รายงานวิจัยฉบับสมบูรณ์

โครงการ: ปัจจัยทางพันธุกรรมทางภูมิคุ้มกันที่มีผลต่อการเกิดโรคและการดำเนินโรคของการติดเชื้อไวรัสตับอักเสบบีเรื้อรัง

โดย

รศ.พญ.ดร.ณัฏฐิยา หิรัญกาญจน์ ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์
จุฬาลงกรณ์มหาวิทยาลัย

ธันวาคม พ.ศ. 2549

สัญญาเลขที่ RSA43-8-0013

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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รหัสโครงการ: RSA43-8-0013, **ชื่อโครงการ :** ปัจจัยทางพันธุกรรมทางภูมิคุ้มกันที่มีผลต่อการเกิดโรคและการดำเนินโรคของการติดเชื้อไวรัสตับอักเสบบีเรื้อรัง

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จากหลักฐานการศึกษาในปัจจุบันพบว่าปัจจัยทางพันธุกรรมของผู้ป่วยโรคไวรัสตับอักเสบบีเรื้อรัง โดยเฉพาะอย่างยิ่งยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันมีบทบาทสำคัญในการเกิดโรคและการดำเนินของโรค จากการศึกษาของเราก่อนหน้านี้พบว่า ในกลุ่มผู้ที่เคยได้รับเชื้อไวรัสตับอักเสบบีและสามารถกำจัดเชื้อไวรัสได้เอง (จำนวน 100 คน) มีความถี่ของรูปแบบ HLA-DR13 สูงกว่าในกลุ่มผู้ป่วยโรคไวรัสตับอักเสบบีเรื้อรัง (จำนวน 150 คน) ถึงแม้ว่าในประชากรไทยจะมีความถี่ของยีนนี้อยู่ต่ำก็ตาม (ร้อยละ 3) จากข้อมูลดังกล่าวแสดงให้เห็นความสำคัญของรูปแบบ HLA-DR13 ในการต้านทานการเกิดโรคไวรัสตับอักเสบบีเรื้อรังในประชากรไทยซึ่งสอดคล้องกับการศึกษาในประชากรชาวผิวขาวและชาวแอฟริกัน ซึ่งแสดงให้เห็นบทบาทและความสำคัญของรูปแบบ HLA-DR13 นี้ต่อการกำจัดไวรัส ในการศึกษาความหลากหลายของไซโตไคน์ยีนที่เกี่ยวข้องกับกระบวนการอักเสบ (Pro-inflammatory genes) ได้แก่ TNF-alpha (-863, -308 และ -238), IL-1 beta (-511) และ IL-1RA (VNTR) พบว่า รูปแบบ -863A ของยีน TNF-alpha และ -511C ของยีน IL-1 beta ซึ่งมีความสัมพันธ์กับการสร้างไซโตไคน์ที่เพิ่มสูงขึ้นในตับ สามารถนำมาใช้เป็นเครื่องหมายทางพันธุกรรมของการเกิดมะเร็งตับ (Hepatocellular carcinoma) ในประชากรไทยที่เป็นโรคไวรัสตับอักเสบบีเรื้อรัง ปัจจุบันพบว่า T helper 1 cells มีบทบาทในการยับยั้งการเพิ่มจำนวนของไวรัส ผ่านกลไกที่ไม่ทำลายเซลล์ (non-cytolytic antiviral mechanism) โดยการสร้าง IFN-gamma และพบว่าไซโตไคน์ IL-12 และ IL-18 ที่สร้างจาก activated macrophages สามารถกระตุ้นการสร้าง IFN-gamma และ TNF alpha และช่วยเพิ่มความสามารถในการทำงานของ NK-cell และเพิ่มการแสดงออกของ FasL จากความสำคัญของไซโตไคน์ทั้ง 3 ชนิดดังกล่าว จึงสนใจศึกษาความสัมพันธ์ระหว่างความหลากหลายของยีนซึ่งมีผลต่อการแสดงออกของยีนกับความเสี่ยงในการเกิดโรคไวรัสตับอักเสบบีเรื้อรังในประชากรไทย จากการศึกษาไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของความหลากหลายของยีน IFN-gamma ที่ตำแหน่ง +874 ใน intron ที่ 1, ยีน IL-12 ที่ตำแหน่ง +1159 ใน 3' UTR และยีน IL-18 ที่ตำแหน่ง -137 ในบริเวณ promoter ระหว่างกลุ่มควบคุมและกลุ่มผู้ป่วย (จำนวน 140 คนต่อกลุ่ม) อย่างไรก็ตามพบว่าความถี่ของ genotypes AA ที่ตำแหน่ง -607 ใน promoter ของยีน IL-18 ในผู้ป่วยโรคไวรัสตับอักเสบบีเรื้อรังสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ การเปลี่ยนนิวคลีโอไทด์ที่ตำแหน่ง -607 จาก C เป็น A ซึ่งเปลี่ยนแปลง cyclic AMP-responsive element (CRE) consensus sequence ส่งผลให้การสร้าง IL-18 และ IFN-gamma ลดลง จากผลการศึกษาแสดงให้เห็นถึงความสำคัญของ IL-18 กับความความเสี่ยงในการเกิดโรคไวรัสตับอักเสบบี

คำสำคัญ โรคไวรัสตับอักเสบบีเรื้อรัง, มะเร็งตับ, HLA-DR, TNF-alpha, IL-1 beta, IL-1RN, IL-10, IL-12, IL-18, IFN-gamma, ความหลากหลายของยีน

Project Code: RSA43-8-0013 **Project Title**: Immunogenetic factors influencing susceptibility and disease progression of chronic hepatitis B infection

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Considerable evidence suggests that host genetic factor, especially genes related to immunity, might play an important role in the pathogenesis and clinical outcome of chronic hepatitis B virus (HBV) infection. We have previously reported that the frequency of HLA-DR13 in the 100 recovered patients was significantly higher than 150 chronic HBV patients. Although DR13 present with low frequency in Thai population (~3%), the protective role of DR13 can be clearly demonstrated in Thai population similar to Caucasian and African study supporting the important role of this HLA allele in clearing the virus. For the study of pro-inflammatory genes, three markers at position -863, -308, and -238 of TNF alpha gene and one marker each for IL-1 beta (-511) and IL-1RA (VNTR) were analyzed. We found that the TNF-alpha-863A allele and IL-1B-511C allele which is likely to be associated with high cytokine production in liver, is a genetic marker for HCC development in chronic hepatitis B patients in Thai population. There are growing evidences that T helper 1 cells play a predominant role in suppressing viral replication mainly by IFN-gamma through noncytolytic antiviral mechanism. IL-12 and IL-18 are novel cytokines that are mainly produced by activated macrophages and are able to induce IFN-gamma and TNF-alpha, as well as enhancing the cytotoxicity of NK cells and FasL expression. Therefore, polymorphisms within these 3 cytokine genes, previously reported to affect expression level were analyzed for the association with chronic HBV susceptibility in this study. There was no significant difference in the genotype distribution or allele frequency of +874 SNP in the first intron of IFN gamma gene, +1159 SNP in 3'UTR of IL-12 gene and -137 SNP in the promoter region of IL-18 gene between controls and patients. However, chronic HBV patients had significantly higher frequency of AA genotype at position -607 in the promoter region of IL-18 gene when compared with healthy individuals. In the promoter of IL-18 gene, a change from nucleotide C to A at -607 position disrupts the cyclic AMP-responsive element (CRE) consensus sequence and results in markedly lower transcription activity of IL-18 and potentially also to IFN-gamma. This result suggests the concrete involvement between IL-18 and susceptibility to HBV.

Keywords Chronic hepatitis B, Hepatocellular carcinoma, HLA-DR, TNF-alpha, IL-1 beta, IL-1RN, IL-10, IL-12, IL-18, IFN-gamma, polymorphism

INTRODUCTION

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.

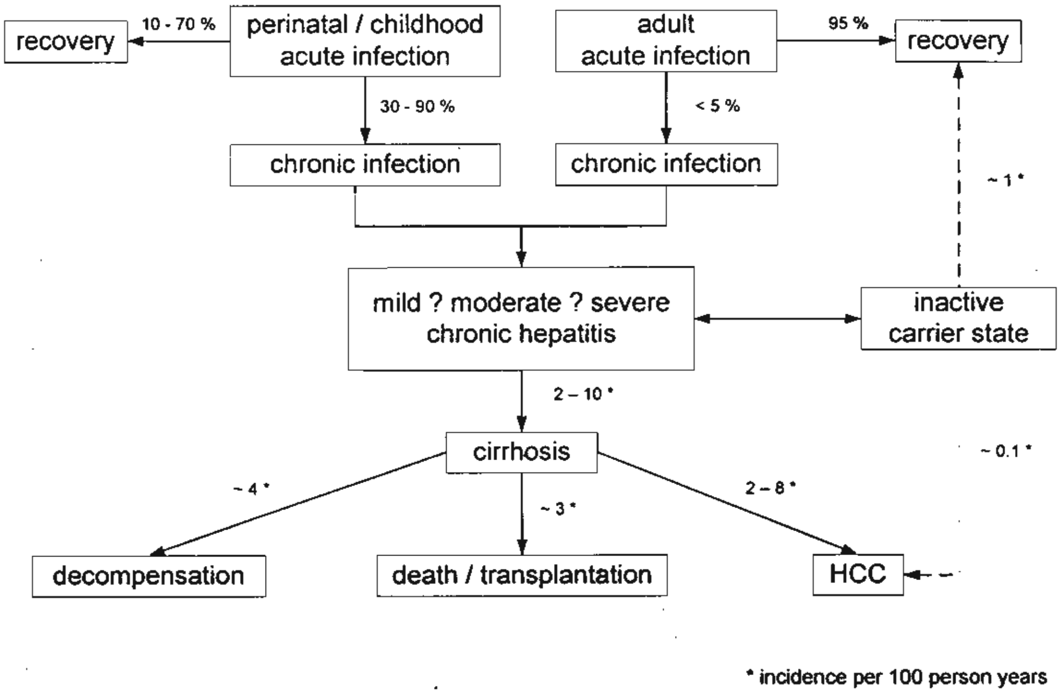

Figure 1. Outcomes of HBV infection

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 1998). 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).

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.

Tumor necrosis factor-α

TNF-α is a cytokine with a wide range of pro-inflammatory activities (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. 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-KB 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).

Interleukin-1

Interleukin-1 (IL-1) is a proinflammatory cytokine with multiple biological effects (Dinarello 1996). The IL-1 gene family (including IL-1A, IL-1B and IL-1RN) on chromosome 2q13-21 encoded three proteins, which comprise the agonists IL-1α, IL-1β, and their naturally occurring inhibitor, IL-1 receptor antagonist (IL-1RN) (Nicklin, Weith et al. 1994; Dinarello 1996). Recently, allele 2 of IL-1RN intron 2 has been reported as a resistant marker of HBV infection suggesting the role of IL-1 polymorphisms in the pathogenesis of developing chronic hepatitis B (Zhang, Li et al. 2004). This allele is associated with enhanced IL-1β production *in vitro*(Santtila, Savinainen et al. 1998) and *in vivo* (Hwang, Kodama et al. 2002). Therefore, Zhang et al. hypothesized that high production of IL-1β may help increase the production of other cytokines such as IL-2, IL-6 and TNF-α and trigger the complex immunological processes to eliminate the virus. Interestingly, besides its major role as proinflammatory cytokine, IL-1β has been implicated as important factor for tumor growth (Roshak, Jackson et al. 1996; Bamba, Ota et al. 1998; Rahman, Dhar et al. 2001). Several independent evidences have also suggested that genetic polymorphisms within IL-1β gene are associated with gastric cancer and HCC in hepatitis C virus (HCV) infection (Tanaka, Furuta et al. 2003; Wang, Kato et al. 2003; Lee, Ki et al. 2004).

Interleukin-18

IL-18, which is predominantly secreted by activated monocytes/macrophages, is a proinflammatory cytokine that plays an important role in both innate and adaptive immune responses against viruses and intracellular pathogens. IL-18 in the presence of IL-12 was identified as an IFN-γ-inducing factor that can stimulate Th1 cells (Okamura, Tsutsi et al. 1995). In addition, to its potent induction of IFN-γ, IL-18 can activate CD8$^+$ T cells and NK cells, which play central role in viral clearance (Dinarello 1999). CD8$^+$ T cells can induce apoptosis in HBV-infected cell, and secrete IFN-γ and TNF-α that lead to intercellular inactivation of HBV (Mahoney 1999). Several lines of evidence support an important role of IL-18 in HBV clearance. Kimura et al. found that IL-18 can inhibit HBV replication in the liver of transgenic mice probably mediated by IFN-γ and IFN-α/ß which are produced by hepatic NK cells and NKT cells (Kimura, Kakimi et al. 2002). The IFN-γ level in the patients who were stimulated by HBcAg alone was much lower than the levels in the patients who were stimulated by HBcAg, IL-12 and IL-18 protein at various concentrations (Sun, Chen et al. 2004; Szkaradkiewicz, Jopek et al. 2005). On the other hand, HBsAg has been reported to regulate IFN-g production by inhibiting IL-18 and IL-12 production which may be important for persistent HBV infection in chronic HBV carrier (Cheng, Imanishi et al. 2005).

IL-18 expression level is regulated by the IL-18 promoter gene. Two SNPs at position -607 (rs1946518) and -137 (rs187238) in the promoter region are found to be associated with various inflammatory diseases e.g, diabetes (Mojtahedi, Naeimi et al. 2006; Szeszko, Howson et al. 2006), sarcoidosis (Zhou, Yamaguchi et al. 2005), and rheumatoid arthritis (Gracie, Koyama et al. 2005). One report from Zhang et al (2005) suggested the association of genetic marker in IL-18 gene and chronic HBV susceptibility in Han Chinese population (Zhang, Wu et al. 2005).

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 150 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

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

Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) analysis of HLA-DR12 and HLA-DR13 were performed as previously described (Olerup and Zetterquist 1992). Internal control primers were used to check for successful PCR amplification. These primers amplify a human growth hormone sequence (Pravica, Perrey et al. 2000). Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/ GeneAmp PCR system 9600. The DRB1 full typing of the 8 patients that carry DR13 was performed using SSP UniTray (Pel-Freeze, Wisconsin, USA).

Analysis of TNF-alpha polymorphism

Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) was used to analyze TNF-alpha gene 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). Negative controls without DNA template were included in each experiment.

Genotyping for the SNPs of IL-1B and IL-1RN genes

Molecular genetic analysis was performed on genomic DNA obtained from peripheral blood leukocytes by using standard salting-out method as previously described (Miller, Dykes et al. 1988). SNP at position -511 of IL-1B was genotyped by the polymerase chain reaction with sequence specific primer (PCR-SSP) (F-5' CTCATCTGGCATTGATCTGG-3'; R-5'GGTGCTGTTCTCTGCCTCG/A-3') (Hutyrova, Pantelidis et al. 2002). The PCR conditions were established according to a previously described method (Bunce, O'Neill et al. 1995).

The VNTR of IL-1RN gene was assessed by a PCR-based assay. Oligonucleotides F-5' CTCAGCAACACTCCTAT-3'; and R-5' TCCTGGTCTGCAGGTAA-3' flanking this region were used as primers (Hutyrova, Pantelidis et al. 2002). The PCR conditions consist of the initial denaturation at 94 ^{o}c for 2 min, followed by 35 cycles of 94 ^{o}c for 20 sec, 59 ^{o}c for 50 sec and 72 ^{o}c for 20 sec, and final extension at 72 ^{o}c for 7 min. Each allele was identified according to its size (Hutyrova, Pantelidis et al. 2002).

Genotyping for the SNPs of IL-18 gene

SNP at position -607 of IL-18 were genotyped by the polymerase chain reaction with sequence specific primer (PCR-SSP). The PCR conditions were established according to a previously described method (Kretowski, Mironczuk et al. 2002).

Genotyping for the SNPs of IL-10 gene

The genomic DNA were analyzed by Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) method for genotyping the polymorphism of IL-10 at position -3575(T/A), -2763(A/C), -1082(G/A) and -592(C/A) as previously described (Edwards-Smith, Jonsson et al. 1999). The PCR reaction were amplied for -3575, -2763, -1082 and -592 under specific PCR condition. The amplification PCR products were subjected to specific restriction endonuclease and were separated on agarose gel electrophoresis as previously described (Edwards-Smith, Jonsson et al. 1999).

Genotyping for the SNPs of IFN-gamma gene

Polymorphisms at +874T/A within intron 1 of the IFN-γ gene were identified by using the PCR-SSP method. The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the IFN-γ gene specific primers described by Pravica and colleagues (Pravica, Perrey et al. 2000).

Genotyping for SNPs of IL-12 gene

Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) was used to analyze polymorphisms at +1159 (A/C) on 3' UT region (Davoodi-Semiromi, Yang et al. 2002).

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. In addition, linkage disequilibrium was calculated using the LDPlotter Tool.

RESULTS

Protective effect of DR13 allele against chronic hepatitis B infection

The distribution of HLA-DR12 and HLA-DR13 alleles in chronic HBV patients and transient HBV infection patients were shown in table 4. DR13 allele was not found in any chronic HBV patients. In contrast, DR13 allele was identified with high frequency of 8% in the transient HBV infection patients which was significantly higher than chronic HBV patients (0%) (Pc = 0.0008, OR = 0.04, 95%CI = 0.00-0.26). HLA-DR12 allele was increased in chronic HBV patients (32. 7%) compared with transient hepatitis B patients (27%), but did not reach statistical significance. Additional DRB1 typing was performed to identify another allele for 8 individuals that carried DR13 and revealed that all 8 patients are heterozygous for DR13. There was no significance in distribution of HLA-DR12 in chronic HBV patients with HCC compared with chronic HBV patients without HCC.

TNF-α gene polymorphisms associated with clearance of chronic HBV infection

The distribution of genotypes of TNF-α gene polymorphism at all 3 position -863(C/A), -308 (G/A), -238 (A/G) among healthy controls were in agreement with the prediction under the condition of Hardy-Weinberg equilibrium. The -863 C/C genotype and C allele was found to be significantly decreased in chronic HBV patients compared with healthy control (Pc = 0.01, OR = 0.46, 95% CI = 0.27-0.77 and Pc = 0.03, OR =

0.54, 95% CI = 0.35-0.84, respectively) (table 5). There were no significant differences in allele frequency of -308A/G and -238A/G polymorphism at the promoter of TNF-α gene between chronic HBV patients and transient HBV patients. The haplotype frequencies of the TNF- α promoter polymorphism were determined by PHASE program. In this study, CGG haplotype were the most common haplotype in Thai population. No significant differences in haplotype frequencies could be demonstrated between chronic HBV patients and transient HBV patients/healthy control.

Association of TNF-α gene polymorphisms with decreased risk of HCC in chronic hepatitis B patients

When stratified chronic HBV patients into patients without HCC and with HCC, the -863 A allele was found to be significantly increased in the HCC group compared to healthy control (Pc = 0.003, OR = 2.61, 95% CI = 1.49-4.60). By analysis of mode of inheritance, -863 A/A, A/C genotypic frequencies were significantly increased in the HCC group compared to healthy control (Pc = 0.001, OR = 3.62, 95% CI = 1.77-7.46). The CGG haplotype and CGG/CGG genotype of -863/-308/-238 haplotype were found to be significantly decreased in chronic HBV patients with HCC, as compared with healthy control (Pc = 0.03, OR = 0.39, 95% CI = 0.18-0.80 and Pc = 0.02, OR = 0.50, 95%CI = 0.30-0.83, respectively) (table 5). The homozygous CGG haplotype (CGG/CGG) also significantly decreased in chronic HBV patients with HCC compared to chronic HBV patients without HCC (Pc = 0.03, OR = 0.37, 95% CI = 0.17-0.79)

Association of IL-1β gene polymorphisms with increased risk of HCC in chronic hepatitis B patients

The genotype and allele frequencies of IL-1B and IL-1RN in healthy control subjects and patients with chronic hepatitis B, which comprises patients with and without HCC are shown in Table 6. All three groups were in Hardy-Weinberg equilibrium with no significant Chi-square values comparing the observed and expected genotype frequencies of each of tested polymorphisms. The heterozygous C/T of IL-1B was the most common genotype in all three groups (51.97% in healthy controls, 45.65% in patients with HCC and 56.67% in patients without HCC). The homozygous T/T was the second most common genotype in healthy controls (27.63%) and patients without HCC (24.44%), followed by the C/C genotype that were found 20.39% and 18.89%, respectively. In contrast to these two groups, the C/C was the second most common genotype in patients with HCC (36.96%), followed by the T/T genotype that was found 17.39%. Comparison of IL-1B-511 genotype revealed that IL-1B-511 C/C genotype was significantly increased in patients with HCC compared with healthy controls (p=0.036; OR=2.29; 95%CI=1.05-4.97) and patients without HCC (p=0.036; OR=2.52; 95%CI=1.05-6.04). Analysis of allele frequencies of IL-1B-511 showed that IL-1B-511 C allele was also significantly increased in patients with HCC, compared to that in the healthy control (p=0.033; OR=1.72; 95%CI=1.04-2.84). The effect of IL-1B-511 C allele was similar to autosomal recessive mode of inheritance. The presence of two C alleles (CC) is required to increase the likelihood of HCC development.

Four genotypes of IL-1RN (1/1, 1/2, 2/2, 1/4) were found in this study. The IL-1RN 1/1 genotype was the most common genotype in all the three groups (79.61% in healthy controls, 82.61% in patients with HCC and 82.22% in patients without HCC), followed by 1/2 genotype that were found 19.08%, 17.39% and 16.67%, respectively. The 2/2 genotype was found in healthy control (0.66%) and patients without HCC (1.11%), whereas the 1/4 genotype was especially found in healthy controls (0.66%). There were no significant differences in genotype or allele frequencies of IL-1RN between patients with chronic hepatitis B and healthy controls.

Association of interleukin 18 gene polymorphism (-607A/A genotype) with susceptibility to chronic hepatitis B virus infection

The genotype and allele frequencies of position -607 and -137 in healthy control subjects and patients with chronic hepatitis B are shown in Table 7. The genotype frequencies among healthy controls were in agreement with the prediction under the condition of Hardy-Weinberg equilibrium. The -607 A/A genotype is significantly higher in the patients with chronic hepatitis B compared to those in the controls (OR (95% CI) = 2.62 (1.36-5.09), p_c = 0.009). There were no significant differences in allele frequency of -137 polymorphism between chronic HBV patients and normal control. The haplotype frequencies of the IL-18 promoter polymorphism were determined by PHASE program. There was no statistically significance in haplotype frequency between chronic HBV patients and normal control.

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.

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

Table 3. Demographic and clinical data of subjects

Characteristics	chronic group		transient HBV	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. Distribution of specific HLA-DRB1 (DR12 and DR13) alleles in patients with chronic hepatitis B (with HCC and without HCC), transient HBV infection patients and healthy control.

HLA-DRB1 allele	Chronic hepatitis B				
	With HCC n = 50 (%)	Without HCC n =100 (%)	Total n = 150 (%)	Transient hepatitis B n =100 (%)	Healthy control n = 150 (%)
DR13	0(0)	0(0)	**0(0)**[a]	8(8)	7(4.7)
DR12	18(36)	31(31)	49(32.7)	27(27)	42(28)

[a] Pc= 0.0008 *vs* transient hepatitis B, OR (95% CI) =0.04 (0.00-0.26).

Table 5. Genotype and allele frequencies of TNF- α gene polymorphisms in chronic hepatitis B patients (with HCC and without HCC), transient hepatitis B patients and healthy control.

SNP of TNF- α gene		Total chronic hepatitis B n = 150 (%)	With HCC n = 50 (%)	Without HCC n =100 (%)	Transient hepatitis B n = 100 (%)	Healthy control n = 150 (%)
-863						
	genotype					
	A/A	7(4.7)	**3(6)** [c]	4(4)	2(2)	5(3.3)
	A/C	58(38.7)	**25(50)** [c]	33(33)	28(28)	34(22.7)
	C/C	**85(56.6)** [a]	22(44)	63(63)	70(70)	111(74)
	allele					
	A	72(24)	**31(31)** [d]	41(20.5)	32(16)	44(14.7)
	C	**228(76)** [b]	69(69)	159(79.5)	168(84)	256(85.3)
-308						
	genotype					
	A/A	0(0)	0(0)	0(0)	0(0)	1(0.6)
	A/G	22(14.7)	8(16)	14(14)	18(18)	26(17.3)
	G/G	128(85.3)	42(84)	86(86)	82(82)	123(82)
	allele					
	A	21(7.3)	8(8)	14(7)	18(9)	28(9.3)
	G	278(92.7)	92(92)	186(93)	182(91)	272(90.7)
-238						
	genotype					
	A/A	1(0.7)	1(2)	0(0)	1(1)	0(0)
	A/G	9(6)	5(10)	4(4)	7(7)	10(6.7)
	G/G	140(93.3)	44(88)	96(96)	92(92)	140(93.3)
	allele					
	A	11(3.7)	7(7)	4(2)	9(4.5)	10(3.3)
	G	289(96.3)	93(93)	196(98)	191(95.5)	290(96.7)
-863/-						

308/-238	genotype					
	CGG/CGG	69(46)	**15(30)** [e]	54(54)	49(49)	79(52.7)
	allele					
	CGG	199(66.3)	**57(57)** [f]	142(71)	121(60.5)	218(72.7)
	AGG	69(23)	29(29)	40(20)	33(16.5)	45(15)
	CAG	20(6.7)	7(7)	13(6.5)	20(10)	28(9.3)
	CGA	12(4)	7(7)	5(2.5)	7(3.5)	9(3)

[a] Pc = 0.01 vs healthy control, OR (95%CI) = 0.46 (0.27-0.77)

[b] Pc = 0.03 vs healthy control, OR (95%CI) = 0.54 (0.35-0.84)

[c] Pc = 0.001 vs healthy control, OR (95%CI) = 3.62 (1.77-7.46)

[d] Pc = 0.003 vs healthy control, OR (95%CI) = 2.61 (1.49-4.60)

[e] Pc = 0.03 *vs* chronic hepatitis B without HCC, OR (95%CI) = 0.37 (0.17-0.79);
Pc = 0.03 vs healthy control, OR (95%CI) = 0.39 (0.18-0.80)

[f] Pc = 0.02 vs healthy control, OR (95%CI) = 0.50 (0.30-0.82)

Table 6. **IL-1B and IL-1RN polymorphisms in patients with HBV and healthy controls**

Polymorphisms	Patients with HBV without HCC n = 90 (%)	Patients with HBV with HCC n = 46 (%)	Healthy controls n = 152 (%)
IL-1B-511			
Genotype frequencies			
C/C	17 (18.89)	**17 (36.96)**[a,b]	31 (20.39)
C/T	51 (56.67)	21 (45.65)	79 (51.97)
T/T	22 (24.44)	8 (17.39)	42 (27.63)
Allele frequencies			
C	85 (47.22)	**55 (59.78)**[c]	141 (46.38)
T	95 (52.78)	37 (40.22)	163 (53.62)
IL-1RN			
Genotype frequencies			
1/1	74 (82.22)	38 (82.61)	121 (79.61)
1/2	15 (16.67)	8 (17.39)	29 (19.08)
2/2	1 (1.11)	0	1 (0.66)
1/4	0	0	1 (0.66)
Allele frequencies			
1	163 (90.56)	84 (91.30)	272 (89.47)
2	17 (9.44)	8 (8.70)	31 (10.20)
4	0	0	1 (0.33)

[a] Versus healthy controls, OR (95% CI) = 2.29 (1.05-4.97), p = 0.036.

[b] Versus patients without HCC, OR (95% CI) = 2.52 (1.05-6.04), p = 0.036.

[c] Versus healthy controls, OR (95% CI) = 1.72 (1.04-2.84), p = 0.033.

Table 7. IL-18 position -607 and -137 polymorphisms in patients with HBV and healthy controls

Polymorphisms	Patients with HBV	Healthy controls
IL-18-607	n = 140 (%)	n = 140 (%)
Genotype frequencies		
A/A	**39 (27.86)**[a]	18 (12.80)
C/A	68 (48.57)	83 (59.30)
C/C	33 (23.57)	39 (27.90)
Allele frequencies		
A	146 (52.14)	119 (42.50)
C	134 (47.85)	161 (57.50)
IL-18-137		
Genotype frequencies		
G/G	105 (75.00)	102 (72.86)
G/C	29 (20.71)	35 (25.00)
C/C	6 (4.29)	3 (2.17)
Allele frequencies		
G	239 (85.36)	239 (85.36)
C	41 (14.64)	41 (14.64)
IL-18 -607:-137 combined genotypes		
Genotype frequencies		
A:G, C:G	47 (33.57)	51 (36.43)
C:G, C:G	33 (23.57)	39 (27.86)
A:G, A:G	24 (17.14)	12 (8.57)
A:C, C:G	20 (14.29)	29 (20.71)
A:C, A:G	11 (7.86)	5 (3.57)
Allele frequencies		
C:G	133 (47.50)	157 (56.07)
A:G	106 (37.86)	82 (29.29)
A:C	41 (14.64)	36 (12.86)

[a] Versus healthy controls, OR (95% CI) = 2.62 (1.36-5.09), p_c = 0.009

DISCUSSION

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. Our preliminary result screening T cells from one DR13 individual that recovered from HBV infection with overlapping peptides reveal positive response to peptide from polymerase, not from pre-core or core protein (data not shown).

It was proposed that a genetically increased capacity to produce TNF α would result in more effective inhibition of HBV; thus the resolution of HBV infection is associated with high TNF α promoter allele. However, an extensive review of all previous analysis of TNF α promoter polymorphism association with HBV infection summarized in table 1 showed that although there were clearly some associations, different markers were involved in each study. This fact suggests that these associations might be due to linkage disequilibrium with other genes. There was report that -308A allele was in strong linkage disequilibrium with DR13 (Kim, Lee et al. 2003). The present study demonstrated that the -863C allele and -863C/C genotype of TNF-α gene was increased in healthy control compared to chronic HBV patients. There are trend for association when compared to transient HBV patients although it is not statistically significant when corrected for multiple comparison (uncorrected P value = 0.04). This -863C allele was not in linkage disequilibrium with HLA-DR13 suggesting that other genetic markers linked with -863C independent of DR13 might influence clearance of chronic HBV infection in Thai.

Besides role of TNF-α polymorphism in the pathogenesis of chronic HBV infection, its association with the development of HCC was reported (Ho, Wang et al. 2004). That study in Taiwan showed that carriers of -308A allele, either homozygous or heterozygous subjects (associated with high TNF-α production), had a higher risk of developing HCC. 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. Although no significant association at -308 position was found, another high TNF-α production genetic marker, -863A allele was shown to associate with an increased risk of HCC with autosomal mode of inheritance in this study. Interestingly, the haplotype (-863/-308/-238) analysis revealed that the homozygosity of the low-production haplotype (CGG/CGG) was a protective marker for HCC in this study.

TNF-α has been found in high concentration in patients with cancer (Abrahamsson, Carlsson et al. 1993; Partanen, Koskinen et al. 1995). The existing evidence implicates the role of TNF-α inflammatory pathway that increased tumorigenesis (Szlosarek and Balkwill 2003). More convincing 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.

The association between the development of chronic hepatitis B and the polymorphisms of IL-1B gene (-511C/T) and IL-1RN gene (VNTR at intron 2), as suggested by Zhang et al., 2004, were not observed in this study. However, the -511C allele of IL-1B gene was identified as a genetic marker for hepatocellular carcinoma development in patient with chronic HBV infection. The hypothesis regarding IL-1 genetic polymorphism and hepatocarcinogenesis are based on the assumption that carriers of these genotypes are associated with increased levels of IL-1B in the liver in response to HBV infection and induce hepatocyte damage that may finally lead to HCC development. This hypothesis was supported by the observation that IL-1B level was increased in the liver tissue surrounding tumor tissue (Bortolami, Venturi et al. 2002). IL-1B is a proinflammatory cytokine as well as a tumor growth factor. There are several evidences that support its role in tumor growth development. First, IL-1B can increase production of prostaglansin E2 and hepatocyte growth factor (Bamba, Ota et al. 1998). Second, IL-1B can induce angiogenesis which is an important step in promoting tumor growth by either upregulate COX2 and inducible nitric oxide (Rahman, Dhar et al. 2001) or via the induction of vascular endothelial growth factor (VEGF) (Bortolami, Venturi et al. 2002). Third, IL-1B is also reported to attenuate interferon-induced antiviral activity and STAT1 activation in the liver (Tian, Shen et al. 2000).

However, the report of the IL-1B polymorphism in the promoter area in association with cancer gave controversial results. Briefly, a number of study mostly in Caucasian population support that -511T in linkage disequilibrium with -31C is a risk haplotype for gastric cancer development (El-Omar, Carrington et al. 2000; El-Omar, Carrington et al. 2001; Machado, Pharoah et al. 2001; Figueiredo, Machado et al. 2002; Machado, Figueiredo et al. 2003). There have been more conflicting data regarding the effect of IL-1B-511/-31 haplotype on the risk of gastric cancer and HCV-related HCC in Asian population. While some studies support the result from Caucasian group (Furuta, El-Omar et al. 2002; Hwang, Kodama et al. 2002; Takada, Suzuki et al. 2002), a number of studies report the opposite that -511C /-31T is instead a risk haplotype for cancer development (Hamajima, Matsuo et al. 2001; Kato, Onda et al. 2001; Matsukura, Yamada et al. 2003; Wang 2003; Yang, Hu et al. 2004; Chang, Jang et al. 2005). Interestingly, functional studies of IL-1B genotype seem to support that -511C/-31T haplotype is associated with high-production of IL-1B. First, the IL-1B -31 polymorphism involves a TATA sequence in the promoter and the -31T allele is associated with a five-fold elevated binding activity with the transcription initiation factor (El-Omar, Carrington et al. 2000; El-Omar, Carrington et al. 2001). Second, mucosal IL-1B level in H. pylori-infected gastric cancer patients were higher in patient homozygous for IL-1B -31T compare with other genotypes (Chang, Jang et al. 2005). Although one study reports that IL-1B -511T/-31C is associated with high level of IL-1B in the plasma (Hulkkonen, Laippala et al. 2000), it is likely that gene expression in each organ are differently regulated and the assessment of IL-1B level in targeted organ is more reliable. The functional study in liver tissue is also required to better understand the role of IL-1B genotype in chronic hepatitis and HCC development.

Previous report by Zhang et al (2005) found no significant relationship between polymorphism at position -607 and disease susceptibility (Zhang, Wu et al. 2005). They reported that C allele at position -137 plays a protective role in the development of HBV infection. They discuss that -137C was related with high production of IL-18, which result in better elimination of HBV. However, a functional study performed by IL-18 promoter cloning into Hela cell line and reporter gene analysis showed that promoter sequence containing -607A/-137C haplotype have low promoter activity while -607C/-137G have high activity (Giedraitis, He et al. 2001). Their result suggesting an important role of -137 position which is concordant with the prediction from TRANSFAC database that a change from G to C alters the H4TF-1 nuclear factor binding site to a binding site for an unknown factor found in the GM-CSF promoter. However, the major cells that produce IL-18 are monocyte/macrophage and the regulation of IL-18 production could be different in different cell types. A number of studies also suggest strong disease association with -607 but not -137 position (Takada, Suzuki et al. 2002; Sivalingam, Yoon et al. 2003). The SNP at -607 was predicted to lie within a potential cAMP-responsive element binding protein (CREB) binding site. A change from C to A at this position disrupts this potential binding site. It is possible that the regulation of IL-18 production in chronic HBV patients, the CREB, not the H4TF-1, is an important positive regulation of IL-18 transcript. Although the exact characteristic of IL-18 promoter need to be further clarified, the -607 A/A genotype likely results in markedly lower transcription activity and lower production of IL-18 from hepatic macrophage. Low production of IL-18 in the liver leads to decreased inhibition of HBV replication (Kimura, Kakimi et al. 2002). Therefore, a strong association between -607 A/A polymorphism and the susceptibility of chronic hepatitis B demonstrated in this study is consistent with the proposed functional significance of this SNP.

In conclusion, our study showed that HLA-DR13 allele is a strong protective genetic marker in chronic hepatitis B infection. The functional significance of this particular HLA molecule in HBV immune response may provide insight to develop novel prophylactic and therapeutic tools. Furthermore, the –863A 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 hepatocyte 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. This study also reports that IL-1B-511C allele, which is likely to be associated with high IL-1B production in liver, is a genetic marker for HCC development in chronic hepatitis B patients in Thai population. Lastly, this study proposes that A/A genotype at position -607 in IL-18 gene can be used as a new genetic maker in Thai population for predicting chronic hepatitis B development.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

1.1 Netsawang J, Tangwattanachuleeporn M, **Hirankarn N**, Wongpiyabovorn J . The distribution of IL-10 Promoter Polymorphism in Thais. J Med Assoc Thai 2004; 87 Suppl2:S117-S122

1.2 Kimkong I, Tangkijvanich P, Poovorawan Y, **Hirankarn N**. 2004 Interleukin-1β Gene Polymorphism Associated With Hepatocellular Carcinoma in Hepatitis B Virus Infection. Tissue Antigens 2004 Oct; 84:383-384 (abstract)

1.3 **Hirankarn N**, Kimkong I, Kummee P, Tangkijvanich P, Poovorawan Y. Interleukin-1β Gene Polymorphism Associated With Hepatocellular Carcinoma in Hepatitis B Virus Infection. World Journal of Gastroenterology 2006 Feb; 12(5):776-779.

1.4 Kummee P, Tangkijvanich P, Poovorawan Y, **Hirankarn N**. Association of HLA-DRB1*13 and TNF-alpha Gene Polymorphisms with Clearance of Chronic Hepatitis B Infection and Risk of Hepatocellular Carcinoma in Thai Population. (submitted to Journal of Viral Hepatitis, IF2005=2.55)

1.5 **Hirankarn N**, Tangkijvanich P, Poovorawan Y. Association of interleukin 18 gene polymorphism (-607A/A genotype) with susceptibility to chronic hepatitis B virus infection. (submitted to Tissue Antigen, IF2005=2.747)

2. การนำผลงานวิจัยไปใช้ประโยชน์

- **เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)**

2.1 รับนิสิตปริญญาโทหลักสูตรสหสาขาวิชาจุลชีววิทยาทางการแพทย์ จุฬาลงกรณ์มหาวิทยาลัย ชื่อ นางสาวพิทยา คำมีทำวิทยานิพนธ์เรื่อง The Association between HLA-DRB1 and TNF gene and Susceptibility to Chronic Hepatitis B เริ่มเรียนปีการศึกษา พ.ศ. 2546 และสำเร็จการศึกษา ปี พ.ศ. 2548

3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 การไปเสนอผลงานในที่ประชุมวิชาการ

3.1.1 Ingorn Kimkong, Pittaya Kummee, Pisit Tangkijvanich, Yong Poovorawan, **Nattiya Hirankarn**. Interleukin-1β Gene Polymorphism associated with Chronic Hepatitis B Virus Infection. The Fifth Princess Chulabhorn International Science Congress: Evolving Genetics and Its Global Impact, Bangkok, Thailand, August 16-20, 2004.

3.1.2 Ingorn Kimkong, Pisit Tangkijvanich, Yong Poovorawan, **Nattiya Hirankarn.** Interleukin-1β Gene Polymorphism Associated With Hepatocellular Carcinoma in Hepatitis B Virus Infection. The First International Conference on Basic and Clinical Immunogenomics, Budapest, Hungary, October 3-7, 2004.

3.1.3 Pittaya Kummee, Pisit Tangkijvanich, Yong Poovorawan, **Nattiya Hirankarn.** Association between polymorphisms of HLA-DRB1 gene with susceptibility and/or disease progression of chronic hepatitis B infection in Thai population. The 30th Australian and South East Asian Tissue Typing Association Meeting, Chiangmai, Thailand, Nov 22-24, 2006.

3.2 การได้รับเชิญเป็นวิทยากร

3.2.1 นำเสนอเรื่อง Immunogenetics and Chronic Hepatitis B ณ ห้องประชุมหน่วยโรคติดเชื้อ ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย วันที่ 3 กันยายน 2546

3.2.2 นำเสนอเรื่อง Host and Viral Factors Associated with Chronic Hepatitis B ณ ห้องประชุมภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย วันที่ 13 สิงหาคม 2547

3.2.3 นำเสนอเรื่อง "The association of genetic polymorphism within proinflammatory cytokine gene and risk of hepatocellular carcinoma with chronic hepatitis B infection" ณ The HIV Netherlands Australia Thailand Research Collaboration Thai Red Cross AIDS Research Centre วันที่ 11 กรกฎาคม 2548

SUGGESTION FOR FURTHER WORK

Candidate gene-based association studies rely on having predicted the identity of the correct gene or genes, usually on the basis of biological hypotheses or the location of the candidate within a previously determined region of linkage. Even if these hypotheses are broad, they will, at best, identify only a fraction of genetic risk factors for diseases in which the pathophysiology is relatively well understood. When the fundamental physiological defects of a disease are unknown, the candidate-gene approach is clearly inadequate to fully explain the genetic basis of the disease. With the advent of new genetic markers and automated genotyping, genetic mapping can be conducted extremely rapid. This approach has been successful in some infectious diseases, but no report on such similar scans for HBV viral persistence is available. Therefore, whole genome scan is required which makes it possible to find chromosome regions containing determinant(s) of persistent HBV infection.

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