistance to chronic infection in Caucasians (Hohler, Gerken et al., 1997). In Asian studies also found an association between HLA-DR13 alleles and clearance of HBV infection; (Ahn, Han et al., 2000; Akcam, Sunbul et al., 2002). In other populations, clearance of HBV has been associated with the HLA-DR2 in Qatar (Almarri and Batchelor 1994), DRB1*1101/1104 in China (Jiang, Wang et a., 2003), DRB1*1201 in China (Meng, Chen et al., 2003), DRB1*0406 in Taiwan (Han Chinese) (Wu, Wang et al., 2004). However, the association between DQA1*0501 and DQB1*0301 in USA (African American) and susceptibility to chronic hepatitis B was also reported (Thio, Carrington et al., 1999). These data show that the difference of HLA susceptibility or protective alleles in populations of distinct ethnic background, and HLA-DR13 show strong association with the clearance of HBV in several ethnic groups. The beneficial effect of HLA-DR13 phenotype on the outcome of HBV infection may be due to the induction of a vigorous HBc-specific $CD4^+$ T cell response which might be either a more proficient antigen presentation by HLA-DR13 molecules themselves or due to a linked polymorphism in a neighboring immunoregulatory gene (Diepolder, Jung et al., 1998).

Cytokines play an importance role in the defense against viral infection, both indirectly, through the determination of the predominant pattern of the host response, and directly, through inhibition of viral replication. The maximum capacity of cytokine production varies among individuals and correlates with the polymorphism in the cytokine gene promoters. Several cytokines such as IL-RN (Zhang, Li et al., 2004), IL-10 (Miyazoe, Hamasaki et al., 2002), TGF-β (Kim, Lee et al., 2003), IFN-γ (Ben-Ari, Mor et al., 2003) and TNF-α (Hohler, Kruger et al., 1998; Kim, Lee et al, 2003; Lu, Li et al., 2004) have been identified to participate in the process of viral clearance via host immune response to HBV. Furthermore, TNF-α is an important cytokine in the immune pathogenesis of HBV infection particularly with regard to the non cytolytic control of viral replication. TNF-α plays a critical role not only in protection from HBV infection but also in the pathogenesis of chronic HBV infection and associated with the development of HCC (Ho, Wang et al. 2004). The existing evidence implicates the role of TNF-α inflammatory pathway that increased tumorigenesis (Szlosarek and Balkwill., 2003). TNF-α has been found in high concentration in patients with cancer (Abrahamsson, Carlsson et al., 1993; Partanen, Koskinen et al., 1995). Recent evidence suggests that the TNF-α, which is a key player in inflammation can also activate signalling pathways, in both cancer cells and tumor-asscociated inflammatory cells, that promote malignancy (Balkwill and Coussens.,2004; Pikarsky, Porat et al., 2004).

Thus the aim of this study, HLA-DRB1 and TNF-α genes were analyzed by candidate gene approach. Population-based case-control studies were used to identify HLA-DRB1 and TNF-α genes polymorphisms in patients with chronic HBV compared with control group and determine the association with chronic HBV infection in Thai population. The genotyping method for HLA-DRB1 and TNF-α genes polymorphisms were done by the PCR-sequence specific primer (PCR-SSP) and the PCR-Restriction fragment length polymorphism (PCR-RFLP), respectively. Then analysis genotype and allele frequencies were compared between patients and control subjects.

We hypothesized that the specific polymorphism of HLA-DRB1 and TNF-α genes that determine risk for development and/or severity of chronic HBV infection in Thai population will be found. The knowledge from this research might lead to better understanding of

pathogenesis and/or disease progression of chronic HBV infection in Thai population and development of new treatment and prevention. In addition, it will provide the frequency of HLA-DRB1 and TNF-α genes polymorphisms in Thai population which are basic knowledge for study these markers in other disease in the future.

CHAPTER II

OBJECTIVE

The objective of this study was:

To identify the polymorphisms of HLA-DRB1 gene and TNF-alpha gene in patient with chronic HBV compared with control group and to determine the association with disease susceptibility and/or progression of chronic HBV infection in Thai population.

CHAPTER III

LITERATURE REVIEW

Chronic hepatitis B virus (HBV) infection

Chronic hepatitis B virus (HBV) infection is one of the most important chronic viral diseases and is a major public health problem. The clinical course of HBV infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to cirrhosis or hepatocellular carcinoma (Figure 1) and leads to high morbidity and mortality.

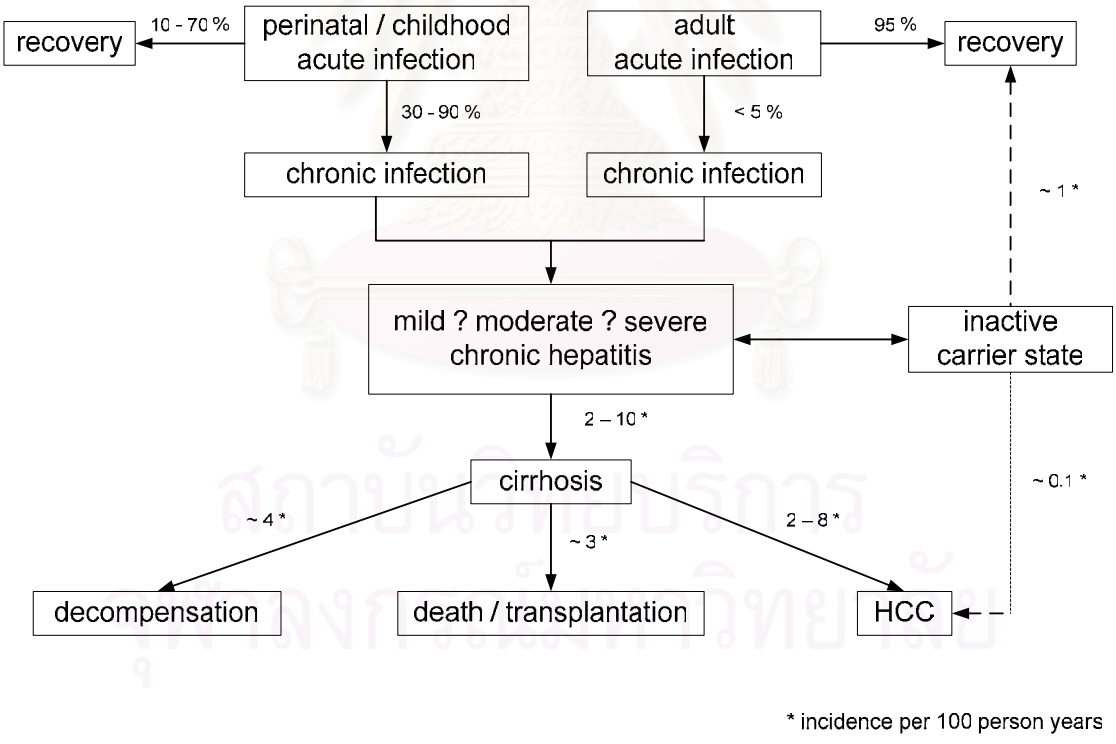

Figure 1. Outcomes of HBV infection

Epidemiology

The distribution of hepatitis B infection varies greatly throughout the world. In area where the prevalence is high, such as Southeast Asia, China, and Central Africa, the major mode of infection in HBV-endemic areas is perinatal transmission. Ninety-five percent of infected neonates with immature immune systems become asymptomatic chronic HBV carriers, as compared with 30 percent of children infected after the neonatal period but before six years of age. Only 3 to 5 percent of adult are persistent carrier (Lee .,1997). Approximately 350 million people are estimated to be chronically infected with hepatitis B virus. The number continue to increase until the World Health Organization recommends hepatitis B vaccination systemically at birth. This vaccination program has begun to induce a rapid decrease in the number of acute hepatitis B virus (HBV) infection in newborn infant (Ranger-Rogez, Alain et al., 2002). In Thailand, the hepatitis B vaccine recommendation has been extended to all neonates in 1992. The consequence was a decrease in the number of HBV carriers from 6-8 % in 1887 to 2.7 % in 2002 (Thai red cross) and prevalence in children (<5-year olds) decreased from 2-3% t0 <1%(Department of Communicable Disease Control).

Hepatitis B virus

Virologic Characteristic

Hepatitis B virus (HBV) is the prototype member of the *hepanaviridae* family. The viral genome of HBV is a partially double-stranded circular DNA that contain approximately 3200 base pairs that encodes four overlapping open reading frames (ORFs): *S*, for the surface, or envelope; gene *C*, for the core gene; *X* for the *X* gene; and *P*, for the polymerase gene. The *S* and *C* genes have upstream regions termed *preS* and *preC*.

The whole virion (Dane particle) is a 42-nm sphere that contains a core, or nucleocapsid that enclose the DNA and small spheres and rods in circulation which excess of envelope material is 22 nm.

HBV transmission

HBV transmission may be classified into three major modes of infection: parenteral inoculation such as drug use, exposure to blood, blood products, or blood derivative transfusions; Sexual transmission through contact with an infected person (horizontal transmission) and transmission from HBV carriers mother to her child (vertical transmission) (Ranger-Rogez, Alain et al., 2002). In Thailand, the major mode of transmission is via vertical transmission or horizontal transmission in the childhood (Poovorawan, Sripattanawat et al.,1998; Lolekha, Warachit et al., 2002; Taketa, Ikeda et al., 2003).

HBV genotype

Based on sequence divergence in the entire genome, HBV genomes have been classified into eight genotypes designated A to H. The genotypes of HBV have distinct geographical distributions. Genotypes A and D have global distributions. Genotypes B and C are predominantly in East and South East Asia. Genotype E is predominant in West Africa. The most divergent genotype HBV/F is found exclusively amongst indigenous peoples in Central and South America. The geographical location HBV H is restricted to Central and South America (Weber., 2005). In Thailand, HBV genotype C and B were predominant, accounting for 73% and 21%, respectively (Tangkijvanich, Mahachai et al., 2005).

Host immune response to HBV infection

The experimental approach to HBV pathogenesis have been difficult because the host range of HBV is limited to man and chimpanzees. In cell culture, only primary hepatocyts can be infected. Therefore, HBV transgenic mouse models were developed to study the interaction between the virus and the host' immune system (Chisari .,1996). Animal experiments suggest that pathological hepatitis B infection are not cause by the virus directly but are mediated by host immunity (Chisari and Ferrari .,1995; Hilleman .,2001).

After exposure to the virus, the first stage infection is controlled by non-specific immunity such as IFN-alpha and beta that activate NK cells and antigen presenting cells. These cells trigger specific immunity which consist of humoral immunity and cellular immunity. Antibodies and T lymphocytes are main antigen specific effectors of humoral immunity and cellular immune system, respectively. In cellular immune response, $CD8^+$ T cells recognize HBV peptide fragments derived from intracellular processing and presentation on the hepatocyte surface by MHC class I molecules. This process leads to direct cell killing by the $CD8^+$ cytotoxic T lymphocyte (CTL). However, some studies demonstrated that CTLs can eliminated intracellular virus not only by the destruction of infected cells but also by noncytopathic antiviral mechanism. For example, Guidotti et al found that IFN-γ and TNF-α secreted by HBV-specific CTLs can abolish HBV gene expression and replication in the liver of HBV transgenic mice without killing the hepatocytes including contribute to viral clearance during acute viral hepatitis (Guidotti, Ishikawa et al., 1996; Guidotti, Borrow et al., 1999; Pasquetto, Wieland et al., 2002; Thimme, Wieland et al., 2003).

Besides $CD4^+$ T cells recognize HBV peptide fragments derived from extracellular processing and presentation on antigen presenting cells surface by MHC class II molecules. This process leads to the stimulation of T cell proliferation and cytokine synthesis. The differentiation into Th1 or Th2 cells influenced from type of cytokines which determine whether the adaptive immune response is biased towards a cellular or humoral immune response. Th1 cytokines include IL-2, interferon IFN-γ, TNF-α, IL-12 and IL-18. These cytokine lead to the activation of CTL, which effect clearance infected cell. Th2 cytokines include IL-4-6 and IL-10, which regulated Th1 cytokines production and lead to activation and differentiation of antibody by B cell. Several studies reported that IL-12, IL-18 and a synergistic of IL-12 and IL-18 produced by antigen-presenting cells stimulate natural killer (NK) cells and T cell to secrete IFN-γ and to generate normal Th1 response. These properties suggested that IL-12 and IL-18 can induce cytokines and suppress HBV replication in the liver of HBV transgenic mice that may play an important role in the clearance in the immune response to HBV infection (Cavanaugh, Guidotti et al., 1997; Nakanishi, Yoshimoto et al., 2001; Kimura, Kakimi et al.,2002).

In addition, the different immune response in patients in whom virus is cleared successfully depended on the match between the HBV peptide presented by host major-compatibility-complex molecules and the specific T cell receptor repertoire of the host. In contrast, this response is absent or weak in chronically infected patients who do not clear the virus (Chisari and Ferrari., 1995).

Life cycle of HBV in the human host (Lee., 1997)

Stage 1 Immune tolerance stage: In healthy adult, an incubation period is about two to four weeks. In contrast, in neonatal infection, this period often lasts for decades. In this stage, most cases of HBV infection have active viral replication but no elevation in the aminotransferase levels and no symptoms of illness.

Stage 2 Immune clearance stage: An immunologic response develops or improves, leading to cytokine stimulation and direct cell lysis and the inflammatory process. Secretion of HBeAg still occurs in stage 2, but HBV DNA levels in serum drop as the number of infected cells declines. In patients with acute HBV infection, stage 2 is the period of symptomatic hepatitis and typically lasts three to four weeks. In patients with chronic disease, stage 2 may persist for 10 or more years, leading to cirrhosis and its complications.

Stage 3 Immune latent phase: HBeAg is no longer present, and the antibody to HBeAg becomes detectable. A marked decrease in viral DNA is observed, although many patients remain positive for HBV DNA as detected by PCR. In stage 3, the infection has cleared, and aminotransferase levels become normals. However, patients remain positive for HBsAg.

Stage 4 Immune phase: Most patients eventually become negative for HBsAg and positive for antibody to HBsAg, HBV DNA can no longer be detected by any means, and the patients is unlikely to become reinfected or to have to have a reactivated infection.

Etiology

The outcome of hepatitis B virus infection is highly variable, the factor which determine the outcome in an individual patient are unclear. They may be classified into three categories: (1) environmental factors (2) virological factors such as viral load, genotype and genetic divergence due to viral gene mutations. HBV mutates very rapidly and used high genetic variability as an effective mechanism for escaping the host immune response (Wang .,2003). (3) Host factors are believed to be responsible for clinical outcome of the disease particularly immunological factor including the innate and adaptive immune response against viral infection, which play important roles in modulating both the antiviral immune response and host susceptibility to HBV. The evidence that identifies host genes, which influence the outcome of HBV infection and support of genetic factor arise from twin studies conducted in Taiwan (Lin, Chen et al., 1989). In these studies it was demonstrated that the degree of concordance for hepatitis B surface antigen (HBsAg) status was significantly higher in monozygotic twins than in dizygotic twins.

Most of the reports of human genes associated with HBV infection have currently focused on HLA associations and cytokines. For example, some investigators reported the association of HLA class II alleles such as DRB* 1302 or HLA-DR13 (Thursz, Kwiatkowski et al. 1995; Hohler, Gerken et al., 1997; Ahn, Han et al., 2000) or DQA1* 0501- DQB1* 1102 haplotype (Thio, Carrington et al., 1999) with acute and/or chronic hepatitis B virus infection, respectively. In addition, several pro-inflammatory cytokines such as Th1 cytokines: IFN-γ (Ben-Ari, Mor et al., 2003) and TNF-α (Hohler, Kruger et al., 1998; Kim, Lee et al., 2003; Lu, Li et al., 2004) have been identified to participate in the process of viral clearance and host immune to HBV. In contrast, the Th2 cytokines : IL-10 serves as a potent inhibitor of Th1 effector cells in HBV disease (Miyazoe, Hamasaki et al., 2002). Thus, reports from various laboratories have shown some inconsistencies with regard to the effects of host genetic factors on HBV clearance and persistence.

Study Approaches

Study approach for susceptibility loci can be classified into two main approaches: family-based studies and population-based studies.

Family-based studies

This approach is a tool for searching susceptibility loci that cosegregate with disease in families with Mendelian pattern of inheritance. The principle of family-based is based on the fact that two genes or markers which are closely on a chromosome will be cosegregate together with disease in families due to a recombination between genes is low. Linkage analysis has been performed at various regions or one regions of interest. Strategies to identified linkage analysis can use either genome-wide scaning, microsatellite, SNPs or candidate gene. The approach of family-based studies is a power tool for detecting major genes in complex disease. However, this method has limited power to detect less influential genes and it is difficult to collect samples from families that include both parents particularly in late-onset diseases.

Population-based studies

Population-based studies is used to investigate whether a marker allele is associated with susceptibility to the disease by comparing the frequency of the allele in a disease population with unrelated control. Similar to the strategies to identified linkage analysis, this approach can use either genome-wide scaning, microsatellite, SNPs or candidate gene. The association between genetic marker and susceptibility to the disease may be due to that marker itself or due to another gene in linkage disequilibrium in a neighboring gene. Moreover, false positive arising from population stratification or small data sets can be found (Gough, Saker et al., 1995). However, this method have a lot of advantage since the identification and collection of samples from subjects is easier and more efficient than the collection of family samples. Besides, population-based studies is sensitive method that can be detect less influential genes in complex diseases and late-onset diseases.

Genetic association studies in chronic HBV infection

The number of association studies of candidate genes in infectious diseases has increased rapidly as more polymorphisms are identified in gene considered to have important roles in pathogenesis or protection. These candidate genes have been identified in several ways. HLA is a critical genetic factor that determines individual variation of immune response have been clearly elucidated. In addition to the control of transplant acceptance and immune responsiveness, it has been recognized for many years that the MHC in the human plays an important role in the development of autoimmune diseases and the variation in outcome from infectious diseases. Many studies have reported the role of HLA in the infectious diseases. For example, the association in bacterial disease leprosy and tuberculosis and HLA DR2 in Asian population (Singh, Mehra et al., 1983; Todd, West et al., 1990; Brahmajothi, Pitchappan et al., 1991). The largest HLA association studies of an infectious disease have been of malaria. In the Gambia HLA-B* 5301 and HLA-DRB* 1302 were independently associated with a reduce risk of severe malaria in childhood (Hill, Allsopp et al., 1991). From the studies in European, there is evidence supporting an association between HLA-DR5 and clearance of HCV infection (Peano, Menardi et al., 1994; Tibbs, Donaldson et al., 1996; Zavaglia, Bortolon et al., 1996). Moreover, a large number of studies of HLA genes and; various manifestations of HIV infection have now been reported association between HLA-B35 antigen and the HLA-A1-B8-DR3 haplotype have been associated several times with more rapid disease progression (Kaslow, Duquesnoy et al., 1990; McNeil, Yap et al.,1996) and HLA-B27 may be associated with slow progression (Kaslow, Carrington et al., 1996; McNeil, Yap et al., 1996).

There are many reports of the significant associations between HLA-DRB1 and chronic HBV infection, three studies demonstrated independently that HLA-DR13 is involved in the elimination of HBV. In Gambia, the HLA allele DRB*1302 was associated with protection against persistent HBV infection in both children and adults (Thursz, Kwiatkowski et al., 1995). A European study has confirmed that DRB*1302 and DRB*1301 alleles also conferred resistance to chronic infection in Caucasians (Hohler, Gerken et al., 1997). In Asian studies also found an association between HLA-DR13 alleles and clearance of HBV infection (Ahn,

Han et al., 2000; Akcam, Sunbul et al., 2002). In other populations, clearance of HBV has been associated with the HLA-DR2 in Qatar (Almarri and Batchelor., 1994), DRB1*1101/1104 in China (Jiang, Wang et al., 2003), DRB1*1201 in China (Meng, Chen et al., 2003), DRB1*0406 in Taiwan (Han Chinese) (Wu, Wang et al., 2004). However, the association between DQA1*0501 and DQB1*0301 in USA (African American) and susceptibility to chronic hepatitis B was also reported (Thio, Carrington et al., 1999) (table 1).

In addition the association in certain cytokines were reported, due to the role they play in HBV pathogenesis. Several cytokines such as IL-RN (Zhang, Li et al., 2004), IL-10 (Miyazoe, Hamasaki et al., 2002), TGF-β (Kim, Lee et al., 2003) IFN-γ (Ben-Ari, Mor et al., 2003) have been identified to participate in the process of viral clearance, host immune response to HBV. Particularly, TNF-α (Hohler, Kruger et al., 1998; Kim, Lee et al., 2003; Lu, Li et al., 2004) have strong supported evidences influencing in various population as summarized in table 2.

Mutation in the mannose binding protein (MBP) gene (codon 52, 54 and 57), as well as in its promoter lead to low serum concentrations of MBP, preventing both its ability to activate complement and to act as an opsonin (Summerfield, Ryder et al., 1995). The HBV envelope has a mannose-rich oligosaccharide to which MBP could bind. Therefore, these mutations may be important in HBV pathogenesis (Thomas, Foster et al., 1996). The mutation of codon 52 in the MBP gene has been correlated with persistent HBV infection in British Caucasians but not in Chinese Asians (Thomas, Foster et al., 1996).

The vitamin D receptor is expressed on monocytes and lymphocytes and stimulation of this receptor is though to influence the immune response. There are a number of polymorphisms in the vitamin D receptor some of which appear to influence transcription efficiency of this gene. An allele of the vitamin D receptor that increase transcription efficiency has been associated with control of viral replication in HBV infection (Bellamy and Hill., 1998).

Table 1 HLA genes and chronic hepatitis B infection

Study	Country	*n*	specificity	Odds ratio	*P*
(Almarri and Batchelor., 1994)	Qatar	34	DR2	0.1	0.013
			DR7	3.73	0.05
(Thursz, Kwiatkowski et al., 1995)	Gambia	185 (age <10 year)	DRB1* 1302	0.53	0.01
		40 (Adult)	DRB1* 1302	0.24	0.01
(Hohler, Gerken et al., 1997)	Germany	70	DRB1* 1301/2	0.12	0.0004
(Thio, Carrington et al., 1999)	USA (African American)	31	DQA1* 0501	2.6	0.05
			DQB1* 0301	3.9	0.001
(Ahn, Han et al., 2000)	Korea	83	DR13	0.14	0.002
(Akcam, Sunbul et al., 2002)	Turkey	30	B73, DR4, DR13	< 1 , protection	< 0.05
(Jiang, Wang et al., 2003)	China (Chongqing province)	52	DRB1* 1101/1104	< 1 , protection	0.0145
			DRB1* 0301	4.15	0.0074
			DQA1* 0501	2.87	0.0157
			DQB1* 0301	4.07	0.0075
(Meng, Chen et al., 2003)	China (Zhejiang Province)	30	DRB1* 1201	< 1 , protection	0.025
(Wu, Wang et al., 2004)	Taiwan - Han Chinese	98	DRB1* 0406	0.057	0.001

Table 2. Cytokine gene polymorphism and chronic hepatitis B infection

Study	Country	N	specificity	Odds ratio	*p*
Hohler* et al., 1998	Germany	71 chronic HBV vs. 32 recovered HBV patients	-238 (A) , low level of TNF-alpha	> 1 , susceptibility to develop chronic HBV infection	0.04
		71 chronic HBV vs.99 healthy control	-238 (A) , low level of TNF-alpha	> 1, susceptibility to develop chronic HBV infection	0.003
Kim et al., 2003	Korea	1109 chronic HBV vs. 291 recovered HBV patients	-308 (A) , high level of TNF-α	0.56-0.57 , protection	0.01
			-863 (A) ,low level of TNF-α	1.52-1.58 , susceptibility to develop chronic HBV infection	0.003-0.004
Lu et al., 2004	China (Beiging)	207 chronic HBV vs.148 recovered HBV patients	-238 (G) ,low level of TNF-α	> 1, susceptibility to develop chronic HBV infection	0.02
Ho et al., 2004	Taiwan	74 HCC vs. 289 healthy controls	-308A, high level of TNF-α	3.5, susceptibility to develop HCC	<0.01
Xu et al., 2005	China (Changsha)	56 chronic severe HBV vs. 90 healthy controls	-308A/G, high level of TNF-α	> 1, susceptibility to develop chronic HBV infection	0.015
Miyazoe** et al., 2002	Japan	213 chronic HBV vs. 52 healthy controls	None		
		66 asymptomatic carriers vs. 147 chronic progressive liver	ATA haplotype at -1082, -819, -592 , low level of IL-10	<1, less progressive	<0.02
			GCC, high level of IL-10	>1, more progresssve	<0.05
		46 definite ASC vs. 58 chronic hepatitis	ATA, low level of IL-10	<1, less progressive	<0.05
		46 definite ASC vs. 58 chronic hepatitis	ATA, low level of IL-10	<1, less progressive	<0.01
Ben-Ari et al., 2003	Israel	77 chronic HBV vs. 48 healthy controls	+874 (A/A), low level of IFN-γ	>1, susceptibility to develop chronic HBV	0.003
Kim et al., 2003	Korea	228 HCC vs. 773 non-HCC	-504 (C/T, T/T), high level of TGF-β	0.67, less progressive	0.02
		1040 chronic carriers vs. 283 recovered HBV	-308 (A), high level of TNF-α	0.56-0.57, clearance	0.01
Zhang et al.,2004	China (Hubei)	190 chronic HBV vs. 249 healthy controls	1/2, 2 of IL-1RN	<1, less progressive	0.016, 0.029

***Also analyzed SNP of** TNF-α at position -308 but no significant association were detected.

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The Major Histocompatibility Complex

The human major histocompatibility complex (MHC) region on chromosome 6p21 is called the human leukocyte antigen (HLA) system. The MHC-HLA system has a crucial role not only in the immune response to foreign material but also to self-antigen. It can be subdivided into three main gene clusters. The class I region genes include those encoding the alpha peptide chains of HLA-A, -B, and -C antigens, which are membrane-bound proteins expressed on the cell surface of nucleated cells involved in the presentation of endogenous peptides to cytotoxic ($CD8^+$) T lymphocytes. The class II region genes encode molecules largely expressed on specialized antigen-presenting cells, including macrophages, B lymphocytes, and, under certain circumstances, other cell types. In this way, the class II genes are likely to be involved in the autoimmune process. Class II molecules bind peptides generated by the degradation of proteins in the endocytic pathway. As a result, the class II antigen complex is an important mechanism for stimulating the T-cell receptor on $CD4^+$ and T lymphocytes. The class III region contains many genes encoding immune regulator proteins, including some of the cytokines.

The Genetics of the HLA

The HLA complex is a gene cluster of various loci grouped together on single region. Gene mapping studies have indicated that the HLA occupies 3.5 megabases of DNA on the short arm of chromosome 6 in the distal portion of the 6p21.3 band. The loci of the HLA complex may be divided into three classes based on certain functional characteristics of genes within each class: class I, class II and class III. The organization of the genes that encode HLA molecules is shown in Figure 2 (Klein and Sato., 2000).

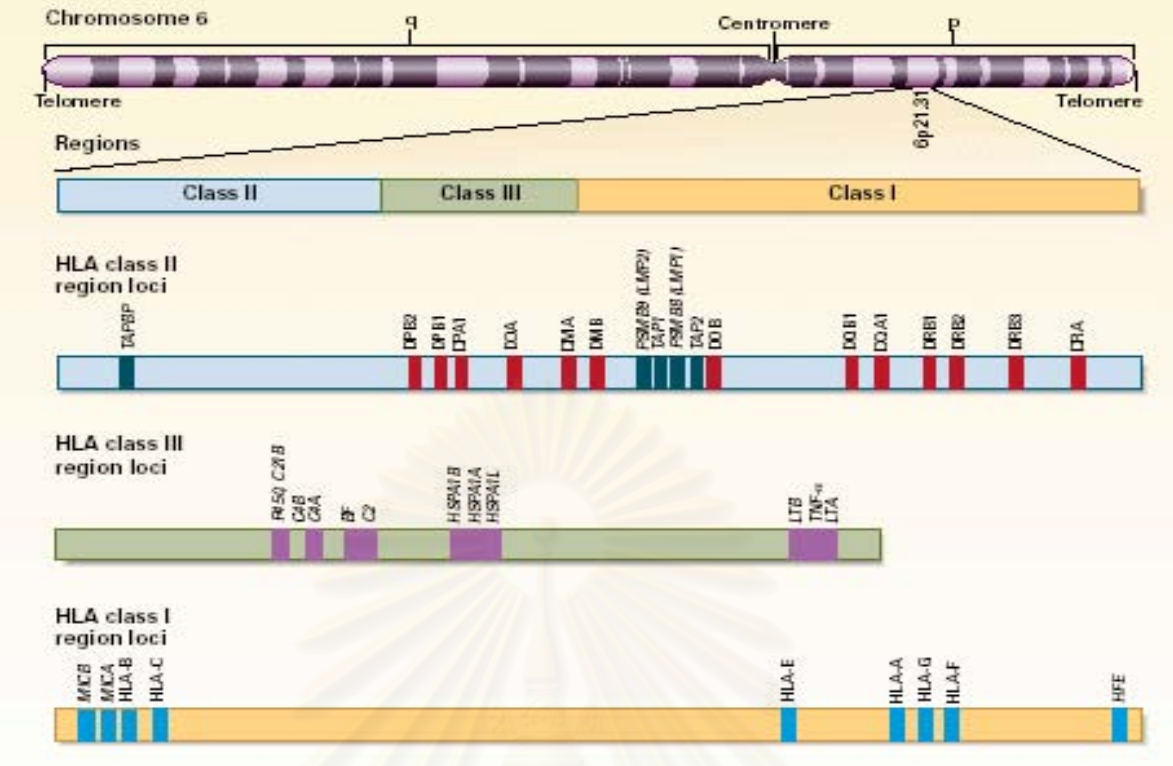


Figure 2 Location and organization of the HLA complex on chromosome 6 (Klein and Sato ., 2000)

1. Class I HLA genes

The class I genes of the HLA complex can divided into 2 types. There are classical HLA class Ia and non-classical HLA class Ib. The classical HLA class Ia contains three loci, called HLA-A, HLA-B and HLA-C loci, spread over a region of 2 Mb. The HLA-E, -F and –G genes encode non-classical HLA class Ib proteins. The class I HLA genes appear to be coordinately controlled with the beta two-microglobulin gene located on chromosome two. Although the beta two-microglobulin gene is not an HLA gene, the gene does code for a subunit associated with the alpha chain of HLA I molecule. Studies indicate that the expression of the class I molecule is dependent upon the expression of the beta two-microglobulin gene. A class I gene is comprised of eight exons. The first exon encodes a signal peptide that directs the insertion of the HLA I molecule into the endoplasmic reticulum during translation. Exon two through four encodes the three external domains of the protein

(alpha 1-3). The fifth exon encodes the transmembrane domain, while exons six through eight encode cytoplasmic domains. Polymorphisms are on exons two and three.

2. Class II HLA genes

The genetic locus encoding class II molecules is known as the D region in humans. The D region is further subdivided into DP, DQ, DR, DO and DN (Bell, Denny et al., 1985). The class II genes are made up of both alpha and beta genes. Similar to class I genes, the genomic arrangement of class II genes reflects the domain structure of class II proteins. The first exon of both alpha and beta chain genes encode the 5' untranslated region, the leader or signal sequences. Exons two and three code for either the alpha one and alpha two or the beta one and beta two domains. Exon four of the alpha genes encodes the transmembrane cytoplasmic domain and part of the 3 untranslated regions, while exon four of the beta genes encodes the transmembrane and a portion of the cytoplasmic domain. Exon five encodes the rest of the untranslated region in the alpha genes and the rest of the cytoplasmic domain of the beta genes.

3. Class III HLA genes

The class III HLA genes encode complement components (C2, C4 and factor B) that show no structural similarity to either class I or class II molecules. These genes, along with genes encoding heat-shock protein-70 components and the peptide transporters that function in the loading of HLA class I molecules.

HLA polymorphism

One of most striking features of the HLA is the extensive polymorphism of the class I and II genes and their encoded products. The number of definable allelic products of a given locus in a species has been increased by development of new methodology. The HLA Sequence Database which holds information on HLA sequences currently contains 2,083 allele sequences. In addition to the physical sequences, the database contains detailed information concerning the material from which the sequence was derived and data on the validation of the sequences. To date (September 2005), some 396 HLA-A, 699 HLA-B, 198 HLA-C, 8 HLA-E, 2 HLA-F and 15 HLA-G class I alleles have been named. A total of 3 HLA-DRA, 494 HLA-DRB (413 HLA-DRB1), 28 HLA-DQA1, 66 HLA-DQB1, 23 HLA-DPA1, 119 HLA-DPB1, 4 HLA-DMA, 7 HLA-DMB, 12 HLA-DOA and 9 HLA-DOB class II sequences have also been assigned (http://www.ebi.ac.uk/imgt/hla/stats.html)

Tumor necrosis factor-α

TNF-α is a cytokine with a wide range of pro-inflammatory activities (Vassalli., 1992); (Beutler. 1995). It is produced predominantly by activated macrophages and less so by other cell types. Human TNF-α is synthesized as a pro-protein comprising 233 amino acids, with a molecular mass of 26 kDa. The pro-protein is cleaved by a specific metalloprotease (also named TNF-α converting enzyme, TACE) to yield a monomeric form of 17 kDa comprising 157 nonglycosylated amino acids. Under physiological conditions, TNF-α forms a noncovalently bound cone-shaped homotrimer (Jones, Stuart et al., 1989). The human TNF-α gene is located 850 kb telomeric of the class II HLA-DR locus and 250 kb centromeric of the class I HLA-B locus of the short arm of chromosome 6 (Kamizono, Hiromatsu et al., 2000). Polymorphism in the promoter region of the TNF-α gene may be important for TNF-α gene expression and protein. The maximum capacity of TNF-α production varies among individuals and correlates with the polymorphism in the TNF-α gene promoters.

TNF-α plays a critical role in protection from HBV infection. Biermer et al reported that TNF-α inhibit HBV replication by non-cytopathic suppression mediated by NF-κB pathway (Biermer, Puro et al., 2003). Considerable evidence suggests that TNF-α gene polymorphisms associated with chronic hepatitis B development (table 2). Kim et al found an association between -308A allele and -863A allele were significant associated with HBV clearance and with persistence of HBV infection, respectively (Kim, Lee et al., 2003). From the studies in TNF-α gene polymorphisms at position -238, susceptibility to develop chronic HBV infection has been associated with -238A allele in German (Hohler, Kruger et al., 1998). By contrast, the -238G allele was associated with develop chronic HBV infection in Chinese (Wu, Wang et al., 2004). However, the association between TNF-α and chronic HBV infection is controversial in each population. This difference may be due to the fact that the TNF-α promoter polymorphism at position -238, likely serving as a marker, was in linkage disequilibrium with neighboring genes encoding HLA or other undefined genes, thus possibly influencing the outcomes of disease (Wu, Wang et al., 2004).

Furthermore, TNF-α gene plays a critical role not only in protection from HBV infection but also in the pathogenesis of chronic HBV infection and associated with the development of HCC (Ho, Wang et al.2004). The existing evidence implicates the role of TNF-α inflammatory pathway that increased tumorigenesis (Szlosarek and Balkwill.,2003). TNF-α has been found in high concentration in patients with cancer (Abrahamsson, Carlsson et al., 1993; Partanen, Koskinen et al., 1995). Recent evidence suggests that the TNF-α, which is a key player in inflammation can also activate signaling pathways, in both cancer cells and tumor-asscociated inflammatory cells, that promote malignancy (Balkwill and Coussens., 2004; Pikarsky, Porat et al., 2004). A genetic polymorphism of TNF-α at promoter region has been found to be associated with susceptibility to various cancer. For example, the association in -863A polymorphism of TNF-α and HPV clearance in cervical cancer in Caucasian (Deshpande, Nolan et al., 2005). In Korean, Jung et al reported the -308 A/G in the TNF-α promoter was associated with increase TNF-α serum level than -308G/G which effect the development of bladder tumors grade (Jeong, Kim et al., 2004). In other cancers -308A polymorphism of TNF-α appeared to be a protective genotype for the intestinal type in gastric cancer (Lee, Kim et al., 2004).

TNF-α gene polymorphism

Polymorphisms in the cytokine gene can be found within coding regions, introns and promoter region that characterized by single nucleotide polymorphisms (SNPs), microsatellite repeats and minisatellite or variable number of tandem repeats (VNTRs). These polymorphisms within gene may be associated with differences in cytokine production individual. For example, polymorphism within the promoter region may alter binding affinity of transcription factor which influence protein levels (Holmes, Russell et al., 2003). In addition, the effect of cytokine gene polymorphism on gene expression can be study in *vitro* and in *vivo* (Bidwell, Keen et al., 1999).

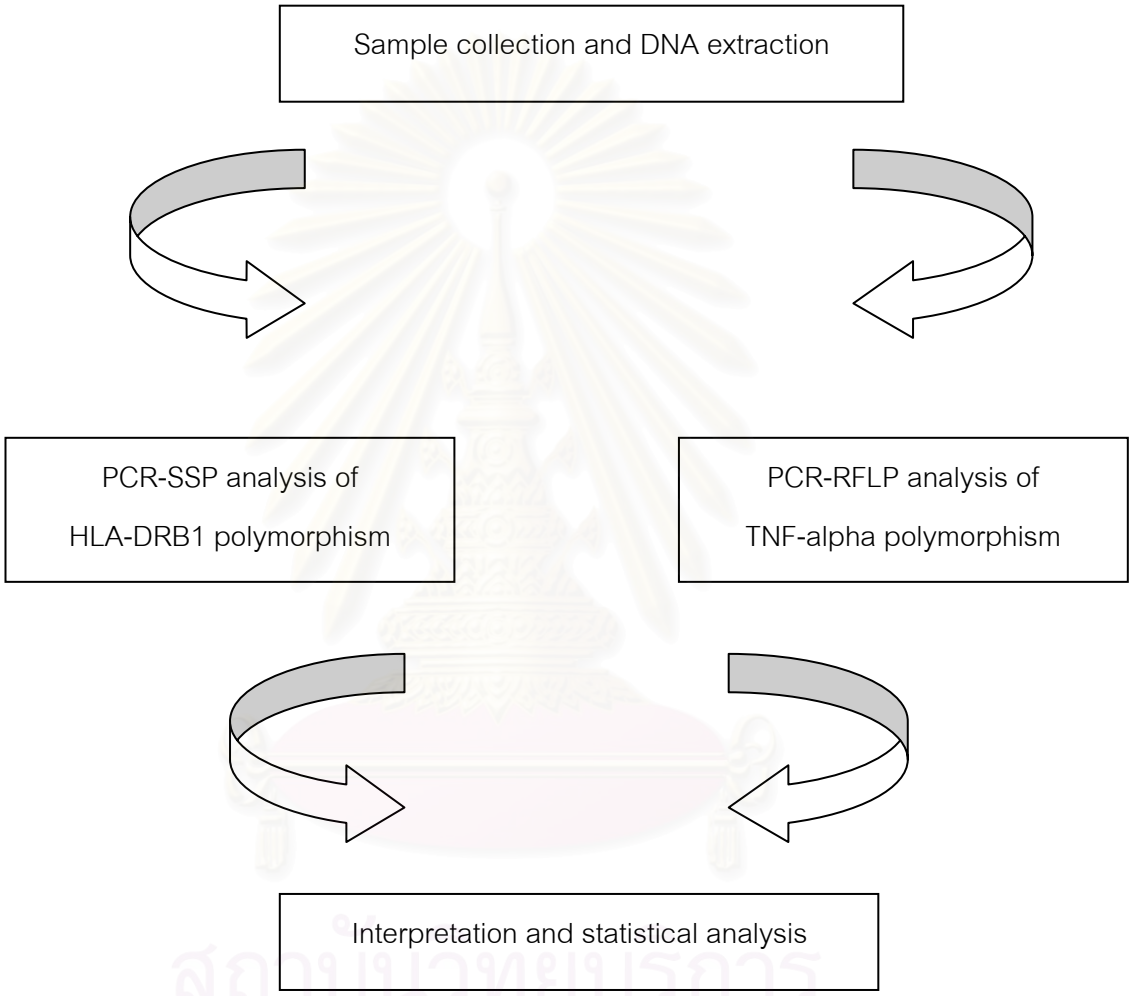
TNF-α gene polymorphisms are characterized by several single nucleotide polymorphisms (SNPs) in promoter region. Interestingly some of these SNPs within this gene appear to influence TNF-α expression and susceptibility to autoimmune and infectious diseases (Rink and Kirchner., 1996). The maximum capacity of TNF-α production varies among individuals and correlates with the polymorphism in the TNF-α gene promoters. Evidence functional analysis of TNF-α gene promoters reported the effects of TNF-α gene polymorphism on transcriptional regulation.

Udalova et al demonstrated that the p50-p50 homodimeric form of NF-κB complex acts as a transcriptional repressor on binding to its regulatory site in the promoter region of the TNF gene. It was shown that p50-p50 homodimeric form had a significantly decreased affinity to TNF-863A which associated with increasing TNF gene expression and TNF-α production (Udalova, Richardson et al., 2000). Furthermore, the high level of TNF-α production by concavalin A (con A) activated peripheral blood mononuclear cells was related -863A (Higuchi, Seki et al., 1998). The study by Hohjohi et al also reported -863A allele specific binding of the transcription factor OCT-1 to the SNP sites at position -863 in the promoter which effect TNF-α expression (Hohjoh and Tokunaga., 2001). In contrast, Skoog et al demonstrated that the -863A allele was associated with lower transcriptional activity in chloramphenical acetyltransferase (CAT) reporter gene studies in human hepatoblastoma (HepG2) cells and -863A allele was associated with significantly lower serum TNF-α concentrations in healthy middle-age men in Sweden (Skoog, van't Hooft et al., 1999).

Recent studies have demonstrated that the -308 polymorphism affects transcription factor binding and enhances transcription from the TNF promoter in cell lines after stimulation with various inducers of TNF-α synthesis. Although the actual biological effect of this polymorphism in vivo has not been clearly demonstrated, the -308A allele has been shown to be associated with higher TNF-α production (Kroeger, Carville et al., 1997; Wilson, Symons et al., 1997; Galbraith, Steed et al., 1998; Huang, Pirskanen et al., 1999; Maurer, Kruse et al., 1999). Besides, Grove et al suggested that -238A allele which falls within a putative Y regulation box of the TNF-α promoter, was associated with increased TNF-α expression (Grove, Daly et al., 1997).

CHAPTER IV

MATERIALS AND METHODS



Subjects

Thai patients from outpatients and inpatients service of King Chulalongkorn Memorial hospital were included in the study. Subjects were categorized into two different groups: chronic carrier group and transient hepatitis B group: (1) chronic carrier group contained 150 subjects (50 with hepatocellular carcinoma and 100 without hepatocellular carcinoma), who had been hepatitis B surface antigen (HBsAg)-positive for at least 6 months and did not have any type of liver disease such as chronic hepatitis C or alcoholic liver disease. Moreover, all patients had elevated serum ALT and AST level; (2) transient hepatitis B group to serve as control for the population-based case-control study contained 100 subjects, who tested hepatitis B surface antigen (HBsAg) negative and both HBV core antibody (anti-HBc) and HBV surface antibody (anti-HBs) positive. In addition, healthy subjects group contained 100 subjects were recruited from volunteer unrelated donors from Thai red cross, who tested both hepatitis B surface antigen (HBsAg) and HBV surface antibody (anti-HBs) negative. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bankok, Thailand approved the study and the subjects gave their informed consent. Demographic data of the subjects was summarized in table 3.

DNA extraction

DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as anticoagulant, using a salting out method (Miller, Dykes et al., 1988). For the genomic DNA extraction, 1 ml of red lysis buffer (RCLB) was added to 0.5 ml of buffy coat, vortex for 30 seconds. This solution was centrifuged at 10,000-12,000 rpm for 30 seconds and the supernatant was discarded to obtain the pellet. The pellet remaining should be white to pink. This step may be repeated if necessary. To this pellet, 200 μl nuclei (NLB) and 50 μl 10% SDS were added. Pellet was broken up with pipette tip and vortex to get powdery, tiny flakes. The solution, 150 μl of NLB and 10 μl of proteinase K (10 mg/ml in H_2O stored frozen) were added, followed by incubation at 65 ^{0} C for 2 hours. Precipitation of proteins was obtained by adding 175 μl of 5.3 M NaCl. This solution was centrifuged at 10,000-12,000 rpm for 15 minutes in micro centrifuge. After centrifugation, the DNA in the supernatant was

precipitated in 1 ml of cold absolute ethanol. Invert 6-10 times to precipitate DNA, it will appear as a white to translucent stringy mass. This solution was centrifuged at 10,000-12,000 rpm for 10 minutes and the supernatant was discarded to obtain the pellet. After removal of the ethanol, the pellet was dissolved in 200 μl of sterile distilled water, followed by incubation at 65 ^{0}C for 15 minutes. Use gentle vortexing to resuspend. If clumps of undissolved DNA are present, it will be in 65 ^{0}C until completely resuspended.

Genotyping methodology

Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP)

Analysis of HLA-DRB1 (HLA-DR-12, HLA-DR13) polymorphism

We performed the polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) analysis of HLA-DR12 and HLA-DR13 as previously described (Olerup and Zetterquist., 1992). The genomic DNA of 150 chronic HBV patients (50 with HCC, 100 without HCC) and 100 transient HBV patients were amplified with the use of the HLA-DRB1 (HLA-DR12, HLA-DR13) specific primers as shown in table 4.

The reaction volume for the amplification reaction was 20 μl, containing 100 ng/μl genomic DNA, 0.1 μl of 5.0 U Taq polymerase (Promega or Gibco), 2 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 1.2 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM deoxynucletide triphosphates, 1 μl (20 pmol) of each specific primers and 0.1 μl (20 pmol) of internal control primers. Internal control primers were used to check for successful PCR amplification. These primers amplify a human growth hormone sequence (Pravica, Perrey et al. 2000) (table 4). Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/ GeneAmp PCR system 9600. The PCR cycling parameters of HLA-DR13 allele consisted of an initial denaturation at 95°C for 1 minutes, followed by 10 cycles of denaturation (95°C, 15 seconds), annealing (60°C, 50 seconds) and extension (72°C, 40 seconds) and 20 cycles of denaturation (95°C, 20 seconds), annealing (56°C, 50 seconds) and extension (72°C, 50 seconds) final extension at 72°C for 7 minute (Olerup and

Zetterquist 1992). The PCR cycling parameters of HLA-DR12 allele consisted of an initial denaturation at 95°C for 1 minutes, followed by 10 cycles of denaturation (95°C, 15 seconds), annealing (62°C, 50 seconds) and extension (72°C, 40 seconds) and 20 cycles of denaturation (95°C, 20 seconds), annealing (56°C, 50 seconds) and extension (72°C, 50 seconds) final extension at 72°C for 7 minute (Olerup and Zetterquist 1992). The resulting products were further analyzed by electrophoresis in 1.5 % Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 100 volts for 40 minutes and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD). Negative controls without DNA template were included in each experiment. The positive results of HLA-DR13 allele, HLADR12 allele and human growth hormone gene showed band of 130, 248 and 428 bp fragment, respectively. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

For DRB1 full typing of the 8 patients that carry DR13 were perform and using SSP UniTray (Pel-Freeze, Wisconsin, USA). (see Appendix F)

Polymerase Chain Reaction-Restriction fragment Length Polymorphism

Analysis of TNF-alpha

We performed the Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) analysis of TNF-alpha at promoter position -308 (A/G), -863 (A/C) and -238 (G/A) as previously described (Allen, Lee et al., 2001; Wennberg, Nordstrom et al., 2002; Lu, Li et al., 2004). The genomic DNA of 150 chronic HBV patients (50 with HCC, 100 without HCC) and 100 transient HBV patients were amplified with the use of the TNF-alpha gene specific primers as shown in table 4.

The reaction volume for the amplification reaction was 30 μl, containing 100 ng of genomic DNA, 0.15 μl of 5.0 Taq polymerase (Promega or Gibco), 3 μl of 10x PCR buffer (20nM Tris-HCL pH 8.0, 100 mM KCL), 1.8 μl of 25 mM $MgCl_2$, 0.6 μl of 10 mM dNTP and

1.5 μl (20 pmol) of each oligonucleotide primer. PCR was carried out using Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystem/ GeneAmp PCR system 9600 under specific PCR condition. At position -863 (A/C), the PCR protocal consisted of an initiation denateration at 94 ^{0} C for 2 minutes, followed by 30 cycles of denateration (94 ^{0} C, 20 seconds), annealing (59 ^{0} C, 50 seconds) and extension (72 ^{0} C, 20 seconds) and final extension at 72 ^{0} C, 7 minutes. At position -308 (G/A), the PCR protocal consisted of an initiation denateration at 94 ^{0} C for 5 minutes, followed by 35 cycles of denateration (94 ^{0} C, 1 minutes), annealing (60 ^{0} C, 1 minutes) and extension (72 ^{0} C, 1 minutes) and final extension at 72 ^{0} C, 7 minutes. At position -238(G/A), the PCR protocal consisted of an initiation denateration at 94 ^{0} C for 2 minutes, followed by 30 cycles of denateration (94 ^{0} C, 1 minutes), annealing (59 ^{0} C, 1 minutes) and extension (72 ^{0} C, 1 minutes) and final extension at 72 ^{0} C, 5 minutes. The resulting products were further analyzed by electrophoresis in 1.5% Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 100 volts for 40 minitues and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD). Negative controls without DNA template were included in each experiment. A molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments. Successful amplification PCR products for 3 positions:

1. At position -863 (C/A)

PCR product size is 124 bp fragment and then, 10 μl of amplified DNA were digested with 5U of specific restriction enzyme *HpyCH4IV* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 1 in a total volume of 15 μl at 37 ^{0} C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 40 minutes. If an A was present at this position, the enzyme would cut the 124 bp PCR product into two fragment; 102 and 22 bp. No digestion would occur if a C was present. A molecular ladder of 100-bp (promega) was used to estimated the size of the PCR fragment.

2. At position -308 (G/A)

PCR product size is 134 bp fragment and then, 10 μl of amplified DNA were digested with 5U of specific restriction enzyme *NcoI* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 4 in a total volume of 15 μl at 37 ^{0} C for 14-16 hours, followed by

3% agarose gel electrophoesis at 100 volts for 40 minutes. If an A was present at this position, the enzyme would cut the 134 bp PCR product into two fragment; 112 and 20. No digestion would occer if a C was present. A molecular ladder of 100-bp (Promega) was used to estimated the size of the PCR fragment.

4. At position -238 (G/A)

PCR product size is 152 -bp fragment and then, 10 μl of amplified DNA were digested with 5U of specific restriction enzyme *MsP-1* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 2 in a total volume of 15 μl at 37 ^{0}C for 14-16 hours, followed by 3% agarose gel electrophoesis at 100 volts for 40 minutes. If an A was present at this position, the enzyme would cut the 152 bp PCR product into two fragment; 132 and 20 bp. No digestion would occer if a C was present. A molecular ladder of 100-bp (Promega) was used to estimated the size of the PCR fragment.

Statistical Analysis

We compared chronic hepatitis B group with transient hepatitis B group in disease susceptibility and chronic hepatitis B with HCC group were compared with chronic hepatitis B without HCC group in disease progression. In addition, we compared the genotype distribution between normal control Thai population and other population. The genotype frequencies were checked by consistency among normal controls with those expected from Hardy-Weinberg equilibrium. Allele and genotype frequencies were compare between groups using the Chi-square (χ^2) test or Fisher' extract probability test, where appropriate. Gene frequencies were determined by gene counting. A *P* value of < 0.05 was considered significant. Odd ratio (OR) with 95% confidence interval (CI) were calculated using the statistical program Epi Info version 6 (Centers for Disease Control and Prevention [CDC], 1994). The mode of inheritance analysis was also included. Furthermore, when one element in the 2x2 table (Chi-square (χ^2) test) was zero, OR was calculated with the Haldane' s modified formula [RR_h= (2a+1)(2d+1)/(2b+1)(2c+1)]. Haldane' s correction for the OR was used when either all patients were positive or all controls were negative for a particular specificity or allele (Haldane S., 1956).

Haplotype and linkage disequilibrium analysis

The program PHASE were used to reconstructing haplotypes from population genotype data (Stephens and Donnelly., 2003). The software can deal with SNP, microsattellite, and other multi-allelic loci, in any combination and missing data are allowed. The remaining ambiguous sites are assigned by PHASE, and the uncertainly associated with each PHASE assignment is calculated (see Appendix C). In addition, linkage disequilibrium was calculated using the LDPlotter Tool (see Appendix D).

Table 3 Demographic and clinical data of subjects

Characteristics	chronic group		transient HBV infection group	healthy control
	with HCC	without HCC		
n	50	100	100	100
Sex (m/f)	41/9	65/35	48/52	40/60
Age ± SD(yr)	54±13.7	46±13.8	46±14.3	23±11.4
ALT(U/L)*	107	132	-	-
% HBeAg positive*	37.5%	71.43%	-	-
Genotype*				
A	-	7.1%	-	-
B	18.75%	28.6%	-	-
C	81.25%	64.3%	-	-

* data can be obtained only from a subset of patients (16 HCC patients and 14 chronic HBV patients without HCC)

Table 4 Primers used for analysis of the HLA-DRB1 gene and TNF-α gene polymorphism

Gene polymorphism and primer	Sequence of primers (5' →3')	References
HLA-DRB1*1301-2cviop -Forward -Reverse Internal control primer -Forward -Reverse	 TAC TTC CAT AAC CAG GAG GAG A CCC GCT CGT CTT CCA GGA T GCCTTCCCAACCATTCCCTTA TCACGGATTTCTGTTGTGTTTC	(Olerup and Zetterquist., 1992) (Pravica, Perrey et al., 2000)
HLA-DR12 -Forward -Reverse Internal control primer -Forward -Reverse	 AGT ACT CTA CGG GTG AGT GTT CAC TGT GAA GCT CTC CAC AG GCCTTCCCAACCATTCCCTTA TCACGGATTTCTGTTGTGTTTC	(Olerup and Zetterquist., 1992) (Pravica, Perrey et al., 2000)
TNF-α (-863A/C) -Forward -Reverse	 GGCTCTGAGGAATGGGTTAC CTACATGGCCCTGTCTTCGTTACG	(Wennberg, Nordstrom et al., 2002)
TNF-α (-308A/G) -Forward -Reverse	 AGG CAA TAG GTT TTG AGG GCC AT CAT CAA GGA TAC CCC TCA CAC TC	(Allen, Lee et al., 2001)
TNF-α (-238A/G) -Forward -Reverse	 AGA AGA CCC CCC TCG GAA CC ATC TGG AGG AAG CGG TAG TG	(Lu, Li et al., 2004)

CHAPTER V

RESULT

1. Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) Analysis of HLA-DRB1 (HLA-DRB1*1301-2, HLA-DR12)

1.1 PCR-SSP analysis of HLA-DRB1*1301-2

HLA-DRB1*1301-2 allele was identified using the PCR-SSP method. The positive result of HLA-DRB*1301-2 allele and human growth hormone gene (internal control) showed band of 130 and 428 bp fragment, respectively (Olerup and Zetterquist 1992) (Figure 3).

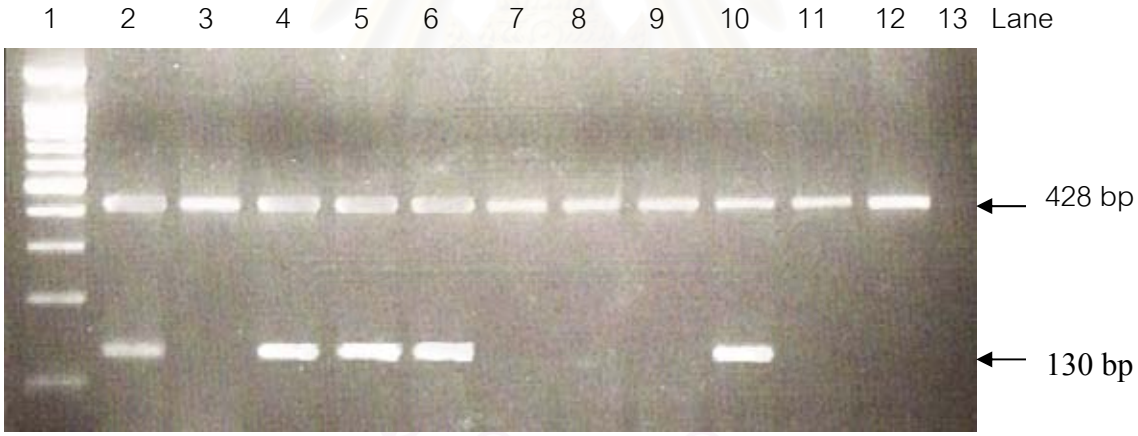


Figure 3 The representative of PCR-SSP results from samples with HLA-DRB1*1301-2 specific primers amplification.

Lane 1 is 100 bp molecular markers.

Lane 2-12 samples all show positive internal control band.

Lane 2, 4-6, 10 are specific band that interpreted for HLA-DRB1*1301-2 band (130 bp).

Lane 13 is negative control (no DNA sample).

1.2 PCR-SSP analysis of HLA-DR12

HLA-DR12 allele was identified using the PCR-SSP method. The positive result of HLA-DR12 allele and human growth hormone gene (internal control) showed band of 248 and 428 bp fragment, respectively (Olerup and Zetterquist 1992) (Figure 4).

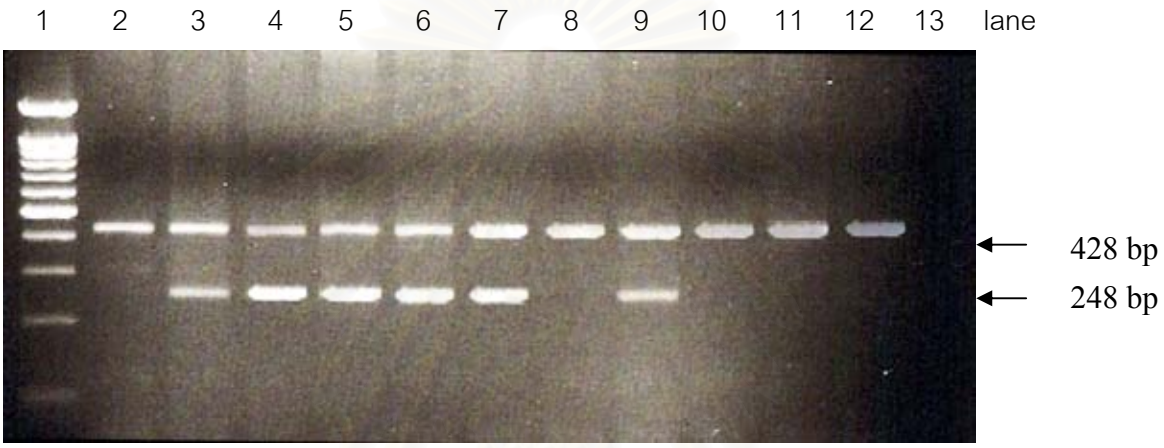


Figure 4 The representative of PCR-SSP results from samples with HLA-DR12 specific primers amplification.

Lane 1 is 100 bp molecular markers.

Lane 2-12 samples all show positive internal control band.

Lane 3- 7, 9 are specific band that interpreted for HLA-DR12 band (248 bp).

Lane 13 is negative control (no DNA sample).

2. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

Analysis of TNF-α

2.1 PCR-RFLP analysis of TNF-α promoter at position -863

Polymorphism at -863A/C in the promoter region of the TNF-α were identified by the PCR-RFLP method. If an A was present at this position, the *HpyCH4IV* restriction enzyme would cut the 124 bp PCR product into two fragment; 102 and 22 bp. No digestion would occur if a C was present (Wennberg, Nordstrom et al. 2002) (Figure 5).

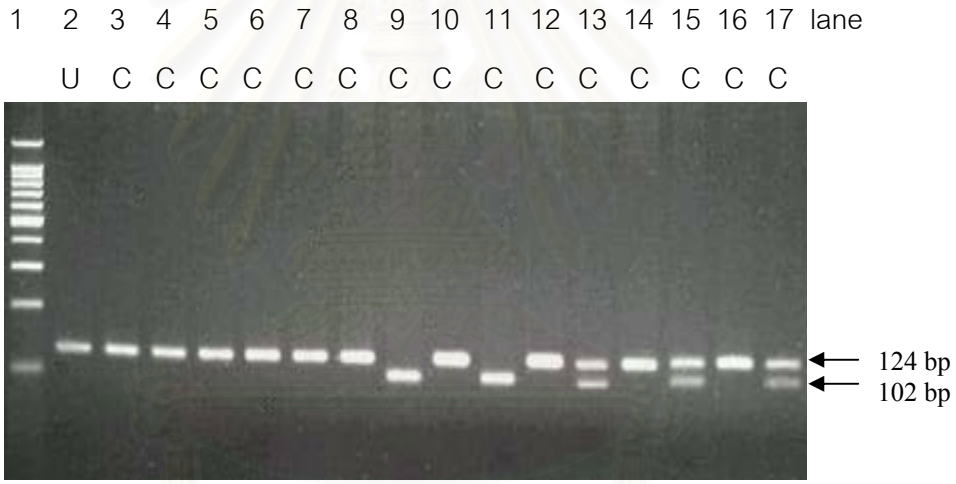


Figure 5 The representative of PCR-RFLP results from samples with homozygous of –863C, homozygous of –863A and heterozygous –863C/A.

Lane 1 is 100 bp molecular marker.

Lane 3-8, 10, 12, 14, 16 are homozygous of -863C.

Lane 9, 11 are homozygous of –863A.

Lane 13, 15, 17 are heterozygous –863C/A.

Under these electrophoresis condition the 22 bp product is not visible. U = not add restriction enzyme, C = add restriction enzyme.

2.2 PCR-RFLP analysis of TNF-α promoter at position -308

Polymorphism at -308A/G in the promoter region of the TNF-α were identified by the PCR-RFLP method. If an G was present at this position, the *NcoI* restriction enzyme would cut the 134 bp PCR product into two fragment; 114 and 20 bp. No digestion would occur if a A was present. (Allen, Lee et al. 2001) (Figure 6).

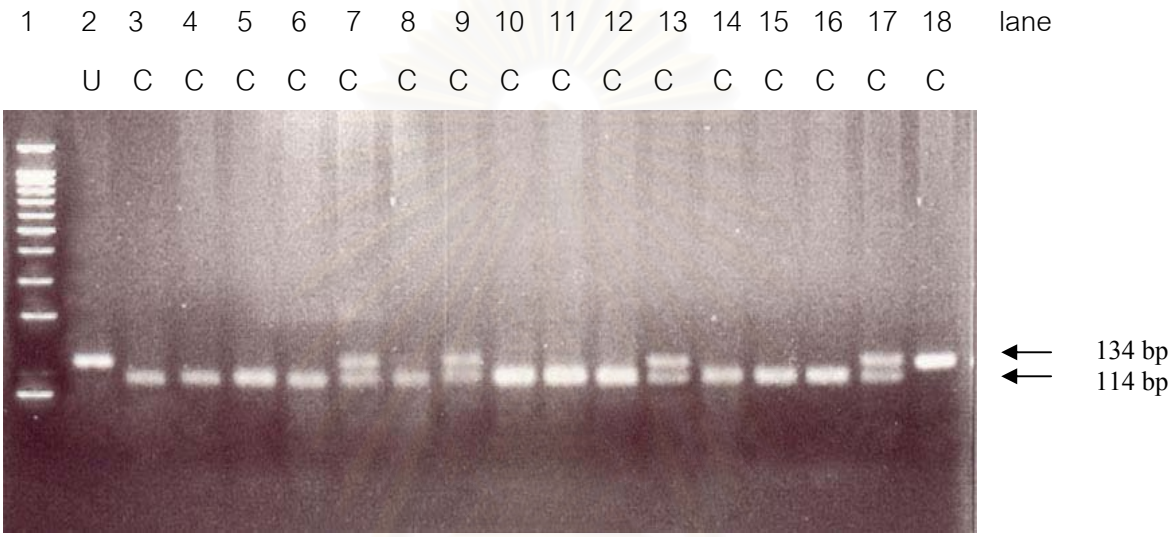


Figure 6 The presentative of PCR-RFLP result from samples with homozygous of -308A, homozygous of -308G and heterozygous -308A/G.

Lane 1 is 100 bp molecular markers.

Lane 18 is homozygous of -308A.

Lane 3-6, 8, 10-12, 14-16 are homozygous of -308G.

Lane 7, 9, 13, 17 are heterozygous of -308A/G.

Under these electrophoresis condition the 20 bp product is not visible. U = not add restriction enzyme, C = add restriction enzyme.

2.3 PCR-RFLP analysis of TNF-α promoter at position -238

Polymorphism at -238A/G in the promoter region of the TNF-α were identified by the PCR-RFLP method. If an G was present at this position, the *MsP-1* restriction enzyme would cut the 152 bp PCR product into two fragment; 130 and 22 bp. No digestion would occur if a A was present (Lu, Li et al. 2004) (Figure 7).

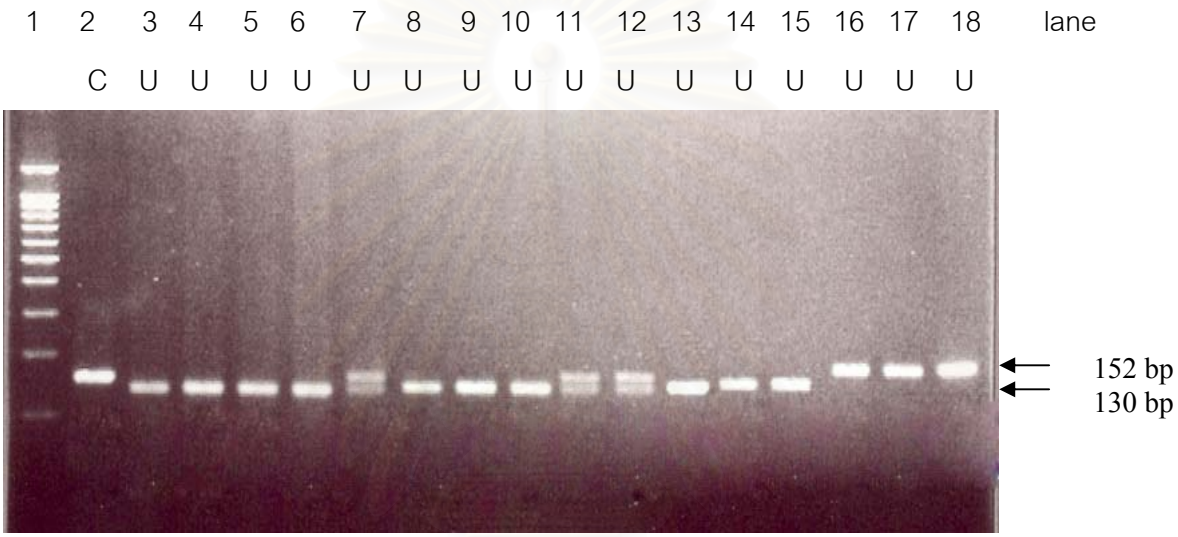


Figure 7 The representative of PCR-RFLP result from samples with homozygous of -238A, homozygous of -238G and heterozygous -238A/G.

Lane 1 is 100 bp molecular markers.

Lane16, 17, 18 are homozygous of -238A.

Lane 3-6, 8-10, 13-15 are homozygous of -238G.

Lane 7, 11-12 are heterozygous of -238A/G.

Under these electrophoresis condition the 22 bp product is not visible. U = not add restriction enzyme, C = add restriction enzyme.

3. The association results of HLA-DRB1 gene and TNF-α gene polymorphisms with susceptibility to chronic hepatitis B infection

3.1. HLA-DRB1 gene polymorphisms in patients with chronic hepatitis B and transient hepatitis B

The distribution of HLA-DRB1*1301-2 and HLA-DR12 phenotypes in chronic HBV patients and transient HBV infection patients were shown in table 5. HLA-DRB1*1301-2 phenotype was not found in any chronic HBV patients. In contrast, HLA-DRB1*1301-2 phenotype was identified with high frequency of 8% in the transient HBV infection patients was significantly higher than chronic HBV patients (0%) (p=0.0004, OR=0.04, 95%CI=0.00-0.26). The frequencies of HLA-DR13 phenotype in healthy Thai individual is 2% (table 25). HLA-DR12 phenotype was increased in chronic HBV patients (32.67%) compared with transient hepatitis B patients (27%), but did not reach statistical significance.

For DR13 genotype was identify using SSP Unitray (Pel-Freeze, Winconsin, USA) (see Appendix F). DRB1 typing was performed to identify another allele for 8 individuals that carried DR13 using All 8 patients are heterozygous for DR13 (table 6).

Table 5 Distribution of specific HLA-DRB1 (DR12 and DRB1*1301-2) phenotypes in patients with chronic hepatitis B and transient HBV infection patients

HLA-DRB1	chronic hepatitis B n=150	transient hepatitis B n=100
DRB1*1301-2	0(0%)[a]	8(8%)
DR12	49 (32.67%)[b]	27(27%)

[a] p=0.0004, OR=0.04, 95%CI=0.00-0.26

[b] p=0.41

Table 6 DRB1 typing in 8 individuals who transient from HBV infection

Individuals	DRB1 phenotype
1	DRB13, DR14
2	DRB13, DR4
3	DRB13, DR15
4	DRB13, DR7
5	DRB13, DR9
6	DRB13, DR14
7	DRB13, DR16
8	DRB13, DR11

* DRB1 typing was identify using The PEL-FREEZ SSP Unitray™ (see Appendix F).

3.2 TNF-α gene polymorphisms in chronic hepatitis B patients and transient hepatitis B patients

3.2.1 TNF-α gene polymorphism at position -863 (A/C)

The distribution of genotypes of TNF-α gene polymorphism at position -863(C/A) among healthy controls were in agreement with the predict under the condition of Hardy-Weinberg equilibrium (Appendix E)

Genotype and allele frequencies for -863 at the promoter of TNF-α gene in patients with chronic hepatitis B and transient hepatitis B patients were shown in table 7 and 8. Two of 100 transient hepatitis B patients (2%) were homozygous for the A/A genotype, 28 (28%) were heterozygous and 70 (70%) were homozygous for the C/C genotype. The allele frequencies were 16% for A allele and 84% for C allele. In comparison, 7 of 150 patients with chronic hepatitis B (4.67%) were homozygous for the A/A genotype, 57(38.67%) were heterozygous and 86 (56.66%) were homozygous for the C/C genotype. The allele frequencies were 23.67% for A allele and 76.33% for C allele.

The -863A allele was found to be significantly increased in chronic hepatitis B patients compared with transient hepatitis B patients (P=0.0495, OR=1.63, 95% CI=1 to 2.65) (table 7).

3.2.2 TNF-α gene polymorphism at position -308 (A/G)

The distribution of genotypes of TNF-α gene polymorphism at position -308(G/A) among healthy controls were in agreement with the predict under the condition of Hardy-Weinberg equilibrium (Appendix E)

Genotype and allele frequencies for -308 at the promoter of TNF-α gene in patients with chronic hepatitis B and transient hepatitis B patients were shown in table 9 and 10. The homozygous A/A genotype was not found in both groups. Eighteen of 100 transient hepatitis B patients (18%) were heterozygous and 82 (82%) were homozygous for the G/G genotype. The allele frequencies were 9% for A allele and 91% for G allele. In comparison,

21 of 150 patients with chronic hepatitis B (14%) were heterozygous and 129 (86%) were homozygous for the G/G genotype. The allele frequencies were 7% for A allele and 93% for G allele.

There were no significant differences in allele frequency of -308A/G polymorphism at the promoter of TNF-α gene between chronic hepatitis B patients and transient hepatitis B patients.

3.2.3 TNF-α gene polymorphism at position -238(A/G)

The distribution of genotypes of TNF-α gene polymorphism at position -238(G/A) among healthy controls were in agreement with the predict under the condition of Hardy-Weinberg equilibrium (Appendix E)

Genotype and allele frequencies for -238 at the promoter of TNF-α gene in patients with chronic hepatitis B and transient hepatitis B patients were shown in table 11 and 12. One of 100 transient hepatitis B patients (1%) were homozygous for the A/A genotype, 7 (7%) were heterozygous and 92 (92%) were homozygous for the G/G genotype. The allele frequencies were 4.5% for A allele and 95.5% for G allele. In comparison, 1 of 150 patients with chronic hepatitis B(0.67%) were homozygous for the A/A genotype, 9(6%) were heterozygous and 140 (93.33%) were homozygous for the G/G genotype. The allele frequencies were 3.67% for A allele and 96.33% for G allele.

There were no significant differences in allele frequency of -238A/G polymorphism at the promoter of TNF-α gene between chronic hepatitis B patients and transient hepatitis B patients.

3.3 Haplotype analysis of TNF- α promoter polymorphism at position (-863A/C, -308A/G, -238A/G) in patients with chronic hepatitis B and transient hepatitis B patients

The haplotype frequencies of the TNF- α promoter polymorphism were also determined by PHASE program. The genotype frequencies of the haplotype and haplotypes frequencies in patients with chronic hepatitis B and transient hepatitis B patients were shown in table 13 and 22. In haplotype analysis, we found 4 haplotypes; CGG, AGG, CAG and CGA and also found 9 genotypes of haplotypes; AGG/AGG, CAG/AGG, CGA/AGG, CGACAG, CGA/CGA, CGG/AGG, CGG/CAG, CGG/CGA and CGG/CGG. In this study, CGG/CGG genotype of haplotype and CGG haplotype were found the most common haplotype in chronic hepatitis B patients and transient hepatitis B patients. However, no significant differences in genotype frequencies of the haplotype could be demonstrated between chronic hepatitis B patients and transient hepatitis B patients.

Table 7 Genotype and allele frequencies for the -863 at the promoter of TNF- α gene in chronic hepatitis B patients and transient hepatitis B patients.

	chronic hepatitis B *n* = 150	transient hepatitis B *n* = 100
Genotype frequencies		
A/A	7(4.67%)	2(2%)
A/C	57(38.67%)	28(28%)
C/C	86(56.66%)	70(70%)
Allele frequencies		
A	71(23.67%) [a]	32(16%)
C	229(76.33%)	168(84%)

[a] P=*0.0495, OR=1.63, 95% CI=1 to 2.65*

Table 8 Risk of chronic hepatitis B infection associated with TNF- α (-863C/A) genotype according to different models of inheritance.

	chronic hepatitis B *n* = 150	transient hepatitis B *n* = 100
A dominance, C wild type		
C/C	86(57.33%)	70(70%)
A/A or A/C	64(42.67%) [a]	30(30%)
A recessive, C wild type		
C/C or A/C	143(95.33%)	98(98%)
A/A	7(4.67%) [b]	2(2%)

[a] *P*= 0.058

[b] *P*= 0.322

Table 9 Genotype and allele frequencies for the -308 at the promoter of TNF- α gene in chronic hepatitis B patients and transient hepatitis B patients.

	chronic hepatitis B *n* = 150	transients hepatitis B *n* = 100
Genotype frequencies		
A/A	0(0%)	0(0%)
A/G	21(14%)	18(18%)
G/G	129(86%)	82(82%)
Allele frequencies		
A	21(7%) [a]	18(9%)
G	279(93%)	182(91%)

[a] *P*= 0.51

Table 10 Risk of chronic hepatitis B infection associated with TNF- α (-308G/A) genotype according to different models of inheritance.

	chronic hepatitis B *n* = 150	transient hepatitis B *n* = 100
A dominance, G wild type		
G/G	129(86%)	82(82%)
A/A or A/G	21(14%) [a]	18(18%)
A recessive, G wild type		
G/G or A/G	150(100%)	100(100%)
A/A	0(0%)	0(0%)

[a] *P*= 0.499

Table 11 Genotype and allele frequencies for the -238 at the promoter of TNF- α gene in chronic hepatitis B patients and transient hepatitis B patients.

	chronic hepatitis B *n* = 150	transient hepatitis B *n* = 100
Genotype frequencies		
A/A	1(0.67%)	1(1%)
A/G	9(6%)	7(7%)
G/G	140(93.33%)	92(92%)
Allele frequencies		
A	11(3.67%) [a]	9(4.5%)
G	289(96.33%)	191(95.5%)

[a] $P = 0.815$

Table 12 Risk of chronic hepatitis B infection associated with TNF- α (-208G/A) genotype according to different models of inheritance.

	chronic hepatitis B *n* = 150	transient hepatitis B *n* = 100
A dominance, G wild type		
G/G	140(93.33%)	92(92%)
A/A or A/G	10(6.67%) [a]	8(8%)
A recessive, G wild type		
G/G or A/G	150(100%)	100(100%)
A/A	0(0%)	0(0%)

[a] $P = 0.49$

Table 13 Haplotype frequencies of TNF- α promoter polymorphism at position (-863A/C, -308A/G, -238A/G) between chronic hepatitis B patients and transient hepatitis B patients.

Haplotype	chronic hepatitis B $2n$ = 300	transient hepatitis B $2n$ = 200
Haplotype frequencies		
CGG	199(66%)[a]	121(60.5%)
Other haplotype	101(33.67%)	79(39.5%)
AGG	69(23%)[b]	33(16.5%)
Other haplotype	231(77%)	167(83.5%)
CAG	20(6.67%)[c]	20(10%)
Other haplotype	280(93.33%)	180(90%)
CGA	12(4%)[d]	7(3.5%)
Other haplotype	288(96%)	193(96.5%)

[a] P= 0.21

[b] P= 0.09

[c] P= 0.23

[d] P= 0.96

4. The association result of HLA-DRB1 gene and TNF-α gene polymorphisms with development of HCC

4.1. HLA-DRB1 gene polymorphisms in chronic hepatitis B patients with HCC and without HCC

The distribution of HLA-DRB1*1301-2 and HLA-DR12 phenotypes in chronic HBV patients with HCC and without HCC were shown in table 14. HLA-DRB1*1301-2 phenotype was not found in any chronic HBV patients. There was no significant in distribution of HLA-DR12 phenotype in chronic HBV patients with HCC compared with chronic HBV patients without HCC.

4.2. TNF-α gene polymorphisms in chronic hepatitis B patients with HCC and without HCC

4.2.1 TNF-α gene polymorphism at position -863 (C/A)

Genotype and allele frequencies for -863 at the promoter of TNF-α gene in chronic hepatitis B patients without HCC and with HCC were shown in table 15 and 16. Four of 100 patients without HCC (4%) were homozygous for the A/A genotype, 33 (33%) were heterozygous and 63 (63%) were homozygous for the C/C genotype. The allele frequencies were 20.5% for A allele and 79.5% for C allele. In comparison, 3 of 50 patients with HCC (4.67%) were homozygous for the A/A genotype, 25(50%) were heterozygous and 22 (44%) were homozygous for the C/C genotype. The allele frequencies were 31% for A allele and 69% for C allele.

The effect of –863A allele of TNF-α gene was similar to autosomal dominance mode of inheritance. The presence of one A allele (AA or AC) conferred the significant OR of 2 (*P*=0.041, OR=2.17, 95% CI=1.03 to 4.59) (table 16).

4.2.2 TNF-α gene polymorphism at position -308 (A/G)

Genotype and allele frequencies for -308 at the promoter of TNF-α gene in chronic hepatitis B patients without HCC and with HCC were shown in table 17 and 18. The homozygous A/A genotype was not found in both groups. Fourteen of 100 patients without HCC (14%) were heterozygous and 86 (86%) were homozygous for the G/G genotype. The allele frequencies were 7% for A allele and 93% for G allele. In comparison, 7 of 50 patients with HCC (14%) were heterozygous and 43 (86%) were homozygous for the G/G genotype. The allele frequencies were 7% for A allele and 93% for G allele.

There were no significant differences in allele frequency of -308A/G polymorphism at the promoter of TNF-α gene between patients without HCC and with HCC.

4.2.3 TNF-α gene polymorphism at position -238(A/G)

Genotype and allele frequencies for -238 at the promoter of TNF-α gene in chronic hepatitis B patients without HCC and with HCC were shown in table 19 and 20. The homozygous A/A genotype was not found in patients without HCC. Four of 100 patients without HCC (4%) were heterozygous and 96 (96%) were homozygous for the G/G genotype. The allele frequencies were 2% for A allele and 98% for G allele. In comparison, 1 of 50 patients with HCC (2%) were homozygous for the A/A genotype, 5(10%) were heterozygous and 44 (88%) were homozygous for the G/G genotype. The allele frequencies were 7% for A allele and 93% for G allele.

The -238A allele was found to be significantly increased in chronic hepatitis B patients with HCC compared with chronic hepatitis B patients without HCC (P=0.046, OR=3.69, 95% CI=0.94 to 15.42) (table 19).

Table 14 Distribution of specific HLA-DRB1 (DR12 and DRB1*1301-2) phenotypes in patients with chronic hepatitis B chronic hepatitis B patients without HCC and with HCC

HLA-DRB1	Patients without HCC n=100	Patients withHCC n=50
DRB1*1301-2	0(0%)	0(0%)
DR12	31(31%)[a]	18(36%)

[a] *p*= 0.66

Table 15 Genotype and allele frequencies for the -863 at the promoter of TNF- α gene in chronic hepatitis B patients without HCC and with HCC

	Patients without HCC *n* = 100	Patients with HCC *n* = 50
Genotype frequencies		
A/A	4(4%)	3(6%)
A/C	33(33%)	25(50%)
C/C	63(63%)	22 (44%)
Allele frequencies		
A	41(20.5%)	31(31%)[a]
C	159(79.5%)	69(69%)

[a] ***P*=** 0.062

Table 16 Risk of chronic hepatitis B infection associated with TNF- α (-863C/A) genotype according to different models of inheritance.

	Patients without HCC *n* = 100	Patients with HCC *n* = 50
A dominance, C wild type		
C/C	63(63%)	22(44%)
A/A or A/C	37(37%)	28(56%)[a]
A recessive, C wild type		
C/C or A/C	96(96%)	47(94%)
A/A	4(4%)	3(6%)[b]

[a] *P*=0.041, OR=2.17, 95% CI=1.03 to 4.59

[b] *P*= 0.89

Table 17 Genotype and allele frequencies for the -308 at the promoter of TNF- α gene in chronic hepatitis B patients without HCC and with HCC.

	Patients without HCC *n* = 100	Patients with HCC *n* = 50
Genotype frequencies		
A/A	0(0%)	0(0%)
A/G	14(14%)	8(16%)
G/G	86(86%)	42(84%)
Allele frequencies		
A	14(7%)	8(8%)[a]
G	186(93%)	92(92%)

[a] *P*= 0.93

Table 18 Risk of chronic hepatitis B infection associated with TNF- α (-308G/A) genotype according to different models of inheritance.

	Patients without HCC *n* = 100	Patients with HCC *n* = 50
A dominance, G wild type		
G/G	86(86%)	42(84%)
A/A or A/G	14(14%)	8(16%)[a]
A recessive, G wild type		
G/G or A/G	100(100%)	100(100%)
A/A	0(0%)	0(0%)

[a] *P*= 0.93

Table 19 Genotype and allele frequencies for the -238 at the promoter of TNF- α gene in chronic hepatitis B patients without HCC and with HCC.

	Patients without HCC *n* = 100	Patients with HCC *n* = 50
Genotype frequencies		
A/A	0(0%)	1(2%)
A/G	4(4%)	5(10%)
G/G	96(96%)	44(88%)
Allele frequencies		
A	4 (2%)	7(7%)[a]
G	196(98%)	93(93%)

[a] P=0.046, OR=3.69, 95%CI=0.94 to 15.42

Table 20 Risk of chronic hepatitis B infection associated with TNF- α (-238G/A) genotype according to different models of inheritance.

	patients without HCC *n* = 100	patients with HCC *n* = 50
A dominance, G wild type		
G/G	96(96%)	44(88%)
A/A or A/G	4(4%)	6(12%)[a]
A recessive, G wild type		
G/G or A/G	100(100%)	49(98%)
A/A	0(0%)	1(2%)[b]

[a] *P*= 0.08

[b] *P*= 0.33

4.3 Haplotype analysis of TNF- α promoter polymorphism at position (-863A/C, -308A/G, -238A/G) in chronic hepatitis B patients without HCC and with HCC.

The haplotype frequencies of the TNF- α promoter polymorphism were also determined by PHASE program. The haplotypes frequencies and genotype frequencies of haplotype in patients with chronic hepatitis B with HCC and without HCC were shown in table 21 and table 22. In haplotype analysis, we found 4 haplotypes; CGG, AGG, CAG and CGA and also found 9 genotypes of haplotypes; AGG/AGG, CAG/AGG, CGA/AGG, CGACAG, CGA/CGA, CGG/AGG, CGG/CAG, CGG/CGA and CGG/CGG. In this study, CGG/CGG genotype of haplotype and CGG haplotype were found the most common haplotype in both groups. In addition, CGG haplotype and CGG/CGG genotype of haplotype were found to be significantly increased in chronic HBV patients without HCC, as compared with chronic HBV patients with HCC (*P*= 0.02, OR=0.54, 95%CI=0.32 to 0.92 (table 21) and *P*= 0.009, OR=0.37, 95%CI=0.17 to 0.79 (table 23), respectively).

Haplotype analysis revealed that the homozygosity of the most common haplotype (CGG/CGG) was a protective marker for HCC in agreement with the positive association of -863A and -238A genotype.

Table 21 Haplotype frequencies of TNF- α promoter polymorphism at position (-863A/C, -308A/G, -238A/G) in chronic hepatitis B patients without HCC and with HCC.

Haplotype	patients without HCC $2n$ = 200	patients with HCC $2n$ = 100
Haplotype frequencies		
CGG	142(71%)	57(57%) [a]
Other haplotype	58(29%)	43(43%)
AGG	40(20%)	29(29%) [b]
Other haplotype	160(80%)	71(71%)
CAG	13(6.5%)	7(7%) [c]
Other haplotype	187 (93.5%)	93(93%)
CGA	5(2.5%)	7(7%) [d]
Other haplotype	195(97.5%)	193(93%)

[a] P= 0.02, OR=0.54, 95%CI=0.32 to 0.92

[b] P= 0.1

[c] P= 0.9

[d] P= 0.11

Table 22 Genotype frequencies of haplotype for TNF- α promoter polymorphism at position (-863A/C, -308A/G, -238A/G) between chronic hepatitis B patients (with HCC and without HCC) and transient hepatitis B patients.

	chronic hepatitis B		transient hepatitis B
	with HCC (*n*=50)	without HCC (*n*=100)	*n* = 100
Genotype frequencies of haplotype			
AGG/AGG	3	4	2
CAG/AGG	2	4	5
CGA/AGG	3	3	1
CGACAG	-	1	1
CGA/CGA	1	-	1
CGG/AGG	19	25	23
CGG/CAG	5	8	14
CGG/CGA	2	1	4
CGG/CGG	15	54	49

χ^2 =6.87, p <0.55; compare between the overall distribution of haplotype frequencies in chronic hepatitis B patients with transient hepatitis B patients

χ^2 =11.28, p <0.186; compare between the overall distribution of haplotype frequencies in chronic hepatitis B patients with HCC and without HCC

Table 23 Genotype frequencies of haplotype for TNF- α promoter polymorphism at position (-863A/C, -308A/G, -238A/G) in chronic hepatitis B patients without HCC and with HCC.

Haplotype	patients without HCC *n* = 100	patients with HCC *n* = 50
CGG/CGG	54(54%)	15[a] (30%)
CGG/ -	34(34%)	26(52%)
-/-	12(12%)	9(24%)

[a] *P*= 0.009, OR=0.37, 95%CI=0.17 to 0.79

4.4 The analysis of synergistic effect between -863(C/A) and -238(G/A) of TNF- α promoter polymorphism in development of HCC

Since both -863(C/A) and -238(G/A) of TNF-α promoter gene was significantly increase in chronic hepatitis B patients with HCC compared with without HCC. We hypothesized that individuals carrying both specific genotype of TNF- α at position -863(C/A) and -238(G/A) were at higher risk of developing of HCC than those carrying only one genotype. Then, we analyzed by comparing 3 groups using chi-square test, and the odds ratios were calculated, as shown in table 24;

1. Individuals carrying both specific genotype of TNF- α gene at position -863 (AA or AC) and -238 (AA or AG) compared with individuals, who did not carry specific genotype of TNF- α gene at position -863 (CC) and -238 (GG).

2. Individuals carrying specific genotype of TNF- α gene at position -863 (AA or AC) but not carrying -238 (AA or AG) compared with individuals, who did not carry specific genotype of TNF- α gene at position -863 (CC) and -238 (GG).

3. 2. Individuals carrying specific genotype of TNF- α gene at position -238 (AA or AG) but not carrying -863 (AA or AG) compared with individuals, who did not carry specific genotype of TNF- α gene at position -863 (CC) and -238 (GG).

Table 24 The analysis of synergistic effect between -863(C/A) and -238(G/A) of TNF- α promoter polymorphism in development of HCC

Group	Chronic HBV patients		OR
	With HCC	Without HCC	
1	3	2	4.57[a]
	20	61	
2	24	35	2.09[b]
	20	61	
3	3	2	4.57[c]
	20	61	

[a] p= 011, [b] p=0.067, [c] p=0.11

According to table 24, synergistic effect between -863(C/A) and -238(G/A) of TNF- α promoter polymorphism in development of HCC was not observed in this study.

5. Pattern of TNF- α and HLA-DRB1 gene polymorphisms in various populations

This study will provide the basic knowledge of allele distribution for HLA-DRB1 gene and TNF- α gene polymorphisms in healthy controls Thai individuals, as compared with other populations from previous reports.

5.1 Pattern of HLA-DRB1 gene polymorphisms

The analysis of HLA-DR12 gene showed no significant differences in the distribution between study population in Thai and Chinese populations. There were significant differences in allele frequencies between Thai population with Han-Chinese, German, Brazilian, Japanese and African American population (table 25).

The analysis of HLA-DR13 gene, Thai population showed no significant differences in the distribution with Chinese, Han-Chinese and Japanese populations. In contrast, there were significant differences in allele frequencies in Thai population compared with German and Brazilian and African American population (table 25).

5.2 Pattern of TNF- α gene polymorphisms

Allele and haplotype frequencies of TNF- α between study group and previous report groups that consist of Caucasians, African-American, and Asians population are reflected in table 27-29.

5.2.1 Pattern of TNF- α gene polymorphisms at position -863C/A

The analysis polymorphism at –863C/A in the promoter region of the TNF-α gene showed no significant differences in allele and genotype frequencies between Thai population with Swedish, Caucasians, Japanese, Chinese-Singporeans, Korean (table 26).

5.2.2 Pattern of TNF- α gene polymorphisms at position -308G/A

The analysis polymorphism at –308G/A in the promoter region of the TNF-α gene showed no significant differences in allele and genotype frequencies between Thai population with Chinese and Korean. In contrast, there were significant difference in Thai compared with Swedish, Caucasians, and Japanese (table 27).

5.2.3 Pattern of TNF- α gene polymorphisms at position -238G/A

The analysis polymorphism at –238G/A in the promoter region of the TNF-α gene showed no significant differences in allele and genotype frequencies between Thai population with Caucasians, American, Japanese, Korean (table 28).

Table 25 Comparison between HLA-DRB1 allele frequencies distribution in the different population

HLA-DRB1	control						
Author	This study	Jiang et al.	Wu et al.	Tillmann et al.	Liphaus et al.	Mignot et al.	Mignot et al
Year		2003	2004	2001	2002	2001	2001
Ethic group	Thai	Chinese	Han-chinese	German	Brazillian	Japanese	African-American
N	100	106	98	170	308	1,396	486
HLA-DR12	30%	32%	16.32%*	5.3%*	1.3%*	4.37%*	4.32%*
HLA-DR13**	4%	3.8%	8.16%	12.4% *	27.6%*	7.66%	16.46% *

- HLA-DR12: * The *p* value was lower than 0.05 when compared with Thais
- HLA-DR13: * The *p* value was lower than 0.05 when compared with Thais

** With in DR13 group, only 1% (n=1/100) has DR1301-2 allele

Table 26 Comparison between genotype and allele frequencies of TNF-α -863 gene polymorphism in the different population

TNF-α -863	control					
	Skoog et al	Wennberg et al	Higuchi T et al	Park et al	Lee SG et al	This study
Year	1999	2002	1998	2000	2004	
Ethic group	Swedish	Caucasian	Japanese	Chinese-Singaporeans	Korean	Thai
N	156	97	575	190	261	100
Genotype						
A/A	6(3.8%)	2(2%)	10(1.7%)	6(3.2%)	8(3.1%)	4(4%)
A/C	44(28.2%)	23(24%)	141(24.5%)	51(26.8%)	72(27.6%)	26(26%)
C/C	106(68%)	72(74%)	424(73.7%)	133(70%)	181(69.4%)	70(70%)
Allele						
A	56(18%)	27(14%)	161(14%)	63(16.6%)	88(16.9%)	34(17%)
C	256(82%)	167(86%)	989 (86%)	317(83.4%)	434(89.1%)	166(83%)

Not significant: compare between genotype and allele frequencies in Thai with Swedish, Caucasian, Japanese, Chinese-Singaporeans and Korean

Table 27 Comparison between genotype and allele frequencies of TNF-α -308 gene polymorphism in the different population

TNF-α-308	control					
Author	Skoog et al.	Allen et al.	Higuchi T et al	Xu et al	Lee SG et al	This study
Year	1999	2001	1998	2005	2004	
Ethic group	Swedish*	Caucasian **	Japanese***	Chinese	Korean	Thai
N	156	250	575	90	261	100
Genotype						
A/A	7(4.5%)	5(2%)	1(0.2%)	0(0%)	1(0.4%)	1(1%)
A/G	50(32%)	75(30%)	18(3.1%)	10(11.1%)	42(16.1%)	19(19%)
G/G	99(63.5%)	170(68%)	556(96.7%)	80 (88.9 %)	218(83.5%)	80(80%)
Allele						
A	64(20.5%)	85(17%)	4(2.1%)	10(5.6%)	44(8.4%)	21(10.5%)
G	248(79.5%)	415(83%)	188(97.9%)	170(94.4%)	478(91.6%)	179(89.5%)

* The genotype distribution is significantly different when compared with Thai (χ^2 = 8.61, *p*=0.01)

*The allele distribution is significantly different when compared with Thai (χ^2 = 8.12, *p*=0.004)

** The allele distribution is significantly different when compared with Thai (χ^2 = 4.20, *p*=0.040)

*** The genotype distribution is significantly different when compared with Thai (χ^2 = 43.62, *p*=0.00)

***The allele distribution is significantly different when compared with Thai (χ^2 =10.26, *p*=0.001)

Table 28 Comparison between genotype and allele frequencies of TNF-α -238 gene polymorphism in the different population

TNF-α -238	control					
Author	Hohler et al	Allen et al.	Jazrawi et al.	Lee SG et al.	Higuchi T et al.	This study
Year	1998	2001	2003	2004	1998	
Ethic group	Caucasian	Caucasians	American	Korean	Japanese	Thai
N	99	250	168	261	575	100
Genotype						
A/A	0(0%)	1(0.4%)	2(1.2%)	0(0%)	0(0%)	0(0%)
A/G	7(7%)	29(12%)	13(7.7%)	25(9.6%)	23(4%)	6(6%)
G/G	92(93%)	220(88%)	153(91.1 %)	236(90.4%)	552(96%)	
94(94%)						
Allele						
A	7(3.5%)	30(6%)	17(5.1%)	25(4.8%)	23(2%)	6(3%)
G	191(96.5%)	469(94%)	319(94.9%)	497 (95.2%)	1,127(98%)	194(97%)

Not significant: compare between genotype and allele frequencies in Thai with Caucasian, American, Korean and Japanese

CHAPTER VI

DISCUSSION

Chronic infection by hepatitis B virus results from an inability to clear the virus, which is associated with liver disease and liver cancer. The etiology of chronic HBV infection is remain unclear. However, several evidence suggested that host factors, especially genes related to immunity, particularly HLA gene and TNF-α gene might play an important role in the pathogenesis and the clinical outcome of HBV infection. Individuals with different polymorphisms of these genes may differ in susceptibility or resistance to disease. This study was designed to analyze the association between polymorphisms of HLA gene and TNF-α gene with susceptibility to chronic HBV infection in Thai population.

HLA gene is an important role in immune pathogenesis of HBV infection. Interestingly, the clearance of HBV infection was associated with HLA-DR13 in several ethnic groups. For example, HLA-DR13 was associated with protection against persistent HBV infection in Gambian (Thursz, Kwiatkowski et al., 1995), in Caucasians (Hohler, Gerken et al., 1997) and in Asian (Ahn, Han et al., 2000). However, the frequency of HLA-DR13 phenotype was quite low (3%) in Thai population, where as HLA-DR12 phenotype was high frequency (16.9%) in Thai population (Wongsurawat., 2003). In addition, some study reported that HLA-DR12 was associated with protection against chronic hepatitis B in Chinese (Meng, Chen et al., 2003) which related with Thai. Thus, we analyze the association of both HLA-DR13 and HLA-DR12 and susceptibility to chronic HBV infection in Thai population in this study.

In the present study, we found that the distribution of HLA-DRB1*1301-2 in patients who transient from HBV infection was statistically higher than chronic hepatitis B patients (p=0.0004, OR=0.04, 95%CI=0.00-0.26). There was no significant difference in the distribution of HLA-DR12 between chronic hepatitis B patients and transient hepatitis B patients. The result of the present study show strong association between HLA-DR13 and clearance of HBV which support other studies in several ethnic groups. This similarity in

various independent studied help indicate the important role of HLA-DR13 in chronic HBV infection. It has been suggested that the beneficial effect of HLA-DR13 phenotype on the outcome of HBV infection may be due to the induction of a vigorous HBc-specific $CD4^+$ T cell response, which might be either a more proficient antigen presentation by HLA-DR13 molecules themselves or due to a linked polymorphism in a neighboring immunoregulartory gene (Diepolder, Jung et al., 1998). The identification of the specific peptide epitopes derive from the virus presented by these HLA molecules may provide suitable vaccine candidates both for prophylactic and therapeutic use. Cao et al. reported that HBc-specific $CD4^+$T cell clone and T cell lines derived from subjects carry DR13 who spontaneously recovered from acute HBV infection show a dominant recognition of HBcAg peptide spanning aa 1-20 (P1), 11-30 (P2), 41-60 (P5), 111-131 (P12) and 141-160 (P15). Most T cell generated from these subjects recognized a single epitope within HBcAg at aa 147-156 (^{147}TVVRRRGRSP ^{156}) (Cao, Desombere et al., 2002). Diepolder showed that patients with acute hepatitis B who carrying HLA-DR13 mount a more vigorous HBc- specific $CD4^+$ T cell than patients without HLA-DR13. However, peptide epitopes aa 50-69, aa 61-85, and aa 81-105 were recognized most frequently by both group (Diepolder, Jung et al., 1998). However, additional study is needed to validate these finding and to further explore the role of HLA-DR13 phenotype in antigen presentation of HBV core epitopes to HBc-specific $CD4^+$ T cell responses in patients with acute, self-limited HBV infection.

In addition, TNF-α is an important cytokine involved in noncytotoxic antiviral mechanisms. It is located on the HLA class III region in the short arm of chromosome 6. Considerable evidence suggests that TNF-α gene polymorphisms associated with chronic hepatitis B development (table 2). The present study demonstrated that the -863A allele was found to be increased in chronic HBV patients compared to transient HBV patients. No significant association in TNF-α gene polymorphism at position -308 and -238 were found between the two groups. Besides we also analyzed haplotype of TNF-α gene polymorphisms, haplotype analysis in this study did not reveal any significant association with outcomes of chronic HBV infection when compared chronic HBV patients with transient HBV patients.

The lack of strong association between susceptibility to chronic HBV infection and TNF-α gene markers in the present study compare to other previous studies may be due to the limitation of sample recruitment. The major limitation of this study is the fact that we did not know the exact onset of infection of the patients in both groups. Since the differences in the mode of viral transmission may influence the clinical outcomes of patients with chronic HBV infection. However, despite this limitation, we found a weak association supporting the role of TNF-α (-863A) gene as risk factor for chronic HBV infection.

Moreover, TNF-α is also an important role in the progression of chronic HBV infection. TNF-α plays a critical role in the pathogenesis of chronic HBV infection and associated with the development of HCC (Ho, Wang et al., 2004). The existing evidence implicates the role of TNF-α inflammatory pathway that increased tumorigenesis (Szlosarek and Balkwill., 2003). TNF-α has been found in high concentration in patients with cancer (Abrahamsson, Carlsson et al., 1993; Partanen, Koskinen et al., 1995). Thus, the association analysis between TNF-α polymorphisms and chronic HBV infection risk was performed with the stratified chronic HBV patients according to progression to HCC and non-HCC. Interestingly, the -863A and -238A alleles were found to be significantly increased in chronic HBV patients with HCC compared to chronic HBV patients without HCC in this study. Haplotype analysis revealed that the homozygosity of the significant most common haplotype (CGG/CGG) was highly significant as a protective marker for HCC supporting positive association of -863A and -238A genotype.

The functional analysis of TNF-α polymorphism at position-863(C/A) has revealed the effects of TNF-α gene polymorphism on transcriptional regulation. Udalova et al demonstrated a clear effect of this nucleotide change on the relative binding affinities of different forms of the NF-κB complex. It was shown that the p50-p50 homodimeric form of this complex had a significantly decreased affinity to its DNA binding site for -863A. As the p50-p50 homodimer acts as a transcriptional repressor on binding to its regulatory site in the promoter region of the TNF gene, decreased binding is thought to result in an inadequate down-regulation of TNF gene expression, and thus increased TNF-α production (Udalova,

Richardson et al., 2000). However, the functional studies of TNF-α polymorphism at position -238(G/A) and -308(G/A) remain unclear. Recent evidence suggests that the TNF-α, which is a key player in inflammation can also activate signaling pathways, in both cancer cells and tumor-asscociated inflammatory cells, that promote malignancy (Balkwill and Coussens., 2004; Pikarsky, Porat et al., 2004).

Although the above explanation supports a pathophysiological mechanism for the association of this TNF-α variant with chronic hepatitis B infection, it is also possible that this association is not due to the TNF-α, but to another gene in linkage disequilibrium in a neighboring immunoregulartory gene. Especially, haplotype analysis showed a strong protective haplotype. It has been suggested that self-elimination of HBV infection may be due to the influence from another protective allele.

In conclusion, our study showed that HLA-DRB1*1301-2 alleles is a host factor that might be a protective allele in chronic hepatitis B infection. Furthermore, the –863A allele and -238A allele of TNF-α gene were identified as a genetic marker for hepatocellular carcinoma development in patient with chronic HBV infection. The hypothesis regarding TNF-α genetic polymorphism and hepatocarcinogenesis are based on the assumption that carriers of these genotypes are associated with increased levels of TNF-α in the liver in response to HBV infection and induce hapatocyte damage that may finally lead to HCC development. Additional study is needed to validate these finding and to further explore the genetic pathogenesis of HBV infection.

CHAPTER VII

CONCLUSION

In this study, we investigated whether HLA-DRB1 gene and TNF-α gene polymorphisms are associated with susceptibility and/or progression of chronic HBV infection. The result demonstrated that HLA-DRB1*1301-2 allele is related with resistance to chronic hepatitis B infection that may be a protective marker in chronic hepatitis B infection. Moreover, HLA-DRB1*1301-2 showed a strong association with the clearance of HBV which similar to another study in several ethic groups. This effect could be due to more proficient antigen presentation by the HLA-DR13 molecules themselves or a link polymorphisms in neighboring immunoregulatory gene. Moreover, we also found an association between TNF-α -863A allele (A/C or A/A genotype) and -238A allele and the development of HCC in chronic hepatitis B patients. These finding suggested that TNF-α may play an important role in the carcinogenesis of HCC and served as a genetic marker for HCC development in chronic hepatitis B infection. Carriers of these genotypes are proposed to be associated with increased levels of TNF-α in the liver in response to HBV infection and induced hapatocyte damage that may lead to HCC development. Furthermore, haplotype analysis revealed that the homozygosity of the significantly most common haplotype (CGG/CGG) was a protective marker for HCC in agreement with the positive association of -863A and -238A genotype.

These results indicated that polymorphisms of HLA-DRB1 gene and TNF-α might be one of the host factors, which influence the immune response to HBV and may be associated with the outcome of HBV infection in Thai population.

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APPENDICES

APPENDIX A

Reagent for agarose gel electrophoresis

1. 50x Tris-acetate buffer (TAE)

Tris base	*424.0*	*g*
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	100	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

2. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the solution and store in the dark at 4°C.

3. 1.5% Agarose gel

Agarose	0.3	g
1x TAE	20	ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

4. 5x Loging buffer 100 ml

Tris HCL	*0.6*	*g*
EDTA	1.68	g
SDS	0.5	g
Bromphenol Blue	0.1	g
Sucrose	40	g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5 microtube and store at 4°C.

APPENDIX B

Reagent for DNA extraction

1. Red Cell Lysis Buffer (RCLB)

NH_4Cl	1.875	g
Tris-HCl	0.25	g

Dissolve NH_4Cl and Tris-HCL in 500 ml of distilled water. Adjust pH to 7.2. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated. Shelf life is approximately 6 months.

2. Nuclei Lysis Buffer (NLB)

1 M Tris (pH 8.0)	10	ml
5 M NaCl	0.5	ml
0.5 M EDTA (pH 8)	0.4	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.

3. 1 M Tris

Tris base	12.11	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

4. 5 M NaCl

NaCl	29.22	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

5. EDTA

EDTA	37.22	g
Distilled water	200	ml

Adjust volume to 200 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated.

6. 5.3 M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

7. Proteinase K 10 mg/ml

Proteinase K	100	mg
Distilled water	10	ml

Mix the solution and store at -20°C.

8. 10% SDS

SDS	10	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

APPENDIX C

Haplotype analysis by PHASE

PHASE program is produced by the Mathematical Genetics Group, University of Oxford, Oxford, UK. The software is available online at http://www.stats.ox.ac.uk/ mathgen/ (Stephens and Donnelly 2003).

Input file format

The input file is supplied by the user to specify how many individuals there are to be analysed, how many loci/sites each individual has been typed at, what sort of loci/ sites these are (SNP or microsatellite), and the genotypes for each individual. The default format input file, as illustrated in the accompanying file test.inp. The default structure for the input file can be represented as follows:

NumberOfIndividuals
NumberOfLoci
P Position(1) Position(2) Position(NumberOfLoci)
LocusType(1) LocusType(2) ... LocusType(NumberOfLoci)
ID(1)
Genotype(1)
ID(2)
Genotype(2)
:
:
:
:
ID(NumberOfIndividuals)]
Genotype(NumberOfIndividuals)

Where the quantities above are as follows:

- Number of individuals An integer specifying the numbers of individuals who have been genotyped.

- Number of Loci An integer specifying the number of loci or sites at which each individual has been typed.

- P The character 'P' (upper case, without quotation marks).

- Position(i) A number indicating the position of locus i, relative to some arbitray reference point (typically in units of base pairs, but any units can be used: if you use a unit other than base pairs, see the documentation on the -R option). The loci must be in their physical order along the chromosome (ie these Positions must be increasing).

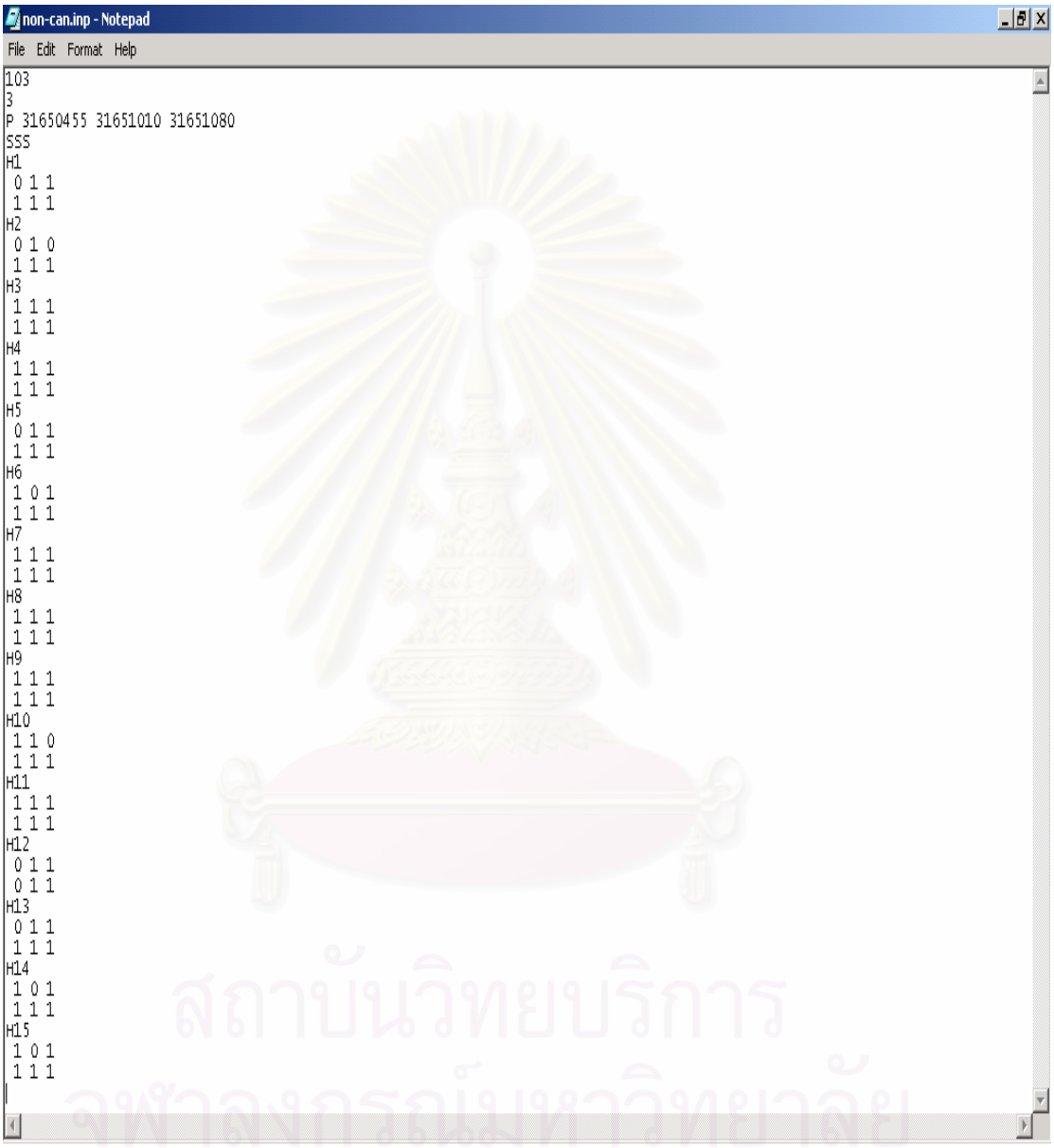
- LocusType(i) A letter indicating the type of locus i. The options are (a) S for a biallelic (SNP) locus, or biallelic site in sequence data. (b) M for microsatellite, or other multi-allelic locus (eg triallelic SNP, or HLA allele). The default assumption is that this denotes a microsatellite locus with stepwise mutation mechanism.

- ID(i) A string, giving a label for individual i.

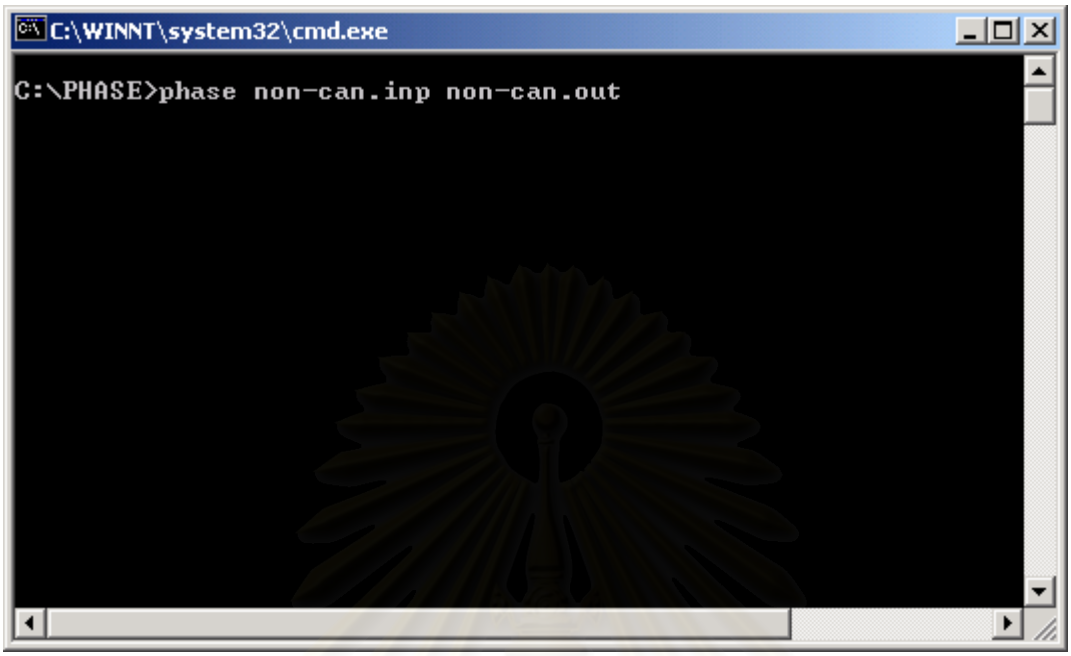
- Genotype(i) The genotypes for the ith individual. This is given on two consecutive rows. At each locus, one allele is entered on the first row, and one on the second row. It does not matter which allele is entered on each row. For biallelic loci, any two characters (e.g. A/C, G/T, 0/1) can be used to represent the two alleles, and they do not need to be separated by a space. Missing alleles at SNP loci should be entered as ?. For multiallelic loci a positive integer must be used for each allele (representing the number of repeats at microsatellite loci), and data for each locus should be separated by aspace. Missing alleles at multiallelic loci should be represented by -1.

This study, consider the example input file, non-can.inp, which is as follows:

The example of input file (non-can.inp) was shown below.



Running of PHASE program was shown below.



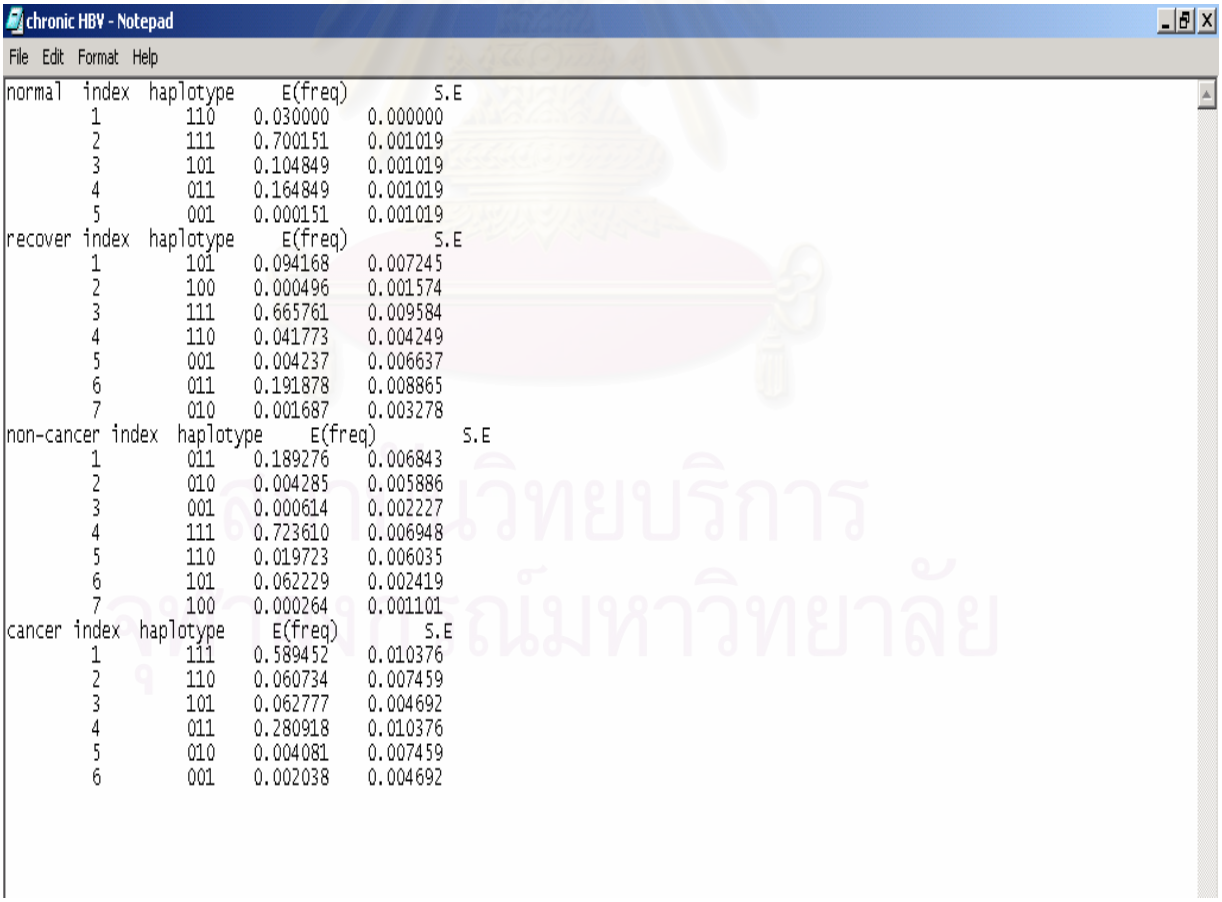
Output file

When run, the program initially outputs the data it has read from the input file. The program produces a number of output files. The first, which has the user-specified name, and a similar format to previous versions of PHASE, contains a summary of individual haplotype estimates for each individual.

Type of output file, which were used in this study, consists of two types.

1. Output frequencies: defined as how many haplotype in this study group and used the number (frequencies) to estimate how many the people, who have these haplotypes.

The example of output file (output frequencies) was shown below.



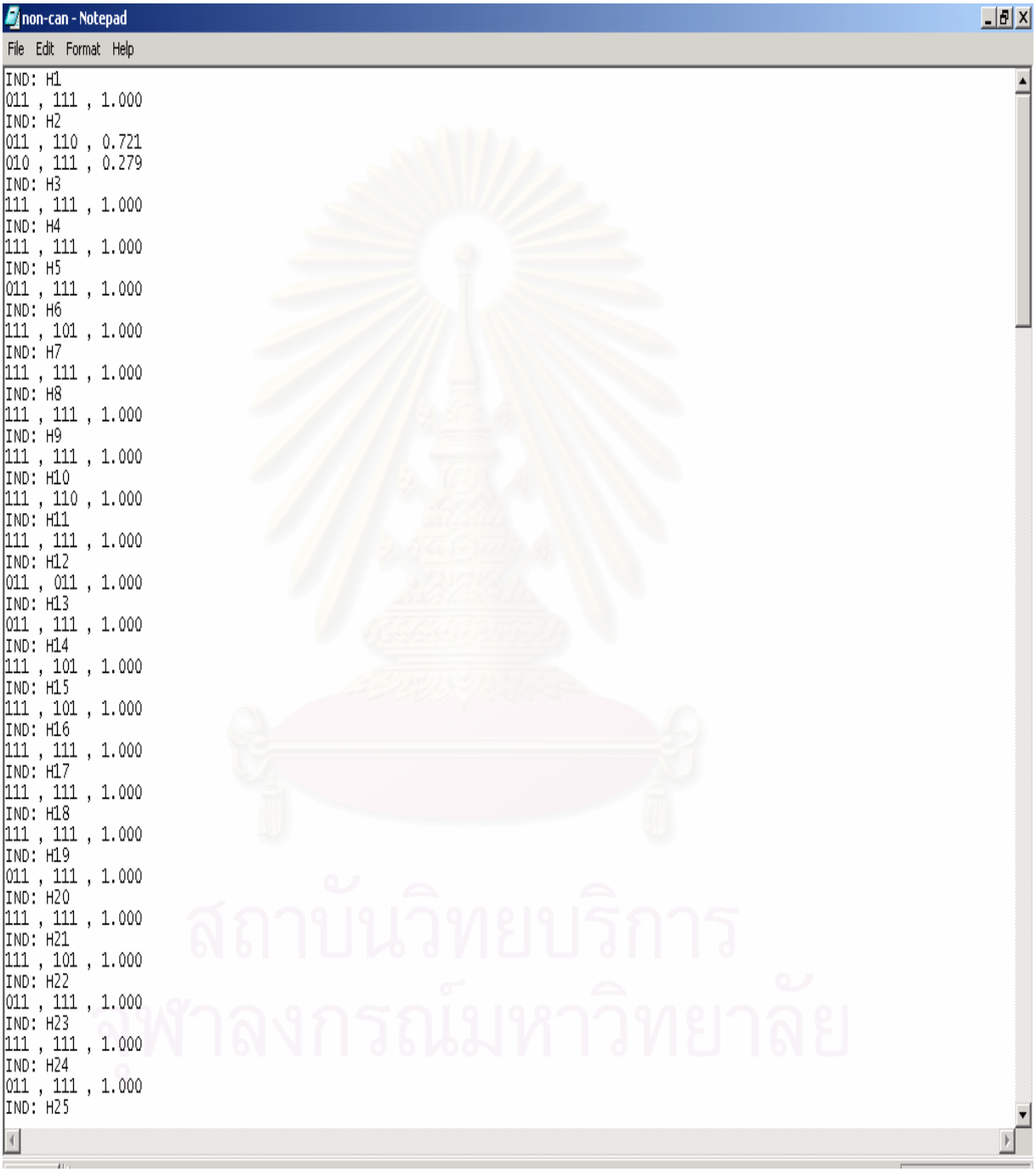
chronic HBV - Notepad

File Edit Format Help

```
normal  index  haplotype     E(freq)         S.E
         1         110    0.030000    0.000000
         2         111    0.700151    0.001019
         3         101    0.104849    0.001019
         4         011    0.164849    0.001019
         5         001    0.000151    0.001019
recover index  haplotype     E(freq)         S.E
         1         101    0.094168    0.007245
         2         100    0.000496    0.001574
         3         111    0.665761    0.009584
         4         110    0.041773    0.004249
         5         001    0.004237    0.006637
         6         011    0.191878    0.008865
         7         010    0.001687    0.003278
non-cancer index  haplotype     E(freq)         S.E
         1         011    0.189276    0.006843
         2         010    0.004285    0.005886
         3         001    0.000614    0.002227
         4         111    0.723610    0.006948
         5         110    0.019723    0.006035
         6         101    0.062229    0.002419
         7         100    0.000264    0.001101
cancer index  haplotype     E(freq)         S.E
         1         111    0.589452    0.010376
         2         110    0.060734    0.007459
         3         101    0.062777    0.004692
         4         011    0.280918    0.010376
         5         010    0.004081    0.007459
         6         001    0.002038    0.004692
```

2. Output pairs: defined as the individuals haplotype

The example of output file (outputpairs) was shown below.



APPENDIX D

Linkage Disequilibrium Analysis

The LDPlotter Tool allows conversion of a Nickerson Lab prettybase format file into a plot showing pairwise LD of various type (r^2 , r, D and abs(D)). r^2 values are calculated using an iterative EM algorithm taken from " Estimation of LD in randomly mating populations", WG Hill, Heredity: 33(2), 229-239 (1974).

The software is available online at http://innateimmunity.net/IIPGA2/Bioinformatics/

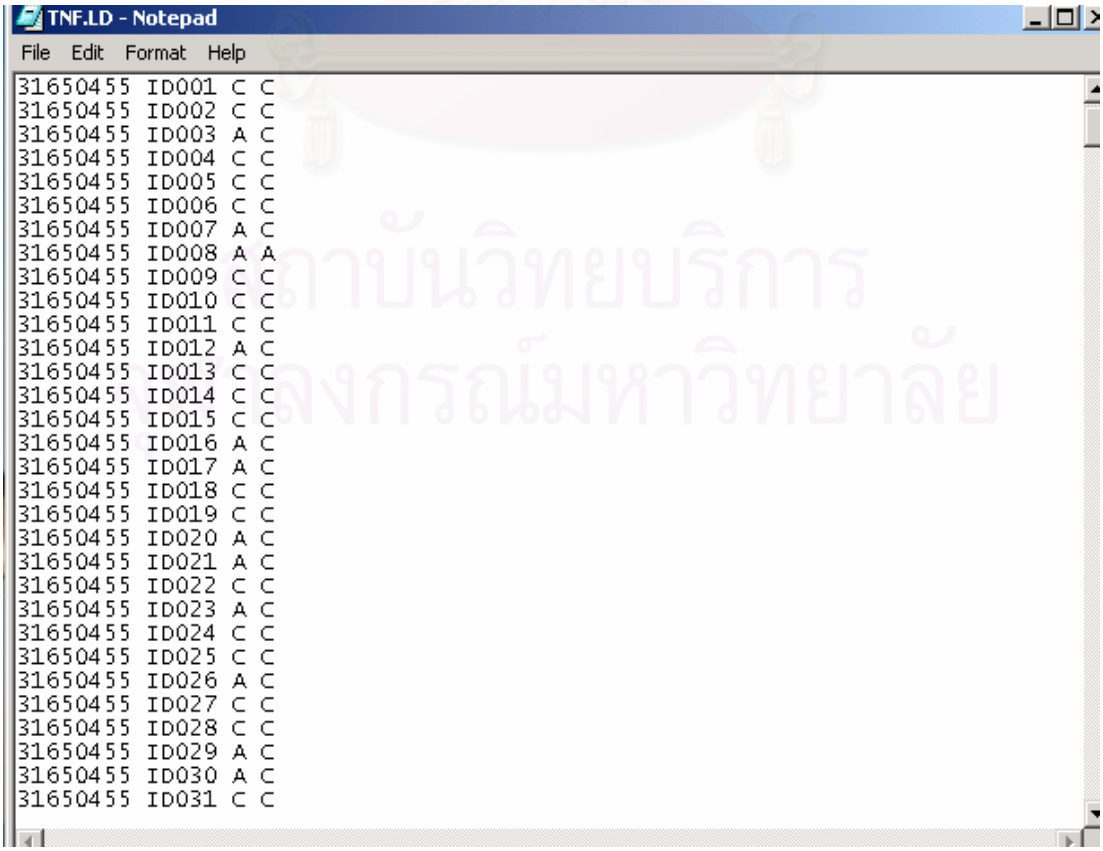
I Genename

II Prettybase

Input is a standard prettybase file. This study, consider the example input file, TNF.LD.txt, which is as follows:

The example of input file (TNF.LD.txt) was shown below.

TNF-α (-863C/A)



TNF-α (-308G/A)

TNF.LD - Notepad

File Edit Format Help

```
31651010 ID001 G G
31651010 ID002 G G
31651010 ID003 G G
31651010 ID004 G G
31651010 ID005 A G
31651010 ID006 G G
31651010 ID007 G G
31651010 ID008 G G
31651010 ID009 G G
31651010 ID010 G G
31651010 ID011 G G
31651010 ID012 G G
31651010 ID013 G G
31651010 ID014 G G
31651010 ID015 G G
31651010 ID016 G G
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31651010 ID019 G G
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31651010 ID022 G G
31651010 ID023 A G
31651010 ID024 A G
31651010 ID025 A G
31651010 ID026 A G
31651010 ID027 G G
31651010 ID028 G G
31651010 ID029 G G
31651010 ID030 G G
31651010 ID031 G G
```

TNF-α (-238G/A)

TNF.LD - Notepad

File Edit Format Help

```
31651080 ID001 A G
31651080 ID002 G G
31651080 ID003 G G
31651080 ID004 G G
31651080 ID005 A G
31651080 ID006 G G
31651080 ID007 G G
31651080 ID008 G G
31651080 ID009 G G
31651080 ID010 G G
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31651080 ID022 G G
31651080 ID023 G G
31651080 ID024 G G
31651080 ID025 G G
31651080 ID026 G G
31651080 ID027 G G
31651080 ID028 G G
31651080 ID029 G G
31651080 ID030 G G
31651080 ID031 A G
```
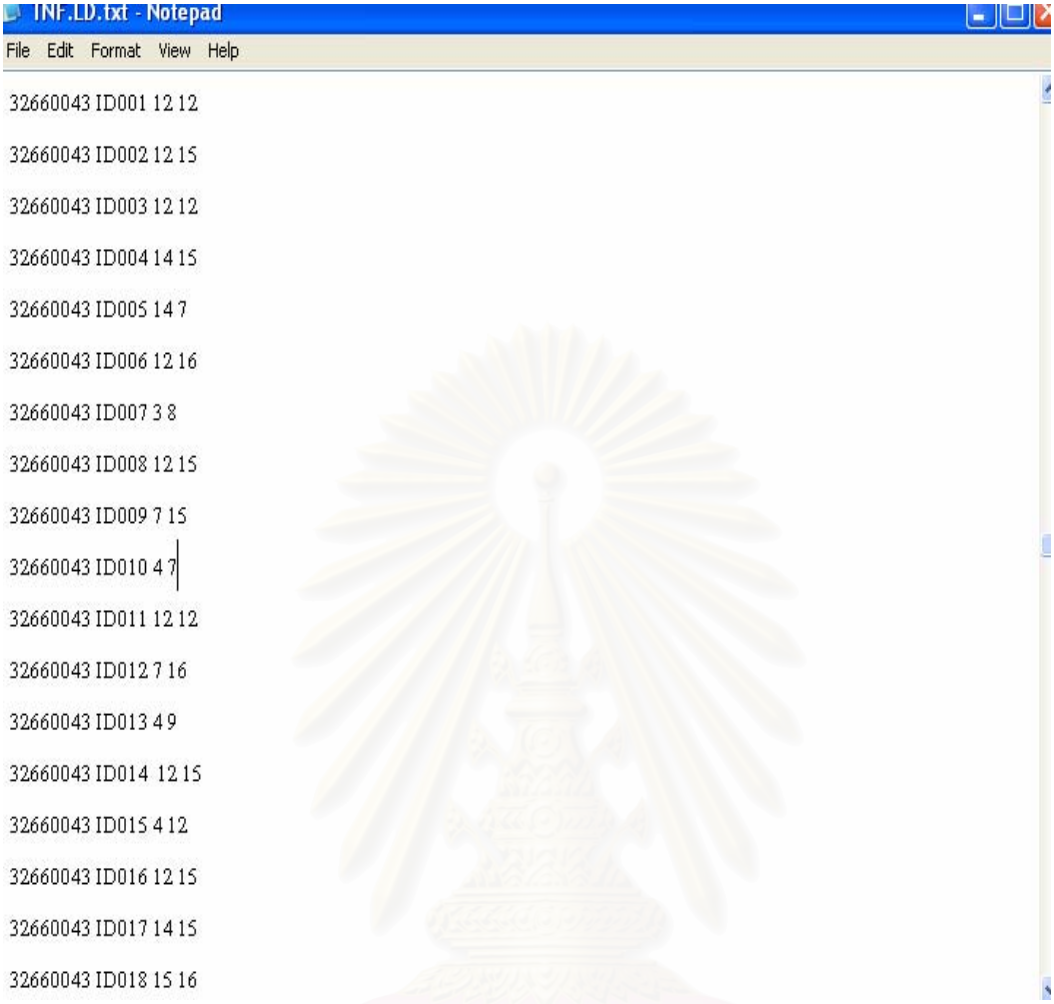

HLA-DRB1



III. LD Type

Indicates which measure of LD would like to plot

- ☐ $r^2 = D^2 / P_A * P_B * (1 - P_A) * (1 - P_B)$
- ☐ $r = \mathrm{sqrt}(r^2)$
- ☐ $D' = D / D_{max}$
- ☐ $\mathrm{Abs}(D) = \left| P_{AB} - (P_A * P_B) \right|$

IV. Configure Populations

This text area allows you to configure how the LD Plotter will split the sample in the field should have one line for each population represented in dataset. Each line should be a population identifier, followed by a colon, followed by a description of the population. Leaving this textarea empty will indicate that not wish to partition sample set, but instead, would like to consider all of the samples to be part of a single population.

V. Plot Title

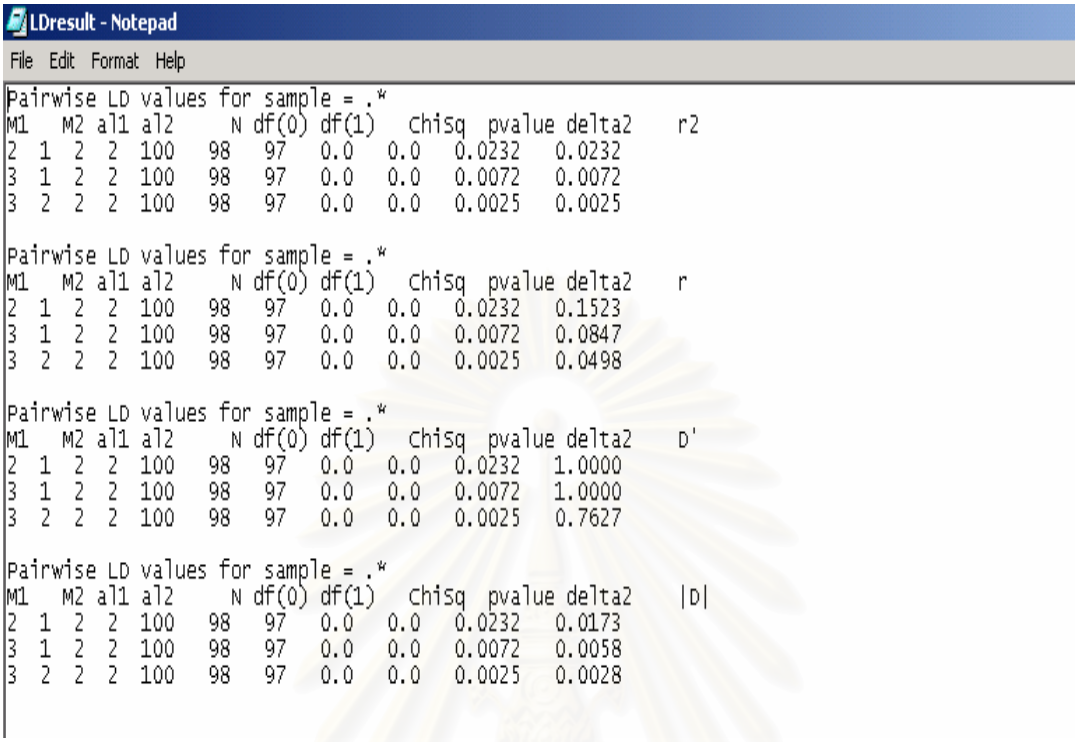
The plot title has two modes of operation, one for simple use, and the other for more advanced purposes for users who are familiar with Phython format strings, and would to specify exactly what the plot title should look like.

- **Simple**: With a simple plot title, we begin with an arbitrary string such as: Pairise LD and then add information about the plot to the heading:
    1. ☐ Append Gene name to plotTitle: The gene name entered above will to the title of the plot.
    2. ☐ Append Population to plotTitle: Each plot will be labeled with the population. If you are splitting your dataset by populations using the **Population Configuration** above, it is highly recommend that you keep this option checked, otherwise you will not know which plot belongs to which population.
    3. ☐ Append LD type to plotTitle: The type of LD measure calculated will be displayed in the plot title.
    4. ☐ Appened minfaf to plotTitle: The minraf used for the run will be displayed in the plot title.
- **Advanced**: The advanced title option allows you to input the plot title as a legal python format string. This format string is evaluated against a dictionary of variables/values (plotTitle% variables). At the current time, the dictionary is populated with the variables *miniraf, population, and ldtype*. These are the same variables which are available in the "Simple" option above, but you have the flexibility of arranging the items however you would link in the title. Errors in the title will be displayed in the title itself if at all possible.

VI. Miscellaneous

- **Miniraf**: You can set an arbitrary threshold for minimum allele frequence. The default value of 0.0 will not exclude SNPs based on rare frequency. If you specify a higher value, SNPs with frequency for the rare allele below this threshold in any for the rare population will NOT appear in the plot.
- **Color Scheme**: Several color schemes can be used to indicate the extend of LD between two loci.
  - **Circle**: The extend of LD between two loci is draw by default as a colored square at the intersection point of the two loci.
  - **SNP Map**: The SNPs map is a representation of the gene running along the diagonal of the half matrix plot which show the relative position of each SNP locus in the gene. It is only useful if you are using SNP IDs which directly correlate the position of the SNP in the gene.
  - **Full-matrix**:
  - **Numerical**: This option allowed you to download a spreadsheet of LD *values* instead of a graphical plot. This is useful if you would like to process the infprmation using another program, or if you have another plotting program.
  - **Download**: The download option will indicate that instead of displaying the result in the browser window, you would like to be prompted for a location where the fill will be saved on your computer.

The output file: The output file (TNF.LD.txt) was shown below.



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```
Pairwise LD values for sample = .*
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    r2
2  1  2  2  100   98   97   0.0   0.0   0.0232   0.0232
3  1  2  2  100   98   97   0.0   0.0   0.0072   0.0072
3  2  2  2  100   98   97   0.0   0.0   0.0025   0.0025

Pairwise LD values for sample = .*
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    r
2  1  2  2  100   98   97   0.0   0.0   0.0232   0.1523
3  1  2  2  100   98   97   0.0   0.0   0.0072   0.0847
3  2  2  2  100   98   97   0.0   0.0   0.0025   0.0498

Pairwise LD values for sample = .*
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    D'
2  1  2  2  100   98   97   0.0   0.0   0.0232   1.0000
3  1  2  2  100   98   97   0.0   0.0   0.0072   1.0000
3  2  2  2  100   98   97   0.0   0.0   0.0025   0.7627

Pairwise LD values for sample = .*
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    |D|
2  1  2  2  100   98   97   0.0   0.0   0.0232   0.0173
3  1  2  2  100   98   97   0.0   0.0   0.0072   0.0058
3  2  2  2  100   98   97   0.0   0.0   0.0025   0.0028
```

Linkage disequilibrium coefficients ($|D'|$ and r^2) among TNF- α SNPs

		$\|D'\|$		
		-863	-308	-238
r^2	-863	-	1	1
	-308	0.0232	-	0.7627
	-238	0.0072	0.0025	-

In this study, linkage disequilibrium was not found among -863(C/A), -308(G/A) and -238(G/A) of TNF- α promoter polymorphism.

The output file (TNF-α (-863, -308, -238) and HLA-DRB1) was shown below.

Result 4 position. LD.txt - Notepad

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Pairwise LD values for sample = .*

M1 M2 al1 al2 N df(0) df(1) ChiSq pvalue delta2 r

2 1 2 2 100 98 97 0.0 0.0 0.0232 0.1523

3 1 2 2 100 98 97 0.0 0.0 0.0072 0.0847

3 2 2 2 100 98 97 0.0 0.0 0.0025 0.0498

4 1 2 2 100 98 97 0.0 0.0 0.0000 -1.0000

4 2 2 2 100 98 97 0.0 0.0 0.0000 -1.0000

4 3 2 2 100 98 97 0.0 0.0 0.0000 -1.0000

Pairwise LD values for sample = .*

M1 M2 al1 al2 N df(0) df(1) ChiSq pvalue delta2 r2

2 1 2 2 100 98 97 0.0 0.0 0.0232 0.0232

3 1 2 2 100 98 97 0.0 0.0 0.0072 0.0072

3 2 2 2 100 98 97 0.0 0.0 0.0025 0.0025

4 1 2 2 100 98 97 0.0 0.0 0.0000 0.0000

4 2 2 2 100 98 97 0.0 0.0 0.0000 0.0000

4 3 2 2 100 98 97 0.0 0.0 0.0000 0.0000

Pairwise LD values for sample = .*

M1 M2 al1 al2 N df(0) df(1) ChiSq pvalue delta2 D'

2 1 2 2 100 98 97 0.0 0.0 0.0232 1.0000

3 1 2 2 100 98 97 0.0 0.0 0.0072 1.0000

3 2 2 2 100 98 97 0.0 0.0 0.0025 0.7627

4 1 2 2 100 98 97 0.0 0.0 0.0000 0.0000

4 2 2 2 100 98 97 0.0 0.0 0.0000 0.0000

Pairwise LD values for sample = .*

M1 M2 al1 al2 N df(0) df(1) ChiSq pvalue delta2 |D|

2 1 2 2 100 98 97 0.0 0.0 0.0232 0.0173

3 1 2 2 100 98 97 0.0 0.0 0.0072 0.0058

3 2 2 2 100 98 97 0.0 0.0 0.0025 0.0028

4 1 2 2 100 98 97 0.0 0.0 0.0000 0.0000

4 2 2 2 100 98 97 0.0 0.0 0.0000 0.0000

4 3 2 2 100 98 97 0.0 0.0 0.0000 0.0000

In this study, linkage disequilibrium was not found between TNF- α promoter polymorphism (-863, -308, -238) and HLA-DRB1.

APPENDIX E

Exact Hardy-Weinberg Equilibrium Test

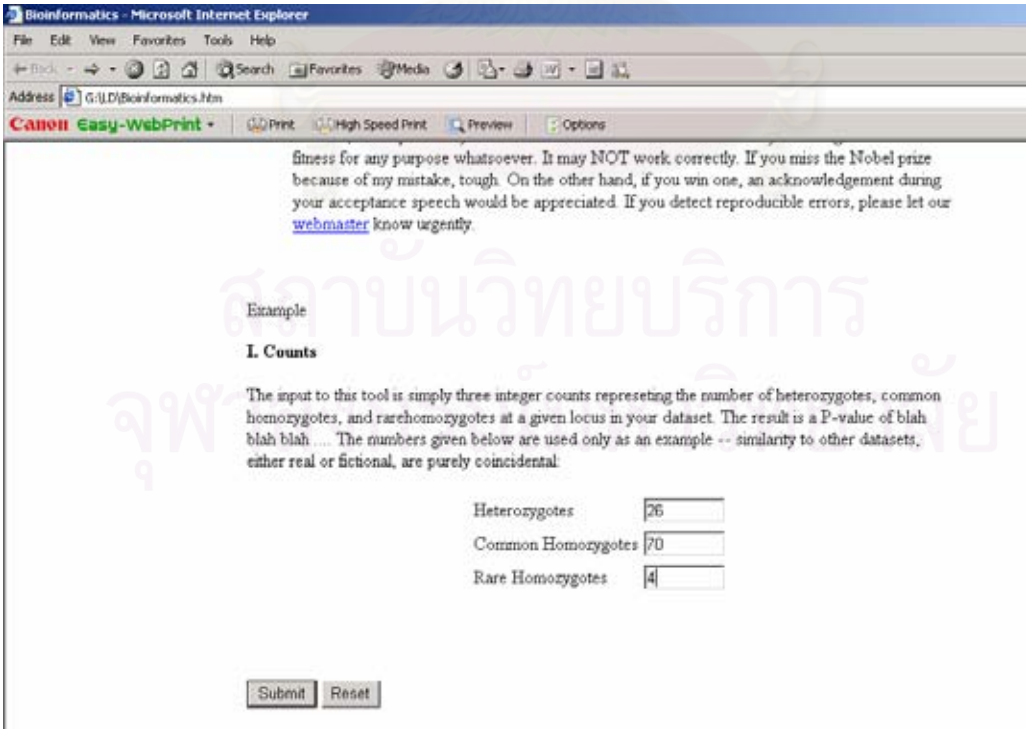
Exact Hardy-Weinberg Equilibrium Test for genotype counts, biallelic locus. This program will estimate the statistical significance of a test that the genotype counts are in Hardy-Weinberg equilibrium.

The software is available online at http://innateimmunity.net/IIPGA2/Bioinformatics/.

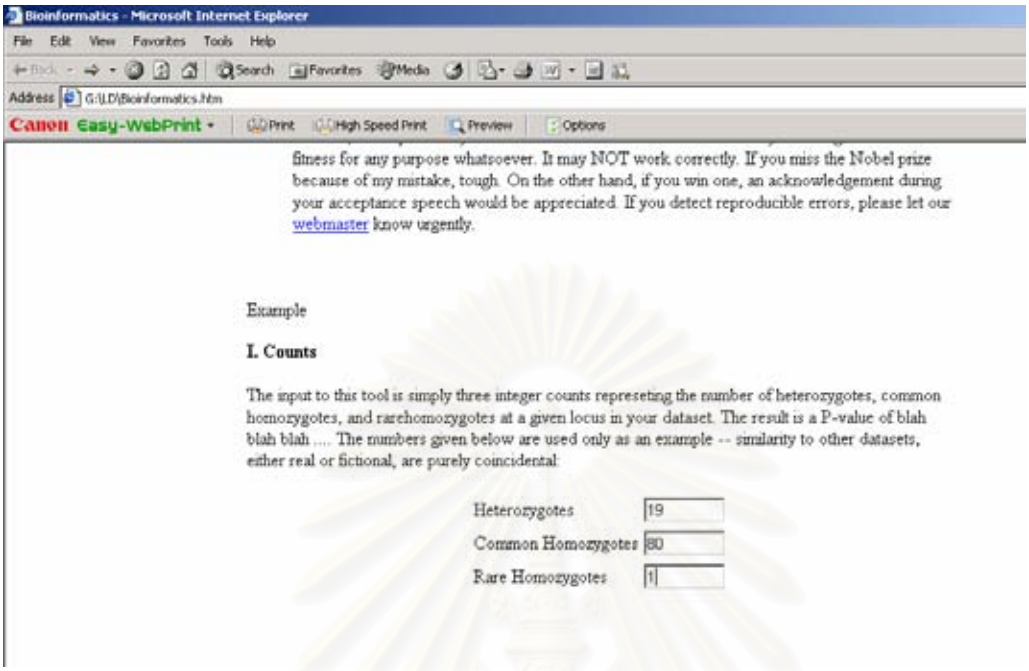
I Counts

The input to this tool is simply three interger counts representing the number of heterozygotes, common homozygotes, and rare homozygotes at a given locus in your data set.

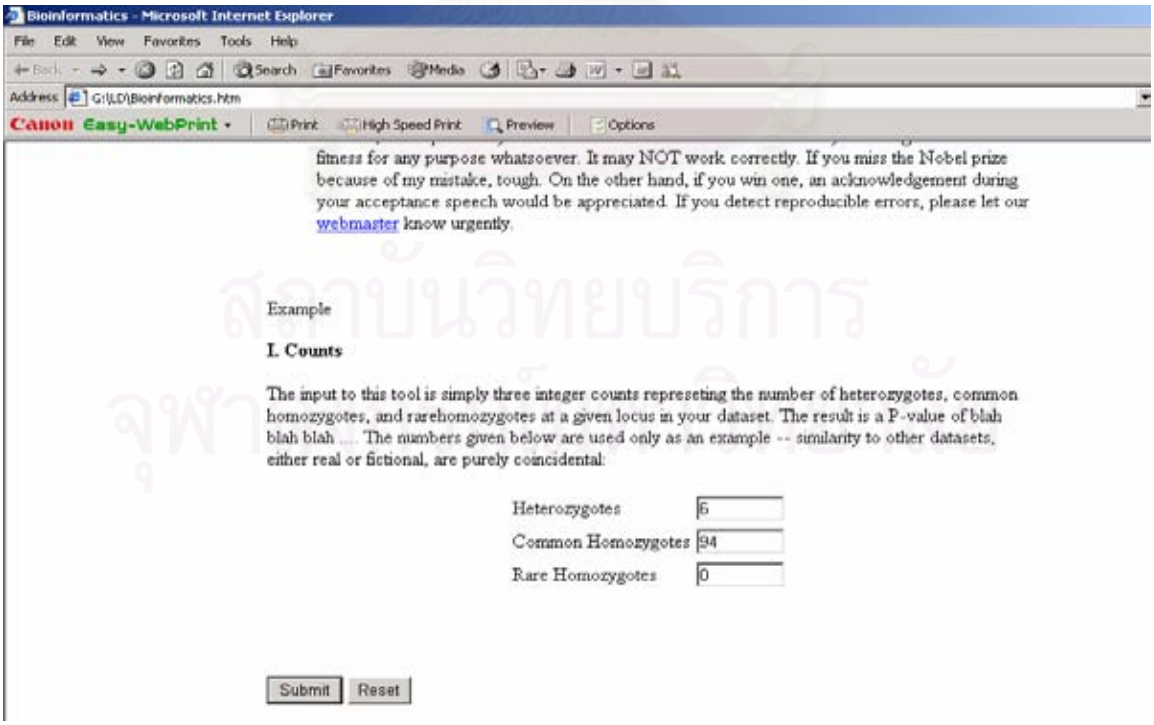
This study, the genotype counts of TNF-α (-863C/A) was shown below.



The genotype counts of TNF-α (-308G/A) was shown below.

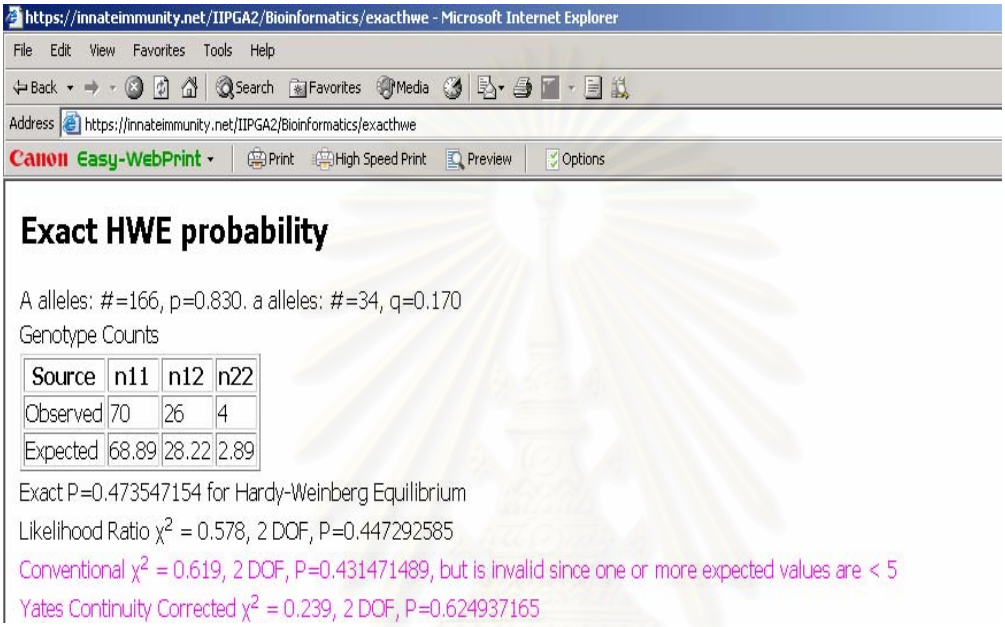


The genotype counts of TNF-α (-308G/A) was shown below.



When run, the program will estimate the statistical significance of a test that the genotype counts are in Hardy-Weinberg equilibrium. The output file of TNF-α (-863C/A) was shown below.

The output of the genotype counts of TNF-α (-863C/A) was shown below.



https://innateimmunity.net/IIPGA2/Bioinformatics/exacthwe - Microsoft Internet Explorer

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Exact HWE probability

A alleles: #=166, p=0.830. a alleles: #=34, q=0.170

Genotype Counts

Source	n11	n12	n22
Observed	70	26	4
Expected	68.89	28.22	2.89

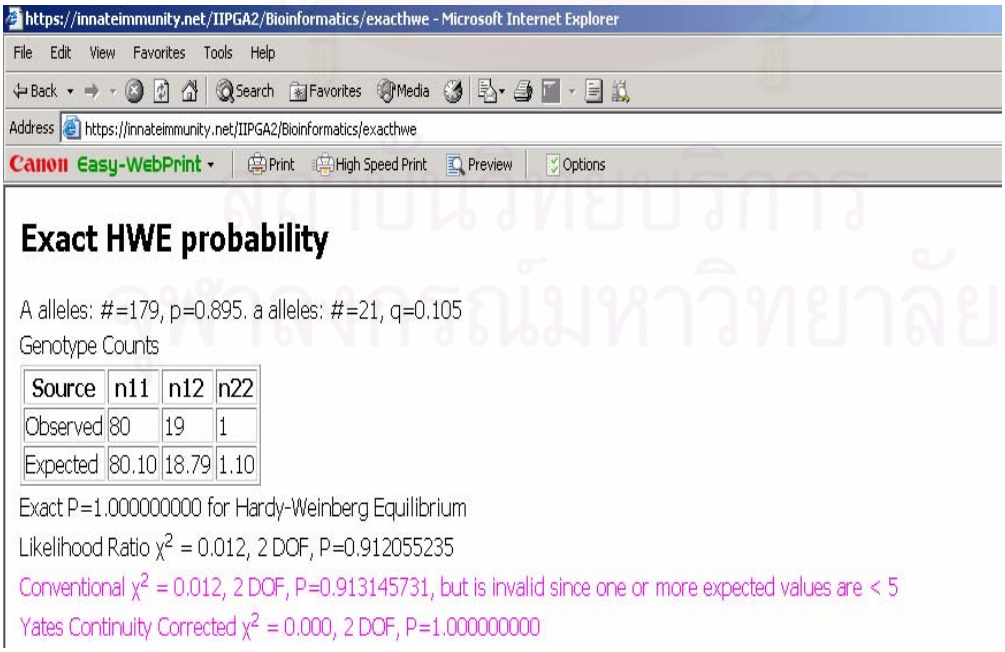
Exact P=0.473547154 for Hardy-Weinberg Equilibrium

Likelihood Ratio χ^2 = 0.578, 2 DOF, P=0.447292585

Conventional χ^2 = 0.619, 2 DOF, P=0.431471489, but is invalid since one or more expected values are < 5

Yates Continuity Corrected χ^2 = 0.239, 2 DOF, P=0.624937165

The output of the genotype counts of TNF-α (-308G/A) was shown below.



https://innateimmunity.net/IIPGA2/Bioinformatics/exacthwe - Microsoft Internet Explorer

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Exact HWE probability

A alleles: #=179, p=0.895. a alleles: #=21, q=0.105

Genotype Counts

Source	n11	n12	n22
Observed	80	19	1
Expected	80.10	18.79	1.10

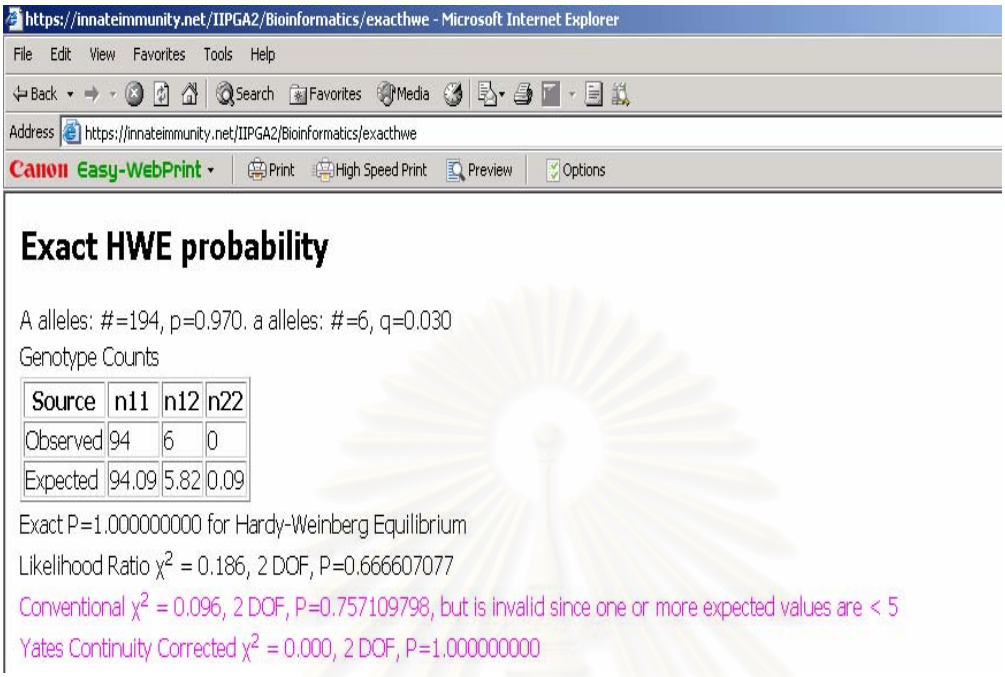
Exact P=1.000000000 for Hardy-Weinberg Equilibrium

Likelihood Ratio χ^2 = 0.012, 2 DOF, P=0.912055235

Conventional χ^2 = 0.012, 2 DOF, P=0.913145731, but is invalid since one or more expected values are < 5

Yates Continuity Corrected χ^2 = 0.000, 2 DOF, P=1.000000000

The output of the genotype counts of TNF-α (-238G/A) was shown below.



APPENDIX F

SSP UniTray™

The PEL-FREEZ SSP UniTray™ is a PCR-based method designed to provide low to medium resolution of HLA Class I A, B, and C loci and/or Class II DRB and /or DQB loci. The PEL-FREEZ SSP UniTray™ consist of various formulations of allele or group specific primer mixs that are used to amplify genomic DNA using a 96 well thermal tray. Setup includes mixing a reaction buffer with a genomic DNA sample and Taq DNA polymerase, dispensing the mixture to the UniTray™ , sealing and thermal cycling. After cycling is complete, the PCR products are loaded onto a 2%agarose gel for electrophoresis. After electrophoresis, the ethidium bromide stained gel is photographed and interpreted using a worksheet for specific amplification patterns. The UniTray™ method is based on sequence specific primer amplification methods (SSP). The primer sets amplify the alleles described by the international nomenclature committee of WHO.

The PEL-FREEZ SSP UniTray™

1. Kit Components:

1.1 96 well polycarbonate PCR trays containing 5 μl/well of optimized primer solution overlaid with paraffin oil

1.2 Optimized PCR buffer containing DNTPs

1.3 Plastic sealers for sealing PCR trays

1.4 Gel Loading Buffer

1.5 Gel Document Form

1.6 Worksheets

1.7 Primer Mix Specificity Table

2. Materials, reagents and equipment not supplied:

2.1 Taq DNA polymerase (for kits without Taq)

2.2 Pipettors and tips: 1-10 μl, 10-200 μl, and 100—1000 μl

2.3 8 channel pipettor

2.4 96 well thermal cycler with heated lid

2.5 PEL-FREEZE Heat Equalizing Block, Product Code#90000

2.6 TBE eletrophoresis buffer (at 0.5X concentration)

2.8 DNA Molecular Weight markers to cover of 50-2000 bp

2.9 Grade Agarose

2.10 Ethidium bromide (10mg/ml)

3. Sample

3.1 DNA Sample in TE buffer or sterile water. The $OD_{260/280}$ is between 1.7 and 1.9 on diluted sample measured by UV spectrophotometry.

SSP UniTray™- Set Up Instructions

Product Name	DRDQ2T
Number of Tests per Tray	2
Number of Primer mixes per test	40
Lane number location of each test	Test 1: Lane 1-40 Test 2: Lane 57-96
Step 1: Thaw buffer	Thaw buffer, Vortex (use 1 vial of buffer per test)
Step 2: Prepare Unitray	Place in tray holder and remove seal
Step 3: Add water to the PCR buffer	94 μl
Step 4: Add Taq polymerase and vortex	3.8 μl
Step 5: Prepare contamination control	Pipette 8 μl into contamination control well
Step 6: Add DNA (75-125 ng/μl) and vortex	31 μl
Step 7: Dispense into remaining wells	8 μl

4. Thermal cycler: SSP Amplification Profile: The following thermal cycling profile was optimized and validated using various thermal cycling equipment:

Step 1	1 minute at 96 ^{0}C
Step 2 5 cycles of	96^{0}C 25 seconds
	70^{0}C 50 seconds
	72^{0}C 45 seconds
Step 3 21 cycles of	96^{0}C 25 seconds
	65^{0}C 50 seconds
	72^{0}C 45 seconds
Step 4 4 cycles of	96^{0}C 25 seconds
	55^{0}C 60 seconds
	72^{0}C 120 seconds
Hold	4^{0}C specify time*

Total reaction volume in each well = 23 μl

After thermal cycling, remove tray and proceed to gel electrophoresis. If not performing electrophoresis immediately, store tray at 4^{0}C for up to two weeks.

6. Gel Electrophoresis

Use a high quality agarose, capable of resolving 50-200 base pair fragments of DNA. Prepare the gel in 0.5X TBE buffer, add 2 μl of 10 mg/ml ethidium bromide for each 100 ml agarose solution and mix well. Use 0.5X TBE buffer in gel chamber as a running buffer. Gel can be run at 140 volts about 10-12 minutes.

7. Interpretation: Affix the gel photo to the Gel Documentation Form. Examine gel photo Carefully and determine the positive lanes.

BIOGRAPHY

Miss Pittaya Kummee was born on September 19, 1974 in Krabi, Thailand. She graduated Bachelor of Science (Medical Technology), Faculty of Medical Technology, Chulalongkorn University in 1998, and attended to particulate in Medical Microbiology program, Graduate School, Chulalongkorn University for her master degree.