



รายงานวิจัยฉบับสมบูรณ์

โครงการปัจจัยของผู้ป่วยและเชื้อไวรัสที่สัมพันธ์กับการดำเนินโรค
และการรักษาโรคตับอักเสบแบบเรื้อรังจากไวรัสตับอักเสบบีและซี

Host and Viral Factors Associated with Clinical Outcome
and Treatment Response of Chronic Viral Hepatitis

โดย...ศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์และคณะ

กรกฎาคม 2557

สัญญาเลขที่ BRG5580005

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หัวหน้าโครงการ

ศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์

ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและจุฬาลงกรณ์มหาวิทยาลัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "ปัจจัยของผู้ป่วยและเชื้อไวรัสที่สัมพันธ์กับการดำเนินโรคและการรักษาโรคตับอักเสบแบบเรื้อรังจากไวรัสตับอักเสบบีและซี" ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ BRG5580005 ระยะเวลาดำเนินการ 2 ปี ตั้งแต่ 31 กรกฎาคม 2555 ถึงวันที่ 30 กรกฎาคม 2557 ผู้รับทุนขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้การสนับสนุนอย่างเต็มที่จนทำให้โครงการวิจัยนี้สำเร็จลุล่วงด้วยดี ผู้รับทุนขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญในความสำเร็จของโครงการ ได้แก่ ศ.นพ.ยง ภู่วรวรรณ ศ.นพ.เกียรติ รักษ์รุ่งธรรม ศ.พญ.ณัฐธิยา หิรัญกาญจน์ ผศ.ดร.สัณชัย พยุงบวร (คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย) พญ.อัญชลี อวิหิงสานนท์ (ศูนย์วิจัยโรคเอดส์ สภากาชาดไทย) รศ.ดร.ปรัชญา คงทวีเลิศ (คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่) ดร.อิงอร กิมก (คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์) ผู้ช่วยวิจัยที่มีส่วนช่วยเหลือให้โครงการวิจัยสำเร็จลงได้แก่ นางสาวภัทริดา สงวนหมู่ นางสาวศรัณย์ธร อัครธำรงสิน นางสาวณัฐธยาน์ ช่วยเพ็ญและนางสาวอภิษฐา คล้ายพึงสินธุ์ รวมทั้ง ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในฐานะสถาบันต้นสังกัดที่ให้การสนับสนุนโดยอำนวยความสะดวกในโครงการเป็นอย่างดี

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รหัสโครงการ: BRG5580005

ชื่อโครงการ: ปัจจัยของผู้ป่วยและเชื้อไวรัสที่สัมพันธ์กับการดำเนินโรคและการรักษาโรคตับอักเสบแบบเรื้อรังจากไวรัสตับอักเสบบีและซี

ชื่อนักวิจัยและสถาบัน: ศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์
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โครงการวิจัย:

ไวรัสตับอักเสบบีและไวรัสตับอักเสบซีเป็นสาเหตุสำคัญของเกิดโรคตับอักเสบแบบเรื้อรัง ตับแข็งและมะเร็งตับ ปัจจุบันเชื่อว่าปัจจัยต่างๆของผู้ติดเชื้อและปัจจัยที่เกี่ยวข้องกับเชื้อไวรัสมีผลต่อการดำเนินโรคและการตอบสนองต่อการรักษาที่แตกต่างกัน ดังนั้นโครงการวิจัยนี้จึงมีจุดมุ่งหมายเพื่อศึกษาความสำคัญของปัจจัยดังกล่าวในผู้ที่มีการติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง การศึกษาแบบ case-control study พบว่าความหลากหลายทางพันธุกรรมของยีนในหลายตำแหน่งเช่น type I interferon receptor 2, interleukin-10 receptor subunit beta และ human leukocyte antigen genes มีความสัมพันธ์กับการหายขาดจากการติดเชื้อไวรัสตับอักเสบบี ส่วนความหลากหลายทางพันธุกรรมของยีน signal transducers and activator of transcription (STAT) genes และการกลายพันธุ์แบบ A1762T/G1764A ของเชื้อไวรัสมีความสัมพันธ์กับการเกิดมะเร็งตับ ในขณะที่การได้รับสารอะฟลาทอกซินจากเชื้อราซึ่งเป็นสารก่อมะเร็งพบได้น้อยในประชากรไทยที่เป็นโรคมะเร็งตับ

การศึกษาในผู้ติดเชื้อไวรัสตับอักเสบซีพบว่าปัจจัยที่ทำให้โรคตับมีความรุนแรงมากได้แก่มีการติดเชื้อเอชไอวีร่วมด้วยและมีภาวะขาดวิตามินดี นอกจากนี้ยังพบว่าไวรัสตับอักเสบซีต่างสายพันธุ์มีการตอบสนองต่อการรักษาด้วยยาต้านไวรัสที่แตกต่างกัน การศึกษาความหลากหลายทางพันธุกรรมของยีนบางตำแหน่งของผู้ที่ติดเชื้อเช่น Interferon lambda-3 (IFNL3 หรือ IL-28B) และ IFNL4 genes มีประโยชน์ในการทำนายผลการรักษาด้วยยาต้านไวรัสสำหรับเชื้อไวรัสสายพันธุ์ 1 แต่มีประโยชน์น้อยสำหรับเชื้อไวรัสสายพันธุ์ 3 และ 6 นอกจากนี้การศึกษาความหลากหลายทางพันธุกรรมของยีน inosine triphosphatase (ITPA) ยังสามารถทำนายการเกิดภาวะโลหิตจางในระหว่างการรักษาได้เป็นอย่างดี ดังนั้นการศึกษาปัจจัยต่างๆของผู้ติดเชื้อและปัจจัยที่เกี่ยวข้องกับเชื้อไวรัสจึงเป็นประโยชน์ในการพยากรณ์ความรุนแรงของโรคและทำนายผลของการรักษาสำหรับผู้ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง

ABSTRACT

Project Code: BRG5580005

Project Title: Host and Viral Factors Associated with Clinical Outcome and Treatment Response of Chronic Viral Hepatitis

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Project Period: 31 July 2012-30 July 2014

Project Description:

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are associated with a high lifetime risk of developing cirrhosis and hepatocellular carcinoma (HCC). Several viral and host factors appear to strongly influence the clinical outcome and treatment response of infected patients. This project was aimed at studying the clinical significance of these factors in diverse groups of chronically infected individuals. In case-control studies, our data showed that single nucleotide polymorphisms (SNPs) in several candidate genes, including the *type I interferon receptor 2*, *interleukin-10 receptor subunit beta* and *human leukocyte antigen* genes, were associated with HBV clearance, while SNPs at the *signal transducers and activator of transcription (STAT)* genes were associated with HCC development. In addition, A1762T/G1764A mutations of HBV, but not aflatoxin B1-induced R249S mutation of the *p53* gene, were associated with HCC development in Thai patients.

Our data showed that a high proportion of HCV-infected patients had advanced liver fibrosis. Based on multivariate analysis, strong predictors for advanced fibrosis were HIV co-infection and low serum levels of vitamin D. Our data showed that SNPs in the *interferon lambda-3 (IFNL3 or IL-28B)* and *IFNL4* genes were equally associated with the treatment outcome in genotype 1 but their roles were attenuated in genotype 3 or genotype 6. We also demonstrated for the first time that the early viral kinetics of genotype 6 during therapy correlated with the IFNL3 polymorphism. In addition, SNP in the *inosine triphosphatase (ITPA)* gene influenced the degree of treatment-induced anemia. Identification of these host and viral factors may therefore provide promising predictors of clinical outcome and treatment response in patients with HBV or HCV infection.

Keywords: Viral hepatitis, polymorphisms, molecular virology, clinical outcome, treatment

หน้าสรุปโครงการ (Executive Summary)

ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา

1. ชื่อโครงการ ปัจจัยของผู้ป่วยและเชื้อไวรัสที่สัมพันธ์กับการดำเนินโรคและการรักษาโรคตับอักเสบบรรเทา
เรื้อรังจากไวรัสตับอักเสบบีและซี

Host and Viral Factors Associated with Clinical Outcome and Treatment
Response of Chronic Viral Hepatitis

2. ชื่อหัวหน้าโครงการ นพ.พิสิฐ ตั้งกิจวานิชย์

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3. สาขาที่ทำการวิจัย อณูชีววิทยาของไวรัสตับอักเสบบี

4. งบประมาณทั้งโครงการ 2,000,000 บาท

5. ระยะเวลาดำเนินงาน 2 ปี

6. จุดประสงค์ของโครงการ

The aims of this research project were to

- 1) Determine host and viral factors in predicting treatment response of patients with chronic HBV infection
- 2) Determine host genetic variations associated with clearance of HBV infection
- 3) Determine host and viral factors and p53-R249S mutation associated with HCC
- 4) Determine molecular epidemiology and natural history of chronic HCV genotype 6 by comparison to those of genotype 1 and 3
- 5) Determine host genetic variations and viral factors associated with treatment response in patients with chronic HCV infection

7. ข้อมูลจากโครงการวิจัยโดยสรุป

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are major public health problems worldwide, with approximately 400 and 170 million people are persistently infected with the viruses, respectively. Chronic HBV and HCV infections are associated with a diverse clinical spectrum of liver damage ranging from mild chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC). Studies to date suggest that host genetic variations and

molecular virological factors have important influences on the clinical outcome and treatment response to antiviral therapy, but additional studies in certain aspects are needed.

Regarding HBV-related chronic liver disease, we investigated the effects of two single nucleotide polymorphisms (SNPs), *type 1 interferon receptor 2 gene (IFNAR2)-F8S* and *interleukin-10 receptor subunit beta gene (IL10RB)-K47E*, on chronic HBV infection. The data suggested that IFNAR2-F8S polymorphisms might be involved in the susceptibility to chronic HBV infection, while AA genotype of IL10RB-K47E might provide a protective role. We also found the interaction between IFNA5 (-2529) and IFNA1 (-1823) genes within promoter regions of IFN- α 1 and the risk of developing chronic HBV infection. In addition, we determined associations of variations at the *human leukocyte antigen (HLA)-DP* gene with outcome in HBV infected Thai patients and the major homozygous genotypes of rs3077 and rs9277378 were significantly associated with chronic HBV infection. These data are consistent with many genome-wide association studies (GWAS) performed to seek associations between human genetic variation and the outcome of HBV infection in Asian populations.

Regarding HBV-related HCC, previous GWAS have shown that SNP at the *kinesin family member 1B (KIF1B)* gene locus, rs17401966, is strongly associated with the development of HBV-related HCC in Chinese populations. Unlike previous reports, the SNP showed no significant association with HBV-related HCC in Thai patients indicating that there might be other mechanisms or pathways involved in the development of HCC. Instead, SNPs in the *signal transducers and activator of transcription (STAT)* genes such as rs2293152 and rs1053004 at STAT3 and rs7574865 at STAT4 were associated with HBV-induced HCC. In addition, we showed that A1762T/G1764A mutations of HBV, but not aflatoxin B1-induced R249S mutation of the *p53* gene, were associated with the development of HCC in Thai patients.

Regarding antiviral treatment, pegylated interferon (PEG-IFN)-based therapy in chronic HBV infection is still far from optimal and has potential side effects. Theoretically, the combination therapy of an immunomodulator (PEG-IFN) and a potent oral antiviral drug (e.g. entecavir) might improve the response rate in patients with chronic hepatitis B. To address this hypothesis, we compared the efficacy of PEG-IFN plus entecavir and PEG-IFN alone in Thai patients with HBeAg-negative chronic hepatitis B. Our interim data showed that the combination therapy led to a higher rate of HBV DNA suppression than PEG-IFN monotherapy at the end of treatment (week 48). However, both treatment regimens were comparable on the basis of viral suppression, HBsAg reduction and HBsAg clearance at 24

week of the follow-up. Thus, the combination therapy might not superior to PEG-IFN monotherapy in patients with HBeAg-negative chronic hepatitis B.

Approximately 2.2% of Thai population has been chronically infected with HCV. Based on phylogenetic analyses of sequences in core and NS5B region, subtype 3a was most predominant followed by 1a, 1b and genotype 6. Regarding molecular evolutionary analysis, it was estimated from our study that HCV genotype 3a had been present in Thailand for approximately 100 years. This particular strains spread during the 1960s-1970s, which coincided with the period of the Vietnam War (1955-1975) and the widespread use of injection stimulants introduced by the US army. Factors associated with disease progression in HCV mono-infection and HIV/HCV co-infection in Asian populations have not been well characterized. Our data showed that a high proportion of HCV mono-infected and co-infected patients had advanced liver fibrosis assessed by transient elastography. Patients infected with genotype 6 tended to have higher HCV RNA levels but less advanced liver fibrosis compared to those infected with genotype 1 or genotype 3. Based on multivariate analysis, strong and independent predictors for advanced fibrosis were HIV co-infection and low serum levels of vitamin D.

So far very little is known from both the virological and therapeutic points of view for HCV genotype 6, which has been found locally in Southeast Asia. In particular, the predictor of treatment response to combined PEG-IFN and ribavirin (RBV) therapy. Recently, independent GWAS have identified that SNPs in the *interferon lambda-3 (IFNL3 or IL-28B)* and *IFNL4* genes are associated with response to combined therapy in individuals infected with HCV genotype 1. These data suggest that host genetics may be useful for the prediction of drug response. Our results showed that the two polymorphisms were equally associated with the treatment outcome in genotype 1 but their roles in predicting treatment response were attenuated in genotype 3 or genotype 6. We also demonstrated for the first time that the early viral kinetics of genotype 6 correlated with the IFNL3 polymorphism in patients treated with PEG-IFN plus RBV therapy. In addition, our data showed that inosine triphosphatase (ITPA) polymorphism influenced the degree of RBV-induced anemia during therapy but not correlated with clinical outcomes. Thus, identification of these polymorphisms prior to therapy may provide an opportunity for clinicians to optimize the decision making with standard of care regimens.

Project Description

1. Introduction and Rationale

Molecular virology of hepatitis B virus

Hepatitis B virus (HBV) is a member of the family Hepadnaviridae, which includes a variety of avian and mammalian viruses that share similar genomic organization, organ tropisms, and a unique strategy of genome replication.¹ Its genome comprises a partially double stranded 3.2 kb DNA organized into four open-reading frames (ORF).² The pre-S/S ORF codes for the three surface proteins (hepatitis B s antigen; HBsAg). The pre-C/C ORF codes for the capsid protein (C region) and, when the full pre-C/C region is translated, for a non-structural protein bearing the hepatitis B e antigen (HBeAg) determinant, which is exported to the peripheral circulation after post-translational processing. Nucleotide substitutions in the pre-C region may abrogate the production of the HBe protein, whereas mutations in the core promoter region appear to regulate its expression. The polymerase ORF spans a large part of the HBV genome and encodes the HBV polymerase that has several properties, including a reverse transcriptase activity and an RNase H activity. Finally, the X-ORF encodes a 154 amino acid protein called hepatitis B virus X protein. This protein is a multifunctional regulator that modulates host transcription, cell cycle progress, protein degradation, apoptosis and signal transduction pathways.³

After infected the hepatocytes, the viral DNA is transferred to the nucleus, where the viral polymerase is removed, and the double-stranded, open circular DNA is converted to a covalently closed circular DNA molecule (cccDNA).⁴ CccDNA is transcribed by cellular RNA polymerases into messenger RNAs for viral protein synthesis, and into a pre-genomic (pg) RNA, which is subsequently encapsidated in the cell cytoplasm together with a molecule of HBV DNA polymerase. The latter has a reverse transcriptase function that catalyzes the synthesis of the negatively stranded genomic DNA, while the pgRNA is gradually degraded by the RNase H activity of the polymerase in the nucleocapsid. A positive DNA strand is then synthesized by the polymerase, using the negative-strand as template. Newly generated nucleocapsids can be recycled to yield additional cccDNA molecules in the nucleus, but most of them bud into the endoplasmic reticulum to form mature virions that are subsequently released into the pericellular space by exocytosis.^{1, 5}

HBV is currently classified into at least eight genotypes, designated A to H based on genomic sequence diversity of more than 8%.⁶ These genotypes have a distinct global

geographical distribution. For examples, genotypes A and D are common in Europe and North America, whereas genotypes B and C are highly prevalent in Asia. Genotypes F and H are restricted to Central and South America. Genotype E is found predominantly in West Africa and genotype G is found in the USA and Europe. In Thailand, HBV genotypes C and B are predominant, accounting for approximately 75% and 20%, respectively.⁷

HBV has a high mutation rate compared with other DNA viruses because it lacks of proofreading capacity during the replication via reverse transcription of its pgRNA.⁸ The frequency of HBV mutation has been estimated to be approximately 1.4 to 3.2×10^{-5} nucleotide (nt) substitutions per site per year, around 10-fold higher than that of other DNA viruses.⁹ The magnitude and rate of virus replication are also important in the process of mutation generation; the total viral load in serum frequently approaches 10^{11} virions/mL. Most estimates place the mean half-life of the serum HBV pool at approximately 1 to 2 days, translating to a rate of de novo HBV production approaching 10^{11} virions/day. The high viral loads and turnover rates coupled with poor replication fidelity influence mutation generation and the complexity of the HBV quasi-species pool. These mutations arise during active replication and mutant strains can become dominant if they offer an advantage to the fitness of the virus. They may therefore contribute to viral persistence as a result of their escape from host immune surveillance and replication efficiency. The well-known naturally occurring HBV variants include the precore (PC) stop codon mutation (G1896A), which abolishes HBeAg production, and the dual mutation in the basal core promotor (BCP) region (A1762T/G1764A), which down-regulates HBeAg production.¹⁰ Such mutations have been reported in up to 50-80% of patients with HBeAg-negative chronic hepatitis B in Europe and Asia.¹¹

Natural history of chronic HBV infection

Chronic HBV infection is a major health problem worldwide, affecting approximately 400 million people.² The clinical course of chronic HBV infection is different between Asian and Western patients. Asian HBV carriers mostly acquire the virus in the perinatal period or early childhood, which leads almost invariably to persistent infections, while Western HBV carriers usually acquire the virus at older ages through horizontal transmission.² On the basis of the virus and host interactions, the natural history of perinatally acquired HBV infection can be divided into four dynamic phases: immune tolerance, immune clearance, low or non-replication, and reactivation.¹² The initial immunotolerant phase, persisting for 2-3

decades, is characterized by the presence of HBeAg, high serum levels of HBV-DNA, but normal or minimally elevated serum alanine aminotransferase (ALT) and only minimal histological activity and fibrosis. After a variable period of HBeAg positivity, immune tolerance to the virus is lost and the immune system mounts an attack on infected hepatocytes. This second immunoactive phase is characterized by fluctuating HBV-DNA levels, elevated ALT and hepatic necroinflammation. Serum HBV-DNA levels in this phase generally exceed 20,000 IU/mL or 10^5 copies/mL (the so-called HBeAg positive chronic hepatitis). Such responses may lead to reduced virus replication and decreasing serum concentrations of HBeAg. Ultimately, HBeAg may become undetectable with seroconversion to antibody (anti-HBe), signaling the third low or non-replication phase with undetectable or low levels of viremia and little inflammation. This inactive HBsAg carrier state is characterized by HBeAg negativity and anti-HBe positivity, undetectable or low levels of HBV-DNA (less than 2000 IU/mL or 10^4 copies/mL), persistently normal ALT levels and inactive liver histology with a usually minimal amount of fibrosis.

A number of inactive HBsAg carriers may eventually develop HBV reactivation with recrudescence of liver disease. Reactivation of viral replication may occur due to reactivation with the wild type virus with reversion back to the HBeAg positive state, or much more frequently with replication-competent HBV variants that prevent HBeAg expression, such as PC stop codon mutation or the dual mutation in the BCP region. This fourth reactivation phase is characterized by HBeAg negativity with anti-HBe positivity, detectable serum HBV-DNA levels (exceed 2,000 IU/mL or 10^4 copies/mL), ALT elevation and moderate or severe necroinflammation with variable amounts of fibrosis on liver biopsy (the so-called HBeAg negative chronic hepatitis). Without treatment, high levels of virus replication may persist in the face of a vigorous immune response in immune clearance or reactivation phases, leading to severe inflammation and fibrosis, and ultimately, cirrhosis, and hepatocellular carcinoma (HCC).¹² It has been estimated that approximately 15 to 40% of people who develop chronic HBV infection are expected to progress to cirrhosis and HCC.¹³

Viral factors associated with disease severity in chronic HBV infection

The clinical outcome of HBV infection is rather complex and is influenced by many factors, including host factors (gender, age, and immune status), viral factors and exogenous factors such as concurrent infection with other hepatotropic viruses (e.g. HCV), alcohol and aflatoxin B1 (AFB1) exposure. AFB1 is the major metabolite of the molds *Aspergillus*

fumigatus and *Aspergillus parasiticus*. AFB1 induces a single nucleotide substitution in codon 249 in the *p53* tumor suppressor gene, which results in the change of the amino acid arginine to serine (R249S). This mutation is present in up to 50% of patients with HCC who are indigenous to geographic regions with high exposure to AFB1.¹⁴

The clinical spectrums of chronic HBV infection range from mild hepatitis to the most severe liver diseases including cirrhosis, and HCC. In Thailand, chronic HBV infection is the most common risk factor for development of HCC, accounting for approximately 65% of cases.^{15, 16} Chronic HBV infection exerts its pro-oncogenic properties through both indirect and direct mechanisms.¹⁷ The indirect mechanisms are related to its propensity to induce continuous or recurrent phases of liver necroinflammation and to promote the progression of chronic hepatitis to cirrhosis, which is the step preceding the development of HCC in most cases. The direct carcinogenic mechanisms have been related to the capacity of HBV to integrate into the host's genome and to produce proteins provided of potential transforming properties.

Regarding viral factors, recent prospective cohort data have shown that HBV DNA above 2,000 IU/mL in persons above age 40 is associated with increased risk of disease progression to cirrhosis and HCC.¹⁸ In addition, there are now growing evidence suggesting that viral genotypes may influence the clinical outcomes of patients with chronic HBV infection.¹⁹ Among Asian patients who constitute approximately 75% of HBV carriers worldwide, it has been shown that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis, as well as HCC compared to genotype B.¹⁹ Considering natural genetic variants, while the role of the PC mutant to the course of chronic HBV infection is still controversial, the BCP mutants have been linked to the severity of liver diseases, particularly HCC.^{20, 21} Apart from these variants, other mutations such as T1753C/A/G in the BCP region and C1653T in the enhancer II region (EnhII) have become increasingly recognized as being associated with the outcome of chronic HBV infection, including HCC development.^{22, 23} In addition, several lines of evidence have suggested that mutants, particularly deletions, occurring naturally in the preS region correlate with the development of HCC.²⁴ Finally, it has also been shown that mutations in the X gene may contribute to the development of HCC in HBV-infected patients.^{25, 26} Although such mutations may be associated with an increased risk of HCC, whether there are any additive or synergistic effects on hepatocarcinogenesis with combinations of HBV mutations are unclear. In addition, interactions of these HBV mutations with host genetic

factors, as well as environmental factors, such as the exposure to aflatoxin remain to be established.

Host factors associated with HBV infection

In addition to viral factors, host genetic factors are considered to govern the disease development, progression or regression in HBV infection. The evidence in support of genetic factors arises from twin studies conducted in Taiwan.²⁷ In this report, it was demonstrated that the degree of concordance for HBsAg status was significantly higher in monozygotic twins than in dizygotic twins. As HBV is generally believed not to have a direct cytopathic effect, most available evidence suggested that liver damage in HBV infection is produced 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 the association between human leukocyte antigen (HLA) class II genes and the susceptibility of chronic HBV infection.²⁸ Previously, we conducted an association study included patients with chronic hepatitis B, those with resolved infection and healthy individuals with similar ethnic background. Interestingly, *HLA-DR13* showed a strong association with the clearance of the virus, which was similar to reports from several ethnic groups.²⁹

Recently, a genome-wide association study (GWAS) conducted in Japan show that genetic variations in the *HLA-DQ* genes were strongly associated with chronic hepatitis B infection, and this association was independent from the *HLA-DP* genes.³⁰ Another GWAS conducted in China investigating over 400,000 single nucleotide polymorphisms (SNPs) in chronic HBV carriers with or without HCC found that a region in the *KIF1B* gene on chromosome 1 was highly associated with HCC.³¹ This region of chromosome 1 has previously been of interest in HCC as loss of heterozygosity was reported in tumor tissue. The results from these GWAS provide a novel insight that host genetic variations might be critical for virus clearance and play an important role in the development of liver cancer. How these host genetic factors contribute to clinical outcome among Thai populations are yet to be determined.

Treatment of chronic HBV infection

Treatment of chronic hepatitis B is aimed at driving viral replication to the lowest possible level, and thereby to halt the progression of liver disease and prevent the onset of

complications. The currently approved agents for treatment of chronic hepatitis B are interferon alfa (IFN) and nucleoside or nucleotide analogues (NA), such as lamivudine, adefovir, entecavir, telbivudine and tenofovir.³² NA directly inhibits reverse transcriptase and thereby impairs viral replication, whereas IFN has marked immunomodulatory and enhances the cell-mediated immune response in the process of clearing the virus. Thus, response to IFN-based therapies tends to be more sustained than to NA.³² IFN is effective after a relatively short course of treatment (6 months to 1 year) and, unlike NA, has not been associated with drug resistance.^{32, 33} Currently, peginterferon alfa (PEG-IFN), created by attaching a polyethylene glycol molecule to IFN, significantly improves pharmacokinetics and results in more convenient dosing interval than conventional IFN.³²

However, PEG-IFN therapy can result in only 30-40% HBeAg seroconversion in HBeAg-positive chronic hepatitis, which is still far from satisfactory.³⁴ Considering the treatment is expensive and has potential side effects, it is important to identify pretreatment and on-treatment parameters for predicting response and non-response of patients treated with this agent. Our recent data have shown that pretreatment quantitative HBsAg determination is useful for predicting response to PEG-IFN therapy. In addition, the presence of double BCP and pre-S mutation/deletions at entry may be associated with a high rate of antiviral response in HBeAg-positive and HBeAg-negative chronic hepatitis, respectively.³⁵ In addition, our data have shown that quantitative measurement of serum HBeAg during therapy may be superior to serum HBsAg and HBV DNA as a prediction of HBeAg seroconversion. Kinetics of HBsAg levels on therapy may help predict HBsAg clearance after treatment.³⁶ It has been clearly shown in chronic HCV infection that the study of viral kinetics during treatment has resulted in improved understanding of viral dynamics and has led to improved therapeutic options. However, although chronic HBV infection has long been treated with IFN-based therapy, most of the studies regarding the viral kinetics so far have been reported only during NA therapies.³⁷ Thus, in the case of HBV infection, for which treatment is still far from optimal, it is of special importance to understand viral dynamics and antiviral mechanisms associated with all possible therapies, particularly PEG-IFN-based therapy.

There is a renewed interest in use of combined treatment of NA and IFN-based therapy in patients with chronic hepatitis B because of limitations of monotherapies.³⁸ This hypothesis is based on the fact that IFN-based therapy have only mild virus-suppressive activity, but can induce an effective host immune response in susceptible patients, whereas NA have a marked virus-suppressive activity in a majority of patients, but have no

immunomodulatory effects. Therefore, the combination of the two could possibly provide both viral suppression and immunomodulation and hence increase the response rate. Although the results of the combination with IFN-based therapy and lamivudine have been mixed,³⁸ additional trials of combination therapies with PEG-IFN and a more potent NA such as entecavir are necessary. In this respect, studies of pretreatment viral genetic variability and viral kinetics during such combination therapies may help to predict and optimize therapeutic response.

Molecular virology of hepatitis C virus

HCV is a small, enveloped RNA virus that belongs to the family of flaviviruses. Its genome consists of a single-strand of positive-sense RNA of approximately 9.6 kb, which contains an ORF coding for a polyprotein precursor of approximately 3000 residues.³⁹ The precursor is cleaved into at least ten different proteins, including the structural proteins, the p7 ion channel and the nonstructural proteins. The structural proteins, which form the viral particle, include a highly conserved viral core protein and two envelope proteins designated E1 and E2, which coat the virus. The E1 and E2 proteins appear to be heterodimers on the surface of the virus and contain important neutralizing epitopes, including a hypervariable portion of the E2/NS1 region that seems to mutate rapidly under immune pressure resulting in escape from neutralization and facilitating viral persistence. The nonstructural proteins seem to be critical for viral replication, which include the NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase.⁴⁰

As with most other RNA viruses, HCV is characterized by significant genetic heterogeneity, which results from the high viral replication during its life cycle and the accumulation of genetic mutations due to the error-prone replication system. This so-called quasi-species of mixed virus populations may confer a significant survival advantage, because the simultaneous presence of multiple variants allows rapid selection of mutants better suited to new environmental conditions. Currently, seven major genotypes of HCV have been identified along with more than 80 sub-genotypes. Genotypes are classified based on nucleotide sequence diversity of more than 30%.⁴¹ Genotypes 1, 2, and 3 are widely distributed around the world; genotypes 4 and 5 have been identified mainly in Africa, while genotype 6 has been found locally in Southeast Asia.^{41, 42} In Thailand, approximately 2.15% of the general populations have been infected chronically with HCV. Regarding HCV

genotypes, approximately 50-60% of patients are infected with HCV genotype 3, while approximately 30-40% and 10-20% are infected with genotype 1 and 6, respectively.⁴³

HCV genotype 6 displays pronounced genetic diversity. Novel subtypes of genotype 6 have been discovered continuously and now comprise at least 22 subtypes, 6a–6v. These may represent the evolution of this genotype for a considerable time span.⁴⁴ Accumulation of nucleotide mutations may drive continual development of HCV genetic diversity. This genotype variability appears to occur at a higher frequency in Vietnam and Myanmar than in Thailand. Currently, epidemiological data on the subtypes of HCV-6 are still limited. Thus, it is important to determine the genetic variability of these variants among Thai patients chronically infected with the virus.

Natural history of chronic HCV infection

Chronic HCV infection is a global health problem, affecting approximately 170 million individuals worldwide. In the last few years, the natural history of chronic HCV infection has been better understood. Chronic HCV infection is characterized by inflammation of the liver, progressive fibrosis of variable degrees, long-term progression to cirrhosis and eventually HCC development.⁴⁵ Indeed, the progression of fibrosis determines the ultimate prognosis of the patients and thus needs effective antiviral therapy. The major factors known to be associated with fibrosis progression in patients with chronic HCV infection are older age at infection, male gender and excessive alcohol drinking. In addition, progression of fibrosis is more rapid in immunocompromised patients. Recently, the importance of hepatic steatosis associated with obesity and diabetes has been increasingly recognized and studies are in progress to elucidate the relationship between metabolic disorders, insulin resistance, HCV replication and progression of fibrosis. In contrast to chronic HBV infection, viral load and genotype do not seem to significantly influence the progression rate in patients with chronic HCV infection.⁴⁶

Regarding HCV genotypes, studies on the effect of viral genotype on disease progression have always been conflicting. For example, some studies showed that genotype 1b posed a higher risk for developing HCC,⁴⁷ while other reports showed the risk of HCC among genotypes 1–3 to be rather similar.⁴⁸ Most studies, however, very few patients with HCV genotype 6 were recruited. Thus, there is scarcity in literature on the risk factors and natural history of HCV genotype 6, and its comparison to other HCV genotypes.

Treatment of chronic HCV infection

Prevention of HCV-associated complications can be achieved by antiviral therapy based on the use of a combination of PEG-IFN and ribavirin.⁴⁹ While patients with genotype 2 and 3 have higher sustained virological response (SVR) rates to 80-90% after a 24-week treatment, patients with genotype 1 generally have low SVR rates of only 40-50% after a 48-week course of therapy.⁴⁹ Response to treatment in patients with genotype 6 may be at an intermediate level between that seen with genotype 1 and genotype 2 or 3.⁵⁰ To date, however, no large randomized trials of genotypes 6-infected patients have been conducted. Furthermore, the optimal duration of treatment (24 vs 48 weeks) for HCV genotype 6 is unclear. Despite the overall improved response to this combination therapy, more than 75% of patients suffer from treatment-related adverse events and the costs remain high, which make individualized therapy of paramount importance to maximize treatment response and minimize adverse events.

HCV viral kinetics with IFN-based therapies has been studied recently to evaluate patients' responses.⁵¹⁻⁵³ Rapid early viral kinetics has been shown to have favorable SVR rates, which make shorter treatment duration possible. Typically, treatment with IFN-based therapy produces a biphasic decline in serum HCV RNA levels. The first phase noted in most patients is depending on the IFN dose and lasts approximately 1-2 days.⁵⁴ The second phase varies from days to months, and is assumed to reflect clearance of productively infected cells. Although the second-phase decline slope is considered the best predictor of an SVR, it has been suggested that a strong viral drop during the first phase is necessary to achieve a rapid second-phase decline.⁵⁵ During the initial first phase, HCV RNA may fall by 1-2 log₁₀ IU/mL in genotype 1 infected patients and by as much as 3-4 log₁₀ IU/mL in genotype 2 infected patients. Non-responders to the therapy may have no first-phase or second-phase decline (null response), or instead may have a first-phase decline followed by little or no second-phase decline (flat response).⁵⁶

From these concepts,⁵⁷ it is possible to calculate the kinetic parameters of HCV by mathematic models. Patients who fail to reach an early virological response (EVR), which is defined as a drop in HCV RNA levels of at least 2 log₁₀ IU/mL after 12 weeks of therapy, are unlikely to have an SVR.⁵⁸ Recently, testing for rapid virological response (RVR), which is defined as an detectable level of HCV RNA at 4 weeks of treatment, has been shown to offer further prediction for the individualization of therapy according to treatment-related viral kinetics.⁵⁹ A better kinetic pattern (a rapid, pronounced decline in viral load) could be

interpreted as a good virus-host-drugs interaction whereas a worse pattern (a slow decline) could require other therapeutic approaches, such as the prolongation of treatment.⁶⁰ Currently, there is limited information on the viral kinetics of HCV genotype 6. Therefore, it seems to be very useful to evaluate the HCV kinetics during PEG-IFN and ribavirin therapy in order to define the optimal duration of treatment for patients infected with genotype 6 by comparison to those of genotype 1 and 3.

Host factors associated with HCV infection

The dominant role of the immunomodulator of IFN-based therapy in treating HCV infection has driven extensive investigations for genetic associations between the immune system and outcome of therapy. Many studies have focused on the HLA class I and II genes, and most, but not all, studies have found associations between particular alleles and outcome of IFN-based therapy. However, there is little overlap in the alleles identified by the various groups.⁶¹

A breakthrough in understanding host genetic associations with outcome of therapy occurred in 2009 when three independent GWAS demonstrated that polymorphisms around the *interleukin (IL)-28B* gene, which encodes IFN- λ 3, are strongly associated with treatment outcome for patients with HCV genotype 1.⁶²⁻⁶⁴ The strongest association is found for SNP rs12979860, located about 3 kb upstream of the IL28B coding region. Patients with a CC genotype at this SNP are more than twice as likely to achieve SVR as patients with a CT or TT genotype. The favorable association of the CC genotype is found in both European and African-American patients, and differential prevalence of the CC genotype explains approximately half of the twofold poorer response rate to treatment found in African Americans.⁶⁴ The same polymorphisms are subsequently associated with spontaneous clearance of HCV in untreated patients.⁶⁵ The link between *IL28B* genotype and HCV clearance may impact decisions regarding initiation of current therapy and the economics of treatment in patients with genotype 1. In contrast to the data on genotype 1, the available data on *IL-28B* polymorphisms as predictors of SVR in patients with genotype 2 or 3 HCV infection are rather conflicting.⁶¹

The association of SNPs with ribavirin-induced reduction in hemoglobin levels has also been assessed because treatment-induced hemolytic anemia is one of the most serious side-effects of IFN-based therapy. Genotypes of SNPs rs1127354 within the *inosine triphosphatase (ITPA)* gene that decrease the activity of the enzyme have been found to be

strongly associated with protection against anemia.⁶⁶ Other SNPs of interest identified by GWAS in patients with chronic HCV infection are the *ITPA/DDR1* variants, which are associated with IFN-induced thrombocytopenia in patients with genotype 1.⁶⁷ The roles of these SNPs in Thai patients treated with standard therapy, particularly those infected with genotype 6, are currently unknown.

2. Objectives

The aims of this research project were to

- 1) Determine host and viral factors in predicting treatment response of patients with chronic HBV infection
- 2) Determine host genetic variations associated with clearance of HBV infection
- 3) Determine host and viral factors and p53-R249S mutation associated with HCC
- 4) Determine molecular epidemiology and natural history of chronic HCV genotype 6 by comparison to those of genotype 1 and 3
- 5) Determine host genetic variations and viral factors associated with treatment response in patients with chronic HCV infection

3. Methodology

Blood and tissue samples

Blood samples (serum and peripheral blood mononuclear cells, PBMC) and tissue samples used in this project were obtained from patients with chronic viral hepatitis and HCC, who attended at King Chulalongkorn Memorial Hospital, Bangkok. All blood samples were collected and stored at -80°C until tested. Liver tissues from liver biopsy or surgical resection were stored in liquid nitrogen until used. In addition, formalin-fixed, paraffin embedded liver specimens obtained from patients with HCC were included.

The diagnosis of chronic hepatitis was based on the presence of prolonged elevation of serum ALT and/or histologically proven. The diagnosis of HCC was based on typical imaging studies and/or histopathology (fine needle aspiration, core liver biopsy or surgical resection) according to American Association for the Study of Liver Diseases (AASLD) guideline.⁶⁸ Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyper-attenuation at the arterial phase, hypo-attenuation at the portal phase in dynamic CT or MRI. In cases without typical imaging features, liver biopsy was performed to confirm the diagnosis of HCC.

Serological and virological assays

Qualitative HBsAg, HBeAg, anti-HBc and anti-HBs were determined by commercially available enzyme-linked immunosorbent assays (ELISA) (Abbott Laboratories, Chicago, IL). Anti-HCV was tested with a commercially available ELISA assay (Murex anti-HCV version 4.0, Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions. The quantification of HBsAg was performed using the ARCHITECT i2000SR (Abbott Diagnostic, Abbott Park, Chicago, IL) according to the manufacturer's specifications, as described previously.³⁵ The sensitivity of the assay ranges from 0.05 to 250 IU/mL. Samples with HBsAg titers beyond the upper range were diluted with phosphate buffered saline (PBS) into 1:10, 1:1000 and 1:10000 prior to further analysis. The quantification of HBeAg was performed using the ARCHITECT i2000SR (Abbott Diagnostic), a two-step immunoassay based on the use of chemiluminescence microparticles (CIMA), according to the manufacturer's instructions.⁶⁹

Serum HBV DNA was quantified using real-time polymerase chain reaction (PCR) (COBAS TaqMan HBV assay; Roche Diagnostics, Basel, Switzerland). Serum HCV RNA was assessed by real-time quantitative reverse transcription (RT)-PCR (COBAS TaqMan HCV assay; Roche Diagnostics, Basel, Switzerland), in accordance with the manufacturer's instructions.

DNA/RNA extraction from liver specimens

Extraction of the nucleic acids (DNA/RNA) from formalin-fixed, paraffin embedded liver specimens and PCR were performed as described previously.⁷⁰

HBV DNA preparation, amplification and direct sequencing

HBV DNA extracted from serum or tissue sample was incubated in lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/mL proteinase K) and phenol–chloroform-isoamyl alcohol extraction. The DNA pellet was re-suspended in 30 µl sterile distilled water and subjects to be amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ), using primers from interest gene regions as appropriate. For detecting the pre-S1, pre-S2 and S genes, the primers consisted of a forward primer Pre-S1 F (nt 2817–2838: 5'-TCACCATATTCTTGGGAACAAGA-3') and a reverse primer R4 (nt 689–668: 5'-ATGGCACTAGTAA CCTGAGCC-3'). For detecting the EnhII/BCP/PC (nt 1552–2053) regions, the primers consisted of a forward primer X101 5'-TCTGTGCCTTCTCATCTG-3' and a reverse primer CO2 5'-GTGAGGTGAACAATGTTCCG-3'. Two microlitres of DNA sample were combined

with a reaction mixture containing 20 μL of 2.5X Eppendorf MasterMix (Hamburg, Germany), 1 μM P1, 1 μM P2 and sterile water, in a final volume of 50 μL . PCR was performed under the following conditions: after an initial 2 min denaturation step at 94°C, 35 cycles of amplification were performed, each including 30 sec denaturation at 94°C, 30 sec annealing at 55°C and 30 sec extension at 72°C, followed by a final 10 min extension at 72°C. Each amplified DNA sample (10 μL) was added to loading buffer and run on a 2% agarose gel (FMC Bioproducts, Rockland, ME) at 100 Volt for 60 min. The 479-bp product stained with ethidium bromide on preparation was visualized on a UV transilluminator.

For automated DNA sequencing, the PCR products of interest were purified from the gel using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg, Germany) according to the manufacturer's specifications. The sequencing reaction was performed using the Gene Amp PCR System 9600 (Perkin-Elmer, Boston, USA). The sequencing products were subjected to a Perkin Elmer 310 Sequencer (Perkin-Elmer, Boston, USA). The results were analyzed and HBV genotypes were determined by BLAST analysis. HBV DNA sequences were also subjected to phylogenetic analysis.

For phylogenetic analysis, nucleotide sequences were multiply aligned by using the program CLUSTAL_X (version 1.83). Alignments were then fed into phylogenetic trees that were constructed for each subalignment by using the neighbor joining methods implemented by the MEGA program. The statistical validity of the neighbor joining methods was assessed by bootstrap re-sampling with 1000 replicates, as described previously.^{71, 72}

RT-PCR amplification and direct sequencing for HCV

Total RNA was extracted by the guanidinium method⁷³ and reverse transcribed into cDNA using random primers (Promega, Madison, WI) and M-MLV reverse-transcriptase (Promega, Madison, WI). For HCV RNA detection, the RNA fragment of the 5'UTR was amplified. Viral RNA positive samples were then selected for amplification of core and NS5B regions. Nested PCR amplification of 5'UTR and core region was performed as previously described.⁷³⁻⁷⁵ The NS5B region was amplified by nested PCR resulting in a 471-bp fragment, with outer primers, NS5B_F1 (CAATWSMMACBACCATCAT GGC, position 7999-8020), NS5B_R1 (CCAGGARTTTRACTGGAGTGTG, position 8805-8825) and inner primers, NS5B_F2 (GATGGGHHSBKCM TAYGGATTCC, position 8159-8181), NS5B_R2 (CATAGCNTCCGTGAANGCTC, position 8611-8630) (nucleotide numbering was according to reference strain H77, GenBank

accession number AF00906). First round-PCR was performed by mixing 3 µl of cDNA to a final volume of 25 µl PCR reaction mixture containing 5 pmol each of NS5BF1 and NS5BR1 primers, 200 µM dNTP, 1.5 mM Mg²⁺, and 1.25 units of Tag DNA polymerase. Samples were amplified under the following conditions: 3 min at 94 °C for initial denaturation followed by 40 cycles at 94 °C for 1 min., 49 °C for 1 min. and 72 °C for 1.30 min, and concluded by a final extension step at 72 °C for 7 min. For the second round, 0.5 µl of first round PCR product were added to a reaction mixture identical to the first round, except for the inner primer set, NS5BF2 and NS5BR2 (5 pmol each). The amplification reaction was performed under identical conditions to the first round, except for increasing the melting temperature for primer annealing to 52 °C. The PCR products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide and subsequently visualized under UV light.

After gel purification (Perfectprep Gel Cleanup Kit, Eppendorf, Hamburg, Germany), the amplicons from both core and NS5B were subjected to sequencing as described elsewhere.⁷⁴ The sequences were edited and assembled using Chromas LITE (v.2.01) (www.technelysium.com.au), BioEdit (v.5.0.9) (Ibis Therapeutics, Carlsbad, CA) and SeqMan (DNASTAR, Madison, WI). Sequence similarities between the sequences generated in this study were examined by using the BLASTN web program (<http://www.ncbi.nlm.nih.gov>).

The edited sequences were analyzed for HCV genotypes using the Viral Genotyping Tool (<http://www.ncbi.nlm.nih.gov>) and samples designated to genotype 6 were subjected to further analysis. Multiple sequence alignment was performed with the program CLUSTAL_X (version 1.83). Phylogenetic trees of HCV genotype 6 based on both core and NS5B sequences were constructed with MEGA software (v.4).⁷⁶ Neighbor-joining trees were generated using Kimura's two parameters and the confidence values were calculated based on bootstrap re-sampling tests multiplied by 1000.

Determination of SNPs genotypes

Genotypes of SNPs located within or around *IL-28B*, *HLA-DQ* and *HLA-DP* loci were determined based on PCR and sequencing as described previously.⁷⁷ For example, SNPs typing around *IL-28B* locus were determined by the following protocol. Human genomic DNA was extracted from 100 mL of PBMC samples using a QIAamp blood kit according to the manufacturer's instruction. To determine SNP genotype of rs8103142 and rs11881222 as well as rs8099917, specific sets of primers were designed within the *IL28B* gene and approximately 8-kb upstream of the non-coding region of the gene, respectively. Nested PCR

amplified a short fragment containing rs8103142, rs11881222 or rs8099917 using specific primer pairs. The amplification conditions for the three SNPs spanning regions was carried out under the following conditions: pre-incubation for 5 min at 94°C, followed by 40 cycles of three step holds (94°C for 30 s, 65°C for 30 s, and 72°C for 45 s) before final extension at 72°C for 7 min. The amplification products were subjected to electrophoresis in 2.5% agarose gel. Amplified fragments (~200 bp) were sequenced directly in both forward and reverse directions with Prism Big Dye (Applied Biosystems) on an ABI 3100 DNA automated sequencer. Genotyping was based on the chromatograms of nucleotide bases in positions 502 and 685 of rs8103142 and rs11881222, respectively (nucleotide numbering started at the ATG start codon of the *IL28B* gene). SNPs typing of rs8099917 were analyzed in the same criteria. The superimposed chromatogram signals at those three SNPs positions were interpreted as heterozygous genotype.

Liver stiffness measurement

After fasting for at least 2 hours, liver stiffness measurement was obtained from each patient using transient elastography (FibroScan, Echosens, Paris, France) according to the manufacturer's instructions. Results were recorded in kilopascals (kPa) as the median value of all measurements. The procedure was based on at least 10 validated measurements: the success rate (ratio between numbers of validated and total measurements) was over 60% and interquartile range was less than 30%.⁷⁸

Liver Histology

Liver biopsy was obtained before the start of therapy. Necroinflammation activity (A0=absent, A1=mild, A2=moderate, and A3=severe) and fibrosis stage (F0=absent, F1=portal fibrosis, F2=portal fibrosis with few septa, F3=septal fibrosis, and F4=cirrhosis) were assessed according to the METAVIR score.⁷⁹

Statistical analysis

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 21.0 for Windows, SPSS Inc., Chicago, IL). Student t test and Mann-Whitney U test were used to test continuous variables with normal and skewed distribution, respectively. Correlation analyses were performed after logarithmic transformation of data with skewed distributions and tested by Pearson's correlation analysis. Categorical variables

were tested by Chi-square test or Fisher's exact test. Statistical significance was defined by a P value of less than 0.05.

4. Results and Discussion

Interim analysis of randomized study comparing efficacy of PEG-IFN monotherapy versus combination with entecavir in HBeAg-negative chronic hepatitis B

(Abstract presented in APASL 2014)

The combination therapy of PEG-IFN and a potent antiviral drug might improve the response rate in patients with chronic hepatitis B. The aim of this study was to compare the efficacy of PEG-IFN plus entecavir and PEG-IFN alone in Thai patients with HBeAg-negative chronic hepatitis B. Total 106 treatment-naïve patients with HBeAg-negative chronic hepatitis B were randomly assigned to either combination of PEG-IFN alpha-2b (1.5 microgram/kg weekly for 48 weeks) and entecavir (0.5 mg/day for 48 weeks) (group 1) or PEG-IFN alpha-2b monotherapy (1.5 microgram/kg weekly for 48 weeks) (group 2). Serum HBV DNA and HBsAg levels were quantified using commercially available assays (Roche Diagnostics). Virological response (VR) was defined as HBV DNA level $<2,000$ IU/mL at week 72. There were 51 and 55 patients in groups 1 and 2, respectively. At baseline, no significant difference between groups 1 and 2 was observed with respect to sex, mean age (41.3 vs. 40.6 years), ALT level (58 vs. 60 IU/L), HBV DNA level (5.0 vs. 5.1 $\log_{10}$ IU/mL) and HBsAg level (3.3 vs. 3.4 $\log_{10}$ IU/mL). At week 48, the mean HBV DNA level in group 1 was significantly lower than that of group 2 (0.2 vs. 1.5 $\log_{10}$ IU/mL, respectively) ($p < 0.001$). However, there was no difference in mean HBsAg level between both groups (2.5 vs. 2.4 $\log_{10}$ IU/mL, $p = 0.532$). At week 72, there was no significant difference between groups 1 and 2 with respect to mean HBV DNA level (2.9 vs. 3.4 $\log_{10}$ IU/mL, $p = 0.144$) and HBsAg level (2.8 vs. 2.7 $\log_{10}$ IU/mL, $p = 0.503$). VR at week 72 was achieved in 29 (56.9%) of group 1 and 27 (49.1%) of group 2 ($p = 0.443$). HBsAg clearance was detected in 3 (5.9%) and 5 (9.1%) in groups 1 and 2, respectively ($p = 0.717$). In conclusion, PEG-IFN plus entecavir led to a higher rate of HBV DNA suppression than PEG-IFN monotherapy at the end of treatment. However, both treatment regimens were comparable on the basis of viral suppression, HBsAg reduction and HBsAg clearance at 24 week after the end of therapy.

Association of interferon-alpha gene polymorphisms with chronic HBV infection

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In this study, the association between the risk of chronic hepatitis B virus infection and the polymorphisms within promoter regions of IFN- α 1 and five genes was explored. This association study was performed on 180 Thai patients with chronic HBV infection (HCC=65 and non-HCC=115), 173 individuals with self-limited HBV infection and 140 healthy controls. Our results showed that the A allele of -1823G/A SNP within IFNA1 gene was significantly associated with an increased risk of chronic HBV infection as compared to healthy individuals and self-limited HBV group [OR (95% CI) =2.20 (1.51–3.19), $P=0.000014$ and OR (95% CI)=1.61 (1.12–2.33), $P=0.0073$, respectively]. The effect of A allele was similar to autosomal recessive in which the presence of AA genotype when compared to GG and GA conferred the OR of 2.79 (95% CI =1.72–4.52, $P=0.0000085$). By multifactor dimensionality reduction analysis, we found the interaction between IFNA5 (-2529) and IFNA1 (-1823) genes that gave the risk to chronic HBV infection, with the OR (95% CI) of the high-risk to low-risk group was 2.79 (1.77–4.40), $P<0.0001$. However, further study in functional significance is required.

Association of IFNAR2 and IL10RB genes in chronic HBV infection

(Published in Tissue Antigens 2013; 82: 21-5)⁸¹

In this study, we investigated the effects of two functional polymorphisms, type I interferon receptor 2 gene (IFNAR2)-F8S and interleukin-10 receptor subunit beta gene (IL10RB)-K47E, on chronic HBV infection. We included 227 Thai patients with chronic HBV infection (100 with HCC and 127 non-HCC), 170 individuals with self-limited HBV infection and 150 healthy controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to analyze these two SNPs. In this study, the C allele of IFNAR2-F8S was found to be significantly increased in chronic HBV patients when compared with healthy controls [odds ratio, OR (95% confidence interval, CI)=3.31 (2.11–5.21), $P=6.214 \times 10^{-9}$ and corrected P-value, $P_c=1.864 \times 10^{-8}$]. The effect of this allele was similar to that of an autosomal dominant gene in the presence of CC and CT genotype, when compared to TT with an OR of 4.02 ($P=4.631 \times 10^{-9}$ and $P_c=1.389 \times 10^{-8}$). Furthermore, AA genotype of IL10RB-K47E was found to be significantly decreased in chronic HBV patients compared with individuals with self-limited HBV infection ($P=0.006$, $P_c=0.018$ and OR=0.45). For haplotype analysis, we found CA and CG haplotypes were associated with susceptibility to chronic HBV ($P=0.014$, OR=6.84 and $P=0.002$, OR=3.75, respectively) when compared with healthy individuals. This study suggests that IFNAR2-F8S polymorphisms might be involved in

the susceptibility to chronic HBV infection. Moreover, AA genotype of IL10RB-K47E may provide a protective effect in this disease. However, an association study using a larger sample size should be performed to confirm these findings.

Genetic association of Human Leukocyte Antigens (HLA) with chronicity or resolution of HBV infection

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Previous studies showed that SNPs in the HLA-DP, TCF19 and EHMT2 genes may affect the chronic hepatitis B (CHB). To predict the degree of risk for chronicity of HBV, this study determined associations with these SNPs. The participants for this study were defined into 4 groups; HCC (n = 230), CHB (n = 219), resolved HBV infection (n = 113) and HBV uninfected subjects (n = 123). The HLA-DP SNPs (rs3077, rs9277378 and rs3128917), TCF19 SNP (rs1419881) and EHMT2 SNP (rs652888) were genotyped. Due to similar distribution of genotype frequencies in HCC and CHB, we combined these two groups (HBV carriers). The genotype distribution in HBV carriers relative to those who resolved HBV showed that rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, p<0.001 and OR = 0.47, p<0.001). The other SNPs rs3128917, rs1419881 and rs652888 were not associated with HBV carriers. In conclusion, genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population.

Decline in serum 25-OH vitamin D levels in HIV- HBV co-infected patients after long term antiretroviral therapy

(Abstract presented in 7th IAS Conference on HIV Pathogenesis and Treatment 2014 and full article published in Antivir Ther 2014; 19: 41-9)⁸³

Vitamin D insufficiency plays an important role in the development of fibrosis in chronic liver disease. We assessed vitamin D status in HIV/HBV co-patients prior to and after HBV-active ART that included tenofovir. Correlation of Vitamin D levels and liver fibrosis was also assessed. HIV/HBV co-infected patients were enrolled in a prospective cohort study in Bangkok, Thailand. Liver fibrosis was assessed by transient elastography, Significant fibrosis was defined as a fibrosis score of >7.1 kPa. Serum 25 hydroxy vitamin D [25 (OH) D] < 30ng/ml was defined as hypovitamin D. Factors related to 25(OH)D levels or significant liver fibrosis were determined by logistic regression analysis. A total of 158 HIV/HBV patients (33% female) were included with a median age of 42 years old. 92% were on TDF-containing ART

(96%, 35%, 65 % with 3TC, bPI, NNRTI, respectively) with a median CD4 of 509 cells/mm³ and 89% had HIV RNA < 50 copies/ml. Overall, liver disease was mild with 12% having a fibrosis score > 7.5 kPa and 2% > 14 kPa. Median duration on tenofovir was 5 (IQR 3-7) years. The median eGFR was 94.2 ml/min/1.732. The median serum 25(OH)D level prior to and following tenofovir was 24.8 (IQR 21.3-30.6) ng/ml, and 22.8(IQR 18.0-27.7; p=< 0.001) ng/ml respectively. The proportion of patients with hypovitamin D significantly increased from 72.2% prior to tenofovir to 84.2% after tenofovir, p 0.004. Factors associated with hypovitamin D by multivariate analysis were female gender [adjusted odd ratio: OR (95% confidential interval: 95% CI : 3.6 (1.2-11.1), p=0.026); and duration of ART >5 years OR 2.6 (IQR 1.1-6.4), p=0.039. Vitamin D levels were not associated with significant liver fibrosis. In conclusions, although, our cohort of HIV/HBV co-infected patients live in the tropics , there was a high prevalence of hypovitamin D, especially in female patients and with prolonged ART. Given HIV/HBV co-infection requires long term HBV-active drugs including tenofovir that can also contribute to bone loss, routine vitamin D assessment and supplementation as necessary should be considered in all HIV-HBV co-infected patients.

Single nucleotide polymorphism of KIF1B (rs17401966) was not associated with the development of HBV-related HCC in Thai patients

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HBV infection can become chronic, and if left untreated, it can progress to HCC. Thailand is endemic for HBV and HCC is one of the top five cancer causing deaths among Thai HBV-infected males. A SNP at the KIF1B gene locus, rs17401966, has been shown to be strongly associated with the development of HBV-related HCC. There are no Thai data on genotypic distribution and allele frequencies of rs17401966. Thai HBV patients seropositive for HBsAg (n=398) were divided into two groups: Case group (chronic HBV with HCC; n=202) and Control group (HBV carriers without HCC; n=196). rs17401966 was amplified by polymerase chain reaction (PCR) and analyzed by direct nucleotide sequencing. The genotypic distribution of rs17401966 for homozygous major genotype (AA), heterozygous minor genotype (AG) and homozygous minor genotype (GG) in the case group was 49.5% (n=100), 40.1% (n=81) and 10.4% (n=21), respectively. As for the control group, the genotypic distribution of rs17401966 was 49.5% (n=97), 42.3% (n=83) and 8.2% (n=16) for the homozygous major genotype (AA), heterozygous minor genotype (AG) and homozygous minor genotype (GG), respectively. Binary logistic regression was used to assess the

association between rs17401966 and the risk of developing HCC. rs17401966 was not statistically associated with the risk of HCC development in Thai chronic HBV patients (p -value = 0.998, OR = 1.00 and 95% CI = 0.68-1.48). In conclusion, the *KIF1B* gene SNP (rs174019660) investigated in this study showed no significant association with HBV-related HCC in Thai patients infected with HBV indicating that there must be other mechanisms or pathways involved in the development of HCC.

Associations of SNPs in signal transducer and activator of transcription 3 (STAT3) and STAT4 for risk of HCC in Thai patients with chronic hepatitis B

(Abstract presented in AASLD Meeting 2014)

Signal transducers and activator of transcription (STAT) proteins, a family of transcription factors that play pivotal roles in cytokine signaling pathways. Several evidences indicated that SNPs in STATs gene such as rs2293152 and rs1053004 at STAT3 and rs7574865 at STAT4 have been associated with chronic hepatitis B (CHB) induced (HCC. This study aims to describe the association between these SNPs and HCC in Thai patients with CHB. Study subjects were enrolled and divided into 3 groups including CHB-related HCC (n=192), CHB without HCC (n=200) and healthy controls (n=190). The rs2293152 and rs7574865 SNPs were genotyped using PCR-RFLP whereas the rs1053004 SNP was genotyped using allelic discrimination assays based on TaqMan real-time PCR. Data analysis revealed that the distribution of rs2293152 and rs1053004 at STAT3 and rs7574865 at STAT4 genotypes were in Hardy-Weinberg equilibrium ($P>0.05$). rs2293152 SNP on STAT3 gene was not significantly associated with the risk of HCC ($P>0.05$) whereas the CC genotype of rs1053004 SNP was significantly associated with an increased risk of HCC compared with the CHB without HCC (odds ratio=1.85, 95% confidence interval=1.00-3.43, $P=0.049$). In addition, the genotype of rs7574865 SNP at STAT4 (GG versus TT+GT) was significantly associated with an increased risk of HCC when compared with the healthy controls (odds ratio=1.71, 95% confidence interval=1.13-2.59, $P=0.011$). In conclusion, these findings provided important evidence that the rs1053004 SNP at STAT3 and rs7574865 SNP at STAT4 were significantly associated with HCC risk and might be used as a novel genetic marker for HCC in Thai population.

HBV genetic variations and TP53 R249S mutation in patients with HCC in Thailand

(Published in Asian Pac J Cancer Prev 2013; 14: 3555-9)⁸⁵

Chronic HBV infection and dietary exposure to aflatoxin B1 (AFB1) are major risk factors for HCC. The aim of this study was to evaluate the role of HBV genetic variations and R249S mutation of the p53 gene, a marker of AFB1-induced HCC, in Thai patients chronically infected with HBV. Sixty-five patients with HCC and 89 patients without HCC were included. Viral mutations and R249S mutation were characterized by direct sequencing and restriction fragment length polymorphism (RFLP) in serum samples, respectively. The prevalence of T1753C/A/G and A1762T/G1764A mutations in the basal core promotor (BCP) region was significantly higher in the HCC group compared to the non-HCC group. R249S mutation was detected in 6.2% and 3.4% of the HCC and non-HCC groups, respectively, which was not significantly different. By multiple logistic regression analysis, the present of A1762T/G1764A mutations were independently associated with the risk of HCC. Overall, the data suggested that A1762T/G1764A mutations were associated with the development of HCC in Thai patients.

Prevalence of aflatoxin induced p53 mutation at codon 249 (R249s) in HCC patients with and without HBsAg

(Published in Asian Pac J Cancer Prev 2013; 14: 7675-9)⁸⁶

A missense mutation in exon 7 (R249S) of the p53 tumor suppressor gene is characteristic of aflatoxin B1 (AFB1) exposure. AFB1 is believed to have a synergistic effect on HBV carcinogenesis. However, results of studies comparing R249S prevalence among patients are conflicting. The aim of this study was to determine the prevalence of the R249S mutation in HCC patients with or without positive HBsAg. Materials and Methods: Paraffin embedded liver tissues were obtained from 124 HCC patients who underwent liver resection and liver biopsy in King Chulalongkorn Memorial Hospital. Restriction fragment length polymorphism (RFLP) was utilized to detect the R249S mutation. Positive results were confirmed by direct sequencing. Results: Sixty four (52%) patients were positive for HBsAg and 18 (15%) were anti-HCV positive. 12 specimens tested positive by RFLP. Ten HCC patients (8.1%) were confirmed to be R249S positive by Sanger sequencing (AGG to AGT). Out of these 10, six were HBsAg positive, and out of the remaining 4, two were anti-HCV positive. The R249S prevalence among HCC patients with positive HBsAg was 9.4% compared to 6.7% for HBsAg negative samples. Patients with the R249S mutation were younger (55 ± 10 vs 60 ± 13 year-old) and tended to have a more advanced Edmonson-Steiner grade of HCC, although differences did not reach statistical significance. In conclusion, our study shows

moderate prevalence of aflatoxin B1-related p53 mutation (R249S) in HCC with or without HBsAg. HBsAg positive status was not associated with R249S prevalence.

Advanced liver fibrosis by transient elastography, Fibrosis 4, and ALT/ platelet ratio index among Asian hepatitis C with and without HIV infection: Role of vitamin D levels

(Published in J Gastroenterol Hepatol 2014; 29; 1706-14)⁸⁷

Vitamin D insufficiency plays an important role in liver fibrosis in HCV-infected patients. We assessed liver fibrosis by transient elastography and 25 hydroxy vitamin D [25(OH)D] status in HCV-infected patients, with (HIV/HCV) or without HIV co-infection (HCV) from Thailand. Fibrosis stage was defined as mild (<7.1 kPa); moderate (7.2–9.4 kPa); severe (9.5–14 kPa), and cirrhosis (>14 kPa). Hypovitamin D was defined as 25(OH)D<30 ng/mL. Logistic regression analyses were used to assess predictors for significant fibrosis. Serum 25(OH) D levels, HCV genotypes (GT), IL28B and HCV-RNA were assessed. A total of 331 HCV and 130 HIV/HCV patients were enrolled (70% male, 35% people who inject drugs [PWIDs]). HCV GT distribution was as follows: GT3 47%, GT1 34%, GT6 17%. IL-28B CC genotype (rs12979860) were found in 88% of HIV/HCV and 85% of HCV. In HCV, liver fibrosis was mild in 56.5%; moderate in 18.4%; severe in 12.4%; and cirrhosis in 12.7%. In HIV/HCV, these figures were 30.6%, 27.8%, 17.6%, and 24.1%, respectively. Patients with significant fibrosis were more often male, older, with HIV infection, hypovitamin D, and less likely to be infected with GT6. Factors associated with significant fibrosis by multivariate analysis were HIV infection (adjusted odd ratio [95% confidential interval]: 2.67, 1.20–5.93), $P=0.016$, Fib-4 score>1.45 (6.30, 2.70–14.74), $P<0.001$, and hypovitamin D (2.48, 1.09–5.67), $P=0.031$. GT 6 was less likely to have advanced liver fibrosis (0.17, 0.05–0.65), $P=0.01$. Conclusions: HIV infection, Fib-4 score>1.45, and hypovitamin D are strong and independent predictors for the presence of advanced fibrosis in our HCV-infected patients. These data highlight the urgent need of HCV treatment and vitamin D supplement in resource-limited settings.

Genetic History and Evolution of HCV 3a Infection in Thailand

(Published in Intervirology 2013; 56:284-94)⁸⁸

Approximately 2.2% of Thai population has been infected with HCV, 50% with the predominant subtype 3a (HCV-3a). This study has investigated the molecular epidemiology and epidemic history of HCV-3a in Thailand. Based phylogenetic analyses of 356 sequences in core and NS5B region, subtype 3a (38.5%) was most predominant followed by 1a (21%),

1b (13.8%), genotype 6 (19.9%) which comprises subtypes 6e (0.3%), 6f (11%), 6i (1.9%), 6j (1.9%) and 6n (4.8%) and 3b (5.6%). Patients most at risk of infection have a history of blood transfusion (33.1%) followed by tattooing (27.7%), IVDU (26.9%), unsafe medical injection (23.8%), and shaving performed by a barber (23.1%). The phylogenetic analysis indicated the existence of a specific cluster of HCV-3a strains in the Thai population. Molecular evolutionary analysis estimates the date of the most recent common ancestor (tMRCA) to the year 1914 (1874-1950) indicating that HCV-3a has been present in this area for approximately 100 years. The population genetic model of HCV-3a investigated by the Bayesian skyline plot showed that this particular strains spread during the 1960s-1970s, which coincides with the period of the Vietnam War (1955-1975) and the widespread use of injection stimulants introduced by the US army. The estimated history of HCV-3a epidemics in Thailand suggests that the future burden of HCV-related disease may increase over the next few decades.

Distribution of HCV genotype and SNPs of interleukin-28B (IL-28B) gene in HIV/HCV-co-infected Thai populations

(Abstract presented in the Eleventh International Congress on Drug Therapy in HIV Infection)

HCV infection remains a major silent killer, worldwide, particularly in resource poor settings where treatment of hepatitis C is mainly impossible. PEG-IFN plus RBV are the recommended treatment for patients with chronic hepatitis C genotype 2/3. Recent study revealed that treatment responses against HCV infection by PEG-IFN and RBV are significantly associated with the SNPs of IL-28B gene. There is limited data about the HCV genotype and SNPs of IL-28B in HIV-infected Thai population. Therefore, we aimed to investigate HCV genotype and the SNP patterns of the IL-28B gene in our HIV/HCV co-infection. Quantification of HCV RNA was done by a real-time polymerase chain reaction assay (Abbott with lower limit of detection of B12 copies/mL). HCV RNA-positive samples based on reverse transcriptase-polymerase chain reaction (RT-PCR) of the 5'UTR were amplified with primer specific for the core and NS5B regions. Nucleotide sequences of both regions were analyzed for the genotype by phylogenetic analysis. DNA sample was extracted from PBMCs or sera. Then SNPs within IL-28B gene were detected by TaqMan real-time PCR (rs8099917 and rs12979860). The data were analyzed by allelic discrimination (AD) software on the ABI-7900HT. Totally 60 HIV/HCV-co-infected patients were studied. Median HCV RNA were 5.8 log₁₀copies/mL, 70% of them had HCV RNA >100,000 copies/mL. After sequencing, the

phylogenetic analyses in this study showed that genotype 3 was the most prevalent in this population (56%); following by genotype 1 (30%) and 6 (13%). Approximately 4% of them had infected for both genotypes 1 and 3. For IL-28B at rs8099917 and rs12979860 position, 95% of them were major allele (T/T or C/C) and 5% were heterozygous (T/G or C/T). In conclusion, HCV genotype 3 is the most prevalent in our HIV/HCV co-infection. 95% of our patients have favorable IL-28B gene. These data are useful for further chronic hepatitis C treatment in Thailand.

IFNL3 (IL28B) and IFNL4 polymorphisms are associated with treatment response in Thai patients with hepatitis C genotype 1, but not with genotypes 3 and 6

(Published in J Med Virol 2014; 86: 1482-90)⁸⁹

Recent studies have shown an association between SNPs in the interferon lambda-3 (IFNL3 or IL-28B) and IFNL4 genes and treatment response to hepatitis C virus genotype 1 (HCV-1) infection. However, the importance of these SNPs for HCV genotype 3 (HCV-3), and particularly HCV genotype 6 (HCV-6) remains to be elucidated. We analyzed a cohort of 225 Thai individuals with chronic HCV infection treated with pegylated-interferon and ribavirin, of whom 69 (30.7%), 114 (50.7%) and 42 (18.6%) patients were infected with HCV-1, HCV-3 and HCV-6, respectively. DNA extracted from serum samples was analyzed for the SNPs: rs12979860 and rs469415590. Sustained virological response (SVR) rates to HCV-1, HCV-3 and HCV-6 were 68.1%, 85.1% and 78.6%, respectively. The distribution of CC, CT and TT genotypes of rs12979860 was 189(84%), 28 (12.4%) and 8 (3.6%), respectively, while the distribution of TT/TT, Δ G/TT and Δ G/ Δ G genotypes of rs469415590 was 192(85.3%), 28(12.5%) and 5(2.2%), respectively. The two SNPs were found to be in strong linkage disequilibrium. Significantly lower frequencies of the favorable genotypes of rs12979860 and rs469415590 (CC and TT/TT, respectively) were found in the HCV-1 group in comparison with the other groups. The favorable genotypes were significantly associated with rapid virological response (RVR) and SVR in patients with HCV-1. However, these genotypes were associated with only RVR but not SVR in the patients with HCV-3 and HCV-6. In conclusion, the SNPs: rs12979860 and rs469415590 were equally associated with the treatment outcome in patients with HCV-1. In contrast, the role of these SNPs in predicting treatment response was attenuated in patients with HCV-3 and HCV-6. Thus, identification of these SNPs may be useful only in patients with relatively difficult-to-treat HCV-1 infection.

Early viral kinetics during hepatitis C virus genotype 6 treatment according to IL28B polymorphisms

(Published in World J Gastroenterol 2014; 20: 10599-605)⁹⁰

To investigate interleukin-28B polymorphisms and early viral kinetics of hepatitis C genotype 6 in patients treated with PEG-IFN/RBV therapy. Sixty-five patients with chronic HCV infection treated with PEG-IFN/RBV were included, of whom 15 (23.1%), 16 (24.6%) and 34 (52.3%) patients were infected with hepatitis C genotype 1 (HCV-1), genotype 3 (HCV-3) and genotype 6 (HCV-6), respectively. Serum HCV-RNA levels were measured frequently during the first 4-week of therapy. DNA extracted from samples was analyzed for the IL28B SNP rs12979860 by polymerase chain reaction and direct sequencing. During the first 4-week of therapy, the mean viral decline in patients with HCV-6 ($5.55 \pm 1.82 \log_{10} \text{IU/mL}$) was comparable to that of patients with HCV-3 ($5.55 \pm 1.82 \log_{10} \text{IU/mL}$ vs $5.86 \pm 1.02 \log_{10} \text{IU/mL}$, $P=0.44$) and was significantly higher than patients with HCV-1 ($5.55 \pm 1.82 \log_{10} \text{IU/mL}$ vs $4.23 \pm 1.99 \log_{10} \text{IU/mL}$, $P=0.04$). In the HCV-6 group, the first phase (days 0-2) viral decline was significantly higher in patients with the favorable rs12979860 CC than with non-CC allele (2.46 ± 1.01 and $1.70 \pm 0.67 \log_{10} \text{IU/mL}$, respectively, $P=0.045$). A statistically insignificant decrease in the second-phase (days 7-28) was also found in patients with the CC than those with the non-CC allele (1.24 ± 0.64 and $0.80 \pm 0.65 \log_{10} \text{IU/mL/week}$, respectively, $P=0.172$). At baseline, the SNP genotype was an independent predictor of rapid virological response (RVR) but not of sustained virological response (SVR). In conclusion, in PEG-IFN/RBV therapy, the IL28B polymorphisms affect the early viral kinetics of HCV-6.

Role of interferon lambda-4 (IFNL4) and inosine triphosphatase (ITPA) polymorphisms in the treatment of Thai patients with chronic hepatitis C virus genotype 3

(Abstract presented in the Viral Hepatitis Congress 2014)

Recent studies have shown that IFNL4 and ITPA polymorphisms are associated with PEG-IFN plus RBV treatment response and RBV-induced anemia, respectively, in chronic hepatitis C virus (HCV) infection. However, their roles in Asian populations infected with HCV genotype 3 (HCV-3) remain unclear. In this study, 123 Thai individuals with chronic HCV-3 infection treated with PEG-IFN plus RBV for 24 weeks were included. DNA extracted from blood samples was tested for the IFNL4 ss469415590 and ITPA rs1127354. The associations of the polymorphisms and treatment response and hemoglobin reduction ($> 3 \text{ g/dL}$ at week 4 of therapy) were analyzed. The distribution of TT/TT, $\Delta\text{G}/\text{TT}$ and $\Delta\text{G}/\Delta\text{G}$ genotypes of

ss469415590 was 111(90.3%), 11(8.9%) and 1(0.8%), respectively, while CC, CA and AA genotypes of rs1127354 was 93 (75.6%), 26(21.1%) and 4 (3.3%), respectively. The overall rapid virological response (RVR) and sustained virological response (SVR) rates were 84.6% and 79.7%, respectively. 30 (24.4%) patients required reduction in RBV dosages. ss469415590 TT/TT genotype, when compared to non-TT/TT genotype, was significantly associated with an increased RVR rate (93.3% and 73.7%, respectively, $p=0.047$) but not with an increased SVR rate. rs1127354 CC genotype, when compared to non-CC genotype, was associated with higher proportion of hemoglobin reduction (29.0% and 10.0%, respectively, $p=0.035$). The ITPA polymorphisms showed no association with RVR and SVR rates. In Thai patients with chronic HCV-3 infection, IFNL4 polymorphism was associated with RVR but not SVR. ITPA polymorphism influenced the degree of anemia during therapy but not improved clinical outcomes.

The correlation between hepatitis C core antigen and HCV RNA levels with respect to HIV status, HCV genotype and IFNL4 polymorphism

(Abstract presented in the Viral Hepatitis Congress 2014 and full article submitted to Intervirology 2014)

Serum HCV core antigen (HCVcAg) concentrations correlate with HCV RNA levels in HCV mono-infected patients. Data in HCV/HIV co-infected patients are still limited. We aim to compare the use of HCVcAg measurement with respect to HIV status, HCV genotypes, interferon lambda-4 (IFNL4) polymorphism and clinical parameters. We analyzed an untreated cohort of 104 patients with HCV mono-infection and 85 patients with HCV/HIV co-infection. Serum HCVcAg was measured by a commercial chemiluminescent microparticle immunoassay. The presence of IFNL4 polymorphism ss469415590 was identified by real time PCR. $\log_{10}$ HCVcAg levels were significantly correlated with corresponding $\log_{10}$ HCV RNA levels ($r=0.889$, $p<0.001$), but not correlated with ALT levels and liver stiffness. The correlation between HCV RNA and HCVcAg was particularly high in co-infected patients and those with high viremia. Mean $\log_{10}$ HCVcAg concentration was significantly higher in co-infected patients than in mono-infected patients. Patients harboring TT/TT genotype of ss469415590 had significantly higher levels of $\log_{10}$ HCVcAg than those with non-TT/TT genotype. HCVcAg levels were similar across HCV genotypes. HCVcAg concentrations had an excellent correlation with HCV RNA levels, particularly in HCV/HIV co-infected individuals and

might be associated with *IFNL4* polymorphism. HCVcAg testing could be used as an alternative to HCV RNA assays in resource-limited settings.

HCV genotype 6: Virology, epidemiology, genetic variation and clinical implication

(Review article: Published in World J Gastroenterol 2014; 20: 2927-40)⁹¹

HCV is a serious public health problem affecting 170 million carriers worldwide. It is a leading cause of chronic hepatitis, cirrhosis, and liver cancer and is the primary cause for liver transplantation worldwide. HCV genotype 6 (HCV-6) is restricted to South China, South-East Asia, and it is also occasionally found in migrant patients from endemic countries. HCV-6 has considerable genetic diversity with 23 subtypes (a to w). Although direct sequencing followed by phylogenetic analysis is the gold standard for HCV-6 genotyping and subtyping, there are also now rapid genotyping tests available such as the reverse hybridization line probe assay. HCV-6 patients present with similar clinical manifestations as patients infected with other genotypes. Based on current evidence, the optimal treatment duration of HCV-6 with pegylated interferon/ribavirin should be 48 wk, although shortened treatment duration of 24 wk could be sufficient in patients with low pretreatment viral load who achieve rapid virological response. In addition, the development of direct-acting antiviral agents is ongoing, and they give high response rate when combined with standard therapy.

5. Conclusion

Chronic viral hepatitis is a major public health problem worldwide that can lead to serious liver diseases such as cirrhosis and HCC. Several viral and host factors appear to strongly influence the clinical outcome and treatment response of infected individuals. In case-control studies, our data showed that SNPs in several candidate genes were associated with HBV clearance and HCC development. In addition, viral genetic factors such as mutations were associated with hepatocarcinogenesis in Thai patients. These findings provide important evidences for recognizing genetic markers of HBV-associated HCC and clearance. However, further studies of different cohorts are warranted to validate these findings.

Our study showed that HIV infection, and hypovitamin D were strong and independent predictors for the presence of advanced fibrosis in HCV-infected patients. These data highlight the urgent need of HCV treatment and vitamin D supplement in resource-limited settings. Regarding treatment of chronic HCV infection, SNPs in the *IFNL3* and *IFNL4* genes were strongly associated with the treatment response in genotype 1, but

not genotypes 3 and 6. Analysis of these SNPs may therefore be useful to guide treatment for Thai patients infected with HCV genotype 1 who will benefit from shorten or extended treatment duration and identify those who will encounter a treatment failure. In addition, ITPA genetic variants associated with adverse treatment effects could influence the efficacy of the current standard of care. Thus, these findings may have the potential to support the individualized treatment strategy in Thai patients with chronic HCV infection.

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Output

Publications from the project: 15 published articles, 1 revised manuscript and 8 abstracts

1. Tangkijvanich P, Sa-nguanmoo P, Avihingsanon A, Ruxrungtham K, Poovorawan K, Poovorawan Y. Characterization of Hepatitis B Virus Mutations in Untreated Co-infected Patients with HIV and HBV Based on Complete Genome Sequencing. *J Med Virol* 2013; 85: 16-25. [impact factor (IF) 2013=2.217]
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3. Thongbai C, Sa-nguanmoo P, Kranokpiruk P, Poovorawan K, Poovorawan Y, Tangkijvanich P. Hepatitis B Virus Genetic Variations and TP53 R249S Mutation in Patients with Hepatocellular Carcinoma in Thailand. *Asian Pac J Cancer Prev* 2013; 14: 3555-9. (IF=1.500)
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9. Chittmittrapap S, Chieochansin T, Chaiteerakij R, Treeprasertsuk S, Klaikaew N, Tangkijvanich P, Komolmit P, Poovorawan Y. Prevalence of Aflatoxin Induced p53

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 11. Thong VD, Akkarathamrongsin S, Poovorawan K, Tangkijvanich P, Poovorawan Y. Hepatitis C virus genotype 6: virology, epidemiology, genetic variation and clinical implication. *World J Gastroenterol* 2014; 20: 2927-40. (IF=2.433)
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 15. Akkarathamrongsin S, Payungporn S, Thong VD, Poovorawan K, Prapunwattana P, Poovorawan Y, Tangkijvanich P. Early viral kinetics during hepatitis C virus genotype 6 treatment according to IL28B polymorphisms. *World J Gastroenterol* 2014; 20: 10599-605. (IF=2.433)
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17. Avihingsanon A, Mek-A-nantawat W, Apornpong T, Akkarathamrongsin S, Ubolyam S, Chomhong P, Tangkijvanich P. Distribution of HCV genotype and single nucleotide polymorphisms (SNPs) of IL-28B gene in HIV/HCV-co-infected Thai populations (Abstract).
18. Tangkijvanich P, Chittmittraprap S, Poovorawan K, Poovorawan Y. Interim analysis of randomized study comparing efficacy of peginterferon-alpha-2b monotherapy versus combination with entecavir in HBeAg-negative chronic hepatitis B (Abstract).
19. Tangkijvanich P, Chittmittraprap S, Poovorawan K, Poovorawan Y. A randomized study comparing efficacy of peginterferon-alpha-2b monotherapy versus combination with entecavir in HBeAg-negative chronic hepatitis B: Analysis of week 72 (Abstract).
20. Poovorawan K, Chirathaworn C, Tangkijvanich P, Komolmit P, Poovorawan Y. Serum levels of interferon-gamma-inducible protein 10 (IP-10) in each phases of chronic hepatitis B and response to peginterferon therapy in HBeAg-positive and HBeAg-negative chronic hepatitis B (Abstract).
21. Khemnark S, Avihingsanon A, Tangkijvanich P, Kullavanijaya P, Treeprasertsuk S. Higher Rate of liver fibrosis progression in untreated chronic hepatitis C with high baseline FIB-4 $\geq$ 1.45: A prospective study (Abstract).
22. Chanthra N, Payungporn S, Chuaypen N, Tangkijvanich P. Associations of single nucleotide polymorphism rs1053004 in signal transducer and activator of transcription 3 (STAT3) for risk of hepatocellular carcinoma in Thai patients with chronic hepatitis B (Abstract).
23. Akkarathamrongsin S, Thong VD, Poovorawan Y, Tangkijvanich P. Role of interferon lambda-4 and inosine triphosphatase polymorphisms in the treatment of Thai patients with chronic hepatitis C virus genotype 3 (Abstract).
24. Limothai U, Akkarathamrongsin S, Poovorawan Y, Tangkijvanich P. IFNL3 (IL28B) and IFNL4 polymorphisms are not associated with treatment response to pegylated interferon-based therapy in Thai patients with HBeAg-negative chronic hepatitis B (Abstract).

รางวัลที่เกี่ยวข้องกับโครงการวิจัย

ศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์ หัวหน้าโครงการวิจัยได้รับรางวัล

“นักวิจัยดีเด่นแห่งชาติ สาขาวิทยาศาสตร์การแพทย์” ปี พ.ศ. 2556 จากสภานักวิจัยแห่งชาติ

การนำผลงานวิจัยไปใช้ประโยชน์

ข้อมูลจากงานวิจัยบางส่วนได้นำมาเรียบเรียงเป็นหนังสือสำหรับบุคลากรทางการแพทย์และประชาชนทั่วไปเรื่อง “ไวรัสตับอักเสบบี: รู้เท่าทัน ป้องกันมะเร็งตับ” และ “การตรวจทางวิทยาภูมิคุ้มกันของไวรัสตับอักเสบบี”

การได้รับเชิญเป็นวิทยากร

1. วิทยากรบรรยายเรื่อง “Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH): What’s the difference? What’s the treatment?” ให้แก่เภสัชกรและนักวิทยาศาสตร์ของบริษัท Rottapharm (Thailand) ในวันที่ 24 สิงหาคม 2555 กรุงเทพฯ
2. วิทยากรบรรยายเรื่อง “โรคตับกับการดื่มสุรา” ในการฝึกอบรมแพทย์ พยาบาลและเจ้าหน้าที่ทางด้านการแพทย์ บริษัทไทยประกันชีวิต จำกัด ในวันที่ 26 กันยายน 2555 สำนักงานใหญ่ บริษัทไทยประกันชีวิต จำกัด กรุงเทพฯ
3. วิทยากรบรรยายเรื่อง “High toward cure for management of HBeAg positive patients” ในงานประชุม “Table discussion: Treatment challenges of chronic hepatitis B in a modern era” ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในวันที่ 28 กันยายน 2555 โรงแรมเดอะเซนตริจีส กรุงเทพฯ
4. วิทยากรบรรยายเรื่อง “Management of hepatocellular carcinoma: An interesting case” ในงานประชุม “The Third HCC Management: Medical, surgical and radiology Integration Asia Pacific Live Virtual Exchange Meeting” ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในวันที่ 3 พฤศจิกายน 2555 โรงแรมพลาซ่า แอทธินี กรุงเทพฯ
5. วิทยากรบรรยายเรื่อง “Practical guideline for management of chronic hepatitis B” ให้แก่หน่วยงานเภสัชกรรม โรงพยาบาลจุฬาลงกรณ์ ในวันที่ 30 พฤศจิกายน 2555 โรงพยาบาลจุฬาลงกรณ์
6. วิทยากรบรรยายเรื่อง “Nucleos(t)ide therapy: When to stop therapy?” ในงานประชุม Optimising clinical outcomes in the management of naïve CHB patients ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในวันที่ 2 ธันวาคม 2555 โรงแรม Cap Dara พัทยา จังหวัดชลบุรี
7. วิทยากรบรรยายเรื่อง “Epidemiology and natural history of HCV and HIV-HCV co-infection” ในงานประชุมอบรมระดับนานาชาติ TREATASIA: Regional Training on Management of HIV-Hepatitis C co-Infection ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร แพทย์ผู้เชี่ยวชาญโรคติดเชื้อและพยาธิวิทยา ในวันที่ 20 กุมภาพันธ์ 2556 โรงแรม Grande Centre Point Hotel and Residence กรุงเทพฯ
8. วิทยากรบรรยายเรื่อง “รู้เท่าทันป้องกันไวรัสตับอักเสบบี” ให้แก่บุคลากรขององค์การสะพานปลาและประชาชนผู้สนใจ ในวันที่ 8 มีนาคม 2556 องค์การสะพานปลา กรุงเทพฯ

9. วิทยากรบรรยายเรื่อง “Interim analysis of randomized study comparing the efficacy of PEG-IFN α -2b monotherapy versus combination with entecavir in HBeAg-negative CHB: Role of host and viral factors associated with treatment response” ในการประชุมวิชาการประจำปี ครั้งที่ 8 “Common and Challenging Consultation in Hepatology” ของสมาคมโรคตับแห่งประเทศไทย ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในวันที่ 22 มีนาคม 2556 ณ โรงแรมระยอง แมริออท รีสอร์ท แอนด์ สปา จังหวัด ระยอง
10. วิทยากรบรรยายเรื่อง “HBV Reactivation in Patients Receiving Chemotherapy” ในการประชุมวิชาการประจำปี ครั้งที่ 8 “Common and Challenging Consultation in Hepatology” ของสมาคมโรคตับแห่งประเทศไทย ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในวันที่ 22 มีนาคม 2556 ณ โรงแรมระยอง แมริออท รีสอร์ท แอนด์ สปา จังหวัด ระยอง
11. วิทยากรบรรยายเรื่อง “ไวรัสตับอักเสบบี: รู้เท่าทันป้องกันมะเร็งตับ” ให้แก่บุคลากรของการสื่อสารแห่งประเทศไทย (กสท.) และประชาชนผู้สนใจ ในวันที่ 10 พฤษภาคม 2556 ที่ กสท. บางรักกรุงเทพ
12. วิทยากรบรรยายเรื่อง “The role of silymarin in liver disease” ให้แก่เภสัชกรของสมาคมร้านขายยาและประชาชนผู้สนใจ ในวันที่ 19 พฤษภาคม 2556 ที่โรงแรม แม่น้ำ ริเวอร์ไซด์ กรุงเทพ
13. วิทยากรบรรยายเรื่อง “การให้คำปรึกษาผู้บริจาคโลหิตที่ติดเชื้อ HBV และ HCV” ให้แก่เจ้าหน้าที่ภาคบริการโลหิตแห่งชาติ ในวันที่ 13 กรกฎาคม 2556 ที่ศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทย กรุงเทพ
14. วิทยากรบรรยายเรื่อง “Hepatitis Testing and Donor Counseling” ให้แก่เจ้าหน้าที่ภาคบริการโลหิตแห่งชาติ ในการประชุม The 5th NBC and RBC Laboratory Leaders Scientific Meeting ในวันที่ 23 สิงหาคม 2556 ที่โรงแรมวิรันดา อ. ชะอำ จังหวัดเพชรบุรี
15. วิทยากรบรรยายเรื่อง “Case Studies of Hepatitis B and Hepatitis C” ให้แก่แพทย์ประจำบ้านสาขาอายุรศาสตร์ ในการประชุม GI Board Review for Internist ในวันที่ 25 สิงหาคม 2556 อาคาร อบรม คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
16. วิทยากรบรรยายเรื่อง “Start Strong Stay Strong: Can We Reach the Ultimate Goal of CHB Therapy?” ให้แก่หน่วยงานเภสัชกรรม โรงพยาบาลจุฬาลงกรณ์ ในวันที่ 20 กันยายน 2556 โรงพยาบาลจุฬาลงกรณ์
17. วิทยากรบรรยายเรื่อง “Clinical Applications of HBsAg Quantification in Chronic Hepatitis B” ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในงานประชุมวิชาการประจำเดือน “Faculty Lecture 2013”ของหน่วยโรคทางเดินอาหาร ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในวันที่ 22 พฤศจิกายน 2556
18. วิทยากรบรรยายเรื่อง “Hepatic inflammation and fibrosis” ในการอบรม Basic science ของสมาคมแพทย์ระบบทางเดินอาหารแห่งประเทศไทย ให้แก่แพทย์ผู้เชี่ยวชาญโรคระบบทางเดินอาหาร ในวันที่ 17 มกราคม 2557 อาคารศูนย์การแพทย์โรงพยาบาลวิชัยยุทธ กรุงเทพฯ

19. วิทยากรบรรยายเรื่อง “ไวรัสตับอักเสบบี การรักษาและการป้องกัน” ในการประชุม “ไวรัสตับอักเสบบี ภัยเงียบใกล้ตัวคุณ” ให้แก่ผู้ติดเชื้อไวรัสตับอักเสบบีและผู้สนใจทั่วไป ในวันที่ 19 มีนาคม 2557 ที่ห้องประชุมอุดมสังวรญาณ ชั้น 23 รพ. เจริญกรุงประชารักษ์ กรุงเทพฯ
20. วิทยากรบรรยายเรื่อง “What’s new in management of chronic HBV infection” ในการประชุมวิชาการเรื่อง “Chula ID Weekend 2014” ให้แก่แพทย์สาขาโรคติดเชื้อและแพทย์ทั่วไป ในวันที่ 29 มีนาคม 2557 ห้องประชุมจรัญพัฒน์ ตึกโนติงเกล ชั้น 3 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
21. วิทยากรบรรยายเรื่อง “The Role of Combination Therapy with Pegylated Interferon and Nucleos(t)ide Analogues for Chronic Hepatitis B” ในการประชุมวิชาการ “Asian Pacific Hepatitis Summit (APHS) 2014” ให้แก่แพทย์นานาชาติ ในวันที่ 24 และ 25 พฤษภาคม 2557 ที่โรงแรม Le Royal Meridien Shanghai เมืองเซี่ยงไฮ้ ประเทศสาธารณรัฐประชาชนจีน
22. วิทยากรบรรยายเรื่อง “Liver Disease Research in Thailand: Opportunities & Challenges” ในการประชุมวิชาการ “10 ปี ชีวเคมีทางการแพทย์ จุฬา” จัดโดยภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในวันที่ 10 กรกฎาคม 2557 ห้อง 230 อาคารแพทยพัฒน์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
23. วิทยากรบรรยายเรื่อง “Treatment outcomes of Current HCV (PEG IFN/Ribavirin) Therapy” ในการประชุมสัมมนาเรื่อง “ความก้าวหน้าในการรักษาไวรัสตับอักเสบบี” จัดโดยศูนย์วิจัยโรคเอดส์ สภากาชาดไทย ในวันที่ 29 กรกฎาคม 2557 ณ อาคารเฉลิมพระเกียรติ ชั้น 7 ห้อง 7A ศูนย์วิจัยโรคเอดส์ สภากาชาดไทย
24. วิทยากรบรรยายเรื่อง “Overview of Liver Research at Chulalongkorn” ในการอภิปรายพิเศษ “University of Sydney and Faculty of Medicine, Chulalongkorn University International Symposium 2014-Joint Research towards Clinical Excellence” ในวันที่ 31 กรกฎาคม 2557 ถึงวันที่ 1 สิงหาคม 2557 ณ ห้อง 210 ตึกอนันตมหิตล ชั้น 2 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

การเชื่อมโยงทางวิชาการกับนักวิชาการต่างประเทศ

1. Professor Yasuhito Tanaka, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Science, Nagoya, Japan
2. Professor Masashi Mizokami, Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan
3. Professor Jacob George, Sydney University, Australia

บทความสำหรับการเผยแพร่

ไวรัสตับอักเสบบีและไวรัสตับอักเสบบีซีเป็นสาเหตุสำคัญของเกิดโรคตับอักเสบบแบบเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับตามลำดับ ในปัจจุบันประชากรทั่วโลกมีการติดเชื้อไวรัสตับอักเสบบีและไวรัสตับอักเสบบีซีมากกว่า 400 และ 170 ล้านคนตามลำดับ ในประเทศไทยมีความชุกของการติดเชื้อไวรัสตับอักเสบบีและไวรัสตับอักเสบบีซีประมาณร้อยละ 4 และ 2 ตามลำดับ ภาวะแทรกซ้อนของโรคตับที่เกิดจากการติดเชื้อไวรัสตับอักเสบบแบบเรื้อรัง เป็นปัญหาที่สำคัญอย่างยิ่งทางสาธารณสุขของประเทศเพราะก่อให้เกิดความสูญเสียทางด้านเศรษฐกิจและสังคมเป็นอย่างมาก ดังนั้นองค์ความรู้พื้นฐานต่างๆของไวรัสตับอักเสบบีและไวรัสตับอักเสบบีซี จึงมีความสำคัญต่อความเข้าใจธรรมชาติของโรคอย่างถูกต้อง ซึ่งจะนำไปสู่การวินิจฉัย การรักษาและการป้องกันโรคที่มีประสิทธิภาพยิ่งขึ้น

ผู้ติดเชื้อไวรัสตับอักเสบบีและไวรัสตับอักเสบบีซีแต่ละคนมีการดำเนินของโรคแตกต่างกัน ทั้งนี้อาจขึ้นอยู่กับปัจจัยของเชื้อไวรัสและของผู้ป่วย องค์ความรู้ต่างๆที่ได้จากการศึกษาวิจัยของโครงการเรื่อง "ปัจจัยของผู้ป่วยและเชื้อไวรัสที่สัมพันธ์กับการดำเนินโรคและการรักษาโรคตับอักเสบบแบบเรื้อรังจากไวรัสตับอักเสบบีและซี" ซึ่งได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัยเป็นเวลา 2 ปี ตั้งแต่ ตั้งแต่ 31 กรกฎาคม 2555 ถึงวันที่ 30 กรกฎาคม 2557 นี้ แสดงให้เห็นว่าความหลากหลายทางพันธุกรรมของยีนบางตำแหน่งของผู้ที่ติดเชื้อไวรัสตับอักเสบบีมีความสัมพันธ์กับการหายขาดหรือการดำเนินโรคต่อไปจนเป็นมะเร็งตับ นอกจากนี้การกลายพันธุ์ในบางตำแหน่งของเชื้อไวรัสมีความสัมพันธ์กับการเกิดมะเร็งตับ ส่วนการได้รับสารอะฟลาทอกซินจากเชื้อราซึ่งเป็นสารก่อมะเร็งพบได้น้อยในประชากรไทยที่เป็นโรคมะเร็งตับ การศึกษาในผู้ติดเชื้อไวรัสตับอักเสบบีพบว่าสายพันธุ์ของไวรัสที่พบได้บ่อยในประชากรไทยได้แก่สายพันธุ์ 3, 1 และ 6 ตามลำดับ ผู้ที่มีการติดเชื้อเอชไอวีร่วมด้วยมักมีการดำเนินที่รุนแรงกว่าผู้ติดเชื้อไวรัสตับอักเสบบีอย่างเดียว โดยมีความรุนแรงของโรคแตกต่างกันขึ้นกับสายพันธุ์ของไวรัส นอกจากนี้ยังพบว่าไวรัสตับอักเสบบีซีต่างสายพันธุ์มีการตอบสนองต่อการรักษาด้วยยาต้านไวรัสที่แตกต่างกัน การศึกษาความหลากหลายทางพันธุกรรมของยีนบางตำแหน่งของผู้ที่ติดเชื้อสามารถทำนายผลการรักษาและผลข้างเคียงจากการรักษาได้

ผลของการศึกษาวิจัยจากโครงการนี้นอกจากจะเป็นประโยชน์ในประชากรไทยโดยใช้เป็นข้อมูลในการเปรียบเทียบกับประชากรต่างๆทั่วโลกแล้ว ยังก่อให้เกิดองค์ความรู้ใหม่ๆเกี่ยวกับกลไกการเกิดโรค การพยากรณ์ความรุนแรงของโรคและการรักษาผู้ป่วยตับอักเสบบเรื้อรัง รวมทั้งจะเป็นประโยชน์ในการป้องกันและรักษาโรคมะเร็งตับในอนาคต

Characterization of Hepatitis B Virus Mutations in Untreated Patients Co-Infected With HIV and HBV Based on Complete Genome Sequencing

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Co-infection of HBV with HIV results in an accelerated course of HBV-associated chronic liver disease. Several studies have shown that viral mutations are related to disease progression in mono-infection with HBV. However, it is unclear whether HBV mutation patterns might differ between co-infected and mono-infected patients. To compare the frequencies and mutation patterns in the HBV genome between co-infection and mono-infection. Twenty-four treatment-naïve co-infected and 31 treatment-naïve mono-infected Thai patients were included. HBV mutations were characterized by whole genome sequencing of virus serum samples. The clinical features and frequency of known clinically significant mutations were compared between the two groups. No significant difference between the groups was found with respect to sex, age and HBeAg. However, HBV DNA levels were significantly higher in co-infected patients. The distribution of HBV genotypes was comparable between the two groups and restricted mostly to sub-genotypes C1 and B2. An isolate with recombinants of genotypes G/C1 was also identified in a patient with co-infection. There was no difference in the prevalence of mutations in the enhancer II/basal core promoter/precore region, *pre-S/S* and polymerase genes between the two groups. In conclusion, dual infections tend to engender increased HBV DNA levels. There was no major difference in the frequencies of common HBV mutations between co-infected and mono-infected patients. Thus, HBV mutations may not contribute to disease pathogenesis in Thai patients with co-infection. **J. Med. Virol. 85:16–25, 2013.** © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis B virus; HIV; mutations; genotypes; genome sequencing; recombination

INTRODUCTION

Co-infection with HIV and HBV is common because both viruses share routes of transmission through blood or blood products and sexual contact. There are approximately 40 and 400 million people worldwide infected chronically with HIV and HBV, respectively [McGovern, 2007; Thio, 2009]. An estimated 10% of HIV-infected individuals have chronic hepatitis B, with a higher prevalence found in areas where HBV infection is endemic including countries in Africa and

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Ethical approval: The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

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Asia. Following the introduction of HAART, liver disease has emerged as one of the most important causes of morbidity and mortality in HIV–HBV co-infected populations [Thio, 2009; Joshi et al., 2011]. It is well recognized that HIV infection can accelerate the course of HBV-associated liver disease, such as the progression of cirrhosis in co-infected individuals [Price and Thio, 2010]. In contrast, the association between HIV infection and the occurrence of HBV mutations is less well known.

HBV shows remarkable genetic variability and is classified currently into 10 genotypes, designated A–J based on genome sequence analysis [Liaw and Chu, 2009]. The virus has a high mutation rate compared with other DNA viruses as it lacks proofreading capacity during its replication via reverse transcription [Kay and Zoulim, 2007]. Several studies have shown that mutation patterns in the viral genome are associated significantly with progression of liver disease in HBV mono-infection. The most well known occurring naturally mutations include PC stop codon mutation (G1896A), which abolishes HBeAg production, and double BCP mutations (A1762T/G1764A), which down regulate HBeAg production. Another PC mutation is G1899A, which often occurs either alone or with the G1896A mutation. Recent data in HBV mono-infection have shown that the double BCP mutations are associated with more aggressive liver disease and the development of HCC [Wai and Fontana, 2004; Lin and Kao, 2008]. Other mutations such as T1753C/A/G in the BCP region and C1653T in the EnhII region have also been recognized as being associated with the outcome of chronic HBV mono-infection [Tanaka et al., 2006; Yuen et al., 2008]. Furthermore, mutations or deletions in the *pre-S1/pre-S2* gene have been shown to accumulate frequently in HBV mono-infected patients and are associated with an increased risk of HCC [Chen et al., 2007; Choi et al., 2007]. Whether these variants might be more found frequently in HIV–HBV co-infection has been investigated poorly. The aim of this study was to identify mutation patterns by complete genome sequencing of HBV among Thai patients co-infected with HIV compared to sex- and age-matched mono infected individuals.

MATERIALS AND METHODS

Study Patients

The co-infected patients enrolled in this study were part of a prospective observational study of all HIV-infected patients who participated in any clinical/observational trial of the HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT), Bangkok, Thailand from April 1999 to October 2008. All 24 individuals recruited for this study were chosen on the basis of serum availability and detectable HBV DNA level. To compare HBV genome sequences between co-infected and mono-

infected individuals, serum samples were also obtained from 31 patients with chronic HBV mono-infection, who were matched for age (± 5 years) and gender. These patients were selected randomly from a pool of patients with chronic liver disease who were followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during the same period.

None of the patients enrolled in this study had hepatitis C virus infection or received any antiviral therapy for chronic HBV infection when the serum sample was obtained. All patients were informed about the purpose of the study, and subsequently gave their written informed consent. Serum samples were collected and frozen at -70°C until further use. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

HBV Genome Sequencing

HBV DNA was extracted from 100- μl plasma samples using proteinase K in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation. The DNA pellets were dissolved in 30 μl sterile distilled water. Subsequently, serum samples were subjected to complete HBV genome amplification by PCR using four primer sets selected from conserved regions as previously described [Sa-Nguanmoo et al., 2008]. The primer sequences were as follows:

Set 1, PreS1F+ (5'-GGGTCACCATATTCTTGGAAC-3': position 2814 to 2835) and R5 (5'-AGCCCCAAA-GACCCACAATTC-3': position 1015 to 995);
Set 2, F6 (5'-ATATGGATGATGTGGTATTGGG-3': position 737 to 758) and X102 (5'-ACCTTTAACCTAATCTCC-3': position 1764 to 1748);
Set 3, X101 (5'-TCTGTGCCTTCTCATCTG-3': position 1552 to 1569) and CORE2 (5'-CCCACCTTATGAGTCCAAGG-3': position 2476 to 2457);
Set 4, CORE1 (5'-GAGTGTGGATTGCACTCCTCC-3': position 2268 to 2289) and R1 (5'-TGTAACACGAGCAGGGGTCCTA-3': position 201 to 180).

The PCR reaction mixture and the amplification method were performed as described previously [Sa-Nguanmoo et al., 2012]. The sequencing was performed by a commercial sequencing assay (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia). Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and submitted to the GenBank database (accession no. JQ801470–JQ801524).

Genotyping, Phylogenetic Analysis, and Mutation Analysis

The sequences were characterized for the genotyping assay by phylogenetic analysis as previously described [Sa-Nguanmoo et al., 2010]. Briefly, each sample's sequences from *pre-S1/pre-S2/S* and *preC/*

C regions were aligned with each available human genotype stored at the GenBank database (National Center for Biotechnology Information, Bethesda, MD) by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Based on these alignments, phylogenetic trees were constructed for genotyping using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ). Some sequences were genotyped by the Viral Genotyping Tool (National Center for Biotechnology Information). Genetic recombinants were further determined by SimPlot program and bootscanning analysis (Simplot Version 3.5.1, Baltimore, MD). The nucleotides were translated into amino acid sequences using the translation tool in ExPASy Proteomics Server (<http://www.expasy.ch/tools/dna.html>).

Complete genome sequences were evaluated for mutations and deletions in the EnhII/BCP/PC region, *pre-S/S* and polymerase genes. The amino acids at positions 120 and 160 of the S protein were indicative for “a” determinant mutations.

HBV DNA Quantitation

Serum HBV DNA level was measured by a commercial real-time method with a detection limit as low as 10 IU/ml (Roche Diagnostics, Tokyo, Japan) in accordance with the manufacturer’s instructions.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD), and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher’s exact test for categorical variables and by the Mann–Whitney *U*-test or Student’s *t*-test for quantitative variables. *P*-values < 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 17.0 (SPSS, Inc., Chicago, IL).

RESULTS

Demographic and Clinical Data of the Patients

The demographic data and clinical features of patients with co-infection and mono-infection are shown in Table I. Compared with the mono-infection group, patients with co-infection had higher significantly mean levels of HBV DNA. However, there was no significant difference between groups with respect to sex, age, serum ALT level and status of HBeAg (positive/negative).

Distribution of HBV Genotypes

Phylogenetic analysis was performed based on the *pre-S1/pre-S2/S* and *preC/C* genes (Fig. 1A,B). Among co-infected patients, 18 (75%) samples were determined as genotype C (sub-genotype C1) and 5 (20.8%) samples were identified as genotype B (sub-genotype B2). As for mono-infected patients, 24 (77.4%) and 7 (22.6%) samples were identified as genotype C (sub-genotype C1) and genotype B (sub-genotype B2), respectively. There was no statistical difference in the distribution of HBV genotypes between the two groups (Table II).

Based on the whole genome sequence, recombination of genotypes G and C1 was identified in a patient with co-infection. The recombination breakpoints were estimated at positions 46 (*pre-S2/pol* overlapping region), 760 (*S/pol* overlapping region), 1540 (*X/pol* overlapping region), and 2780 (*pol* gene; Fig. 2).

Prevalence and Characterization of EnhII/BCP/PC Mutations

Common mutations were found at nucleotides 1653, 1753, 1762, 1764, 1858, 1896, and 1899. The C1653T mutation/deletion was observed in 2 (8.3%) samples of co-infected patients but was not detected in mono-infected patients. The T1753C mutation/deletion was observed in 5 (20.8%) and 5 (16.1%) samples obtained from co-infected and mono-infected patients,

TABLE I. Demographic and Clinical Characteristics of Co-Infected and Mono-Infected Patients

Characteristics	Co-infection (n = 24)	Mono-infection (n = 31)	<i>P</i>
Age, years	36.4 $\pm$ 8.3	37.8 $\pm$ 7.7	0.517
Sex			
Male	18 (75)	23 (74.2)	0.598
Female	6 (25)	8 (25.8)	
ALT, U/L	100.4 $\pm$ 134.2	96.7 $\pm$ 61.1	0.902
HBeAg status			
Positive	13 (54.2)	16 (51.6)	0.534
Negative	11 (45.8)	15 (48.4)	
HBV DNA level, log ₁₀ IU/ml	7.0 $\pm$ 1.9	6.0 $\pm$ 1.5	0.039
CD4 count, cell/ μ l	243.0 $\pm$ 195.5		
HIV RNA level, log ₁₀ copies/ml	4.2 $\pm$ 1.3		
Risk factors for HIV infection			
Heterosexual	18 (75)		
Homosexual	6 (25)		

Data were expressed as mean $\pm$ SD, no. (%).

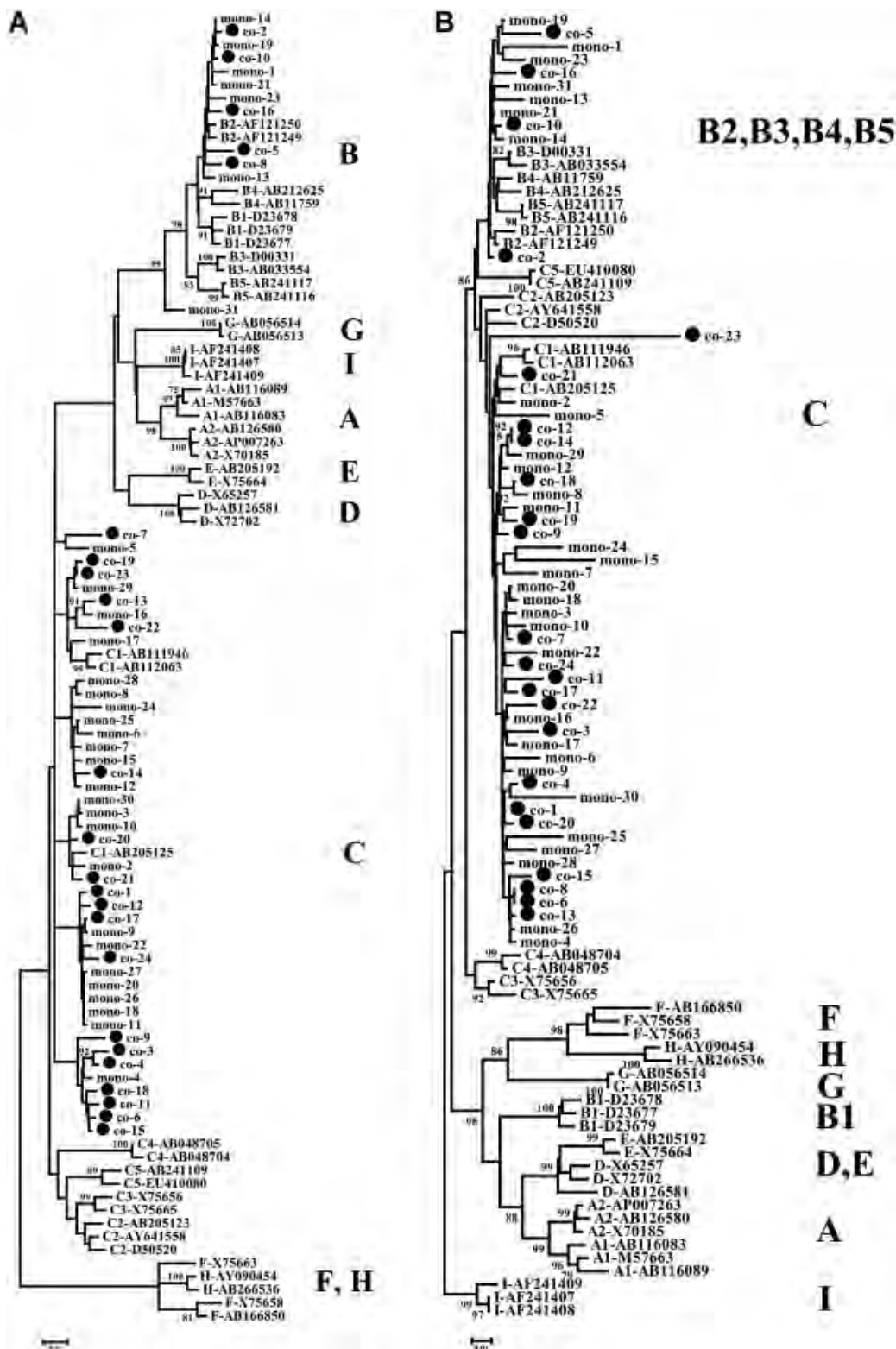


Fig. 1. Phylogenetic relationship of the sequence obtained in the present study and representative sequences of human HBV strains from GenBank. Regions included in the comparison were: (A) the *pre-S1/pre-S2/S* gene; (B) the *preC/C* gene, including nucleotide positions 1814–2452. Percentage bootstrap values (>75%) are shown at the respective nodes. The scale bar at the bottom indicates the genetic distance. The sequences in this study are indicated by symbols (●co-xx, co-infection; mono-xx, mono-infection).

TABLE II. Prevalence of HBV Mutations in Co-Infected and Mono-Infected Patients

Characteristics	Co-infection (n = 24)	Mono-infection (n = 31)	P
HBV genotype			0.517
B2	5 (20.8)	7 (22.6)	
C1	18 (75)	24 (77.4)	
G/C1	1 (4.2)	0	
C1653T/deletion	2 (8.3)	0	0.102
T1753C/deletion	5 (20.8)	5 (16.1)	0.654
A1762T/deletion	11 (45.8)	13 (41.9)	0.773
G1764A/deletion	12 (50)	13 (41.9)	0.551
T1858C/deletion	1 (4.2)	1 (3.2)	0.853
G1896A/deletion	6 (25)	11 (35.5)	0.404
G1899A/deletion	1 (4.2)	2 (6.5)	0.711
Pre-S1 mutation/deletion	4 (16.7)	4 (12.9)	0.695
Pre-S2 mutation/deletion	5 (20.8)	6 (19.4)	0.892
"a" determinant mutation	1 (4.2)	0	0.251
Drug resistance mutation			0.790
rtI233V	1 (4.2)	1 (3.2)	
rtV173L	0	1 (3.2)	

Data were expressed as no. (%).

respectively. The A1762T and G1764A mutations were found in 11 (45.8%) and 12 (50%) samples of co-infected patients, while the double mutations were found in 13 (41.9%) samples of mono-infected patients. The T1858C mutation was found in 1 (4.2%) and 1 (3.2%) sample of co-infected and mono-infected patients, respectively. The G1896A mutation was found in 6 (24.6%) and 11 (35.5%) samples of co-infected and mono-infected patients, respectively. In addition, The G1899A mutation was detected in 1 (4.2%) sample of co-infected patients and 2 (6.5%)

samples of mono-infected patients. There was no significant difference in the prevalence of these mutations between the two groups (Table II). Nucleotide sequence alignment of the EnhII/BCP/PC region of these 55 samples is shown in Figure 3.

Prevalence and Characterization of *pre-S/S* Mutations/Deletions

Pre-S1 mutations/deletions were detected in 4 (16.7%) and 4 (12.9%) samples of co-infected and

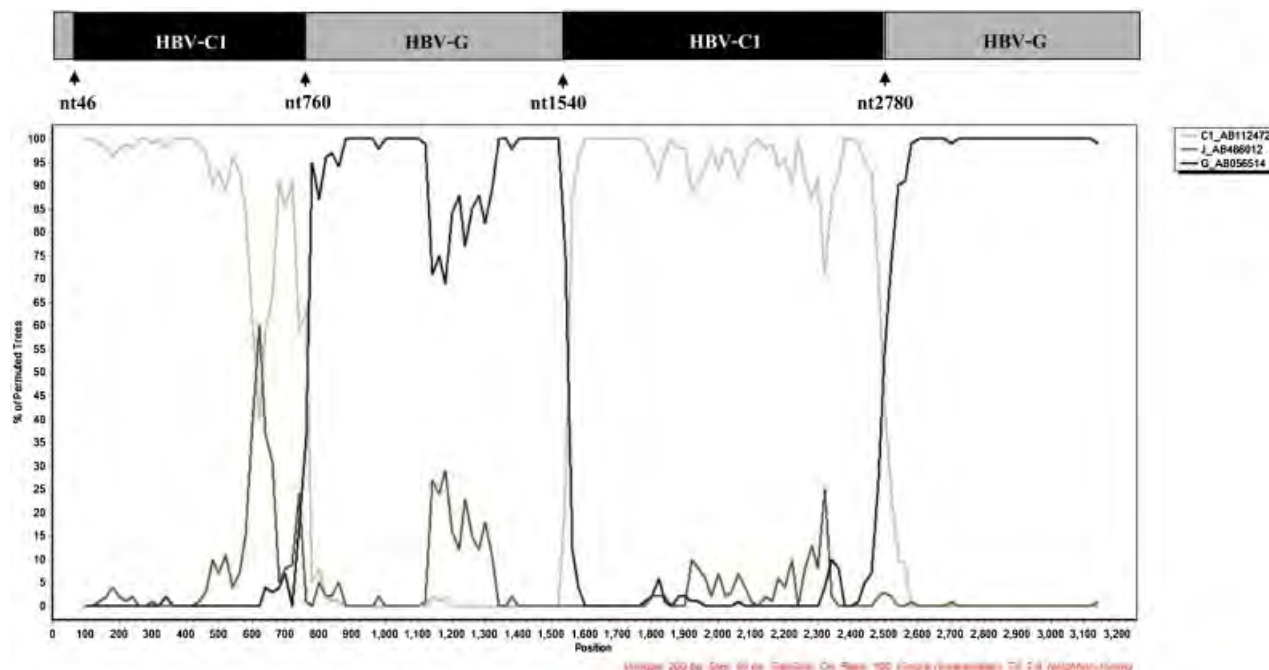


Fig. 2. Bootscanning analysis of the recombinant isolate. Dashed line(s) indicate(s) the breaking point(s) of recombination. The number above the dashed line indicates the nucleotide position of each isolate compared with the reference strain (accession no. C1-AB112472, G-AB056514) and the out-group strain (accession no. J-AB486012).

				nt 1651-1660	nt 1751-1780				nt 1851-1860	nt 1891-1900
		Genotype	Sex	C1653T	T1753C	A1762T/G1764A	TACTGGGAGG	T1858C	G1896A/G1899A	
Genotype C1				TACATAAGAG	GATTAGGTTA	AAGGTCTTTG		TACGTCCCAC	GCTTTGGGGC	
Genotype B2				.G.....			A.....	C.T....T..	A....	
CO 1	1	C1	M					C.T....T..		
CO 2	2	B2	F	.G.....	.G.....	.T.A.....	A.....	C.T....T..		
CO 3	3	C1	M	.T.....		.T.A.....		C.T....T..	A....	
CO 4	4	C1	M				AAG.....	C.T....T..		
CO 5	5	B2	M	.G.....	.G.....		A.....	C.T....T..	A....	
CO 6	6	C1	M					C.T....T..		
CO 7	7	G/C1	M		C.....			C.T....T..		
CO 8	8	B2	F					C.T....T..		
CO 9	9	C1	M		C.C.....	.T.A.....		C.T....T..	A....	
CO 10	10	B2	M	.G.....	.G.....		A.....	C.T....T..		
CO 11	11	C1	M		C.....			C.T....T..	A....	
CO 12	12	C1	M		C.C.....	.T.A.....		C.T....T..		
CO 13	13	C1	F			.T.A.....		C.T....T..		
CO 14	14	C1	M		C.C.....	.T.A.....		C.T....T..		
CO 15	15	C1	M			.T.A.....		C.T....T..		
CO 16	16	B2	M	.G.....	.G.....		A.....	C.T....T..	A....	
CO 17	17	C1	M					C.T....T..		
CO 18	18	C1	M				G.....	C.T....T..		
CO 19	19	C1	F		C.....			C.T....T..		
CO 20	20	C1	M		C.....	.T.A.....	A.....	C.T....T..		
CO 21	21	C1	M		G.....	.T.A.....		C.T....T..		
CO 22	22	C1	M	.T.....		A...AC.		C.T....T..	A....	
CO 23	23	C1	F	C.				C.T....T..		
CO 24	24	C1	M					C.T....T..	A....	
Mono 1	1	B2	M	.G.....	.G.....		A.....	C.T....T..	A....	
Mono 2	2	C1	M			.T.A.....		C.T....T..		
Mono 3	3	C1	M		C.....			C.T....T..		
Mono 4	4	C1	M			.T.A.....		C.T....T..		
Mono 5	5	C1	M		C.....	.T.A.....		C.T....T..	A....	
Mono 6	6	C1	M		C.....	.T.A.....		C.T....T..	A....	
Mono 7	7	C1	M		C.....	.T.A.....		C.T....T..	A....	
Mono 8	8	C1	M			.T.A.....	A.....	C.T....T..		
Mono 9	9	C1	M					C.T....T..		
Mono 10	10	C1	M		C.....	.T.A.....		C.T....T..	A....	
Mono 11	11	C1	M					C.T....T..		
Mono 12	12	C1	M					C.T....T..		
Mono 13	13	B2	M	.G..C.....	.G.....		A.....	C.T....T..	A..A..	
Mono 14	14	B2	M	.G.....	.G.....		A.....	C.T....T..		
Mono 15	15	C1	M					C.....T..	A....	
Mono 16	16	C1	M		C.....	.T.A.....	A.....	C.T....T..		
Mono 17	17	C1	M		A.....			C.T....T..	A....	
Mono 18	18	C1	M					C.T....T..		
Mono 19	19	B2	M	.G.....	.G.....		A.....	C.T....T..		
Mono 20	20	C1	M					C.T....T..		
Mono 21	21	B2	M	.G.....			A.....	C.T....T..		
Mono 22	22	C1	M		GC.....	.T.A.....		C.T....T..	A....	
Mono 23	23	B2	M	.G.....	.G.....		A.....	C.T....T..	A....	
Mono 24	24	C1	M			.T.A.....		C.T....T..		
Mono 25	25	C1	F		C.....	.T.A.....		C.T....T..	A....	
Mono 26	26	C1	F					C.T....T..		
Mono 27	27	C1	F					C.T....T..		
Mono 28	28	C1	F			.T.A.....		C.T....T..		
Mono 29	29	C1	F			.T.A.....		C.T....T..		
Mono 30	30	C1	F		AG.....			C.T....T..		
Mono 31	31	B2	F	.G.....			A.....	C.T....T..	A....	

Fig. 3. Nucleotide sequence alignment of the EnhII/BCP/PC region of the samples in this study.

mono-infected patients, respectively. The prevalence of *pre-S2* mutations/deletions among co-infected and mono-infected patients was 20.8% and 19.4%, respectively. There was no significant difference in the prevalence of such mutations between the two groups (Table II). As for the prevalence of site-specific *pre-S/S* mutations, *pre-S1* deletion and *pre-S2* start codon mutation was the most common (18.2% each), followed by *pre-S2* deletion (14.5%) and *pre-S1* start codon mutation (10.9%). The mean age of patients with *pre-S* mutations/deletions was higher

significantly than that of patients without the mutants (39.5 ± 9.7 years vs. 31.4 ± 11.2 years, $P = 0.035$). Amino acid sequence alignment of the *pre-S1/pre-S2* region of these 55 samples is shown in Figure 4.

Prevalence and Characterization of the “a” Determinant and Drug Resistance Mutations

A point mutation (Gly145Arg) in the “a” determinant region was detected in one (4.2%) sample

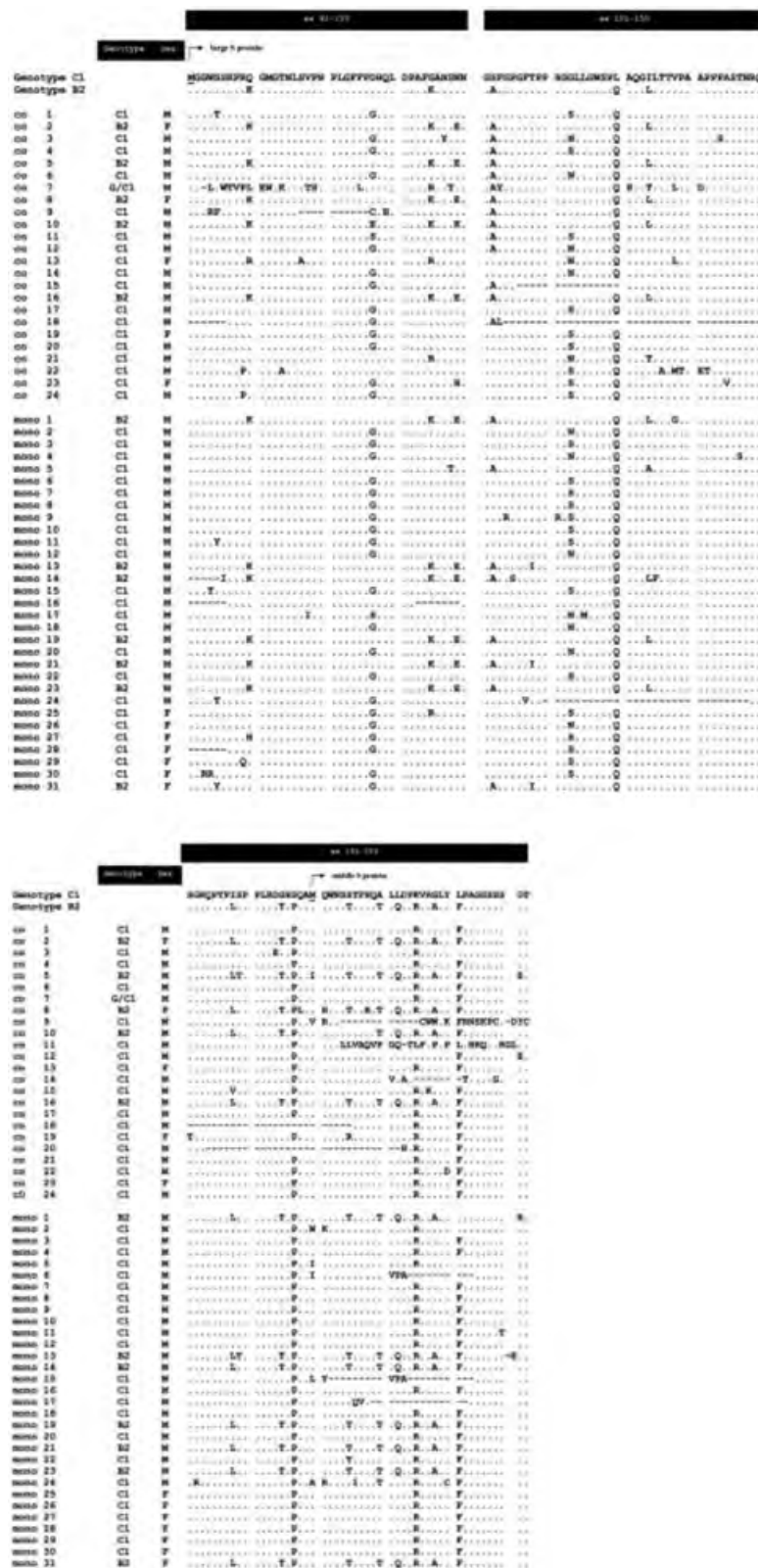


Fig. 4. Amino acid sequence alignment of the pre-S1/pre-S2 region of the samples in this study.

obtained from co-infected patients. As for mutations in the polymerase gene, a point mutation associated with adefovir resistance (rtI233V) was found at similar frequency among co-infected and mono-infected patients (4.2% and 3.2%, respectively). Moreover, a mutant associated with lamivudine resistance (rtV173L) was detected in one (3.2%) of mono-infected patients but was not found in co-infected patients (Table II).

Comparison of Mutations in Relation to HBV Genotypes

To determine whether the differences in frequencies of EnhII/BCP/PC and *pre-S* mutations/deletions between the co-infected and mono-infected patients were influenced by HBV genotypes, the mutations within each group were compared with respect to genotypes B and C. There was no significant difference in the prevalence of these mutations between the two groups depending on HBV genotype (Table III).

DISCUSSION

Although HIV–HBV co-infection prevails in Southeast Asia, it is unclear whether the complete HBV genome may differ between co-infected and mono-infected individuals. Previous data have demonstrated that an HBV mutant encoding truncated core/precursor proteins (–1G mutant) was associated with high HBV DNA levels in co-infected patients with genotype A [Revill et al., 2007]. A recent study from several sites including Australia, the United States, and Asian countries showed that HBV mutations varied significantly between co-infected and mono-infected patients when compared with the same HBV genotype. In particular, double BCP mutations were more frequent significantly in HBV genotype C mono-infection and the –1G mutant was more frequent significantly in co-infection and was observed only in genotype A. Furthermore, *pre-S*2 deletions were detected more frequently in co-infected samples [Audsley et al., 2010]. A previous prospective study from Argentina showed that isolates of HBV genotypes A and F from mono-infected patients tended to have BCP mutations comparable to those of co-infected patients [Cassino et al.,

2009]. These data suggest HBV mutation patterns might be associated with HIV co-infection and might be contributing to disease pathogenesis in co-infected individuals.

The current study was designed to exclude the possibility of cohort effect by sex- and age-matching between Thai patients with co-infection and mono-infection. In addition, confounding factors of HBV genotypes and HBeAg status were minimized as they were also matched between the two groups. This data confirmed previous reports that HBV viral load is higher typically in co-infection than mono-infection [Colin et al., 1999]. In contrast, this study showed that there was no significant difference in the frequencies of double BCP mutations between the two groups although such mutations were more found commonly in genotype C. In addition, the prevalence of G1896A, T1753C, and C1653T was not significantly different between the two groups. It should be mentioned that HBV genotypes in this study were restricted mostly to sub-genotypes C1 and B2, which reflected their high frequencies in Southeast Asia and Thailand [Tangkijvanich et al., 2005; Sa-Nguanmoo et al., 2010]. As a result, the occurrence of –1G mutant, which is restricted to genotype A, could not be detected in this study.

It has been shown that *pre-S* deletion mutants tend to accumulate during a later stage of persistent HBV infection, including cirrhosis and HCC [Chen et al., 2006; Choi et al., 2007]. Data from in vitro studies have demonstrated that intracellular accumulation of pre-S mutant proteins can induce ER stress and alter HBV protein expression, which are associated with increased oxidative stress and DNA damage [Hsieh et al., 2004]. This study showed rather similar prevalence of these mutants in both groups, although they were more found commonly in genotype C. Moreover, patients with *pre-S* mutations/deletions were older than those infected with the wild-type, which confirmed previous observations that the frequency of the mutants tends to increase in direct relation to the patient's age [Chen et al., 2006; Choi et al., 2007]. Taken together, this study suggests that the emergence of common mutations in the EnhII/BCP/PC and *pre-S* regions might not lead to pathogenesis in

TABLE III. Prevalence of HBV Mutations According to HBV Genotypes

Characteristics	Co-infection (n = 24)		Mono-infection (n = 31)	
	B2 (n = 5)	C1 (n = 19) ^a	B2 (n = 7)	C1 (n = 24)
C1653T	0	2 (10.5)	0	0
T1753C	0	5 (26.3)	0	5 (20.8)
A1762T	2 (40)	9 (47.4)	0	13 (54.2)
G1764A	2 (40)	10 (52.6)	0	13 (54.2)
T1858C/deletion	0	1 (5.3)	0	1 (4.2)
G1896A	2 (40)	4 (21.1)	3 (42.9)	8 (33.3)
G1899A/deletion	0	1 (5.3)	1 (14.3)	1 (4.2)
<i>Pre-S</i> mutations/deletions	0	7 (36.8)	1 (14.3)	8 (33.3)

Data were expressed as no. (%).

^aIncluded one case of genotype G/C1 recombination.

co-infected patients. Instead, these mutants might occur during a long-standing inflammatory process of chronic HBV infection among Thai populations.

Amino acid substitutions within the “a” determinant domain lead to conformational changes which may interfere with immunization against HBV infection [Coleman, 2006]. Indeed, the G145A mutant in the second loop of the “a” determinant is considered the most common mutation causing vaccine escape. The prevalence of the G145A mutant among co-infected patients in this study was 4% approximately, which was lower slightly than that reported previously in mono-infected individuals (6–12%) [Coleman, 2006]. Regarding polymerase gene mutations, several signature mutants associated with drug resistance have been characterized. Such mutations are classified as primary (being responsible for decreased drug susceptibility) or compensatory (being responsible for restoring replication fitness of the mutants) [Ghany and Liang, 2007]. In this study, a primary mutation associated with adefovir resistance (rtI233V) was found at similar frequency in the two groups. However, the primary mutation associated with lamivudine resistance was not detected; instead, a compensatory mutant (rtV173L) was found in a patients with mono-infection. This finding was in contrast to most reports but rather similar to a recent study conducted in co-infected patients [Tuma et al., 2011].

HBV strains resulting from genome recombination between different genotypes have been recognized increasingly [Simmonds and Midgley, 2005]. In this study, recombination between genotypes G and C1 was identified in a co-infected patient based on its unusual genotyping pattern upon sequencing and phylogenetic analysis. Indeed, recombination between genotypes C and G appears unusual in Asia as these genotypes are subject to distinct geographical distributions. Interestingly, the site of breakpoints of this recombinant differed from that found in a hybrid of genotypes G/C previously described [Suwannakarn et al., 2005]. It has been proposed that HBV genotype G requires dual infections with other strains to sustain chronicity, although replication competence of this genotype has been documented [Li et al., 2007]. A recent report from the United States has shown that non-hybrid HBV genotype G infection was found frequently in co-infected patients and was associated with more rapid disease progression [Dao et al., 2011]. Whether the recombinant strain of genotypes G/C does enhance the pathogenic potential of the viruses through inter-genotype recombination is unclear and requires further investigation.

In conclusion, this study has shown that dual infections tend to engender increased HBV DNA levels. There was no major difference in the frequencies of common HBV mutations between co-infected and mono-infected patients and these mutants might not be contributing to disease pathogenesis in co-infected patients. However, further large-scale prospective studies, which offer advantages over cross-sectional

investigations, are required to confirm these observations.

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Original article

Diagnostic accuracy of liver stiffness measurement and serum hyaluronic acid for detecting liver fibrosis in chronic hepatitis B with respect to ALT levels

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Background: The assessment of liver fibrosis in chronic hepatitis B is crucial in clinical practice.

Objective: We compared the diagnostic accuracy of liver stiffness measurement (LSM) using transient elastography and serum hyaluronic acid (HA) in detecting liver fibrosis (METAVIR) in chronic hepatitis B, with respect to ALT levels.

Method: One hundred fifty-six Thai patients with chronic hepatitis B who had undergone a liver biopsy were enrolled, and included 112 (71.8%) men and 44 (28.2%) women. The mean age of the patients was 40.1±12.2 years. The predictive accuracy was analyzed by comparing the areas under the receiver-operating characteristic curves (AUROCs).

Result: LSM was superior to HA in predicting fibrosis stages of ≥F2 (AUROCs were 0.820 vs 0.727, $p=0.009$), ≥F3 (0.910 vs 0.848, $p=0.015$) and F4 (0.938 vs 0.876, $p=0.031$). There was significant correlation between ALT level and LSM value, while such correlation between ALT and HA was not detected. Regarding the subgroup of patients with ALT levels >80 IU/L ($2 \times$ ULN), AUROCs of LSM and HA for predicting fibrosis stages of ≥F2 (0.733 vs 0.696), ≥F3 (0.892 vs 0.844) and F4 (0.934 vs 0.893) were not significantly different.

Conclusion: LSM was superior to HA in predicting liver fibrosis and cirrhosis in patients with chronic hepatitis B. However, in patients with ALT elevation, the diagnostic performance of LSM was reduced and its accuracy was comparable to that of HA. Thus, HA could be an alternative method in assessing liver fibrosis in patients with high ALT levels.

Keywords: ALT level, AUROC, cirrhosis, diagnostic accuracy, hepatitis B, hyaluronic acid, liver stiffness, transient elastography

Hepatitis B virus (HBV) infection is a major public health problem worldwide, with approximately 400 million people chronically infected. Chronic HBV infection is associated with a diverse clinical spectrum of liver damage ranging from mild chronic hepatitis to cirrhosis with hepatic decompensation and hepatocellular carcinoma (HCC) [1]. An accurate assessment of fibrosis stages is essential for predicting the prognosis and therapeutic decisions for patients with chronic hepatitis B. Although percutaneous liver

biopsy has been a criterion standard to assess liver histopathology, this procedure has some limitations because of its invasive nature and risk of potentially life-threatening complications [2]. In addition, its accuracy is restricted as a consequence of sampling errors and variations in interpretation [3]. Therefore, this procedure is being gradually replaced by various noninvasive methods for the assessment of liver fibrosis.

Liver stiffness measurement (LSM) using transient elastography has recently been introduced as a new, noninvasive tool for assessing liver fibrosis with high reproducibility [4]. This ultrasound-based technique allows the assessment of approximately

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1/500 of the liver's total mass, thus ensuring a reduction in the sampling error compared with liver biopsy. In patients with chronic hepatitis C virus (HCV) infection, several studies have shown significant positive correlation between LSM and the stage of hepatic fibrosis, as evaluated by the METAVIR score system [5]. Data from the use of LSM to assess the severity of liver fibrosis in patients with chronic hepatitis B are increasing. Recent studies have suggested that LSM exhibits comparable diagnostic performances in chronic hepatitis B compared with chronic hepatitis C [6-9]. However, one limitation is that LSM values can be increased significantly with higher alanine aminotransferase (ALT) levels regardless of fibrosis staging [8, 9]. Thus, additional studies are required to define the accuracy of LSM for predicting liver fibrosis and cirrhosis in patients with chronic hepatitis B, with respect to ALT levels.

Several clinical studies have identified blood tests as surrogate markers of liver fibrosis, which would greatly reduce the necessity to perform liver biopsy. Indirect serological markers on the basis of common laboratory tests, including aspartate aminotransferase (AST)-to-platelet-ratio-index (APRI), FIB-4, Forns' index, Fibrotest, and FibroSpect have been used to stage chronic liver disease [10]. Additionally, several direct serum markers of liver fibrogenesis including hyaluronic acid (HA), serum collagenases and their inhibitors (tissue inhibitor of metalloproteinase [TIMP]), and profibrogenic cytokines such as transforming growth factor β 1 have been investigated [10, 11]. Currently, there are few studies directly comparing the diagnostic accuracy between LSM and serum markers in patients with chronic hepatitis B.

HA, a high-molecular-weight glycosaminoglycan that is an essential component of the extracellular matrix, appears to be the most suitable test for the noninvasive assessment of liver fibrosis [11, 12]. In the liver, HA is mainly synthesized by hepatic stellate cells and degraded by hepatic sinusoidal endothelial cells [13]. It has been shown that serum HA levels are low in healthy subjects, but elevated levels occur in patients with various etiologies of fibrotic liver disease, including chronic viral hepatitis and alcohol induced liver disease¹¹. Previous studies demonstrated that serum HA concentrations were significantly related to the histological degree of liver fibrosis, but there was no correlation between this marker and the

histological activity of necroinflammation [12, 14]. However, the accuracy of HA for predicting liver fibrosis and cirrhosis in conjunction with ALT levels has never been investigated.

This study was aimed at comparing the diagnostic accuracy of LSM and serum HA in detecting liver fibrosis and cirrhosis in patients with chronic hepatitis B. In particular, we evaluated the impact of serum ALT elevations on the diagnostic accuracy of these noninvasive tests.

Patients and methods

Patients

This cross-sectional study included consecutive patients with chronic hepatitis B who had undergone a liver biopsy at King Chulalongkorn Memorial Hospital, Bangkok, Thailand between January 2010 and September 2011. Chronic hepatitis B was diagnosed based on hepatitis B surface antigen (HBsAg) in the patient's serum for at least 6 months and detectable serum HBV DNA.

A total of 156 patients were enrolled in this study, and included 112 (71.8%) men and 44 (28.2%) women. The mean age of the patients was 40.1 ± 12.2 years. Patients with the following conditions were excluded from the study: presence of HCV-coinfection or other cause of liver disease, seropositive for anti-HIV, presence of decompensated cirrhosis and HCC and prior antiviral therapy. All patients gave written informed consent for the study and the protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Laboratory tests

A serum sample was obtained from each patient for analysis at the time of performing TE. Liver biochemistry tests [AST, ALT, total bilirubin (TB), alkaline phosphatase (AP), albumin] were performed using commercially available assay kits in an automated analyzer (Hitachi 912). HBsAg and hepatitis B e-antigen (HBeAg) were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abbott Laboratories, Chicago, IL, USA). Serum HBV DNA level was quantified using a commercially available kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan).

Measurement of serum HA concentration

Serum HA was measured by a modified competitive ELISA-like method using an HA-test kit

(Allswell Singapore) according to the manufacturer's specifications as previously described^[15]. Briefly, microtiter plates (Maxisorp, Nunc) were coated with umbilical cord HA (100 μ l/well) in coating buffer at 4°C overnight. Wells were blocked with 150 μ l of 1% (w/v) BSA in phosphate-buffered saline (PBS) for 60 minutes at room temperature. After washing, 100

l of the mixture, either sample or standard competitor (HA Healon: range 39.06–10,000 ng/ml) in B-HABPs (1:100), were added. After incubation for 60 minutes at room temperature, plates were washed and then peroxidase-mouse monoclonal anti-biotin (100 μ l/well; 1:2,000) was added and incubated for 60 minutes at room temperature. The plates were washed again and the peroxidase substrate (100 μ l/well) was added and incubated at room temperature for 15 minutes to allow the color to develop. The reaction was stopped by the addition of 50 μ l of 4 M H₂SO₄. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 microplate reader.

Liver stiffness measurement

LSM values were obtained from each patient using transient elastography (FibroScan, Echosens, Paris, France) according to the manufacturer's instructions [4]. All patients underwent LSM within 30 days of their liver biopsy. Results were recorded in kilopascals (kPa) as the median value of all measurements. The procedure was based on at least 10 validated measurements: the success rate (ratio between numbers of validated and total measurements) was over 60% and interquartile range was less than 30%.

Histopathology assessment

Liver biopsy specimens were obtained using 16 G disposable needles (Hepafix; B. Braun, Melsungen, Germany) applying an ultrasound-guided technique. The specimens were fixed in formalin, embedded in paraffin blocks, and stained with hematoxylin and eosin, and Masson's trichrome stain. Histopathology was performed by an experienced pathologist, who was blinded to the patients' clinical data. A liver biopsy specimen was considered adequate if it was longer than 15 mm (except when cirrhosis was present). The stage of fibrosis was scored according to the METAVIR classification [16]: no fibrosis (F0), portal fibrosis without septa (F1), portal fibrosis with few septa (F2), numerous septa without cirrhosis (F3), and cirrhosis (F4). Significant liver fibrosis was defined as METAVIR fibrosis stage of at least F2 ($\geq$ F2), while

advanced liver fibrosis was defined as METAVIR fibrosis stage of at least F3 ($\geq$ F3).

Statistical analyses

Data were expressed as mean standard deviation (SD) and percentages as appropriate. Comparisons between groups were analyzed using a χ^2 or Fisher's exact test for categorical variables and using a Mann–Whitney test or Student's *t* test when appropriate for quantitative variables. Pearson's correlation coefficient was used. Univariate and multiple regression analysis were used to determine variables that significantly correlated in the univariate analysis. The diagnostic performance of each test was assessed by using receiver operating characteristics (ROC) curves. The area under the ROC curves (AUROC) and 95% confidence intervals (CI) were used as indices of accuracy, with values close to 1.0 indicating high diagnostic accuracy. Optimal cutoff values for each test were chosen to maximize both sensitivity and specificity. A two-sided probability value of $p < 0.05$ was considered statistically significant. Data were analyzed using SPSS software for Windows version 17.0 (SPSS, Chicago, IL).

Results

Characteristics of the patients

In this study, there were 19 (12.2%), 50 (32.0%), 45 (28.8%), 21 (13.5%), and 21 (13.5%) patients with METAVIR fibrosis stage F0, F1, F2, F3, and F4 respectively. Compared with patients with absent (F0) and mild fibrosis (F1), patients with significant liver fibrosis ($\geq$ F2) had higher average age. No significant difference between groups was observed in respect to sex, body mass index (BMI), AST, ALT, TB, AP, albumin, platelet count, HBeAg immunoreactivity, and HBV DNA level (**Table 1**).

LSM and HA according to liver fibrosis stages

LSM and HA values of all enrolled patients according to their fibrosis stages are shown in **Table 2**. The mean LSM value was 8.1 kPa (ranging from 3.3 to 31 kPa) and the mean HA level was 120.4 ng/mL (ranging from 8.3 to 1327.8 ng/mL). There were significant differences in the mean LSM between F0–F1 and F2–F4 fibrosis stages (5.7 $\pm$ 1.9 kPa vs 9.9 $\pm$ 5.7 kPa, $p < 0.001$), between F0–F2 and F3–F4 (6.2 $\pm$ 2.2 kPa vs 13.1 $\pm$ 6.5 kPa, $p < 0.001$), and between F0–F3 and F4 (6.9 $\pm$ 3.1 kPa vs 15.7 $\pm$ 7.1 kPa, $p < 0.001$).

Table 1. Demographic and clinical characteristics of the patients

Characteristics	All patients (n=156)	Patients with F0–F1 (n=69)	Patients with F2–F4 (n=87)	<i>p</i>
Age (years)	40.1±12.2	37.3±10.4	42.4±13.1	0.009
Male	112 (71.8)	49 (71.0)	63 (72.4)	NS
Female	44 (28.2)	20 (28.9)	24 (27.6)	NS
Body mass index (kg/m ²)	23.6±3.1	23.0±2.6	24.1±3.5	NS
Total bilirubin (mg/dL)	0.6±0.4	0.6±0.4	0.7±0.3	NS
AST (IU/L)	46.8±33.2	44.7±36.9	48.5±30.1	NS
ALT (IU/L)	75.4±55.4	69.8±59.5	79.8±51.9	NS
Alkaline phosphatase (IU/L)	69.7±21.8	65.0±14.7	72.7±25.0	NS
Albumin (g/dL)	4.4±0.4	4.5±0.3	4.4±0.4	NS
Platelet count (10 ⁹ /L)	215.2±56.6	223.8±54.4	208.2±57.8	NS
HBeAg-positive	56 (35.9)	23 (33.3)	33 (37.9)	NS
HBeAg-negative	100 (64.1)	46 (66.7)	54 (62.1)	NS
HBV DNA (log ₁₀ IU/mL)	6.0±1.4	5.8±1.5	6.2±1.3	NS

Data are express as mean ± SD; no (%); NS = not significant

Table 2. Mean values of LSM and HA according to METAVIR fibrosis stages

Fibrosis stage	LSM (kPa)	HA (ng/mL)
F0 (n=19)	5.2±1.0	43.9±27.6
F1 (n=50)	5.9±2.1	64.9±48.9
F2 (n=45)	7.0±2.4	79.8±89.3
F3 (n=21)	10.4±4.8	181.8±146.6
F4 (n=21)	15.7±7.1	346.9±222.7

Data are express as mean ± SD; LSM, liver stiffness measurement; HA, hyaluronic acid

Likewise, there were significant differences in the mean HA levels between F0–F1 and F2–F4 stages (59.1±44.9 ng/mL vs 168.9±212.7 ng/mL, $p<0.001$), between F0–F2 and F3–F4 (67.3±66.5 ng/mL vs 264.4±261.3 ng/mL, $p<0.001$) and between F0–F3 and F4 (85.1±93.1 ng/mL vs 346.9±322.7 ng/mL, $p=0.001$).

ROC curve analyses for predicting the fibrosis stages

The AUROCs of LSM for predicting fibrosis stages of ≥F2, ≥F3, and F4 were 0.820 (95% CI, 0.752–0.888), 0.910 (0.851–0.968), and 0.938 (0.896–0.980), respectively. The AUROCs of HA for predicting fibrosis stages of ≥F2, ≥F3, and F4 were 0.727 (0.649–0.804), 0.848 (0.780–0.917) and 0.876 (0.806–0.947), respectively. The AUROCs of LSM for predicting fibrosis stages of ≥F2, ≥F3, and F4 were significantly higher than those of HA ($p=0.009$, $p=0.015$, and $p=0.031$, respectively) (**Figure 1**).

The optimal cutoff values of LSM for predicting stages of ≥F2, ≥F3, and F4 were 6.8 kPa, 8.5 kPa and 10.0 kPa, respectively. The optimal cutoff values of HA for predicting stages of ≥F2, ≥F3, and F4 were 65 ng/mL, 95 ng/mL, and 110 ng/mL, respectively. The cutoff values and the corresponding sensitivities and specificities are summarized in **Table 3**.

Factors associated with the performance of LSM and HA

Table 4 shows the results of the correlation of LSM and HA with various clinical, pathological, and laboratory parameters, including METAVIR fibrosis, BMI, platelet count, ALT, TB, albumin, AP, HBeAg, and HBV DNA. A multiple regression analysis was further performed on LSM and HA by comparison of all significant parameters. The data of multiple regression analysis showed that LSM was significantly correlated with serum ALT [odds ratio (OR), 2.116; 95% CI, 1.053–4.250; $p=0.008$] and METAVIR

fibrosis (OR, 3.929; 95% CI, 1.906–7.092; $p=0.001$), while HA was significantly correlated with METAVIR fibrosis (OR, 3.349; 95% CI, 1.678–6.681; $p=0.001$).

LSM and HA with respect to ALT levels

LSM values were significantly lower in patients with serum ALT levels ≤ 80 IU/L ($2 \times$ upper limit of normal; ULN) than in patients with serum ALT levels > 80 IU/L in the subgroups of F0–F1 patients (5.2 ± 1.0 kPa vs 6.8 ± 2.7 kPa, $p=0.001$), but they did not differ significantly in the subgroup of F2 patients (6.5 ± 1.5

kPa vs 7.5 ± 3.1 kPa, $p=0.155$), F3 patients (8.5 ± 2.2 kPa vs 12.6 ± 6.0 kPa, $p=0.068$) and F4 patients (15.0 ± 7.6 kPa vs 17.4 ± 5.9 kPa, $p=0.504$). In contrast, HA levels were not significantly different between patients with serum ALT levels ≤ 80 IU/L and levels > 80 IU/L in subgroups of F0–F1 patients (59.1 ± 41.4 ng/mL vs 59.2 ± 25.5 ng/mL, $p=0.993$), F2 patients (94.4 ± 116.2 ng/mL vs 63.2 ± 38.4 ng/mL, $p=0.247$), F3 patients (171.1 ± 119.6 ng/mL vs 193.5 ± 177.6 ng/mL, $p=0.736$), and F4 patients (308.2 ± 314.6 ng/mL vs 443.8 ± 351.6 ng/mL, $p=0.398$).

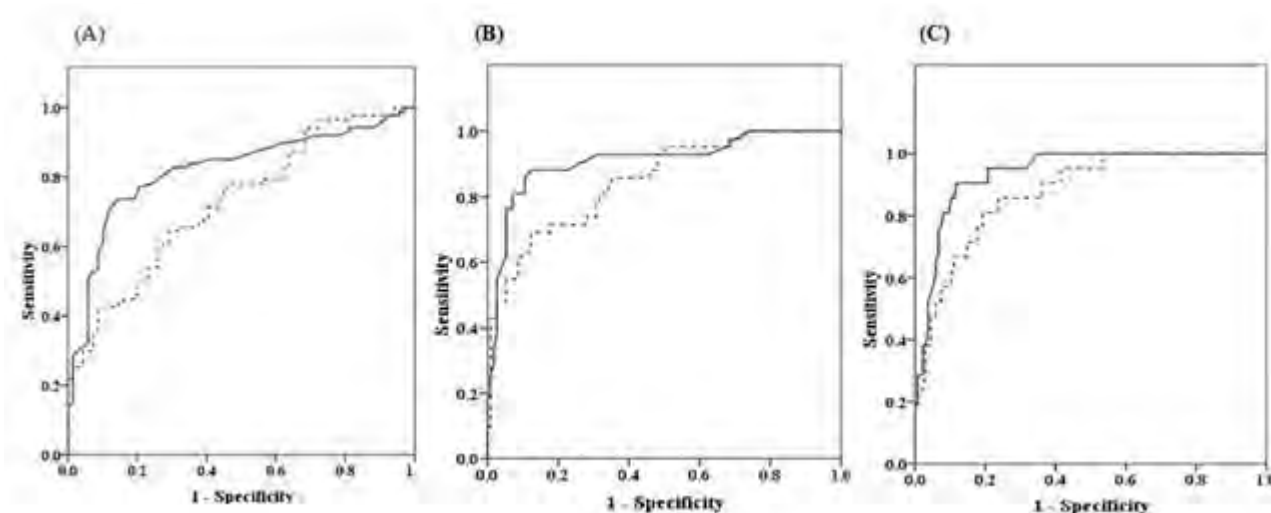


Figure 1. Receiver-operating characteristic (ROC) curves of liver stiffness measurement (LSM) and hyaluronic acid (HA) for predicting significant fibrosis (F0–F1 vs F2–F4), (B) advanced fibrosis (F0–F2 vs F3 or F4), and (C) cirrhosis (F0–F3 vs F4). —, LSM; ---, HA

Table 3. Area under the receiver-operating characteristic curve (AUROCs) and cutoff values for predicting fibrosis stages

		LSM	HA
$\geq F2$	AUROC (95% CI)	0.820 (0.752–0.888)	0.727 (0.649–0.804)
	Cutoff values	6.8 kPa	65 ng/mL
	Sensitivity (%)	73.6	64.4
	Specificity (%)	85.5	71.0
$\geq F3$	AUROC (95% CI)	0.910 (0.851–0.968)	0.848 (0.780–0.917)
	Cutoff values	8.5 kPa	95 ng/mL
	Sensitivity (%)	88.1	71.4
	Specificity (%)	87.7	82.5
F4	AUROC (95% CI)	0.938 (0.896–0.980)	0.876 (0.806–0.947)
	Cutoff values	10.0 kPa, 110 ng/mL	
	Sensitivity (%)	90.5	81.0
	Specificity (%)	88.1	80.7

LSM = liver stiffness measurement, HA = hyaluronic acid, PPV = positive predictive value, NPV = negative predictive value

Table 4. Parameters correlated with LSM and HA

Parameter	LSM		HA	
	r	p	r	p
Body mass index (kg/m ²)	0.218	0.030	0.001	0.990
METAVIR fibrosis	0.636	<0.001	0.516	<0.001
Total bilirubin (mg/dL)	0.056	0.504	0.242	0.004
ALT level (IU/L)	0.553	<0.001	-0.013	0.871
Alkaline phosphatase (IU/L)	0.467	<0.001	0.248	0.007
Albumin (g/dL))	-0.292	<0.001	-0.431	<0.001
Platelet count (10 ⁹ /L)	-0.245	0.002	-0.268	0.001
HBeAg	-0.078	0.330	-0.119	0.140
HBV DNA (log ₁₀ IU/mL)	0.092	0.255	0.031	0.704

ROC curve analyses of LSM and HA with respect to ALT levels

The AUROCs of LSM for predicting fibrosis stage of $\geq$ F2 were significantly higher in patients with serum ALT levels $\leq$ 80 IU/L than in patients with serum ALT levels $>$ 80 IU/L [0.864 (0.788–0.941) vs 0.733 (0.602–0.864), $p=0.040$]. However, there were no statistically significant differences for predicting fibrosis stages of $\geq$ F3 [0.936 (0.872–1.000) vs 0.892 (0.786–0.999), $p=0.121$] and F4 [0.967 (0.925–1.000) vs 0.934 (0.869–1.000), $p=0.458$]. By contrast, the AUROCs of HA values for predicting fibrosis stages of $\geq$ F2, $\geq$ F3, and F4 were not significantly different between patients with serum ALT levels $\leq$ 80 IU/L and patients with serum ALT levels $>$ 80 IU/L [0.757 (0.663–0.851) vs 0.686 (0.548–0.823), $p=0.167$; 0.845 (0.755–0.935) vs 0.844 (0.732–0.956), $p=0.844$ and 0.862 (0.780–0.958) vs 0.893 (0.744–1.000), $p=0.741$, respectively].

Regarding only patients with serum ALT levels $\leq$ 80 IU/L, the AUROCs of LSM for predicting fibrosis stages of $\geq$ F2, $\geq$ F3, and F4 were significantly higher

than those of HA values with the same fibrosis stages. By contrast, in patients with serum ALT levels $>$ 80 IU/L, the AUROCs for predicting fibrosis stages of $\geq$ F2, $\geq$ F3, and F4 did not differ significantly between LSM and HA values (**Table 5**).

Discussion

The discrimination between absent/mild fibrosis (F0–F1) and significant fibrosis to cirrhosis (F2–F4) in chronic viral hepatitis has essential clinical implications for clinicians in deciding therapeutic options, monitoring disease progression, and to determine prognosis of the patients. Our study demonstrates that LSM is an accurate noninvasive technique for the assessment of fibrosis in patients with chronic hepatitis B. For example, LSM was able to accurately discriminate between patients with METAVIR F0–F1 and F2–F4 (AUROC 0.82) and even better between patients with F0–F2 and F3–F4 (AUROC 0.91) and between patients with F0–F3, and F4 (AUROC 0.94). These data are consistent with recent studies conducted on Asian and Caucasian

Table 5. Area under the receiver-operating characteristic curve (AUROCs) with respect to serum ALT levels

		LSM	HA	p
ALT $\leq$ 80 IU/L	$\geq$ F2	0.864 (0.788–0.941)	0.757 (0.663–0.851)	0.009
	$\geq$ F3	0.936 (0.872–1.000)	0.845 (0.755–0.935)	0.002
	F4	0.967 (0.925–1.000)	0.862 (0.780–0.958)	0.006
ALT $>$ 80 IU/L	$\geq$ F2	0.733 (0.602–0.864)	0.696 (0.558–0.823)	0.626
	$\geq$ F3	0.892 (0.786–0.999)	0.844 (0.732–0.956)	0.778
	F4	0.934 (0.869–0.999)	0.893 (0.744–1.000)	0.907

Data are expressed as AUROCs (95% confidence intervals), LSM = liver stiffness measurement, HA = hyaluronic acid.

patients with chronic hepatitis B^[6-8, 17-21]. In a French multicenter study, Marcellin et al. found that the AUROCs in differentiating F0–F1 vs F2–F4, F0–F2 vs F3–F4 and F0–F3 vs F4 were 0.81, 0.93, and 0.93, respectively^[6]. Similarly, a study conducted in Taiwan by Wang et al. showed that the AUROCs in differentiating METAVIR F0–F1 vs F2–F4, F0–F2 vs F3–F4, and F0–F3 vs F4 were 0.86, 0.88 and 0.89, respectively^[18]. A meta-analysis of 50 studies evaluating LSM in chronic liver disease of various etiologies has shown that the mean AUROCs for predicting significant fibrosis, advanced fibrosis and cirrhosis were 0.84, 0.89, and 0.94, respectively [22].

By maximizing sensitivity and specificity, the optimal cutoff values of LSM for $\geq$ F2 (6.8 kPa), $\geq$ F3 (8.5 kPa) and F4 (10.0 kPa) in this study were also comparable with most previous reports, ranging from 6.0–8.0 pKa, 8.1–8.8 pKa and 9.0–14.0 pKa, respectively^[6-8, 17-21]. Using a cutoff level of 10.0 kPa, our data showed that LSM exhibited high sensitivity and specificity (90.5% and 88.1%, respectively) for estimating the presence of cirrhosis. This was in agreement with the French study in which the sensitivity and specificity for diagnosing cirrhosis using a cutoff of 11.0 kPa were 93% and 87%, respectively^[6]. However, it should be mentioned that the cutoff points for estimating the presence of cirrhosis vary significantly among different studies (9.0–14.0 pKa). This discrepancy might be related to several factors including the different populations studied and different study design methodology.

Previous studies have demonstrated that acute severe flares of hepatitis, as defined by ALT $>10 \times$ ULN, could significantly affect LSM values [23-25]. Subsequent studies have also shown that even milder degrees of ALT elevation are associated with significantly higher values of LSM, and therefore might reduce the diagnostic accuracy of the test^[8, 19, 26-28]. In the current study, there was a significant correlation between serum ALT levels and LSM scores. Moreover, ALT level was associated with LSM in addition to histological fibrosis in multiple regression analysis. Our results were in accordance with previous data that ALT elevation ($>2 \times$ ULN) significantly reduced the AUROCs of LSM, particularly in patients with F0–F1 fibrosis stages, while the diagnostic accuracy did not differ significantly in the subgroups of patients with F3–F4. In this study, the influence of biochemical activity on LSM was noticeable in the subset of patients with F0–F1 fibrosis in whom mean

LSM values were significantly lower than in patients with ALT elevation, but with the same histological stages (5.2 ± 1.0 kPa vs 6.8 ± 2.7 kPa, $p=0.001$). These results indicate that patients with mild degree of fibrosis might more likely be overestimated by LSM to the extent of advanced fibrosis or cirrhosis because of their high ALT levels.

To minimize the risk of overestimating fibrosis by LSM during hepatitis flares, different strategies have been proposed. First, it is recommended to perform or repeat LSM after ALT normalization is achieved. By delaying LSM until ALT is normal or near normal, the false-positive results in diagnosing cirrhosis would be greatly reduced [19, 27]. In cases where ALT is persistently elevated, use of an algorithm that will stratify different cutoff LSM scores according to ALT levels is another option. In this respect, a recently published ALT-based algorithm has been developed for patients with chronic hepatitis B, using higher LSM values for optimal cutoffs in those with elevated ALT levels^[8]. An alternative approach would be to utilize serum markers of liver fibrosis alone or in combination with LSM values to improve the diagnostic accuracy [29-32]. However, until now, only limited studies have taken into consideration the ALT levels in the assessment of available serum markers.

Several serum markers have been developed in recent years, including APRI, FIB-4, Forns' index, Fibrotest, and FibroSpect. However, the clinical applicability of Forns' index, Fibrotest, and FibroSpect is rather limited because these markers involve complex mathematical calculations. Likewise, although APRI and FIB-4 can be easily calculated from simple biochemical parameters, these tests take serum aminotransferase into account and are likely be affected by ALT elevation. In this study, we chose to compare the accuracy of LSM against serum HA in conjunction with ALT levels for two reasons. First, HA is generally considered to be the best individual serum marker available. Second, previous studies demonstrated that HA concentrations were not confounded by the grade of necroinflammation activity [12, 14]. Thus, the advantage of HA over the other simple noninvasive markers was that HA might not be affected by an increase in ALT level. This anticipation was supported by our current data showing that ALT levels were not correlated with HA concentrations and, as a result, did not affect its accuracy.

In the current study, determination of HA levels was accurate in predicting significant fibrosis, advanced fibrosis, and cirrhosis, with AUROCs of 0.73, 0.85, and 0.88, respectively.

These results are consistent with another study conducted in patients with chronic hepatitis C in which the AUROCs of the same fibrotic stages were 0.75, 0.82, and 0.89, respectively [33]. Our results are also comparable to another recent study in patients with chronic viral hepatitis (54% were chronic hepatitis B) showing the AUROCs of 0.72, 0.81 and 0.86, respectively^[29]. Although HA was diagnostically inferior to LSM at identifying significant and advanced fibrosis, the performance of HA and LSM was not statistically significant if considering only the subgroup of patients with high ALT levels. HA yielded AUROCs of 0.84 and 0.89 for predicting advanced fibrosis and cirrhosis, respectively, which were considered satisfactory and comparable to those of LSM (AUROCs of 0.89 and 0.93, respectively). These data suggest that the sole measurement of HA may be appropriate and adequate to predict advanced fibrosis and cirrhosis in patients with high ALT levels. Thus, this method can be adopted without delay during ALT elevation and facilitates the clinical management of patients when LSM is not applicable.

In conclusion, our data showed that LSM was superior to HA in predicting liver fibrosis and cirrhosis in patients with chronic hepatitis B. There was significant correlation between ALT level and LSM value, while such correlation between ALT and HA was not found. As a consequence, the performance of LSM was reduced in patients with ALT elevation and its diagnostic accuracy was rather comparable to that of HA in this subgroup of patients. Thus, HA could be an alternative and accurate method for assessing liver fibrosis in patients with ALT elevation.

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RESEARCH ARTICLE

Hepatitis B Virus Genetic Variation and **TP53 R249S** Mutation in Patients with Hepatocellular Carcinoma in Thailand

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Abstract

Chronic hepatitis B virus (HBV) infection and dietary exposure to aflatoxin B1 (AFB1) are major risk factors for hepatocellular carcinoma (HCC). The aim of this study was to evaluate the role of HBV genetic variation and the **R249S** mutation of the *p53* gene, a marker of AFB1-induced HCC, in Thai patients chronically infected with HBV. Sixty-five patients with and 89 patients without HCC were included. Viral mutations and **R249S** mutation were characterized by direct sequencing and restriction fragment length polymorphism (RFLP) in serum samples, respectively. The prevalences of **T1753C/A/G** and **A1762T/G1764A** mutations in the basal core promotor (BCP) region were significantly higher in the HCC group compared to the non-HCC group. **R249S** mutation was detected in 6.2% and 3.4% of the HCC and non-HCC groups, respectively, which was not significantly different. By multiple logistic regression analysis, the presence of **A1762T/G1764A** mutations was independently associated with the risk of HCC in Thai patients.

Keywords: Hepatitis B - aflatoxin - mutation - hepatocellular carcinoma - Thailand

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Introduction

Hepatocellular carcinoma (HCC) is a major public health problem worldwide, accounting for at least 500,000 new cases diagnosed annually. The prevalence of HCC is geographically variable, with the highest frequencies observed in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) is endemic (Gao et al., 2012). HBV, a member of the family Hepadnaviridae, is a partially double-stranded DNA virus that contains four overlapping open reading frames (ORFs) encoding the surface, core, polymerase and X genes. The virus shows remarkable genetic variability and is currently classified into at least eight genotypes (genotypes A-H). HBV also has a high mutation rate resulting from its lacking of proofreading capacity during the viral replication (Kay et al., 2007). The well-known naturally occurring HBV variants include the precore (PC) stop codon mutation (G1896A) and double mutations in the basal core promotor (BCP) region (A1762T/G1764A). Several studies have showed that A1762T/G1764A have higher risks of liver cirrhosis and HCC (Wai et al., 2004). However, BCP mutations are also frequently found among patients without HCC. Apart from these variants, other mutations such as **T1753C/A/G** in the BCP region, **C1653T** in the enhancer II region (EnhII) and pre-S mutations/deletions have been recognized as being associated with

the outcome of chronic HBV infection, including HCC development (Chotiyaputta et al., 2009).

In addition to chronic HBV infections, dietary exposure to aflatoxin B1 (AFB1) is an important risk factor for HCC development (Yu et al., 2004). AFB1 is a mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminates staple foods during storage under conditions that promote fungal growth and toxin production. The molecular hallmark of AFB1-induced HCC is a missense mutation at codon 249 in the *p53* tumor suppressor gene (**TP53**), AGG to AGT, leading to a substitution of an arginine for a serine (**R249S** mutation) (Wild et al., 2009). This mutation is extremely high in HCC in areas with high prevalence of chronic HBV infection and AFB1 exposure such as Qidong, China. In contrast, such mutation is uncommon in regions with low or negligible levels of AFB1 exposure such as the United States and Japan. Recent studies have shown that **R249S** mutation is detectable in free DNA extracted from serum or plasma of patients with HCC, which is identical to those detected in tumor tissues and can be served as a biomarker of AFB1 exposure (Gouas et al., 2009). The prevalence of this mutation in the serum of Thai patients with HCC and its interaction with HBV mutations are uncertain. Thus, the aim of this study was to assess the role of HBV genetic variation, **R249S** mutation, and their interactions in HCC risk in Thai patients with chronic HBV infection.

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Materials and Methods

Patients

Serum samples obtained from patients with HBV-related HCC and positive for HBV DNA were randomly selected from a pool of patients with chronic liver disease who were seen and followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between January 2009 and December 2012. The diagnosis of HCC was based on typical imaging studies and/or histology (fine needle aspiration or surgical resection) according to American Association for the Study of Liver Diseases (AASLD) guideline (Bruix et al., 2005). Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyperattenuation at the arterial phase and hypoattenuation at the portal phase in dynamic CT or MRI. In cases without typical imaging features liver biopsy was performed to confirm the diagnosis of HCC.

To examine the role of *R249S* mutation and molecular virological factors in the development of HCC, patients with chronic hepatitis B were randomly selected as controls. These patients visited our clinic every 4-6 months during the same period of recruitment of the study and none had liver cancer development during follow-up. None of the patients enrolled in this study had a history of hepatitis C virus (HCV) infection or human immunodeficiency virus (HIV) co-infection. In addition, none of the patients had a history of heavy alcoholic drinking, or received any antiviral therapy when the serum sample was obtained. All patients were informed about the purposes of the study, and subsequently gave their written informed consent. Serum samples were collected from each patient at the time of their evaluation and frozen at -70°C until use. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Serological and virological assays

Sera tested for hepatitis B s antigen (HBsAg) was determined using commercially available ELISA assay (Abbott Laboratories, Chicago, IL). Serum HBV DNA level was quantified using a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection range of this assay was 2.4 to 8.7 log IU/mL.

HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 100 μL serum sample by incubation in lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/mL proteinase K) and phenol-chloroform-isoamyl alcohol extraction. The DNA pellet was resuspended in 30 μL sterile distilled water and subjected to amplification of the *pre-S1/pre-S2/S* and *EnhII/BCP/PC* regions by polymerase chain reaction (PCR). The *pre-S1/pre-S2/S* regions were amplified using the primers PreS1F+: 5'-GGGTACCATAT TCT TGGG AAC-3' [forward primer, nucleotide (nt.) 2814 to 2835] and R5: 5'-AGCCCAAAGACCCACAATTC-3' (reverse primer, nt. 1015 to 995). The *EnhII/BCP/PC* regions were amplified using the primers X101:5'-TCTGTGCCTTCTCATCTG-3' (forward primer, nt.1552 to 1569) and CORE2:

5'-CCCACCTTATGAGTCCAAGG-3' (reverse primer, nt. 2476-2457).

Briefly, the reaction mixture comprised 2 μL resuspended DNA, 0.5 μL of 25 mmol of each primer, 10 μL of 2.5X MasterMix® (Eppendorf, Hamburg, Germany) and sterile distilled water to a final volume of 25 μL reaction. The reaction was performed in a PCR thermocycler (Eppendorf, Hamburg, Germany) with the initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s (denaturing), at 55°C for 30 s (annealing), at 72°C for 1 min (extension) and concluded by a final 7 min extension at 72°C . The PCR products were segregated by 2% agarose gel electrophoresis. The PCR products were extracted from the agarose gel using the Perfectprep® Gel cleanup kit (Eppendorf, Hamburg, Germany). The sequencing reaction was performed using the AmpliTaq™ DNA Polymerase FS dye terminator from the ABIPRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with CLUSTALX (version 1.83) program as previously described (Suwannakarn et al., 2008).

HBV genotyping

For the genotyping, phylogenetic analysis based on *pre-S1/pre-S2/S* and *preC/C* regions was performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ) as previously described (Sa-Nguanmoo et al., 2010).

Detection of *R249S* in serum

Serum specimens were subjected for the amplification of exon 7 of the *TP53* gene by nested-PCR. The first PCR reaction consisted of 0.5 μL of DNA template, 10 μL of PerfectTaq plus MasterMix Kit (5 prime GmbH, Hamburg, Germany), 1.25 mM outer forward primer (*TP53*-OS: 5'-CTTGCCACAGGTCTCCCCAA-3'), 1.25 mM outer reverse primer (*TP53*-OAS: 5'-AGGGGTCAGAGGCAAGCAGA-3'), and distilled water to final volume as 25 μL . The second PCR reaction was performed using nested primers, 1.25 mM inner forward primer (*TP53*-IS: 5'-AGGCGCACTGGCCTCATCTT-3'), 1.25 mM inner reverse primer (*TP53*-IAS: 5'-TGTGCAGGGTGGCAAGTGGC-3') and 0.2 μL of first PCR product as DNA template. The PCR cycles was initially denatured at 94°C for 3 min, followed by 40 cycles at 94°C for 18 s (denaturing), at 50°C for 21 s (annealing), at 72°C for 1.30 min (extension) and concluded by final 10 min extension at 72°C . The PCR-amplified products were examined by 2% agarose gel electrophoresis then stained with ethidium bromide and visualized under UV light. The size of the final PCR fragments were 237 bp (first PCR) and 177 bp (second PCR), respectively.

TP53 mutation was analyzed by restriction fragment length polymorphism (RFLP) using a modified method described by Szymanska et al. (2004). Briefly, 15 μL of second PCR product was digested by *HaeIII* restriction endonuclease which recognized sequence CCGG and

encompassed the 249 codon (AGG). The total 20 μ L RFLP reaction mixture comprised of 1 unit of *Hae*III (New England BioLabs inc, Ipswich, MA), 2 μ L of 10X Buffer4 (New England BioLabs inc, Ipswich, MA) and sterile distilled water. The mixture was incubated at 37°C overnight and segregated by 3% agarose gel electrophoresis. The PCR product of wild-type DNA generated 3 bands of 12, 24 and 116 bp, whereas in mutant, in which the restriction site was destroyed, yielded only two band of 12 and 153 bp. Sample with positive RFLP for *R249S* were further confirmed by direct sequencing.

Statistical analysis

Data were presented as percentage, mean and standard deviation. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann-Whitney test or Student's t test when appropriate for quantitative variables. Multiple logistic regression analysis was used to assess the influence of each factor on the risk of HCC development. *P* values below 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 17.0 (SPSS Inc., Chicago, IL).

Results

The clinical features of all patients in this study are showed in Table 1. There were 65 patients with HCC and 89 patients in the control group. The mean age of patients with HCC (58.1 $\pm$ 12.0 years) was significantly higher than that of the controls (41.3 $\pm$ 8.8 years) (p <0.001). In addition, the prevalence of HBV genotype C was significantly higher in the HCC group than in the control group. However, there was no significant difference between groups in respect to sex, and HBV DNA levels.

Pre-S1 mutations/deletions were detected in 27.3% and 19.6% samples of patients with HCC and controls, respectively. The prevalence of *pre-S2* mutations/deletions among the HCC and control groups was 21.8% and 25%, respectively. There was no significant difference in the prevalence of such mutations/deletions between the two groups. Also, there was no significant difference between groups regarding *S* start codon mutation. However, patients with HCC had significantly lower frequency of *S* deletion compared with the controls (23.6% and 44.6%, respectively, p =0.019) (Table 1).

Base on direct sequencing of EnhII/BCP/PC regions, mutational spots were found at nt 1653, 1753, 1762, 1764, 1896 and 1899. Patients with HCC had higher frequencies of T1753A/G/C mutations compared with the controls (59.1% and 35.5%, respectively, p =0.008). Moreover, patients with HCC had higher frequencies of A1762T/G1764A mutations compared with the controls (88.6% and 68.4%, respectively, p =0.003). In contrast, G1896A mutation was less frequently detected in patients with HCC than in the controls (34.1% and 65.8%, respectively, p =0.003). No significant difference between groups was found in respect to C1653T and G1899A mutations (Table 1).

Base on RFLP and direct sequencing, *R249S* mutation was present in 6.2% serum samples of the HCC group,

Table 1. Demographic and Characteristics of Patients with or without HCC

Characteristics	HCC group (n=65)	Non-HCC group (n=89)	<i>p</i>
Age (years)	58.1 $\pm$ 12.0	41.3 $\pm$ 8.8	<0.001
Sex			0.376
Male	53(81.5)	66(74.2)	
Female	12(18.5)	23(25.8)	
HBV genotype			0.003
B	8(12.3)	17 (19.1)	
C	48(73.8)	42 (47.2)	
Unknown	9(13.9)	30(33.7)	
HBV DNA level (Log IU/mL)	5.4 $\pm$ 1.0	5.3 $\pm$ 1.0	0.505
<i>Pre-S1/pre-S2/S</i> mutations/deletions			
PCR positive	55(84.6)	56(62.9)	
<i>Pre-S1</i> start codon mutation	10(18.2)	11(19.6)	1
<i>Pre-S1</i> deletion	5(9.1)	0(0)	0.07
<i>Pre-S2</i> start codon mutation	6(10.9)	5(8.9)	0.895
<i>Pre-S2</i> deletion	6(10.9)	9(16.1)	0.435
<i>S</i> start codon mutation	5(9.1)	7(12.5)	0.676
<i>S</i> deletion	13(23.6)	25(44.6)	0.019
EnhII/BCP/PC mutations			
PCR positive	44(67.7)	76(85.4)	
C1653T	9(20.5)	11(14.5)	0.575
T1753A/G/C	26(59.1)	27(35.5)	0.008
A1762T/G1764A	39(88.6)	52(68.4)	0.003
Precore start codon mutation	3(6.8)	6(7.9)	1
G1896A	15(34.1)	50(65.8)	0.003
G1899A	12(27.3)	12(15.8)	0.12
<i>TP53 (R249S)</i> mutation			0.669
Wild type	61 (93.8)	86 (96.6)	
<i>R249S</i> mutant	4(6.2)	3(3.4)	

*Data were expressed as mean $\pm$ SD, no (%)

which was slightly higher than that found in the control group (3.4%), but did not reach statistical significance. Two of patients with HCC contained both A1762T/G1764A and *R249S* mutations in their sera and the median age of HCC diagnosis in these patients was 52.5 $\pm$ 9.2 years compared with 58.2 $\pm$ 12.1 years for the other HCC cases (p =0.510).

To determine the independent contribution of clinical and virological factors to the development of HCC, multiple logistic regression analysis was performed by using the significant factors identified in the univariate analysis. The significant factor associated with HCC development by multivariate analysis was A1762T/G1764A mutations (odds ratio: 6.51, 95% confidence interval: 1.41-30.16, p =0.017).

Discussion

Viral genetic variations such as genotypes and mutations may play important roles in causing different disease profiles in chronic HBV infection, including HCC development. Moreover, epidemiological studies in high prevalent areas have showed significant interactions between chronic HBV infection and dietary AFB1 in relation to HCC risk. To better understand the contributions of virus-chemical interactions to hepatocarcinogenesis, data from various ethnic groups and countries are needed for comparison. To this end, this case-control study was

conducted to assess the role of HBV genetic variations, including genotypes and mutations, *R249S* mutation in the serum as a marker of mutagenesis by AFB1 and their interactions in HCC risk.

In agreement with previous data, our study showed that HBV genotypes B and C are the two common genotypes in Thailand. We found that the prevalence of genotype C was significantly higher among patients with HCC than those without liver cancer. In fact, several studies reported that genotype C was related to a higher risk of HCC development as compared to genotype B, although such association was not confirmed by other studies (Kim et al., 2011). Additionally, we found significantly higher frequencies of A1762T/G1764A and T1753A/G/C mutations in the HCC group than in controls, which are consistent with previous reports (Chen et al., 2008; Yuen et al., 2008). In fact, T1753A/G/C mutations are usually present along with the existence of A1762T/G1764A mutations. Previous data also demonstrated that T1753A/G/C mutations occurred later than the double BCP mutations in the natural course of chronic HBV infection (Takahashi et al., 1999). Regarding *pre-S* deletion, it has been shown in several reports that such mutant is an important risk factor of HCC (Chen et al., 2006; Choi et al., 2007). In vitro studies have also showed that intracellular accumulation of *pre-S* mutant proteins can modify HBV protein expression, induce ER stress and increased DNA damage (Hsieh et al., 2004). However, *pre-S* deletion was not considered to be a significant factor associated with HCC in this study.

Our data demonstrated that the double BCP mutations were the only independent risk factor for the development of HCC. A prospective cohort of approximately 1600 high-risk individuals in Qidong, China, showed that the double BCP mutations were identified in 50% of HCC cases before cancer detection, suggesting that the variants might indicate a high potential risk for HCC (Kuang et al., 2004). It has also been reported that such mutations is usually associated with HBV genotype as their prevalence is higher in genotype C than genotype B (Kao et al., 2003). Although the mechanism of double BCP mutations in hepatocarcinogenesis remains uncertain, several hypotheses have been proposed. The dual BCP mutants may enhance viral replication by creating a specific transcription factor binding site or modulating the relative levels of precore and core RNAs (Hunt et al., 2000). Furthermore, the presence of the mutants may be associated with decreasing T-cell immune responses (Malmassari et al., 2007). In addition, mutations in the BCP region, which overlaps the coding sequence for the X gene, may result in amino acid changes in the X protein (Malmassari et al., 2007). For example, A1762T/G1764A mutations are corresponding to K130M/V131I in the X protein. These genomic variations could modify the oncogenic potential of the X protein and induce inactivation of *p53*-mediated apoptosis or impairment of DNA repair (Hussain et al., 2007).

The risk of HCC associated with AFB1 is mainly reported in countries with high prevalence of HBV infection. Both double BCP mutations and *R249S* mutation are considered to be surrogate markers of AFB1-induced

HCC in the context of chronic HBV infection. In fact, HCC containing *R249S* mutation can occur in the absence of significant recent AFB1 exposure, which may allow the detection of the mutant DNA in blood specimens several months ahead of clinical diagnosis of HCC (Jackson et al., 2003). In a case-control study in The Gambia, another area of high prevalence of HCC and common exposure to HBV and AFB1, it was reported that *R249S* mutation was detectable in control subjects or patients with cirrhosis, although with a significantly lower prevalence than in HCC patients (Kirk et al., 2005). A previous study conducted in northern Thailand reported the detection rates of A1762T/G1764A and *R249S* mutations in plasma of HCC patients in approximately 40% and 25%, respectively (Kuang et al., 2005). Recent data of case-control studies from Thailand also suggested that the development of HCC might be enhanced by the cooperation of chronic HBV infection and of AFB1 induced *R249S* mutation, particularly among HCC patients with young age and without pre-existing cirrhosis (Villar et al., 2012; Ortiz-Cuaran et al., 2013). Data from our cohort, however, did not support the interactions of HBV and AFB1 in promoting hepatocarcinogenesis, because the presence of *R249S* mutations in patients with or without HCC was rather similar. In addition, the mean age of HCC patients who had both A1762T/G1764A and *R249S* mutations did not significantly differ from that of the others.

It should be noted that the prevalence of *R249S* mutation in our study was relatively low compared to other reports from Thailand. The low prevalence of such mutant in this study also explained why there was no association between AFB1 exposure and HCC risk among chronic HBV carriers. The basis of this discrepancy between our study and the other reports was unclear but might be related to the following hypotheses. First, the method using RFLP assay might not be sensitive for detecting *R249S* mutation in some specimens containing trace amounts of the mutant DNA. In this regard, it has been shown that, at high blood levels of *R249S* mutation, RFLP provides good results in agreement with more sensitive methods such as short oligonucleotide mass analysis (SOMA) (Szymanska et al., 2009). At low levels, however, the signals generated by RFLP are below the detection threshold and might be responsible for the false-negative results. Thus, based on RFLP assay, serum specimens negative for *R249S* mutation might contain levels that were too low for detection by this method. Second, the presence and amount of *R249S* mutation in serum may fluctuate among individuals according to season, with a pattern differs from the well-known seasonal variations in exposure to AFB1 (Villar et al., 2011). In addition, individual exposure to AFB1 is likely to fluctuate among different geographic areas and ecological zones (Liu et al., 2010). Nonetheless, little research has been conducted on comparisons of the seasonal and geographical influences on AFB1 contamination in Thailand.

In conclusion, our case-control study demonstrated that the dual BCP mutations were an independent virological factor associated with the risk of HCC. Identification of these HBV mutants may be helpful for predicting the development of HCC. In contrast, our cohort failed to

identify an AFB1-associated risk for HCC. Further large-scale prospective studies using more sensitive methods for detecting R249S mutation are needed to elucidate these observations.

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RESEARCH ARTICLE

The *KIF1B* (rs17401966) Single Nucleotide Polymorphism is not Associated with the Development of HBV-related Hepatocellular Carcinoma in Thai Patients

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Abstract

Hepatitis B virus (HBV) infection can become chronic and if left untreated can progress to hepatocellular carcinoma (HCC). Thailand is endemic for HBV and HCC is one of the top five cancers, causing deaths among Thai HBV-infected males. A single nucleotide polymorphism (SNP) at the *KIF1B* gene locus, rs17401966, has been shown to be strongly associated with the development of HBV-related HCC. However, there are no Thai data on genotypic distribution and allele frequencies of rs17401966. Thai HBV patients seropositive for HBsAg (n=398) were therefore divided into two groups: a case group (chronic HBV with HCC; n=202) and a control group (HBV carriers without HCC; n=196). rs17401966 was amplified by polymerase chain reaction (PCR) and analyzed by direct nucleotide sequencing. The genotypic distribution of rs17401966 for homozygous major genotype (AA), heterozygous minor genotype (AG) and homozygous minor genotype (GG) in the case group was 49.5% (n=100), 40.1% (n=81) and 10.4% (n=21), respectively, and in controls was 49.5% (n=97), 42.3% (n=83) and 8.2% (n=16). Binary logistic regression showed that rs17401966 was not statistically associated with the risk of HCC development in Thai chronic HBV patients (p-value=0.998, OR=1.00 and 95% CI=0.68-1.48). In conclusion, the *KIF1B* gene SNP (rs17401966) investigated in this study showed no significant association with HBV-related HCC in Thai patients infected with HBV, indicating that there must be other mechanisms or pathways involved in the development of HCC.

Key words: *KIF1B* - rs17401966 - chronic hepatitis B infection (HBV) - HCC - Thailand

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Introduction

Hepatitis B virus (HBV) is a major public health problem worldwide. In untreated chronic patients, HBV infection can cause liver cirrhosis and can progress to Hepatocellular Carcinoma (HCC) (Wong et al., 2006). In Thailand, HBV infection is the major cause of HCC: 65% of HCC patients are infected with HBV (Tangkijvanich et al., 1999), and patients with HBV are 20 times more likely to develop HCC (Tangkijvanich et al., 2003). More than 85% of primary liver cancers are HCC, and over 80% of these cancer patients are located in sub-Saharan Africa and Eastern Asia with China having the highest HCC prevalence (El-Serag et al., 2007). HCC is also the most common cancer and cause of death in Thai males (Srivatanakul et al., 2004).

Recently, a genome-wide association study (GWAS) conducted in 5 different locations in China discovered that a single nucleotide polymorphism (SNP) known as rs17401966 was strongly associated with the development of HCC among chronic HBV patients (Zhang et al., 2010). This SNP is located within the 24th intron of the kinesin family member 1B (*KIF1B*) gene on human chromosome 1p36.22. In several types of cancers - including HCC - *KIF1B* has been deleted (Bagchi et al., 2008), which indicates that this gene may have tumor suppressor potential.

In order to understand the activity of *KIF1B* gene, its structure has been fully investigated. The gene encodes two spliced isoforms of kinesin protein, denoted as *KIF1B α* and *KIF1B β* , which play a role in organelle and vesicle transportation in many different cell types

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(Nangaku et al., 1994). Both *KIF1B α* and *KIF1B β* share the same first 660 residues in the N-terminal motor domain but their C-terminal cargo-binding domains are completely different (Nangaku et al., 1994; Zhao et al., 2001). Aside from its transportation activity, *KIF1B β* has also been shown to suppress tumor in several cancers by interacting with inhibitors of the cell cycle or activators of apoptosis (Munirajan et al., 2004).

The major allele (A) of rs17401966 has been reported to be associated with HBV-related HCC and occurs at a frequency of 71% whereas its minor allele (G) is protective against the development of HBV-related HCC and has a frequency of 29% (Zhang et al., 2010). In contrast, two other studies involving Koreans, Japanese, Hong Kong Chinese and Saudi Arabians (Al-Qahtani et al., 2012; Sawai et al., 2012) showed that there was no significant association between rs1740966 and the development of HBV-related HCC.

Currently, it is unclear why two separate studies conducted in China showed different results. However, because of this discrepancy - and lack of data in Thais - the authors decided to investigate the genotypic distributions and allele frequencies of rs1740966 in Thai HBV patients with and without HCC. In addition, the authors assessed whether rs1740966 is associated with the development of HBV-related HCC. The information obtained from this study will help with the management of surveillance programs of HBV-related HCC patients.

Materials and Methods

Study population

HBV-infected patients from Chulalongkorn King Memorial Hospital, Thailand between January 2012 and December, 2012 were divided into two groups: case group (with HCC) and control group (without HCC). There were 202 and 197 patients in the case and control groups, respectively. All patients, regardless of group, were HBs antigen positive. HCC was diagnosed based on clinical symptoms, ultrasonogram and/or computed tomography and an alpha fetoprotein concentration of more than 400 ng/ml. Patients who had any evidence of liver mass by ultrasonography were excluded from the control group. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 534/54). Informed consent was obtained from all patients before any procedures were performed.

Sample collection

Blood samples were collected from HBV patients with and without liver cancer. For each patient, 2-3 ml of blood was collected into an EDTA tube. PBMCs were obtained by centrifugation and put in a separation medium (Wisent Inc., Quebec, Canada). Red blood cells were removed using Red Cell Lysis Buffer (1M Tris-HCl pH 7.6, 5 M NaCl, 1 M MgCl₂). PBMCs were re-suspended in Phosphate Buffer Saline (PBS) at pH

7.4 and stored at -20°C until further analysis.

DNA extraction

DNA was extracted from 100 μ L of PBMC suspension from each patient. The cells were lysed with lysis buffer (proteinase-K/SDS in Tris buffer) followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 30 μ L sterile distilled water and stored at -20°C until further analysis.

Amplification of the *KIF1B* gene flanking rs17401966 region

The DNA region flanking rs17401966 of the *KIF1B* gene was amplified by polymerase chain reaction (PCR) using specific primers. The forward primer was rs17401966_F: 5'-ATTTCATCCCACCTTTAGTCATTGCAAG-3' and the reverse primer was rs17401966_R: 5'-CACAACTACTATGACTTCAGCAACT-3'. The PCR reaction was composed of 0.5 μ L of 10 μ M forward and reverse primers (0.2 μ M final concentration for each), 10 μ L of 2.5X PerfectTaq Plus MasterMix (5 PRIME Inc., Hamburg, Germany), 1 μ L of DNA sample and distilled water up to a final volume of 25 μ L. The PCR reaction was performed under the following thermal conditions: initial denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds; then ending with the final extension at 72°C for 7 minutes. To assess the size of the PCR product, electrophoresis was performed on a 2% w/v agarose gel stained with ethidium bromide. The desired PCR product size of 292 base pairs was confirmed and visualized on a UV transilluminator.

DNA purification and nucleotide sequencing

The PCR products of interest were purified from the gel using the GelExtract Mini Kit (5PRIME, Hamburg, Germany) and were used as a template for nucleotide sequencing. DNA sequencing was performed by FirstBASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia). Nucleotide sequences of the samples were analyzed by using Chromas Lite (Technelysium Pty Ltd) and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis

The association between SNP rs17401966 and the risk of developing HCC were assessed by using binary logistic regression. Statistical significant difference was accepted when p-value was below 0.05. The odds ratio was considered if both upper and lower 95% CI did not cover 1. Statistical parameters were calculated using SPSS for Windows v13.0 (SPSS Inc., Chicago, IL).

Results

Age and sex ratio between case and control groups

In this study, there were a total of 398 HBV-infected

patients. All of the patients were confirmed to be HBsAg positive and were later divided into 2 groups based on whether they had HCC or not. There were 202 HBV-infected patients with HCC (case group) and 196 asymptomatic HBV carriers (control group). The mean age and standard deviation for case and control groups were 59.8±12.3 years and 46.3±9.9 years, respectively. The sex ratio (female/male) in the case group was 44/158 whereas in control group it was 65/131 (Table 1).

Genotypic distributions of rs17401966 and its association to HBV-related HCC

The genotypic distributions of rs17401966 between both groups were comparable (Table 2, Figure 1A). In the case group, 49.5% (n=100), 40.1% (n=81) and 10.4% (n=21) had homozygous major genotype (AA), heterozygous minor genotype (AG) and homozygous minor genotype (GG), respectively. Similarly, in the control group, 49.5% (n=97), 42.3% (n=83) and 8.2% (n=16) had homozygous major genotype (AA), heterozygous minor genotype (AG) and homozygous minor genotype (GG), respectively. rs17401966 was not significantly associated with the development of HCC for the heterozygous genotype (AG) [odd ratio (95% CI)=0.95 (0.79-1.31) with p=0.795], homozygous minor genotype (GG) [odd ratio (95% CI)=1.27 (0.63-2.58) with p=0.503] or heterozygous (AG)+homozygous (GG) genotypes [odd ratio (95% CI)=1.00 (0.68-1.48) with p=0.998] (Table 2).

Table 1. Basic Demographic Data of the Patients in this Study

Characteristics	Case	Controls
Total	202	196
Age		
Mean (Standard Deviation)	59.8 (12.3)	46.3 (9.9)
Sex		
Female, n (%)	44 (21.8)	65 (33.2)
Male, n (%)	158 (78.2)	131 (66.8)
Ratio (female/male)	44/158	65/131

Allele frequencies of rs17401966 and its association to HBV-related HCC

The frequencies of the major (A) and minor (G) alleles were comparable between both groups. In the case group, 69.6% and 30.4% had major (A) and minor (G) alleles, respectively. In the control group, 70.7% and 29.3% had major (A) and minor (G) alleles, respectively (Table 3, Figure 1B). After adjusting for variables such as age and sex in the logistic regression analysis, minor allele (G) of rs17401966 was not significantly associated with the development of HCC [odd ratio (95% CI)=0.95 (0.70-1.28) with p=0.733] (Table 3).

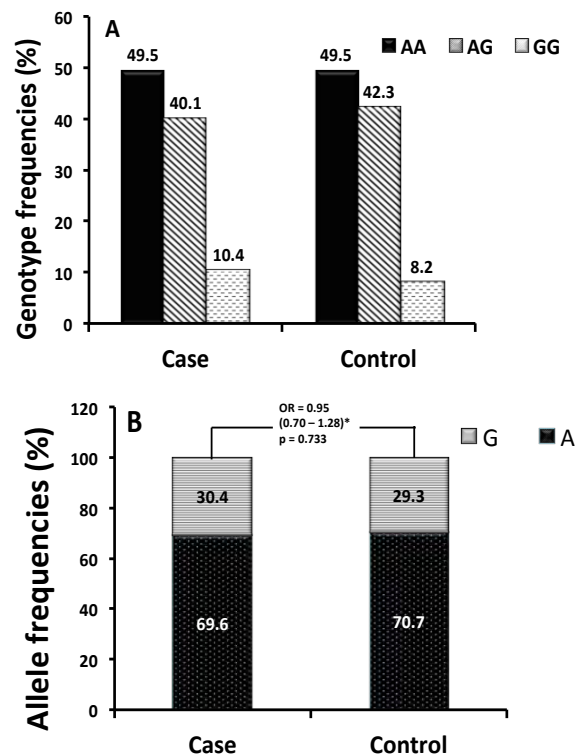


Figure 1. Genotype (A) and Allele (B) Frequencies for rs17401966 between Case and Control Groups. *95%CI (lower-upper)

Table 2. Genotypic Distribution of rs17401966 and Its Association with HBV-related HCC

SNPs	Genotype	Cases (n=202)	Controls (n=196)	OR*	95%CI	p-values*
rs17401966	AA	100 (49.5%)	97 (49.5%)	1**	—	—
	AG	81 (40.1%)	83 (42.3%)	0.95	0.63-1.43	0.795
	GG	21 (10.4%)	16 (8.20%)	1.27	0.63-2.58	0.503
	AG+GG	102 (50.5%)	99 (50.5%)	1.00	0.68-1.48	0.998

*Cases compare with controls; ** Reference

Table 3. Allele Frequencies of rs17401966 and Association to HBV-related HCC

SNP	Allele	Cases (2n=404)	Controls (2n=392)	OR*	95%CI	p-values*
rs17401966	Major (A)	281 (69.6%)	277 (70.7%)	1**	—	—
	Minor (G)	123 (30.4%)	115 (29.3%)	0.95	0.70-1.28	0.733

*Cases compare with controls; **Reference

Table 4. Comparison of Genotypic Distribution of rs17401966 among Different Populations

Populations	Total			Case, n (%)			Control, n (%)			OR	95%CI	p-value	Reference			
	Total	AA	AG	AA	AG	GG	A	G	Total					AA	AG	GG
Chinese (Total)	2310	1497 (64.8)	736 (31.9)	77 (3.3)	3730 (80.7)	890 (19.3)	1789	936 (52.3)	715 (40.0)	138 (7.7)	2587 (72.3)	991 (27.7)	1.61	1.45-1.78	<0.001	Zhang et al., 2010
Japanese (Total)	321	196 (61.0)	107 (33.3)	18 (5.6)	499 (77.7)	143 (22.3)	1020	609 (59.7)	352 (34.5)	59 (5.8)	1570 (77.0)	470 (23.0)	0.94	0.76-1.16	0.572	Sawai et al., 2012
Korean	164	88 (53.6)	59 (36.0)	17 (10.4)	235 (71.6)	93 (28.4)	144	74 (51.4)	55 (38.2)	15 (10.4)	203 (70.5)	85 (29.5)	1.06	0.75-1.50	0.751	Sawai et al., 2012
Hong Kong Chinese	93	44 (46.8)	39 (41.5)	10 (10.6)	127 (68.3)	59 (31.7)	187	94 (50.3)	80 (42.8)	13 (6.9)	268 (71.7)	106 (28.3)	0.85	0.58-1.25	0.409	Sawai et al., 2012
Thai	202	100 (49.5)	81 (40.1)	21 (10.4)	281 (69.6)	123 (30.4)	196	97 (49.5)	83 (42.3)	16 (8.2)	277 (70.7)	115 (29.3)	0.95	0.70-1.28	0.733	This study
Saudi Arabian	183	127 (69.4)	47 (25.7)	9 (4.9)	301 (82.2)	65 (17.8)	72	47 (65.3)	22 (30.6)	3 (4.2)	116 (80.6)	28 (19.4)	1.12	0.68-1.83	0.657	Al-Qahtani et al., 2012

Discussion

There are no data on the genotypic distributions and allele frequencies of rs17401966 in Thai HBV-infected patients, and this study is the first to report such data in Thais. The genotypic distributions between both groups were comparable for the homozygous major genotype (AA), heterozygous minor genotype (AG) and homozygous minor genotype (GG). Similar results were also observed in Japanese, Korean, Hong Kong Chinese and Saudi Arabian populations (Al-Qahtani et al., 2012; Sawai et al., 2012). The genotypic distributions of rs17401966 among different populations are summarized in Table 4.

In this study, the frequency of homozygous major genotype (AA) was 49.5% for both groups while frequency of heterozygous minor genotype (AG) was 40.1% and 42.3% for the case and control groups, respectively, and the frequency of homozygous minor genotype (GG) was 10.4% and 8.2% for case and control groups, respectively. The distribution of rs17401966 genotypes in the Thai population was closely related to the Hong Kong Chinese population but highly different from those found in Saudi Arabians. The genotypic distributions of rs17401966 between the case and control group were significantly different in Chinese populations from Guangxi, Beijing, Jiangsu, Guandong and Shanghai (Zhang et al., 2010). The (AA) homozygous major genotype in the case group was significantly higher than the control group, whereas the (AG) heterozygous and (GG) heterozygous minor genotypes in the case group were lower than those found in the control group. This discrepancy indicates that there are other factors aside from ethnicity that are involved in the development of HCC. It is been previously shown that factors such as age, sex, alcohol abuse, folate intake, aflatoxin B1, mutations of *p53*, other viral factors, viral mutation and different genotypes of HBV may be associated with HCC development (Montesano et al., 1997; Lee et al., 2007).

In addition, based on binary logistic regression analysis, rs17401966 was not significantly associated with the development of HCC in Thai patients chronically infected with HBV (p -value=0.998, OR=1.00 and 95% CI=0.68-1.48). This finding is in agreement with results reported in Japanese, Korean, Hong Kong Chinese and Saudi Arabian populations (Al-Qahtani et al., 2012; Sawai et al., 2012). Although the study conducted by Zhang et al. indicated otherwise, this may be due to other factors as previously mentioned.

Thus, it is likely that a combination of other factors contribute to the development of HCC. For example, age has been shown to be positively correlated with HCC. Likewise, males are 4-5 times more likely to develop HCC compared to females (Tangkijvanich et al., 1999). However these factors cannot be controlled for, unlike alcohol and folate consumption. Alcohol abuse will increase the risk of HCC development by 2-4 folds (Fattovich et al., 2004; Morgan et al., 2004) whereas high folate intake may help reduce the risk of developing alcohol-associated HCC (Person et al., 2013). The correlation among these factors indicates the complexity of hepato-carcinogenesis. Another factor that may affect the development of HCC is aflatoxin B1, which is a carcinogen associated with R249S mutation in the *p53* tumor suppressor gene (Groopman et al., 2005). The *p53* mutation is extremely prevalent in sub-Saharan Africa and China because of high exposure to aflatoxin (Montesano et al., 1997). Other factors that may also contribute to the development of HCC are certain genotypes and mutations of HBV. For example, HBV genotype C was positively correlated with HCC incidence. The risk of HCC development increases if patients with HBV genotype C also have a double Basal Core Promoter mutation (Yang et al., 2008). The prevalence of HBV genotype C in Korea, Japan, Thailand and China are 100%, 82.3%, 73% and 50.99%, respectively (Tangkijvanich et al., 2005; Kim et al., 2007; Matsuura et al., 2009) while Saudi Arabians are predominantly infected with genotype D (Abdo et al., 2006). Thus, the different viral genotypes that predominate in each country are associated with the development of HCC in patients. Therefore, not only the allele frequency is responsible for the differences in association between rs17401966 and the risk of HBV-related HCC development. The authors speculate that there must be several factors aside from rs17401966 interacting with each other to

cause discrepancies among the studies. Furthermore, the difference of mean age between case and control groups may be a limitation for the comparison of genotype and allele frequency. In conclusion, SNP rs17401966 was not associated with the risk of developing HCC in Thai HBV-infected patients. This indicates that there must be other mechanisms or pathways involved in the development of HCC.

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Association of interferon-alpha gene polymorphisms with chronic hepatitis B virus infection

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Summary

In this study, the association between the risk of chronic hepatitis B virus infection and the polymorphisms within promoter regions of *IFN- α 1* and five genes was explored. This association study was performed on 180 Thai patients with chronic HBV infection [hepatocellular carcinoma (HCC) = 65 and non-HCC = 115], 173 individuals with self-limited HBV infection and 140 healthy controls. Our results showed that the A allele of -1823G/A SNP within *IFNA1* gene was significantly associated with an increased risk of chronic HBV infection as compared to healthy individuals and self-limited HBV group [OR (95% CI) = 2.20 (1.51–3.19), $P = 0.000014$ and OR (95% CI) = 1.61 (1.12–2.33), $P = 0.0073$, respectively]. The effect of A allele was similar to autosomal recessive in which the presence of AA genotype when compared to GG and GA conferred the OR of 2.79 (95% CI = 1.72–4.52, $P = 0.0000085$). By multifactor dimensionality reduction analysis, we found the interaction between *IFNA5* (-2529) and *IFNA1* (-1823) genes that gave the risk to chronic HBV infection, with the OR (95% CI) of the high-risk to low-risk group was 2.79 (1.77–4.40), $P < 0.0001$. However, further study in functional significance is required.

Introduction

Although the number of HBV infection has been reduced by the use of an effective HBV vaccine, there

remain 350 million individuals worldwide infected chronically and become carriers of the virus. Some of them develop liver cirrhosis and hepatocellular carcinoma. Approximately one million people die each year from these complications (Lavanchy, 2004). Thailand has been classified as a region of intermediate endemicity as well as Taiwan, India, Pakistan, Korea and Philippines (Mohamed *et al.*, 2004). Twin studies in Chinese showed a higher concordance rate for HBeAg persistence in monozygotic twins compared with dizygotic twins (Lin *et al.*, 1989). This provides the strong evidence for a host genetic effect. Therefore, the difference of host genetic might affect the different outcomes of patients with HBV infection. Several association studies reported that various human leukocyte antigen (HLA) class I and II loci, many cytokine and cytokine receptor genes were associated with HBV susceptibility and/or HBV persistence or disease severity (Thursz *et al.*, 2011).

Interferon-alpha (*IFN- α*) is a cytokine that mainly involved in innate immune response against viral infection. The introduction of *IFN- α* by several stimuli has been shown to inhibit HBV gene expression and/or HBV replication in the livers of HBV-transgenic mice (Cavanaugh *et al.*, 1998). Furthermore, it has been widely used in the treatment of chronic HBV infection (Karayiannis, 2003). There are 13 subtypes in the *IFN- α* family that composed of *IFNA1*, *IFNA2*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA7*, *IFNA8*, *IFNA10*, *IFNA13*, *IFNA14*, *IFNA16*, *IFNA17* and *IFNA21*. These genes are located in a cluster on the short arm of human chromosome 9 (Diaz *et al.*, 1994). *IFNA2* is a major subtype of *IFNA* species that is produced in response to many viruses. In addition, it is often used to treat hepatitis B in combination with the other antiviral drugs (Bonino *et al.*, 2007). An association study of *IFNA2* gene found that a deletion in the promoter was significantly more frequent in HBV-infected Vietnamese patients than in control individuals. Moreover, the deletion in promoter of *IFNA2* gene reduces the transcription of this gene *in vitro* (Song le *et al.*, 2006). However, there is no association study between HBV infection and the other major subtypes such as *IFNA1* and *IFNA5*, which are interesting subtypes in

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response to viral infection. *IFNA1* and *IFNA5* have been reported to be expressed in the liver tissue, especially *IFNA5* which is the predominant subtype (Castelruiz *et al.*, 1999). Furthermore, the expression of *IFNA5* is higher than other IFNA subtypes in peripheral blood mononuclear cell (PBMC) of patients infected with some viruses such as hepatitis C virus (Castelruiz *et al.*, 1999; Larrea *et al.*, 2001). Polymorphisms in the promoter region of these genes might influence such gene expression.

Therefore, we investigated the association between single nucleotide polymorphism (SNP) within the promoter region of *IFNA1* and *IFNA5* genes with susceptibility to chronic HBV infection and the development of HCC.

Materials and methods

Subjects

One hundred and eighty Thai patients with chronic HBV infection from Chulalongkorn Memorial Hospital were recruited in this study. The diagnosis of chronic hepatitis B was established by seropositivity for HBsAg over a 6-month period and did not have any other type of liver diseases such as chronic hepatitis C or alcoholic liver disease. In addition, all the patients had elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Patients with chronic HBV infection were further divided into two groups: without ($N = 115$; 34 women and 81 men: mean age $\pm$ SD = 44.44 ± 14.63 years) and with HCC ($N = 65$; 10 women and 55 men: mean age $\pm$ SD = 53.96 ± 16.97 years) according to the absence or presence of concurrent HCC. Diagnosis of HCC was based on histopathology and/or a combination of mass lesion in the liver from hepatic imaging and serum alpha fetoprotein level >400 ng mL $^{-1}$. Moreover, self-limited HBV group served as control for the population-based case-control study contained 173 subjects (91 women and 82 men: mean age $\pm$ SD = 48.41 ± 13.76 years), who tested HBsAg negative and both HBV core antibody (anti-HBc) and HBV surface antibody (anti-HBs) positive, with normal liver function tests, and no history of HBV vaccination. Moreover, 140 ethnically and geographically matched controls (83 women and 57 men: mean age $\pm$ SD = 23.09 ± 12.58 years) from healthy blood donor of the Thai Red Cross Society were recruited as control group. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, approved the study, and the subjects gave their informed consent.

Moreover, chronic HBV patients with AST and ALT level of ≥ 100 U L $^{-1}$ were defined as high AST and ALT groups, while the patients with AST and ALT <100 U L $^{-1}$ were classified as low liver enzyme groups. Patients with HBV DNA levels of $\geq 10^5$ copies per mL were characterized as high viral load

group, whereas the patients with HBV DNA levels of $<10^5$ copies per mL were considered as low viral replication subjects (Li *et al.*, 2011).

DNA extraction and genotyping study

DNA was extracted from the buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, using a salting-out method (Miller *et al.*, 1988). DNA was aliquoted and stored at -20 °C until used. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to analyse the polymorphism of *IFNA1* gene (-1823G/A, rs1332190) and *IFNA5* gene (-2529A/T, rs3758236) as previously described (Hirankarn *et al.*, 2008). Negative controls without DNA template were included in each experiment. Ten per cent of the samples were confirmed by direct sequencing of PCR products to verify the accuracy of genotyping.

Statistical analysis

Genotype frequencies were checked for consistency among normal controls with those expected from the Hardy–Weinberg equilibrium (HWE). Allele and genotype frequencies were compared between groups using the chi-squared (χ^2) test or Fisher's exact probability test, where appropriate. The PLINK v1.07 program was used to calculate HWE, P values, odds ratios and 95% confidence intervals, as well as for haplotype analysis (Purcell *et al.*, 2007). A P value of <0.05 was considered statistically significant. In addition, gene–gene interaction was analysed by multifactor dimensionality reduction (MDR) approach (Hahn *et al.*, 2003).

Results

The distribution of genotype and allele frequencies of *IFNA1* and *IFNA5* gene polymorphisms in chronic HBV patients (with HCC and without HCC), self-limited HBV patients and healthy controls are shown in Table 1. In this study, both SNPs were in Hardy–Weinberg equilibrium when compared the observed and expected genotype frequencies of each SNP ($P > 0.05$). In this study, we found statistically significant differences in the allele frequency of -1823G/A SNP (rs1332190) within *IFNA1* gene between patients with chronic HBV and healthy controls. The A allele of this SNP was significantly associated with an increased risk in chronic HBV infection as compared to healthy individuals [OR (95% CI) = 2.20 (1.51–3.19), $P = 0.000014$], whereas the comparison between patients with chronic HBV and self-limited HBV group showed lower statistical significance with the OR (95% CI) of 1.61 (1.12–2.33) and $P = 0.0073$. The effect of A allele was similar to autosomal recessive in which the presence of AA genotype when compared to GG and GA conferred the OR of

Table 1. Genotype and allele frequencies of *IFNA1* and *IFNA5* gene polymorphisms in chronic HBV patients (with HCC and without HCC), self-limited HBV patients and healthy controls

SNP	Genotype/allele	Total chronic HBV N = 180 (%)	With HCC N = 65 (%)	Without HCC N = 115 (%)	Self-limited HBV N = 173 (%)	Healthy control N = 140 (%)
<i>IFNA1</i> (-1823) (rs1332190)	GG	7 (3.89)	2 (3.08)	5 (4.35)	16 (9.25)	13 (9.29)
	GA	56 (31.11)	20 (30.77)	36 (31.30)	65 (37.57)	71 (50.71)
	AA	117 (65.00) ^b	43 (66.15) ^a	74 (64.35) ^h	92 (53.18)	56 (40.00)
	G	70 (19.44)	24 (18.46)	46 (20.00)	97 (28.03)	97 (34.64)
	A	290 (80.56) ^{a,c}	106 (81.54) ^{d,f}	184 (80.00) ^{g,i}	249 (71.97)	183 (65.35)
<i>IFNA5</i> (-2529) (rs3758236)	AA	46 (25.56)	15 (23.08)	31 (26.96)	37 (21.39)	37 (26.43)
	AT	83 (46.11)	30 (46.15)	53 (46.09)	92 (53.18)	64 (45.71)
	TT	51 (28.33)	20 (30.77)	31 (26.96)	44 (25.43)	39 (27.86)
	A	175 (48.61)	60 (46.15)	115 (50.00)	166 (47.98)	138 (49.29)
	T	185 (51.39)	70 (53.85)	115 (50.00)	180 (52.02)	142 (50.71)

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SNP, single nucleotide polymorphism.

^aA compared with G allele (total chronic HBV vs. healthy control); OR (95%CI) = 2.20 (1.51–3.19), $P = 0.000014$; ^bAutosomal recessive model AA compared with GA + GG genotype (total chronic HBV vs. healthy control); OR (95%CI) = 2.79 (1.72–4.52), $P = 0.0000085$; ^cA compared with G allele (total chronic HBV vs. self-limited HBV); OR (95%CI) = 1.61 (1.12–2.33), $P = 0.0073$; ^dA compared with G allele (chronic HBV with HCC vs. healthy control); OR (95%CI) = 2.34 (1.37–4.02), $P = 0.00083$; ^eAutosomal recessive model AA compared with GA + GG genotype (chronic HBV with HCC vs. healthy control); OR (95%CI) = 2.93 (1.52–5.69), $P = 0.00049$; ^fA compared with G allele (chronic HBV with HCC vs. self-limited HBV); OR (95%CI) = 1.72 (1.01–2.93), $P = 0.033$; ^gA compared with G allele (chronic HBV without HCC vs. healthy control); OR (95%CI) = 2.12 (1.39–3.25), $P = 0.00025$; ^hAutosomal recessive model AA compared with GA + GG genotype (chronic HBV without HCC vs. healthy control); OR (95%CI) = 2.71 (1.58–4.66), $P = 0.0001$; ⁱA compared with G allele (chronic HBV without HCC vs. self-limited HBV); OR (95%CI) = 1.56 (1.03–2.37), $P = 0.029$.

2.79 (95% CI = 1.72–4.52, $P = 0.0000085$). In addition, we classified total chronic HBV as with HCC ($N = 65$) and without HCC ($N = 115$). Our finding still showed a significant difference of A allele of -1823G/A SNP (rs1332190) between chronic HBV patients with HCC and healthy controls as well as chronic HBV patients without HCC vs. healthy controls [OR (95%CI) = 2.34 (1.37–4.02), $P = 0.00083$ and OR (95%CI) = 2.12 (1.39–3.25), $P = 0.00025$, respectively]. When we compared both chronic HBV groups (with and without HCC) vs. self-limited HBV patients, this A allele frequency was also found to be significantly different [OR (95% CI) = 1.72 (1.01–2.93), $P = 0.033$ and OR (95% CI) = 1.56 (1.03–2.37), $P = 0.029$, respectively]. The effect of A allele seems to be autosomal recessive model with the OR of 2.93 (95% CI = 1.52–5.69, $P = 0.00049$). However, we did not find any significant associations of -2529A/T SNP in *IFNA5* gene between patients with chronic HBV and control groups ($P > 0.05$).

Furthermore, we performed haplotype analysis of these two SNPs (-2529A/T SNP in *IFNA5* and -1823G/A SNP in *IFNA1*). There were four haplotypes including AG, TG, AA and TA. To test the association of *IFNA* haplotype and disease development, we compared each tested haplotype with other two haplotypes between patients and controls. In this study, we found significant association between TA haplotype and risk to chronic HBV infection [total chronic HBV (OR = 1.69, $P = 0.0065$), with HCC (OR = 1.91, $P = 0.0091$) and without HCC (OR = 1.64, $P = 0.0314$)]. In contrast, TG was observed to be protective haplotype [total chronic HBV (OR = 0.331, $P = 0.0004$), with HCC (OR = 0.328, $P = 0.0112$) and without HCC (OR = 0.333, $P = 0.0022$)]. The results of haplotype analysis are shown in Table 2. These haplotype results were consistent with the additional analysis of gene–gene interaction using MDR approach. In this study, the combination of TT genotype of *IFNA5* gene with AA genotype of *IFNA1* gene

Table 2. Haplotype analysis for -2529A/T (rs3758236) of *IFNA5* gene and -1823G/A (rs1332190) of *IFNA1* gene

Haplotype	Haplotype frequency					Total vs. Healthy control		With HCC vs. Healthy control		Without HCC vs. Healthy control	
	Total chronic HBV	With HCC	Without HCC	Self-limited HBV	Healthy control	OR	P value	OR	P value	OR	P value
AG	0.0973	0.0856	0.0999	0.1328	0.1513	0.579	0.0378	0.473	0.0838	0.631	0.1048
TG	0.0972	0.0990	0.1001	0.1475	0.1952	0.331	0.0004	0.328	0.0112	0.333	0.0022
AA	0.3888	0.3759	0.4001	0.3469	0.3416	1.230	0.2192	1.150	0.5565	1.280	0.1997
TA	0.4167	0.4394	0.3999	0.3727	0.3120	1.690	0.0065	1.910	0.0091	1.640	0.0314

HBV, hepatitis B Virus; HCC, hepatocellular carcinoma; OR, odds ratio.

Table 3. Analysis of gene–gene interaction using MDR approach for -2529A/T (rs3758236) of *IFNA5* gene and -1823G/A (rs1332190) of *IFNA1* gene

Combination	Chronic HBV	Healthy control	Case/control ratio	Predicted class
TT, AA	34	9	3.7778	High risk
AT, AA	53	28	1.8929	High risk
AA, AA	30	19	1.5789	High risk
TT, GA	16	26	0.6154	Low risk
AT, GA	28	33	0.8485	Low risk
AA, GA	12	12	1	Low risk
TT, GG	1	4	0.25	Low risk
AT, GG	2	3	0.6667	Low risk
AA, GG	4	6	0.6667	Low risk

MDR, multifactor dimensionality reduction.

(TT, AA) was classified as high-risk group (ratio of case: control >1) as well as AT, AA and AA, AA. Whereas the combination between TT and GA, AT and GA, AA and GA, TT and GG, AT and GG, AA and GG of *IFNA5* and *IFNA1* genes, respectively, was classified as low-risk group (ratio of case: control ≤ 1) (Table 3). By whole statistical analysis, the OR (95% CI) for the high-risk to low-risk group was 2.79 (1.77–4.40), $P < 0.0001$.

In addition, the association between allele, genotype and haplotype frequencies of *IFNA1* and *IFNA5* gene polymorphisms with HBV viral load and liver enzymes in a subset of the patients was analysed. In this study, there was no significant association between these gene polymorphisms with the level of HBV DNA, AST and ALT liver enzymes (Table 4).

Discussion

In this study, we determined the association between the *IFNA* gene polymorphisms and the susceptibility to chronic HBV infection. We focused on two polymorphisms in the promoter region comprising -1823G/A SNP (rs1332190) within *IFNA1* gene and -2529A/T SNP (rs3758236) within *IFNA5* gene. Our results suggest that *IFN* polymorphism might be important for the risk of chronic HBV infection. In this study, we found that A allele of -1823G/A SNP within *IFNA1* had a higher risk of developing chronic hepatitis B. By model of inheritance analysis, the association of this SNP seems to be recessive effect model. In addition, we divided chronic HBV patients into patient with HCC and without HCC groups. Our results showed that SNP within *IFNA1* was associated with both groups. This suggests that there may be other genetic

Table 4. Allele, genotype and haplotype frequencies in chronic hepatitis B patients stratified by HBV DNA levels, aspartate aminotransferase and alanine aminotransferase

	HVL N = 40 (%)	LVL N = 19 (%)			HAST N = 42 (%)	LAST N = 74 (%)			HALT N = 45 (%)	LALT N = 72 (%)		
SNPs			OR	P value			OR	P value			OR	P value
Alleles												
IFNA1												
rs1332190												
G	17 (21.25)	4 (10.53)	2.29	0.1547	18 (21.43)	30 (20.27)	1.07	0.8342	21 (23.33)	28 (19.44)	1.26	0.4769
A	63 (78.75)	34 (89.47)			66 (78.57)	118 (79.73)			69 (76.67)	116 (80.56)		
IFNA5												
rs3758236												
A	41 (51.25)	17 (44.74)	1.3	0.5084	42 (50.00)	72 (48.65)	1.06	0.8431	44 (48.89)	70 (48.61)	1.01	0.967
T	39 (48.75)	21 (55.26)			42 (50.00)	76 (51.35)			46 (51.11)	74 (51.39)		
Genotypes												
IFNA1												
rs1332190												
GG	1 (2.50)	0 (0)	1	1	2 (4.76)	2 (2.70)	1.8	0.5591	2 (4.44)	2 (2.78)	1.63	0.638
GA	15 (37.50)	4 (21.05)	2.25	0.2065	14 (33.33)	26 (35.14)	0.92	0.8444	17 (37.78)	24 (33.33)	1.21	0.624
AA	24 (60.00)	15 (78.95)	0.4	0.1508	26 (61.91)	46 (62.16)	0.99	0.9781	26 (57.78)	46 (63.89)	0.77	0.5086
IFNA5												
rs3758236												
AA	11 (27.50)	4 (21.05)	1.42	0.5951	11 (26.19)	18 (24.32)	1.1	0.8235	12 (26.67)	17 (23.61)	1.18	0.7096
AT	19 (47.50)	9 (47.37)	1.01	0.9925	20 (47.62)	36 (48.65)	0.96	0.9151	20 (45.00)	36 (50.00)	0.8	0.5584
TT	10 (25.00)	6 (31.58)	0.72	0.5953	11 (26.19)	20 (27.03)	0.96	0.922	13 (28.89)	19 (26.39)	1.13	0.7679
Haplotypes												
AG	0.1153	0.0417	4.74	0.1952	0.1328	0.1218	1.13	0.8076	0.129	0.1188	1.12	0.8166
TG	0.0972	0.0636	2.14	0.5435	0.0815	0.0809	1.01	0.9882	0.1043	0.0757	1.76	0.4484
AA	0.3972	0.4057	0.97	0.9299	0.3647	0.3647	1.01	0.9699	0.3599	0.3673	0.97	0.9083
TA	0.3903	0.4891	0.65	0.3102	0.4326	0.4326	0.94	0.8351	0.4068	0.4382	0.87	0.6361

HBV, hepatitis B virus; SNPs, single nucleotide polymorphisms; HVL, high viral load, HBV DNA level $\geq 10^5$ copies per ml; LVL, low viral loads, HBV DNA level $<10^5$ copies per ml; HAST, high aspartate aminotransferase, AST level ≥ 100 U L $^{-1}$; LAST, low aspartate aminotransferase, AST level <100 U L $^{-1}$; HALT, high alanine aminotransferase, ALT level ≥ 100 U L $^{-1}$; LALT, low alanine aminotransferase, ALT level <100 U L $^{-1}$; OR, odds ratio.

factors responsible for the development of HCC. Recent studies reported that some cytokine polymorphisms such as SNPs in *IL-1B*, *TNF- α* and *IL-28B* affected the progression of HCC (Hirankarn *et al.*, 2006; Kummee *et al.*, 2007; Ren *et al.*, 2012). Our significant finding was not seen between -2529A/T SNP in *IFNA5* gene and any groups of patients with chronic HBV. However, haplotype analysis revealed significant association between haplotype TA and risk to chronic HBV infection. This result corresponded to the analysis of genotype combination using MDR program (Hahn *et al.*, 2003). The genotype having two T allele of *IFNA5* gene and two A allele of *IFNA1* gene had higher risk effect than the other combinations. This is consistent to our previous study in *IFNA1* and *IFNA5* genes interaction (Hirankarn *et al.*, 2008).

IFN- α is widely used in patients infected with HBV to provide the lack of this cytokine. However, there are no data for different patterns of IFNA subtypes that produced in patients with HBV. The activity of IFN- α is mediated by type I interferon receptor, which composed of IFNAR1 and IFNAR2 (Pestka *et al.*, 2004). A mechanism to control the level of IFN- α was studied by *in vitro* analysis of the promoter variants in *IFNA2* gene (Song *et al.*, 2006). For *IFNA1* and *IFNA5* genes, our previous report showed that these two genes were associated with some diseases such as systemic lupus erythematosus (SLE) (Hirankarn *et al.*, 2008). However, there were no any previous reports in association between polymorphisms of these two genes and HBV infection. There was only one report in type I interferon receptor genes such as *IFNAR1* and *IFNAR2*. Several studies found that polymorphisms in promoter, intron, exon and 3'UTR regions of *IFNAR1* gene were associated with HBV infection (Zhou *et al.*, 2007; Song *et al.*, 2008; Zhou *et al.*, 2009; He *et al.*, 2010; Zhou *et al.*, 2012). For *IFNAR2* gene, a study in polymorphism of this gene showed that non-synonymous SNP located on signal peptide region was associated with outcome of persistent HBV infection in Gambians (Frodsham *et al.*, 2006). However, this association was not observed in Chinese patients (Chen *et al.*, 2010).

In conclusion, our finding showed the association between polymorphisms in promoter region of *IFNA1* and *IFNA5* genes and the risk to chronic HBV infection. However, until now, there is still no any report in functional significance of polymorphisms in these two genes. Thus, functional analysis involving with the effect of promoter polymorphism on the gene expression is required in the future.

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Association of *IFNAR2* and *IL10RB* genes in chronic hepatitis B virus infection

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Key words

chronic hepatitis B; hepatocellular carcinoma; interleukin-10 receptor subunit beta; single nucleotide polymorphisms; type I interferon receptor 2

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Abstract

In this study, we investigated the effects of two functional polymorphisms, type I interferon receptor 2 gene (*IFNAR2*)-F8S and interleukin-10 receptor subunit beta gene (*IL10RB*)-K47E, on chronic hepatitis B virus (HBV) infection. We included 227 Thai patients with chronic HBV infection [100 with hepatocellular carcinoma (HCC) and 127 non-HCC], 170 individuals with self-limited HBV infection and 150 healthy controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to analyze these two single nucleotide polymorphisms (SNPs). In this study, the C allele of *IFNAR2*-F8S was found to be significantly increased in chronic HBV patients when compared with healthy controls [odds ratio, OR (95% confidence interval, CI) = 3.31 (2.11–5.21), $P = 6.214 \times 10^{-9}$ and corrected P -value, $P_c = 1.864 \times 10^{-8}$]. The effect of this allele was similar to that of an autosomal dominant gene in the presence of CC and CT genotype, when compared to TT with an OR of 4.02 ($P = 4.631 \times 10^{-9}$ and $P_c = 1.389 \times 10^{-8}$). Furthermore, AA genotype of *IL10RB*-K47E was found to be significantly decreased in chronic HBV patients compared with individuals with self-limited HBV infection ($P = 0.006$, $P_c = 0.018$ and OR = 0.45). For haplotype analysis, we found CA and CG haplotypes were associated with susceptibility to chronic HBV ($P = 0.014$, OR = 6.84 and $P = 0.002$, OR = 3.75, respectively) when compared with healthy individuals. This study suggests that *IFNAR2*-F8S polymorphisms might be involved in the susceptibility to chronic HBV infection. Moreover, AA genotype of *IL10RB*-K47E may provide a protective effect in this disease. However, an association study using a larger sample size should be performed to confirm these findings.

Introduction

Hepatitis B virus (HBV) infection is the most common cause of acute and chronic hepatitis. Although the number of HBV infection has been reduced by the use of an effective HBV vaccine, there remain 350 million individuals worldwide infected chronically and who become carriers of the virus. In addition, chronic HBV infection is associated with the development of cirrhosis and hepatocellular carcinoma (HCC) leading to the death of approximately 1 million people each year (1). Thailand has been classified as a region of intermediate endemicity as well as Taiwan, India, Pakistan, Korea and Philippines (2). Chinese twin studies showed a higher concordance rate for hepatitis B e antigen (HBeAg) persistence in monozygotic twins as compared with dizygotic twins (3). Therefore, the difference of host genetics might

affect the different outcomes of patients with HBV infection. Several studies indicate an association between human leukocyte antigen gene (HLA) with this disease and/or the ability to eliminate HBV (4–6). However, recent studies showed that non-HLA genes are likely to be involved more than HLA genes. In a whole genome study of Frodsham et al., a cluster of class II cytokine receptor gene on chromosome 21q22 was identified as a major susceptibility locus (7). Further study of Frodsham et al. found *IFNAR2*-F8S and *IL10RB*-K47E that are nonsynonymous single nucleotide polymorphisms (SNPs) were significantly associated with outcome of persistent HBV infection in Gambians (7). Moreover, several studies in Chinese populations reported that these two functional SNPs were associated with susceptibility to chronic HBV infection (8, 9). However, these studies have shown controversial results. In

the Chen study (9), IL10RB SNP, A allele was found to be at risk for chronic HBV infection, whereas Gong *et al.*'s study (8) showed the protective effect of this allele. For IFNAR2 SNP, the study of Gong *et al.* found that CC (SS) genotype was associated with HBeAg negative patients and the T (F) allele was associated with the risk to high viral loads. However, these results were not found in Chen *et al.*'s study. Therefore, we investigated the effects of these two polymorphisms on chronic HBV infection in a Thai population.

Materials and methods

Subjects

Two hundred and twenty-seven Thai patients with chronic HBV infection from Chulalongkorn Memorial Hospital were recruited in this study. The diagnosis of chronic hepatitis B was established by seropositivity for hepatitis B surface antigen (HBsAg) over a 6-month period and did not have any other types of liver diseases such as chronic hepatitis C or alcoholic liver disease. In addition, all patients had elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Patients with chronic HBV infection were further divided into two groups: without ($N = 127$; 50 women and 77 men; mean age $\pm$ SD = 44.44 ± 14.63 years) and with HCC ($N = 100$; 15 women and 85 men; mean age $\pm$ SD = 53.96 ± 16.97 years) according to the absence or presence of concurrent HCC. Diagnosis of HCC was based on histopathology and/or a combination of mass lesion in the liver from hepatic imaging and serum alpha fetoprotein level >400 ng/ml. Moreover, self-limited HBV group served as control for the population-based case-control study contained 170 subjects (89 women and 81 men; mean age $\pm$ SD = 48.41 ± 13.76 years), who tested HBsAg negative and both HBV core antibody (anti-HBc) and HBV surface antibody (anti-HBs) positive, with normal liver function tests, and no history of HBV vaccination. Moreover, 150 ethnically and geographically matched controls (86 women and 64 men; mean age $\pm$ SD = 24.27 ± 7.19 years) from healthy blood donor of the Thai Red Cross Society were recruited as healthy control group. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent. Demographical characteristics and clinical profiles of subjects are shown in Table 1.

DNA extraction and genotyping study

DNA was extracted from the buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, using the salting-out method (10). DNA was aliquoted and stored at -20°C until used. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to analyze the polymorphisms

of IFNAR2 gene [F8S, rs2229207 (T/C)] and IL10RB gene [K47E, rs2834167 (A/G)]. The primer sequences for IFNAR2-F8S were newly designed in this study. The forward primer was 5'-CTATTCCTTACAGGTCTCTC-3' and the reverse primer was 5'-GCAGCACTTACCCATGAG-3'. PCR conditions were as follows: predenaturation at 95°C for 2 min, followed by 35-step cycles of denaturation at 95°C for 30 s, annealing at 55°C for 50 s and extension at 72°C for 30 s, with a final extension at 72°C for 7 min; 10 μl of PCR products were digested with *Xmn*I for 16 h at 37°C . The digestion products were separated on 4% agarose gel and visualized under ultraviolet light with ethidium bromide staining. For IL10RB-K47E, analysis of this SNP was previously described by Chen *et al.* (9). Ten percent of the samples were confirmed by direct sequencing of PCR products to verify the accuracy of genotyping.

Statistical Analysis

Genotype frequencies were checked for consistency among normal controls with those expected from the Hardy-Weinberg equilibrium (HWE). Allele and genotype frequencies were compared between groups using the chi-squared (χ^2) test or Fisher's exact probability test, where appropriate. The PLINK v1.07 program was used to calculate HWE, *P*-values, odds ratios (ORs) and 95% confidence intervals (CIs), as well as for haplotype analysis (11). A *P*-value of <0.05 was considered statistically significant. In the case of multiple comparisons, corrected *P*-value (P_c) for a number of comparisons (Bonferroni correction) was applied.

Results

The distribution of genotype and allele frequencies of IFNAR2-F8S (rs2229207, T/C) and IL10RB-K47E (rs2834167, A/G) polymorphisms in chronic HBV patients (with HCC and without HCC), self-limited HBV patients and healthy controls is shown in Table 2. In this study, both SNPs were in HWE when comparing the observed and expected genotype frequencies of each SNP ($P > 0.05$).

In our study, we found statistically significant differences in the allele frequency of IFNAR2-F8S between patients with chronic HBV and healthy controls. The C allele of this SNP was significantly associated with an increased risk in chronic HBV infection as compared with healthy individuals [OR (95% CI) = 3.31 (2.11–5.21), $P = 6.214 \times 10^{-9}$ and $P_c = 1.864 \times 10^{-8}$]. The effect of C allele was similar to autosomal dominant in which the presence of CC and CT genotype when compared with TT conferred the OR of 4.02 (95% CI = 2.41–6.75, $P = 4.631 \times 10^{-9}$ and $P_c = 1.389 \times 10^{-8}$). In addition, we classified the total chronic HBV group into those with HCC ($N = 100$) and those without HCC ($N = 127$). Our finding showed a significant difference of C allele of

Table 1 Demographical characteristics and clinical profiles of subjects

	Healthy subjects	Self-limited HBV infection	Chronic HBV infection	
			Without HCC	With HCC
Number of subjects	150	170	127	100
Gender (female/male)	86/64	89/81	50/77	15/85
Age (mean $\pm$ SD)	24.27 $\pm$ 7.19	48.41 $\pm$ 13.76	44.44 $\pm$ 14.63	53.96 $\pm$ 16.97
ALT (U/l) (mean $\pm$ SD)	NA	NA	160.59 $\pm$ 225.95	61.86 $\pm$ 50.26
AST (U/l) (mean $\pm$ SD)	NA	NA	110.34 $\pm$ 136.84	87.6 $\pm$ 90.88
HBV DNA (log ₁₀) (mean $\pm$ SD)	NA	NA	5.53 $\pm$ 1.77	5.75 $\pm$ 2.19

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NA, not applicable.

Table 2 Genotype and allele frequencies of IFNAR2-F8S and IL10RB-K47E gene polymorphisms in chronic HBV patients (with HCC and without HCC), self-limited HBV patients and healthy controls

SNP	Genotype/allele	Total HBV N = 227 (%)	HBV with HCC N = 100 (%)	HBV without HCC N = 127 (%)	Self-limited HBV N = 170 (%)	Healthy control N = 150 (%)
IFNAR2-F8S (rs2229207,T/C)	CC	13 (5.73) ^a	7 (7) ^d	6 (4.72) ^f	10 (5.88)	2 (1.33)
	CT	96 (42.29) ^a	42 (42) ^d	54 (42.52) ^f	86 (50.59)	26 (17.33)
	TT	118 (51.98)	51 (51)	67 (52.76)	74 (43.53)	122 (81.33)
	C	122 (26.87) ^b	56 (28) ^e	66 (25.98) ^g	106 (31.18)	30 (10.00)
	T	332 (73.13)	144 (72)	188 (74.02)	234 (68.82)	270 (90.00)
IL10RB-K47E (rs2834167,A/G)	AA	22 (9.69) ^c	13 (13)	9 (7.09) ^h	33 (19.41)	19 (12.67)
	AG	115 (50.66)	51 (51)	64 (50.39)	67 (39.41)	69 (46.00)
	GG	90 (39.65)	36 (36)	54 (42.52)	70 (41.18)	62 (41.33)
	A	159 (35.02)	77 (39)	82 (32.28)	133 (39.12)	107 (35.67)
	G	295 (64.98)	123 (62)	172 (67.72)	207 (60.88)	193 (64.33)

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SNP, single nucleotide polymorphism.

^aAutosomal dominant model CC + CT compared with TT genotype (total HBV vs healthy control); OR (95% CI) = 4.02 (2.41–6.75), $P = 4.631 \times 10^{-9}$, $P_c = 1.389 \times 10^{-8}$.

^bC compared with T allele (total HBV vs healthy control); OR (95% CI) = 3.31 (2.11–5.21), $P = 6.214 \times 10^{-9}$, $P_c = 1.864 \times 10^{-8}$.

^cAA compared with AG + GG genotype (total HBV vs self-limited HBV); OR (95% CI) = 0.45 (0.24–0.83), $P = 0.006$, $P_c = 0.018$.

^dAutosomal dominant model CC + CT compared with TT genotype (HBV with HCC vs healthy control); OR (95% CI) = 4.19 (2.29–7.70), $P = 6.734 \times 10^{-7}$, $P_c = 2.020 \times 10^{-6}$.

^eC compared with T allele (HBV with HCC vs healthy control); OR (95% CI) = 3.50 (2.09–5.87), $P = 2.630 \times 10^{-7}$, $P_c = 7.890 \times 10^{-7}$.

^fAutosomal dominant model CC + CT compared with TT genotype (HBV with HCC vs healthy control); OR (95% CI) = 3.90 (2.20–6.94), $P = 3.814 \times 10^{-7}$, $P_c = 1.144 \times 10^{-6}$.

^gC compared with T allele (HBV without HCC vs healthy control); OR (95% CI) = 3.61 (1.93–5.20), $P = 9.169 \times 10^{-7}$, $P_c = 2.751 \times 10^{-6}$.

^hAA compared with AG + GG genotype (HBV without HCC vs self-limited HBV); OR (95% CI) = 0.32 (0.13–0.723), $P = 0.003$, $P_c = 0.009$.

IFNAR2-F8S (rs2229207) between chronic HBV patients with HCC vs healthy control [OR (95% CI) = 3.50 (2.09–5.87), $P = 2.630 \times 10^{-7}$ and $P_c = 7.890 \times 10^{-7}$] and the patients without HCC vs healthy controls [OR (95% CI) = 3.61 (1.93–5.20), $P = 9.169 \times 10^{-7}$ and $P_c = 2.751 \times 10^{-6}$]. The effect of C allele seems to be autosomal dominate with an OR of 4.19 (95% CI = 2.29–7.70, $P = 6.734 \times 10^{-7}$ and $P_c = 2.020 \times 10^{-6}$) for patients with HCC and the OR of 3.90 (95% CI = 2.20–6.94, $P = 3.814 \times 10^{-7}$, $P_c = 1.144 \times 10^{-6}$) in the patients without HCC.

For IL10RB-K47E, AA genotype was found to be significantly decreased in chronic HBV patients compared with those with self-limited HBV infection [OR (95% CI) = 0.45 (0.24–0.83), $P = 0.006$ and $P_c = 0.018$]. When we classified total chronic HBV patients into those with HCC and those without HCC, we found that only the chronic HBV patients

without HCC still showed a significant decrease in the frequency of AA genotype as compared with the self-limited HBV group [OR (95% CI) = 0.32 (0.13–0.72), $P = 0.003$, $P_c = 0.009$].

Furthermore, we performed haplotype analysis of these two SNPs of IFNAR2-F8S and IL10RB-K47E. The results of haplotype analysis are shown in Table 3. There were four haplotypes including CA, TA, CG and TG. To test the association of IFNAR2-IL10RB haplotype and disease development, we compared each tested haplotype with the other three haplotypes between patient groups and healthy controls. In this study, we found significant association between CA and CG haplotypes and risk to chronic HBV infection [total chronic HBV (OR = 6.84 and $P_c = 0.014$), with HCC (OR = 7.7 and $P_c = 0.016$), without HCC (OR = 5.99 and $P_c = 0.039$); total chronic HBV (OR = 3.75 and $P_c = 0.002$), with HCC

Table 3 Haplotype analysis for *IFNAR2-F8S* (rs2229207) and *IL10RB-K47E* (rs2834167)

Haplotype	Haplotype frequency				HBV without HCC vs Healthy control		HBV with HCC vs Healthy control		Total HBV vs Healthy control	
	Healthy control	HBV without HCC	HBV with HCC	Total HBV	OR	<i>P_c</i> value	OR	<i>P_c</i> value	OR	<i>P_c</i> value
CA	0.0248	0.0730	0.0841	0.0835	5.99	0.039	7.7	0.016	6.84	0.014
TA	0.3416	0.2498	0.2896	0.2902	0.63	0.116	0.80	0.887	0.69	0.732
CG	0.0779	0.1868	0.1852	0.1858	3.72	0.001	3.48	0.002	3.75	0.002
TG	0.5557	0.4904	0.4412	0.4406	0.72	0.414	0.59	0.048	0.66	0.059

HBV, hepatitis B Virus; HCC, hepatocellular carcinoma; OR, odds ratio; *P_c*, corrected *P*-value.

(OR = 3.48 and *P_c* = 0.002), without HCC (OR = 3.72 and *P_c* = 0.001), respectively]. In contrast, TG was observed to be a protective haplotype. The protective association of TG haplotype was found only in chronic HBV patients with HCC when compared with healthy controls (OR = 0.59 and *P_c* = 0.048).

Discussion

In this study, we investigated the association between of *IFNAR2-F8S* (rs2229207, T/C) and *IL10RB-K47E* (rs2834167, A/G) polymorphisms and the susceptibility to chronic HBV infection. Our results suggest that the *IFNAR2-F8S* polymorphism might be important in the susceptibility to chronic HBV infection. In this study, we found that the C allele (S allele) of *IFNAR2-F8S* was higher in the chronic hepatitis B group when compared with healthy controls, but not when compared with the self-limited HBV group. The association of this SNP seems to be a dominant effect by model of inheritance analysis. Our results were consistent with previous Chinese studies of Chen *et al.* (9) and Gong *et al.* (8) who found no differences in the allele frequency in chronic HBV infection patients when compared to patients with self-limited HBV. However, these studies did not compare chronic HBV patients with healthy individuals. Our study is the first to report comparisons with healthy controls, which indicates susceptibility to chronic HBV. It is interesting that the genotype frequency between healthy controls and self-limited HBV differ. Our finding suggests that this SNP might be influential in individuals susceptible to HBV infection and lead to chronic HBV infection. The negative association in patients with self-limited HBV suggests that this SNP might not be important for viral clearance. In addition, we divided chronic HBV patients into patients with HCC and patients without HCC groups. Our results showed that *IFNAR2-S* allele was associated with both groups when compared with healthy controls. This suggests that the *IFNAR2* polymorphism affect susceptibility to the chronic HBV infection but not the progression of HCC. *IFNAR2* gene is a member of class II cytokine receptor family located on chromosome 21 (12). The *IFNAR2-F8S* (rs2229207, T/C) is nonsynonymous SNP altering amino acid from phenylalanine to serine. This SNP is located on

signal peptide region, which is important in *IFNAR2* protein trafficking to the membrane. The risk S allele found in this study associates with lower cell surface protein expression of *IFNAR2* (7). Furthermore, the enhancement of signal transduction and antiviral response induced by interferon- α (IFN- α) is lesser in cells expressing the S than the F allele (7). These observations suggest that S allele of *IFNAR2* gene might be responsible for the risk of chronic HBV infection and the development of HCC by affecting the protein expression of *IFNAR2* on the cell surface, leading to impaired signal transduction and antiviral response. However, the functionality of this polymorphism in our Thai patients should be further determined in more like studies.

For *IL10RB-K47E* (rs2834167, A/G), we did not find the association of allele frequency of this SNP between chronic HBV patients and healthy controls. However, when we analyzed genotype frequencies, the AA (KK) genotype was significantly decreased in chronic HBV patients when compared with patients with self-limited HBV infection. In addition, we observed that only the chronic HBV without HCC group showed a significant decrease in the frequency of KK genotype compared with patients with self-limited HBV infection when the total chronic HBV was classified into with and without HCC subgroups. Our results are consistent with a previous study suggesting that the KK might be a protective genotype in patients with persistent infection (8). However, these results differed from the study of Chen *et al.* (9) who found that the A allele may be a risk for chronic HBV infection. However, further study in other populations might confirm these finding. *IL10RB-K47E* is a nonsynonymous SNP changing amino acid from lysine to glutamic acid. This SNP is located on the first extracellular FnIII domain repeat region. There has been report that K allele of *IL10RB* gene affected lower receptor expression, resulting in the decrease of signal transduction (7). The reduced IL-10/IL-10R signal might cause the blockade of immunoregulatory role of IL-10 and lead to the resolution of chronic HBV infection. Further study is required to prove this hypothesis.

In haplotype analysis, we found that all haplotypes having risk C allele from *IFNAR2* gene (CA and CG haplotypes) showed significant association with susceptibility to chronic HBV infection in both the patients with HCC and without HCC. This confirmed the importance of C allele in disease

susceptibility and the progression of HCC. However, these results are inconsistent with the study of Gong et al. (8) in which no significant associations were found by haplotype analysis. Increased sample sizes and further studies are needed to confirm this finding.

In conclusion, we found an association between IFNAR2-F8S polymorphisms and susceptibility to chronic HBV infection. Furthermore, a protective effect of AA genotype of IL10RB-K47E was observed in this study. Nevertheless, an association study using a larger sample size should be performed to further verify our findings.

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Conflict of Interests

The authors have declared no conflicting interests.

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Molecular Epidemiology and Genetic History of Hepatitis C Virus Subtype 3a Infection in Thailand

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Key Words

Hepatitis C virus subtype 3a · Epidemic · Genetic history · Evolution · Molecular clock

Abstract

Objective: Among all hepatitis C virus (HCV) infections, subtype 3a is the most common genotype in Thailand. This study investigates the molecular epidemiology and epidemic history of HCV subtype 3a in Thailand. **Methods:** Three hundred and fifty-six serum samples were collected from HCV-infected Thai patients. The virus was isolated, after which the core and NS5B regions were sequenced. Subsequently, the HCV genotype was classified by phylogenetic analysis based on the core and NS5B regions. Molecular evolution analysis of HCV subtype 3a was estimated using BEAST (Bayesian Evolutionary Analysis by Sampling Trees) v.1.5.4. **Results:** Based on our phylogenetic analyses, subtype 3a (38.5%) was the most prevalent, followed by 1a (21%), 1b (13.8%), genotype 6 (19.9%) [comprised of subtypes 6e (0.3%), 6f (11%), 6i (1.9%), 6j (1.9%) and 6n (4.8%)] and 3b (5.6%). Our phylogenetic tree indicates the existence of a specific group of HCV subtype 3a strains in the Thai popula-

tion. Molecular evolutionary analysis dated the most recent common ancestor of the Thai HCV subtype 3a strains as existing approximately 200 ago, and a Bayesian skyline plot showed that this particular strain spread to Thailand during the mid-1970s and early 1980s. This period overlaps with the Vietnam War (1955–1975) and the widespread use of injection stimulants introduced by the US Army during this time. **Conclusion:** The estimated history of HCV subtype 3a infection in Thailand may help to predict the future burden of HCV-related diseases and facilitate better public health control and surveillance.

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Introduction

Hepatitis C virus (HCV) is a major public health problem affecting approximately 170 million people worldwide [1], and persistent HCV infection may eventually lead to cirrhosis and culminate in hepatocellular carcinoma (HCC). In addition, serious HCV complications often occur without clinical symptoms until after two or three decades of viral incubation [2, 3]. More than 20

years of epidemiological surveillance have proven that HCV-related HCC is increasing in developed countries [4]. Although in Europe and the United States an average of 3.7% of cirrhosis cases per year contribute to HCC incidence, the growing incidence in Japan is expected to reach a plateau in the near future [5, 6].

HCV is a member of Flaviviridae which can be classified into six major genotypes and many subtypes [7, 8]. Genotype distribution differs according to geographic regions, mode of transmission and treatment responses [2, 9, 10]. Genotypes 1, 2 and 3 are distributed worldwide and contribute to the majority of HCV infection. Subtypes 1a and 1b are most common in Europe, the USA and Japan, and subtypes 2a and 2b are predominant in Japan, North America and Europe, while subtype 3a is most prevalent in the Indian subcontinent and Thailand [11–13]. On the other hand, the less common genotypes 4, 5 and 6 are restricted to certain geographic areas. Genotype 4 is prevalent in the Middle East, genotype 5 in South Africa and genotype 6 in South China and Southeast Asia [13–16].

The mode of HCV transmission is associated with genotype distribution; for example, evolutionary analysis has suggested that HCV subtype 1a may be spread via blood transfusion and unsafe medical practices [17]. In addition, subtypes 1a and 3a are prevalent in young individuals and particularly in injecting drug users (IDUs) [18] in the USA, UK, Uzbekistan, Russia and Thailand [10, 19–21]. Past and present knowledge of HCV prevalence is important in order to be able to predict the future burden of HCV-related liver diseases. However, due to its asymptomatic nature and long-term complications, it is difficult to estimate the past prevalence of HCV infection from medical records. Thus, other methods are required. Coalescence theory is based on the hypothesis that all individuals originated from one common ancestor, and that populations which have evolved independently tend to genetically diverge over the time. Applying the coalescence theory and a molecular clock to phylogenetic reconstruction of present day HCV sequences allows HCV population dynamics to be estimated prior to the virus's discovery and investigation [9, 12], and can be used to estimate the modification of viral population size and viral epidemic behavior [22, 23]. A fine example of this method's efficacy comes from the elucidation that the high prevalence of HCV subtype 4a in Egypt was due to unsterile needle injection during mass treatment campaigns against schistosomiasis from the 1930s to the 1950s [9].

The distribution of HCV genotypes in Thailand has been investigated in this study and previously, and it has

been found that subtype 3a is the dominant strain (39–51%) in all regions and populations. In addition, subtype 3a has a very high prevalence in IDUs (75–83%) and our phylogenetic analysis shows a specific clustering of Thai HCV, which indicates a specific transmission route. Other subtypes are also present in Thailand and the Thai population such as the 1a, 1b, 2a and 6 variants, but at a lower prevalence in comparison to subtype 3a [12, 24]. In Myanmar, Laos and Cambodia, Thailand's neighbors, HCV genotype 6 variants are predominant rather than 3a [14, 25, 26]. Thus, due to its high prevalence in the Thai population and geographic specificity, this study focused on the analysis of subtype 3a from Thai isolates along with reference strains from other countries.

Population-based surveys have shown that the HCV seroprevalence is 2.2% in Thailand [12]; however, HCC incidence caused by HCV infection is low in this country. Previous studies have demonstrated that the initial spread time of HCV infection is associated with the prevailing socioeconomic conditions, reflecting the present incidence as well as predicting the future development of HCC [11, 27–30]. Because HCC is associated directly with the incubation period of HCV infection and HCV subtype 3a is the predominant genotype [11, 30], the initial spread of HCV subtype 3a among the Thai population may occur later than in other countries.

Due to the epidemic history of HCV transmission, which reflects the present viral population and disease burden, the objective of the study is to estimate the time of HCV subtype 3a introduction and spread in the Thai population. The suspected mode of transmission history of HCV infection has also been described. This estimation may be useful for predicting the incidence of HCV-related diseases in the country and also used as data for HCV prevention and public health control.

Materials and Methods

Sample and Clinical Data Collection

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University before using the specimens. Serum samples were collected from HCV-infected patients at Chulalongkorn Hospital (Bangkok), the hospitals in Phetchabun Province (Phetchabun General Hospital, Lomsak General Hospital and Lomkao Crown Prince Hospital), as well as the National Blood Center, Thailand. Patients who were tested for anti-HCV and had positive results were sent to the Center of Excellence in Clinical Virology in order to investigate the viral RNA and genotype for treatment. Only samples with HCV RNA positive by RT-PCR were recruited for this study. Coded anonymous samples were collected from 2003 to 2009. All specimens

were stored at -70° until tested. Demographic data of patients such as age, sex, nationality, habitat and risk factors were also collected.

DNA Extraction and Sequencing

Viral RNA was extracted from 356 anti-HCV-positive serum specimens by using guanidine thiocyanate extraction [31]. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Madison, Wisc., USA). Partial core and NS5B regions were amplified by RT-PCR using specific primer pairs (core: primers 410/951 and 953/951, NS5B: primers NS5BF1/R1 and NS5BF2/R2) as previously described [32]. The resulting cDNA fragments were purified (HiYield Gel/PCR DNA Fragment Extraction Kit, RBC Bioscience, Taipei, Taiwan) and subjected to direct sequencing (First BASE Laboratories, Selangor, Malaysia). Nucleotide sequences were edited and assembled using Chromas LITE (v.2.0.1), BioEdit (v.5.09; Ibis Therapeutics, Carlsbad, Calif., USA) and SeqMan (DNASTAR, Madison, Wisc., USA).

HCV Genotyping

The genotype of each sample was determined by phylogenetic analysis of both the core and NS5B sequences. All sequences were aligned with reference genotypes retrieved from the Los Alamos HCV database [33] using CLUSTALW v1.83. Neighbor-joining trees of partial core and NS5B sequences were constructed using the Gojobori-Ishi-Nei 6-parameter method with bootstrap resampling tests using 1,000 replicates (<http://clustalw.ddbj.nig.ac.jp>). Sequences clustering on the same branch as the reference strain were assigned the reference genotype. Some of the samples with HCV genotype 6 had been previously published elsewhere [32]. The core region represents nucleotide positions 377–669 and the NS5B region represents nucleotide positions 8282–8591 relative to the sequence M62321. There were discrepant subtypes between the core and NS5B trees; therefore, we reanalyzed all sequences by using another method. All sequences were subjected to FindModel (<http://hcv.lanl.gov/content/sequence/findmodel/findmodel.html>) to find the best substitution model. Then, the HCV genotype classification was confirmed by constructing maximum likelihood trees with the GTR+ Γ model and bootstrapped 1,000 times using MEGA version 5.05.

Phylogenetic Analysis of HCV Subtype 3a

In total, 136 core sequences and 132 NS5B sequences clustering in subtype 3a were obtained. To analyze subtype 3a of Thailand, the available subtype 3a sequences of the core and NS5B genes were retrieved from the Los Alamos HCV database and aligned with the respective subtype identified in this study [33]. Redundant sequences originating from the same individual or from an individual with unknown nationality were excluded as were those of insufficient length in nucleotide sequence. Multiple alignments were calculated using CLUSTALW v1.83 and the phylogenetic relationships of the core and NS5B were constructed using the neighbor-joining method based on the 6-parameter model with a bootstrap test performed on 1,000 replicates (<http://clustalw.ddbj.nig.ac.jp>). Particular clusters originating from Thai subtype 3a strains were investigated.

Evolutionary Analysis of HCV Subtype 3a

The HCV subtype 3a sequences of the partial core and NS5B regions originating from the same sample, which grouped in the

Thai cluster, were selected for further analysis. This left 74 sequences of the NS5B dataset which was 325 nucleotides long (representing nucleotide positions 8286–8610 relative to the sequence M62321) and subjected to evolutionary analysis. The most appropriate nucleotide substitution model for the NS5B sequences dataset was determined using the jModelTest version 0.1. The best-fitting model of these data sets was GTR+ Γ . HCV subtype 3a population dynamics were estimated using the strict molecular clock and the uncorrelated lognormal model under the substitution model mentioned above. Since the samples were not systemically collected and contained insufficient temporal data to directly estimate the evolutionary rate, the external rate was used in both the strict and relaxed clock model. The previously estimated substitution rate of 5.8×10^{-4} substitutions per site per year obtained from the NS5B gene was applied as a prior to generate an evolutionary timescale of years [34]. The most appropriate clock model was selected based on the Bayes factors computed by Tracer v1.5. The Bayesian skyline plot approach implemented in BEAST (Bayesian Evolutionary Analysis by Sampling Trees) v1.5.4 (<http://evolve.zoo.ox.ac.uk/beast/>) was used to calculate the epidemic history. Each Markov chain Monte Carlo analysis was run for 50,000,000 states and sampling every 5,000 states. Markov chain Monte Carlo convergence, effective sample size, the date of the most common ancestor of the cluster and Bayes factors were computed and investigated using Tracer v1.5, and annotated phylogenetic trees were displayed by FigTree v. 1.2.2 (<http://evolve.zoo.ox.ac.uk/beast/>).

Nucleotide Accession Number

All nucleotide sequences were submitted to the GenBank database. The accession numbers of partial core and NS5B sequences were HQ229038 and HQ229604, respectively. HCV genotypes in this study were classified according to the reference strains as follows: 1a: NC004102, EF407419, AB301742, AF511950, EU234064, DQ155558, EU256041, EU781772, D10749; 1b: HM041987, HM041997, EF032894, AB016785, EU155228, U16362, D11355, FJ217354, EF032894, AF145454, EU155305, AB429050, AY587016, D90208, AF176573; 1c: AY051292; 2a: AB047639; 2b: D10988; 3a: D17763, AF046866, X76918, AF525902, AB472164, D14308, D10079, EF543248, AM423015, EF543249, X76918, AY003973, AF506583, D10078, AB327107, AB444431, AB444489; 3b: D37840, HM042021, D37854, D49374, D37853; 3k: D63821; 4a: NC009825, DQ418788; 4d: DQ418786; 5a: Y13184, NC009826; 6a: DQ480513, AY859526; 6b: D84262; 6c: EF424629; 6d: D84263; 6e: DQ314805; 6f: DQ835760, DQ835764; 6g: D63822, DQ314806; 6h: D84265; 6i: DQ835770, DQ835762; 6j: DQ835761, DQ835769; 6k: DQ278893, D84264; 6l: EF424628; 6m: DQ835765, DQ835763; 6n: AY878652, DQ835768; 6o: EF424627; 6p: EF424626; 6q: EF424625; 6r: EU408328; 6s: EU408329; 6t: EF632071; 6u: EU246940, and 6w: DQ278892.

Results

Clinical Data and Patient Background

A total of 356 serum samples collected from patients with detectable HCV-RNA were subjected to RT-PCR amplification and sequencing of the core and NS5B regions. Subject age ranged between 17 and 73 years and the mean age was 43.01 ± 10.9 . Out of 187 patients with

Table 1. Genotype of HCV determined in this study

Core genotype	NS5B genotype											total
	1a	1b	2a	3a	3b	6e	6f	6i	6j	6n	ND	
1a	75											75 (21.1)
1b	1	48										49 (13.8)
2a			2									2 (0.6)
3a				130	1						5	136 (38.2)
3b					17						1	18 (5.1)
6e						1						1 (0.3)
6f							37				2	39 (11)
6i								7				7 (2.0)
6j									7			7 (2.0)
6n										17		17 (4.8)
ND		1		2	2							5 (1.4)
Total, n (%)	76 (21.3)	49 (13.3)	2 (0.6)	132 (37.1)	20 (5.6)	1 (0.3)	37 (10.4)	7 (2.0)	7 (2.0)	17 (4.8)	8 (2.2)	356 (100)

Values are given as n (%). Genotypes are classified based on core and NS5B sequences. ND = not detected.

clinical reports, 92% were diagnosed with chronic liver disease, 5.3% with cirrhosis and 2.7% with HCC. The mean age of the patients in the HCC group was higher than in the chronic hepatitis and cirrhosis group (57, 43.7 and 46.3 years, respectively). Among patients in the chronic hepatitis group, males were predominant with a 2:1 male-to-female ratio. Approximately 92% of the patients lived in the central area of Thailand. One hundred and thirty (36.5%) subjects had known risk factors such as blood transfusion (33.1%), tattooing (27.7%), intravenous drug use (26.9%), unsafe medical injection (23.8%), shaving by a barber (23.1%), prostitution (13%), needle stick injury (11%), HIV coinfection (1.5%) and a spouse with HIV/HCV (1.5%) (see online supplementary tables 1, 2; for all online suppl. material, see www.karger.com/doi/10.1159/000351621). Among all risk factors, intravenous drug use incurred the greatest diversity of HCV genotypes identified (genotypes 1, 2, 3 and 6). In cases where the patient's background showed that more than one risk factor could be associated with multiple routes of transmission, the exact route could not be specified in this study (suppl. table 3).

HCV Genotype

Phylogenetic analysis based on partial core and NS5B sequences (n = 356 sequences) showed that most Thai patients carried subtype 3a (n = 137, 38.5%), followed by subtype 1a (n = 75, 21.1%), 1b (n = 49, 13.8%), genotype 6 (n = 71, 19.9%) and subtype 3b (n = 20, 5.6%). Genotype

6 was classified as 6e (n = 1, 0.3%), 6f (n = 39, 11%), 6i (n = 7, 2.0%), 6j (n = 7, 2.0%) and 6n (n = 17, 4.8%) (suppl. fig. 1). Genotype 2 was detected in only 2 cases. Two samples, CUTH45 and CUTH233, showed discordant subtypes based on the core and NS5B, in that one sample was 1b/1a and the other was 3a/3b, respectively (table 1). To test that the discordant core and NS5B genotypes were not an artifact of a selected substitution model, the genotype was reclassified by other phylogenetic and nucleotide substitution models. The results showed the same classification was given to both discordant samples by the maximum likelihood trees and the neighbor-joining trees (suppl. fig. 2, 3).

HCV genotype 3 could be detected in all regions of the country; genotype 2 was identified in 2 patients residing in the central area, while genotypes 1 and 6 were not found in the southern and western regions, (suppl. table 2). These results may be due to the fact that most of the patients in this study were recruited from Chulalongkorn Hospital, hence central Thailand. In addition, HCV genotypes 1 and 3 were found in all stages of liver disease, while genotype 2 was found only in chronic hepatitis and genotype 6 was not detected in any HCC patients (suppl. table 1).

Phylogenetic Analysis of HCV Subtype 3a

HCV subtype 3a isolated in this study was analyzed in comparison with reference sequences available from the Los Alamos HCV database. Figure 1 shows the neighbor-

joining tree estimated from the partial core and NS5B sequences. In total, 136 core and 132 NS5B sequences clustered together and were classified as subtype 3a (table 1). In this clade, subbranches exhibited a phylogeographic structure by grouping samples from the same origin together, e.g. HCV strains from the former Soviet Union, Pakistan and Thailand (fig. 1a, b).

The phylogenetic tree of partial core sequences isolated in this study had 92 isolates in the Thai cluster, while the tree constructed from NS5B sequences had 82 isolates (fig. 1a, b). To define these Thai clusters more clearly, 74 samples from both the core and NS5B sequences from these same clusters were selected for further evolutionary analysis.

Evolutionary Analysis of HCV Subtype 3a

The NS5B dataset was analyzed using BEAST under a strict and uncorrelated lognormal relaxed molecular clock with a coalescence bayesian skyline plot model. Model comparison was undertaken through comparison of estimated marginal likelihoods of each model calculated by the Tracer v. 1.5 program. The results show that the relaxed molecular clock had a higher marginal likelihood than the strict model, $\ln P(\text{model}|\text{data})$ was $-3,028.84$ and $-3,039.35$, respectively ($\log_{10}$ Bayes factor of >4.5). Using the data from the relaxed clock model, the origin of the most recent common ancestor of the HCV subtype 3a strains in Thailand existed around 200 years ago (95% credible interval, 573–15 years ago). The mean rate under relaxed clock (ucl.d.mean) was 9.28×10^{-4} (95% credible region: 1.23×10^{-6} to 2.04×10^{-3}) and the standard deviation (ucl.d.stdev) was 0.29 (95% credible region: 6.51×10^{-2} to 0.49). If the ucl.d.stdev is zero, it means no variation in the rate among branches. Rate heterogeneity among the site (α) of NS5B was 0.267 while the coefficient of variation (COV) was 0.3 (95% credible region: 0.05–0.49), which showed heterogeneity among branches. COV measures the rate variation among lineages. A small value of COV represents less rate variation and close to clock-like evolution. So, the evolution will be the strict molecular clock if the COV is equal to zero. Our COV was close to the previous reported value for HCV genotype 6 and Dengue virus [35, 36]. Covariance parameter of the relaxed clock was -0.0079 (95% credible region: -0.1642 to 0.151).

Figure 2 shows the bayesian skyline plot estimated from the NS5B dataset. The effective population size was relatively constant from the early 20th century before changing to exponential growth in the mid-1970s. From the early 1980s to the present the rate has been on a relatively slow decline and seems to be steady after the year 1990.

Discussion

In this study, we have described the genotypic and evolutionary analysis of HCV-infected patients in Thailand. While the severity of liver disease was associated with advanced age, the number of cirrhotic and HCC cases remained small. However, previous studies have found that the mean age of patients with HCC is significantly higher than that of chronic hepatitis patients, suggesting that protracted infection may increase the likelihood of severe complications [13, 15, 30].

In Thailand, HCV genotype 3 was the predominant genotype with subtype 3a being the most common subtype. Genotype 1 and 6 variants were also prevalent, followed by subtype 3b and subtypes 2a and 2c [12, 37]. However, although the genotypic distribution of HCV found in this study is similar to previous reports, these results likely do not accurately represent Thailand's overall genotype distribution because most of the examined samples were obtained from patients living in the central region of Thailand. Also, two samples, 1b/1a and 3a/3b, showed discordance between subtypes based on the core and NS5B regions (table 1, suppl. fig. 1, 2). Intragenotypic recombination of HCV has been reported before, thus additional analysis should be performed to identify the recombination breakpoint of these discrepant samples to confirm the recombination event [38].

Some of the HCV genotypes show phylogeographic distribution, e.g. genotype 2 is common in Africa and genotype 6 is common in East Asia [29, 35]. Phylogenetic analysis indicated that there was a HCV subtype 3a-specific cluster for Thai strains (fig. 1), which may be attributable to a specific route of transmission. In addition, these strains shared a most recent common ancestor that existed approximately 200 years ago. Based on our phylogenetic trees (fig. 1), the Thai subtype 3a strains did not form a monophyletic cluster, thus various 3a strain ancestors may have been introduced into the country in varying regions and at different time points. However, because most of the HCV subtype 3a strains were collected from central Thailand, we were unable to analyze the possible entry point of the original HCV strain into Thailand, while the emergence of subtype 3a in Thailand may be related to the introduction of Western medical practices such as vaccinations and operations [39].

Phylogenetic reconstruction (fig. 1) showed the Thai and Indian/Pakistani clusters in close proximity, which supports the argument of a close relationship between the HCV subtype 3a viruses circulating in those countries. Historically, the Indian culture has had a profound influ-

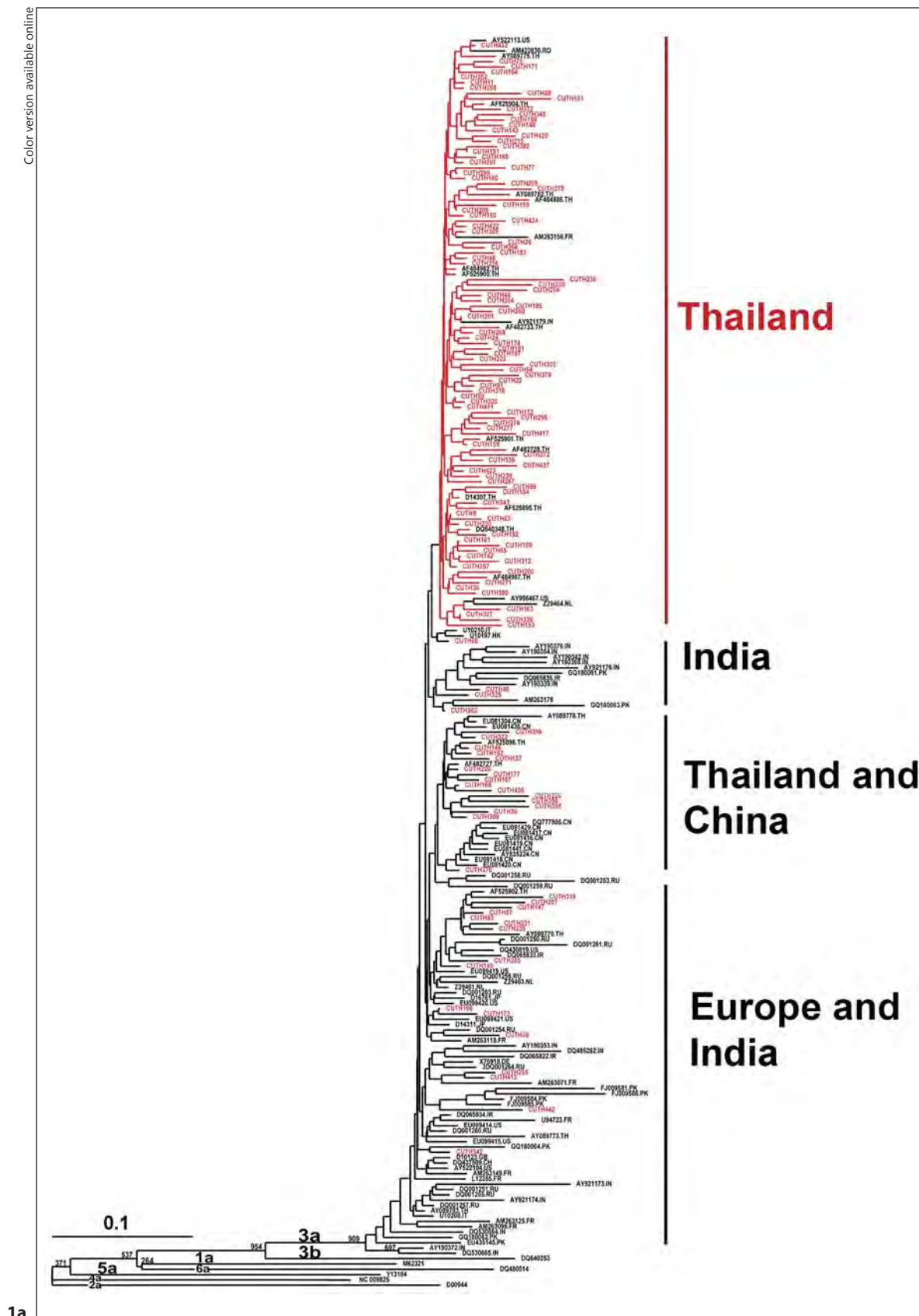
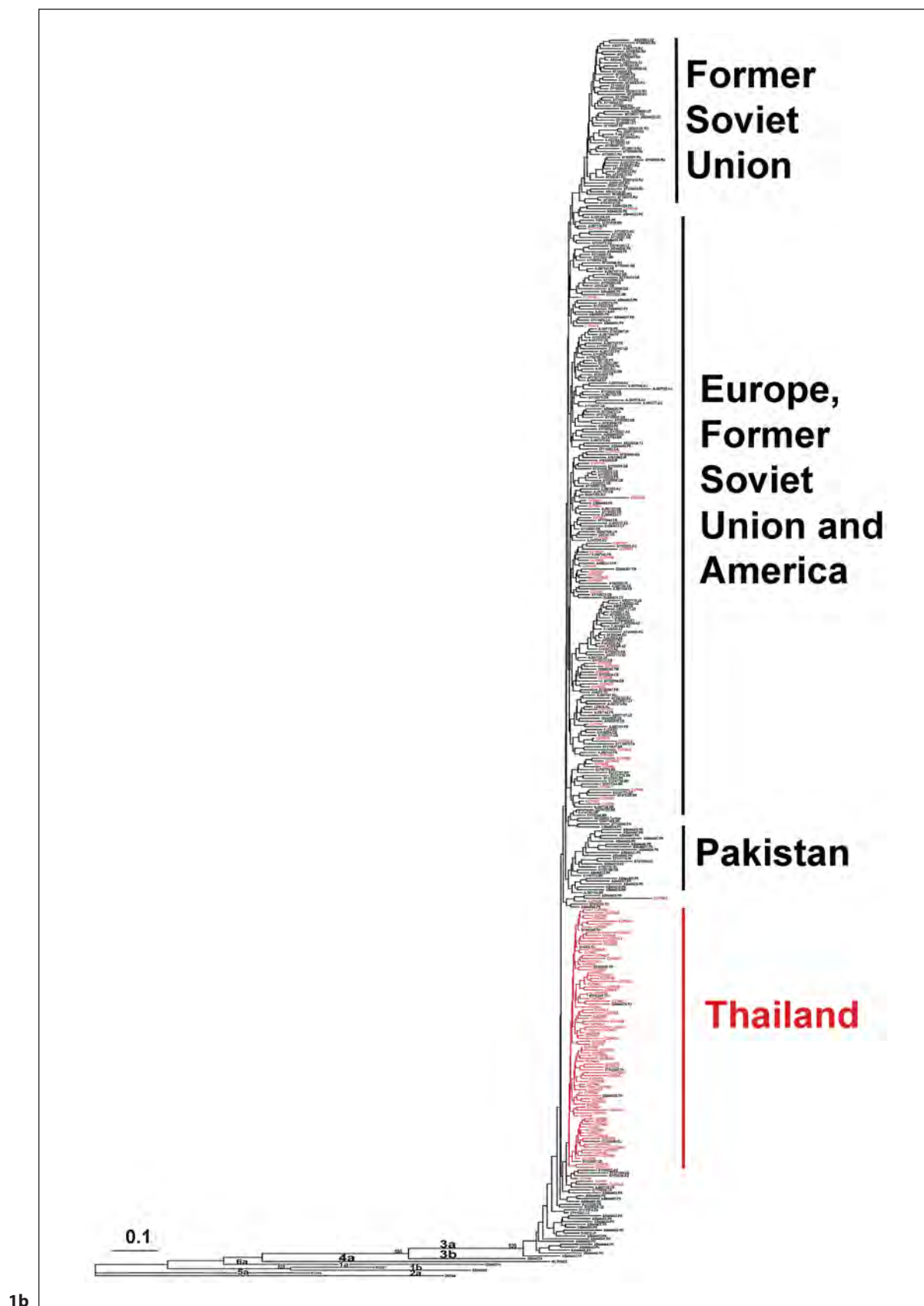


Fig. 1. Phylogenetic tree constructed based on the core (a) and NSSB (b) region of HCV subtype 3a. Thai isolates identified in this study are labeled in red. The red branches represent a particular cluster of Thai which subjected to evolutionary analysis. Isolates of the same origin tend to cluster together and their countries are shown as abbreviation names. AU = Australia; AZ = Azerbaijan; BR = Brazil; CA = Canada; CH = Switzerland; CN = China; CY = Cyprus; EE = Estonia; ES = Spain; FR = France; GB = United Kingdom; IN = India; IR = Iran; JP = Japan; LK = Sri Lanka; LT = Lithuania; NL = The Netherlands; PK = Pakistan; RU = Russia; TH = Thailand; TJ = Tajikistan; TN = Tunisia; TW = Taiwan; US = United States; UZ = Uzbekistan.



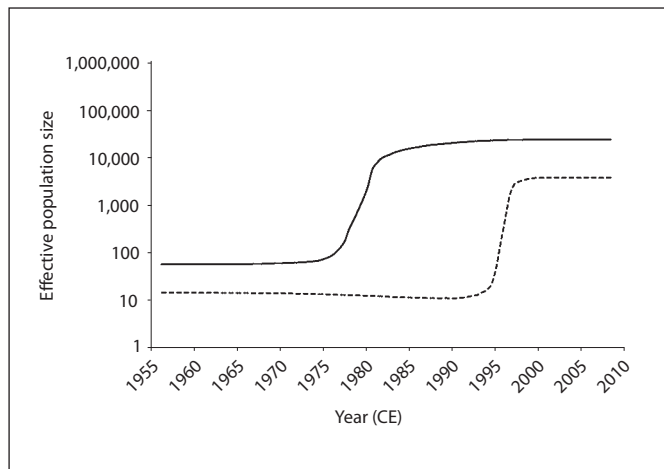


Fig. 2. Bayesian skyline plot for HCV subtype 3a of the Thai cluster. The black line indicates the estimated median of effective numbers of infection based on the NS5B gene and the dotted lines represent 95% highest posterior density confidence interval.

ence on Thai culture, language, ceremony and food. In addition, there has been a history of trade migration between both countries, which may have resulted in a long period of mutual HCV transmission. The widespread introduction of HCV subtype 3a in the Thai population occurred around the mid-1970s and was later introduced to Pakistan [11]. A prior study suggested that the subtype 3a population in Pakistan is older than it is in other countries, and helps contribute to the high number of HCC patients in that country. Khan et al. [11] estimated that the 3a subtype was introduced to the Pakistani population around the 1950s and thus prior to its emergence in the former Soviet Union (ca. 1960s) [30] and Thailand (ca. 1970s). So, it is possible that the most recent common ancestor may have existed in this area.

While 81% of HCV patients in Pakistan carry HCV subtype 3a [11], the prevalence of subtype 3a in India varies upon the sampling population and location. For example, in the northeastern part of India, HCV subtype 3a constitutes around 8% of all genotypes [40]; however, in North and Central India the prevalence of genotype 3 is around 80% [41]. Also, in Bangladesh, an analysis of 168 samples showed that subtype 3b was most prevalent (45.5%), followed by subtype 3a (18.7%) [42]. The prevalence of HCV subtype 3a in the Indian subcontinent is in contrast to reports on Southeast Asian countries – except Thailand – in which the most prevalent genotypes are genotypes 1 and 6 [25, 32, 43–46].

Bayesian phylogeography has provided evidence that HCV subtype 3a was transmitted via heroin trafficking and IDU stemming from the Golden Triangle and Golden Crescent to the Chinese provinces of Yunnan in 1981 and Xinjiang in 1990, which are adjacent to the Golden Triangle and Golden Crescent, respectively. Those two provinces served as a hub for the drug trade and also played an important role in the viral transmission of viruses such as HIV and HCV to other regions of China and Asian [47]. In addition, subtypes 1a and 3a were proposed to have been introduced from an endemic area to the industrial nations by IDU [18]. According to all of these studies, HCV subtype 3a may circulate in an endemic area for a long period and then migrate to other parts of the world through trading, population migration and drug trafficking with subsequent IDU.

As evidenced by our molecular clock estimation, Thai HCV subtype 3a strains have experienced a rapid expansion of their effective population from the mid-1970s to the early 1980s (fig. 2). This time period of expansion is consistent with an influx of IDUs in Thailand as a result of US Army deployment in Southeast Asia during the course of the Vietnam War (1955–1975). At that time, the US Army set up camp at several locations throughout the North and Northeast of Thailand, and the use of stimulants via intravenous administration was widespread [48]. More recently, however, the trend of drug administration has changed from needle to noninjection (non-IDU) practices such as inhaling or oral uptake. Specifically, methamphetamine use has dramatically risen since the 1990s and has largely replaced intravenous drug use. HCV prevalence among IDUs (approx. 86%), however, is still proportionally much higher than non-IDUs (approx. 5%) [21, 49].

Thus, even though we propose that needle sharing may be implicated as the major route of transmission of subtype 3a in Thailand, other factors may have a part in maintaining the present day transmission rates. Risk factor profiles of our patients showed that blood transfusion represented the most profound risk factor in this study (suppl. table 3). Thus, we hypothesize that while intravenous drug use may have been the major cause of the viral expansion in the Thai population during the mid-1970s through the 1980s, contaminated blood products likely play a role in maintaining the current and new viral strains in the population.

In the past, anonymous blood donation was not a popular practice among Thai people, who traditionally preferred to receive blood from their friends or relatives (<http://www.blooddonationthai.com/>, in Thai). Howev-

er, by 1985 this behavior had changed and blood transfusions from anonymous donors gradually replaced the old tradition. In 1986, donors from convicts were excluded, and in 1990 the National Blood Bank, Thai Red Cross Society implemented universal screening for anti-HCV. At this time, it was found that 2% of first-time donors were positive for HCV antibodies, though this gradually declined to 0.5% by 2009 [50]. Presently, the blood screening policy of the National Blood Center consists of questionnaires to exclude high-risk donors, anti-HCV serology screening of all blood units using chemiluminescent immune assay with some negative samples being further screened by nucleic acid amplification technology [50]. Furthermore, coverage of nucleic acid amplification technology testing throughout the country has been increased from 34% in 2009 to 80% in 2012 (The 21st Academic Annual Meeting of National Blood Centre 2013, Bangkok, Thailand). However, even though the rate of HCV infection among current donors in Thailand is low due to the blood screening policy implemented, the HCV seroprevalence survey was still as high as 2.2% in 2004 [12].

In 2007, the Bureau of Epidemiology reported that HCV was endemic in Thailand's Phetchabun Province and proffered that the route of transmission was due to an unhygienic medical injection procedure [51] given by inadequately trained medical personnel [52]. The infection rate was 70% for intravenous drug users compared to a rate of 0.98% in new blood donors [21]. Thus, initially, needle sharing combined with receiving contaminated blood products significantly escalated the risks of HCV transmission; however, transfusion-related HCV infection has been under control since the implementation of the screening program in 1990. In addition, the first 2 cases of HIV were first reported in Thailand in 1984, and since then health and biosafety education concerning HIV includes information on HCV also [53].

In 2009, the Thailand National Cancer Institute reported that liver and bile duct cancer was the third most common cause of cancer in Thailand, ranking as the third most common cause in males (12.4%) and as the fifth most common cause in females (4%) [54]. The report also showed that most hepatitis cases were the result of infection with HBV (approx. 51.9%), followed by HCV (approx. 11.8%) and, to a lesser extent, HAV, HDV, HEV and others [55]. Therefore, although HCV is generally the major cause of new HCC cases in developed countries [13], HBV is the most predominant cause of HCC in Thailand [56]. Due to the course of disease progression,

HCV-related liver disease may take 20–30 years before progressing to liver cirrhosis or HCC [2, 3, 27]. Thus, the spread of HCV in Thailand during the 1970s and 1980s may soon manifest itself phenotypically. Fortunately, however, since 2012 the National Health Security Office (NHSO) has launched a policy of free HCV treatment by registered specialists for chronic hepatitis patients with HCV genotype 2 or 3, which are the genotypes most likely to be cured by the standard viral therapy of pegylated interferon plus ribavirin. Accordingly, if most of the favorable HCV genotype patients can be accessed and receive treatment, the incidence of HCV-related diseases may not be as high as expected in the future.

Similar to previous reports, our study indicates that HCV subtype 3a is the most prevalent strain in Thailand followed by genotypes 1 and 6. Evolutionary analysis based on the coalescence theory estimated that common HCV subtype 3a was introduced and spread into the Thai population during the mid-1970s to the early 1980s. This event coincided with the Vietnam War period which is also when intravenous drug use and needle sharing was widespread. Sharing needles may have been a primary source of the HCV epidemic in the country, and receiving contaminated blood products may have been the primary factor for high HCV prevalence in the Thai population. However, HCV prevalence seems to have steadily declined since anti-HCV antibody screening was implemented in blood donation centers. Our results suggest that the cirrhosis and HCC incidence of this population may increase in the future unless effective treatment and public health policy is introduced to help prevent further disease progression.

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RESEARCH ARTICLE

Prevalence of Aflatoxin Induced p53 Mutation at Codon 249 (R249S) in Hepatocellular Carcinoma Patients with and without Hepatitis B Surface Antigen (HBsAg)

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Abstract

Background: A missense mutation in exon 7 (R249S) of the p53 tumor suppressor gene is characteristic of aflatoxin B1 (AFB1) exposure. AFB1 is believed to have a synergistic effect on hepatitis virus B (HBV) carcinogenesis. However, results of studies comparing R249S prevalence among patients are conflicting. The aim of this study was to determine the prevalence of the R249S mutation in hepatocellular carcinoma (HCC) patients with or without positive HBsAg. **Materials and Methods:** Paraffin embedded liver tissues were obtained from 124 HCC patients who underwent liver resection and liver biopsy in King Chulalongkorn Memorial Hospital. Restriction fragment length polymorphism (RFLP) was utilized to detect the R249S mutation. Positive results were confirmed by direct sequencing. **Results:** Sixty four (52%) patients were positive for HBsAg and 18 (15%) were anti-HCV positive. 12 specimens tested positive by RFLP. Ten HCC patients (8.1%) were confirmed to be R249S positive by Sanger sequencing (AGG to AGT). Out of these 10, six were HBsAg positive, and out of the remaining 4, two were anti-HCV positive. The R249S prevalence among HCC patients with positive HBsAg was 9.4% compared to 6.7% for HBsAg negative samples. Patients with the R249S mutation were younger (55 ± 10 vs 60 ± 13 year-old) and tended to have a more advanced Edmonson-Steiner grade of HCC, although differences did not reach statistical significance. **Conclusions:** Our study shows moderate prevalence of aflatoxin B1-related p53 mutation (R249S) in HCC with or without HBsAg. HBsAg positive status was not associated with R249S prevalence.

Keywords: Aflatoxin B1 - R249S - p53 mutation - 249ser - Hepatocellular carcinoma - HBsAg

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Introduction

Aflatoxin is carcinogenic and associated with Hepatocellular carcinoma (HCC) (Yoo, 2010). HCC is a common cancer worldwide and especially in the Asia-Pacific region (Srivatanakul et al., 2004). Cirrhosis from chronic hepatitis B and C, alcohol induced liver disease and non-alcoholic steatohepatitis are common risk factors for HCC. Aflatoxin B1 (AFB1) exposure is also considered a risk factor for HCC (El-Serag and Rudolph, 2007; Williams et al., 2004).

Mutation at the third base pair of codon 249 of exon 7 of the p53 gene from guanine to thymine (R249S) is thought to be specifically correlated to AFB1 exposure. This missense mutation is the most common p53 mutation detected in HCC patients from high AFB1 exposure areas (Bressac et al., 1991; Hsu et al., 1991; Yang et al., 1997).

Aflatoxin exposure can induce the equivalent mutation in an in-vitro liver cell culture model (Smela et al., 2002; Besaratinia et al., 2009; Gouas et al., 2010). Previous studies have shown that the R249S mutation occurs almost exclusively in HCC and is extremely rare in other cancers. For example, a large number of lung cancers and breast cancer tissues were tested for p53 tumor suppressor gene mutations, and none of them were the aflatoxin-specific R249S mutation (Behn et al., 1998; Cuny et al., 2000).

Several in vitro studies have found synergistic effects between HBV infection and aflatoxin exposure on the TP53 mutation (Qu et al., 2005). In addition, mutation in the X protein of HBV (HBx) has also been correlated with R249S-related carcinogenesis (Jiang et al., 2010; Ortiz-Cuaran et al., 2013). Prospective cohort and nested case-control studies from China and Taiwan (Ross et al., 1992; Wang et al., 1996; Wu et al., 2009) indicate a

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multiplicative risk of developing HCC in patients with concomitant aflatoxin exposure and HBV infection. For these reasons, HBV vaccination has been proposed as a means to prevent aflatoxin-induced HCC (Khlanguiswet and Wu, 2010).

However, the result from a meta-analysis did not conclude whether HBV is associated with increased R249S mutation rate (Stern et al., 2001). Out of 15 studies analyzed from the US (Hoque et al., 1999), Europe (Volkmann et al., 1994) and Asia, only 1 R249S mutation was documented in patients without HBV infection. Furthermore, data from developing countries with moderate aflatoxin exposure showed similarly low rates of the mutation (3 R249S mutations from 11 studies) (Buetow et al., 1992; Soini et al., 1996). However, although the number of patients included in meta-analysis was large, the study population and method of R249S testing were heterogeneous.

Recent studies from non HBV endemic areas have demonstrated significant R249S mutation rates in HCV-related HCC and non-HCV-related HCC (El-Kafrawy et al., 2005; Kirk et al., 2005; Nogueira et al., 2009). However, the number of HBV-related HCC patients was too low for a statistical comparison between HBsAg positive and negative subgroups. A more recent study in Thailand demonstrated that the R249S mutation was more common in HBsAg positive group: The R249S mutation was identified in 19 of 48 (40%) HBsAg positive HCC patients and 9 of 35 (26%) HBsAg negative HCC patients. However, this study also lacked an adequate number of cases to make statistical conclusions (Villar et al., 2012). Thus, although the risk of HCC attributed to aflatoxin in Thai HBsAg-positive patients is estimated to be 30-fold more than HBsAg negative individuals (15.9-21.9 versus 0.53-0.73/10,000 person-years) (Liu and Wu, 2010) no previous study has been conclusive. Therefore, the aim of this study is to determine if a statistical association exists between HBV surface antigen (HBsAg) status and R249S mutation status in HCC patients using a patient population of adequate sample size.

Materials and Methods

Patients

Patients with pathologically confirmed HCC were identified using a computerized database. Surgical specimens were obtained from patients that had undergone hepatic resection and percutaneous liver biopsy in King Chulalongkorn Memorial Hospital, Bangkok, Thailand. HBsAg, alpha-fetoprotein (AFP) and other serology tests were extracted from patients' records. Pathological data including diagnosis, grade, pattern and tumor differentiation were reported by a specialized pathologist. All patients and respective specimens were anonymous. The protocol of the study was approved by the ethical committee, faculty of Medicine, Chulalongkorn University (IRB No. 496/52)

Inclusion criteria consisted of pathologically proven HCC along with available HBsAg results. Exclusion criteria included unavailable paraffin embedded tissue, secondary liver tumors and/or a concomitant cancer

diagnosis.

Tissue samples and preparation of DNA

The materials for genetic studies was extracted from paraffin embedded tissue from patients with HCC and were retrieved from the department of pathology, faculty of medicine, Chulalongkorn University. DNA was extracted using a commercial DNA extraction kit (5 prime GmbH, Hamburg, Germany) according to the protocol provided by the manufacturer. The extracted DNA was eluted in 50 µl of distilled water and stored at -200C until polymerase chain reaction (PCR) was run.

PCR amplification and restriction enzyme digestion

Nested PCR was used to amplify the DNA region of interest. The first round PCR mixture contained 2 µl of DNA template, 10 µl of PerfectTaq Plus MasterMix Kit (5 prime GmbH, Hamburg, Germany) 1.25 mM of the outer forward primer (TP53-OS: 5'-CTT GCC ACA GGT CTC CCC AA -3'), 1.25 mM of the outer reverse primer (TP53-OAS: 5'-AGG GGT CAG CGG CAA GCA GA-3') and distilled water (DW) to a final volume of 25 µl. The first round PCR program was the following: 94°C for 3 minute, 40 cycle of 94°C for 18 second, 50°C for 21 second, and 72°C for 1.30 minute concluded by 72°C for 10 minute. The PCR mixture and PCR program of the second round were the same as those of the first round except for the following: an inner forward primer (TP53-OS: 5'-AGG CGC ACT GGC CTC ATC TT-3') and innner reverse primer (TP53-OAS: 5'-TGT GCA GGG TGG CAA GTG GC-3') were used and 0.5 µl of first round PCR product was used as the template for the second round of PCR. The amplification result was then run on a 2% agarose gel and visualize by UV transilluminator after staining with ethidium bromide. The TP53 mutation was analyzed by a modified version of restriction fragment length polymorphism (RFLP) as described by Szymanska (Szymanska et al., 2004). Briefly, second round PCR products were subjected to digestion with the restriction endonuclease, HaeIII, which recognizes the sequence CCGG that encompasses the 249 codon. The RFLP mixture contained 1 unit of HaeIII (New England BioLabs inc, Ipswich, MA), 2 µl of 10X Buffer 4 (New England BioLabs inc, Ipswich, MA), 15 µl of second round PCR product and distilled water to a final volume of 20 µl. This mixture was then incubated at 37°C overnight and subsequently run on a 3% agarose gel. RFLP should cut wild-type DNA at three sites thus producing three bands (12, 61, and 92 bp), whereas RFLP should only cut and produce two bands in the mutant (12 and 153 bp). Specimens with positive RFLP for R249S were then confirmed by Sanger sequencing.

Statistical analysis

An adequate sample size to reach statistical significance was determined based an estimated mutation rate of 30% for HBsAg positive samples and 10% for HBsAg negative samples (Dupont and Plummer, 1990). It was determined that at least sixty-two cases and 62 controls were required

Results are expressed as mean±SD. The difference in the R249S mutation rate between subgroups was

determined by a chi-square test. Statistical significance was established as a P value of less than 0.05. Calculations were performed using SPSS version 17.0 (SPSS, Inc., Chicago, Ill)

Results

Patients' characteristics, yield of DNA extraction and RFLP

169 formalin fixed paraffin embedded tissue blocks were included in the study. Their source was from liver resection or liver biopsy specimens collected between 2007 and 2010 that were labeled as HCC according to the pathology department. Eight cases were excluded due to the unavailability of HBsAg results (2 cases) and the final pathological diagnosis not being HCC (6 cases). In addition, 13 pairs of samples belonged to the same patients. Thus, 148 samples were eligible for the study.

DNA could be successfully extracted and tested for the R249S mutation by RFLP in 124 HCC cases. DNA extraction or RFLP processes failed on 24 specimens, most of which were obtained by percutaneous liver biopsies. Sixty-four of the 124 cases (51.6%) were positive for HBsAg. Eighteen patients (14.5%) were positive for HCV antibody. Three cases were positive for both HBV and HCV. The median AFP level was 20.1 IU/ml. Details are described in Table 1.

R249S mutation rate determined by RFLP and confirmed by sequencing

RFLP revealed the R249S mutation in 12 out of the 124 specimens. Those 12 specimens were sent for sequencing and revealed AGG to AGT missense mutation (R249S) in 10 specimens (8.1%) and a silent mutation (AGA) in 2 specimens. No R249S mutation was detected in 10 control liver tissue specimens without HCC.

Prevalence of R249S mutation in HCC with versus without HBsAg

R249S mutation was found in 9.4% (6 of 64) of HBsAg positive HCC specimens versus 6.7% (4 of 60) of HBsAg negative HCC patients. Categorized by serologic etiology, R249 prevalence was 9.8% (6/61) in HBV-related HCC specimens, 13.3% (2/15) of HCV-related HCC specimens, 4.4% (2/45) of non-B non-C HCC specimens and 0% (0/3)

Table 1. Characteristics of HCC Patients Included in the Study

Study population	(n=124)
Male (%)	103 (83)
Mean age, year	59.3±12.7
HBsAg positive (%)	64 (51.6)
Anti-HCV positive (%)	18 (14.5)
Liver biopsy specimen (%)	38 (30.6)
Tumor differentiation	
-well differentiated %	25
-moderately differentiated %	55
-poorly differentiated %	20
Tumor pattern	
-mixed (%)	48 (39)
-trabecular (%)	51 (41)
Mean AFP (IU/ml)	6004±26811
Median AFP (IU/ml)	20.1
R249S mutation (%)	10 (8.1)

*Character (% of R249S mutation among those subgroup)

Table 2. Comparison between Characteristics of HCC Patients with Mutant Versus Patients with Wild Type Codon 249

Character (% with mutation)	R249S Mutant (n=10)	Wild type (n=114)	p value
Male (%)	9 (89 %)	94	>0.05
Mean age, year	55±10	60±13	>0.05
Serology			
HBsAg positive	6 (9.4)	58	>0.05
Anti-HCV positive*	2 (11.1)	16	>0.05
Etiology by serology (%)			
HBV-HCV coinfection	0 (0)	3	
HBV (HBsAg)	6 (9.8)	55	
HCV (anti-HCV)	2 (13.3)	13	
Non B Non C	2 (4.4)	43	
Tumor differentiation			
Well	2 (6.7)	28	>0.05
Moderately	5 (7.6)	61	>0.05
Poorly	3 (12.0)	22	>0.05
Tumor pattern			
Mixed (%)	4 (8.3)	44	>0.05
Trabecular (%)	4 (7.8)	47	
Mean AFP (IU/ml)	2486±7828	6327±27911	>0.05
Median AFP (IU/ml)	9.1	23.7	
Log AFP	1.2±1.2	1.67±1.4	>0.05
Mean CA 19-9	99.6±14.3	39.7±19.7	>0.05
Cirrhosis by pathology (%)	4 (10.3)	35	>0.05
Liver biopsy specimen (%)	2 (5.3)	36	>0.05
Positive tumor at margin of resection (%)	1 (11.1)	8	>0.05

*Anti-HCV serology were available for 108 cases

of HBV-HCV co-infected HCC specimens. HBsAg status was not significantly associated with R249S mutation (p=0.58).

Prevalence of R249S mutation and other variables

Patients with R249S mutation were younger (55±10 vs 60±13 year-old) and tended to have a more advanced Edmonson-Steiner grade of HCC, though differences did not reach statistical significance.

Discussion

The study objective was to compare the differences R249S mutation rate between HBsAg positive and negative patients. Our results demonstrate that HBsAg status was not statistically related to an increased or decreased rate of R249S mutation. Studies from Brazil (Nogueira et al., 2009) and Egypt have also reached similar conclusions (El-Kafrawy et al., 2005). These results continue to suggest the importance of decreasing aflatoxin contamination and exposure in the general population regardless of HBsAg status (Nogueira et al., 2009; Liu and Wu, 2010; Hamid et al., 2013).

In this study, the prevalence of the R249S mutation in Thailand was found to be 8.1%. In addition, the average Thai person's exposure to aflatoxin contamination is 53 ng/kg body weight/day aflatoxin B1 intakes. Thus, although universal Hepatitis B vaccine programs can decrease incidence of HCC (Wichajarn et al., 2008) - with both immune memory and antibody persisting for up to 20 years (Poovorawan et al., 2012) - aflatoxin exposure may have still made significant contributions to some hepatocarcinogenesis. We therefore believe that the

reduction of aflatoxin intake in the Asia Pacific region is a sensible policy to help mitigate risk of HCC (Bridges et al., 2011).

However, besides peanuts, corn and red chili are both common sources of aflatoxin exposure and these foods are difficult to avoid in the Asia Pacific region. Moreover, without access to these foods nutritional status may suffer and it has been shown that good nutritional status in chronic liver disease is associated with improved outcome (O'Brien and Williams, 2008). Thus a recommendation to avoid specific foods such as peanuts and corn seem like an impractical suggestion.

The R249S mutation rate reported in our study and our recently report (Thongbai et al., 2013) are lower than those reported in a previous Thai study. The explanations may be the following: i) Compared to past levels, (Sripathomswat and Thasnakorn, 1981), aflatoxin contamination may be lower due to improved food storage and processing (Waenlor and Wiwanitkit, 2003); ii) Foods in Bangkok and the central part of Thailand may have lower concentration of aflatoxin than the northern part of Thailand where the previous study was conducted; iii) HCC patients from northern Thailand likely have a different genetic background compared to patients from central Thailand and Bangkok, and genetics may play a role in aflatoxin related carcinogenesis (Kirk et al., 2005). However, the difference in the R249S detection method is likely not an explanation for the lower prevalence reported in our study. The previous study used Short Oligonucleotide Mass Analysis (SOMA) to determine R249S prevalence in patients, and this method has been demonstrated to be 1.40 fold more sensitive than RFLP in discovering R249S mutations in a sample population (Qian et al., 2002). Thus, even if the R249S prevalence was 1.40 fold greater than what we found in our study, the prevalence would still only be 11.3%, which is a considerably lower value than what the previous study in Thailand reported. Finally, a limitation of our study may be the use of paraffin embedded tissue because paraffin preservation may fragment or denature DNA strands.

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Genetic Association of Human Leukocyte Antigens with Chronicity or Resolution of Hepatitis B Infection in Thai Population

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Abstract

Background: Previous studies showed that single nucleotide polymorphisms (SNPs) in the *HLA-DP*, *TCF19* and *EHMT2* genes may affect the chronic hepatitis B (CHB). To predict the degree of risk for chronicity of HBV, this study determined associations with these SNPs.

Methods: The participants for this study were defined into 4 groups; HCC (n = 230), CHB (n = 219), resolved HBV infection (n = 113) and HBV uninfected subjects (n = 123). The *HLA-DP* SNPs (rs3077, rs9277378 and rs3128917), *TCF19* SNP (rs1419881) and *EHMT2* SNP (rs652888) were genotyped.

Results: Due to similar distribution of genotype frequencies in HCC and CHB, we combined these two groups (HBV carriers). The genotype distribution in HBV carriers relative to those who resolved HBV showed that rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, $p < 0.001$ and OR = 0.47, $p < 0.001$). The other SNPs rs3128917, rs1419881 and rs652888 were not associated with HBV carriers.

Conclusions: Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population.

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Introduction

The hepatitis B virus (HBV) is one of the most common causes of chronic hepatitis B (CHB), liver cirrhosis and hepatocellular carcinoma (HCC). Globally more than 2 billion people have been infected with HBV and 378 million are suffering from chronic hepatitis. Over 600,000 people die each year because of HBV infection. In high prevalence areas such as the central Asian republics, Southeast Asia, Sub-Saharan Africa and the Amazon basin over 8% of the population may be HBV carriers [1]. The main route of HBV infection is vertical transmission from mother to infant and horizontal transmission between children, whereby 90% will develop chronic hepatitis as infants or in early childhood and never clear the virus [1–3]. In contrast, 15% of HBV

infections in adulthood develop into chronic hepatitis with viral persistence.

The frequency of HBV infection which develops into chronic hepatitis depends on the age at which the person is infected [1,2]. However, the factors determining HBV persistence or clearance are not clearly understood [4–6]. Risk factors for viral persistence include the following: virological factors (viral load, genotype, viral gene mutations and co-infection with another virus), host factors (age at infection, gender, immune status and genetic variability) and extrinsic factors (e.g. alcohol consumption and chemotherapy) [7]. Whether viral infection results in acute or chronic infection also depends on cellular immune responses influenced by human leukocyte antigen (*HLA*) class I and II molecules which must present the viral antigens to CD8⁺ T cells and CD4⁺ T cells, respectively [8]. The genes encoding *HLA* are the most

polymorphic in the human genome, presumably in order to be able to respond to all potential foreign antigens [9].

Recently, many genome-wide association studies (GWAS) have been performed to seek associations between human genetic variation and the outcome of HBV infection [10–15]. Studies in the Japanese population showed that 11 single nucleotide polymorphisms (SNPs) located within or around the *HLA-DPA1* and *HLA-DPB1* loci are significantly associated with the occurrence of CHB. Of these 11 SNPs, the most strongly associated with the outcome of HBV infection were rs9277535 and rs3128917 in *HLA-DPB1* and rs3077 in *HLA-DPA1* [10].

Thereafter, GWAS studies in the Korean population confirmed the presence of these host factors related to HBV outcome and reported two new SNPs significantly associated with CHB within the *HLA* region, namely rs1419881 and rs652888 in transcription factor 19 (*TCF19*) and euchromatic histone-lysine methyltransferase 2 (*EHMT2*), respectively [16]. *TCF19* (or transcription factor SC1) is a *trans*-activating factor that mainly influences the transcription of genes required for late growth regulation at the G1-S checkpoint and during S phase [17]. *EHMT2* is a histone methyltransferase responsible for mono- and di-methylation of H3K9 (lysine at 9th residue of histone subunit 3) in euchromatin [18], which modifies the conformation of chromatin from its tightly packed form, heterochromatin, and thus influences gene repression or transcriptional silencing [19].

In the present study, we determined associations between the SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), *TCF19* (rs1419881) and *EHMT2* (rs652888) in HBV infected patients compared to those with resolved infections and those who had never been infected.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of the Faculty of Medicine, University (Bangkok, Thailand) code IRB.455/54. Written informed consent was obtained from each patient and all samples were anonymized.

Sample Collection

All blood samples were negative for hepatitis C virus and human immunodeficiency virus. Subjects were defined into 4 groups: 230 hepatitis B surface antigen (HBsAg)-positive HCC, and 219 CHB who had been HBsAg-positive for at least 6 months were recruited at the King Chulalongkorn Memorial Hospital, whereas patients with resolved HBV and uninfected subjects were from the Thai Red Cross Society and from the north-eastern part of Thailand (age >40 years) which had been screened by Immunoassay (Architect i2000SR, Abbott, USA.) for HBsAg, antibody to hepatitis B surface antigen (anti-HBs) and antibody to hepatitis B core protein (anti-HBc). Of these subjects, 113 were negative for HBsAg but positive for anti-HBc and/or positive for anti-HBs after resolution of infection, while 123 uninfected subjects were all negative for HBsAg, anti-HBc and anti-HBs. All samples in this study were collected from subjects who have lived at the same area in Thailand, suggesting that the genetic background would be balanced between a case and control.

Genotyping assays

DNA was extracted from peripheral blood mononuclear cell using phenol-chloroform DNA extraction. The concentration of DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). We determined SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), and

the genes *TCF19* (rs1419881) and *EHMT2* (rs652888) by commercial TaqMan PCR assays (Applied Biosystems, USA). In this study we investigated *HLA-DPB1* (rs9277378) because this SNP had a high level of linkage disequilibrium with rs9277535 ($D' = 1.00$, $R^2 = 0.954$) [20] and was clearly detectable by the TaqMan assay rather than rs9277535.

Statistical analyses

In this study, Hardy-Weinberg equilibrium was performed on each SNP. The Chi-square test of independence and Odds Ratio (OR) from two-by-two tables for comparisons between case and control groups was performed using Microsoft Excel. Statistical significance was defined by $P < 0.05$. The calculated of possibility level was established using Chi-square contingency table analysis.

Results

Subjects were defined into 4 groups: group 1) HCC (age = 58.2 ± 12 years, 190/230 (82.6%) male); group 2) CHB (age = 46.6 ± 10 years, 144/219 (65.7%) male); group 3) those with resolved HBV (age = 48.2 ± 6 years, 83/113 (73.5%) male); and group 4) HBV uninfected subjects (age = 46.7 ± 6 years, 73/123 (59.3%) male). The details are given in Table 1. To find the genetic factor associated with chronicity of HBV infection, however, the two groups (group 1 and 2) were combined (designated “HBV carriers”). Indeed, according to the frequencies of minor alleles of the SNPs in the *HLA-DP*, *TCF19* and *EHMT2* genes listed in Table 2, the frequencies of minor alleles of these 5 SNPs in HCC and CHB were similar (data shown in Table S1). The composite HBV carriers group had a minor allele frequency for rs3077 and rs9277378 lower than in groups 3 and 4 (OR = 0.57, 95% CI = 0.42–0.78, $p < 0.001$ and OR = 0.63, 95% CI = 0.47–0.85, $p = 0.008$ for rs3077, OR = 0.59, 95% CI = 0.44–0.81, $p = 0.001$ and OR = 0.56, 95% CI = 0.42–0.75, $p < 0.001$ for rs9277378, respectively). In contrast, the minor allele frequency for rs1419881 in HBV carriers was similar to group 3 (OR = 0.80, 95% CI = 0.60–1.08, $p = 0.142$) but lower than in group 4 (OR = 0.64, 95% CI = 0.48–0.85, $p = 0.002$). Moreover, minor allele frequency for rs3128917 and rs652888 in HBV carriers was comparable to groups 3 and 4 (OR = 1.14, 95% CI = 0.85–1.53, $p = 0.371$ and OR = 1.06, 95% CI = 0.80–1.41, $p = 0.673$ for rs3128917; OR = 1.14, 95% CI = 0.84–1.55, $p = 0.400$ and OR = 1.12, 95% CI = 0.83–1.50, $p = 0.471$ for rs652888, respectively).

The results of Hardy-Weinberg equilibrium analysis of each SNPs were shown in Table 3. All data were over 0.01 ($p > 0.01$), indicating that the frequencies did not deviate from Hardy-Weinberg equilibrium. The genotype distribution in HBV carriers compared to subjects with HBV resolution showed that both rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, 95% CI = 0.30–0.69, $p < 0.001$ for rs3077 and OR = 0.47, 95% CI = 0.31–0.72, $p < 0.001$ for rs9277378, are described in Table 3), suggesting that major homozygous genotypes were risk factors with the chronicity of HBV. The other SNPs rs3128917, rs1419881 and rs652888 were not associated against HBV carrier status (OR = 1.22, 95% CI = 0.76–1.97, $p = 0.413$ for rs3128917, OR = 0.67, 95% CI = 0.42–1.06, $p = 0.084$ for rs1419881 and OR = 1.31, 95% CI = 0.87–2.00, $p = 0.198$ for rs652888, respectively).

The genotype frequencies for 5 SNPs are shown in Table 3. Comparing HBV carriers with uninfected subjects showed that rs3077, rs9277378 and rs1419881 were all protectively associated with chronic HBV infection (OR = 0.63, 95% CI = 0.42–0.95,

Table 1. Characteristics of participants in HCC, CHB, resolved HBV and HBV uninfected subjects in Thailand.

	HCC (n = 230)	CHB ^a (n = 219)	Resolved ^b (n = 113)	Uninfected ^c (n = 123)
Age (years)	58.2±12	46.6±10	48.2±6	46.7±6
Male	190 (82.6%)	144 (65.7%)	83 (73.5%)	73 (59.3%)
HBsAg positive	230 (100%)	219 (100%)	0	0
ALT>40 (IU/L)	43 (18.7%)	61 (27.8%)	-	-
Alb (g/dl)	3.7 (2.5–5.6)	4.5 (3–5.2)	-	-
TB (mg/dl)	1.2 (0.17–14.8)	0.56 (0.2–2.67)	-	-

Abbreviation: HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; ALT, Alanine transaminase; Alb, Albumin; TB, Total bilirubin.

^aDefined as chronic hepatitis B includes chronic HBV infection but not cirrhosis and HCC.

^bDefined as HBsAg negative but anti-HBc or/and anti-HBs positive.

^cDefined as any HBV serological markers negative.

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$p = 0.025$ for rs3077 and $OR = 0.55$, 95% $CI = 0.36–0.82$, $p = 0.003$ for rs9277378 and $OR = 0.57$, 95% $CI = 0.36–0.90$, $p = 0.015$ for rs1419881, respectively). Comparing HBV carriers and uninfected subjects rather than those with resolved infection regarding rs1419881 was significantly protective association against CHB, but rs3128917 and rs652888 were not associated against CHB ($OR = 1.58$, 95% $CI = 1.02–2.46$, $p = 0.042$ for rs3128917 and $OR = 1.09$, 95% $CI = 0.65–1.82$, $p = 0.080$ for rs652888). When we consider the Bonferroni corrections (5 SNPs), however, the P value for rs1419881 did not reach the level of significant difference ($0.015 > 0.05/5$) between HBV carriers and HBV uninfected subjects. These data suggested that other SNPs, rs1419881, rs3128917 and rs652888 were not associated with HBV carriers in this study.

Results of meta-analysis for 3 SNPs (rs3077, rs9277378 and rs3128917) in the *HLA* gene were shown in Table S2 and S3; HBV carriers were compared to HBV resolved or HBV uninfected subjects, respectively. While the other 2 SNPs were published only from Korean population, thus the meta-analysis appeared only between HBV carriers and HBV uninfected subjects. All SNPs analyzed by the meta-analysis were significantly associated with HBV carriers.

The associations between these 5 SNPs and HBV status are depicted graphically in Figure S1. Each histogram compares HBV carriers with subjects that have resolved HBV infection or were never infected. The results showed that the minor dominant model of rs3077 and rs9277378 was highly protective associated against chronic HBV, while no significant associations were observed with rs3128917 and rs652888. Furthermore, comparing the frequency of rs1419881 between HBV carriers and uninfected subjects also revealed its association against chronic HBV infection but the association with resolved HBV did not achieve statistical significance.

Discussion

Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population. In the human genome, single nucleotide polymorphisms are found in every 300–570 nucleotides. Many SNPs have no effect on the function of the encoded proteins, but some variants do appear in regulatory or coding part of the gene and affect gene expression level or protein function which can give rise to disease [21] such as the 3 SNPs including rs3077, rs9277378 and rs3128917 in *HLA*-

Table 2. Minor allele frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

SNPs	Gene	Minor alleles ^a	HBV carriers ^b (2n = 898)	Resolved (2n = 226)	Uninfected (2n = 246)	HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
						OR (95% CI)	P values	OR (95% CI)	P values
rs3077	<i>HLA-DPA1</i>	T	227 (25.3%)	84 (37.2%)	86 (35.0%)	0.57 (0.42–0.78)	<0.001	0.63 (0.47–0.85)	0.008
rs9277378	<i>HLA-DPB1</i>	A	237 (26.4%)	85 (37.6%)	96 (39.0%)	0.59 (0.44–0.81)	0.001	0.56 (0.42–0.75)	<0.001
rs3128917	<i>HLA-DPB1</i>	G	459 (51.1%)	108 (47.8%)	122 (49.6%)	1.14 (0.85–1.53)	0.372	1.06 (0.80–1.41)	0.673
rs1419881	<i>TCF19</i>	C	361 (40.2%)	103 (45.6%)	126 (51.2%)	0.80 (0.60–1.08)	0.142	0.64 (0.48–0.85)	0.002
rs652888	<i>EHMT2</i>	C	329 (36.6%)	76 (33.6%)	84 (34.1%)	1.14 (0.84–1.55)	0.400	1.11 (0.83–1.50)	0.478

Abbreviation: CI, confidence interval; OR, odds ratio.

^aDefined by using data from public database (NCBI).

^bDefined as the combination between HCC and CHB.

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Table 3. Genotype frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

SNP	Genotype	HBV carriers ^a (n = 449)	Resolved (n = 113)	Uninfected (n = 123)	HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
					OR (95% CI)	P values	OR (95% CI)	P values
rs3077	CC	259 (57.7%)	43 (38.1%)	57 (46.3%)	1.00	-	1.00	-
HLA-DPA1	CT	153 (34.1%)	56 (49.6%)	46 (37.4%)	0.45 (0.29–0.71)	<0.001	0.73 (0.47–1.13)	0.161
	TT	37 (8.2%)	14 (12.4%)	20 (16.3%)	0.44 (0.22–0.88)	0.018	0.41 (0.22–0.75)	0.003
	Dominant^b				0.45 (0.30–0.69)	<0.001	0.63 (0.42–0.95)	0.025
	HWEp	0.038	0.516	0.049				
rs9277378	GG	242 (53.9%)	40 (35.4%)	48 (39.0%)	1.00	-	1.00	-
HLA-DPB1	AG	177 (39.4%)	61 (54.0%)	54 (43.9%)	0.48 (0.31–0.75)	0.001	0.65 (0.42–1.00)	0.051
	AA	30 (6.7%)	12 (10.6%)	21 (17.1%)	0.41 (0.20–0.87)	0.018	0.28 (0.15–0.54)	<0.001
	Dominant				0.47 (0.31–0.72)	<0.001	0.55 (0.36–0.82)	0.003
	HWEp	0.757	0.110	0.390				
rs3128917	TT	99 (22.0%)	29 (25.7%)	38 (30.9%)	1.00	-	1.00	-
HLA-DPB1	TG	241 (53.7%)	60 (53.1%)	48 (39.0%)	1.18 (0.71–1.94)	0.525	1.93 (1.19–3.13)	0.008
	GG	109 (24.3%)	24 (21.2%)	37 (30.1%)	1.33 (0.73–2.44)	0.355	1.13 (0.67–1.92)	0.648
	Dominant				1.22 (0.76–1.97)	0.413	1.58 (1.02–2.46)	0.042
	HWEp	0.117	0.496	0.015				
rs1419881	TT	162 (36.1%)	31 (27.4%)	30 (24.4%)	1.00	-	1.00	-
TCF19	TC	213 (47.4%)	61 (54.0%)	60 (48.8%)	0.67 (0.41–1.08)	0.097	0.66 (0.41–1.07)	0.088
	CC	74 (16.5%)	21 (18.6%)	33 (26.8%)	0.67 (0.36–1.25)	0.210	0.42 (0.24–0.73)	0.002
	Dominant				0.67 (0.42–1.06)	0.084	0.57 (0.36–0.90)	0.015
	HWEp	0.778	0.349	0.792				
rs652888	TT	169 (37.6%)	50 (44.2%)	57 (46.3%)	1.00	-	1.00	-
EHMT2	TC	231 (51.4%)	50 (44.2%)	48 (39.0%)	1.37 (0.88–2.12)	0.162	1.62 (1.05–2.50)	0.027
	CC	49 (10.9%)	13 (11.5%)	18 (14.6%)	1.12 (0.56–2.22)	0.756	0.92 (0.49–1.70)	<0.001
	Dominant				1.31 (0.87–2.00)	0.198	1.09 (0.65–1.82)	0.080
	HWEp	0.022	0.926	0.142				

Abbreviation: CI, confidence interval; OR, odds ratio ; HWEp, Hardy-Weinberg equilibrium analysis.

^aDefined as the combination between HCC and CHB.

^bDefined as a minor dominant according to the comparison between heterozygous+minor homozygous genotype and major homozygous genotype (eg. rs3077; CT+TT vs. CC).

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DP region of MHC class II. The function of HLA-DP is to present bound peptide antigens, e.g. from HBV, at the surface of antigen-presenting cells. CD4+ T cells recognize these antigens and initiate the adaptive immune response. They assist the MHC class I-restricted CD8+ T cells which are the primary cellular effectors mediating HBV clearance from the liver during acute viral infection [22]. HBV infection will either be cleared by these means, or establish itself as a chronic infection. The reason for the latter is unclear but may be related to variation of *HLA-DP* alleles. Thus, the position of *HLA-DP* SNPs might be associated with possibility of clearance or chronicity. The rs3077 and rs9277535 SNPs are located within the 3' untranslated region (UTR) of *HLA-DPA1* and *HLA-DPB1*, respectively while rs3128917 is located downstream of *HLA-DPB1*.

Recent investigations have identified 11 risk alleles for CHB related to mRNA expression of *HLA-DPA1* and *HLA-DPB1* [23]. The results showed that only these two alleles, rs3077 and rs9277535 were strongly associated with the risk of CHB and decreased expression of *HLA-DPA1* and *HLA-DPB1*, respectively. In contrast, while rs3128917 was associated with CHB, it was not associated with the level of *HLA-DPB1* expression [23]. Variation

at 5' and 3' UTRs can alter the binding sites of regulatory proteins which protect and stabilize newly synthesized RNA, either increasing or decreasing binding [24,25]. Nevertheless, the present study showed that rs3128917 was not associated with HBV carrier status in Thailand. Because rs3128917 is located downstream of the direction of transcription of the gene, this suggests that it does not affect regulation or coding of the gene and would have no effect on HLA protein expression.

The results from the present study not only establish the importance of variation at the *HLA-DP* gene but also explore two new SNPs, rs1419881 located in *TCF19* and rs652888 in the *EHMT2* gene [16]. *TCF19* (or transcription factor SC1) is a late growth regulatory gene like histone, thymidine kinase etc, maximally expressed at the onset of DNA synthesis at the G1-S boundary and S phase of cell cycle. This protein is also involved in regulations of growth and transcription factors controlling the number and development of peripheral-blood monocytes and erythrocytes [26]. The *EHMT2* gene is a histone methyltransferase [18] mainly responsible for mono- and di-methylation of H3K9 in euchromatin. This changes the conformation of chromatin from euchromatin to heterochromatin and then affects gene repression

[19]. Histone methylation has a critical role in gene transcription and epigenetic events [27–30].

According to recently published GWAS data [11], two SNPs associated with the risk for CHB in the Korea population were identified. These were the top signals in the genome-wide significance level analysis and were independently associated with *HLA-DP* and *HLA-DQ*, respectively. The authors then confirmed the results in a replication sample, showing that the frequency of their two SNPs strongly associated with CHB; OR = 0.76, 95% CI = 0.68–0.86, $p = 4.51\text{E-}11$ for rs1419881 and OR = 1.26, 95% CI = 1.07–1.47, $p = 2.78\text{E-}06$ for rs652888 [16]. Furthermore, another GWAS study focused on HLA, of hepatitis B vaccinated people in Indonesia, showed that rs652888 was also associated with risk of CHB ($p \leq 0.0001$) in that population [31].

In the present study, however, we found that rs1419881 tended to be associated with chronic HBV infection, based on the results of a comparison between HBV carriers and uninfected subjects. Nonetheless, it did not reach the significance by the Bonferroni corrections, as well as when HBV carriers were compared with patients who had their HBV infection resolved, no association with rs1419881 was observed. The second SNP, rs652888, was not associated with chronic HBV infection in the Thai population. Although our study had sampling error due to small samples, it might be another effect that the result between rs652888 in *EHMT2* gene and chronic hepatitis B in Thai population was not associated. The reason for these negative findings for the two SNPs might be due to the affected gene functions that were not involved with the immune system or processes of persistent infection. Data supporting this notion are to be found in the GWAS data for the Korean population, where pathway analysis of genes involved in the regulation of immune function showed that *TCF19* and *EHMT2* genes are not significantly involved in human immunity [16].

Mapping the position of the two new SNPs showed that rs1419881 located at the 3' UTR of exon 4, with a tendency towards association with CHB and rs652888 which is not associated with CHB located on an intron. The position of each SNP might affect the phenotype of gene expression and susceptibility to disease, explaining why some are associated with chronic HBV infection, and others not. According to previous publications, the 3' UTR of the *HLA-DP* region is strongly involved with regulating HLA-DP expression and influences the outcome of HBV infection [32]. In addition, another study showed that variation of the 3' UTR of HLA-C was strongly associated with HLA-C expression levels and with control of human immunodeficiency virus [33]. This illustrated the general principle that the position of SNPs affects association with diseases.

The prevalence of HBV in Eastern countries, i.e. Asia, sub-Saharan Africa and the Pacific is much higher than in Western Europe and America. Most people in Eastern countries are infected with HBV during childhood and 8–10% of these develop CHB. In contrast, the frequency of chronic carriers in Western Europe and North America is $\leq 1\%$. Furthermore, previous GWAS and meta-analysis reported that A alleles at rs3077 and rs9277353 have protective effects against CHB. Asian and African populations, especially Chinese, have lower frequencies of A alleles than European and American populations [10,34,35]. Moreover, the previous study showed no associations of rs3077 and rs9277353 with progressive CHB infection; however rs3077 was highly significant associated with HBV infection but not associated with rs9277353 in Caucasian populations [36].

While the frequency of alleles at rs3128917 and rs1419881 in Asian and African populations are quite similar, Northern and Western European populations have high frequencies of the protective T allele at rs3128917 but have low T allele frequencies

(a risk allele for CHB) at rs1419881. The allele frequencies of populations in the worldwide for conspicuous details came from dbSNP Short Genetic Variations available at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi. Lastly, both ethnic Eastern and Western populations have similar allele frequencies at rs652888, carrying a risk for CHB, with T allele frequencies very much higher than C allele frequencies, which has a protective effect. In addition, evolution of genomic characteristics, the migratory history of different populations, as well as HBV genotypes [37], HBV carrier rate [38] and pathological procession of liver disease [39] in each country may affect the distribution of *HLA* alleles. This was illustrated by a recent report in two Han Chinese populations (southern and northern) having different distributions of *HLA-DP* genes [39]. Thus, the genetics of the host is one of the factors influencing and predicting disease outcome [40].

According to less number of samples, it might influence statistical power in this study. Thus, we made another statistic meta-analysis of data obtained from previous reports and this study in Table S3. We compared HBV carriers with HBV uninfected subjects, because most previous studies also compared CHB with HBV clearance and/or healthy (negative for any HBV serological markers). Interestingly, all SNPs analyzed by the meta-analysis were significantly associated with HBV carriers. These results could support our data in Thailand. Additionally, no heterogeneity was observed between HBV carriers and HBV-resolved subjects ($P_{\text{het}} = 0.10$ for rs3077, 0.79 for rs9277378, and 0.07 for rs3128917), as well as between HBV carriers and HBV uninfected subjects ($P_{\text{het}} = 0.10$ for rs3077, 0.02 for rs9277378, 0.91 for rs1419881, and 0.04 for rs652888) except for rs9277378 ($P_{\text{het}} = 0.000$), for the minor allele frequency (MAF) of only rs9277378 was different between HapMap-CHB (MAF = 46.3% of G allele) and HapMap-JPT (MAF = 44.8% of T allele).

In the present study, we determined associations of variations at the *HLA-DP* gene with outcome in HBV infected Thai patients and the major homozygous genotypes of rs3077 and rs9277378, but not rs3128917, were significantly associated with HBV carrier status. Although genetic variation of two new SNPs, rs1419881 in the *TCF19* gene and rs652888 in the *EHMT2* gene, were not associated with the outcome of HBV infection in the Thai population, a large-scale study should be required.

Supporting Information

Figure S1 Association of 5 SNPs with HBV carriers, resolved HBV and uninfected subjects in Thailand. The results were compared between percentages of combination of heterozygous genotypes and minor homozygous genotypes (White square) with percentages of major homozygous genotypes (Grey square). Five SNPs applied in this study were rs3077, rs9277378 and rs3128917 in *HLA-DP* gene, rs1419881 in *TCF19* gene and rs652888 in *EHMT2* gene. OR, odds ratio; (lower-upper), 95% confidence interval. (PPTX)

Table S1 Minor allele frequencies in HCC, CHB, resolved HBV and uninfected subjects in Thailand. (DOC)

Table S2 The meta-analysis of minor allele frequencies in HBV carriers and resolved HBV. (DOC)

Table S3 The meta-analysis of minor allele frequencies in HBV carriers and uninfected subject. (DOC)

Author Contributions

Conceived and designed the experiments: SP TW YP YT. Performed the experiments: NP. Analyzed the data: NP SP SI KM NS. Contributed reagents/materials/analysis tools: PT SO SM. Wrote the paper: NP.

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WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Hepatitis C virus genotype 6: Virology, epidemiology, genetic variation and clinical implication

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Abstract

Hepatitis C virus (HCV) is a serious public health problem affecting 170 million carriers worldwide. It is a leading cause of chronic hepatitis, cirrhosis, and liver cancer and is the primary cause for liver transplantation worldwide. HCV genotype 6 (HCV-6) is restricted to South China, South-East Asia, and it is also occasionally found in migrant patients from endemic coun-

tries. HCV-6 has considerable genetic diversity with 23 subtypes (a to w). Although direct sequencing followed by phylogenetic analysis is the gold standard for HCV-6 genotyping and subtyping, there are also now rapid genotyping tests available such as the reverse hybridization line probe assay (INNO-LiPA II; Innogenetics, Zwijnaarde, Belgium). HCV-6 patients present with similar clinical manifestations as patients infected with other genotypes. Based on current evidence, the optimal treatment duration of HCV-6 with pegylated interferon/ribavirin should be 48 wk, although a shortened treatment duration of 24 wk could be sufficient in patients with low pretreatment viral load who achieve rapid virological response. In addition, the development of direct-acting antiviral agents is ongoing, and they give high response rate when combined with standard therapy. Herein, we review the epidemiology, classification, diagnosis and treatment as it pertains to HCV-6.

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Key words: Hepatitis C virus; Genotype 6; Epidemiology; Clinical; Treatment

Core tip: Hepatitis C virus (HCV) genotype 6 is restricted to South China, South-East Asia, and it is occasionally found in migrant patients from endemic countries. Treatment response rates are lower than those of genotype 3 but higher than those of genotype 1. Based on current evidence, the optimal treatment duration of HCV-6 should be 48 wk. Shortened treatment duration of 24 wk could be sufficient in patients with low pretreatment viral load who achieve rapid virological response. The development of direct-acting antiviral agents is ongoing, and they give high response rate when combined with standard therapy. We review the epidemiology, classification, diagnosis and treatment.

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INTRODUCTION

Hepatitis C virus (HCV) infection is an important worldwide public health problem. Most HCV cases become chronic hepatitis C (CHC), which may advance to liver fibrosis, cirrhosis, and hepatocellular carcinoma. The global prevalence of HCV infection is estimated at more than 170 million people^[1-3], and some studies estimate that mortality related to HCV infection (death from liver failure or hepatocellular carcinoma) will continue to increase over the next two decades^[4].

Hepatitis C virus is a member of the *Flaviviridae* family and belongs to the genus *Hepacivirus*. HCV is classified into six major genotypes (1-6) and subdivided into various subtypes named in alphabetical order from a to z. Currently, sequencing of HCV isolates has identified more than 83 subtypes from the six genotypes^[5]. The genomes among HCV genotypes differ from each other by approximately 30%-35% while the genomes among the subtypes differ by 15%-20%. The prevalence of HCV genotypes varies geographically: HCV-1 is found worldwide including developed regions such as North America and Europe. HCV-2 has high prevalence in Central and West Africa as well as some western countries, while HCV-3 is predominantly found in the Far Eastern countries and the Indian subcontinent^[6]. Meanwhile, HCV genotypes 4, 5 and 6 are endemic to specific geographical areas: HCV-4 is mainly found in Egypt and Sub-Saharan Africa, HCV-5 in South Africa^[7], and HCV-6 in South China and South-East Asian countries^[8-10] (Figure 1)^[10-34].

HCV-6 is highly diverse with 23 subtypes currently known^[8,9,30]. This genetic diversity may be the result of a long period of viral circulation^[14]. In addition, repeated viral exposure through activities such as intravenous drug use leads to more than one viral strain circulating in the host, which in turn increases the chance of viral recombination events among circulating HCV genotypes and strains. The high variation and accumulation of HCV-6 in Southeast Asia also supports the idea that this area may be the origin and worldwide distribution center of this genotype.

Various factors determine treatment response. In the case of host factors, age, sex, race, fibrosis and steatosis level all have important influences on treatment outcome^[35-37], while the most important viral factors for predicting response to IFN-based therapy are genotype and viral load at baseline. In the past 10 years, the standard treatment of HCV patients has been a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) with a 24 to 48 wk regimen depending on the viral genotype of each infected individual. Thus, HCV genotype is

considered one of the most robust independent predictors for sustained virological response (SVR). Most clinical studies on PEG-IFN/RBV efficacy have been based on common genotypes such as genotype 1, 2 and 3, while scant clinical data has been generated concerning genotype 6. However, due to this genotype's extreme genetic diversity, it may be important to study it in a controlled, clinical setting in order to gauge the standard therapy's efficacy^[5].

Currently, the few available studies suggest that treatment with the longer 48-wk regimen may lead to a higher rate of SVR. On the other hand though, treatment with the 24-wk regimen may also lead to a similar SVR rate in subgroups of patients, as in the case of patients with genotypes 2 and 3^[38]. In 2011, newer agents known as direct-acting antivirals (DAA) were approved for use in conjunction with PEG-IFN and RBV for the treatment of HCV-1. However, the efficacy and safety of DAA in the treatment of the HCV-6 patients still needs to be assessed.

This study aimed to review the virology, epidemiology, genetic variation and clinical implication of HCV genotype 6. All data were retrieved and selected from related HCV-6 topics from PubMed database.

EPIDEMIOLOGY

Within endemic countries, HCV-6 shows variability in subtype prevalence. Vietnam has reported HCV-6 prevalence of 51%-54.4% in Ho Chi Minh City and 47.1% elsewhere with the most common subtypes being 6a followed by 6e and 6l. In total, 12 subtypes have thus far been identified in Vietnam (6a, 6c, 6e, 6f, 6h, 6k, 6l, 6n, 6o, 6p, 6r and 6t)^[39-41]. Although there is limited information regarding HCV epidemiology in Laos and Cambodia, a few studies have reported a high proportion of HCV-6 in these countries also. Among Laos blood donors, 95.6% of the HCV RNA positive samples were classified as HCV-6 with various subtypes being found including 6b, 6h, 6k, 6l, 6n, 6o, 6q and unclassified subtypes^[42,43]. A study of Cambodian migrants in Thailand reported that HCV seroprevalence in this group is similar to their guest country (2.3%). It was also found that HCV-6 is the most dominate genotype in Cambodian migrants with 52% of the HCV-RNA carriers testing positive for this genotype with the subtype breakdown as the following: 6e (20%), 6r (20%), 6f (8%) and 6p (4%)^[42]. Similar to its neighboring countries, Thailand also has several endemic HCV-6 subtypes (6b, 6c, 6e, 6f, 6i, 6m and 6n) along with novel subtypes (6u and 6v), which are found in the North and Northeast^[43]. However, unlike the Cambodian migrant population in Thailand, this genotype contributes a lower proportion (20.1%) of overall infection in comparison with HCV-3 and HCV-1^[32]. In Myanmar, HCV-6 prevalence has gone from being the third most prevalent HCV genotype in 2004 to the first after analysis of a large number of blood samples from 2007. HCV-6 is especially prevalent in the Northern part of Myanmar

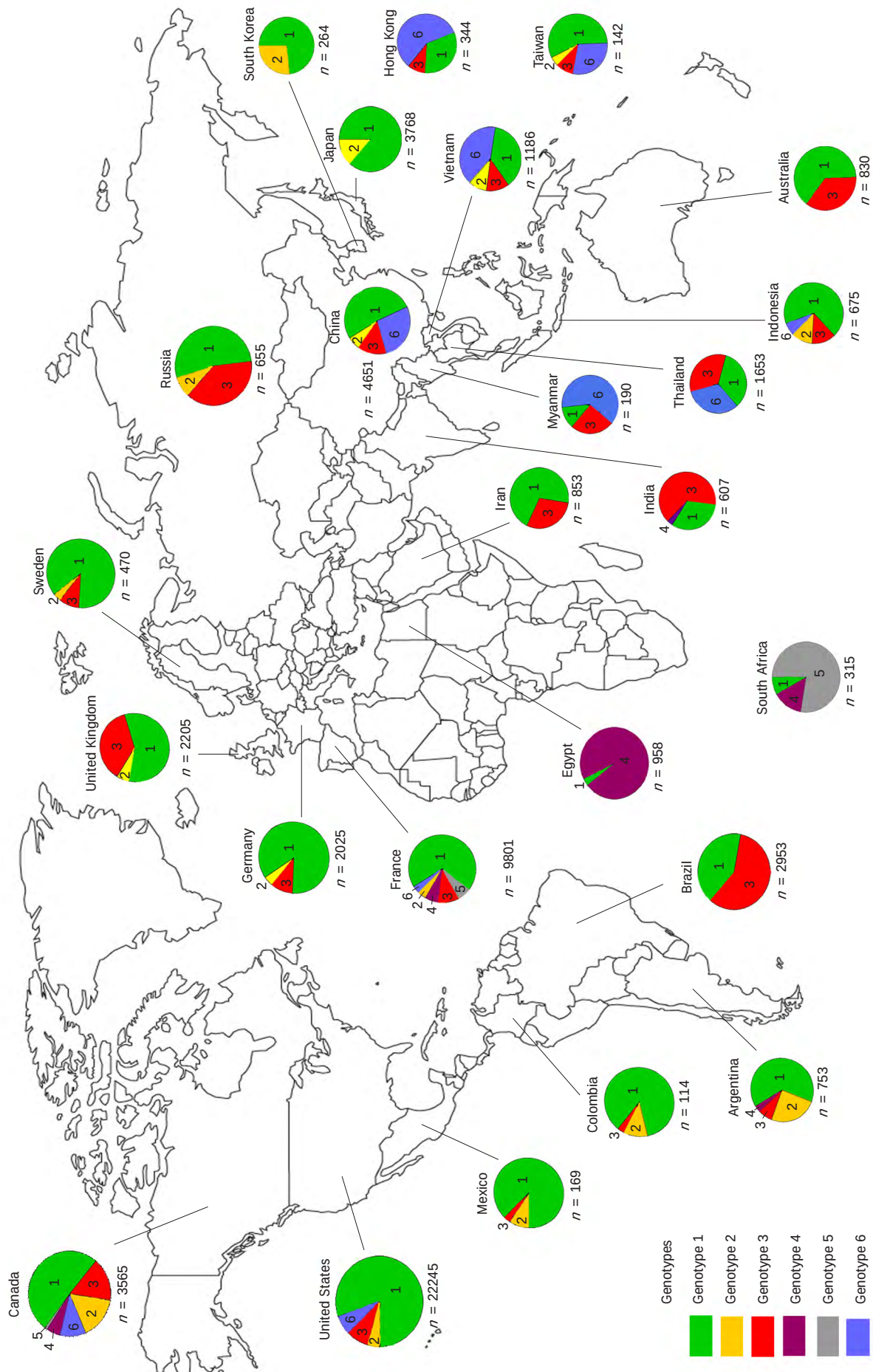


Figure 1 The prevalence of the various hepatitis C virus genotypes varies considerably between countries and regions^[10-34].

with subtypes 6f, 6n and 6m predominating^[44-47]. Despite its ubiquitous presence in the above-mentioned countries, however, HCV-6 is only rarely reported in other proximal countries. For example, only three samples of subtype 6g (previously designated as genotype 1a) have been reported in Indonesia since 1996^[48], while only a single recent report of subtype 6n has been found in Kuala Lumpur, Malaysia, and notably, this patient was co-infected with HIV-1 and had a history of IVDU^[49].

HCV-6 can also be found outside of the immediate South East Asian region in countries such as South China, Hong Kong and Taiwan (Figure 2)^[28,40,43,46-48,50-55].

Intravenous drug abuse is the transmission route suspected of being most responsible for the high frequency of this genotype in certain parts of Asia and the factor driving a continuous discovery of new subtypes. In China, HCV is frequently found in the South in patients with HIV-1 co-infection and IDU history^[56]. For example, prevalence of HCV-6 in chronic hepatitis cases is 12.9% to 14.2% while being 28.2% to 51.5% in IDUs. In addition, similar to Vietnam, 6a is the major subtype in IDUs from all study groups^[17,57,58]. A similar trend of HCV-6 infection can also be observed in Hong Kong. More than half of infected IDU have this genotype (53.2% to 58.5%), which is much higher than the prevalence in the general population (23.6%), and subtype 6a is the most common subtype^[52,59]. In Taiwan, there has been no report of HCV-6 until 2010, but since then there is a growing prevalence of HCV-6 and subtype 6a along with novel subtypes being reported in prisoners and IDUs^[53].

EVOLUTION OF HEPATITIS C VIRUS GENOTYPE 6

There is now considerable evidence to support the hypothesis that HCV-6 originated in Southeast Asia. First, this genotype is mainly observed in countries such as Vietnam, Cambodia, Laos, Myanmar and Thailand with the prevalence of HCV-6 in these countries ranging from approximately 20% to more than 50% of all genotypes. Second, there are a great number of known and novel subtypes circulating within these populations^[41]. Third, when HCV-6 is found outside of its endemic area, such as in countries like US, Canada and Australia, the virus is almost exclusively isolated from Asian immigrants^[7,60]. Fourth, a study in Laos showed that HCV-6 is highly divergent from other genotypes, and that it has distinct genetic differences from other strains, which suggests that there may yet be unclassified subtypes existing in this area. Thus, the accumulation of such genetic heterogeneity suggests that this genotype has circulated, adapted and evolved in this area for a long period of time (Table 1)^[39,46,61-64].

Evolutionary analysis of HCV-6 subtypes hypothesizes that these subtypes evolved from a common ancestor more than 1000 years ago, and that some subtypes may have maintained their endemicity via local epidemics during the 20th century initiated and propagated by modern medicine, blood transfusion and IVDU^[42]. However, each

of the subtypes seems generally restricted to different locations such as subtype 6d in Vietnam, 6f in Thailand, 6g in Indonesia and 6r in Cambodia. In addition, strains isolated from the same country tend to cluster together in a HCV-6 phylogenetic tree (Figure 3)^[42,47].

HCV-6 has spread through other East Asian countries by multiple transmission routes with one of the most effective routes being IDU. In the general population, HCV-1 is still the predominant genotype across Asia. However, HCV-6 - and especially subtype 6a - is the dominant genotype among IDUs^[53,58,59]. An example of this is the phylogeographic and coalescent analysis of HCV-6's spread through China. Analysis shows that subtype 6a and some other Chinese strains may have originated in Vietnam and spread to neighboring Guangxi and Yunnan provinces^[65,66]. Finally, since Guangdong is a major gateway to China, this province may have been the origin of subtype 6a dispersion throughout other regions of the country^[58].

Similar to other RNA viruses, HCV has a high tendency for genetic heterogeneity due to a lack of proof reading activity by its viral RNA polymerase. This genetic drift results in an accumulation of viral mutations, which will then be selected for by forces of environmental pressure. Thus, only the fittest strains survive and become the major circulating viral population. Of course, however, this process of fixation requires a relatively long period of time. Meanwhile, viral recombination can result in novel strains overnight, although the drastic nature of recombination can be a danger to viral survival. So far, the evidence suggests that novel HCV strains mainly accumulate through genetic drift by collecting viral mutation instead of recombination, as there have only been two reported cases of HCV-6-specific recombination occurring^[67,68]; recombinants RF_2i/6p and RF_2b/6w from a Vietnamese blood donor and an IDU in Taiwan, respectively^[53,69].

METHODS USED FOR GENOTYPING

Since HCV genotype is such an important consideration for predicting an effective treatment regimen, several different genotyping methods have been developed. These methods include direct nucleic acid sequencing^[70], a reverse hybridization line probe assay (LiPA)^[71], subtype-specific reverse transcription (RT)-PCR^[72], DNA restriction fragment length polymorphism^[73], heteroduplex mobility analysis^[74], primer-specific and mispair extension analysis^[75], melting curve analysis with fluorescence resonance energy transfer probes^[76] and serologic genotyping (Figure 4)^[5,77,78].

However, not every method is equal. The Asian Pacific Association for the Study of the Liver states that genotype discrimination based on primers from the 5' untranslated region (5'UTR) do not distinguish some of the HCV-6 subtypes prevalent in Southeast Asia, and that these subtypes should instead be classified as genotype 1 or 1b^[79], available methods generally use distinct motifs

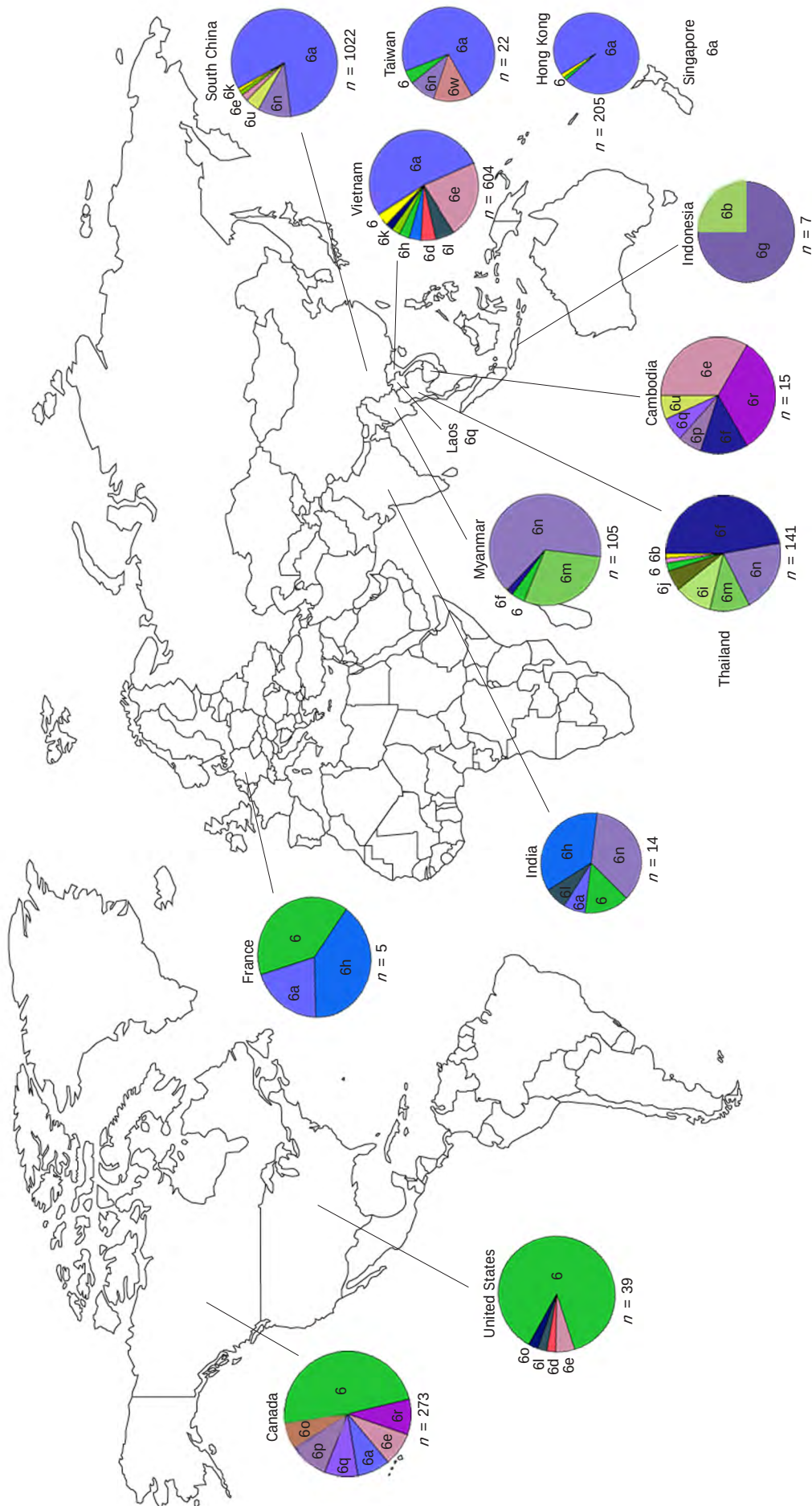


Figure 2 Distribution of the prevalence of subtype of hepatitis C virus-6 in the world [28,40,43,46-48,50-55]

Table 1 Prevalence of hepatitis C virus-6 in Asians *n* (%)

Country	Ref.	Genotype 6 prevalence
Myanmar	Lwin <i>et al</i> ^[46]	1333 (49)
Vietnam (Hanoi, Vietnam)	Pham <i>et al</i> ^[39]	238 (47.1)
Thailand		
(blood donors throughout country)	Kanistanon <i>et al</i> ^[61]	NR (18)
(blood donors in northern Thailand provinces)	Jutavigittum <i>et al</i> ^[62]	326 (31)
(blood donors from central Thailand)	Akkarathamrongsin <i>et al</i> ^[63]	375 (30)
Hong Kong, China (Blood donors)	Leung <i>et al</i> ^[64]	910 (27)

NR: Not reported.

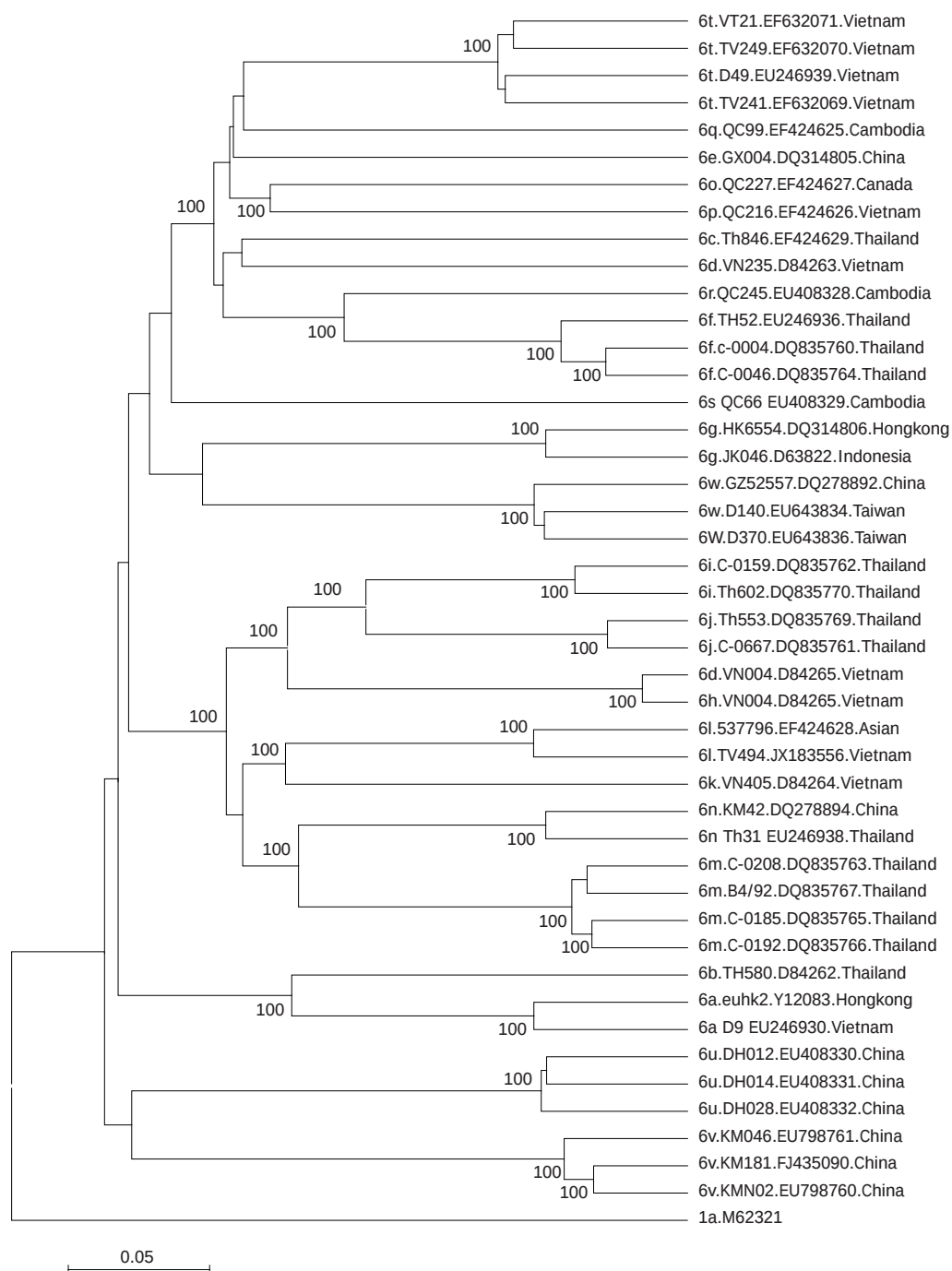
**Figure 3** Phylogenetic relationships among subtypes hepatitis C virus-6. Phylogenetic tree constructed from whole genome sequence of all hepatitis C virus-6 subtypes (subtype 6a to 6w) which was analyzed by K2P model and MEGA version.5. Bootstrap value of 1000 replicates was indicated at each node.



Figure 4 Methods used for genotyping. The 5'-untranslated region (5'-UTR) and Core regions targeted by INNO-LiPA (the 5'-UTR only for INNO-LiPA I or both the 5'-UTR and core regions for INNO-LiPA II), sequencing of NS5B are used the standard methods for classifying hepatitis C virus genotype and subtype^[5,78].

found within the HCV genome for HCV genotype^[5]. In addition, previous studies reported the mistyping of HCV-6 as genotype 1 by the INNO-LiPA I assay (Innogenetics, Zwijnaarde, Belgium)^[80,81]. However, the INNO-LiPA HCV II (Innogenetics, Zwijnaarde, Belgium) genotyping assay seems to overcome the deficiencies demonstrated by the INNO-LiPA I assay, as it has shown remarkable improvement in genotyping accuracy and differentiation between HCV-1 and HCV-6 variants by using core sequencing data as well as 5' UTR data^[82,83].

CLINICAL CHARACTERISTICS

Acute HCV infection is infrequently diagnosed and leads to chronic infection in about 80% of cases^[84]. Clinical manifestations can occur, usually within 7 to 8 wk (range: 2-26 wk) after exposure to HCV, but the majority of persons have either no symptoms or only mild symptoms, and fulminant hepatic failure due to acute HCV infection is very rare. Although clinical features will be present in less than 25% of infected patients, symptoms of acute hepatitis include jaundice, malaise, nausea and right upper quadrant pain^[85]. While the infection becomes chronic in most cases, chronic infection is either asymptomatic or has only mild nonspecific symptoms such as fatigue as long as cirrhosis and hepatocellular carcinoma are not present. Other clinical manifestations are possible, however, such as nausea, weakness, myalgia, arthralgia and weight loss^[86]. Although there have been many papers describing HCV-6's epidemiology, the clinical characteristics have not been well described in those studies. Nguyen *et al.*^[87] reported that patients with HCV-6 presented similar clinical manifestations as those with genotype 1 or 2/3. They also found that people with HCV genotype 1 and 6 had a somewhat higher baseline viral load than those with others genotypes. However, when comparing HCV-6 patients with patients infected with other genotypes, these differences were not statistically significant with regard to host factors (*e.g.*, age, history of smoking, alcohol use, family history of CHC, hepatitis B, hepatocellular carcinoma and liver-related death), baseline laboratory values (*e.g.*, ALT, total bilirubin, albumin, white blood cell count, platelet count), and liver histology^[55,88]. However, steatosis is a chief modulator of clinical course of HCV infection^[89].

TREATMENT

The current standard-of-care for treatment of HCV-infected patients is a combination of PEG-IFN and RBV. Among the various viral and host factors, HCV genotype is one of the most important predictors of response to treatment and is used to guide the duration of treatment. Patients with HCV genotype 1 are typically treated for 48 wk, whereas patients with genotype 2 and 3 are treated for 24 wk^[35,36,90]. Limited studies have suggested that the response rate of HCV-6 may be at an intermediate level between those of genotypes 3 and 1^[87,91-93].

Virological response kinetics during therapy has emerged as an important prognostic factor of treatment outcome in patients with chronic HCV infection^[94,95]. Absence of an early virological response (EVR) at week 12 during therapy is the best negative predictor for non-response to treatment. In contrast, rapid virological response (RVR; defined as undetectable HCV RNA at week 4) is regarded as the most important predictor for SVR (defined as undetectable HCV RNA at week 24 after the end of therapy) and has emerged as an important milestone to guide the appropriate duration of therapy. For example, in patients with genotype 1, an individualized approach to therapy designed according to early viral kinetics has been adopted to optimize therapeutic outcome in patients. Recent clinical trials have used RVR to identify those patients with low baseline viral load that may benefit from shortened treatment duration of 24 wk^[96-98].

TREATMENT OUTCOMES OF HCV-6 VS OTHER GENOTYPES

Currently, although the treatment outcome of patients with HCV-6 has so far not been exhaustively studied, a few studies exist which give hints as to what the standard course of care should be. Most previous studies have reported that genotype 6 behaves more similar to genotypes 2 and 3 (SVR rates of 76%-80%)^[87,99,100] and thus responds better to therapy than genotype 1 (SVR rates of 46%-52%)^[101]. For example, Nguyen *et al.*^[87] demonstrated that patients with HCV-6 had significantly better SVRs to PEG-IFN and RBV combination therapy than patients with genotype 1 (74% *vs* 49%). Furthermore, Tangkijvanich *et al.*^[102]

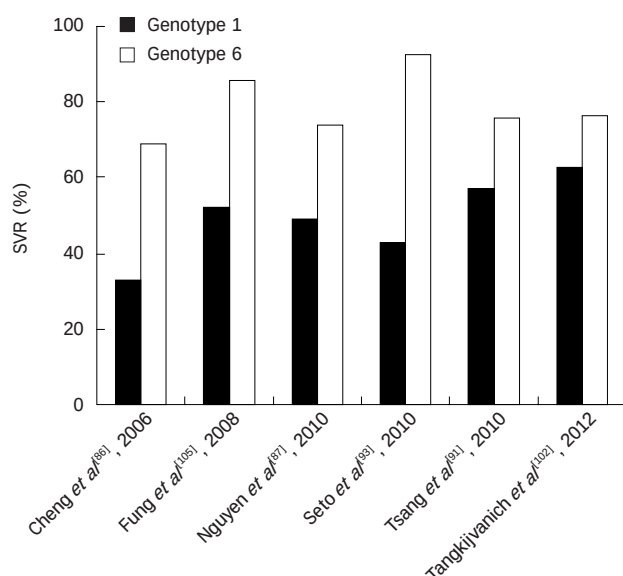


Figure 5 Sustained virological response in genotype 6 vs genotype 1. SVR: Sustained virological response.

reported that the SVR of HCV-6 is higher than that of genotype 1 but lower than that of genotype 3 (Figure 5).

OPTIMAL TREATMENT DURATION OF HCV-6

Most prior studies of HCV-6 included patients treated for 48-52 wk^[103-105]. A small study of Asian-American patients comparing a 48-wk to a shortened 24-wk regimen showed a significantly higher SVR rate in those treated by the 48-wk course (74% *vs* 49%)^[38]. However, the limitation of the study was its retrospective design and the results were not analyzed with regard to an intention-to-treat method. A retrospective study conducted in China showed that the rate of SVR in 22 patients with HCV-6 treated for 24 wk was comparable to that of genotypes 2/3 (82% and 83%, respectively)^[92]. In that study, the positive predictive values of RVR and EVR for HCV-6 were comparable with those for genotypes 2/3 (87% *vs* 91% and 86% *vs* 87%, respectively).

A randomized controlled trial of 60 patients with HCV-6 demonstrated that there was no significant difference in SVR rates in patients treated with 48-wk and 24-wk regimens (79% and 70%, respectively)^[101]. In that study, RVR was a significant predictor of SVR in the 48-wk group and tending towards significance in the 24-wk group, although a sizeable number of patients did not have RVR measurement performed. Recently, Thu Thuy *et al*^[106] conducted an open-label randomized trial in Vietnam, which aimed at assessing the rate of SVR in HCV-6 chronic HCV following 48 and 24 wk of PEG-IFN and RBV combination therapy. They demonstrated that RVR was achieved in the majority of HCV-6 patients and similar and high rates of SVR were noted following 48- and 24-wk therapy (71% *vs* 60%, respectively, $P = 0.24$).

The feasibility of a response-guided therapy by indi-

vidualizing the duration of treatment according to viral kinetics in patients with genotype 6 was first investigated by Fired *et al*^[107]. In that pilot study, more than 70% of patients with HCV-6 achieved RVR and received an abbreviated 24-wk regimen. Among them, the rate of relapse was approximately 10%, and nearly 90% of them eradicated the virus. These data were consistent with observations regarding treatment of HCV genotypes 1, 2, 3 and 4, which suggest that monitoring RVR might be useful to guide treatment duration for patients with HCV-6. In particular, therapy might be shortened to 24 wk in patients with low pretreatment viral load who achieve RVR, whereas a 48-wk course was appropriate for those who cleared the virus after week 4. The abbreviated regimen could offer advantages by reducing unnecessary medication exposure, which may make the treatment of HCV-6 more affordable and maximize the cost effectiveness of therapy. However, further prospective randomized trials are required to evaluate the response-guided strategy in a larger number of patients with HCV-6.

OPTIMAL DOSE OF RBV IN TREATING HCV-6

Although PEG-IFN represents the backbone of treatment, combination with RBV has been shown to help prevent relapse. Current guidelines recommend a weight-adjusted dose of RBV in combination with PEG-IFN for treating patients with genotype 1, while a flat, low dose of RBV (800 mg/d) is recommended for treating patients with genotype 3^[95]. However, a weight-adjusted dose of RBV might be useful to enhance the response rate in patients with genotype 3 who do not achieve RVR and in those with RVR undergoing abbreviated therapy^[108,109]. Currently, the optimal dose of RBV for treatment of patients with HCV-6 is unknown. In previous studies, daily weight-based or fixed doses of RBV had been used, rendering comparisons rather complicated. Nonetheless, recent prospective trials adopted a weight-based dosage of RBV (1000-1200 mg/d) for abbreviated treatment (24 wk), which might result in achieving SVR equivalent to that obtained with longer treatment duration (48 wk)^[101]. These data might reflect the need of a weight-based dosage of RBV in patients with HCV-6 undergoing abbreviated therapy.

SAFETY AND SIDE EFFECT PROFILE OF TREATMENT

Treatment with PEG-IFN and RBV has been shown to be safe in patients with HCV-6, but treatment discontinuation or dose reduction due to side-effects is typical. Although HCV genotypes play a role in achieving SVR, there is no significant difference in the frequency or types of side effects experienced among patients of genotypes 1, 2, 3 or 6^[87,104,105] taking PEG-IFN and RBV, although side effect profiles do appear to differ among patients of

different ethnicities. For example, compared with Caucasians, Asian patients are more likely to decrease their RBV dose or discontinue the therapy due to anemia. In addition, Asian patients reported symptoms of depression, more commonly than Caucasian patients^[110,111]. Other common side effects include flu-like symptoms (fever, fatigue, headache, malaise, and loss of appetite), dyspepsia and some cases with rash, weight loss, arthralgia and alopecia^[110]. However, these symptoms are often mild and tolerable and without the requirement for PEG-IFN and/or RBV dose modification.

PREDICTORS OF SVR

As in studies of patients with other HCV genotypes, pretreatment predictors of response are useful for advising patients on their likelihood of SVR. Pretreatment host and viral characteristics affect early viral kinetics. Once treatment has been initiated, outcome depends on how fast HCV RNA becomes undetectable. Multivariate analyses have identified various predictors of response in HCV-6 such as youth (< 40-50 years)^[100,105], low BMI, treatment adherence and RVR^[35,100,105]. Among these predictors - and concordant with observations in other HCV genotypes - the importance of RVR (undetectable HCV RNA after 4 wk of treatment) in the prediction of SVR has been further substantiated in HCV-6, wherein the positive predictive value to achieve SVR in patients with RVR has been 83%-87%^[87,105].

ROLE OF INTERLEUKIN IL28-B

Recent studies have reported that one of the strongest baseline predictors of SVR in HCV genotype 1 are single-nucleotide polymorphisms (SNPs) on chromosome 19 in or near the interleukin-28B gene (*IL28B*, encoding interferon lambda-3). Following antiviral treatment, patients carrying the CC genotype of one of these predictive SNPs (rs12979860) have a twofold (95%CI: 1.8-2.3) greater rate of SVR than patients who carry the TT alleles (78% for the CC genotype, 38% for the TC genotype, and 26% for the TT genotype). Interestingly, the C allele frequency is much higher in white and Asian populations than in black populations^[112]. More recently, a variant upstream of *IFNL3* creating a novel gene, designated as *IFNL4*, has been discovered^[113]. This region harbors a dinucleotide variant (ss469415590) that is found in two alternative forms (Δ G or TT alleles). The ss469415590 indel is more strongly associated with treatment response of HCV-1 infection in African-American individuals compared to rs12979860^[113].

Data regarding the association of the SNPs with antiviral response in HCV-6 infected patients are still very limited. A recent study from Hong Kong showed that rs8099917, another *IL28B* polymorphism, was associated with response to PEG-IFN/RBV therapy in HCV-6 infected patients^[114]. In that report, the favorable TT genotype of rs8099917, when compared to the unfavorable TG genotype, was significantly associated with an

increased SVR rate (96.2% and 62.5%, respectively) and was the only clinical parameter that predicted SVR.

DAA

In 2011, direct-acting antivirals (telaprevir and boceprevir) were approved by the US Food and Drug Administration for treatment of HCV genotype 1. They are first generation NS3-4A protease inhibitors (PI), targeting the protease enzyme that cleaves the HCV polypeptide thus inhibiting the replication process. The addition of a DAA to PEG-IFN/RBV has reduced treatment duration and side effects, and improve efficacy and cost^[115]. Thus, the development of DAA represents a significant milestone in improving the efficacy of HCV treatment, especially in patients with HCV genotype 1.

Currently, clinical results of the use of DAAs for patients with HCV-6 are limited^[116]. For example, monotherapy with TMC 435, a second-generation NS3/4A PI with pan-genotype antiviral activity, could induce a significant mean viremia decrease of -4.35 log₁₀ UI/mL after 8 days in patients with HCV-6. In addition, five patients with HCV-6 were included in the ATOMIC study and treated with sofosbuvir (GS 7977), a NS5B polymerase inhibitor, plus PEG-IFN/RBV for 24 wk. The RVR rate at week 4 and the SVR rate 12 wk after the end of the treatment were both 100%.

CONCLUSION

Three percent of the world's population is infected with HCV. Of that 3%, HCV-6 accounts for a disproportionately high burden high of prevalence in Southeast Asia and the surrounding areas as well as in infection drug users and people with thalassemia major. Previous literature suggest older tests may have misclassified HCV-6 as genotype 1, but newer line probe assays have shown impressive improvement in genotyping accuracy and differentiation between HCV genotype 1 and HCV-6 variants. Clinical characteristics and predictors of poor response are similar for patients with HCV-6 and other HCV genotypes. Current data suggests that the response rate of HCV-6 may be at an intermediate level between those of genotypes 3 and 1. Thus, the optimal treatment duration of HCV-6 should be 48 wk, although shortened treatment duration of 24 wk could be sufficient in patients with low pretreatment viral load who achieve RVR. In addition, there are currently conflicting data on the role of IL28B testing in predicting treatment response in patients with HCV-6.

Further studies will be required to arrive at a sensitive diagnostic method for HCV-6/subtypes, optimal treatment duration, and early predictors for treatment response and drugs (DAA) to achieve higher SVR rates in patients with HCV-6.

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HEPATOLOGY

Advanced liver fibrosis by transient elastography, Fibrosis 4, and alanine aminotransferase/platelet ratio index among Asian hepatitis C with and without human immunodeficiency virus infection: Role of vitamin D levels

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Key words

APRI, Fib-4, genotype 3, genotype 6, hepatitis C, HIV/hepatitis C co-infection, liver fibrosis, Thailand, vitamin D.

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Abstract

Background and Aim: Vitamin D insufficiency plays an important role in liver fibrosis in hepatitis C virus (HCV)-infected patients. We assessed liver fibrosis by transient elastography and 25 hydroxy vitamin D [25(OH)D] status in HCV-infected patients, with (HIV/HCV) or without HIV co-infection (HCV) from Thailand.

Methods: Fibrosis stage was defined as mild (< 7.1 kPa); moderate (7.2–9.4 kPa); severe (9.5–14 kPa), and cirrhosis (> 14 kPa). Hypovitaminosis D was defined as 25(OH)D < 30 ng/mL. Logistic regression analyses were used to assess predictors for significant fibrosis. Serum 25(OH) D levels, HCV genotypes (GT), interleukin-28B (IL28B) and HCV-RNA were assessed.

Results: A total of 331 HCV and 130 HIV/HCV patients were enrolled (70% male, 35% people who inject drugs [PWIDs]). HCV GT distribution was as follows: GT3 47%, GT1 34%, GT6 17%. IL-28B CC genotype (rs12979860) were found in 88% of HIV/HCV and 85% of HCV. In HCV, liver fibrosis was mild in 56.5%; moderate in 18.4%; severe in 12.4%; and cirrhosis in 12.7%. In HIV/HCV, these figures were 30.6%, 27.8%, 17.6%, and 24.1%, respectively. Patients with significant fibrosis were more often male, older, with HIV infection, hypovitaminosis D, and less likely to be infected with GT6. Factors associated with significant fibrosis by multivariate analysis were HIV infection (adjusted odd ratio [95% confidential interval]: 2.67, 1.20–5.93), $P = 0.016$, Fib-4 score > 1.45 (6.30, 2.70–14.74), $P < 0.001$, and hypovitaminosis D (2.48, 1.09–5.67), $P = 0.031$. GT 6 was less likely to have advanced liver fibrosis (0.17, 0.05–0.65), $P = 0.01$.

Conclusions: HIV infection, Fib-4 score > 1.45, and hypovitaminosis D are strong and independent predictors for the presence of advanced fibrosis in our HCV-infected patients. These data highlight the urgent need of HCV treatment and vitamin D supplement in resource-limited settings.

Introduction

Since the widespread availability of combination antiretroviral therapy (cART), there has been a dramatic decline in HIV/AIDS-related morbidity/mortality, and significant increase in the life expectancies of HIV-infected patients worldwide.¹ However, the improved survival after effective cART has been associated with higher mortality and morbidity rates contributable to chronic

infection with hepatitis C virus (HCV) which is increasing.^{2–4} This is especially problematic for resource-limited settings (RLS), where treatment of HCV is generally not easily accessible. HIV has a negative impact on the natural history of HCV, and compared with HCV mono-infected patients, HIV/HCV co-infected patients have a more rapid progression from chronic-active-hepatitis-to-liver-cirrhosis, end-stage liver disease, liver cancer, and death.⁵

Factors that contribute to the rapid development of liver fibrosis/cirrhosis among HIV/HCV co-infected patients are males, acquiring HCV at an older age, heavy alcohol consumption, low CD4 cell count, HCV genotype 3 and insulin resistance.^{6–12} Studies from HCV genotype 1, HIV/HCV co-infection have found that low serum levels of 25-OH-vitamin D [25(OH)D] are associated with severe liver fibrosis^{13,14} and lower HCV treatment response.^{14,15} However, these data were obtained mostly from resource-rich countries and may not be applicable in Asia where the financial status, behavior, culture, modes of HCV acquisition and HCV genotype distribution are different. Presently, factors associated with liver fibrosis in HIV/HCV co-infection and HCV mono-infection in RLS have not been well-characterized.

Management of HCV-related liver disease is based on staging of liver fibrosis assessed by liver biopsy. This limits the ability to assess the presence of fibrosis in RLS because liver biopsies are not easily accessible. HIV co-infection forms an additional barrier to access to liver biopsy because of widespread HIV stigmatization in many settings in Thailand. Therefore, little is currently known about the prevalence of liver fibrosis in Asia. An alternative non-invasive tool to measure the degree of liver fibrosis is transient elastography (FibroScan, TE, Echosens, Paris, France), which has been validated in both HCV mono-infected and HIV/HCV co-infected patients.¹⁶ This is a promising tool in RLS to assess the presence of liver fibrosis among HCV-infected patients with/without HIV co-infection without the need for liver biopsy.

In the present study, we investigated the prevalence of different stages of liver fibrosis among untreated HCV-infected patients, with/without HIV co-infection. Predictors for the presence of liver fibrosis included serum 25(OH)D levels, HCV genotypes, IL28B and HCV-RNA.

Material and methods

Study design and participants. Chronic HCV (at least two occasions more than 6 months apart) with and without HIV were enrolled from Taksin Hospital, Chulalongkorn Memorial Hospital, and HIV-NAT, Thailand. Study protocol was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University. All patients gave written consent. Basic characteristics included age, sex, risk category for HCV infection and for HIV infection if applicable, history of alcohol use, body weight (BW), body mass index (BMI), history of antiretroviral agents (ARV) use among those co-infected with HIV/HCV, calcium and vitamin D intake, and history of bone fracture. Duration of HCV infection was estimated by using the first reported exposure as the start of infection, e.g. first time the person shared used needles if PWIDs (people who inject drugs), first time of receiving blood products, first time of needle stick injury if health-care workers and/or first time of unprotected sex.

Samples for HCV-RNA, HCV genotyping and IL28B were collected between June 2010 and April 2012. Blood was drawn after 10 h of fasting to assess CD4 count, HIV-RNA, complete blood count, glucose, lipid panels, serum creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), 25(OH)D levels, and HBV. Tests for anti-HCV antibody were repeated using chemiluminescent microparticle immunoassay (CMIA) (Abbott test generation3, Abbott Architect ci4100, Wiesbaden, Germany).

HCV viral load testing. Quantification of HCV-RNA was performed by real-time polymerase chain reaction assay (Abbott Molecular Inc. Des Plaines, IL, USA). Lower (LLD) and upper detection limits (ULD) of the assay were < 12 IU/mL and 100 000 000 IU/mL, respectively. Specimens yielding values above ULD were diluted 100-fold and retested.

HCV Genotyping and IL28B polymorphism. HCV-RNA was reversed by random primer and viral 5'UTR was amplified. Positive 5'UTR were then subjected for further amplification with primers specific for the core and NS5B regions as described previously.¹⁷ Viral genotypes were classified based on the nucleotide sequence using BLAST search and viral genotyping tool (<http://www.ncbi.nlm.nih.gov/>). Human DNA was extracted from PBMCs/sera and used to detect IL28B polymorphism. PCR and direct sequencing were used to detect rs12979860. Genotype rs12979860 was analyzed directly from the chromatogram of the nucleotide sequences.

Assessment of vitamin D levels. Whole blood samples were centrifuged at 2500 r.p.m. for 20 min and plasma was stored at -80°C until use. ARCHITECT CMIA was used to quantitate the levels of 25(OH)D according to the manufacturer's instructions (Abbott, Barcelona, Spain). Hypovitaminosis D was defined as having serum levels of 25(OH)D < 30 ng/mL. Imprecision of the ARCHITECT 25-OH Vitamin D assay is < 10% but within laboratory (total) coefficient variation.

Assessment of liver fibrosis and cirrhosis. Liver stiffness was assessed by transient elastography (TE). All TE were performed by a single-blinded TE specialist to avoid interindividual variation and standard criteria with FS validation.¹⁸ AST/platelet ratio index (APRI) score was calculated by using Wai *et al.*'s formula:¹⁹ (AST/upper limit of normal considered as 40 IU/L)/platelet count $\times 10^9/\text{L}$. Fibrosis 4 Score (Fib-4) was calculated by using Sterling *et al.*'s formula:²⁰ (age $\times$ AST)/[(platelet count $\times$ ALT)^{1/2}].

Fibrosis stage was defined according to fibrosis score as measured by TE: mild (equivalent to Metavir F0–F1), ≤ 7.1 kPa; moderate (F2), 7.2–9.4 kPa; severe (F3), 9.5–14 kPa; and cirrhosis (F4), > 14 kPa. Furthermore, advanced liver fibrosis (F3 and F4) was defined as a fibrosis score of > 9.5 kPa. Cirrhosis was diagnosed based on definitive clinical evidence, fibrosis score > 14 kPa, and/or imaging evidence by abdominal sonogram of the liver showing cirrhosis/splenomegaly with portal hypertension.

Statistical analysis. Analysis was performed by Stata version 12.1 (Stata Corp., College Station, TX, USA). For descriptive analysis, frequencies of categorical variable were calculated. Median and interquartile range (IQR) were calculated for continuous variables. Each variable was correlated to vitamin D status and advanced liver fibrosis. Magnitude of associations was expressed as odds ratio (OR) and 95% confidence intervals (95% CI). Categorical variables were analyzed by using the Pearson's chi square test/Fisher's exact test, as appropriate. Continuous variable was analyzed using Student's *t*-test. Pearson correlation coefficient was used to explore the association between serum vitamin D levels and advanced liver fibrosis. For multivariate analysis, mul-

multiple logistic regression analysis was used. Variables presenting $P < 0.1$ in univariate analysis were tested in the final model.

Results

Characteristics of study population (Table 1). A total of 130 untreated HIV/HCV co-infected and 331 untreated HCV mono-infected patients were enrolled. None of them reported fracture nor taking calcium/vitamin D. Majority were males (70%) and former PWIDs. The HCV mono-infected group had a higher proportion of patients > 50 years (34% vs 18%), longer estimated duration of HCV infection (17 years vs 15 years), and higher proportion of alcohol consumption (77% vs 39%). Median 25(OH)D levels were comparable between the HCV mono-infected and HIV/HCV co-infected patients. The prevalence of vitamin D insufficiency [25(OH)D 20–30 ng/mL] and deficiency [25(OH)D < 20 ng/mL] among mono-infected patients was 53.9% and 8.9%, respectively, thus the prevalence of hypovitaminosis D was 62.8%. The prevalence of vitamin D insufficiency and deficiency among co-infected patients was 47.9% and 13.7%, respectively (hypovitaminosis D 61.6%).

HCV genotype (GT) and IL28B polymorphism. The most prevalent circulating genotype was HCV GT3 (47%), followed by GT1 (34%) and GT6 (17%). HIV/HCV co-infected patients more often had plasma HCV-RNA $> 800\,000$ IU/mL (54% vs 43%).

IL28B at rs12979860 position was available for 94 HIV/HCV and 136 HCV mono-infected patients. The major allele (CC genotypes) of rs12979860 position was found in 88% of HIV/HCV and 85% HCV mono-infected patients. Only 1% of HIV/HCV and 4% of HCV mono were minor TT allele.

HIV parameters for HIV/HCV co-infected patients.

The median CD4 cell count at time of TE was 494 cells/mm³ (IQR 310–625 cells/mm³), only 16 (12.5%) of patients had a CD4 cell count of < 200 cells/mm³. cART was used by 91% of patients. Median duration of cART was 7 years (IQR 5–11). Protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) were used by 36.4% and 59.3%, respectively, and 78% of those on ART had HIV-RNA < 50 copies/mL. Stavudine was used in 77 (59.2 %) patients with the median duration of 33 months (IQR 15–56).

Liver fibrosis and its fibrosis markers based on the transient elastography results.

Median ALT for HIV/HCV co-infected and HCV mono-infected patients were 71 IU/L (IQR 43–112) and 60 IU/L (IQR 31–97), respectively ($P = 0.004$). Figure 1 depicts the distribution of liver fibrosis staging. A greater proportion of the participants from the HIV/HCV co-infected group had advanced liver fibrosis (41.7% vs 25.1%). Also, 24.1% of the participants from the HIV/HCV co-infected group had cirrhosis based on the fibrosis score > 14 kPa, compared with 12.7% in the HCV mono-infected group. The proportion of patients with Fib-4 score > 3.25 and APRI score > 1.5 were significantly greater in HIV/HCV co-infection (21.6% vs 9.1% and 27.2% vs 10.8%, respectively).

Comparisons of HCV genotypes 6 to 1 and 3 (Table 2).

There were no differences in the median age, sex, duration of HCV infection, duration of HIV infection, or transmission route between genotypes. However, patients with HCV GT3 had a significantly higher median ALT (77 IU/L) compared with those with GT1 (61 IU/L) and GT6 (51 IU/L), $P < 0.001$. Only 23% of the participants with HCV GT3 had normal ALT levels. HCV-RNA was significantly higher in GT6 (6.6 log₁₀ IU/mL) compared with GT1 and GT3 (5.9 log₁₀ IU/mL), $P = 0.01$. Furthermore, participants with HCV GT3 had higher median fibrosis scores. In contrast, fewer participants with HCV GT6 had TE > 7.1 kPa (39.7%) compared with GT3 (57.9%) and GT1 (47.5%).

Factors associated with advanced liver fibrosis (metavir F3-F4) (Table 3).

After adjustment in the multivariate analysis, HIV co-infection (aOR 2.67 [95% CI 1.20–5.93], $P = 0.016$), Fib-4 score > 1.45 (aOR 6.30 [95% CI 2.70–14.74], $P < 0.001$), and plasma 25(OH)D level of < 30 ng/mL (aOR 2.48 [95% CI 1.09–5.67], $P = 0.031$) remained significantly associated with advanced liver fibrosis. HCV GT6 was associated with a decreased risk of liver fibrosis (aOR 0.17 [95% CI 0.05–0.65], $P = 0.01$).

In HIV/HCV co-infected patients (Table 4), HIV-related parameters such as CD4 cell count, HIV-RNA, cART use, and duration of ART were not associated with advanced liver fibrosis whereas d4T exposure and 25(OH)D were strongly correlated with advanced liver fibrosis. Importantly, the correlation for 25(OH)D < 30 mg/dL and liver fibrosis was seen at milder levels of liver fibrosis in HIV/HCV co-infection but not in HCV mono-infection (TE > 7.5 kPa vs TE > 9.5 kPa). For the multivariate analysis, d4T exposure (aOR 4.92 [95% CI 1.44–16.8; $P = 0.011$]), Fib-4 score > 1.45 (aOR 5.29 [95% CI 1.52–18.35], $P = 0.009$), and 25(OH)D < 30 mg/dL (aOR 5.04 [95% CI 1.44–17.6; $P = 0.011$]) were strongly correlated with significant liver fibrosis (TE > 7.5 kPa). In addition, from the multivariate analysis, only liver fibrosis > 7.5 kPa, not ARV, was associated with 25(OH)D < 30 mg/dL (OR 3.75 [95% CI 1.22–11.55]; $P = 0.021$).

To further investigate the nature of the relationship between Fib-4 and APRI and the development of advanced liver fibrosis, we examined the correlation between Fib-4, APRI and liver stiffness which revealed positive correlation between liver stiffness and Fib-4 [Pearson correlation (r) = 0.50, $P < 0.001$] and APRI ($r = 0.49$, $P < 0.001$).

A subanalysis was performed including patients who had IL28B testing. IL28B polymorphism was not found to be a predictor for liver fibrosis [rs 12979860 CC: OR 1.27 (95% CI 0.47–3.42, $P = 0.639$)].

Discussion

In this analysis of 130 untreated HIV/HCV and 331 untreated HCV mono-infected patients using TE to measure degree of liver fibrosis, the primary finding was that people co-infected with HCV and HIV have a high prevalence of significant liver fibrosis. HIV co-infected patients were 2.67 times more likely to have advanced liver fibrosis (TE > 9.5 kPa) compared with HCV mono-infected patients. Almost 70% of our HIV/HCV co-infected patients had significant liver fibrosis (TE > 7.1 kPa) and 41.6% had advanced liver fibrosis with TE > 9.5 kPa.

Table 1 Demographic and clinical features of the study participants: 130 HIV/HCV co-infected and 331 HCV mono-infected participants

Characteristics	Total (N = 461)	HIV/HCV (N = 130)	HCV mono (N = 331)	P-value
Sex, N (%)				< 0.001
Male	323 (70.1)	111 (85.4)	212 (64.1)	
Age, years	43 (36–52)	42 (37–48)	44 (36–53)	0.364
Age ≥ 50 years, N (%)	136 (29.50)	24 (18.46)	112 (33.84)	0.001
Duration of HCV exposure, years	17 (10–24)	15 (10–21)	17.5 (10–25)	0.042
Median (IQR) body mass index (BMI), kg/m ²	23.2 (20.8–25.6)	21.6 (19.4–23.8)	23.9 (21.5–26.0)	< 0.001
BMI > 25 kg/m ² , N (%)	116 (29.3)	19 (15.8)	97 (35.1)	< 0.001
HCV risk factors				< 0.001
Heterosexual	25 (5.73)	25 (19.23)	5 (1.63)	
MSM	17 (3.90)	17 (13.08)	0 (0.0)	
IV drug use	154 (35.32)	81 (62.31)	73 (23.86)	
Blood transfusion	91 (20.87)	4 (3.08)	87 (28.43)	
TAS	64 (14.68)	1 (0.77)	63 (20.59)	
Unknown	80 (18.35)	2 (1.54)	78 (25.49)	
Alcohol consumption, N (%)	273 (66)	44 (39)	229 (77)	< 0.001
Current ALT (IU/L)	62 (35–100)	71 (43–112)	60 (31–97)	0.004
Plasma HCV-RNA, log ₁₀ IU/mL [†]	5.9 (5.4–6.6)	6.0 (4.9–6.6)	5.8 (5.6, 6.5)	0.829
Plasma HCV-RNA levels of > 800,000 IU/mL, N (%)	118 (49.2)	70 (54.3)	48 (43.2)	< 0.001
HCV genotype [‡]				
1	128 (34)	32 (31.7)	96 (34.9)	
2	2 (0.5)	0	2 (0.7)	
3	176 (46.8)	50 (49.5)	126 (45.8)	
5	1 (0.3)	0	1 (0.4)	
6	63 (16.8)	13 (12.9)	50 (18.2)	
1 + 3	5 (1.3)	5 (4.9)	0	
1 + 2	1 (0.3)	1 (0.9)	0	
FibroScan, [§] kPa	7.1 (5.2–10.5)	8.5 (6.4–13.85)	6.6 (4.9–9.5)	< 0.001
Fib-4 score, median (IQR) [¶]	1.3 (0.8–2.1)	1.7 (1.0–3.1)	1.1 (0.8–1.9)	0.001
< 1.45, N (%)	241 (57.2)	54 (43.2)	187 (63.2)	< 0.001
1.45–3.25, N (%)	126 (29.9)	44 (35.2)	82 (27.7)	
> 3.25, N (%)	54 (12.8)	27 (21.6)	27 (9.1)	
APRI score, median (IQR) [¶]	0.6 (0.3–1.1)	0.8 (0.4–1.6)	0.5 (0.3–1.0)	< 0.001
< 0.5, N (%)	188 (44.7)	38 (30.4)	150 (50.7)	< 0.001
0.5–1.5, N (%)	167 (39.7)	53 (42.4)	114 (38.5)	
> 1.5, N (%)	66 (15.7)	34 (27.2)	32 (10.8)	
25(OH)D levels, ^{††} ng/mL, median (IQR)	27.4 (22.9–33.6)	27 (22.4–34.3)	27.5 (23.2–33.4)	0.961
> 30 ng/mL, N (%)	112 (37.7)	45 (38.5)	67 (37.2)	
20–30 ng/mL, N (%)	153 (51.5)	56 (47.9)	97 (53.9)	
< 20 ng/mL	32 (10.8)	16 (13.7)	16 (8.9)	
Median (IQR) current CD4 cell counts, cells/mm ³		494 (310–625)		
HIV RNA < 50 copies/mL, N (%)		100 (78.1)		
Current antiretroviral therapy, N (%)		118 (90.8)		
None		12 (9.2)		
2 NRTIs and NNRTI		70 (59.3)		
2 NRTIs and boosted PI		43 (36.4)		
Other		5 (4.2)		

Note: Median and interquartile range (IQR) is presented, unless otherwise indicated. APRI = [(AST/ULN) × 100]/Platelets count 10⁹/L (ULN = the upper limit of normal); Fib-4 = [age (years) × AST (IU/L)]/[platelet count (10⁹/L) × ALT (IU/L)]^{1/2}.

[†]HCV-RNA was available for 129 HCV/HIV co-infected and 111 HCV mono-infected participants.

[‡]HCV genotype was available for 101 HCV/HIV co-infected and 275 HCV mono-infected participants.

[§]FibroScan was available for 108 HIV/HCV co-infected and 331 HCV mono-infected participants.

[¶]Fib-4 and APRI were available for 125HIV/HCV co-infected and 296 HCV mono-infected participants.

^{††}Vitamin D levels were available for 117HIV/HCV co-infected and 180 HCV mono-infected participants.

ALT, alanine aminotransferase; APRI, AST/platelet ratio index; IV, intravenous; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men having sex with men; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; TAS, tattoo, acupuncture, sharing of contaminated needles.

To our knowledge, this study is the first of its kind to report the prevalence of liver fibrosis among HCV-infected Asians with/without HIV co-infection. Even though the participants from the HCV mono-infected group had a longer duration of expected HCV infection (17.5 years vs 15 years) and had a higher proportion of patients over 50 years old (34% vs 18%). The prevalence of advanced liver fibrosis was higher among the HIV/HCV co-infected group (41.7% vs 25.1%). Only 12.7% of the participants from the HCV mono-infected group had cirrhosis, whereas

in the HIV/HCV co-infected group, 24.1% had cirrhosis. These findings are consistent with previous studies that the prevalence of liver fibrosis is much higher in individuals co-infected with HCV and HIV.^{6,21,22} The prevalence of cirrhosis in our HIV/HCV co-infected population (24.1%) was higher than Europe (13%)²¹ and Brazil (8.5%).²³ Given the significant risk for hepatocellular carcinoma (HCC) development in this population, our findings highlight the urgent need for HCV treatment and HCC screening for patients in RLS. Given that 47% of the HCV patients in our patients have HCV GT3 and 90% of them have IL28B rs12979860 CC allele, they are good candidates for HCV treatment with pegylated interferon alfa (PegIFN) and ribavirin.

Our findings disagree with prior studies that patients infected with HCV GT1 tend to have higher plasma HCV-RNA levels than those infected with HCV GT3.^{24,25} In fact, we found that patients infected with HCV GT6 had higher HCV-RNA than those infected with GT1 and GT3. Although patients infected with HCV GT6 had higher median plasma HCV-RNA and a greater proportion of plasma HCV-RNA > 800,000 IU/mL, they had lower median fibrosis score by TE and a lower proportion of patients with advanced liver fibrosis compared with GT3 and 1. By multivariate analysis, HCV GT3 was only marginally significantly associated with advanced fibrosis. In a cross-sectional study from France and Spain, of 314 liver biopsy specimens from HCV mono-infected patients²⁶ and 283 HIV/HCV co-infected patients by TE,¹² the association between HCV GT3 and advanced liver fibrosis was

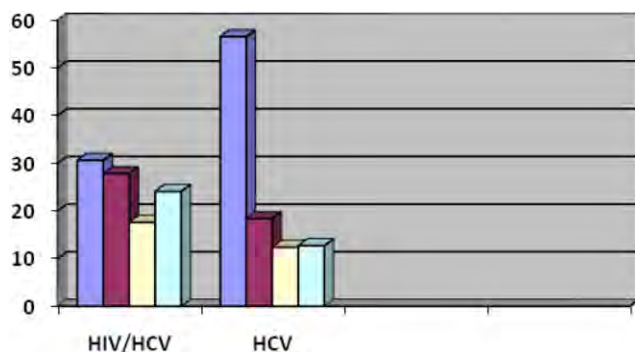


Figure 1 Distribution of liver fibrosis staging among HIV/HCV and HCV mono-infected patients. ■, F1 (< 7.1 kPa); ■, F2 (7.2–9.4 kPa); ■, F3 (9.5–14 kPa); ■, F4 (> 14 kPa).

Table 2 Hepatitis C (HCV) genotypes 1, 3 and 6 are compared with each other as well as to the study participants' characteristics

Characteristics	Total (n = 367)	HCV genotypes, N (%)			P-value
		Genotype 1 (n = 128)	Genotype 3 (n = 176)	Genotype 6 (n = 63)	
Age, years	44 (37–52)	44 (37–52)	44 (37–52)	46 (36–53)	0.987
Median (IQR)					
Male	261 (71.12)	87 (67.97)	130 (73.86)	44 (69.84)	0.518
MSM	14 (4.08)	6 (4.96)	7 (4.19)	1 (1.82)	0.618
History of intravenous drug use	120 (34.99)	36 (29.75)	66 (39.52)	18 (32.73)	0.213
Duration of HCV infection, years	18 (12–25)	16 (10–24)	19 (13–25)	20 (10–25)	0.262
HIV co-infection	95 (25.89)	32 (25.00)	50 (28.41)	13 (20.63)	0.463
HCV-RNA, log ₁₀ IU/mL	6.0 (5.7–6.6)	5.9 (5.7–6.4)	5.9 (5.5–6.6)	6.6 (5.9–6.8)	0.010
Median (IQR)					
ALT, IU/L	66 (42–108)	61 (37–100)	77 (53–114)	51 (32–83)	< 0.001
Median (IQR)					
Normal	109 (32.15)	43 (36.13)	37 (22.84)	29 (50.00)	< 0.001
Abnormal	230 (67.85)	76 (63.87)	125 (77.16)	29 (50.00)	
Platelet count < 100,000/mm ³	22 (6.57)	6 (5.13)	14 (8.75)	2 (3.45)	0.278
Fib-4 score	1.36 (0.88–2.24)	1.35 (0.8–2.16)	1.49 (0.97–2.49)	1.11 (0.86–1.68)	0.034
Median (IQR)					
< 1.45	179 (54.08)	64 (55.17)	77 (48.73)	38 (66.67)	0.064
> 1.45	152 (45.92)	52 (44.83)	81 (51.27)	19 (33.33)	
APRI score					
Median (IQR)	0.63 (0.38–1.18)	0.59 (0.31–1.12)	0.75 (0.5–1.4)	0.42 (0.33–0.85)	< 0.001
< 0.5	128 (38.67)	52 (44.83)	44 (27.85)	32 (56.14)	< 0.001
> 0.5	203 (61.33)	64 (55.17)	114 (72.15)	25 (43.86)	
Fibroscan, kPa					
Median (IQR)	7.6 (5.4–10.95)	7.2 (5.4–11.4)	8.1 (5.6–11.6)	6.5 (5.2–8.4)	0.008
< 7.1	174 (48.88)	64 (52.46)	72 (42.11)	38 (60.32)	0.029
≥ 7.1	182 (51.12)	58 (47.54)	99 (57.89)	25 (39.68)	

ALT, alanine aminotransferase; APRI, AST/platelet ratio index; IQR, interquartile range; MSM, men having sex with men.

Table 3 Univariate and multivariate logistic regression analyses of the factors associated with advanced liver fibrosis (TE > 9.5 kPa)

Variables	Univariate			Multivariate		
	OR	95% CI	P	aOR	95% CI	P
Male gender	1.69	(1.05,2.72)	0.032	1.01	(0.40–2.52)	0.988
Current age of ≥ 50 years	2.96	(1.91,4.6)	< 0.001	—	—	—
Age by 10 years	1.83	(1.46–2.29)	< 0.001	1.51	(0.88–2.58)	0.133
BMI > 25 kg/m ²	1.75	(1.09–2.80)	0.020	2.25	(0.89–5.72)	0.087
HCV-RNA of > 100,000 IU/mL	2.03	(0.94,4.38)	0.072	—	—	—
HCV-RNA of > 800,000 IU/mL	1.12	(0.64–1.95)	0.685	—	—	—
Current abnormal ALT	4.58	(2.68–7.80)	< 0.001	—	—	—
HIV co-infection	2.12	(1.34, 3.36)	0.001	2.67	(1.20–5.93)	0.016
HCV genotype	—	—	< 0.001	—	—	0.010
Genotype 3	1.32	(0.81, 2.16)	—	0.96	(0.44–2.10)	—
Genotype 6	0.32	(0.14, 0.74)	—	0.17	(0.05–0.65)	—
Fib-4 score of > 1.45	8.51	(5.15–14.07)	< 0.001	6.30	(2.70–14.74)	< 0.001
APRI score of > 0.5	10.31	(5.62–18.89)	< 0.001	—	—	—
Estimated duration of HCV infection of > 20 years	1.93	(1.21–3.07)	0.006	1.10	(0.47–2.60)	0.828
Current alcohol consumption	0.86	(0.54–1.35)	0.510	—	—	—
Hypovitaminosis D [25(OH)D < 30 (ng/mL)]	1.78	(1.04–3.06)	0.037	2.48	(1.09–5.67)	0.031
IL28 B						
rs12979860 CC allele	1.4	(0.58–3.37)	0.045	—	—	—

Calculations for Fib-4 and APRI scores are described in the Materials and Methods section.

95% CI, 95% confidence interval; aOR, adjusted odds ratio; APRI, AST/platelet ratio index; HCV, hepatitis C virus; HIV, human immunodeficiency virus; OR, odds ratio.

Table 4 Univariate and multivariate logistic regression analysis of the factors associated with advanced liver fibrosis (TE ≥ 7.5 kPa) only HIV/HCV co-infected patients

Variables	Univariate			Multivariate		
	OR	95%CI	P	aOR	95% CI	P
Male gender	1.24	(0.41–3.73)	0.702	—	—	—
Age increased by 10 years	1.48	(0.81–2.71)	0.208	—	—	—
BMI > 25 kg/m ²	3.88	(0.82–18.23)	0.086	1.34	(0.17–10.84)	0.782
HCV-RNA of > 800,000 IU/mL	0.95	(0.42–2.11)	0.892	—	—	—
Current abnormal ALT	3.50	(1.48–8.25)	0.004	—	—	—
HCV genotype	1.37	(0.47–4.03)	0.335	1.59	(0.42–6.03)	0.419
Genotype 3						
Genotype6	0.52	(0.13–2.04)	—	0.53	(0.09–2.99)	—
Fib-4 score of > 1.45	4.55	(1.92–10.76)	0.001	5.29	(1.52–18.35)	0.009
APRI score of > 0.5	5.18	(2.15–12.47)	< 0.001	—	—	—
Estimated duration of HCV infection of > 20 years	1.30	(0.54–3.13)	0.558	—	—	—
Current alcohol consumption	1.11	(0.48–2.58)	0.800	—	—	—
Hypovitaminosis D [25(OH)D < 30 (ng/mL)]	2.00	(0.88–4.53)	0.096	5.04	(1.44–17.62)	0.011
rs12979860 CC allele	1.38	(0.57–3.33)	0.468	—	—	—
ART Regimen	—	—	0.494	—	—	—
Efavirenz or nevirapine	Ref	1	—	—	—	—
Protease inhibitor	0.96	(0.4–2.28)	—	—	—	—
Stavudine exposure	2.66	(1.17–6.07)	0.020	4.92	(1.44–16.79)	0.011
Didanosine exposure	0.87	(0.35–2.14)	0.756	—	—	—
Indinavir exposure	0.54	(0.17–1.74)	0.301	—	—	—
CD4 ≤ 500 cells/mm ³	0.66	(0.29–1.47)	0.306	—	—	—
HIV-RNA < 50 copies/mL	2.61	(0.95–7.16)	0.063	2.01	(0.45–9.03)	0.362

95% CI, 95% confidence interval; aOR, adjusted odds ratio; APRI, AST/platelet ratio index; ART, antiretroviral therapy; BMI, body mass index; HCV, hepatitis C virus; HIV, human immunodeficiency virus; OR, odds ratio.

highly significant. Surprisingly, we found that patients with HCV GT6 were less likely to have liver fibrosis compared with infection with HCV GT1 and 3. To our knowledge, we are the first to compare HCV-RNA and liver fibrosis between patients infected with HCV GT6 to those infected with GT1 or 3. Data from Caucasians indicate that the progression rate for fibrosis is much faster among patients infected with HCV GT3 compared with those with HCV GT1.^{12,26} However, data from our cohort indicate that HCV GT3 was not significantly correlated with liver fibrosis compared with GT1. Our finding is similar to another HCV study from India where HCV GT3 is the most circulating genotype (75%).²⁷ Hissar *et al.* reported that GT3 was not correlated with liver fibrosis.²⁷ Therefore, it is highly likely that host genetic factors may play an important role in liver fibrosis. Host genetic factors among HCV GT3 Asians and Caucasians should be further explored.²⁷

Although ALT elevations are not a good marker of liver fibrosis especially in HIV co-infected patients, in this study we found that Fib-4 score was strongly correlated with advanced liver fibrosis. Participants with Fib-4 score of > 1.45 had a sevenfold increased risk of having advanced liver fibrosis compared with those with Fib-4 score of < 1.45. Fib-4 is cheap and very easy to perform, requiring only levels of ALT, AST, platelet and age. No additional tests, machines, or skills are required besides laboratory tests routinely given twice a year. From this observation, Fib-4 can be used as a surrogate marker to detect advanced liver fibrosis, especially in RLS where liver biopsy and FibroScan machine are not widely accessible as well as a tool for monitoring liver disease progression.^{28,29}

In this study, patients with hypovitaminosis D(25(OH)D levels < 30 ng/mL) were almost three times more likely to have advanced liver fibrosis. Recently, low serum 25(OH)D levels have been reported in patients with chronic HCV GT1 and were found to be associated with severe liver fibrosis.^{13,14} Vitamin D plays a major role for calcium homeostasis and bone health, but vitamin D is now also widely recognized as a critical factor involved in the immune system, inflammatory response, and fibrogenesis.³⁰ Recent studies have found that low serum levels of 25(OH)D are also associated with low sustained virological response (SVR) to Peg-IFN/ribavirin therapy.^{14,15} Furthermore, vitamin D supplementation improves early virological response (EVR) (94% vs 48%) and SVR (86% vs 42%) in HCV GT1 treated with Peg-IFN/ribavirin.³¹ Therefore, hypovitaminosis D in our HCV population has clinical implication both for liver fibrosis progression and for treatment efficacy. Ensuring adequate vitamin D levels in this population is important.

Almost of our HIV/HCV co-infected patients were on cART, with a median duration of 7 years. Furthermore, only 13% had current CD4 cell counts of 200 cells/mm³. We did not find any association between several HIV-related factors, such as current or nadir CD4 count, HIV-RNA or duration of cART, and the presence of liver fibrosis. This finding is inconsistent with previous studies that showed advanced liver disease progression among HIV/HCV co-infected patients with current CD4 < 500 cells/mm³, and nadir CD4 of < 250 cells/mm³.^{12,32} We found only d4T exposure was positively correlated (sixfold increased) with advanced liver fibrosis, which might be explained by mitochondrial toxicity and/or insulin resistance. The data on the effect of cART on liver fibrosis is conflicting. Macias *et al.* showed that the use of PIs was able to slow down the progression of the liver disease.³³ Moreover, a

recent report on the use of didanosine has been shown to be associated with liver fibrosis,³⁴ whereas another recent publication from the EuroSida group failed to support whether ART could ameliorate the progression of liver disease.²¹

Our study had some limitations. First, we did not perform liver biopsy for the assessment of the presence of liver fibrosis. However, TE has already been validated for both HIV/HCV and HCV mono-infected Caucasian patients in several studies. The positive predictive value of TE for diagnosis of advanced liver fibrosis has been as high as 95%.²⁸ In addition, when we combined the APRI, Fib-4 and TE together, the result of advanced fibrosis was the same. Therefore the combined use of TE and Fib-4 or APRI to evaluate advanced liver fibrosis could avoid a liver biopsy. Second, this was a cross-sectional study that precluded analysis of the progression of liver fibrosis. In order to see the trend of liver fibrosis using the TE, longitudinal studies are recommended. Third, data on bone mass density, parathyroid hormone, calcium and phosphate were not available. Therefore it is unclear whether low levels of vitamin D had any effect on the bones or any calcium homeostasis in this population or not. Lastly, because insulin resistance has not been investigated in our study population, we were not able to confirm the positive association between insulin resistance and advanced liver fibrosis, especially in HIV/HCV co-infected patients with the median of 8 years of cART.s

Regardless of these limitations, the findings from this study did have several important clinical implications. Our results underline the need for HCV treatment in chronically infected patients, especially for those co-infected with HIV. In this study, most of our HIV/HCV co-infected participants were not on HCV therapy and 70% had significant liver fibrosis at a median age of 40 years. Without the availability of HCV treatment, these patients will develop end-stage liver disease within the next decade. In addition, the majority of our patients had HCV non-GT1, along with favorable IL28B (89% CC genotype), and would therefore be good candidates for the treatment with Peg IFN/ribavirin.

In conclusion, advanced liver fibrosis is seen in 41.7% and 25.1% of HIV/HCV co-infected and HCV mono-infected patients from Thailand, respectively. HCV-RNA tends to be higher among patients infected with HCV GT6, but advanced liver fibrosis is seen less often in this group, compared with those infected with GT1 or GT3. Strong and independent predictors for the presence of advanced fibrosis were HIV infection, Fib-4 score > 1.45, d4T use and hypovitaminosis D. These data highlight the urgent need of HCV treatment and HCC screening for HCV patients in RLS. In addition, the role of vitamin D supplementation should be further explored as a relatively cheap therapeutic option to reduce liver fibrosis and improve SVR.

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Authors' contribution

All authors contributed to the study design. AA drafted the manuscript and conducted the study. TA performed all statistical analyses. SA performed all laboratory experiments for genotyping and polymorphisms. RAR, PT, SJ, OP, and ST conducted the study at their perspective sites, helped collect data and necessary specimens. YP, GVM, JMAL and KR provided feedback and finalized the manuscript.

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Risk factors and molecular characterization of acute sporadic symptomatic hepatitis E virus infection in Thailand

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ABSTRACT

Objective: To report clinical outcomes and viral genotypes of acute symptomatic hepatitis E virus (HEV) infection in Thailand. **Methods:** Forty patients with acute symptomatic HEV infection were recruited during 2009–2013. Clinical, demographic and laboratory data were collected. Diagnosis was accomplished by detection of anti-HEV IgM and/or HEV RNA in the serum or stool. HEV genotypes were classified by direct sequencing of RT-PCR products and phylogenetic analysis. **Results:** The high risk group, comprising immune-compromised, liver cirrhosis and very elderly (>80 years) patients (17 cases), had higher levels of serum alkaline phosphatase at presentation compared with the low risk group. Two fatal cases resulted from acute hepatitis E in the high risk group. Initial clinical presentation did not show statistically significant differences. In six cases (6/40), the virus could be detected in serum or stool by RT-PCR and sequencing. Upon molecular characterization, the viruses were classified as HEV genotype 3f and were in the same cluster as Thai swine HEV. **Conclusions:** Our data showed that acute HEV infection has various clinical presentations and outcomes. Higher levels of serum alkaline phosphatase were observed in high risk patients. All isolated viruses were identified as HEV genotype 3f possibly originating from swine.

1. Introduction

Hepatitis E virus (HEV), a member of the genus *Hepevirus* in the family *Hepeviridae* is a small RNA virus[1]. HEV infection occurs in mammals, including humans, domestic pigs, wild boar, deer and rodents. HEV has been classified

into four major genotypes by molecular characterization and HEV genotype 3 has been identified almost worldwide[2]. HEV is an emerging pathogen worldwide including Asia and the Far East[4]. In Thailand, seroprevalence of HEV ranges from 9% to 22% in adult subjects and increases with age[4]. This prevalence is comparable with the data from east Asia[5]. In occupational risk groups such as pig and poultry farmers who have worked in farms for more than 2 years, HEV prevalence amounted to 27.9%[6]. HEV has been known to be transmitted via contaminated water in endemic areas and recent data have demonstrated animal to human transmission in almost every part of the world[7–9].

In developed countries, hepatitis E infection is more common than previously recognized and might be more common than hepatitis A[10]. The majority of patients with acute HEV infection remain asymptomatic[11].

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Clinical manifestations of acute hepatitis E can range from asymptomatic to fulminant with ensuing liver failure[12]. The risk of acute or subacute liver failure from acute HEV infection is exacerbated by pre-existing chronic liver disease[13]. In case of liver cirrhosis, HEV infection can induce rapid liver decompensation and death[14]. In contrast to generally healthy individuals, HEV infection can become chronic in post organ transplant patients[15]. Extra-hepatic presentations, for example, as previously reported neurological symptoms such as aseptic meningitis and neuralgic amyotrophy are unusual[16].

Acute HEV infection can be detected by laboratory tests for anti-HEV IgM with sensitivities and specificities of close to 90% and 100%, respectively[17]. False positives of anti-HEV IgM can appear in some conditions including acute hepatitis A[18]. Molecular techniques appear to be complementary for diagnosing HEV infection during early acute infection and in immunosuppressed patients[19].

Clinical data, outcomes and risk factors of patients with acute HEV infection in Thailand have not been well researched. Our study has aimed at reporting clinical outcomes, distribution of disease and genotypes of acute symptomatic HEV infection in Thailand.

2. Materials and methods

This study was conducted on acute HEV patients who presented at or were referred to King Chulalongkorn Memorial Hospital during 2009 to 2013. The research protocol was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University.

2.1. Patients

Forty patients with acute symptomatic HEV infection, who attended or were hospitalized at King Chulalongkorn Memorial Hospital during 2009–2013 were recruited. Acute HEV infection was diagnosed when the clinical presentation was consistent with acute hepatitis and serum was positive for HEV-IgM. All patients were assessed for other viral hepatitis and chronic liver diseases. Clinical, demographic and laboratory data were collected from the hospital records. Patients were divided into high and low risk groups. The high risk group comprised very elderly patients (>80 years), liver cirrhosis patients or patients who were immune-compromised due to previous organ transplantation, chemotherapy or HIV infection. None of these risk factors applied to patients in the low risk group. This study did not include acute HEV infection during pregnancy. A case of HEV infection with neurological complications has been reported elsewhere[16].

2.2. Clinical assessment

Initial demographic data, clinical presentation and laboratory data were obtained. Duration of hospitalization and clinical outcome were analyzed.

2.3. Laboratory methods

Enzyme linked immunosorbent assay (ELISA) was used to determine anti-HEV IgM antibodies (DIA.PRO Diagnostic Bioprobes Srl, Sesto San Giovanni, Milano, Italy) according to the manufacturer's specifications.

2.4. HEV-RNA detection and genotyping

The serum and/or stool samples obtained in this study were tested for HEV-RNA by one step–semi–nested reverse transcription polymerase chain reaction (RT–PCR). Briefly, the cDNA was amplified by nested PCR using three primers comprising an inner sense forward primer HE5–1 (nt 19–37) 5'–TCG ATG CCA TGG AGG CCC A–3', and two outer antisense primers HE5–4 (nt 541–60) 5'–CAT AGC CTC SGC RAC ATC AG–3' and HE5–5 (nt 541–560) 5'–CAT YGC CTC SGC AAC ATC GG–3' for the first round of PCR amplification and HE5–2 (nt 105–123) 5'–GCC YTK GCG AAT GCT GTG G–3', HE5–3 (nt 450–469) 5'–TCR AAR CAG TAR GTG CGG TC–3' and HE5–6 (nt450–469) 5'–TYA AAA CAG TAG GTT CGA TC–3' for the second round. Each reaction mixture comprised 1.5 μ L and 0.5 μ L of template for the primary and secondary PCR, respectively, combined with 10.0 μ L of 2.5 $\times$ Master Mix (Perfect *Taq* Plus, Hamburg, Germany), five primers with each primer at a final concentration of 0.5 μ mol/L, and nuclease free water to a final volume of 25.0 μ L. Both amplification reactions were carried out in a Mastercycler Personal (Eppendorf, Hamburg, Germany). After an initial denaturation step at 95 $^{\circ}$ C for 3 min, amplification was performed for 35 cycles including denaturation (94 $^{\circ}$ C for 45 s), annealing (50 $^{\circ}$ C for 45 s) and extension (72 $^{\circ}$ C for 78 s), followed by a final extension step at 72 $^{\circ}$ C for 10 min. A total of 25 μ L of amplified product was subjected to 2% agarose gel (Agarose molecular grade; Promega, WI) electrophoresis at 100 V for 1 h and then stained with ethidium bromide. The expected amplified product (approximately 365 bp in length) was visualized on a UV transilluminator (Gel Doc 1000, BIO–RAD, CA).

2.5. HEV sequencing

The cDNA of all samples were amplified by semi–nested PCR with the above primers to determine subtype. The band of interest was excised and purified using the GelElute Extraction kit (5 prime, Hamburg, Germany) according to the manufacturer's specifications for whole genome

sequencing. The purified DNA served as templates for DNA sequencing performed by FirstBASE Laboratories SDNBHD (Selangor Darul Ehsan, Malaysia). The nucleotide sequences were analyzed in both directions using forward and reverse primers to confirm the consistency of the sequencing result and ensure that variations of nucleotide sequences were not due to sequencing errors. The genotype was determined by BLAST/FASTA (<http://www.ncbi.nlm.nih.gov>) analysis. The sequences were edited and assembled by using programs CHROMAS LITE v.2.0 (www.technelysium.com.au) and SeqMan (DNASTAR, Madison, WI). Multiple protein translation and sequence alignments were generated with BioEdit version 7.0.1 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

2.6. Phylogenetic analysis of HEV

All sequences were multiple aligned using Clustal X Version 1.8 and the nucleotide identities with reference sequences were analyzed using the BioEdit Sequence Alignment Editor version 5.0.9. Phylogenetic trees were constructed using the Neighbor-Joining Method of the MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 software. To investigate the relationship between HEV strains, an un-rooted tree topology based on multiple alignments of the ORF1 and ORF2 nucleotide sequences and those of known genotypes from Genbank (genotypes 1, 2, 3a, 3b, 3c, 3d, 3e, 3f and 4) was established by the neighbor-joining method, and calculation was done with MEGA 3.1 (<http://www.megasoftware.net>). Consistency of branching was tested by bootstrap analysis with 1 000 re-samplings of the data using MEGA 3.1. Multiple protein translation and sequence alignments were generated with BioEdit version 7.0.1 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

2.7. Statistical analysis

Continuous variables were compared between groups using unpaired *t*-test and one-way ANOVA. Categorical variables were compared between groups using *chi*-square/Fisher's exact test. All statistical analyses were performed using SPSS version 16.

3. Results

From 2009 to 2013, 40 patients were diagnosed with acute HEV infection. They comprised 32 males and 8 females, and their age ranged from 27 to 92 years with a median age of 49 years. Seven patients were immune-compromised. Five very elderly patients were above the age of 80 years and five patients had liver cirrhosis. Eight cases (20%) have a history of exposure to raw pork (Table 1). Residence and region of exposure of acute HEV cases were in various regions of Thailand (Figure 1). In this study, 16 of 40 patients were classified as high risk group. Clinical presentations were not statistically different between the high risk and low risk group (Table 2). Laboratory data were not statistically different between the high risk and low risk group except for higher levels of serum alkaline phosphatase at presentation in the high risk group, (221 ± 119) vs. (156 ± 57) U/L, $P=0.01$) (Table 3). Mean duration of hospitalization was longer in the high risk group than the low risk group $(31.8$ vs. 10.6 d, $P=0.13$).



Figure 1. Regions of exposure (red) and residence (yellow) of acute HEV cases in Thailand.

Regions of confirmed relevant exposure included Nonthaburi, Ubonratchathani, Kamphaengphet, Krabi, Surin and Bangkok; regions of residence included Ayutthaya, Ratchaburi, Rayong, Prachinburi, Uthaitani, Chanthaburi, Loei, Samutprakarn, Samut Sakhon, Mahasarakham, Chaiyaphum, Saraburi and Songkhla.

Table 1

Demographic data, risk exposure and clinical outcome of acute hepatitis E patients classified by patient risk [*n*(%)].

Factor	Median age ^{††}	Male	Immune-compromise host	Post transplantation	Liver cirrhosis	Very elderly	Raw pork exposure	Admission rate	Mean admission duration (d) ^{†††}	90 day mortality
All cases (<i>n</i> =40)	49(27–92)	32(80.0)	7(17.5)	2(5.0)	5(12.5)	5(12.5)	8(20.0)	21(52.5)	20.7±32.1	2(5.0)
High risk group (<i>n</i> =16) [†]	63(32–92)	11(68.7)	7(43.7)	2(12.5)	5(31.2)	5(31.2)	3(18.7)	10(62.5)	31.8±43.9	2(12.5)
Low risk group (<i>n</i> =24)	47(27–75)	21(87.5)	–	–	–	–	5(20.8)	11(45.8)	10.6±9.5	0(0)
<i>P</i> value	0.13	0.15					0.98	0.30	0.13	0.07

[†]Risk group defined as patients with one of the following clinical risks: very elderly (>80 years), liver cirrhosis or immune-compromised/post transplantation.

^{††}Followed by Min–Max.

Overall mortality rate from acute hepatitis E in Thailand was 5% (2/40). The 90-day mortality rate in patients at high risk was 12.5% (2/16) compared to 0% in the low risk group. Two fatal cases from acute hepatitis E were observed in a very elderly patient and a cirrhosis patient. Cause of death was multi-organ dysfunction related to acute liver failure after acute hepatitis E infection.

In six cases (6/40), the virus could be detected in serum or stool by RT-PCR and sequencing. All sequences were submitted to the GenBank database under accession numbers FJ653660 for complete genome, JX679072–6 for ORF1; GU947815 and JX841316 for ORF2. Phylogenetic analysis of ORF1 and ORF2 sequences in comparison with the known genotypes showed that the viruses were classified as HEV genotype 3f, and they were closely related to swine HEV strains from Thailand (Figure 2).

4. Discussion

This study has reported clinical manifestations of patients who were diagnosed with acute sporadic symptomatic HEV infection and treated at the tertiary hospital between 2009 and 2013. These cases originated from various regions of Thailand which confirmed the sporadic nature of HEV genotype 3. We found that middle-aged and elderly individuals were at higher risk of infection, which is consistent with a previous report[20], and the majority of patients diagnosed with symptomatic acute HEV hepatitis

were male. Acute HEV infection presented with various clinical symptoms, with the most pronounced one being jaundice. These can mimic other viral hepatitis and various causes of hepatitis including drug induced liver injury[21]. None of the HEV patients in this study had a history of epidemic jaundice.

Acute hepatitis E in Thailand presented with clinical symptoms of acute viral hepatitis and tended to be more severe in high risk patients including elderly, cirrhotic and immune compromised patients. This study established that levels of serum alkaline phosphatase at presentation were more pronounced in the high risk group. Multiple conditions such as pregnancy, alcoholic liver disease and pre-existing chronic liver disease were related to high mortality from acute HEV infection[22,23].

Extra-hepatic manifestations, such as acute pancreatitis, hematological abnormalities, autoimmune phenomena, and neurological syndromes have been reported, although the pathogenesis of these manifestations has remained unclear[24]. One patient from our study developed extra-hepatic neurological complications of acute hepatitis E[16]. Our data have shown that acute HEV infection can have various clinical presentations and outcomes.

Longer duration of hospitalization was observed in elderly, cirrhotic and immune-compromised patients. In this study, the 90-day mortality rate in high risk patients was 12.5% compared with 0% in the low risk group. Previously reported mortality rates in HEV epidemics have ranged from 0.2% to 4.0%, potentially increasing to 10%–25% in pregnant

Table 2

Clinical presentation of acute hepatitis E patients classified by patient risk [n(%)].

Clinical presentation	Encephalopathy	Icterus	Nausea	Vomiting	Diarrhea	Abdominal pain	Decolorized stool	Dark urine	Pruritus	Malaise	Headache	Fever
All cases (n=40)	2(5.0)	32(80.0)	14(35.0)	7(17.5)	8(20.0)	12(30.0)	1(2.5)	25(62.5)	9(22.5)	21(52.5)	1(2.5)	16(40.0)
High risk group (n=16) [†]	2(12.5)	11(68.8)	6(37.5)	3(18.8)	5(31.3)	3(18.8)	0(0)	10(62.5)	3(18.8)	6(37.5)	0(0)	8(50.0)
Low risk group (n=24)	0(0)	21(87.5)	8(33.3)	4(16.7)	3(12.5)	9(37.5)	1(4.1)	15(62.5)	6(25.0)	15(62.5)	1(4.1)	8(33.3)
P value	0.07	0.26	0.67	0.79	0.12	0.25	0.42	0.79	0.72	0.17	0.42	0.22

[†]Risk group defined as patients with one of the following clinical risks: very elderly (>80 years), liver cirrhosis or immune-compromised/post transplantation.

Table 3

Laboratory data of acute hepatitis E patients classified by patient risk.

Laboratory data	TB (mg/dL)	DB (mg/dL)	AST (U/L) [‡]	ALT (U/L) [‡]	ALP (U/L)	INR	Peak TB (mg/dL) [‡]	Peak DB (mg/dL) [‡]	Peak INR
All cases (n=40)	10.4±9.9	8.5±8.6	226(24–5 440)	463(32–3 986)	192±96	1.25±0.34	10.4(0.7–56.5)	9.2(0.4–37.5)	1.4±0.4
High risk group (n=16) [†]	9.4±9.5	7.1±7.7	197(117–5 440)	237(54–3 986)	221±119	1.35±0.30	13.1(4.6–56.5)	10.3(2.6–37.5)	1.5±0.6
Low risk group (n=24)	11.1±10.3	9.6±9.2	324(24–3 266)	495(42–3 750)	156±57	1.17±0.30	10.0(0.7–41.0)	7.7(0.4–34.0)	1.2±0.2
P value	0.62	0.40	0.51 [§]	0.18 [§]	0.01*	0.20	0.46 [§]	0.69 [§]	0.07

Plus-minus values are mean ± SD for all comparisons. TB: total bilirubin, DB: direct bilirubin, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: alkaline phosphatase, INR: international normalized ratio. [†]Risk group defined as patients with one of the following clinical risks: very elderly (>80 years), liver cirrhosis or immune-compromised/post transplantation. *Statistically significant, [‡] media (range), [§] Mann-Whitney U test.

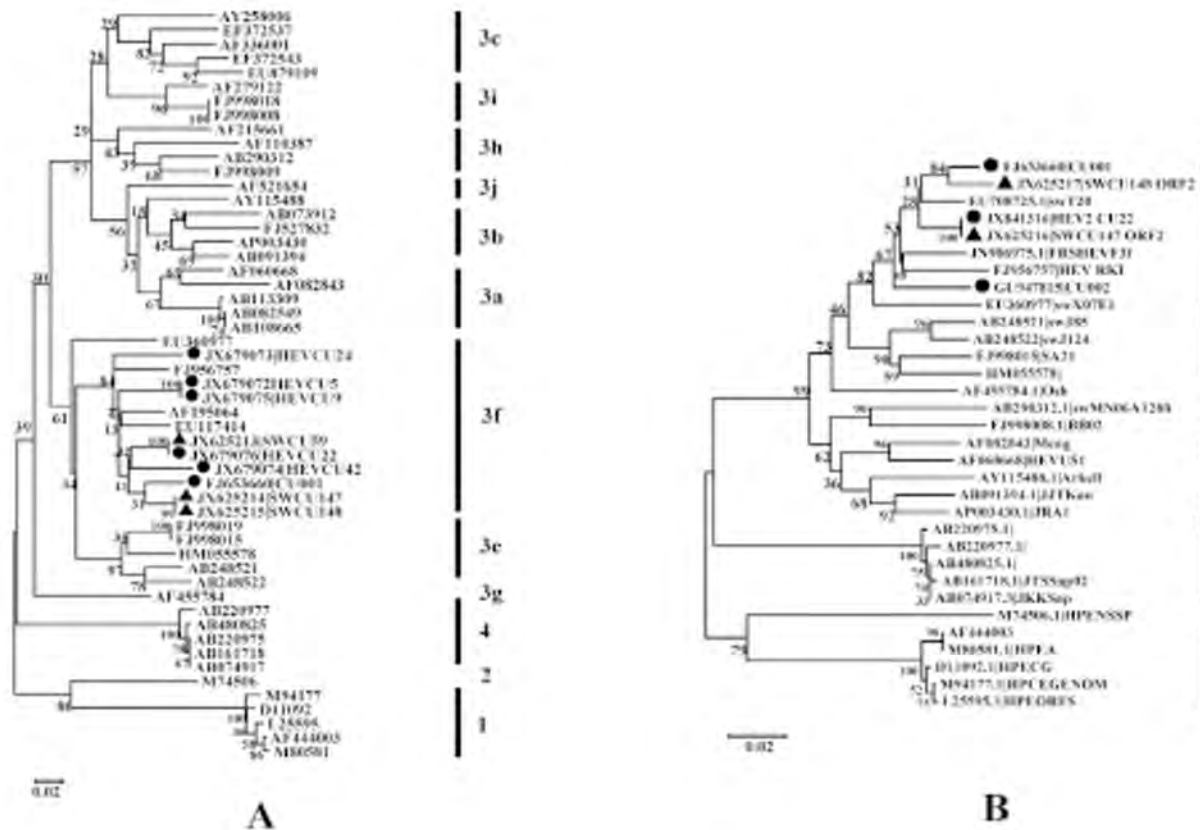


Figure 2. Phylogenetic tree of the ORF1 (A) and ORF2 (B).

The samples from this study were indicated at the respective branches. Symbol ▲ strains were isolated from swine; ● strains were isolated from human.

women[22].

HEV genotype 3 infection has been recognized as zoonotic but the definitive primary source of infection has as yet proven difficult to discover[25]. Eight of our patients had a history of raw pork intake (8/40, 20%). Evidence of pigs as a reservoir for human HEV disease has previously been reported with up to 21.1% seroprevalence[8]. HEV-RNA was detected in six cases from sera or stool. Characterization and phylogenetic analysis of ORF1 and ORF2 sequences of Thai strains showed that they clustered within HEV genotype 3f. One case from our series harbored HEV resembling swine HEV that has been previously reported[8]. Our HEV sequences exhibited the same genotype (3f) closely related to the swine HEV Thailand strains[26]. Infection of the Thai population may have originated from raw pork or contaminated water and food on pig farms.

In conclusion, acute HEV infection in Thailand can be found sporadically and has various clinical presentations and outcomes. Higher mortality rate and longer duration of hospitalization were observed in very elderly, cirrhotic and immune-compromised patients. HEV genotype 3f was detected in all cases and the infection may have originated from swine.

Conflicts of interest statement

No potential conflict of interest relevant to this article was reported.

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***IFNL3 (IL28B)* and *IFNL4* Polymorphisms are Associated With Treatment Response in Thai Patients Infected With HCV Genotype 1, but not With Genotypes 3 and 6**

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Recent studies have shown an association between single nucleotide polymorphisms (SNPs) in the interferon lambda-3 (*IFNL3* or *IL-28B*) and *IFNL4* genes and treatment response to hepatitis C virus genotype 1 (HCV-1) infection. The importance of these SNPs for HCV genotype 3 (HCV-3), and particularly HCV genotype 6 (HCV-6), remains to be elucidated. We analyzed a cohort of 225 Thai individuals with chronic HCV infection treated with pegylated-interferon and ribavirin, of whom 69 (30.7%), 114 (50.7%) and 42 (18.6%) patients were infected with HCV-1, HCV-3, and HCV-6, respectively. DNA extracted from blood samples was analyzed for the SNPs rs12979860 and ss469415590. The distribution of CC, CT, and TT genotypes of rs12979860 was 189 (84%), 28 (12.4%) and 8 (3.6%), respectively, while the distribution of TT/TT, Δ G/TT, and Δ G/ Δ G genotypes of ss469415590 was 192(85.3%), 28 (12.5%), and 5(2.2%), respectively. Significantly lower frequencies of the favorable genotypes CC (for rs12979860) and TT/TT (for ss469415590) were found in the HCV-1 group in comparison with the other groups. The favorable genotypes were associated significantly with rapid and sustained virological response in the HCV-1 group. However, they were only associated with rapid virological response in the HCV-3 and HCV-6 groups. Furthermore, both SNPs were associated equally with the treatment outcome in the HCV-1 group. In contrast, the role of these SNPs in predicting treatment response was attenuated in the HCV-3 and HCV-6 groups. Thus, identification of these SNPs may be useful only in patients with refractory HCV-1 infection. ***J. Med. Virol.* 86:1482–1490, 2014.**

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KEY WORDS: interferon lambda-3; interferon lambda-4; interleukin 28B; single nucleotide polymorphisms; sustained virological response

INTRODUCTION

Hepatitis C virus (HCV) infection is a global public health problem, with an estimated 170 million individuals infected with this virus [Lavanchy, 2009]. HCV has been classified into six major genotypes and several subtypes, which exhibit distinctive patterns of geographic distribution [Argentini et al., 2009;

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Fishman and Branch, 2009; Chao et al., 2011]. In Thailand, approximately 2.2% of the general populations are chronically infected with HCV, most commonly with genotype 3 (HCV-3), genotype 1 (HCV-1) and genotype 6 (HCV-6) [Sunanchaikarn et al., 2007; Akkarathamrongsin et al., 2010a]. The current standard therapy for HCV-1 and HCV-3 is a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) for 48 and 24 weeks, respectively. In Asian populations, up to 80–90% of patients infected with HCV-3 achieve a sustained virological response, while the response rate is approximately 60–70% in HCV-1 infection. With HCV-6, the response rate after 48 weeks of PEG-IFN/RBV therapy is approximately 70–80% [Nguyen and Nguyen, 2013].

Genome wide association studies (GWAS) have reported the associations between different single nucleotide polymorphisms (SNPs) in close proximity to the *interferon lambda-3* (*IFNL3*) gene [formerly known as *interleukin (IL)-28B* gene] and the efficacy of antiviral therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. In addition, several independent studies have confirmed an association between the most studied SNP rs12979860 (located 3 kb upstream of the *IFNL3* gene) and sustained virological response with PEG-IFN/RBV therapy in HCV-1 infection [McCarthy et al., 2010; Montes-Cano et al., 2010; Thompson et al., 2010; Hayes et al., 2011; Scherzer et al., 2011b]. Therefore, it has become obvious that the SNP is a valuable indicator for predicting the response to PEG-IFN/RBV therapy in HCV-1 infection. In contrast, the importance of the SNP in HCV-3 infection is less defined [Mangia et al., 2010; Moghaddam et al., 2011; Sarrazin et al., 2011; Scherzer et al., 2011a; Stattermayer et al., 2011]. Moreover, data regarding the association of the SNP with antiviral response in patients infected with HCV-6 are still very limited.

Recently, a variant in the upstream region of *IFNL3*, designated as *IFNL4*, has been discovered [Prokunina-Olsson et al., 2013]. This region harbors a dinucleotide variant (ss469415590) that is found in two alternative forms (Δ G or TT alleles). The one-base deletion in the Δ G variant results in a frameshift that in turn produces a full-length protein of 179 amino acids. It has been demonstrated that the *IFNL4* Δ G variant is associated with poorer clearance of HCV and response to PEG-IFN/RBV therapy than the TT allele. Compared to rs12979860, the ss469415590 is more associated strongly with treatment response of patients infected with HCV-1 [Prokunina-Olsson et al., 2013]. The importance of this novel polymorphism on predicting treatment response in Thai patients infected with various HCV genotypes is not known. The aim of this study was to investigate the effect of rs12979860 and ss469415590 on treatment response in patients infected with HCV-3 and HCV-6, in comparison with those infected by HCV-1.

METHODS

Patients

Patients with chronic HCV infection were enrolled consecutively between 2007 and 2012 in a single tertiary hospital (King Chulalongkorn Memorial Hospital, Bangkok, Thailand). They were treated and completed a full course of therapy according to well established treatment end points (on the basis of HCV RNA at baseline, weeks 4, 12, 24, end of treatment, and end of follow-up) and achieved treatment adherence of at least 80% of intended dosages of PEG-IFN and RBV. No patient was infected with hepatitis B virus or human immunodeficiency virus. Written informed consent and ethical approval by the Institutional Review Board was obtained from all patients.

The treatment consisted of PEG-IFN-alpha 2a 180 μ g/week or PEG-IFN-alpha 2b 1.5 μ g/kg body weight/week in combination with ribavirin 800–1,200 mg/day according to body weight. Patients infected with HCV-1 and HCV-3 was treated for 48 and 24 weeks, respectively, while patients infected with HCV-6 were treated for 48 weeks. Rapid virological response was defined as undetectable HCV RNA at week 4 during treatment and sustained virological response was defined as HCV RNA negativity at 24 weeks after termination of antiviral therapy. Liver biopsy before treatment was performed in 137 patients and fibrosis was graded according to the METAVIR classification [Bedossa and Poynard, 1996].

Laboratory Tests

HCV genotype was determined by nucleotide sequencing of the core and NS5B regions followed by phylogenetic analysis as described previously [Akkarathamrongsin et al., 2010a]. Serum HCV RNA levels were assessed by real-time-PCR (COBAS TaqMan HCV assay; Roche Diagnostics, Basel, Switzerland).

DNA Extraction and SNP Genotyping

Genomic DNA of HCV patients was extracted from 100 μ l of peripheral blood mononuclear cells (PBMC) or plasma using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The SNPs; rs12979860 and ss469415590 were identified by PCR using specific primers and real time PCR, respectively [Prokunina-Olsson et al., 2013]. According to rs12979860 and ss469415590 genotypes, CC and TT were defined as major alleles and TT and Δ G were defined as minor alleles, respectively.

For rs12979860, nested PCR was performed. The reaction mixture for the first round of rs12979860 amplification consisted of 3 μ l of DNA extracted from PBMC or 5 μ l from plasma and the outer set of primers IL28B-rs12979860_F (5'-GGCGCTGAGGGA-CCGCTACGTAAGTCACCG-3') and IL28B-rs129798-

60_R (5'-CGCTGCCCCCAGCTCAGCGCCTCTTCCT-CC-3'). The PCR mixture contained 5 pmol of each primer, 12.5 µl of 2× Perfect Tag Plus MasterMix (5 PRIME, Gaithersburg, MD) and was adjusted to 25 µl final volume by distilled water. The amplification cycles were as follows: pre-incubation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 30 sec and a final extension step at 72°C for 7 min. Subsequently, the nested PCR reaction contained 1 µl of DNA product from the first PCR and primers; rs12979860_R341 (5'-CTCTTCCTCCTGCGGGACAA-G-3') and rs12979860_F706 (5'-TACACCCGTTTCCTG TCCCAAG-3'). Amplification conditions were identical to the first round. PCR fragment was subjected to 2% agarose gel electrophoresis and visualized under UV light. In order to test its accuracy, we compared this method with the use of a Taqman assay by using ABI Taqman allelic discrimination kit (Assay ID = AH8823E), as described previously [Thomas et al., 2009]. Among 72 sample tested, there was no discrepancy between the results obtained by this method and the ABI Taqman assay (Applied Biosystems, Foster, CA). Thus, the method described above was reliable with 100% accuracy when compared to the standard assay.

For ss469415590, all samples were subjected to real time PCR using the Taqman genotyping assay with MGB probes (Applied Biosystems) as described previously [Prokunina-Olsson et al., 2013]. The real time mixture consisted of 1 µl of DNA extract, 200 nM of each probe (ss469415590_IFNL4_VIC: 5'-ATCGCAGAAGGCC-3' and ss469415590_IFNL4_FAM: 5'-ATCGCAGCGGCCC-3'), 900 nM of each primer (ss469415590_IFNL4_F: 5'-GCCTGCTGCA GAAGCAGAGAT-3' and ss469415590_IFNL4_R: 5'-GCTCCAGCGAGCGGTAGTG-3'), 5 µl of 2× Perfect Tag Plus MasterMix (5 PRIME) adjusted to 10 µl final volume by distilled water. The PCR conditions were as follows: 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 sec at 95°C and 2 min at 60°C. The fluorescent signal was detected at the end of each cycle. The genotype of ss469415590 was analyzed applying the End Point Genotyping method (Light-Cycler 480, Roche Diagnostics, Indianapolis, IN).

Sequencing and Data Analysis

The PCR products were purified using GelExtract Mini Kits (5 PRIME) and subjected to sequencing (First BASE Laboratories, Selangor, Malaysia) using both forward and reverse inner primer pairs. The SNP typing of rs12979860 was analyzed manually based on the chromatograms of nucleotide bases at the SNP positions compared with the reference sequence retrieved from Genbank (<http://www.ncbi.nlm.nih.gov/>). The chromatograms were visualized and analyzed using Chromas LITE (v.2.01), and SeqMan (DNASTAR, Madison, WI). The superimposed chromatogram signals at the SNP position was interpreted as heterozygous genotype [Akkarathamrongsin et al., 2010b].

Statistical Analysis

The Mann-Whitney *U*-test or Student's test were used to compare continuous variables, and the χ^2 test were used to compare categorical variables. Univariate logistic regression was used to assess odd ratios of factors associated with sustained virological response. Multivariate analysis included all parameters from univariate analysis with a *P*-value <0.2. Receiver-operator curves (ROC) were constructed to evaluate the areas under curve regarding virological response associated with the SNPs. All statistical analyses were performed using the SPSS software for Windows version 17.0 (SPSS, Chicago, IL). *P* < 0.05 was considered statistically significant. Linkage disequilibrium between SNPs was assessed by Haploview version 4.2 (<http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview>), which provided *D'* and *r*² values.

RESULTS

Patient Characteristics

A total of 225 patients with chronic HCV infection were included in this study. There were 69 patients with HCV-1, 114 patients with HCV-3 and 42 patients with HCV-6 infection. Table I summarizes the demographic and clinical characteristics of the patients. There were no significant differences in the baseline

TABLE I. Demographics and Characteristics of Patients According to HCV Genotypes

Characteristics	Genotype 1 (n = 69)	Genotype 3 (n = 114)	Genotype 6 (n = 42)	<i>P</i> -value
Age (year)	49.0 ± 10.6	49.5 ± 10.5	42.0 ± 8.5	<0.001
Sex (male)	43 (62.3%)	71 (62.3%)	30 (71.4%)	NS
Body mass index (BMI; kg/m ²)	23.9 ± 3.6	23.9 ± 4.1	23.6 ± 3.8	NS
Baseline ALT (U/L)	89.2 ± 73.2	96.7 ± 12.8	75.4 ± 64.1	NS
Baseline log ₁₀ HCV RNA (IU/ml)	5.9 ± 0.6	5.8 ± 0.8	6.3 ± 0.8	0.002
Advance fibrosis (F3–F4)	19/45 (42.2%)	28/57 (49.1%)	6/35 (17.1%)	0.008
Treatment response				
Sustained virological response	47 (68.1%)	97 (85.1%)	33 (78.6%)	0.025
Relapse	14 (20.3%)	15 (13.2%)	6 (14.3%)	NS
Non-response	8 (11.6%)	2 (1.7%)	3 (7.1%)	0.020

ALT, alanine aminotransferase.

Data described as mean ± SD or n (%).

characteristics between each group with regards to gender distribution, body mass index (BMI), and ALT level. Patients with HCV-6 infection had a significantly lower mean age and lower proportion of advanced fibrosis compared to the other groups ($P < 0.001$ and $P = 0.008$, respectively). HCV-6 infected patients also had a significantly higher baseline HCV-RNA compared to the other groups ($P = 0.002$).

The sustained virological response rate in patients with HCV-1 infection was 68.1%, which was significantly lower than that of patients with HCV-3 (85.1%) and HCV-6 (78.6%; $P = 0.025$). Among the HCV-1 and HCV-3 groups, 14 (20.3%) and 15 (13.2%) patients experienced relapse, while 8 (11.6%) and 2 (1.7%) patients, were non-responders. In the HCV-6 group, 6 patients (14.3%) relapsed, while 3 patients (7.1%) did not respond to treatment.

Prevalence of rs12979860 and ss469415590 Genotypes According to HCV Genotypes

The overall prevalence of rs12979860 genotypes CC, CT and TT in the cohort was 189 (84%), 28 (12.4%), and 8 (3.6%), respectively, while the distribution of ss469415590 genotypes TT/TT, $\Delta G/TT$, and $\Delta G/\Delta G$ in the entire cohort was 192 (85.3%), 28 (12.5%), and 5 (2.2%), respectively. The two SNPs demonstrated strong linkage disequilibrium (D' between rs12979860 and ss469415590 = 0.97, $r^2 = 0.82$). Table II summarizes the characteristics and treatment outcome of patients whose polymorphisms of *IFNL3* and *IFNL4* were discordant. Interestingly, we found a significantly lower frequency of CC genotype in the HCV-1 group (72.5%) in comparison with the HCV-3 group (90.4%) and the HCV-6 group (85.7%) for the rs12979860 ($P = 0.036$). Similarly, TT/TT genotype of ss469415590 was significantly lower in the HCV-1 group (75.4%) than the HCV-3 group (90.4%) and the HCV-6 group (88.1%; $P = 0.033$). Frequencies of rs12979860 and ss469415590 genotypes in conjunction with HCV genotypes are shown in Figure 1a and b, respectively.

Rapid Virological Response Rates According to rs12979860 and ss469415590 Genotypes

Since a relatively small proportion of individuals displayed CT or TT genotype of rs12979860, we grouped these genotypes together for statistical analysis. Our data showed that the decreases in HCV RNA

level during the first 4 weeks of PEG-IFN/RBV therapy were influenced significantly by the SNP genotypes. When considering all viral genotypes, patients with favorable rs12979860 allele (CC genotype) had RVR rates of 78.7%, whereas rapid virological response in non-CC (CT and TT) genotypes was substantially lower (25.0%, $P < 0.001$). Similarly, patients with favorable ss469415590 TT/TT genotype had significantly higher rapid virological response rates than those with unfavorable non-TT/TT genotype ($\Delta G/TT$ and $\Delta G/\Delta G$) (78.1% vs. 24.2%, respectively, $P < 0.001$; Fig. 2a and b). The areas under the receiver operating curve (AUC) values regarding rapid virological response were similar for rs12979860 (0.67) and ss469415590 (0.66) (Supplementary Fig. 1a).

Similar observations were found after stratification by HCV genotypes. The data showed that the patients with the favorable genotypes (the CC genotype of rs12979860 and the TT/TT genotype of ss469415590) in the HCV-1, HCV-3 and HCV-6 groups had significantly higher rapid virological response rates in comparison to those with the unfavorable genotypes (the non-CC genotype of rs12979860 and the non-TT/TT genotype of ss469415590; Fig. 2a and b). There was no significant difference in rapid virological response rate between these two polymorphisms according to HCV genotypes.

Sustained Virological Response Rates According to rs12979860 and ss469415590 Genotypes

Patients in the entire cohort with the favorable genotypes (the CC genotype of rs12979860 and the TT/TT genotype of ss469415590) had significantly higher sustained virological response rates in comparison to those with the corresponding unfavorable genotypes (the non-CC genotype of rs12979860 and the non-TT/TT genotype of ss469415590; 83.6% vs. 52.7%, $P < 0.001$, and 83.9% vs. 48.5%, $P < 0.001$, respectively; Fig. 3a and b). The AUC values regarding sustained virological response were similar for rs12979860 (0.62) and ss469415590 (0.63) (Supplementary Fig. 1b).

After stratification by HCV genotypes, patients in the HCV-1 group with the favorable genotypes had significantly higher sustained virological response rates in comparison to those with the corresponding unfavorable genotypes. For rs12979860, 82% of patients with CC genotype versus 31.6% with non-CC

TABLE II. Characteristics and Treatment Outcome of Patients With Discordant Genotypes at rs12979860 and ss469415590

No.	Age (year)	Sex	HCV genotype	Baseline HCV RNA (log ₁₀ IU/ml)	rs12979860 genotype	ss469415590 genotype	Treatment response
1	37	Male	6	5.68	C/T	TT/TT	SVR
2	53	Female	1	5.26	C/T	TT/TT	SVR
3	61	Female	1	5.50	T/T	TT/TT	Relapse
4	31	Male	6	7.07	T/T	$\Delta G/TT$	Non-response
5	52	Male	1	5.84	C/C	$\Delta G/TT$	Relapse
6	40	Female	3	5.27	T/T	$\Delta G/TT$	SVR

SVR, sustained virological response.

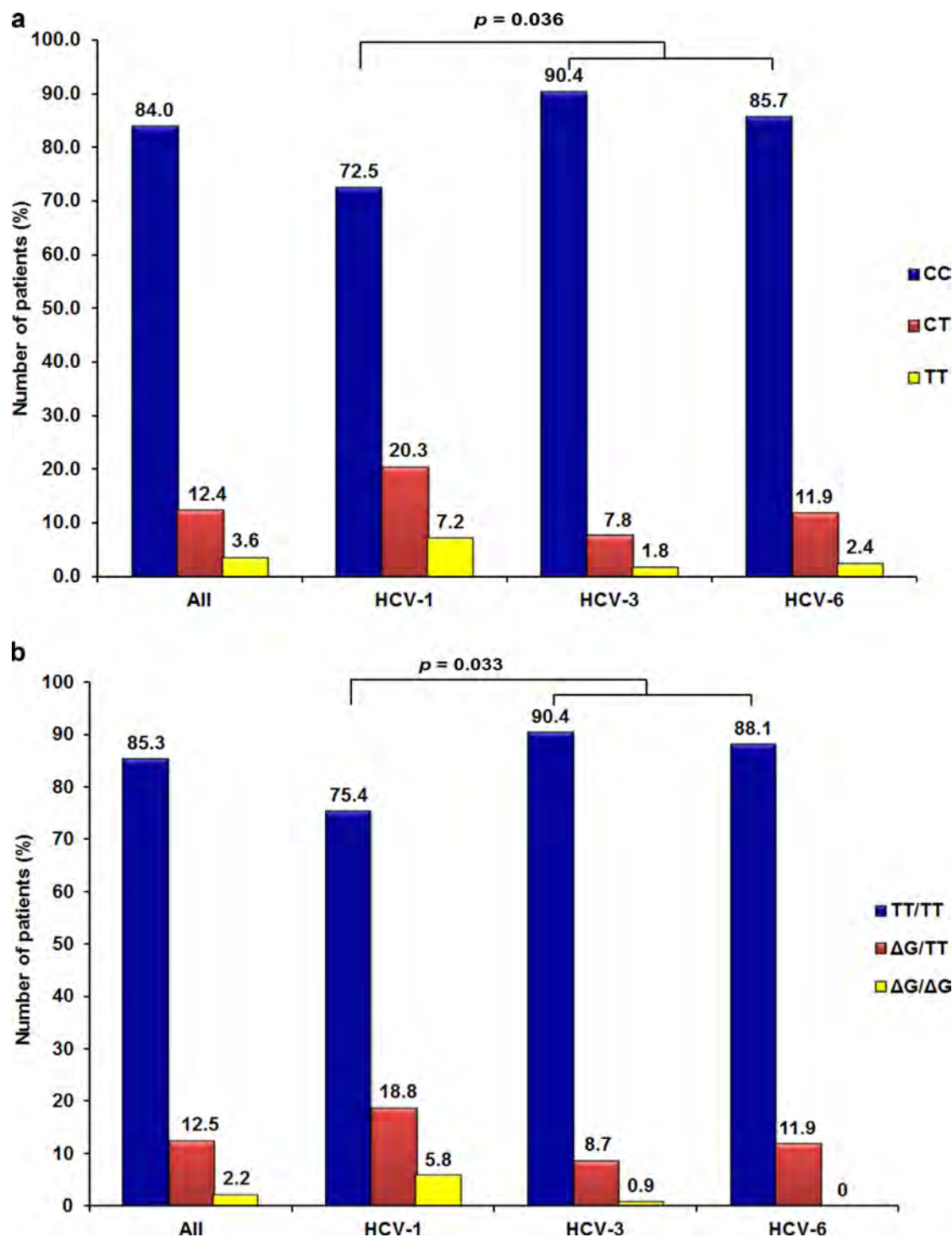


Fig. 1. Distribution of (a) rs12979860 and (b) ss469415590 genotypes in patients with HCV infection.

genotype achieved sustained virological response ($P < 0.001$). For ss469415590, 82.7% of patients with TT/TT genotype versus 23.5% with non-TT/TT genotype achieved SVR ($P < 0.001$). However, there was no such difference in sustained virological response rates among patients infected with HCV-3 and HCV-6 (Fig. 3a and b).

Association of rs12979860 and ss469415590 Genotypes With Clinical Parameters

Baseline serum ALT level, a surrogate marker of inflammation, was significantly higher in patients with CC genotype of rs12979860 compared to patients with non-CC genotype (93.7 ± 64.9 U/L vs.

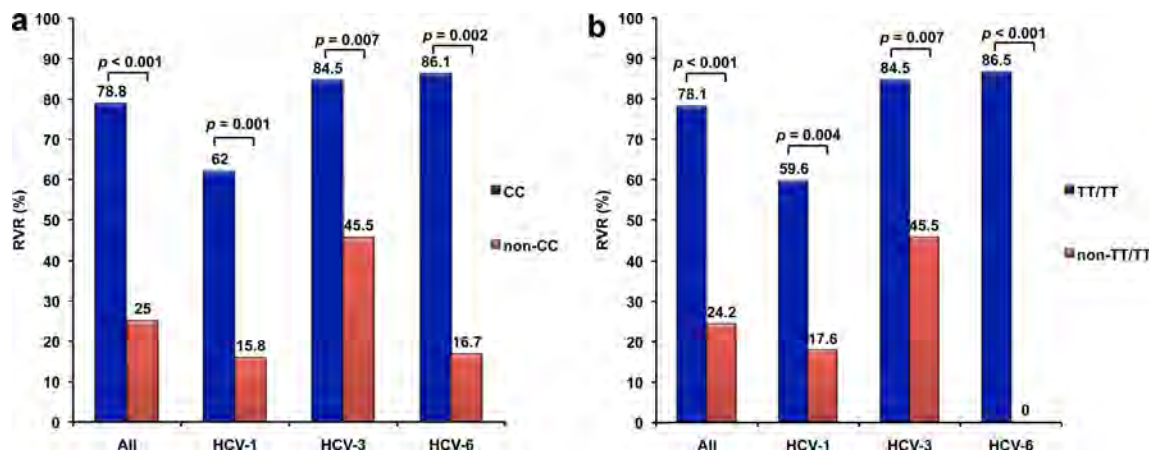


Fig. 2. Association of (a) rs12979860 and (b) ss469415590 genotypes with rapid virological response (RVR) to PEG-IFN/RBV therapy in patients with HCV infection.

72.7 ± 40.5 U/L, respectively, $P=0.013$). Similar observations were found in patients with TT/TT genotype of ss469415590 compared to those with non-TT/TT genotype (93.2 ± 64.3 U/L vs. 74.3 ± 43.5 U/L, respectively, $P=0.037$; Supplementary Table I).

There was no significant difference in the baseline viral load between patients with CC genotype and non-CC genotype of rs12979860 (5.9 ± 0.8 U/L vs. 5.7 ± 0.5 U/L, respectively, $P=0.072$). Similarly, there was no significant difference between patients with ss469415590 TT/TT genotype compared to those with non-TT/TT (5.9 ± 0.8 U/L vs. 5.8 ± 0.5 U/L, respectively, $P=0.081$). Finally, the rs12979860 and ss469415590 polymorphisms were not correlated with advanced fibrosis (F3–F4). Of patients with advanced fibrosis, 38.5% displayed the CC genotype and 39.3% were the non-CC genotype of rs12979860 ($P=0.942$). Similarly, 38.7% and 38.5% of patients with advanced fibrosis expressed TT/TT and the non-TT/TT genotypes of ss469415590, respectively ($P=0.979$; Supplementary Table I).

Factors Associated With Sustained Virological Response

Since both SNPs were found to be in strong linkage disequilibrium, only rs12979860 was selected for subsequent analysis. In patients infected with HCV-1, univariate logistic regression analysis identified significant effects of achieving rapid virological response and the presence of the favorable CC genotype on the sustained virological response (Table III). Both factors remained significant in multivariate logistic regression analysis. The strongest predictor for sustained virological response was rapid virological response (odds ratio [OR], 14.01; 95% confidence interval [CI], 2.74–71.56; $P=0.002$). Excluding rapid virological response, the CC genotype (OR, 20.56; 95% CI, 4.07–103.76; $P<0.001$) and low baseline HCV RNA (OR, 7.39; 95% CI, 1.46–37.4; $P=0.016$) were significant. In patients without rapid virological response, the CC genotype of rs12979860 was identified as having a significant

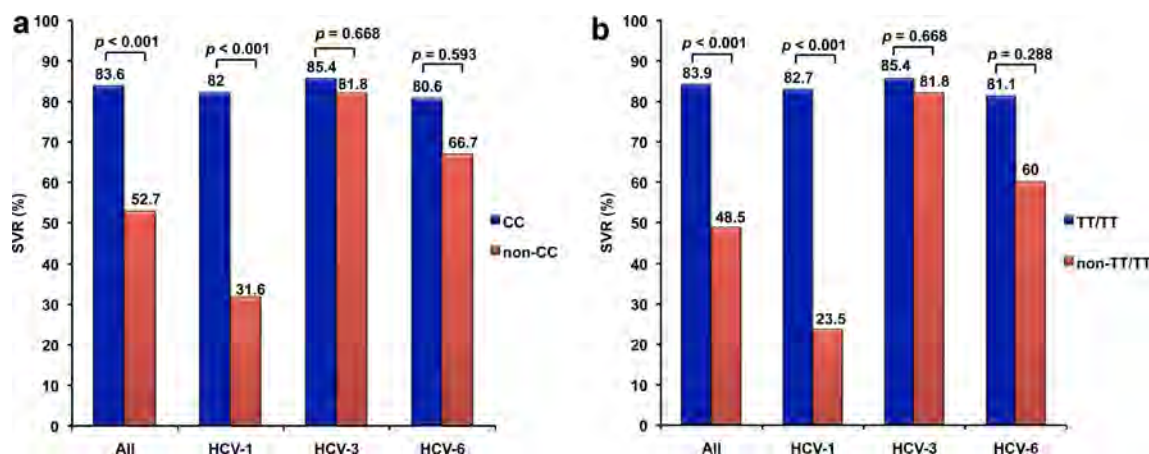


Fig. 3. Association of (a) rs12979860 and (b) ss469415590 genotypes with sustained virological response (SVR) to PEG-IFN/RBV therapy in patients with HCV infection.

TABLE III. Univariate and Multivariate Logistic Regression Analysis of Factors Affecting Sustained Virological Response

	OR	95% CI	P-value	OR	95% CI	P-value
HCV-1						
Age (<40 years)	1.50	0.36–6.19	0.575			
gender (female)	1.45	0.50–4.24	0.493			
BMI (<24 kg/m ²)	1.93	0.69–5.39	0.207			
ALT (<80 U/L)	1.41	0.50–4.00	0.515			
HCV RNA (<600,000 IU/ml)	2.78	0.93–8.35	0.068			
Fibrosis (F0–F2)	1.97	0.56–6.94	0.289			
Rapid virological response	21.33	4.41–103.33	<0.001	14.01	2.74–71.56	0.002
CC genotype	9.87	2.95–32.99	<0.001	5.19	1.33–20.18	0.018
HCV-3						
Age (<40 years)	1.37	0.28–6.04	0.694			
gender (female)	0.84	0.30–2.41	0.750			
BMI (<24 kg/m ²)	0.81	0.28–2.30	0.691			
ALT (<80 U/L)	0.66	0.23–1.92	0.443			
HCV RNA (<600,000 IU/ml)	1.36	0.48–3.81	0.564			
Fibrosis (F0–F2)	2.50	0.66–9.51	0.179	2.11	0.46–9.70	0.338
Rapid virological response	20.88	6.10–71.55	<0.001	13.46	2.83–63.98	0.001
CC genotype	1.30	0.26–6.63	0.749			
HCV-6						
Age (<40 years)	0.52	0.12–2.30	0.389			
gender (female)	4.00	0.44–36.15	0.217			
BMI (<24 kg/m ²)	3.08	0.65–14.52	0.156	0.95	0.67–1.27	0.630
ALT (<80 U/L)	0.54	0.12–2.46	0.429			
HCV RNA (<600,000 IU/ml)	4.00	0.44–36.15	0.217			
Fibrosis (F0–F2)	6.25	0.92–42.50	0.061	3.29	0.25–43.85	0.368
Rapid virological response	7.00	1.38–35.48	0.019	13.30	1.71–103.28	0.013
CC genotype	2.07	0.31–13.68	0.450			

effect on the probability of achieving sustained virological response (OR, 4.13; 95% CI, 1.03–17.63; $P=0.045$). If the analysis was restricted to patients with non-CC genotype, the sustained virological response rates of patients with and without rapid virological response were 66.7% and 25%, respectively ($P=0.154$).

Among patients infected with HCV-3, univariate logistic regression identified significant effects of rapid virological response but not of the favorable CC genotype of rs12979860 on the outcome of sustained virological response (Table III). In a multivariate logistic regression analysis, the only predictor for the sustained virological response was rapid virological response (OR, 13.46; 95% CI, 2.83–63.98; $P=0.001$). If rapid virological response was excluded, no parameter was significant. In patients without rapid virological response, no parameter was associated with sustained virological response.

In patients infected with HCV-6, univariate logistic regression identified a significant effect of rapid virological response but not of favorable genotype of rs12979860 on the outcome of sustained virological response (Table III). In a multivariate logistic regression analysis, the only predictor for the sustained virological response was rapid virological response (OR, 13.30; 95% CI, 1.71–103.28; $P=0.013$). If rapid virological response was excluded, no parameter was significant. In the subset of patients without rapid virological response, no parameter had significant effects on the outcome for sustained virological response.

DISCUSSION

It is generally accepted that both host and virus factors can influence the treatment response in patients infected with HCV. Recent studies have demonstrated that SNPs adjacent to *IFNL3* are promising, host-related predictors of treatment response in patients with HCV-1 infection and more recently in those infected with HCV-4 infection [De Nicola et al., 2012]. However, the association between *IFNL3* genotypes and treatment outcome is less clear and controversial in patients with HCV-3 infection. Some studies have demonstrated that the CC genotype of rs12979860 is associated significantly with sustained virological response in individuals with HCV-3 infection [Mangia et al., 2010; Sarrazin et al., 2011], while other studies have reported no such association [Moghaddam et al., 2011; Scherzer et al., 2011a; Stattermayer et al., 2011]. Moreover, the impact of the polymorphism on viral clearance during therapy in patients with HCV-6 infection is somewhat limited.

Previous reports were confirmed that *IFNL3* polymorphism is useful for predicting treatment response in Thai patients infected with HCV-1. The favorable CC genotype of rs12979860 had approximately two-fold increased sustained virological response rates in comparison to the non-CC genotype [Thomas et al., 2009]. In contrast, no such relationship was found in patients infected with HCV-3 or HCV-6. These data indicate that the impact of the *IFNL3* polymorphism on sustained virological response may be different among Thai patients depending on HCV genotypes. These findings are in agreement with a recent meta-

analysis that the rs12979860 is only associated with the treatment response to PEG-IFN/RBV in patients infected with HCV-1 or HCV-4, but not with HCV-2 or HCV-3 [Jia et al., 2012]. Intriguingly, a recent report from Syria has also demonstrated that *IFNL3* polymorphism did not appear to predict virological response to treatment in patients infected with HCV-5 [Antaki et al., 2012].

A possible explanation of the discrepancy among HCV genotypes is that the effect of the SNPs might be attenuated by a better treatment response in HCV-3 or HCV-6 infection. The lowest sustained virological response rate of combined PEG-IFN/RBV therapy is found generally in patients infected with HCV-1, while a higher sustained virological response rate is observed among patients infected with HCV-3 and the response rate of HCV-6 infection is at an intermediate level [Liang and Ghany, 2013]. Although HCV-6 was regarded initially as a difficult-to-treat genotype, recent data have demonstrated that this genotype has the sustained virological response rate of approximately 80% [Chao et al., 2011], with our study achieving a sustained virological response rate of 78.6%.

Alternatively, the diverse sustained virological response rates might be in part explained by different rs12979860 genotype distribution in different HCV genotypes. In this study, a significantly lower frequency of CC genotype was found in the HCV-1 group in comparison with the other groups. In fact, the prevalence of the CC genotype among the HCV-3 group (90.4%) in this study was comparable to previous reports from other Asian countries (78–90%) [Rangnekar and Fontana, 2013]. In addition, the distribution of favorable *IFNL3* genotype in the HCV-6 group in this study was 85.7%, which was similar to previous reports from Hong Kong (86.7%) [Seto et al., 2013] and Mainland China (92.1%) [Zhang et al., 2013]. Thus, the results of the current study confirmed recent data that significantly lower CC genotype frequencies were detected in patients infected with HCV-1 compared to those infected with HCV-2/3, and also extended the observation to patients with HCV-6 infection. The explanation of these findings remains unclear but might be related to a biological interaction between HCV genotype and *IFNL3* genotype in the development of chronic HCV infection. As shown in a recent study, individuals with CC genotype of *IFNL3* had an increased likelihood of spontaneous clearance after acute infection with HCV-1 compared to those with non-HCV-1 infection [Grebely et al., 2014]. Further studies including different populations and geographical areas would be required to elucidate the differences between *IFNL3* genotype frequencies among patients with different HCV genotypes.

This study also addressed the association of the *IFNL4* ss469415590 genotype with treatment response in Thai patients with HCV infection. This novel polymorphism has been shown to be a better marker for predicting treatment response and clearance than rs12979860 in HCV-infected African-American individ-

uals [Prokunina-Olsson et al., 2013]. Linkage disequilibrium between these two SNPs has been reported to decrease from the highest in Asians (Chinese and Japanese), to intermediate in Caucasians and then the lowest in Africans [Prokunina-Olsson et al., 2013]. In this study, the presence of the ΔG allele of ss469415590 is well-correlated with the unfavorable T allele of rs12979860, thereby indicating strong linkage disequilibrium between these two SNPs in Thai populations. As a result, the absolute difference in sustained virological response rates between these two SNPs was minor and the identification of either SNP may be sufficient in Thai populations.

It should be noted that this study was conducted in a tertiary hospital and selection bias with regards to enrollment of only patients with good compliance to therapy might be possible. However, other studies in Asia have also shown similar sustained virological response rates in the corresponding HCV genotypes [Nguyen and Nguyen, 2013]; hence, this effect was considered to be negligible. Moreover, a recent study from Hong Kong demonstrated that rs8099917, another *IFNL3* polymorphism, was associated with treatment response in HCV-6 infected patients [Seto et al., 2013]. In that report, an increased sustained virological response rate was associated with the favorable TT genotype when compared to the unfavorable TG genotype (96.2% and 62.5%, respectively). This discrepancy between studies has so far not been explained, but might be related to differences between ethnicities. This information emphasizes the necessity to conduct further such association studies in ethnically diverse populations.

In summary, the results demonstrated that these SNPs were associated equally with the treatment outcome in response to PEG-IFN/RBV therapy in patients with HCV-1 infection. In contrast, the role of these polymorphisms in predicting treatment response was attenuated in patients infected with HCV-3 and HCV-6. Identification of these SNPs may be useful prior to treatment in patients with relatively difficult-to-treat HCV-1 infection.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Early viral kinetics during hepatitis C virus genotype 6 treatment according to *IL28B* polymorphisms

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Abstract

AIM: To investigate the early viral kinetics and *interleukin-28B* (*IL28B*) polymorphisms of hepatitis C genotype 6 during pegylated interferon and ribavirin therapy.

METHODS: Sixty-five patients with chronic hepatitis C virus (HCV) infection treated with pegylated interferon and ribavirin (PEG-IFN/RBV) were included, of whom 15 (23.1%), 16 (24.6%) and 34 (52.3%) patients were infected with hepatitis C genotype 1 (HCV-1), genotype 3 (HCV-3) and genotype 6 (HCV-6), respectively. Serum HCV-RNA levels were measured frequently during the first 4-wk of therapy. DNA extracted from samples was analyzed for the *IL28B* single nucleotide polymorphism (SNP) rs12979860 by polymerase chain reaction and direct sequencing.

RESULTS: During the first 4-wk of therapy, the mean viral decline for patients with HCV-6 ($5.55 \pm 1.82 \log_{10} \text{IU/mL}$) was comparable to that of patients with HCV-3 ($5.55 \pm 1.82 \log_{10} \text{IU/mL}$ vs $5.86 \pm 1.02 \log_{10} \text{IU/mL}$, $P = 0.44$) and was significantly higher than patients with HCV-1 ($5.55 \pm 1.82 \log_{10} \text{IU/mL}$ vs $4.23 \pm 1.99 \log_{10} \text{IU/mL}$, $P = 0.04$). In the HCV-6 group, the first phase (days 0-2) viral decline was significantly higher in patients with the favorable rs12979860 CC than non-CC genotypes ($2.46 \pm 1.01 \log_{10} \text{IU/mL/wk}$ vs $1.70 \pm 0.67 \log_{10} \text{IU/mL}$, respectively, $P = 0.045$). A statistically insignificant decrease in the second-phase (days 7-28) decline was also found in patients with the CC genotype than those with the non-CC genotype, though not significantly different ($1.24 \pm 0.64 \log_{10} \text{IU/mL/wk}$ vs $0.80 \pm 0.65 \log_{10} \text{IU/mL/wk}$, respectively, $P = 0.172$). At baseline, the SNP genotype was an independent predictor of rapid virological response but not of sustained virological response.

CONCLUSION: The *IL28B* genotype was linked to an impact on early viral kinetics in response to PEG-IFN/RBV therapy in HCV-6 infected patients.

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Key words: Hepatitis C virus; Genotype 6; Interleukin-

28B; rs12979860; Early viral kinetics

Core tip: Assessment of the early viral kinetics of hepatitis C virus RNA during PEG-IFN/RBV therapy correlates with a specific interleukin-28B polymorphism, potentially revealing insights about the virus-host dynamics. This is the first study to report that single nucleotide polymorphism rs12979860 is well correlated with early viral kinetics in the response to antiviral therapy and leads to a higher rate of RVR in hepatitis C virus-6 infected patients.

Akkarathamrongsin S, Payungporn S, Thong VD, Poovorawan K, Prapunwattana P, Poovorawan Y, Tangkijvanich P. Early viral kinetics during hepatitis C virus genotype 6 treatment according to *IL28B* polymorphisms. *World J Gastroenterol* 2014; 20(30): 10599-10605 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i30/10599.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i30.10599>

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC)^[1]. HCV has been classified into seven major genotypes and several subtypes, which exhibit distinctive patterns of geographic distribution^[2,3]. In Thailand, approximately 2.2% of the general population has been chronically infected with HCV and the most common genotypes are HCV genotype 3 (HCV-3), genotype 1 (HCV-1) and genotype 6 (HCV-6), respectively^[4]. The current standard therapy for all HCV genotypes is a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV). A sustained virological response (SVR) following antiviral treatment can delay disease progression and decrease HCC development. In the Asian population, treatment with the combined therapy results in different SVR depending on the HCV genotype (50%-60% in HCV-1 infected patients, 80%-90% in HCV-3 infected patients, and 70%-80% in HCV-6 infected patients)^[5].

Early viral kinetics as measured by HCV RNA levels during the first few weeks of treatment has been shown to be an important predictor of PEG-IFN-based therapy. Typically, HCV kinetics exhibits a biphasic pattern^[6-8]. The first phase is characterized by a decline in serum HCV RNA from days 0-2, which represents the antiviral action of interferon (IFN) in blocking HCV production. The second phase, defined by the reduction of HCV RNA levels during days 7-28, is thought to correlate with clearance of HCV. However, different HCV genotypes exhibit different viral kinetics^[9,10]. Moreover, it has been shown that polymorphisms near interleukin-28B (*IL28B*) gene are strongly associated with early viral kinetics during PEG-IFN/RBV therapy in patients with chronic HCV-1 infection^[11-14]. However, this correlation has not been investigated in HCV-6 infected individuals. This study aims to determine the early viral kinetics in HCV-6

infected patients undergoing PEG-IFN / RBV treatment and *IL28B* polymorphisms. Results were compared to those of patients infected with HCV-1 and HCV-3.

MATERIALS AND METHODS

Patients

We analyzed data from a cohort of patients recruited at our center (King Chulalongkorn Memorial Hospital, Bangkok, Thailand) as described previously^[15]. Briefly, 66 treatment-naïve adults with chronic HCV infection received PEG-IFN- α 2a (Pegasys, Roche) 180 μ g/wk plus weight-based RBV (Copegus, Roche) according to the following body weights: ≤ 75 kg, 1000 mg/d; and > 75 kg, 1200 mg/d. Patients infected with HCV-1 and HCV-3 were treated for a fixed duration of 48 and 24 wk, respectively. Patients infected with HCV-6 who achieved RVR were assigned to treatment for 24 wk and the remaining patients were treated for 48 wk. One patient who lost follow up was excluded from the current cohort. The study protocol had been approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 511/51), and all participants had provided written informed consent. The study followed the Helsinki Declaration and Good Clinical Practice guidelines.

HCV RNA kinetics

Serum HCV RNA levels were assessed at days 0, 2, 7, 14, 21 and 28 by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)(COBAS TaqMan HCV assay; Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. The first phase decline was characterized as the difference in HCV RNA levels ($\log_{10}$ IU/mL) from day 0 to 2. The second phase decline was defined as the best fit slope of serum HCV RNA ($\log_{10}$ IU/mL/wk) levels from days 7 to 28^[16]. Rapid virological response (RVR) was defined as undetectable HCV RNA at week 4, while SVR was defined as undetectable HCV RNA 24 wk after the end of treatment.

Single nucleotide polymorphism genotyping

Genomic DNA of HCV patients was extracted from 100 μ L of peripheral blood mononuclear cells (PBMC) using the QIAamp DNA Mini Kit according to the manufacturer's instruction (Qiagen, Germany). The single nucleotide polymorphism (SNP); rs12979860 was identified by PCR using specific primers and direct nucleotide sequencing.

The PCR mixture for rs12979860 amplification consisted of 3 μ L of extracted DNA, 5 pmole of each primer including rs12979860_R341 (5'-CTCTTCCTCCTGCGGGACAAG-3') and rs12979860_F706 (5'-TACACCCGTTCTGTCCCAAG-3'), 12.5 μ L of 2 $\times$ Perfect Tag Plus MasterMix (5 PRIME, Gaithersburg, MD) and distilled water to a final volume of 25 μ L. After an initial denaturation at 94 $^{\circ}$ C for 3 min, 40 cycles of amplification were performed, each including

Table 1 Clinical characteristics and single nucleotide polymorphism rs12979860 of the patients according to hepatitis C virus genotypes

Characteristics	Genotype 1 (n = 15)	Genotype 3 (n = 16)	Genotype 6 (n = 34)	P value
Age (yr)	46.6 ± 12.9	42.8 ± 8.2	41.2 ± 8.4	NS
Sex (male)	53.3%	81.3%	67.6%	NS
BMI (kg/m ²)	24.2 ± 12.1	21.3 ± 5.7	23.7 ± 3.7	NS
ALT (U/L)	86.3 ± 57.8	82.6 ± 51.9	62.6 ± 54.5	NS
Log ₁₀ HCV RNA (IU/mL)	6.3 ± 0.8	6.0 ± 0.8	6.5 ± 0.8	NS
Liver fibrosis score				NS
Score 0-2	73.3%	66.7%	71.4%	
Score 3-4	26.7%	33.3%	28.6%	
SNP rs12979860				NS
CC	66.7%	75.0%	82.3%	
CT	20.0%	18.8%	11.8%	
TT	13.3%	6.2%	5.9%	
RVR	46.7%	87.5%	73.5%	0.039
SVR	66.7%	81.3%	76.5%	NS

Data described as mean ± SD or proportions (%). ALT: Alanine aminotransferase; RVR: Rapid virological response; SVR: Sustained virological response; BMI: Body mass index; HCV: Hepatitis C virus.

denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s, then followed by a final extension step at 72 °C for 7 min.

In some cases with insufficient amount of PBMC, nested PCR was performed with samples extracted from plasma. The reaction mixture and amplification condition were the same as described above. The outer set of primers used for the first round of PCR included rs12979860_F (5'-GGCGCTGAGGGACCGCTAC-GTAAGTCACCG-3') and rs12979860_R (5'-CGCT-GCCCCAGCTCAGCGCCTCTTCTCC-3'). Then, 1 µL of the first PCR product was combined with primers including rs12979860_R341 and rs12979860_F706 for the subsequent nested PCR. In order to ensure no contamination occurred, negative control was included in both first round and nested PCR amplification in each experiment. The amplification fragment was separated in 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transilluminator. The PCR products were then purified using GelExtract Mini Kits (5 PRIME, Gaithersburg, MD) and subjected to bi-directional sequencing (First BASE Laboratories, Malaysia) using both forward and reverse primers.

The SNP typing of rs12979860 was analyzed based on the chromatograms of nucleotide bases at the SNP position compared with the reference sequence retrieved from Genbank database (<http://www.ncbi.nlm.nih.gov/>). The chromatograms were visualized and analyzed using Chromas LITE (v2.01) and SeqMan (DNASTAR, Madison, WI). The superimposed chromatogram signals at the SNP position was interpreted as heterozygous genotype^[17]. According to rs12979860 genotypes, CC was defined as major alleles, CT was defined as heterozygous alleles and TT was defined as minor alleles. In samples that the interpretations using sequencing-based assays

were not clear, the results were validated by *TaqMan* SNP Genotyping Assays (Assay ID AH8823E, Applied Biosystems) based on real-time PCR method as described previously^[18].

Statistical analysis

The Mann-Whitney *U* test or Student's *t* test were used to compare continuous variables, and the χ^2 test or Fisher's exact test were used to compare categorical variables. HCV-RNA kinetic throughout the first 4 wk of therapy was analyzed according to HCV genotypes. The effect of the *IL28B* genotypes (CC *vs* non-CC) on HCV viral decline in HCV-1, HCV-3 and HCV-6 patients was also analyzed. Univariate and multivariate logistic regression was used to assess odd ratios associated with RVR and SVR. All statistical analyses were performed using the SPSS software for Windows version 17.0 (SPSS, Chicago, IL, United States). *P* < 0.05 for a two-tailed test was considered statistically significant.

RESULTS

Baseline characteristics and SNP rs12979860

Among 65 patients naïve to PEG-IFN/RBV included in this study, there were 15 patients with HCV-1, 16 patients with HCV-3 and 34 patients with HCV-6. Table 1 summarizes baseline characteristics and rs12979860 genotypes of these patients based on HCV genotypes. There were no significant differences in the baseline characteristics between each group with regards to gender distribution, mean age, body mass index (BMI), ALT level, HCV RNA level and the degree of liver fibrosis assessed by histology. The distribution of rs12979860 genotypes (CC, CT and TT) in the entire cohort was 50 (76.9%), 10 (15.4%) and 5 (7.7%), respectively. There was no significant difference in the distribution of the *IL28B* genotypes between HCV genotypes in this study.

RVR were achieved in 87.5% of patients with HCV-3 and 73.5% of patients with HCV-6, which was statistically more significant than that of patients with HCV-1 (46.7%) (*P* = 0.039). The overall rate of SVR in patients with HCV-3 (81.3%) was higher than that of patients with HCV-6 (76.5%) and was higher than patients with HCV-1 (66.7%), although there was no significant difference (*P* = 0.627). Among patients who attained RVR, SVR was achieved in 90% of patients with HCV-1, 92.9% of patients with HCV-3 and 88% of patients with HCV-6.

Viral decline according to HCV genotypes

The decreases in HCV RNA level during the first 4 wk were significantly correlated to HCV genotypes. We found that the magnitude of viral decline for patients infected with HCV-1 was lower than those infected with HCV-3 or HCV-6 at all time points. After 4 wk of treatment, the HCV RNA levels had declined by a mean of $4.23 \pm 1.99 \log_{10}$ IU/mL in patients infected with HCV-1 compared with 5.86 ± 1.02 and $5.55 \pm 1.82 \log_{10}$ IU/mL

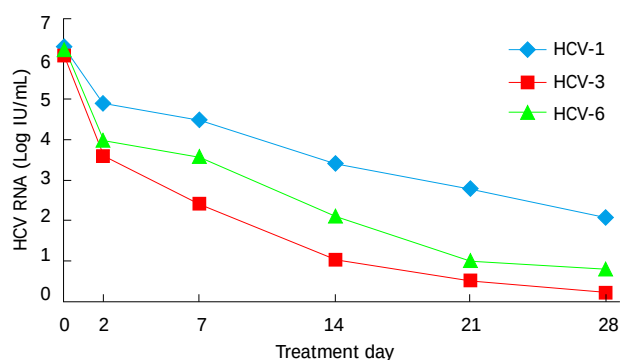


Figure 1 Kinetics of hepatitis C virus based on different viral genotypes. Mean hepatitis C virus (HCV)-RNA levels ($\log_{10}$ IU/mL) at different time points (days 0, 2, 7, 14, 21, 28). During the first 4 wk of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy according to HCV genotypes. HCV-1: HCV genotype 1; HCV-3: HCV genotype 3; HCV-6: HCV genotype 6.

in those infected with HCV-3 and HCV-6 ($P = 0.007$ and $P = 0.040$, respectively). There was no significant difference between patients infected with HCV-3 and HCV-6 ($P = 0.437$). The mean HCV-RNA levels ($\log_{10}$ IU/mL) at different time points during the first 4 wk of PEG-IFN/RBV treatment according to HCV genotypes are shown in Figure 1.

The HCV-3 and HCV-6 groups had a first phase decline of 2.30 ± 1.25 and $2.32 \pm 0.99 \log_{10}$ IU/mL, respectively, which did not achieve statistical difference ($P = 0.958$). However, both were higher than that of the HCV-1 group ($1.38 \pm 0.64 \log_{10}$ IU/mL) ($P = 0.015$ and $P = 0.002$, respectively). The second phase decline was comparable between the HCV-3 and HCV-6 groups were similar ($1.25 \pm 0.66 \log_{10}$ IU/mL/wk *vs* $1.17 \pm 0.65 \log_{10}$ IU/mL/wk, respectively, $P = 0.675$) and higher than that of the HCV-1 group ($0.99 \pm 0.73 \log_{10}$ IU/mL/wk). The differences between HCV-3 and HCV-1 groups, or between HCV-6 and HCV-1 groups, were not statistically significant ($P = 0.310$ and $P = 0.428$, respectively).

Viral decline according to SNP rs12979860

Our data showed that the decreases in HCV RNA level during the first 4 wk of treatment were significantly correlated with the rs12979860 genotypes. Irrespective of viral genotypes, the baseline HCV RNA level in patients with CC genotype was significantly higher than in patients with the non-CC (CT and TT) group (6.42 ± 0.83 and $5.87 \pm 0.88 \log_{10}$ IU/mL, respectively, $P = 0.040$). After 4 wk of treatment, the HCV RNA levels had declined by a mean of $5.77 \pm 1.44 \log_{10}$ IU/mL in patients carrying CC compared with $3.84 \pm 2.08 \log_{10}$ IU/mL in those carrying non-CC genotype ($P < 0.001$).

In the HCV-1 group, the HCV RNA levels had declined after 4 wk of treatment by a mean of $5.34 \pm 1.43 \log_{10}$ IU/mL in patients carrying CC compared with $2.03 \pm 0.50 \log_{10}$ IU/mL in those carrying non-CC genotype ($P < 0.001$). In the HCV-3 group, the corresponding figures were 5.97 ± 1.01 and $5.23 \pm 1.10 \log_{10}$ IU/mL, respectively ($P = 0.504$). In the HCV-6 group, the correspond-

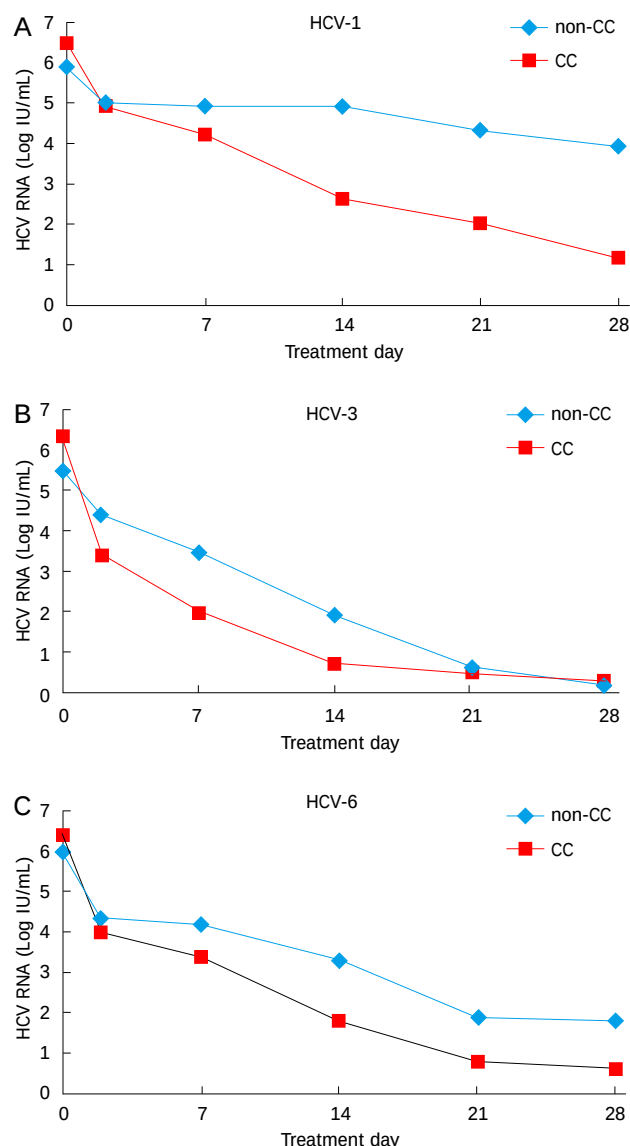


Figure 2 Kinetics of hepatitis C virus based on the SNP rs12979860 alleles. Mean hepatitis C virus (HCV)-RNA levels ($\log_{10}$ IU/mL) at different time points (days 0, 2, 7, 14, 21, 28). During the first 4 wk of pegylated interferon (PEG-IFN) and ribavirin (RBV) treatment according to rs12979860 genotypes. A: HCV genotype 1; B: HCV genotype 3; C: HCV genotype 6.

ing figures were 5.83 ± 1.60 and $4.23 \pm 2.34 \log_{10}$ IU/mL, respectively ($P = 0.163$). The mean HCV-RNA levels ($\log_{10}$ IU/mL) at different time points during the first 4 wk of PEG-IFN/RBV treatment according to *IL28B* genotypes in each HCV genotype are shown in Figure 2.

Irrespective of viral genotypes, the first phase decline was significantly higher in patients with the CC than those with non-CC genotype (2.35 ± 1.04 and $1.28 \pm 0.63 \log_{10}$ IU/mL, respectively, $P < 0.001$) (Figure 3A). In addition, the second phase decline was significantly higher for patients with the CC than for those with non-CC genotype (1.27 ± 0.67 and $0.75 \pm 0.52 \log_{10}$ IU/mL/wk, respectively, $P = 0.004$) (Figure 3B). Patients with CC genotype had RVR rates of 78%, whereas RVR in the non-CC group was substantially lower (46.7%, $P = 0.027$). However, the SVR rates between patients with CC and

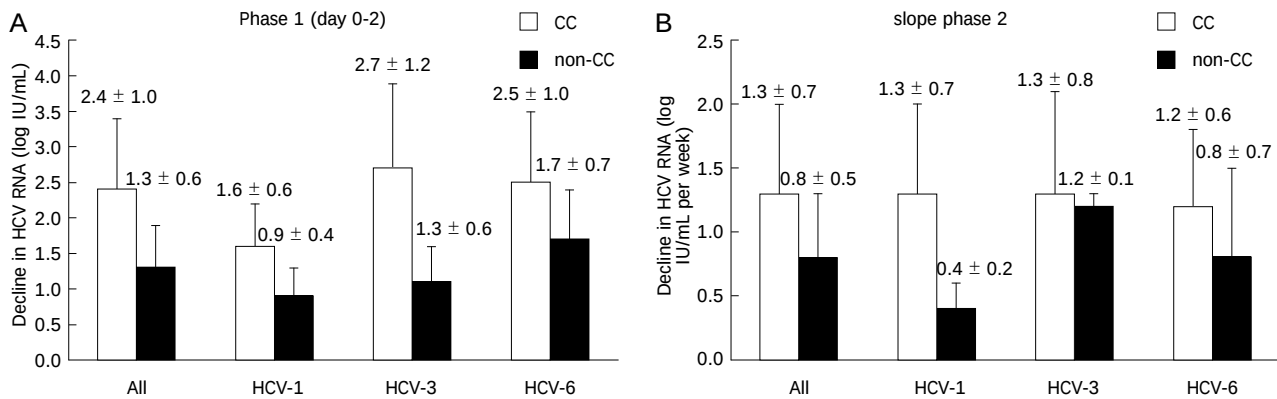


Figure 3 Changes in serum hepatitis C virus RNA concentrations according to rs12979860 genotypes. A: The first phase (days 0-2); B: The second phase (days 7-28) hepatitis C virus (HCV)-1: HCV genotype 1; HCV-3: HCV genotype 3; HCV-6: HCV genotype 6; Data described as mean $\pm$ SD.

non-CC genotypes were not significantly different (76.0% and 73.3%, respectively, $P = 0.833$).

In the HCV-6 group, the first phase decline was 2.46 ± 1.01 and 1.70 ± 0.67 $\log_{10}$ IU/mL in patients with CC and non-CC genotypes, respectively ($P = 0.045$) (Figure 3A). The second phase decline was 1.24 ± 0.64 and 0.80 ± 0.65 $\log_{10}$ IU/mL/wk, respectively, in patients with the corresponding *IL28B* genotypes ($P = 0.172$) (Figure 3B). Patients with the favorable CC genotype achieved significantly higher RVR rates in comparison to those with the unfavorable genotypes (82.1% and 33.3%, respectively, $P = 0.031$). However, the SVR rates between patients with CC and non-CC genotypes were not significantly different (78.6% and 66.7%, respectively, $P = 0.609$).

Factors associated with RVR and SVR in patients with HCV-6

To identify factors associated with RVR and SVR in patients with HCV-6, baseline characteristics of patients and early viral kinetics during therapy were analyzed by univariate and multivariate logistic regression analyses. Potential baseline predictors of RVR and SVR included sex, age, BMI, ALT level, liver fibrosis score, HCV RNA level and rs12979860 genotype.

For RVR, univariate analysis identified significant effects correlated with the presence of the favorable CC genotype (OR = 9.20; 95%CI: 1.30-64.90, $P = 0.026$), the first phase decline (OR = 3.76; 95%CI: 1.29-10.96, $P < 0.015$) and second phase kinetics (OR = 9.50; 95%CI: 1.35-66.99, $P = 0.024$). However, only the first and second phase kinetics remained significant in multivariate logistic regression analysis (the first phase: OR = 6.39; 95%CI: 1.18-34.54, $P < 0.031$, the second phase; OR = 18.15; 95%CI: 1.13-291.50, $P < 0.041$). If only baseline pre-treatment parameters were included in the multivariate model, CC genotype (OR = 16.88; 95%CI: 1.45-169.5, $P = 0.024$) was considered significant.

Variables associated with SVR in univariate analysis were low HCV RNA level (OR = 6.75; 95%CI: 1.11-41.00, $P = 0.030$) and the second phase kinetics (OR = 12.60; 95%CI: 1.93-82.09, $P = 0.008$). In multivariate logistic regression analysis, only the second phase kinetics

was associated with SVR (OR = 12.79; 95%CI: 1.65-99.28, $P = 0.015$). If only baseline characteristics were included in the multivariate model, no parameter was significant.

DISCUSSION

In the present study, we demonstrated for the first time the kinetics of HCV-6 correlates with *IL28B* polymorphisms in patients treated with PEG-IFN/RBV therapy. We found that rs12979860 significantly correlated with the early viral elimination during treatment, with a more rapid reduction of HCV RNA levels in patients carrying the favorable CC allele. The rapid first-phase reduction in patients with the CC allele suggests that the polymorphisms might be linked to the antiviral effect of PEG-IFN/RBV in the blocking of the production or release of HCV. A trend towards a steeper second-phase decline was also found in patients with the CC allele, although this was not statistically significant due to sample size. This finding might indicate that the rs12979860 polymorphism is linked to immune-mediated eradication of infected hepatocytes in addition to the antiviral efficiency.

Our data confirmed previous reports that the decrease in HCV RNA level during the first 4 wk was influenced by HCV genotypes with respect to *IL28B* polymorphisms. Previous data reported that in patients infected with HCV-1, HCV RNA levels declined by 3.8 $\log_{10}$ IU/mL after 4 wk among patients carrying CC compared with 1.1-1.5 $\log_{10}$ IU/mL in patients carrying non-CC allele^[19]. In our study, the decrease observed in the HCV-1 group was approximately 5.3 and 2.0 $\log_{10}$ IU/mL for those carrying CC and non-CC allele, respectively. Among patients infected with HCV-3, the corresponding declines in this study were 5.97 and 5.23 $\log_{10}$ IU/mL, respectively. These observations are in agreement with previous results^[20]. In the HCV-6 group, the corresponding figures were 5.83 and 4.23 $\log_{10}$ IU/mL, for patients with CC and non-CC allele, respectively. We found that the impact of rs12979860 favorable allele on viral reduction during therapy in patients infected with HCV-6 is at an intermediate level compared to those infected with HCV-3 and HCV-1.

In the HCV-6 group, the collective effects of the SNPs on the first- and second-phase viral decline might explain the differences in RVR rates. For instance, approximately 80% of patients with CC allele and 30% of non-CC allele achieved RVR. However, a high likelihood of RVR in patients carrying the favorable CC allele was not associated with a higher SVR rate. As shown in this study, there was no significant difference in SVR rate between CC and non-CC allele (approximately 78% *vs* 67%, respectively). In the multivariate model, our data also confirmed that the SNP rs12979860 was associated with RVR but not SVR. Previous studies in HCV-3 cohorts also showed that RVR but not SVR was influenced by the SNP alleles^[12,21,22]. In contrast, previous reports in HCV-1 infected patients demonstrated that more robust early declines in serum HCV RNA predicted a higher SVR rate during PEG-IFN/RBV therapy^[23]. A possible explanation of the discrepancy among HCV genotypes is that the effect of the SNPs might be attenuated by a better treatment response in HCV-3 or HCV-6 infection. In general, the lowest SVR rate of combined PEG-IFN/RBV therapy is found in HCV-1 infected patients. A much higher SVR rate is observed among HCV-3 infected patients, while the response rate of HCV-6 infection is at an intermediate level^[24]. These results suggest that the underlying mechanisms, which link the host genetic status to early HCV suppression, might be different from those required for virus eradication.

In summary, our results demonstrated that SNP rs12979860 was linked to early viral kinetics in response to PEG-IFN/RBV therapy in HCV-6 infected patients, which might lead to a higher rate of RVR. Despite the recent introduction of direct antiviral agents in patients with HCV-1 infection, combined PEG-IFN/RBV therapy is the only currently approved treatment for HCV-6 infection. Thus, we believe that an improved ability to predict treatment response is an important goal and is worthy of further large-scale studies, particularly in south China and many south-east Asian countries in which HCV-6 genotype is prevalent.

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COMMENTS

Background

The current standard therapy for hepatitis C virus (HCV) genotype 6 (HCV-6) is a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV). However, early viral kinetics of HCV RNA levels during the combined therapy in HCV-6 infected patients with regard to interleukin-28B (*IL28B*) polymorphisms is totally unknown.

Research frontiers

Recently, several genome wide association studies have reported associations of different single nucleotide polymorphisms (SNPs) in close proximity to *IL-28B*

gene with response to antiviral therapy. In addition, several independent studies have confirmed an association between the most studied SNP rs12979860 (located 3 kb upstream of the *IL28B* gene) and sustained virological response with PEG-IFN/RBV therapy in HCV genotype 1 (HCV-1) infection. In contrast, the importance of the SNP in HCV-6 infection is less defined.

Innovations and breakthroughs

This is the first study to report the early viral kinetics of HCV-6 during treatment in correlation with *IL28B* polymorphisms. The data demonstrated that rs12979860 CC genotype correlated with the rapid early viral elimination during treatment, particularly during the first phase. In addition, the impact of the SNP genotypes on viral reduction during therapy in patients infected with HCV-6 is at an intermediate level between those infected with HCV genotype 3 (HCV-3) and HCV-1.

Applications

SNP rs12979860 was linked to an impact on early viral kinetics in response to PEG-IFN/RBV therapy in HCV-6 infected patients, which leads to a higher rate of rapid virological response. This information may be useful for clinicians in counseling individual HCV-6 infected patients who are receiving antiviral therapy, particularly in south China and many south-east Asian countries in which this HCV genotype is highly prevalent.

Terminology

Early viral kinetics of HCV is demonstrated by measurement of HCV RNA levels frequently during the first few weeks of PEG-IFN and RBV treatment. Typically, the early kinetics has a biphasic pattern. The first phase is characterized by a decline in serum HCV RNA from days 0-2, which represents the antiviral action of IFN in blocking HCV production. The second phase, defined by the reduction of HCV RNA levels during days 7-28, indicates the clearance of infected cells by immune response.

Peer review

The paper is well written and gives important information of the viral kinetics of HCV-6 infection, which is considered to be a lesser known HCV genotype. Thus, these data may provide a better understanding of HCV-host dynamics in response to antiviral treatment

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The correlation between hepatitis C core antigen and HCV RNA levels with respect to HIV status, HCV genotype and IFNL4 polymorphism

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Abstract

Objectives: Serum HCV core antigen (HCVcAg) concentrations correlate with HCV RNA levels in HCV mono-infected patients. Data in HCV/HIV co-infected patients are still limited. We aim to compare the use of HCVcAg measurement with respect to HIV status, HCV genotypes, interferon lambda-4 (IFNL4) polymorphism and clinical parameters.

Methods: We analyzed an untreated cohort of 104 patients with HCV mono-infection and 85 patients with HCV/HIV co-infection. Serum HCVcAg was measured by a commercial chemiluminescent microparticle immunoassay. The presence of IFNL4 polymorphism ss469415590 was identified by real time PCR.

Results: $\log_{10}$ HCVcAg levels were significantly correlated with corresponding $\log_{10}$ HCV RNA levels ($r=0.889$, $p<0.001$), but not correlated with ALT levels and liver stiffness. The correlation between HCV RNA and HCVcAg was particularly high in co-infected patients and those with high viremia. Mean $\log_{10}$ HCVcAg concentration was significantly higher in co-infected patients than in mono-infected patients. Patients harboring TT/TT genotype of ss469415590 had significantly higher levels of $\log_{10}$ HCVcAg than those with non-TT/TT genotype. HCVcAg levels were similar across HCV genotypes.

Conclusions: HCVcAg concentrations had an excellent correlation with HCV RNA levels, particularly in HCV/HIV co-infected individuals and might be associated with IFNL4 polymorphism. HCVcAg testing could be used as an alternative to HCV RNA assays in resource-limited settings.

1 Introduction

2 Hepatitis C virus (HCV) infection is a major etiology of liver fibrosis, cirrhosis, and
3 hepatocellular carcinoma, affecting up to 170 million people worldwide [1]. HCV has been
4 classified into seven major genotypes, all of which display different patterns of geographic
5 distribution. In Thailand, approximately 2.2% of the general populations are chronically
6 infected with HCV and the most common genotypes are genotype 3 (HCV-3), genotype 1
7 (HCV-1) and genotype 6 (HCV-6) [2]. The screening tests for HCV infection in clinical
8 practice rely on the detection of anti-HCV antibodies using enzyme-linked immunosorbent
9 assays (ELISA). However, these tests cannot differentiate between resolved HCV infection
10 and an active viral replication. Therefore, the measurement of serum HCV-RNA generally
11 serves to confirm the diagnosis of an ongoing infection [3]. In addition, monitoring of HCV
12 RNA is crucial for assessing the treatment response to anti-viral therapy [4]. Currently, real-
13 time polymerase chain reaction (PCR)-based quantitative determination is the diagnostic gold
14 standard. However, this assay is limited in developing countries due to costs and the
15 requirement for a real-time PCR machine.

16 Recently, a highly sensitive assay for measuring serum HCV core antigen (HCVcAg)
17 concentrations using a fully automated chemiluminescent microparticle immunoassay
18 (CMIA) has become commercially available [5]. A number of studies have demonstrated
19 good correlation between HCVcAg and HCV RNA levels, particularly in HCV mono-
20 infected individuals [6-13]. As a result, HCVcAg testing is considered to be an alternative
21 assay to conventional HCV RNA quantification with the advantages of its rapidity,
22 reproducibility and feasibility in resource limited settings [14]. To date, data regarding the
23 correlation between HCVcAg and HCV RNA levels in patients co-infected with human
24 immunodeficiency virus (HIV) are still limited [15-17]. In addition, the effect of different
25 viral genotypes, particularly HCV-6 prevalent in Southeast Asia [18], on HCVcAg levels and
26 the correlation with HCV RNA levels remains to be established.

27 Genome wide association studies (GWAS) have reported the association between
28 single nucleotide polymorphisms (SNPs) adjacent to the *interleukin-28B* (*IL-28B*) gene
29 (principally rs12979860) and treatment response in patients with chronic HCV infection [19-
30 21]. Recently, a transiently induced region (*interferon lambda-4*; *IFNL4*) harboring a
31 dinucleotide variant ss469415590 (TT or ΔG) showed strong linkage disequilibrium to
32 rs12979860 [22]. So far, this novel ss469415590 SNP is the only functional variant identified
33 in association with HCV clearance [23] and also a good predictor of treatment response to
34 pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy [24]. However, it is unclear

whether there is an association between this SNP and HCVcAg concentrations in patients with chronic HCV infection. Thus, the aims of this study were to evaluate the use of HCVcAg measurement with respect to HIV status, HCV genotypes, ss469415590 polymorphism and clinical parameters.

Materials and Methods

Patients

HCV/HIV co-infected patients, who were seropositive for both anti-HCV and HCV RNA, were enrolled consecutively from the HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT, Bangkok, Thailand). To compare the diagnostic role of HCVcAg between co-infected and mono-infected individuals, serum samples were obtained from patients with chronic HCV mono-infection, who were positive for both anti-HCV antibody and HCV RNA. These mono-infected patients were selected randomly from a pool of patients with chronic liver disease who were followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand). None of the patients enrolled in this study had hepatitis B virus (HBV) infection or received any antiviral therapy for chronic HCV infection when the blood sample was obtained. Written informed consent and ethical approval by the Institutional Review Board was obtained for all patients. All blood samples were stored at -80°C until examined.

Methods

HCV RNA quantification and genotypes

HCV RNA quantification was performed using the real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) (Abbott Molecular Inc. Des Plaines, IL, USA) in accordance with the manufacturer's instructions. The lower and upper detection limits of the assay were <12 IU/ml and 100,000,000 IU/ml, respectively.

HCV genotypes were determined by nucleotide sequencing of the core and NS5B regions followed by phylogenetic analysis as described previously [2].

HCVcAg quantification

Quantification of HCVcAg was performed using a fully automated CMIA according to the manufacturer's instructions (Abbott Diagnostics, Tokyo, Japan). The assay allows the quantitative determination of HCVcAg in a linear range from 3 to 20,000 femtomoles/liter (fmol/l), with the possibility of 1: 9 automated dilutions that extended the assay linearity to 180,000 fmol/l [5].

IFNL4 genotyping

The presence of *IFNL4* polymorphism ss469415590 was identified by RT-PCR assay using Taqman genotyping assay with MGB probes (Apply Biosystem, NY) as described previously [25]. Briefly, genomic DNA of patients with chronic HCV infection was extracted from 100 μ L of peripheral blood mononuclear cells (PBMC) using the QIAamp DNA Mini Kit (Qiagen, Germany). The reaction mixture consisted of 1 μ L of DNA extract, 200 nM of each probe (ss469415590_IFNL4_VIC: 5'-ATCGCAGAAGGCC-3' and ss469415590_IFNL4_FAM: 5'-ATCGCAGCGGCCC-3'), 900 nM of each primer (ss469415590_IFNL4_F: 5'-GCCTGCTGCA GAAGCAGAGAT-3' and ss469415590_IFNL4_R: 5'-GCTCCAGCGAGCGGTAGTG-3') (Applied Biosystems, NY), 5 μ L of 2x Perfect Taq Plus Master Mix (5 PRIME, Gaithersburg, MD) adjusted to 10 μ L final volume by distilled water. The PCR conditions were 2 minutes at 50°C, 10 minutes at 95 °C, 45 cycles of 15 seconds at 95 °C and 2 minutes at 60 °C. The fluorescent signal was detected at the end of each cycle. The genotype of ss469415590 was analyzed applying the End Point Genotyping method (Light Cycler 480, Roche Diagnostics, IN). For this SNP, TT and Δ G are defined as major and minor alleles, respectively.

Liver stiffness measurement

After fasting for at least 2 hours, liver stiffness measurement was obtained from each patient using transient elastography (FibroScan, Echosens, Paris, France) according to the manufacturer's instructions. Results were recorded in kilopascals (kPa) as the median value of all measurements. The procedure was based on at least 10 validated measurements: the success rate (ratio between numbers of validated and total measurements) was over 60% and interquartile range was less than 30% [26].

Data analysis

The Mann-Whitney U test or Student's test were used to compare continuous variables, and the χ^2 test or Fisher's exact test were used to compare categorical variables. The Spearman rank correlation was used to evaluate the relationships between variables. All data were analyzed by using SPSS Statistic Software Package for Windows version 20.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics

A total of 189 patients with chronic HCV infection were included in this study. There were 104 patients with HCV mono-infection and 85 patients with HCV/HIV co-infection. Table 1 summarizes the demographic and clinical characteristics of the patients. There were no significant differences in the baseline characteristics between each group in terms of mean age, body mass index (BMI), mean ALT level, the distribution of HCV genotypes and ss469415590 genotypes. HCV mono-infected patients had higher proportion of female gender compared with HCV/HIV co-infected patients ($p<0.001$). In contrast, HCV/HIV co-infected patients had significantly higher mean liver stiffness, $\log_{10}$ HCV RNA and $\log_{10}$ HCVcAg levels compared with HCV mono-infected patients ($p=0.004$, $p=0.018$ and $p=0.035$, respectively).

Correlation between $\log_{10}$ HCVcAg and $\log_{10}$ HCV RNA levels

In this study, serum HCV RNA and HCVcAg levels were detected in all cases. Mean $\log_{10}$ HCV RNA was 6.1 ± 1.0 IU/ml (range, 3.4-7.9IU/ml) and mean $\log_{10}$ HCVcAg was 3.6 ± 0.6 fmol/l (range, 1.9-4.6fmol/l). In the overall cohort, $\log_{10}$ HCVcAg levels significantly correlated with corresponding $\log_{10}$ HCV RNA levels ($r=0.889$, $p<0.001$) as shown in Fig.1a. Regarding HIV status, there were significant correlation between both markers in mono-infected patients ($r=0.845$, $p<0.001$) (Fig. 1b) and in co-infected patients ($r=0.939$, $p<0.001$) (Fig. 1c). However, mean $\log_{10}$ HCV RNA/HCVcAg ratio was not significantly different between mono-infected and co-infected patients (1.69 ± 0.18 vs. 1.70 ± 0.12 , respectively, $p=0.774$).

Additionally, we investigated the correlation between both markers in terms of HCV viremia. Overall, high viral load samples ($\log_{10}$ HCV RNA ≥ 5.0 IU/ml) showed a better correlation ($r=0.815$, $p<0.001$) than low viral load samples ($\log_{10}$ HCV RNA < 5.0 IU/ml ($r=0.555$, $p<0.001$). The same trends were also observed in the mono-infected group ($r=0.731$, $p<0.001$ and $r=0.462$, $p=0.015$, respectively) and the co-infected group ($r=0.902$, $p<0.001$ and $r=0.724$, $p=0.003$, respectively). Regardless of HIV status, mean $\log_{10}$ HCV RNA/HCVcAg ratio was significantly higher in the high viral load group compared with the low viral load group (1.72 ± 0.13 vs. 1.63 ± 0.23 , respectively, $p=0.002$).

Correlation between $\log_{10}$ HCVcAg levels and HCV genotypes

The correlation between $\log_{10}$ HCVcAg and $\log_{10}$ HCV-RNA levels was also evaluated with respect to HCV genotypes. Our data showed that $\log_{10}$ HCVcAg level significantly correlated with $\log_{10}$ HCV-RNA across HCV genotypes with the best correlation found in HCV-3 [HCV-1 ($r=0.796$, $p<0.001$), HCV-3 ($r=0.919$, $p<0.001$) and HCV-6 ($r=0.895$, $p<0.001$)]. When comparing the levels of $\log_{10}$ HCV-RNA and $\log_{10}$ HCVcAg among different HCV genotypes, we did not observe significant difference although patients with HCV-6 had slightly higher levels of both markers than the other groups (Table 2). In addition, the levels of both markers regarding HIV status were not significantly different across HCV genotypes (data not shown).

The relationship between HCV genotypes and clinical parameters was further examined. Patients with HCV-3 infection had significantly higher mean ALT level than patients with HCV-6 infection (90.8 ± 53.6 vs. 60.0 ± 50.2 U/L, respectively, $p=0.004$), but did not significantly differ from patients with HCV-1 infection (79.8 ± 56.1 U/L, $p=0.229$). There was no significant difference in liver stiffness among patients infected with HCV-1, HCV-3 and HCV-6.

Correlation between $\log_{10}$ HCVcAg levels and ss469415590 genotypes

Since a relatively small proportion of individuals displayed TT/ Δ G or Δ G/ Δ G genotypes of ss469415590, we grouped these genotypes together for statistical analysis. As shown in Fig 2a, patients harboring TT/TT genotype had significantly higher levels of $\log_{10}$ HCV RNA than those with non-TT/TT genotype (6.2 ± 1.0 vs. 5.6 ± 1.0 IU/ml, respectively, $p=0.014$). The similar observation was found for $\log_{10}$ HCVcAg levels (3.6 ± 0.6 vs. 3.3 ± 0.5 fmol/l, respectively, $p=0.004$) (Fig. 2b).

In the mono-infected group, there was no significant difference between patients with TT/TT and non-TT/TT genotypes in term of $\log_{10}$ HCV RNA (6.0 ± 0.9 vs. 5.4 ± 1.2 U/L, respectively, $p=0.119$) and $\log_{10}$ HCVcAg levels (3.6 ± 0.6 vs. 3.3 ± 0.6 fmol/l, respectively, $p=0.193$), although a trend towards increased both markers were observed among the TT/TT genotype. In the co-infected group, patients harboring TT/TT genotype had significantly higher levels of $\log_{10}$ HCV RNA and $\log_{10}$ HCVcAg than those with the non-TT/TT genotype (6.4 ± 1.0 vs. 5.8 ± 0.8 U/L, respectively, $p=0.034$ and 3.8 ± 0.5 vs. 3.3 ± 0.3 fmol/l, respectively, $p=0.002$).

1 The relationship between ss469415590 genotypes and clinical parameters was also
2 explored. Regarding baseline serum ALT level, there was no significant difference between
3 patients with TT/TT and non-TT/TT genotypes (83.8 ± 56.1 vs. 60.7 ± 40.2 U/L, respectively,
4 $p=0.058$), although a trend towards increased ALT levels was observed among the TT/TT
5 genotype group. Similarly, there was no significant difference in liver stiffness between
6 patients with TT/TT and non-TT/TT genotypes (11.9 ± 10.1 vs. 13.9 ± 11.2 kPa, respectively,
7 $p=0.543$).

8 9 *Correlation between HCVcAg levels and clinical parameters*

10 In addition to associations with HCV genotypes and ss469415590 genotypes, the
11 relationship between HCVcAg levels and clinical parameters was examined. In this study,
12 $\log_{10}$ HCVcAg levels were not correlated with serum ALT levels ($r=-0.045$, $p=0.542$) and
13 liver stiffness ($r=-0.122$, $p=0.155$). Similarly, $\log_{10}$ HCV RNA levels were not correlated with
14 serum ALT ($r=-0.031$, $p=0.676$) and liver stiffness ($r=-0.078$, $p=0.365$). We also analyzed the
15 correlation between HCVcAg levels and CD4+T cell counts in the co-infection group. In this
16 study, $\log_{10}$ HCVcAg levels were not correlated with CD4+T cell counts ($r=0.093$, $p=0.396$).
17 Similarly, $\log_{10}$ HCV RNA levels were not correlated with CD4+T cell counts ($r=0.063$,
18 $p=0.569$).

19 20 **Discussion**

21 Detection of HCVcAg levels in the serum has emerged as a potential marker for
22 active HCV infection and may be used to evaluate response to antiviral therapy and disease
23 progression. Several recent data have demonstrated that serum HCVcAg concentrations
24 correlate well with HCVRNA levels in HCV mono-infected patients [6-12]. In the present
25 study, we further investigated the use of HCVcAg measurement in HCV/HIV co-infected
26 patients. In particular, we directly compared the use of HCVcAg measurement between HCV
27 mono-infected and HCV/HIV co-infected individuals, with respect to HCV genotypes, host
28 genetic variations and other disease parameters that could influence the clinical outcome of
29 chronic HCV infection.

30 Previous studies estimated that the sensitivity of the ARCHITECT HCVcAg assay is
31 approximately 3 fmol/l, which corresponds to 500-2500 IU/ml of HCV-RNA [7,8,12]. Thus,
32 the concordance of detectable HCV RNA and HCVcAg of all samples in this cohort likely
33 resulted from high viremia, which is a typical feature of untreated individuals. Our data
34 confirmed previous reports that HCVcAg levels had a good correlation with the

1 corresponding HCV RNA levels in both mono- and co-infection groups. It should be noted
2 that the correlation between both assays appeared to be higher among the co-infected group
3 than the mono-infected group ($r=0.939$ and $r=0.845$, respectively). Indeed, the correlation
4 coefficients between the two markers in co-infected patients exceeded 0.90 in most previous
5 studies [15-17]. For example, a recent cross-sectional survey conducted in China
6 demonstrated that the correlation coefficients between HCVcAg and HCV RNA
7 concentrations was higher in the co-infected than mono-infected group ($r=0.952$ and $r=0.808$,
8 respectively) [17]. Another study conducted in Germany showed that the correlation
9 coefficients between these two markers in HCV/HIV co-infection was 0.97 compared to 0.75
10 in HCV mono-infection [16].

11 It should be mentioned that the correlation between HCVcAg and HCV RNA levels
12 was also typically high among other groups of patients with impaired immune response,
13 including liver and kidney transplant recipients [27] and patients with end-stage kidney
14 disease [28]. In contrast, such correlation was less consistent among immunocompetent
15 individuals. Thus, it appears that the correlation between both markers of HCV replication
16 might be in part influenced by the immune status of infected individuals. The explanation of
17 these interesting findings are unclear, but might be related to an interaction between HCVcAg
18 and anti-HCV antibodies. In general, it was proposed that anti-HCV antibodies might mask
19 the detection of HCVcAg or could lead to an enhanced clearance of circulating antigen.
20 However, among patients with impaired immune response including HIV-infected
21 individuals or patients receiving immunosuppressive therapy, lower antibody levels
22 presumably do not interfere with the measurement of HCVcAg [16,27]. In contrast, patients
23 with HBV/HCV co-infection did not show good correlation between the two markers [16],
24 which might result from the complexity of viral interaction and fluctuation of virological
25 dominance over time [29]. Collectively, these data indicate that HCVcAg measurement is an
26 excellent surrogate marker for monitoring HCV viral replication in HCV/HIV co-infected
27 patients.

28 Of note, mean serum concentrations of HCV RNA and HCVcAg in our study were
29 significantly higher in co-infected than mono-infected groups. These results are consistent
30 with previous reports that HCV/HIV co-infection is associated with persistent HCV viremia
31 and higher HCV viral load [30]. The increased HCV RNA levels among co-infected patients
32 are thought to be in part associated with the decline in CD4⁺ and CD8⁺ T-cell responses to
33 HCV infection. Our data also showed that co-infected patients had significantly higher liver
34 stiffness, representing more advanced liver fibrosis compared to mono-infected patients.

1 These findings are in agreement with previous studies that the prevalence of significant liver
2 fibrosis is usually high among co-infected individuals [31]. In the context of HCV/HIV co-
3 infection, emerging data have suggested that multiple conditions, including increases in pro-
4 fibrogenic cytokine expression and secretion, enhancement of oxidative stress production,
5 and increases in hepatocyte apoptosis all contribute to accelerated fibrosis [32]. In contrast to
6 previous studies, our results did not demonstrate the correlation between HCVcAg
7 concentration and liver inflammatory activity and liver fibrosis [33].

8 Unlike previous reports of HCVcAg, our study included a proportion of patients
9 infected with HCV-6. As mentioned previously, the data on this HCV genotype are sparse
10 due to its restricted distribution in south China and Southeast Asia [18]. In this study, we
11 observed slightly different correlations between HCVcAg and HCV RNA levels across HCV
12 genotypes with the best correlations found in HCV-3, followed by HCV-6 and HCV-1,
13 respectively. However, HCVcAg and HCV RNA levels did not vary according to HCV
14 genotypes. Of note, samples with high viral load showed a better correlation of HCVcAg and
15 HCV RNA concentrations than those with low viral load. In addition, mean HCV
16 RNA/HCVcAg ratio was significantly higher among samples with high viremia compared to
17 those with low viremia. These observations were presumably related to a higher proportion of
18 unmasked HCVcAg detectable by the HCVcAg assay in the setting of high viremia, as
19 described previously in HCV/HIV co-infected patients and transplant recipients [16,27].

20 Recent reports have shown that the ΔG variant of ss469415590 is associated with
21 poorer HCV clearance and response to antiviral therapy than the TT allele [22,24]. However,
22 the association of this polymorphism with HCVcAg levels remains to be elucidated. To our
23 knowledge, our study is the first to assess IFNL4 genetic status in relation to HCVcAg levels
24 in patients with chronic HCV infection. We found that patients harboring TT/TT genotype
25 had significantly higher levels of HCV RNA and HCVcAg than those with the non-TT/TT
26 genotype, regardless of HIV status and HCV genotypes. This finding is consistent with
27 previous data in that the favorable CC genotype of rs12979860, which is associated with
28 better treatment response, is also related to higher baseline viral load [19,34]. The effects on
29 HCV RNA and HCVcAg concentrations might be in part related to biological interaction
30 between HCV replication and IFNL4 protein. As shown in previous studies, the IFNL4 ΔG
31 variant could induce weak expression of interferon-stimulated genes, which in turn provides
32 an antiviral response in reducing HCV viral load. In contrast, the ΔG variant also diminishes
33 the responsiveness to type I and type III interferon required for effective treatment during
34 antiviral therapy [22,35]. More information is needed to understand the mechanisms that

underlie this association, as well as the clinical impact of ss469415590 polymorphisms on HCVcAg detection.

In conclusion, our data showed that there was an excellent correlation between HCV RNA and HCVcAg concentrations, particularly in HCV/HIV co-infected individuals. We also demonstrated that serum levels of HCVcAg were association with ss469415590 polymorphism. As the HCVcAg assay is a reliable test and has the advantages of being rapid and reproducible, its measurement could be used as an alternative to HCV RNA assays in resource-limited settings.

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Table 1.

Demographics and characteristics of HCV mono- and co-infected patients.

Characteristics	Mono-infection (n=104)	Co-infection (n=85)	P value
Age, years	45.6 ± 9.7	44.1 ± 7.2	NS
Sex			<0.001
Males	61 (58.7)	74 (87.1)	
Females	43 (41.3)	11 (12.9)	
BMI, kg/m ²	23.9 ± 4.2	22.6 ± 6.5	NS
ALT, U/L	74.3 ± 55.6	88.8 ± 53.1	NS
Liver stiffness, kPa	9.1 ± 5.6	14.1 ± 12.0	0.004
CD4+T cell counts, cell/μl	441.4 ± 216.6		
HCV RNA, log ₁₀ IU/ml	6.0 ± 1.0	6.3 ± 1.0	0.018
HCVcAg, log ₁₀ fmol/l	3.5 ± 0.6	3.7 ± 0.5	0.035
HCV genotypes			NS
1	38 (36.5)	29 (34.1)	
3	41 (39.4)	44 (51.8)	
6	25 (24.1)	12 (14.1)	
IFNL4 genotypes			NS
TT/TT	92 (88.5)	74 (87.1)	
TT/ΔG	7 (6.7)	10 (11.8)	
ΔG/ΔG	5 (4.8)	1 (1.1)	

BMI, Body mass index; ALT, alanine aminotransferase

Data are presented as mean ± standard deviation or number (%)

Table 2.

Serum levels of log₁₀HCV-RNA and log₁₀HCVcAg according to HCV genotypes.

	Genotype 1 (n=67)	Genotype 3 (n=85)	Genotype 6 (n=37)	<i>P</i> value
HCV RNA, log ₁₀ IU/ml	6.0 ± 0.9	6.1 ± 1.0	6.4 ± 1.1	0.227
HCVcAg, log ₁₀ fmol/l	3.5 ± 0.5	3.6 ± 0.6	3.8 ± 0.6	0.058

Figure Legends

Figure 1. Correlation between log₁₀HCVcAg and log₁₀HCV RNA levels in (a) all patients, (b) HCV mono-infected patients, and (c) HCV/HIV co-infected patients

Figure 2. Mean levels of HCV markers according to ss469415590 genotypes as measured by (a) log₁₀HCV-RNA levels and (b) log₁₀HCVcAg levels

Distribution of HCV genotype and single nucleotide polymorphisms (SNPs) of *IL-28B* gene in HIV/HCV-coinfected Thai populations

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Introduction: Hepatitis C virus (HCV) infection remains a major silent killer, worldwide, particularly in resource poor settings where treatment of hepatitis C is mainly impossible. Pegylated interferon- α (PEG-IFN) plus ribavirin (RBV) are the recommended treatment for patients with chronic hepatitis C genotype 2/3. Recent study revealed that treatment responses against HCV infection by PEG-IFN and RBV are significantly associated with the single nucleotide polymorphisms (SNPs) of interleukin-28B (IL-28B) gene. There are limited data about the HCV genotype and SNPs of IL-28B in HIV-infected Thai population. Therefore, we aimed to investigate HCV genotype and the SNP patterns of the IL-28B gene in our HIV/HCV co-infection.

Methods: Quantification of HCV RNA was done by a real-time polymerase chain reaction assay (Abbott with lower limit of detection of <12 copies/ml). HCV RNA-positive samples based on reverse transcriptase-polymerase chain reaction (RT-PCR) of the 5'UTR were amplified with primer specific for the core and NS5B regions. Nucleotide sequences of both regions were analyzed for the genotype by phylogenetic analysis. DNA sample was extracted from PBMCs or sera. Then SNPs within IL-28B gene were detected by TaqMan real-time PCR (rs8099917 and rs12979860). The data were analyzed by allelic discrimination (AD) software on the ABI-7900HT.

Results: Totally 60 HIV/HCV-co-infected patients were studied. Median HCV RNA were 5.8 log₁₀copies/mL, 70% of them had HCV RNA >100,000 copies/mL. After sequencing, the phylogenetic analyses in this study showed that genotype 3 was the most prevalent in this population (56%); following by genotype 1 (30%) and 6 (13%). Approximately 4% of them had infected for both genotypes 1 and 3. For IL-28B at rs8099917 and rs12979860 position, 95% of them were major allele (T/T or C/C) and 5% were heterozygous (T/G or C/T).

Conclusions: HCV genotype 3 is the most prevalent in our HIV/HCV coinfection. 95% of our patients have favorable IL-28B gene. This data are useful for further chronic hepatitis C treatment in Thailand.

Interim analysis of randomized study comparing efficacy of peginterferon-alpha-2b monotherapy versus combination with entecavir in HBeAg-negative chronic hepatitis B

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Back ground/Aims: The combination therapy of peginterferon (PEG-IFN) and a potent antiviral drug might improve the response rate in patients with chronic hepatitis B. The aim of this study was to compare the efficacy of PEG-IFN plus entecavir and PEG-IFN alone in Thai patients with HBeAg-negative chronic hepatitis B.

Methods: 76 treatment-naïve patients with HBeAg-negative chronic hepatitis B were randomly assigned to either combination of PEG-IFN alpha-2b (1.5 microgram/kg weekly for 48 weeks) and entecavir (0.5 mg/day for 48 weeks) (group 1) or PEG-IFN alpha-2b monotherapy (1.5 microgram/kg weekly for 48 weeks) (group 2). Serum HBV DNA and HBsAg levels were quantified using commercially available assays (Roche Diagnostics). Data were evaluated based on the intention-to-treat analysis.

Results: There were 37 and 39 patients in groups 1 and 2, respectively. At baseline, no significant difference between groups 1 and 2 was observed with respect to sex, mean age (42 vs. 39 years), ALT level (67 vs. 71 IU/L), HBV DNA level (4.9 vs. 5.1 log₁₀IU/mL) and HBsAg level (3.2 vs. 3.5 log₁₀IU/mL). At week 48, HBV DNA was undetectable in 29/37 (78.4%) in group 1 versus 13/39 (33.3%) patients in group 2 (p<0.001). The mean HBV DNA level in group 1 was significantly lower than that of group 2 (0.2 vs. 1.6 log₁₀IU/mL, respectively) (p<0.001). However, there was no significant difference in mean HBsAg level between both groups (2.4 vs. 2.4 log₁₀IU/mL). HBsAg clearance was achieved in 1/37 (2.7%) and 3/39 (7.7%) in groups 1 and 2, respectively (p=0.33).

Conclusions: At the end of treatment, PEG-IFN plus entecavir leads to a higher rate of HBV DNA suppression than PEG-IFN monotherapy. However, the combination therapy might not superior to PEG-IFN monotherapy on the basis of HBsAg reduction and HBsAg clearance.

A randomized study comparing efficacy of peginterferon-alpha-2b monotherapy versus combination with entecavir in HBeAg-negative chronic hepatitis B: Analysis of week 72

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Back ground/Aims: The combination therapy of peginterferon (PEG-IFN) and a potent antiviral drug might improve the response rate in patients with chronic hepatitis B. The aim of this study was to compare the efficacy of PEG-IFN plus entecavir and PEG-IFN alone in Thai patients with HBeAg-negative chronic hepatitis B.

Methods: 106 treatment-naïve patients with HBeAg-negative chronic hepatitis B were randomly assigned to either combination of PEG-IFN alpha-2b (1.5 microgram/kg weekly for 48 weeks) and entecavir (0.5 mg/day for 48 weeks) (group 1) or PEG-IFN alpha-2b monotherapy (1.5 microgram/kg weekly for 48 weeks) (group 2). Serum HBV DNA and HBsAg levels were quantified using commercially available assays (Roche Diagnostics). Virological response (VR) was defined as HBV DNA level <2,000 IU/mL at week 72.

Results: There were 51 and 55 patients in groups 1 and 2, respectively. At baseline, no significant difference between groups 1 and 2 was observed with respect to sex, mean age (41.3 vs. 40.6 years), ALT level (58 vs. 60 IU/L), HBV DNA level (5.0 vs. 5.1 log₁₀IU/mL) and HBsAg level (3.3 vs. 3.4 log₁₀IU/mL). At week 48, the mean HBV DNA level in group 1 was significantly lower than that of group 2 (0.2 vs. 1.5 log₁₀IU/mL, respectively) (p<0.001). However, there was no difference in mean HBsAg level between both groups (2.5 vs. 2.4 log₁₀IU/mL, p=0.532). At week 72, there was no significant difference between groups 1 and 2 with respect to mean HBV DNA level (2.9 vs. 3.4 log₁₀IU/mL, p=0.144) and HBsAg level (2.8 vs. 2.7 log₁₀IU/mL, p=0.503). VR at week 72 was achieved in 29 (56.9%) of group 1 and 27 (49.1%) of group 2 (p=0.443). HBsAg clearance was detected in 3 (5.9%) and 5 (9.1%) in groups 1 and 2, respectively (p=0.717).

Conclusions: PEG-IFN plus entecavir led to a higher rate of HBV DNA suppression than PEG-IFN monotherapy at the end of treatment. However, both treatment regimens were comparable on the basis of viral suppression, HBsAg reduction and HBsAg clearance at 24 week after the end of therapy.

Serum levels of interferon-gamma-inducible protein 10 (IP-10) in each phases of chronic hepatitis B and response to peginterferon therapy in HBeAg-positive and HBeAg-negative chronic hepatitis B.

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Introduction: Serum levels of interferon-gamma inducible protein 10 (IP-10) are a marker for immune activity and might associated with an increased probability of HBeAg loss after pegylated interferon (PegIFN) therapy. This study aimed to determine serum levels of IP-10 in each phase of chronic hepatitis B and after starting treatment with PegIFN.

Materials and Methods: 235 chronic hepatitis B patients without previous treatment and 50 healthy control subjects were recruited. Demographic and clinical data of patients were recorded. All patients were classified into immune tolerance (n=49), immune clearance (n=48), low replication (n=86) and HBeAg negative hepatitis (n=52) base on previous six month data of serum ALT, HBV VL, HBeAg status. Of those, 60 patients with high ALT (HBeAg positive (n=30) and HBeAg negative (n=30)) were treated with PegIFN for 48 weeks. Serum IP-10 was measured at baseline, on-treatment week 12, 24, 48 and week 72 post-treatment.

Results: One hundred and forty-four males and 79 females, median age of 35 years chronic hepatitis B patients and 50 healthy controls were included. Mean level of serum IP-10 at baseline were 240±201, 357±277, 238±153, 288±353 and 202.6±75 pg/ml in IT, IC, LR, ENH and control groups. At baseline IP-10 level were significantly higher in IC compare with IT, LR and control group (Figure 1). However, no statistical difference between mean serum IP-10 level in IC and ENH patients. Dynamic change of post PegIFN treatment serum IP-10 level was difference between HBeAg positive and HBeAg negative patients. In HBeAg positive group, serum IP-10 level was statistically increased in week 12 and 24 after treatment and gradual declining of serum IP-10 level was observed after week 24 (Figure 2). As compare with HBeAg negative group, serum IP-10 level was non-statistical decline after PegIFN treatment. In HBeAg-positive patients, IP-10 level were higher in responders (achieved HBeAg seroconversion at week 72) compare with non-responders (Table 1).

Conclusions: Baseline IP-10 was significantly high in IC phase of disease of chronic hepatitis B. HBeAg-positive patients had higher serum IP-10 response at week 12 and 24 after PegIFN treatment.

Table 1. In HBeAg-positive patients, IP-10 level were higher in responders (achieved HBeAg seroconversion at week 72) compare with non-responders among HBeAg positive patients. *

IP-10 level (pg/ml)	Responders (n=14)	Non-Responder (n=15)	P value
At base line	394±473	318±318	0.61
Week 12	606±342	503±340	0.42
Week 24	579±211	395±210	0.03*
Week 48	428±375	375±246	0.53
Week 72	299±214	214±112	0.35

* Plus-minus values are mean ± SD for all comparisons

Figure 1. Mean level of serum IP-10 in healthy control (n=50) and each phases of chronic hepatitis B included into immune tolerance (IT, n=49), immune clearance (IC, n=48), low replication (LR, n=86) and HBeAg negative hepatitis (ENH, n=52)

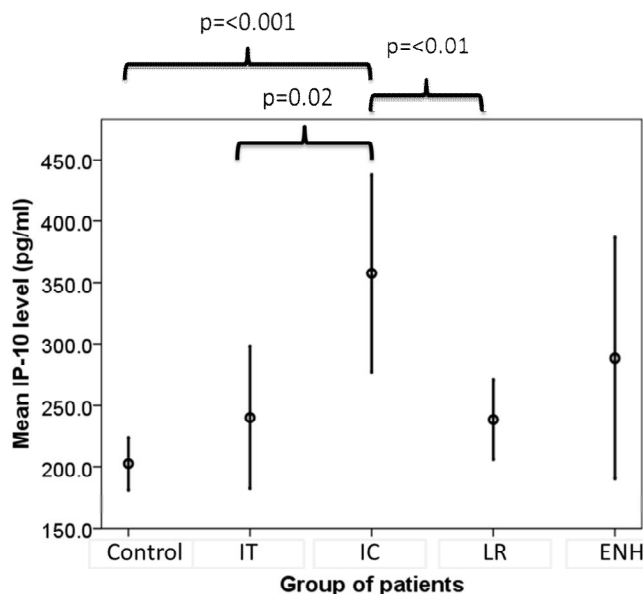
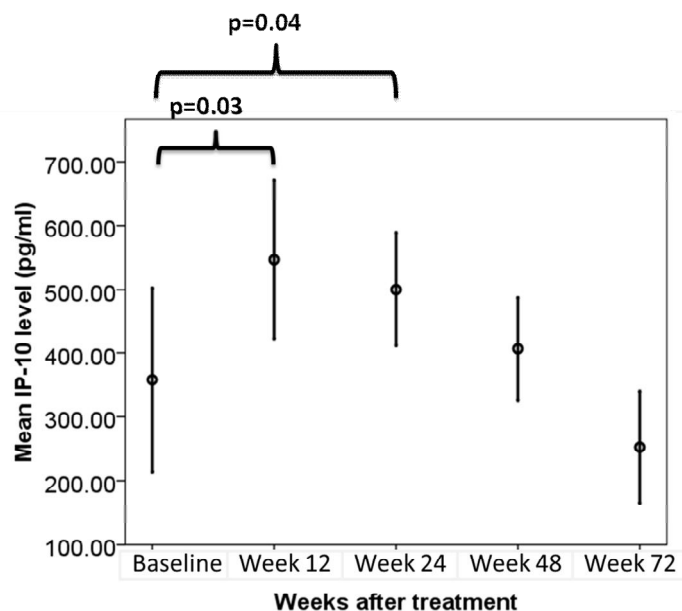


Figure 2. Mean level of serum IP-10 at baseline, after starting treatment week 12, 24, 48 and 72 in HBeAg-positive chronic hepatitis B patients.



Higher Rate of Liver Fibrosis Progression in Untreated Chronic Hepatitis C with High Baseline FIB-4 $\geq$ 1.45: A Prospective Study

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Background: Current noninvasive tools to assess the rate of liver fibrosis progression in patients with chronic hepatitis C (CHC) include aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) ratio, AST: platelet ratio index (APRI), FIB-4 score and liver stiffness (LS) scores obtained from the FibroScan. Little is known about liver fibrosis progression among CHC with and without HIV co-infection from resource limited settings. We aimed to assess the rate and predictors for liver fibrosis progression in untreated CHC patients by using AST/ALT ratio, APRI, FIB-4 score and FibroScan over 1-year of follow-up.

Methods: CHC patients with and without HIV co-infection, were prospectively enrolled between January 1, 2011 and December 31, 2011. LS, AST/ALT ratio, APRI and FIB-4 scores were obtained at baseline and 1 year later (range 10-14 months). Advanced fibrosis (F3-4) was defined as having LS of ≥ 9.5 KPa, AST/ALT.1, APRI > 0.5 or FIB-4 $\geq$ 1.45. Disease progression was defined when the fibrosis becomes advanced or having LS increment of at least 2 KPa in patients with baseline advanced fibrosis. Logistic regression analysis was used to predict the liver fibrosis progression at 1 year of follow-up. Results: A total of 102 untreated CHC patients (52 CHC with HIV co-infection) were enrolled and the mean age was 40.9 ± 8.2 years. At baseline, 72% and 28% of the patients had non advanced and advanced liver fibrosis based on the LS criteria, respectively. Baseline characteristic data of the study population was shown in Table 1. LS scores among CHC patients with HIV were significantly higher than those without HIV (8.1 vs 6.5 KPa, $p=0.05$). Majority of the CHC patients with HIV co-infection (96%) were on HAART and had baseline HIV RNA, 50 copies/ml. The overall median rate of fibrosis progression among untreated CHC patients in nonadvanced patients was 0.2 KPa/year (range -3 to 3.8) and in advanced patients was 4.25 KPa/year (range 2 to 24.2 KPa), $p<0.001$. The rate of progression for liver fibrosis between the untreated CHC with and without HIV infection was not significantly different. After 1 year of observation 38% of the advanced fibrosis patients had liver fibrosis progression, whereas 1.2% of the non-advanced patients developed advanced fibrosis. In univariate analysis, the predictors for liver fibrosis progression in advanced group among untreated CHC patients over 1-year of follow-up were shown in Table 2. The baseline FIB-4 score of ≥ 1.45 was significant predictor (OR 8.2, 95% CI=2.66-25.17, $p=0.001$) (By logistic regression model). FIB-4 scores were positively correlated with LS ($R=0.47$; $p=0.001$).

Conclusion: The median rate of liver fibrosis progression in untreated CHC patients with baseline advanced fibrosis is approximately 4.25 KPa/year. Patients with baseline FIB-4 scores of ≥ 1.45 tend to have faster liver disease progression if left untreated.

TITLE: Associations of single nucleotide polymorphism rs1053004 in *signal transducer and activator of transcription 3 (STAT3)* for risk of hepatocellular carcinoma in Thai patients with chronic hepatitis B

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ABSTRACT BODY:

Background: Signal transducers and activator of transcription (STAT) proteins, a family of transcription factors that play pivotal roles in cytokine signaling pathways. Several evidences indicated that single nucleotide polymorphisms (SNPs) in *STATs* gene such as rs2293152 and rs1053004 at *STAT3* and rs7574865 at *STAT4* have been associated with chronic hepatitis B (CHB) induced hepatocellular carcinoma (HCC).

Objective: This study aims to describe the association between these SNPs and HCC in Thai patients with CHB.

Method: Study subjects were enrolled and divided into 3 groups including CHB-related HCC (n=192), CHB without HCC (n=200) and healthy controls (n=190). The rs2293152 and rs7574865 SNPs were genotyped using polymerase chain reaction – restriction fragment length polymorphism whereas the rs1053004 SNP was genotyped using allelic discrimination assays based on TaqMan real-time PCR.

Results: Data analysis revealed that the distribution of rs2293152 and rs1053004 at *STAT3* and rs7574865 at *STAT4* genotypes were in Hardy-Weinberg equilibrium ($P > 0.05$). rs2293152 SNP on *STAT3* gene was not significantly associated with the risk of HCC ($P > 0.05$) whereas the CC genotype of rs1053004 SNP was significantly associated with an increased risk of HCC compared with the CHB without HCC (odds ratio=1.85, 95% confidence interval=1.00-3.43, $P=0.049$). In addition, the genotype of rs7574865 SNP at *STAT4* (GG versus TT+GT) was significantly associated with an increased risk of HCC when compared with the healthy controls (odds ratio=1.71, 95% confidence interval=1.13-2.59, $P=0.011$).

Conclusion: Therefore, these findings provided important evidence that the rs1053004 SNP at *STAT3* and rs7574865 SNP at *STAT4* were significantly associated with HCC risk and might be used as a novel genetic marker for HCC in Thai population.

Role of interferon lambda-4 and inosine triphosphatase polymorphisms in the treatment of Thai patients with chronic hepatitis C virus genotype 3

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PURPOSE OF THE STUDY: Recent studies have shown that interferon lambda-4 (IFNL4) and inosine triphosphatase (ITPA) polymorphisms are associated with pegylated interferon (PEG-IFN) plus ribavirin (RBV) treatment response and RBV-induced anemia, respectively, in chronic hepatitis C virus (HCV) infection. However, their roles in Asian populations infected with HCV genotype 3 (HCV-3) remain unclear.

METHOD: In this study, 123 Thai individuals with chronic HCV-3 infection treated with PEG-IFN plus RBV for 24 weeks were included. DNA extracted from blood samples was tested for the IFNL4 ss469415590 and ITPA rs1127354. The associations of the polymorphisms and treatment response and hemoglobin reduction (> 3 g/dL at week 4 of therapy) were analyzed.

SUMMARY OF RESULTS: The distribution of TT/TT, ΔG/TT and ΔG/ΔG genotypes of ss469415590 was 111(90.3%), 11(8.9%) and 1(0.8%), respectively, while CC, CA and AA genotypes of rs1127354 was 93 (75.6%), 26(21.1%) and 4 (3.3%), respectively. The overall rapid virological response (RVR) and sustained virological response (SVR) rates were 84.6% and 79.7%, respectively. 30 (24.4%) patients required reduction in RBV dosages. ss469415590 TT/TT genotype, when compared to non-TT/TT genotype, was significantly associated with an increased RVR rate (93.3% and 73.7%, respectively, p=0.047) but not with an increased SVR rate. rs1127354 CC genotype, when compared to non-CC genotype, was associated with higher proportion of hemoglobin reduction (29.0% and 10.0%, respectively, p=0.035). The ITPA polymorphisms showed no association with RVR and SVR rates.

CONCLUSION: In Thai patients with chronic HCV-3 infection, IFNL4 polymorphism was associated with RVR but not SVR. ITPA polymorphism influenced the degree of anemia during therapy but not improved clinical outcomes.

IFNL3 (IL28B) and IFNL4 polymorphisms are not associated with treatment response to pegylated interferon-based therapy in Thai patients with HBeAg-negative chronic hepatitis B

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Objectives: Recent studies have shown an association between single nucleotide polymorphisms (SNPs) in the interferon lambda-3 (IFNL3 or IL-28B) and IFNL4 genes and treatment response to chronic hepatitis C. However, the importance of these SNPs in chronic hepatitis B is unclear. The aim of this study was to investigate whether these SNPs could predict treatment response to pegylated interferon (PEG-IFN)-based therapy in patients with HBeAg-negative chronic hepatitis B.

Methods: We analyzed data from a randomized study comparing efficacy of PEG-IFN monotherapy (group 1) versus simultaneous combination with entecavir (group 2) in Thai patients with HBeAg-negative chronic hepatitis B. All patients were treated for 48 weeks and followed-up for additional 48 weeks to assess virological response, which was defined as HBV DNA level <2,000 IU/mL. DNA extracted from blood samples was analyzed for the SNPs rs12979860 and ss469415590.

Results: A total 126 patients (63 patients in each group) were enrolled in the study. There was no significant difference in rate of virological response between group 1 and group 2 (41.3% vs. 38.1%, respectively, $p=0.856$). HBsAg clearance was detected in 6 (9.5%) and 3 (4.8%) in groups 1 and 2, respectively ($p=0.491$). Overall, the distribution of CC, CT and TT genotypes of rs12979860 was 109 (86.5%), 17 (13.5%) and 0 (0%), respectively, while the distribution of TT/TT, $\Delta G/TT$ and $\Delta G/\Delta G$ genotypes of ss469415590 was identical. There was no association between rs12979860 or ss469415590 genotypes and virological response at the end of follow-up. Similarly, there was no association between these genotypes and HBsAg clearance. In addition, there was no difference in baseline HBV DNA, HBsAg and ALT levels according to the SNP genotypes.

Conclusion: These SNPs were not associated with treatment response to PEG-IFN-based therapy in Thai patients with HBeAg-negative chronic hepatitis B.

Table 1 Baseline characteristics and treatment response at week 96 according to SNP rs12979860

	CC genotype (n=109)	CT genotype (n=17)	P
Baseline characteristics			
Age (year)	39.9 ± 9.5	41.9 ± 9.8	0.444
Sex (male)	74 (67.9%)	15 (88.2%)	0.149
ALT (U/L)	72.5 ± 32.9	83.6 ± 53.3	0.415
Log ₁₀ HBV DNA (IU/ml)	5.4 ± 0.8	5.5 ± 1.0	0.686
Log ₁₀ HBsAg (IU/ml)	3.5 ± 0.5	3.4 ± 0.4	0.585
Liver necroinflammation (A2-A3)	44 (40.4%)	7 (41.2%)	1.000
Liver fibrosis (F3-F4)	18 (16.5%)	1 (5.9%)	0.466
Treatment response			
ALT normalization	77 (70.6%)	15 (88.2%)	0.154
HBV DNA < 2000 IU/ml	44 (40.4%)	6 (35.3%)	0.794
HBV DNA < 10 IU/ml	10 (9.2%)	1 (5.9%)	0.655
HBsAg clearance	7 (6.4%)	2 (11.8%)	0.349
HBsAg seroconversion	6 (5.5%)	1 (5.9%)	0.950