รายงานวิจัยฉบับสมบูรณ์

โครงการอณูไวรัสวิทยาของไวรัสตับอักเสบบีและซี ที่สัมพันธ์กับการเกิดมะเร็งตับและ การตอบสนองต่อการรักษาด้วยยาต้านไวรัส

Molecular Virology of Hepatitis B and C Viruses Associated with Hepatocellular Carcinoma and Treatment Response

โดย

รองศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์และคณะ

กรกฎาคม 2555

สัญญาเลขที่ BRG5380012

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คณะผู้วิจัย
1. รองศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์
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2. ศาสตราจารย์นายแพทย์ยง ภู่วรวรรณ
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและจุฬาลงกรณ์มหาวิทยาลัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย
สกว.และจุฬาลงกรณ์มหาวิทยาลัย ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "อณูไวรัสวิทยาของไวรัสตับอักเสบบีและซีที่สัมพันธ์กับการเกิดมะเร็งตับและการตอบสนองต่อการรักษาด้วยยาต้านไวรัส" ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ BRG5380012 ระยะเวลาดำเนินการ 2 ปี ตั้งแต่ 31 พฤษภาคม 2553 ถึงวันที่ 30 พฤษภาคม 2555 ผู้รับทุนขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย ที่ให้การสนับสนุนอย่างเต็มที่จนทำให้โครงการวิจัยนี้สำเร็จลุล่วงด้วยดี ขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญยิ่งในความสำเร็จของโครงการ ได้แก่ รองศาสตราจารย์แพทย์หญิงวโรชา มหาชัย ผู้ช่วยศาสตราจารย์นายแพทย์ปิยะวัฒน์ โกมลมิศร์ ผู้ช่วยศาสตราจารย์แพทย์หญิงนฤมล วิเศษโอภาส (คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย) รองศาสตราจารย์ ดร. ปรัชญา คงทวีเลิศ (คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่) และผู้ช่วยวิจัยที่มีส่วนช่วยเหลือให้โครงการวิจัยสำเร็จลงได้แก่ นางสาวอภิรดี เทียมบุญเลิศ นางสาวภัทรธิดา สงวนหมู่และนางสาวศรัณย์ธร อัครธำรงสิน รวมทั้งภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในฐานะสถาบันต้นสังกัดที่ให้การสนับสนุนโดยอำนวยความสะดวกในโครงการเป็นอย่างดียิ่ง

สารบัญ

บทคัดย่อ

รหัสโครงการ: BRG5380012

ชื่อโครงการ: อณูไวรัสวิทยาของไวรัสตับอักเสบบีและซีที่สัมพันธ์กับการเกิดมะเร็งตับและการตอบสนองต่อการรักษาด้วยยาต้านไวรัส

ชื่อนักวิจัยและสถาบัน: รองศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์
ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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ระยะเวลาโครงการ: 31 พฤษภาคม 2553 - 30 พฤษภาคม 2555

โครงการวิจัย:

ไวรัสตับอักเสบบีและไวรัสตับอักเสบซีเป็นสาเหตุสำคัญของเกิดโรคตับอักเสบแบบเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับ ปัจจุบันเชื่อว่าปัจจัยต่างๆที่เกี่ยวข้องกับไวรัสเช่นความแตกต่างของสายพันธุ์ การเกิดสายพันธุ์ผสมและการกลายพันธุ์ของยีนในบางตำแหน่ง รวมทั้งปัจจัยต่างๆของผู้ป่วยมีผลต่อการดำเนินโรคที่แตกต่างกัน จุดมุ่งหมายของโครงการนี้เพื่อศึกษาความชุกและความสำคัญทางคลินิกของปัจจัยต่างๆดังกล่าวในผู้ที่มีการติดเชื้อไวรัสตับอักเสบบีหรือไวรัสตับอักเสบซีแบบเรื้อรัง ผลการศึกษาในเชิงระบาดวิทยาพบว่าเชื้อไวรัสสายพันธุ์ซี (genotype C) และสายพันธุ์บี (genotype B) เป็นสายพันธุ์ที่พบได้บ่อยในประเทศไทยและประเทศเพื่อนบ้าน การศึกษาแบบ case-control study พบว่าการกลายพันธุ์แบบ A1762T/G1764A และ G1899A ของไวรัสตับอักเสบบีมีความสัมพันธ์กับความเสี่ยงของการเกิดมะเร็งตับในผู้ป่วยที่ติดเชื้อแบบเรื้อรัง ส่วนการศึกษาระบาดวิทยาของไวรัสตับอักเสบซีพบว่าสายพันธุ์ที่พบบ่อยในประเทศไทยและประเทศเพื่อนบ้านได้แก่สายพันธุ์ 1, 3 และ 6 โดยเฉพาะสายพันธุ์ 6 ซึ่งเป็นสายพันธุ์ที่จำเพาะในภูมิภาคเอเชียตะวันออกเฉียงใต้ มีการกระจายของสายพันธุ์ย่อย (sub-genotype) ในประเทศไทยแตกต่างจากในประเทศเพื่อนบ้าน นอกจากนี้ยังพบว่าไวรัสตับอักเสบซีสายพันธุ์ 6 มีอัตราการตอบสนองต่อการรักษาด้วยยาต้านไวรัสแตกต่างจากสายพันธุ์ 1 และ 3 ผลวิจัยจากโครงการนี้จะเป็นประโยชน์ในทางระบาดวิทยาของการศึกษาไวรัสตับอักเสบในภูมิภาคเอเชียตะวันออกเฉียงใต้ นอกจากนี้ยังทำให้ทราบถึงกลไกการดำเนินของโรค ตลอดจนแนวทางในการรักษาผู้ป่วยตับอักเสบแบบเรื้อรังได้ดียิ่งขึ้น

ABSTRACT

Project Code: BRG5380012

Project Title: Molecular virology of hepatitis B and C viruses associated with hepatocellular carcinoma and treatment response

Investigator: Associate Professor Pisit Tangkijvanich, M.D.

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Project Period: 31 May 2010-30 May 2012

Project Description:

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are major public health problems in Thailand and worldwide. Chronic viral hepatitis is associated with a high lifetime risk of developing cirrhosis and hepatocellular carcinoma (HCC). Several viral and host factors appear to strongly influence the outcome of infected patients. This project was aimed at studying the prevalence and clinical significance of these factors in diverse groups of chronically infected individuals. In epidemiological studies, our data showed that HBV genotypes C and B were the predominant strains found in Thai populations and migrant workers originated from neighboring countries. In a case-control study, our data showed that A1762T/G1764A and G1899A mutations were independent viral factors associated with the risk of developing HCC in Thai patients with chronic hepatitis B. Regarding chronic HCV infection; our epidemiological studies showed that genotype 1, 3 and 6 were the predominant genotypes circulating in Southeast Asia. Among HCV genotype 6, it appeared that the distribution of its sub-genotypes in Thai populations and migrant workers were slightly different. About treatment of chronic HCV infection, the results of our pilot study suggested that the overall response rate of HCV genotype 6 was slightly lower than that of genotype 3 but higher than that of genotype 1. In addition, a response-guided therapy based on viral kinetics might be useful to optimize treatment in patients infected with HCV genotype 6. These data provide useful information regarding the epidemiology and clinical importance of HBV and HCV genetic variability in patients chronically infected with the viruses.

หน้าสรุปโครงการ (Executive Summary)
ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา

1. ชื่อโครงการ อณูไวรัสวิทยาของไวรัสตับอักเสบบีและซีที่สัมพันธ์กับการเกิดมะเร็งตับและการตอบสนองต่อการรักษาด้วยยาต้านไวรัส

Molecular virology of hepatitis B and C viruses associated with hepatocellular carcinoma and treatment response

2. ชื่อหัวหน้าโครงการ น พ.พิสิฐ ตั้งกิจวานิชย์
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3. สาขาที่ทำการวิจัย อณูชีววิทยาของไวรัสตับอักเสบ

4. คำหลัก (Keyword) hepatitis B virus, hepatitis C virus, genotype, mutation, liver cancer, treatment

5. งบประมาณทั้งโครงการ 1,500,000 บาท

6. ระยะเวลาดำเนินงาน 2 ปี

7. ปัญหาที่ทำการวิจัยและความสำคัญของปัญหา

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 with hepatic decompensation and hepatocellular carcinoma (HCC). In Thailand, chronic viral hepatitis affect more than 5 million individuals, of whom an estimated 20% have or will develop cirrhosis and HCC. Studies to date suggest that molecular virological factors of HBV and HCV have important influences on the clinical outcome and treatment response to antiviral therapy, but more studies are needed. For example, the roles of viral genetic variability, including HBV genotypes and mutations, in HCC patients are largely unknown. These data will provide additional information related to viral hepatocarcinogenesis that may lead to future research on prevention and treatment of the cancer. Regarding treatment of chronic HCV infection, little is known about HCV genotype 6, the genotype that has been found locally in Southeast Asia. Specifically, the optimal duration of treatment with pegylated interferon alfa (PEG-IFN) and ribavirin (RBV) for HCV genotype 6 is currently unknown. Recent studies have shown an association between single nucleotide polymorphisms

(SNPs) near *interleukin (IL)-28B* gene and treatment response with PEG-IFN/RBV in HCV genotype 1 infection. However, the importance of these SNPs for HCV genotype 3, and particularly HCV genotype 6 infected patients is less clear. Thus, studies of host genetic factors and viral kinetics during antiviral therapies may help to predict and optimize therapeutic response in patients with chronic HCV infection.

8. วัตถุประสงค์

The aims of this research project were to

1) Determine the molecular epidemiology of HBV and HCV in Thailand and neighboring countries
2) Determine the prevalence and viral genetic variability of HBV, including genotypes and mutations, in serum samples of patients with HCC
3) Study the viral kinetics of HCV (particularly genotype 6) during PEG-IFN/ribavirin therapy for prediction and individualization of treatment
4) Study the roles of host genetic factors associated with treatment response in patients with chronic HCV infection

9. ระเบียบวิธีวิจัย

1) Collecting serum samples from patients with chronic hepatitis B and stores at -80^{0}C
2) Collecting serum samples from patients with chronic hepatitis C and stores at -80^{0}C
3) Collecting serum samples from patients with HBV-associated with HCC and stores at -80^{0}C
4) Collecting serum and PBMC samples from patients with chronic HCV infection, who were treated with PEG-IFN/ribavirin and stores at -80^{0}C
5) Collecting patients' clinical data
6) Determining serological and virological assays of HBV and HCV
7) Performing nested PCR and direct sequencing for HBV and HCV genotyping and mutations
8) Determining SNPs of *IL-28B* by direct sequencing
9) Analyzing the data and preparing manuscripts

Project Description

1. Introduction and Rationale

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 with hepatic decompensation and hepatocellular carcinoma (HCC). In Thailand, chronic viral hepatitis affect more than 5 million individuals, of whom an estimated 20% have or will develop cirrhosis and HCC. Studies to date suggest that molecular virological factors of HBV and HCV have important influences on the clinical outcome and treatment response to antiviral therapy, but more studies are needed. For example, the roles of viral genetic variability, including HBV genotypes and mutations, in HCC patients are largely unknown. These data will provide additional information related to viral hepatocarcinogenesis that may lead to future research on prevention and treatment of the cancer. Regarding treatment of chronic HCV infection, little is known about HCV genotype 6, the genotype that has been found locally in Southeast Asia. Specifically, the optimal duration of treatment with pegylated interferon alfa (PEG-IFN) and ribavirin (RBV) for HCV genotype 6 is currently unknown. Recent studies have shown an association between single nucleotide polymorphisms (SNPs) near interleukin (IL)-28B gene and treatment response with PEG-IFN/RBV in HCV genotype 1 infection. However, the importance of these SNPs for HCV genotype 3, and particularly HCV genotype 6 infected patients is less clear. Thus, studies of host genetic factors and viral kinetics during antiviral therapies may help to predict and optimize therapeutic response in patients with chronic HCV infection.

Molecular virology of hepatitis B virus

Human HBV is the prototype member of the family Hepadnaviridae, which includes a variety of avian and mammalian viruses that share similar genomic organization, organ trophisms, and a unique strategy of genome replication.[1] The human HBV 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), according to translation of the S region only, pre-S2+S region, or pre-S1+pre-S2+S region. 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]

Immediately after infection of 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 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 $x10^{-5}$ 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 positve 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 related with HCC 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) or alcohol. As a result, 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.[14] Chronic HBV infection exerts its pro-oncogenic properties through both indirect and direct mechanisms.[15] 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.[16] In addition, there are now growing evidence suggesting that viral genotypes may influence the clinical outcomes of patients with chronic HBV infection.[17] 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.[17] 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.[18] 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.[19, 20] In addition, several lines of evidence have suggested that mutants, particularly deletions, occurring naturally in the

preS region correlate with the development of HCC.[21] Finally, it has also been shown that mutations in the X gene may contribute to the development of HCC in HBV-infected patients.[22, 23]

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.[24] NA directly inhibit reverse transcriptase and thereby impair 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.[24] 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.[24] Currently, pegylated interferon alfa (PEG-IFN), created by attaching a polyethylene glycol molecule to IFN, significantly improves pharamacokinetics and results in more convenient dosing interval than conventional IFN.[24]

However, PEG-IFN therapy can result in only 30-40% HBeAg seroconversion in HBeAg-positive chronic hepatitis, which is still far from satisfactory.[25] 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.[26] 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.[27] 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.[28] 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,[28] additional trials of combination therapies with PEG-IFN and a more potent NA such as entecavir or tenofovir 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.[29] 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. [30]

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, six 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%.[31] 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.[31] In Thailand, approximately 50-60% of patients are infected with HCV genotype 3, while approximately 30-40% and 10% are infected with genotype 1 and 6, respectively.

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.[32] 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 increasing 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 of do not seem to significantly influence the progression rate in patients with chronic HCV infection.[33]

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 (RBV).[34] 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.[34] 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.[35] 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.[36-38] 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.[39] 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.[40] During the initial first phase, HCV RNA may fall by 1-2 $\log_{10}$ IU/Ml in genotype 1 infected patients and by as much as 3-4 $\log_{10}$ 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).[41]

From these basic concepts, and by using the equation proposed,[42] it is possible to calculate the kinetic parameters of HCV by mathematic models. In practical terms, studies of viral kinetics are characterized by the frequent collection of blood samples and viral quantification at various time points during the initial phases of therapy. This technique allows the recognition of viral decay patterns and can be used to determine the likelihood of treatment success, as well as guide treatment duration in patients with chronic HCV infection. In fact, the primary goal of treatment for chronic HCV infection is to achieve an SVR. Patients who fail to reach an early virological response (EVR), which is defined as a drop in HCV RNA levels of at least 2 $\log_{10}$ IU/mL after 12 weeks of therapy, are unlikely to have an SVR.[43] 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.[44] Moreover, kinetic studies–with only viral load measurements–provide the knowledge on virus-host-drugs interactions. A better kinetic pattern (a rapid, pronounced decline in viral load) could be interpreted as a good interaction whereas a worse pattern (a slow decline) could require other therapeutic approaches, such as the prolongation of treatment.[45] Currently, there is limited (if any) 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.

Although HCV genotype and viral load are two majors factors used to predict the response of patients with chronic HCV infection to IFN-based therapy, host genetic variations have also been reported as useful for predicting the response. Recent genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) near *interleukin (IL)-28B* gene associated with favorable response to treatment in patients infected with HCV genotype 1.[46-48] Overall, 1,137 patients in the first and largest GWAS were enrolled in the genetic association analyses for SVR in three separate ethnic populations, defined by genetic ancestry (Caucasian, Hispanic, and African American). Among seven SNPs identified, the top discovery SNP (rs12979860) was strongly associated with SVR. In addition, regression modeling found that the *IL28B* polymorphism (rs12979860) was the strongest predictor of SVR compared with all other baseline host and viral variables.[46] Other GWAS have independently identified

the *IL28B* region to be an important predictor of SVR. Tanaka et al. revealed that two SNPs (rs8099917 and rs12980275) near the *IL28B* gene were strongly associated with non-response.[48] Suppiah et al. employed a similar GWAS process in a population of European and Australian patients (n=555) and confirmed the association of rs8099917 with SVR. In addition, regression analysis again confirmed rs8099917 to be an independent predictor of response.[47] In summary, a number of SNPs around the *IL28B* gene were found across the studies to be associated with treatment response in patients infected with HCV genotype 1. However, it is not clear at this stage whether these SNPs are associated with the response to PEG-IFN plus ribavirin therapy among patients with HCV genotype 3, and particularly genotype 6.

2. Objectives

The aims of this study were to

1) Determine the molecular epidemiology of HBV and HCV in Thailand and neighboring countries
2) Determine the prevalence and viral genetic variability of HBV, including genotypes and mutations, in serum samples of patients with HCC
3) Study the viral kinetics of HCV (particularly genotype 6) during PEG-IFN/RBV therapy for prediction and individualization of treatment
4) Study the roles of host genetic factors associated with treatment response in patients with chronic HCV infection

3. Methodology

3.1 Subjects

To study the molecular epidemiology of HBV in Thailand and neighboring countries, serum samples were obtained from 6,213 healthy subjects from four provinces including Chiangrai, Udon Thani, Chonburi and Nakhon Si Thammarat, which were chosen as geographical representations of populations in the North, Northeast, Center and South of the country, respectively. This study was a part of a nationwide seroepidemiological survey in Thailand.[49] In addition, 3,009 serum samples collected from 1,119 Cambodians, 787 Laotians and 1,103 Myanmarese migrant workers in Thailand were included as representations of their respective countries.

Serum samples were also obtained from HBV patients with chronic hepatitis, cirrhosis and HCC, who had attended at King Chulalongkorn Memorial Hospital, Bangkok. To evaluate the association between the mutations within the EnhII/BCP/PC and X genes and the risk of HCC, a case–control study among Thai patients was conducted. Serum samples obtained from 60 patients with HBV-related HCC

and positive for HBV DNA were randomly selected from a pool of patients with chronic liver disease. The control group was 60 HBsAg-positive non-HCC patients, who matched for age (±5 years), gender, HBeAg status and HBV genotype with the patients with HCC. The diagnosis of chronic hepatitis was based on the presence of prolonged elevation of serum alanine aminotrasferase (ALT) and/or histologically proven. The diagnosis of cirrhosis was based on histopathology and/or the ultrasonic appearance of cirrhosis plus at least one of the following features: hypersplenism (splenomegaly and thrombocytopenia), ascites, endoscopically confirmed esophageal or gastric varices, or hepatic encephalopathy. The diagnosis of HCC was based on typical imaging studies and/or histopathology (fine needle aspiration, core liver biopsy or surgical resection) according to the American Association for the Study of Liver Diseases (AASLD) guideline.[50] Diagnostic criteria for HCC by imaging modalities were based on reports of focal lesions with hyperattenuation at the arterial phase, hypoattenuation at the portal phase in dynamic computerized tomography (CT) or magnetic resonance imaging (MRI). In cases without typical imaging features liver biopsy was performed to confirm the diagnosis of HCC.

To study the molecular epidemiology of HCV in Thailand and neighboring countries, serum samples were obtained from 419 Thai individuals, who had anti-HCV positive and had resident mainly in the central part of the country. In addition, serum samples collected from 1,431 Cambodians and 1,594 Myanmarese migrant workers in Thailand were included as representations of their respective countries. To evaluate the role of host and viral genetics factors associated with treatment response in chronic HCV infection, male and female patients aged 18-70 years with HCV genotypes 1, 3 and 6 infection who fulfilled the following entry criteria were enrolled: HCV RNA level more than 10,000 IU/mLand increased ALT levels confirming chronic hepatitis. Exclusion criteria were as follows: decompensated liver disease; HBV or human immunodeficiency virus (HIV) co-infection; other causes of liver disease; active injection drug use or alcohol dependence; pregnancy or breast-feeding; serum creatinine level >1.5 mg/dL; haemoglobin concentration, <11 g/dL in women or <12 g/dL in men; neutrophil count, <1,500 cells/mm^3; platelet count, <80,000 platelets/mm^3; a major psychiatric illness; seizure disorder; serious co-morbid conditions and evidence of malignant neoplastic diseases.

All serum samples were collected and stored at -80^0C. The studies of molecular epidemiological aspect had been approved by the Ministry of Public Health and the ethical committee of the Faculty of Medicine, Chulalongkorn University. The studies in patients with HBV and HCV infection in King Chulalongkorn Memorial Hospital had been approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University.

3.2 Serological and virological assays

HBsAg, HBeAg, anti-HBe, 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). The levels of serum HCV RNA were assessed by real-time quantitative RT-PCR (COBAS TaqMan HCV assay; Roche Diagnostics, Basel, Switzerland), in accordance with the manufacturer's instructions.

3.3 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 subjected 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), 1µM 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 (10µL) 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.[51]

3.4 HCV RNA amplification and direct sequencing

Total HCV RNA was extracted by the guanidinium method[52] and reverse transcribed into cDNA using random primers (Promega, Medison, WI) and M-MLV reverse-trancriptase (Promega, Medison, WI). For HCV RNA detection, the DNA 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.[52-54] The NS5B region was amplified by nested PCR resulting in a 471-bp fragment, with outer primers, NS5B_F1 (CAATWSMMAC BACCATCAT GGC, position 7999-8020), NS5B_R1 (CCAGGARTTRACTGGAGTGTG, position 8805-8825) and inner primers, NS5B_F2 (GATGGGHHSBKCMTAYGGATTCC, 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 Mg^{2+}, and 1.25 units of Tag DNA polymerase. Samples were amplified under the following conditions: 3 min at 94 ºC for initial denaturation followed by 40 cycles at 94 ºC for 1 min., 49 ºC for 1 min. and 72 ºC for 1.30 min, and concluded by a final extension step at 72 ºC 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 ºC. 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.[53] 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, Medison, 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).[55] Neighbor-joining trees were generated using Kimura's two parameters and the confidence values were calculated based on bootstrap resampling tests multiplied by 1000.

3.5 SNP genotyping of IL28B

Human genomic DNA was extracted from 100 mL of serum samples using a QIAamp blood kit according to the manufacturer's instruction (QIAGEN, Tokyo, Japan). To determine SNP genotype of rs12979860, 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 rs12979860 using specific primer pairs. The PCR mixture comprised 1 mL DNA, 10 pmol of each primer, 160 mM of deoxyribonucleotide triphosphate, Mg2+ and 1.25 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The amplification conditions for the three SNP spanning regions was carried out under the following conditions: preincubation 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 of rs12979860. Specificity of this method was assessed by comparison with the SNP genotyping of rs12979860 carried out by GWAS.[48]

3.6 Statistical analysis

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 14.0 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

4.1 Molecular epidemiology of HBV in Thailand and neighboring countries

4.1.1 Distribution of HBV genotypes and subtypes

Of 6213 healthy Thai subjects from 4 provinces (Chiangrai, Udon Thani, Chonburi and Nakhon Si Thammarat), 246 (4%) serum samples were seropositive for HBsAg, and 201 serum samples were subjected to further analysis aimed at molecular characterization of HBV. One hundred and forty-seven (73.1%) of the 201 HBsAg-positive subjects were positive for HBV DNA in the sera. Mean age of the subjects was 33.14±14.03 years and 49.7% were male. Of those positive for HBV DNA, 128 (87.1%) cases were determined as genotype C, 17 (11.6%) cases belonged to genotype B, and 2 (1.3%) cases to genotype A. The distribution of the HBV antigen subtypes among these subjects was: adr (84.4%), adw (14.2%) and ayw (1.4%). HBV genotype and subtype prevalence according to geographic distribution is shown in Tables 1 and 2, respectively. Although genotype C was the most common genotype in each geographic area, the prevalence of genotype B was significantly higher in the central part of Thailand compared to other regions (*P =0.007*). Similarly, the prevalence of subtype adw was significantly higher in the central part of Thailand than in other regions (*P =0.001*).

HBsAg positive sera were found in 282 of 3009 (9.4%) samples of Cambodians, Laotians and Myanmarese migrant workers. In these HBV carriers, there were 121 Cambodian (10.8%), 54 Laotian (6.9%) and 107 Myanmareses (9.7%). All sequences that obtained from this study were submitted in GenBank database (accession no. GQ855313-GQ85570 and GQ856585). Phylogenetic analysis of the pre-S1/pre-S2/S and preC/C genes was constructed. Among these subjects, HBV DNA was detected in 102 Cambodian (84.3%), 42 Laotian (77.8%) and 80 Myanmareses (74.8%). Of those positive for HBV DNA, 193 of 224 (86%) cases were determined as genotype C, 26 (11.5%) cases belonged to genotype B, 1 (0.5%) cases to genotype A and 1 (0.5%) cases to genotype D. For antigenic subtype distribution, adr was the most common (68.3%), followed by ayw (8.9%), adw (6.7%) and ayr (0.9%). The prevalence of HBV genotypes and subtypes according to individual's country is shown in Table 1.

Although the entire genome sequence was not performed, three isolates with suspected inter-genotypic recombinants were identified (isolate 31 with genotype B2/C1, accession no. GQ855407; isolate 612 with genotype B3/C1, accession no. GQ855454 and GQ855560; and isolate 3794 with genotype G/C1, accession no. GQ856585). Isolate 31 was shown to be recombined of sub-genotype B2 and C1, with its recombination breakpoints estimated at nucleotide 573 (Fig 1A). Isolate 3794 represented a recombinant of genotypes G/C1 with its recombination breakpoints between nucleotides 2006 and 157 (Fig 1B). Isolate 612 was classified to sub-genotype B3 in pre-S/S gene but showed sub-genotype C1 between nucleotides 1554 and 1974 (figure not shown).

Table 1 Prevalence of HBV genotypes and subtypes in migrant workers

	Cambodia (n = 1119)	Laos (n = 787)	Myanmar (n = 1103)	Total (n = 3009)
No. HBsAg positive	121 (10.8)	54 (6.9)	107 (9.7)	282 (9.4)
No. PCR positive	102 (84.3)	42 (77.8)	80 (74.8)	224 (79.4)
Gender (M : F: ND[a])	81:20:01	31:11:00	46:28:06	158:59:07
Age	29.2±8.6	26.2±7.4	28.3±6.1	28.3±7.6
Genotype				
A	1 (1.0)	0 (0)	0 (0)	1 (0.4)
B	14 (13.7)	11 (26.2)	1 (1.3)	26 (11.6)
C	86 (84.3)	30 (71.4)	79 (98.7)	194 (86.6)
D	0 (0)	0 (0)	1 (1.3)	1 (0.4)
Suspected recombination				
B/C	1 (1.0)	0 (0)	0 (0)	1 (0.4)
B/C	0 (0)	1 (2.4)	0 (0)	1 (0.4)
G/C	1 (1.0)	0 (0)	0 (0)	1 (0.4)
Subtype				
adr	76 (74.5)	20 (47.6)	57 (71.3)	153 (68.3)
adw	9 (8.8)	5 (11.9)	1 (1.3)	15 (6.7)
ayr	1 (1.0)	1 (2.4)	0 (0)	2 (0.9)
ayw	6 (5.9)	12 (28.6)	2 (2.5)	20 (8.9)
Could not be identified	10 (9.8)	4 (9.5)	20 (25.0)	34 (15.2)

Data were expressed as mean ± SD, no (%)

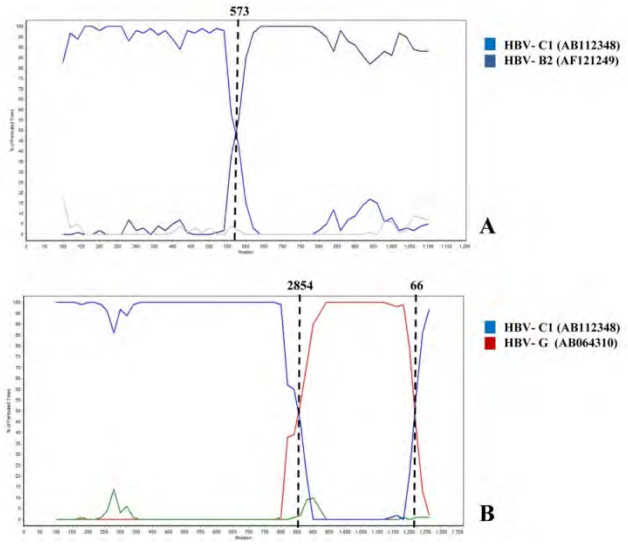

Figure 1 Bootscanning analysis of suspected recombinant isolates. (A) complete *S* gene of isolate 31 was compared with HBV-B2 (AF121249) and HBV-C1 (AB112348); (B) Nucleotide position 2006 – 157 of

isolate 3794 was compared with HBV-C1 (AB112348) and HBV-G (AB064310). Dash line (s) showed the breaking point (s) of recombination. The number over the dash line showed the nucleotide position of each isolate compare with the reference strain (NC_003977)

Genotypes of HBV are generally subtype-specific, although some subtypes are heterogeneous. In general, subtype adw is usually found in genotypes A and B, while adr occurs in genotype C.[56] In this nationwide study in Thailand that included both the identification of the viral genotypes and subtypes in a significant number of HBV carriers, we confirmed the predominance of categories C/adr and B/adw among the HBV strains which accounted for more than 95% of cases. In migrant workers, our data also demonstrated that the major HBV strains belonging to the categories C/adr, which accounted for more than 85% of cases. These data were in agree with previous reports that HBV genotype C was prevalent in Myanmar,[57] and sub-genotypes C1 and B4 were dominant strains in Cambodia.[58] These findings reflect the typical genotypes and subtypes circulating in Thailand and Southeast Asia. Besides the epidemiological data, there is now increasing information suggesting that HBV genotypes may play an important role in causing different disease profiles in chronic HBV infection. It has been shown that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B.[59] Genotype C is also associated with a lower rate of HBeAg seroconversion and a lower response rate to IFN therapy compared to genotype B.[60] The seroprevalence of HBsAg in migrant workers was approximately 7-11%, which was similar to previous reports on seroprevalence in these countries but was higher than the recent nationwide survey in Thailand (4%).[49] The difference in seroprevalence between Thailand and neighboring countries reflects a steady and remarkable decrease of chronic HBV carrier rate in Thai populations after implementation of the universal HBV vaccination since 1992.

HBV strains resulting from genomic recombination between different genotypes have been increasing recognized from various parts of the world. In Asia, recombination of genotypes B/C has been reported in mainland Asia,[61] whereas recombination of genotypes C/D has been detected in Tibet and China.[62, 63] In addition, recombinants between genotypes A/C and genotypes A/D have been documented in Vietnam[64] and India,[65] respectively. Recently, a novel genotype I, with a complex recombination involving genotypes C, A and G has been reported in Vietnam and Laos.[58, 66] In migrant workers, although the whole genome sequence was not performed, we identified three HBV isolates with suspected intergenotypic recombinants. Of note, a hybrid subgenotypes B3/C1 showed the recombination breakpoints occurred in the vincity of the preC/C region, which is the most common site of intergenotypic

recombination as previously described.[61] Another recombinant of genotypes G/C with its recombination breakpoints between nucleotides 2006 and 157 was also demonstrated in this study. Interestingly, the site of breakpoints of this recombinant was different from those found in a hybrid of genotypes G/C previously described by our group in a Thai patient with HCC.[67]

4.1.2 Prevalence of the 'a' determinant mutations

The prevalence and variation of the 'a' determinant mutations among the Thai populations was further studied. Four out of 147 samples were found to have mutations, all of which were Thr126Asn. Of these, 2/43 (4.65%) and 2/104 (1.92%) originated from vaccinated and non-vaccinated subjects, respectively. There were no statistically significant differences between the vaccinated and non-vaccinated groups (*P =0.355*). In migrant workers, various point mutations in the 'a' determinant region were found among Cambodian, Laotian and Myanmareses HBV isolates. For instance, 19 out of 94 (20.2%) of Cambodian samples, 6/38 (15.8%) of Laotian samples and 10/62 (16.1%) of Myanmareses samples were found to have such mutations. Interestingly, the most frequent mutation in Cambodian, Laotian and Myanmareses HBV isolates was Ile126Ser/Asn, which was in concordance with Thai HBV isolates. The alignment of amino acid sequences of the partial S region of these 35 samples is shown in Fig 2.

		Amino acid position 120 - 160
Genotype C		PCRTCTIPAQ GTSMFPSCCC TKPSDGNCTC IPIPSSWAFA R
Genotype B		..K...T... ....T...... .... K
Isolate:	Genotype:	
Cambodia-3	C1	..K...... ....R.... ....
Cambodia-198	B2	..K...T... R..T...... ...T...... .... K
Cambodia-351	C1	..K...... ....R.... ....
Cambodia-385	C1	..K...S... ....
Cambodia-423	C1	..K...N... ....
Cambodia-529	C1	..Q...T... .N.T...... ....
Cambodia-777	C1	..K...S... ....
Cambodia-802	C1	..K...N... ....
Cambodia-812	C1	..K...... ....V .
Cambodia-870	C1	..K...... ....L..... ....
Cambodia-2910	C1	..K..MN... ....
Cambodia-2988	C1	..K...T... .N..Y..... .... K
Cambodia-2997	C1	..K...... ....L....... ....
Cambodia-3198	C1	..K....R.. ....
Cambodia-3282	C1	..K...... ....R.... ....
Cambodia-3342	C1	..K...T... ...T...... ....
Cambodia-3375	B2	..K...T... ...T...... ...T...... .... K
Cambodia-3541	C1	..K...N... ....
Cambodia-3794	G/C1	..K...T... .N..Y..... ....E..... .... -
Laos-1587	C1	..K...... ....A.... .... K
Laos-1694	C1	..K...... .N........ ....
Laos-1893	C1	..K...... R......... ....
Laos-2002	C1	..K...N... ....
Laos-3040	C1	..K...S... ....
Laos-3440	C1	T.......D. ....Y. ....L. .
Myanmar-843	C1	..K...... .P........ ....
Myanmar-862	C1	..K...T... ....G.. .
Myanmar-1071	B3	.....T.... .... I..T...... .... K
Myanmar-1310	C1	..K...... .N........ ....
Myanmar-1529	C1	..K...S... ....
Myanmar-1855	C1	..K...... ....L... .
Myanmar-2283	C1	..K...S... ....
Myanmar-3576	D	......TTV. ....Y..... ....LG K
Myanmar-3905	C1	..K...S... ....
Myanmar-4004	C1	..K...S... ....

Figure 2 The amino acid sequences alignment of the 'a' determinant region of 35 samples

Amino acid substitutions within the ‘a’ determinant domain could lead to conformational changes and may be involved in failures of active and passive immunization for HBV infection.[68] The most common mutation causing vaccine escape involves the mutation at position 145 (Gly145Arg), which is located in the second loop of the ‘a’ determinant[68]. Naturally occurring escape mutants have also been reported in chronic carriers after long-term follow-up. For example, a study in Taiwan showed an increase in the prevalence of ‘a’ determinant mutants in children from 7.8% before to 23.1% 15 years after the introduction of universal vaccination against HBV.[69] In addition, the prevalence of HBsAg mutants was also significantly higher among those fully vaccinated than among those not vaccinated. These data suggest that vaccination might have increased a selection pressure on the emergence of surface mutants in relation to wild-type HBV. In our study among Thai populations, data showed that only 2 vaccinated subjects and 2 non-vaccinated subjects had the same mutant-bearing virus affecting amino acid position 126. As a result, it seems that ‘a’ determinant HBV mutants might be uncommon among chronic carriers from Thailand, and the prevalence of the variants might not be associated with vaccination. However, it should be emphasized that all cases included in the study were HBsAg positive and consequently, those patients with mutations rendering the S protein undetectable with the antibodies tested, were excluded. Moreover, since a viral HBV population infecting a host is usually distributed as a quasispecies,[70] variants are expected to coexist with wild-type strains in most carriers. As such mutations were detected by direct sequencing of the PCR products without cloning; quantitative analysis for the relative amount of mutant or wild-type virus in mixed infection was not feasible in this report. Thus, the true proportion of Thai patients carrying ‘a’ determinant variants could be higher than observed in our study.

In migrant workers, the most common amino-acid substitution found in Cambodian, Laotian and Myanmareses samples was also located at position 126. The prevalence of ‘a’ determinant mutants among chronic carriers from these countries was approximately 15-20%, which was slightly higher than the prevalence among random chronic carriers from recent data (6-12%).[71] Interestingly, this high prevalence of the variants among migrant workers might not be associated with previous vaccination because the coverage rates of HBV vaccine administration in these countries are generally low.[72, 73] Thus, it is speculated that these mutants might have emerged through natural immunoselective pressure of the host, which in turn are infectious and have been circulated among individuals chronically infected with HBV.

4.1.3 Prevalence and characterization of pre-S mutations

Based on direct sequencing, pre-S mutations were detected in 14/147 (9.5%) of Thai carriers. Among these, 13 cases (92.9%) belonged to genotype C. As for the prevalence of pre-S mutations

according to site, pre-S2 deletion was the most common (4.1%), followed by pre-S2 start codon mutation (2.9%), both pre-S2 deletion and start codon mutation (2.0%), and pre-S1 deletion (0.7%). The mean age of patients with pre-S mutations (n=14) was significantly higher than that of patients without the mutants (n=133) (41.2 ± 11.4 years vs 32.3 ± 15.0 years, *P=0.033*). In addition, the mean HBsAg level in patients with pre-S mutations was significantly higher than in those without the mutants (378.8 ± 64.4 vs 305.7 ± 111.0, *P=0.017*). Pre-S mutations were detected in 36 of 209 (17.2%) cases of migrant workers. The prevalence of pre-S mutations/deletions among Cambodian, Laotian and Myanmareses migrant workers was 14.3%, 15.0% and 22.5%, respectively. As for the prevalence of pre-S/S mutations according to site, pre-S2 deletion was the most common (6.7%), followed by pre-S2 start codon mutation (3.8%) and both pre-S2 deletion and start codon mutation (3.3%). The alignment of amino acid sequences of the entire pre-S1/pre-S2 region of the 36 samples is shown in Fig 3.

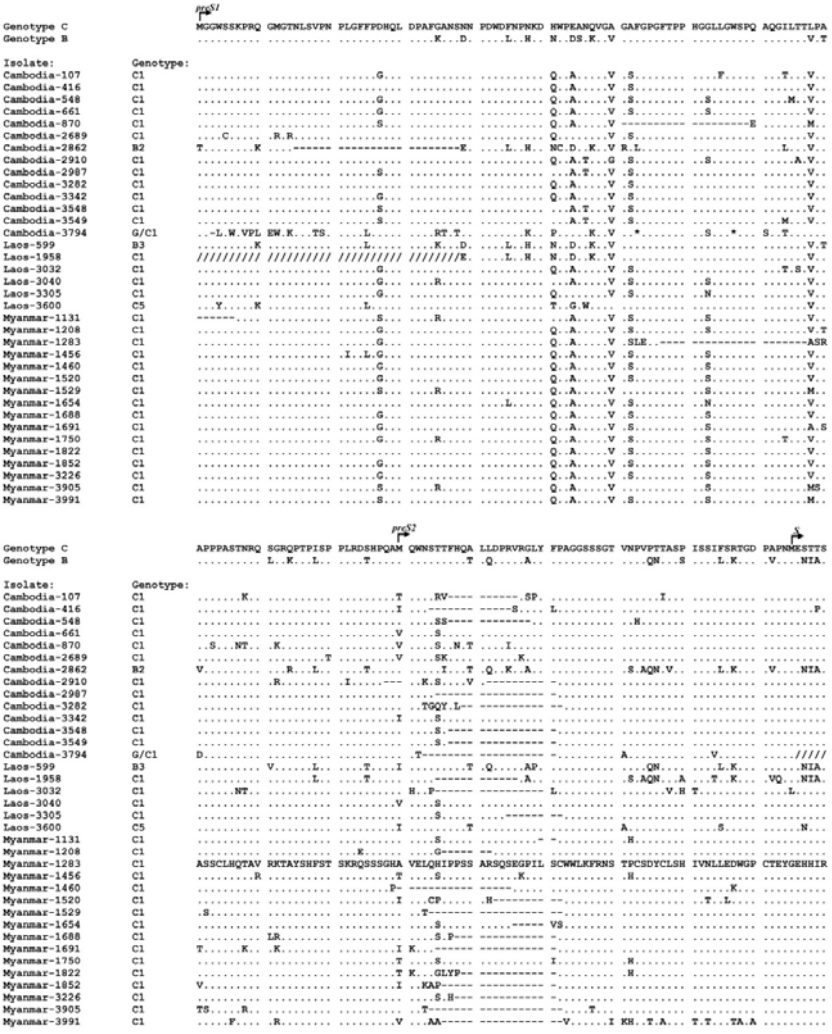

Figure 3 The amino acid sequences alignment of the entire pre-S1/pre-S2 region of 36 samples

The pre-S1 and pre-S2 regions are highly immunogenic and potentially under selective pressure by the immune system because they contain both B- and T-cell epitopes[74] The prevalence of pre-S mutations is variable and considerably different among different geographic areas. For example, Huy et al. reported that the prevalence of HBV pre-S mutants ranged from 0% to 36% in an analysis of HBV-DNA-positive serum samples from individuals residing in 12 countries, including Thailand.[75] In that report, the prevalence of pre-S mutations among Thai patients amounted to 10.5%, which was consistent with the results of our nationwide study (9.5%). The prevalence of pre-S mutations/deletions among Cambodian, Laotian and Myanmareses migrant workers amounted to 14.3%, 15.0% and 22.5%, respectively, which was relatively higher than the results among Thai populations.

Regarding the site of mutations, our data in Thai and neighboring populations showed that pre-S2 deletion was the most common mutation type, followed by pre-S2 start codon mutation, and the combined pre-S2 deletion and start codon mutation. These results are also in agreement with those of recent reports from Japan and Korea, according to which deletion in pre-S2 regions and pre-S2 start codon mutations were among the most prevailing[75, 76] Interestingly, our data showed a higher prevalence of pre-S mutations in Thai patients infected with genotype C than those with genotype B. Taking into consideration that these mutations were predominantly found in genotype C, it is possible that this genotype may be more prone to develop such mutations. Moreover, the mean age of Thai patients with pre-S mutations was significantly higher than that of those without the mutants. This observation also confirmed previous data that the prevalence of pre-S mutations tends to increase in direct relation to the patient's age.[75-77]

4.2 Case-control study on sequence variations of HBV in patients with HCC

The clinical features of patients with HCC and controls are showed in Table 2. Compared with the control group, patients with HCC had higher frequency of cirrhosis. In addition, patients with HCC had significantly poorer liver biochemical parameters (TB and albumin) compared to controls. However, there was no significant difference between groups in respect to ALT and HBV DNA levels.

Table 2 Demographic and clinical characteristics of patients with or without HCC

Characteristics	Control patients (n=60)	Patients with HCC (n=60)	*P*
Age, yr	52.9±8.6	55.7±9.8	0.096
Sex			1
Male	52 (86.7)	52 (86.7)	
Female	8 (13.3)	8 (13.3)	
Total bilirubin, mg/dl	1.5±1.2	2.1±1.4	0.014
Albumin, g/L	3.6±0.6	3.3±0.6	0.005
ALT, U/L	139.7±101.4	161.1±116.9	0.285
Cirrhosis	32 (53.3)	55 (91.7)	<0.001
HBeAg positivity	18 (30.0)	18 (30.0)	1
HBV genotype			1
B	16 (26.7)	16 (26.7)	
C	44 (73.3)	44 (73.3)	
HBV DNA level, log copies/ml	6.1±1.3	5.9±1.4	0.451

Data were expressed as mean ± SD, no (%)

Base on direct sequencing of EnhII/BCP/PC regions, mutational spots were found at nt 1613, 1653, 1753, 1762, 1764, 1766, 1768, 1846, 1858, 1896 and 1899. Compared with the controls, patients with HCC had higher frequencies of T1753C/A, A1762T/G1764A and G1899A mutations. However, no significant difference between groups was found in respect to G1613A, C1653T, C1766T/T1768A, A1846T/C, T1858C and G1896A mutations (Table 3). In addition, four patients with HCC showed the following deletions at or around nt 1762-1764. One patient had deletions at nt 1757-1777, while another had deletions at nt 1756-1764. One additional patient had long deletions at nt 1594-1827, while another case had a deletion at nt 1762-1776. Interestingly, one patient with HCC had a 24-base insertion between nt 1674 and 1675. All these cases belonged to the HBeAg-negative group.

Single codon mutations were present in the X region, but with a generally scattered distribution, and without significant difference between the HCC and control groups. However, three mutational patterns including I127T/N, K130M and V131I, corresponding to T1753C/A and A1762T/G1764A mutations in the BCP region, were found significantly higher frequencies in patients with HCC than in controls. In contrast, no significant difference between groups was found in respect to A36T, P38S, A44L and H94L mutations (Table 3). One patient with HCC had 7 amino acid deletions at codon 129-135, while another patient with HCC had 3 aa deletions at codon 128-130. Another two patients with HCC had 78

and 5 aa deletions at codon 75-152 and 128-132, respectively. One additional patient with HCC had an 8-aa insertion between codon 96 and 97.

Table 3 Virological characteristics of patients with or without HCC

Characteristics	Control patients (n=60)	Patients with HCC (n=60)	*P*
Nucleotide sequences of EnhII/BCP/PC genes			
G1613A	18 (30.0)	24 (40.0)	0.339
C1653T	7 (11.7)	16 (26.7)	0.062
T1753C/A	14 (23.3)	26 (43.3)	0.02
A1762T/G1764A	33 (55.0)	53 (88.3)	<0.001
C1766T/T1768A	3 (5.0)	10 (16.7)	0.075
A1846T/C	14 (23.3)	16 (26.7)	0.833
T1858C	1 (1.7)	3 (5.0)	0.619
G1896A	17 (28.3)	26 (43.3)	0.127
G1899A	5 (8.3)	21 (35.0)	0.001
Amino acid sequences of X gene			
A36T	42 (70.0)	41 (68.3)	0.843
P38S	2 (3.3)	0 (0)	0.496
A44L	14 (23.3)	20 (33.3)	0.311
H94Y	7 (11.7)	16 (26.7)	0.062
I127T/N	18 (30.0)	39 (65.0)	<0.001
K130M	33 (55.0)	51 (85.0)	<0.001
V131I	33 (55.0)	52 (86.7)	<0.001

Data were expressed as mean ± SD, no (%)

To determine the independent contribution of clinical and virological features to the development of HCC, multiple logistic regression analysis was performed by using the significant factors identified in the univariate analysis. These factors included TB and albumin levels, the presence of cirrhosis, and nucleotide sequence variants list in Table 4 (C1653T, T1753C/A, A1762T/G1764A and G1899A mutations). The significant factors associated with HCC development were A1762T/G1764A and G1899A mutations and the presence of cirrhosis.

Table 4 Multivariate analysis of factors associated with HCC

Factor	Odds ratio (95% CI)	*P*
A1762T/G1764A mutations	3.56 (1.16-10.89)	0.026
G1899A mutation	3.54 (1.09-11.47)	0.034
Presence of cirrhosis	8.44 (2.65-26.84)	<0.001

CI, confidence interval; OR, odds ratio

The cumulative effect of the mutations at A1762T/G1764A and/or G1899A, which were the significant factors in multivariate analysis, was further examined. The odd ratio (OR) of HCC with A1762T/G1764A mutations was 6.19, while the OR with G1899A mutation was 5.92. With the presence of both mutations, the OR of HCC increased to 10.23. In setting of cirrhosis, the present of A1762T/G1764A mutations substantially increased the OR of HCC to 15.00, while the present of both A1762T/G1764A and G1899A mutations increased the OR to 13.44 (Table 5). The clinical and virological characteristics of patients with or without A1762T/G1764A mutations, which were the strongest mutations associated with HCC development, are shown in Table 6. Patients with A1762T/G1764A mutations had higher rates of cirrhosis and HBV genotype C than patients without such variants. In addition, patients with A1762T/G1764A mutations had higher frequencies of T1753C/A, C1766T/T1768A and G1899A mutations than patients with the wild type virus. However, no differences between groups were found with regard to other clinical and virological factors, including HBeAg positivity, HBV DNA level, C1653T, G1613A, A1846T/C, T1858C and G1896A mutations.

In this study, we found that double A1762T/G1764A mutations were an independent risk factor for the development of HCC, which was consistent with recent case-control studies conducted in China, Taiwan and Korea.20, 78-80 Also, the magnitude of the OR of HCC associated with the presence of the BCP double mutants in this study was approximately 3-4-fold, which was similar with reports by other studies. In fact, a prospective cohort of approximately 1600 high-risk individuals in Qidong, China, showed that A1762T/G1764A mutations were detected in approximately 50% of HCC cases before cancer development, suggesting that these variants would indicate a high potential risk for hepatocarcinogenesis.81 It has been reported that the development of A1762T/G1764A mutations is associated with HBV genotype and their prevalence is higher in genotype C than genotype B.82 As expected, our data also demonstrated that A1762T/G1764A mutations were genotype C related.

Table 5 Cumulative effect of factors on the risk of HCC

Characteristics	Control patients (n=60)	Patients with HCC (n=60)	Odds ratio (95% CI)	*P*
A1762T/G1764A	33 (55.0)	53 (88.3)	6.19 (2.43-15.83)	<0.001
G1899A	5 (8.3)	21 (35.0)	5.92 (2.06-17.06)	0.001
Cirrhosis	32 (53.3)	55 (91.7)	9.63 (3.38-27.41)	<0.001
A1762T/G1764A and G1899A	3 (5.0)	21 (35.0)	10.23 (2.86-36.67)	<0.001
Cirhosis and G1899A	4 (6.7)	19 (31.7)	6.49 (2.05-20.51)	0.001
Cirhosis and A1762T/G1764A	15 (25.0)	50 (83.3)	15.00 (6.12-36.74)	<0.001
Cirrhosis and A1762/G1764 and G1899A	2 (3.3)	19 (31.7)	13.44 (2.97-60.89)	<0.001

Data were expressed as no (%);

CI, confidence interval; OR, odds ratio

We also showed that the prevalence of T1753C/A mutation was significantly higher among patients with HCC than those without liver cancer, although such mutant was not an independent risk factor of HCC in multivariate analysis. In this study, it should be noted that T1753C/A mutation always existed along with the presence of A1762T/G1764A mutations. Interestingly, previous data also demonstrated that T1753C/A mutation occurred later than A1762T/G1764A mutations in the course of chronic HBV infection.[78] These results suggested that A1762T/G1764A mutations might be the main HBV variants associated with the development of HCC, and T1753C/A mutation might also play an important, albeit lesser, role in hepatocarcinogenesis.

The association between the well-known G1896A mutation in the PC region and the risk of HCC development remains controversial. For instance, a Taiwanese study showed that the presence of the PC mutation significantly increased the risk for HCC,[79] while another community-based cohort study with long-term follow-up conducted in the same country demonstrated that this mutant was associated with a decreased risk of HCC development.80 In this study, our data showed that this common variant might not be account for the increased risk of HCC among Thai populations. In contrast, point mutation at nt. 1899 was an independent viral factor of HCC development. Our results were well-matched with a recent study performed in Taiwan, which demonstrated that the prevalence of G1899A not G1896A mutation was significantly higher among patients with HCC than those without HCC.81 In contrast, G1899A mutation

was found at low prevalence with no clinical association in other previous reports.82, 83 The reasons for these discrepancies among reports remain unclear and merits further studies to clarify the role of G1896A or G1899A mutant in HBV-related hepatocarcinogenesis.

Table 6 Comparison of characteristics of patients with or without A1762/G1764A mutations

Characteristics	No A1762T/G1764A mutations (n=32)	A1762T/G1764A mutations (n=88)	*P*
Age, yr	52.9±9.0	54.9±9.4	0.268
Sex			0.385
Male	28 (87.5)	76 (86.4)	
Female	6 (12.5)	10 (13.6)	
Total bilirubin, mg/dl	1.6±1.2	1.9±1.4	0.215
Albumin, g/L	3.6±0.6	3.4±0.6	0.065
ALT, U/L	145.2±105.5	152.5±111.5	0.74
Cirrhosis	20 (62.5)	67 (76.1)	0.043
HBeAg positivity	9 (28.1)	27 (30.7)	0.663
HBV genotype			<0.001
B	19 (59.4)	13 (14.8)	
C	15 (40.6)	73 (85.2)	
HBV DNA level, log copies/ml	5.9±1.5	6.1±1.2	0.325
Mutations			
G1613A	8 (25.0)	34 (38.6)	0.137
C1653T	4 (12.5)	19 (21.6)	0.303
T1753C/A	0 (0)	40 (45.5)	<0.001
C1766T/T1768A	0 (0)	13 (14.8)	0.019
A1846T/C	8 (25.0)	22 (25.0)	0.815
T1858C	2 (6.3)	2 (2.3)	0.318
G1896A	14 (43.8)	3 (3.4)	0.527
G1899A	2 (6.3)	24 (27.3)	0.007

Data were expressed as mean ± SD, no (%)

Whether there are any additive or synergistic effects on the risk of HCC development with combinations of HBV mutations remains to be established. Recent studies demonstrated that certain complex HBV mutational patterns might be associated with the development of advanced liver diseases, including HCC[77, 79]. In this respect, our study showed that the risk of HCC was significantly increased in patients harboring both A1762T/G1764A and G1899A mutations. Of noted, the risk of HCC was further

increased among cirrhotic patients who had A1762T/G1764A mutations or who had A1762T/G1764A and G1899A mutations in combination. These results suggest that these HBV mutations may serve as helpful virological markers for predicting the development of HCC, particularly in patients who already had cirrhosis. In agreement with our data, a recent prospective study demonstrated that A1762T/G1764A mutations were useful biomarkers for identifying a subset of male patients who were at increased risk of HCC.[80]

Although the precise mechanism of A1762T/G1764A mutations in hepatocarcinogenesis remains uncertain, several hypotheses have been proposed. For instance, it has been shown that A1762T/G1764A mutants may enhance viral replication either by creating a hepatocyte nuclear factor 1 transcription factor binding site or modulating the relative levels of precore and core RNAs.[81] Furthermore, the presence of BCP double mutants may be associated with decreasing T-cell immune responses[82]. 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.[82] Thus, genomic variation in these regions could modify the oncogenic potential of the X protein and induce inactivation of p53-mediated apoptosis or impairment of DNA repair.[83]

In this study, the rate of mutations affecting codons 130 (K130M) and 131(V131I) in the X protein, corresponding to double A1762T/G1764A mutations, significantly differed between patients with or without HCC. In addition, I127T/N mutation in the X protein, which corresponds to T1753C/A mutation, was observed more frequently in patients with HCC than in the control group. These 'hot-spot' mutations are located in the carboxy functional region, and thus might be associated with the transactivating function of the X protein.[84] Previous studies also reported that other amino acid substitutions, such as A36T, P38S, A44L and H94Y were significantly associated with the risk of HCC.[19, 22, 85, 86] However, the prevalence of these mutations, except A36T, was found to be relatively low in our study and there was no significant difference in their prevalence between the HCC and non-HCC group. Thus, our data suggested that the emergence of these mutants might not lead to developing of HCC in Thai patients. Instead, these mutants might occur during a long-standing inflammatory process of vertically-transmitted chronic HBV infection among Thai populations.

4.3 Molecular epidemiology of HCV in Thailand and neighboring countries

Of the 419 anti-HCV positive samples, 375 were positive for viral RNA by RT-PCR amplification of the 5'UTR. These samples were subjected to further amplification of core and NS5B regions followed by nucleotide sequencing. The viral genotypes were investigated employing a viral

genotyping tool (http://www.ncbi.nlm.nih.gov). Among these, 167 (44.5%) samples were genotype 3 [sub-genotypes 3a (39.2%) and 3b (5.3%)], 137 (36.5%) were genotype 1 [sub-genotypes 1a (22.1%) and 1b (14.4%)] and 71 (19%) samples were genotype 6. Seventy-one samples determined as genotype 6 were subjected subsequently to phylogenetic analysis. The nucleotide sequences were submitted to the GenBank database under designated accession numbers FJ859193–FJ859334.

All HCV sequences determined as genotype 6 were confirmed by phylogenetic analysis based on core and NS5B alignments and classified into five specific subtypes. Neighbor-joining trees of the core and NS5B sequences were constructed (Fig. 4) and the sequences clustering closely with the same clade of reference strains were classified as the corresponding genotypes. According to these results, subtypes 6f and 6n are predominant in Thailand, followed by 6i, 6j, and 6e (Table 7).

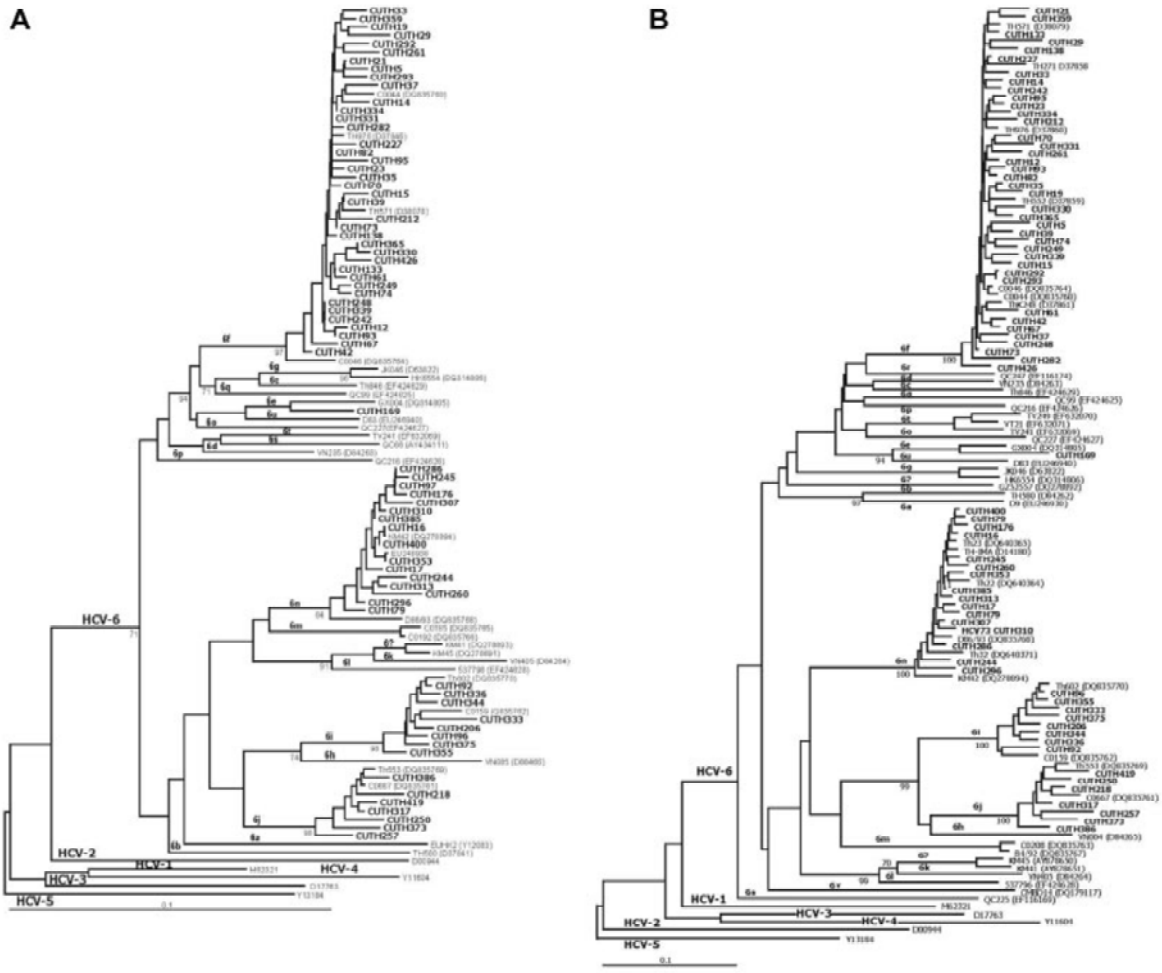

Figure 4 Phylogenetic tree of HCV genotype 6 in Thailand constructed from (A) core and (B) NS5B nucleotide sequences. Boot strap values are shown in the tree root. (Reference strains are represented in bold characters.)

Table 7 HCV genotype 6 subtypes determined by phylogenetic analysis based on core and NS5B regions

	HCV genotype 6 (%)					
Regions	6e	6f	6i	6j	6n	Total
Core	1 (1)	39 (56)	8 (11)	7 (10)	16 (22)	71
NS5B	1 (1)	39 (56)	8 (11)	7 (10)	16 (22)	71

To study the seroprevalence of HCV among immigrant workers, 1,431 and 1,594 serum samples were collected from Cambodia and Myanmar immigrant workers in Thailand, respectively. All subjects were between 15 and 57 years old, with a mean age of 27.13–27.77 years. The majority of the subjects were 24–26 years old. Samples retrieved from Cambodian workers showed 33 (2.3%) positive for HCV antibody by ELISA, as well as 25 (75.8%) samples positive for viral RNA upon RT-PCR of the 5' UTR. Participants aged between 21 and 35 years showed a high rate of HCV infection (Table 8). Among the samples obtained from Myanmar workers, the most numerous immigrants to Thailand, 27 (1.69%) were positive for HCV antibody. The 21–35 years age group showed high infection rate, whereas none from the 36–40 years age group was anti-HCV positive. Fifteen samples proved positive for viral RNA. All RNA-positive samples were subjected to further analysis of the core region and subsequently to direct sequencing.

Table 8 Distribution of anti-HCV-positive samples and genotypes among different age groups

Age group years	Anti-HCV			Genotype										
	male	female	total[3]	1a	1b	3a	3b	6e	6f	6m	6p	6r	6	Total
Cambodia (n = 1,431)														
21–25	5	0	5 (15.2)	0	2	0	0	0	1	0	0	0	0	3
26–30	3	3	6 (18.2)	0	0	1	0	1	0	0	0	1	1	4
31–35	5	2	7 (21.2)	0	2	1	1	0	1	0	0	0	0	5
36–40	2	2	4 (12.1)	0	1	0	0	0	0	0	0	2	0	3
41–45	1	3	4 (12.1)	0	1	0	0	3	0	0	0	0	0	4
46–50	3	1	4 (12.1)	0	0	0	2	0	0	0	1	1	0	3
>50	3	0	3 (9.1)	0	0	0	0	2	0	0	0	2	0	4
Total	22 (2.3)[1]	11 (2.3)[1]	33 (2.3)[1]	0 (0)[2]	6 (24)[2]	4 (16)[2]	1 (4)[2]	5 (20)[2]	2 (8)[2]	0 (0)[2]	1 (4)[2]	5 (20)[2]	1 (4)[2]	25 (75.8)[3]
Myanmar (n = 1,594)														
15–20	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
21–25	4	4	8 (29.6)	0	0	1	4	0	0	1	0	0	0	6
26–30	2	3	5 (18.5)	1	0	1	0	0	1	0	0	0	0	3
31–35	2	5	7 (25.9)	0	1	1	0	0	0	1	0	0	0	3
36–40	0	0	0 (0)	0	0	0	0	0	0	0	0	0	0	0
41–45	1	3	4 (14.8)	0	0	1	1	0	1	0	0	0	0	3
46–50	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
>50	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
Total	9 (33.3)[1]	18 (66.7)[1]	27 (1.7)[1]	1 (6.7)[2]	1 (6.7)[2]	4 (26.7)[2]	5 (33.3)[2]	0 (0)[2]	2 (13.3)[2]	2 (13.3)[2]	0 (0)[2]	0 (0)[2]	0 (0)[2]	15 (55.6)[3]

Figures in parentheses indicate percentages.
[1] Percent calculated with respect to all samples of each country.
[2] Percent calculated with respect to total RNA-positive samples.
[3] Percent calculated with respect to total anti-HCV-positive samples.

HCV genotype of all sequences was determined by phylogenetic analysis based on the core region. HCV genotype 6was predominant in Cambodian workers (56%), followed by 1b (24%), 3a (16%) and 3b (4%). This group showed at least four clusters of HCV-6, -6e, -6f, -6p and -6r (Table 8). One sequence, CBD3571, did not cluster with any of the reference sequences but was grouped close to the clade of 6e and 6u (Fig. 5). Subtype 6e from Vietnam and China was grouped with the Cambodian cluster. It seemed that subtype 6e was transmitted from Cambodia. Based on the cohort study and previous report, subtypes 6p and 6r were found mainly in Cambodia. To analyze the ambiguous isolates, the highly divergent strains, CBD3571 was further subjected to amplification and sequencing of the NS5B region using specific primer sets. Phylogenetic analysis of the neighbor joining tree generated by the 6-parameter model showed that the CBD3751 strain clustered most closely with subtype 6u (61% of 1,000 bootstrap resampling tests. The respective strain occupied a distinct branch of both core and NS5B phylogenetic trees. Phylogenetic analysis (Table 8) showed that samples from Myanmar were mainly genotype 3b (33.2%), the most prevalent genotype in this study. The remaining strains were 3a (26.7%), 6 (26.7%), 1a (6.7%) and 1b (6.7%). Subtypes 6f and 6m were identified in this group. Subtype 6f was grouped with Cambodian and Thai strains. Subtype 6m is generally detected in Myanmar and Thailand. There was no specific cluster of subtype 1b, 3a, 3b and 6m isolates in this study. Subtype 6f from Cambodia and Myanmar has likely migrated from Thailand (Fig. 5).

Phylogenetic analysis based on full-length genome sequences serves as the ‘‘gold standard’’ for HCV genotype or subtype identification. Although this constitutes the most accurate method for virus classification, due to cost and time factors, it is not practical as a routine clinical investigation. Hence, DNA sequencing of sub-genomic regions such as the core, the envelope, and NS5B regions is preferred.[87] Since the 5’UTR is conserved and thus unsuitable for genotype determination, this region has been employed for viral RNA detection. As recombination among HCV strains has emerged, genotyping based on one region may not be effective. To improve accuracy, this study examined the viral genotype by phylogenetic analysis based on the core and NS5B sequences.

Data were collected from the GenBank, EMBL, and DDBJ nucleotide sequence databases revealed that there are at least seven subtypes of HCV genotype 6 circulating in Thailand. In the course of this project, only five subtypes, 6e, 6f, 6i, 6j, and 6n could be identified. Subtypes 6b, 6c, and 6m have also been reported from Thailand, but they were not detected in the cohort study. According to previous studies, the target groups were blood donors and intravenous drug users from the northern part of Thailand.[88, 89]

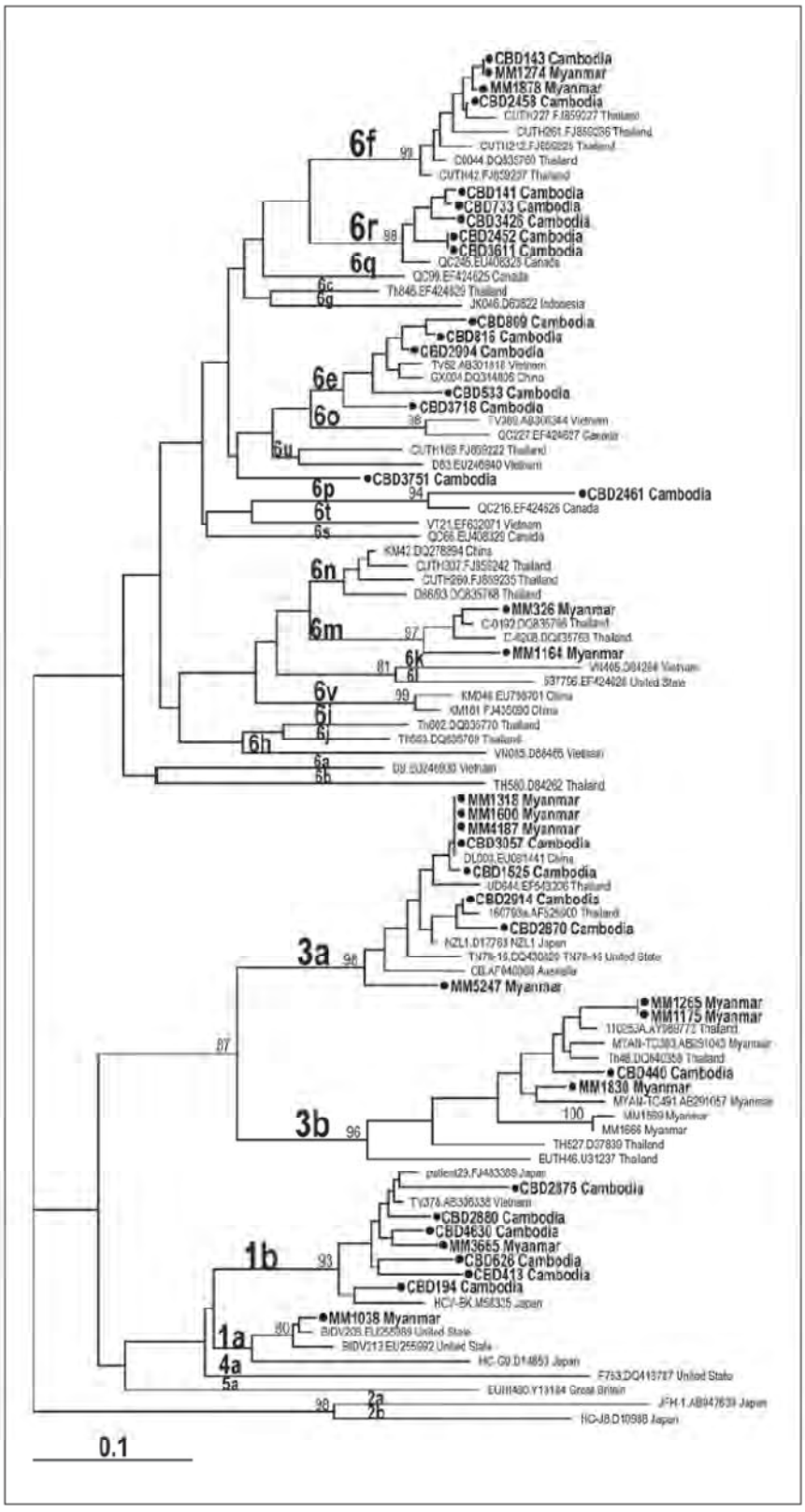

Figure 5 Phylogenetic tree constructed on partial core coding sequences. Sequences determined in the study are in bold. HCV genotypes are indicated on the branch of the individual cluster. Reference sequences were obtained from GenBank database. Bootstrap values > 80% were indicated at each node

Since the geographic location of the respective studies may have an influence on the genotype 6 subtype distributions, samples for this project were collected from Thai people residing in the central area. In correlation with the database, the predominant subtype of genotype 6 was 6f, followed by 6n. Information relating to the southern part of the country is still limited and reports on genotype 6 strains and reports on genotype 6 strains have been very rare, in contrast to the data on HCV genotype distribution in the north and center of the country. Similarly, genotype 6 has as yet not been reported from Malaysia, the country neighboring Thailand to the south. On the other hand, circulation of genotype 6 has been detected in western countries such as Canada. It appears that distribution of genotype 6 is not restricted to south-east Asia, but tends to spread to other regions of the world. Canada also has the highest diversity of HCV genotype 6. Accumulation of Asian immigrants from endemic areas genotype 6 may have contributed to this phenomenon.[90]

Information on HCV infection in some Southeast Asian countries is quite limited, especially Cambodia and Myanmar. Our study was carried out to determine the epidemiology of HCV among foreign immigrant workers from Cambodia and Myanmar. Anti-HCV seroprevalence of Cambodian workers was 2.3%, which was quite similar to the 2.2% determined for Thailand. Myanmar immigrants showed low prevalence of anti-HCV at 1.69%. The subjects from the two countries recruited into the study were mainly young people with a mean age of 26–27 years, which may account for the low prevalence of anti-HCV in this survey, while older age groups tend to show a higher prevalence of HCV infection.[91]

A high level of HCV infection (6.5%) has been detected mainly in adult males from Cambodia. Intravenous injection of various drugs, a popular habit in the Takeo province, may constitute the major source of infection.[92] Another report from rural Cambodia has shown that even in young age groups, HCV prevalence was very high (10.4%).[93] In 2002, a community-based survey suggested that intravenous drug abuse was common and administered at excess rate among the general population. The population knew about HIV transmission associated with dirty needles, but only half were concerned that hepatitis virus could be transmitted by the same route.[94] In contrast to previous studies, the present study demonstrated a lower level of HCV infection (2.3%) mainly representative for healthy male Cambodians. Place of residence in their home country could not be identified. In the meantime, the Cambodian government has made an effort to discourage intravenous drug injection and improve public health. Hence, the decrease in HCV infection rate observed with the samples tested could imply that the health care infrastructure of Cambodia has improved.

Viral RNA was detected in 75.8 and 55.6% of the anti-HCV seropositive samples from Cambodia and Myanmar, respectively. In agreement with various reports, the percentage of anti-HCV positive samples ranged from 50 to 90%. Some individuals who have naturally cleared the virus may remain seropositive without exhibiting viremia. However, owing to low viral load, HCV RNA could not be detected in some infected individuals. Various HCV genotypes were detected among Cambodian immigrants in this survey. Some genotypes are common in Thailand (1b, 3a, 3b, 6e and 6f), while some subtypes of HCV genotype 6 are not found in the native population (6p, 6r and 6u). Subtype 6e is likely transmitted from Cambodia to other countries such as China and Vietnam. Subtype 6r seemed to originate from Cambodia in correlation with a previous study (Fig. 6).

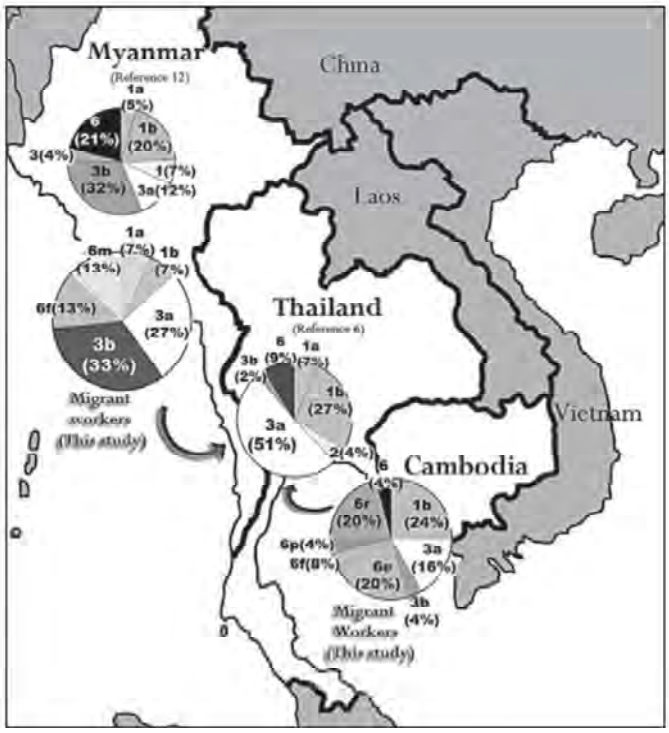

Figure 6 Comparison of HCV genotypes in Thailand and Myanmar

There is a large influx of immigrants from Myanmar and Cambodia to Thailand. In 2007, the annual report from the Office of foreign worker administration Thailand showed that 498,091 and 26,096 people had immigrated from Myanmar and Cambodia, respectively (http://115.31.137.7/workpermit/main/Stat/syear.asp, reported in Thai). As a large sample size was available, healthy workers were included in this study and they may have migrated from different parts of the country. Based on the results of this study, the trend of HCV infection could be extrapolated to the general population. Even though the HCV infection rate was lower than expected, the predominance of genotype 3 (3a; 26.7% and 3b; 33.5%)

of Myanmar immigrant workers in this survey was similar to previous studies.[95] HCV genotype 3 is also the predominant genotype in Thailand followed by genotype 1b and genotype 6. However, we have no data based on the previous study of HCV genotypes in Cambodia for comparison. Subtype 3a from Cambodia and Myanmar had mingled with subtypes from other countries. Genotype 3a is globally prevalent in injection drug users, as well as common in some Asian countries.[91, 96] Therefore, unsafe needle sharing or drug abuse may introduce this genotype to the general population. Furthermore, these two countries are connected by trade, travel and migration all of which may contribute to similar patterns of virus transmission and genotype distribution.

4.3 Response-guided therapy for patients with HCV genotype 6 Infection

The optimal duration of treatment with PEG-IFN plus RBV in patients with HCV genotype 6 is unknown. Our study was aimed at determining treatment response on the basis of rapid virological response (RVR) of HCV genotype 6 in comparison with genotypes 1 and 3. Sixty-six naïve patients received PEG-IFN-α2a (Pegasys, Roche Laboratories) 180 μg/week plus weight-based RBV (Copegus, Roche Laboratories) according to the following body weights: $\leq$75 kg, 1,000 mg/day; and >75 kg, 1200 mg/day. Regarding treatment duration, patients infected with HCV genotype 1 (group 1) and HCV genotype 3 (group 3) were treated for a fixed duration of 48 and 24 weeks, respectively. Patients infected with HCV genotype 6 (group 6) who achieved RVR were assigned to treatment for 24 weeks (group 6A) and the remaining patients were treated for 48 weeks (group 6B).

Patients with undetectable HCV RNA at week 12 were defined as having a complete early virological response (cEVR), whereas those with a minimum 2-$\log_{10}$ decrease from the baseline in HCV RNA at week 12 were defined as having a partial early virological response (pEVR). Patients with no or minimal change in HCV RNA levels (<2-$\log_{10}$ decrease from the baseline at week 12) were defined as non-responders and therapy was discontinued. All patients who completed the treatment were followed up for an additional 24 weeks after the end of therapy to assess sustained virological response (SVR).

A total of 66 patients were included in this pilot study. There were 16 patients in group 1, 16 patients in group 3 and 34 patients in group 6. Table 9 summarizes demographic and baseline characteristics of the patients by HCV genotypes. There were no significant differences in the baseline characteristics between each group in terms of gender distribution, mean age, body mass index (BMI), previous blood transfusion, ALT level, HCV RNA level and the degree of liver fibrosis assessed by histology. However, patients in group 6 had a significantly higher proportion of previous history of intravenous drug use compared with the other groups.

Table 9 Demographic and clinical baseline characteristics of the patients according to HCV genotypes

Baseline characteristics	Genotype 1 (n=16)	Genotype 3 (n=16)	Genotype 6 (n=34)	P
Age (yr)	46.4 ±12.5	42.8 ± 8.2	41.2 ± 8.4	NS
Sex (male)	56.3%	81.3%	67.6%	NS
Body mass index (BMI) (kg/m^2)	23.4 ± 13.1	21.3 ± 5.7	23.7 ± 3.7	NS
Previous blood transfusion	50.0%	53.8%	33.3%	NS
Previous intravenous drug users	15.4%	23.1%	40.7%	0.041
ALT (U/L)	82.7 ± 57.5	82.6 ± 51.9	62.6 ± 54.5	NS
Log_{10} HCV RNA (IU/ml)	6.4 ± 0.8	6.0 ± 0.8	6.5 ± 0.8	NS
Liver fibrosis score				NS
Score 0-2	69.2%	66.7%	71.4%	
Score 3-4	30.8%	33.3%	28.6%	

ALT, alanine aminotransferase; Data described as means ± SD or proportions (%)

Of group 1, 14 (87.5%) patients completed the 48 week-treatment and follow-up. One patient in this group was lost to follow-up by week 24 during therapy. One patient showed minimal changes in HCV RNA levels at week 12 and therapy was discontinued due to non-response. Of group 3, all patients completed the 24 week-treatment and follow-up. Of group 6, 25 (73.5%) patients who achieved RVR were assigned to treatment for 24 weeks (group 6A) and the remaining 9 (26.5%) patients were assigned to treatment for 48 weeks (group 6B). All patients in group 6A completed the 24 week-treatment and follow-up. Of group 6B, three patients were non-responders and therapy was discontinued and the remaining 6 (66.7%) patients completed the 48 week-treatment and follow-up (Fig. 7).

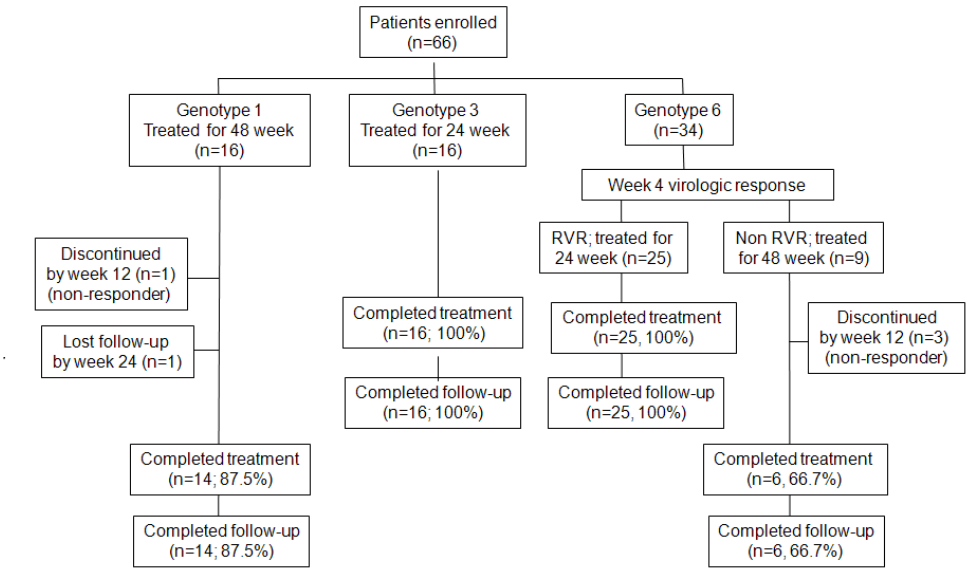

Figure 7 Flow diagram of the patients enrolled in the study.

Figure 8 compares the virological response to the combined therapy within each group. RVR were achieved in 14 of 16 (87.5%) patients in group 3 and 25 of 34 (73.5%) patients in group 6, which was not of statistical significance (p=0.277), but statistically more significant than that in group 1 (7 of 16 patients; 43.8%) (p=0.016 and p=0.045, respectively). The rates of cEVR were comparable between group 3 (15 of 16, 93.8%) and group 6 (30 of 34, 88.2%) (p=0.551), and were higher than in group 1 (12 of 16 patients, 75%), although there was no significant difference (p=0.174 and p=0.243, respectively). The overall rate of SVR in group 3 (13 of 16 patients, 81.3%) was similar to that of group 6 (26 of 34 patients; 76.5%) (p=0.704) and was higher than in group 1 (10 of 16 patients; 62.5%), although there was no significant difference (p=0.245 and p=0.309, respectively).

Among patients who attained RVR, SVR was achieved in 9 of 10 (90%) patients in group 1, 13 of 14 (92.9%) patients in group 3 and 22 of 25 (88%) patients in group 6A. The relapse rates among rapid responders in groups 1, 3 and 6A were 10%, 7.1% and 12%, respectively. There was no statistical difference in terms of SVR and relapse rates among these groups (Fig. 9).

Among patients in group 6A, the rates of cEVR and SVR were 100% and 88%, respectively. In this group, 10 of 10 (100%) patients with pretreatment viral load < 800,000 IU/ml and 12 of 15 (80%) patients with pretreatment viral load > 800,000 IU/ml achieved SVR. For those in group 6B, the rates of cEVR and SVR were 55.5% and 44.4%, respectively, which were significantly lower than those in group 6A (p=0.035 and p=0.025, respectively) (Figure 10). Patients in groups 6A and 6B who achieved cEVR

were likely to achieve SVR (88%, and 80%, respectively) Patients who did not achieve cEVR did not achieve SVR.

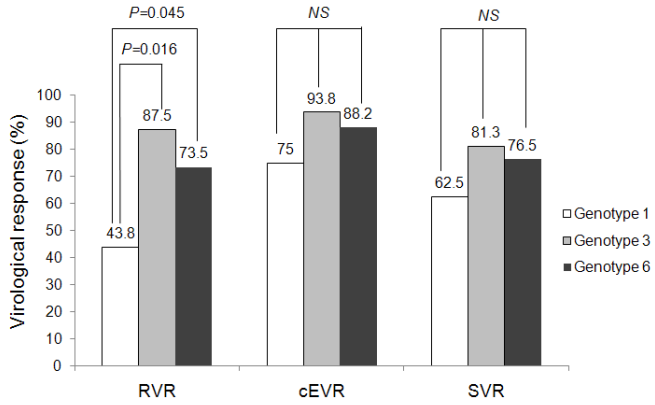

Figure 8 Rates of virological response according to HCV genotypes by intention-to-treat analysis

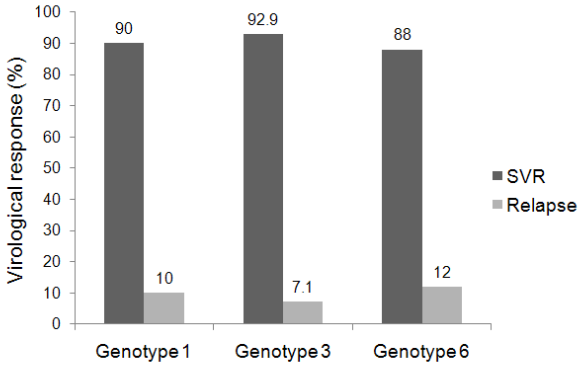

Figure 9 Rates of SVR and relapse in patients achieving RVR according to HCV genotypes

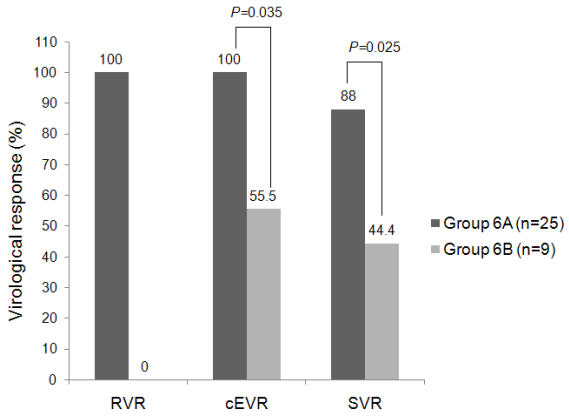

Figure 10 Rates of virological response in groups 6A and 6B

To identify factors associated with SVR, baseline characteristics of patients and early viral kinetics during therapy were analyzed by univariate and multivariate logistic regression analyses. A low HCV RNA level< 800,000 IU/ml (or < $\log_{10}$ 5.9 IU/ml) and achievement of RVR were factors predictive of SVR in univariate analysis. These factors were also independent predictors of SVR in multivariate analysis (Table 10).

Table 10 Univariate and multivariate logistic regression analysis

Factors	N	SVR (%)	Univariate analysis	Multivariate analysis	
			P	Odd ratio (95% CI)	*P*
Age (yr)					
<45	22	68.2	0.21	-	
≥ 45	12	91.7			
Sex					
Male	23	65.6	0.227	-	
Female	11	90.9			
BMI (kg/m2)					
<25	25	80.0	0.649	-	
≥ 25	9	66.7			
ALT (U/L)					
<80	26	80.8	0.355	-	
≥ 80	8	62.5			
Liver fibrosis score					
Score 0-2	26	76.9	0.444	-	
Score 3-4	8	62.5			
Log HCV RNA (IU/ml)					
<5.9	12	100	0.030	2.40 (0.30-4.50)	0.029
≥ 5.9	22	63.6			
RVR					
RVR	25	88.0	0.017	4.51 (1.22-8.79)	0.013
non-RVR	9	44.4			

Currently, the treatment outcome of patients with genotype 6 has not been adequately studied because of the limited number of cases in western countries. However, the optimal treatment duration of HCV genotype 6 is a particularly important consideration in south China and many south-east Asian countries in which this genotype is prevalent.[97, 98] Most prior studies of HCV genotype 6 included patients treated for 48-52 weeks.[99-101] Recently, a small study of Asian-American patients comparing a 48-week to a shortened 24-week regimen showed that a significantly higher SVR rate was achieved in those treated by the 48-week course (75% vs. 49%).[102] 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 genotype 6 treated for 24 weeks was comparable to that of genotypes 2/3 (82% and 83%, respectively).[103] In that study, the positive predictive values of RVR and EVR for HCV genotype 6 were comparable with those for genotypes 2/3 (87% vs 91% and 86% vs 87%, respectively). More recently, a randomized controlled trial of 60 patients with genotype 6 demonstrated that there was no significant difference in SVR rates in patients treated with 48-week and 24-week regimens (79% and 70%, respectively).[104] In that study, RVR was a significant predictor of SVR in the 48-week group and tending towards significance in the 24-week group, although a sizeable number of patients did not have RVR measurement performed. These data indicate that 24 weeks of PEG-IFN plus RBV could effectively treat a subset of patients with genotype 6. However, the feasibility of a response-guided therapy by individualizing the duration of treatment according to viral kinetics in patients with genotype 6 has never been investigated.

To our knowledge, the present report is the first study directly examining the optimal duration of therapy based on RVR in patients with genotype 6. In this study, more than 70% of patients with genotype 6 achieved RVR and received an abbreviated 24-week regimen. Among these patients, the rate of relapse was approximately 10%, and nearly 90% of them eradicated the virus. These data are consistent with observations regarding treatment of HCV genotypes 1, 2, 3 and 4,[105] which suggest that monitoring RVR might be useful to guide treatment duration for patients with genotype 6. In particular, therapy might be shortened to 24 weeks in patients with genotype 6 achieving RVR, whereas a 48-week course was appropriate for those who cleared the virus after week 4. Thus, the integration of RVR into treatment decisions might identify patients with genotype 6 for whom an abbreviated course of therapy has proven to be satisfactory. The abbreviated regimen could offer advantages by reducing unnecessary medication exposure, which may make the treatment of HCV genotype 6 more affordable and maximize the cost effectiveness of therapy.

Several prospective trials of PEG-IFN and RBV have examined the use of RVR to select patients with HCV genotype 1 and non-1 genotypes for abbreviated therapy.[106-109] These studies have shown that a subset of patients with genotypes 2/3 and genotypes 1/4 who achieve RVR may be able to shorten therapy to 12–16 weeks, and 24 weeks, respectively, if certain pretreatment conditions are fulfilled. In recent meta-analyses of randomized controlled trials, it has been demonstrated that abbreviated therapies do not significantly compromise the likelihood of SVR among rapid responders with most favorable characteristics for SVR, including genotype 1 or 2 with low viral load, and genotype 3 with a weight-adjusted RBV regimen.[110] On multivariate analysis, the independent factors associated with SVR among patients with genotype 6 in this study were RVR and low pretreatment viral load. In fact, all rapid responders with low pretreatment viral load eventually eradicated HCV infection after completing 24 weeks of therapy, whereas the relapse rate was relatively high (20%) in rapid responders with high pretreatment viral load. These data suggested that abbreviated therapy for HCV genotype 6 might be particularly effective for rapid responders who had low pretreatment viral load. However, due to the small sample sizes analyzed, the ability to draw conclusions was rather limited, and further studies would be required before an abbreviated course could be generally recommended.

In this study, we found that the rate of RVR in patients with genotype 6 was slightly lower than that of genotype 3 (74% and 88%, respectively), but was significantly higher than that of genotype 1 (44%). These results might reflect a predictive indicator of the subsequent SVR rate of patients with genotype 6, which was at an intermediate level between those of genotypes 3 and 1, as demonstrated in previous reports.[103, 111-113] Also of interest was the observation that, although the proportion of patients achieving RVR varied by genotype, the probability of achieving SVR was consistently high (88-93%) across all genotypes among patients who achieved RVR. This result is consistent with previous data that patients who achieve RVR have the highest rates of SVR (80-90%), regardless of HCV genotype.[105]

Although PEG-IFN represents the backbone of treatment, combination with RBV has been shown to directly influence the outcome of therapy in that it prevents 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/day) is recommended for treating patients with genotype 3.[114] 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.[115, 116] Currently, the optimal dose of RBV for treatment of patients with genotype 6 is unknown. In

previous studies, daily weight-based or fixed doses of RBV had been used, rendering comparisons rather complicated. Nonetheless, a recent prospective trial has adopted a weight-based dosage of RBV for abbreviated treatment (24 weeks), which might result in achieving SVR equivalent to that obtained with longer treatment duration (48 weeks).[104] In our study, all patients, regardless of HCV genotypes, received a weight-adjusted dose of RBV (1000–1200 mg/day). Taken together, these data might reflect the need of a weight-based dosage of RBV in patients with genotype 6 undergoing abbreviated therapy.

4.4 Association of *IL28B* SNPs with treatment response in chronic HCV infection

Recent studies have shown an association SNPs near *IL-28B* gene and SVR with PEG-IFN and RBV in 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) infected patients is less clear. A total of 133 Thai patients with chronic HCV infection treated with PEG-IFN/RBV were included (mean age, 46.6 years; 69.2% were male). Among these, 40 (30.1%), 56 (42.1%) and 37 (27.8%) patients were infected with HCV-1, HCV-3 and HCV-6, respectively. DNA extracted from serum samples was analyzed by direct sequencing of the SNP rs12979860.

In this study, the SVR rates for HCV-1, HCV-3 and HCV-6 were 62.5%, 85.7% and 75.7%, respectively. The distribution of rs12979860 genotype in all patients was as follows: CC, 107 (80.5%); CT, 20 (15%); and TT, 6 (4.5%). There was significant difference in the distribution of CC genotype between HCV-1-infected patients (60%) and those infected with HCV-3 and HCV-6 (94.6% and 81.1%, respectively, $p<0.001$). In HCV-1, the SVR rate of CC genotype was significantly higher than that of non-CC genotype (79.2% and 37.5%, respectively, $p=0.018$). However, there was no such difference regarding the SVR rates in HCV-3 (84.9% and 100% for CC and non-CC genotypes, respectively) and HCV-6 (73.3% and 85.3% for CC and non-CC genotypes, respectively). In summary, the SNP rs12979860 was strongly associated with SVR in patients infected with HCV-1, but not with HCV-3 and HCV-6. Thus, analysis of *IL28B* genotype might not be useful to guide treatment for Thai patients infected with HCV-3 and HCV-6.

5. Conclusion and Future Prospect

Regarding the molecular epidemiological studies of HBV, our data showed that seroprevalence of HBsAg in migrant workers, which may represent the current prevalence of HBV infection in their countries, was higher than in Thailand (7-11% and 4%, respectively). This difference reflects a steady and remarkable decrease of chronic HBV carrier rate in Thai populations after implementation of the universal

HBV vaccination. In addition, our data demonstrated that HBV genotype/subtype C/adr was the predominant strains circulating in Thailand and neighboring countries. Furthermore, the 'a' determinant variants seemed to be more common in migrant workers than in Thai HBV carriers, and might not be attributed to vaccine-induced mutation. Finally, pre-S mutations, especially pre-S2 deletions and pre-S2 start codon mutations were not uncommon among Thai and neighboring populations. These data, along with similar observations in previous reports, could help elucidate the evolutionary pattern of HBV genetic variations in the clinical course of persistent HBV infection circulating in Southeast Asia.

Regarding the case-control study on sequence variations of HBV in patients with HCC, our data showed that A1762T/G1764A and G1899A mutations were independent factors associated with the risk of liver cancer. Thus, identification of these mutants in patients with chronic hepatitis B may be valuable for predicting the development of HCC. Further large-scale prospective studies, which offer advantages over cross-sectional investigations, are needed to verify these findings.

Regarding the epidemiological studies of HCV, our data showed that the prevalence of HCV infection in Cambodia and Myanmar immigrant workers determined in this study was similar to Thailand. Participants were mainly of a young age, which may provide an explanation for lower infection levels than previously reported. Various and as yet unclassified subtypes of HCV genotype 6 may have accumulated in Southeast Asia. Further research should be focusing on HCV genotype distribution, novel subtypes of HCV genotype 6, the evolution of the virus and incidence of HCV-related HCC in Southeast Asian countries.

Regarding treatment of chronic HCV infection, the results of our pilot study suggest that the overall response rate of HCV genotype 6 is slightly lower than that of genotype 3 but higher than that of genotype 1. In addition, a response-guided therapy based on viral kinetics may be useful to optimize treatment in patients with HCV genotype 6. In particular, shortened treatment duration of 24 weeks could be sufficient in patients with low pretreatment viral load who achieve RVR. Further prospective randomized trials are required to evaluate this response-guided strategy in a larger number of patients with genotype 6. In addition, treatment decisions for patients with chronic HCV infection currently are based mainly on their virological clinical characteristics. Although host genetic polymorphisms in the vicinity of *IL-28B* might determine the response rate in HCV genotype 1 infection, analysis of these SNPs might not be useful to guide treatment for Thai patients infected with HCV genotypes 3 and 6. However, further studies of different cohorts are warranted to validate these findings.

6. References

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Output

Publications from the project: 6 published articles, 2 manuscripts and 4 abstracts

1. A case-control study on sequence variations in the enhancer II/core promoter/precore and X genes of hepatitis B virus in patients with hepatocellular carcinoma Hepatol Int 2010; 4: 577-584. (Impact factor; IF=2.645)
2. Molecular epidemiological study of hepatitis B virus among migrant workers from Cambodia, Laos and Myanmar in Thailand. Med Virol 2010; 85: 1341-9. (IF=2.820)
3. Hepatitis C genotype 6 subtypes in Thailand and their geographic distribution. J Med Virol 2010; 82: 257-62. (IF=2.820)
4. Seroprevalence and Genotype of Hepatitis C Virus among Immigrant Workers from Cambodia and Myanmar to Thailand. Intervirol 2010; 54: 10-6 (IF=2.337)
5. Response-guided therapy for patients with hepatitis C virus genotype 6 infection: a pilot study. J Viral Hepatitis 2012; 19: 423-30. (IF=4.088)
6. Molecular Analysis of Hepatitis B Virus Associated with Vaccine Failure in Infants and Mothers: a Case-Control Study in Thailand. J Med Virol 2012; 84:1177-85. (IF=2.820)
7. Genetic history and evolution of hepatitis C virus 3a infection in Thailand (submitted)
8. Diagnostic accuracy of liver stiffness measurement and hyaluronic acid in detecting liver fibrosis and cirrhosis in chronic hepatitis B with respect to ALT levels (submitted)
9. Genotype of hepatitis C virus among blood donor in Udon Thani, the northeast Thailand (abstract presented in the 21st Conference of the Asian Pacific Association for the Study of the Liver; APSL 2011)
10. Prevalence of aflatoxin induced p53 mutation at codon 249 (R249S) in hepatocellular carcinoma with or without HBsAg in Thailand (abstract presented in the 22nd Conference of the Asian Pacific Association for the Study of the Liver; APSL 2012)
11. *IL28B* polymorphism is associated with treatment response in Thai patients with hepatitis C genotype 1, but not with genotype 3 and 6 (abstract presented in the 2nd Asia Pacific Perspectives in Hepatitis Forum; APPH2, 2012)
12. Characterization of HBV mutations in untreated HIV-HBV co-infected patients based on complete genomic sequencing (abstract presented in the 14th International Symposium on Viral Hepatitis and Liver Disease; ISVHLD, 2012)

การนำผลงานวิจัยไปใช้ประโยชน์

ข้อมูลจากงานวิจัยบางส่วนได้ถูกนำมาเรียบเรียงเป็นตำราเรื่อง "ไวรัสตับอักเสบบีและมะเร็งตับ" (Hepatitis B Virus and Liver Cancer) ซึ่งมีการตีพิมพ์เผยแพร่ครั้งที่ 1 เมื่อ เดือนมิถุนายน 2553 (จำนวน 317 หน้า) โดยตำรานี้จะเป็นประโยชน์ต่อนิสิตและนักศึกษาแพทย์ แพทย์ประจำบ้านและแพทย์ทั่วไป ตลอดจนนิสิตมหาบัณฑิตและดุษฎีบัณฑิต เพื่อใช้ประกอบการเรียนการสอนและเพื่อการค้นคว้าวิจัยต่อไป

การได้รับเชิญเป็นวิทยากร

1. วิทยากรบรรยายเรื่อง "Management on chronic hepatitis B and C" ในงานประชุมวิชาการสำหรับพยาบาล ในวันที่ 2 ตุลาคม 2553 ณ โรงแรมฮิลตัน หัวหิน จังหวัดประจวบคีรีขันธ์
2. วิทยากรบรรยายเรื่อง "ไวรัสตับอักเสบหายได้จริงหรือไม่" ในงานประชุมวิชาการประจำปี ครั้งที่ 26 คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ให้แก่แพทย์ทั่วไป ในวันที่ 12 ตุลาคม 2553 คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
3. วิทยากรบรรยายเรื่อง "Molecular virology of viral hepatitis C" ในการอบรม Basic science ของสมาคมแพทย์ระบบทางเดินอาหารแห่งประเทศไทยในวันที่ 17 ตุลาคม 2553 ณ อาคารศูนย์การแพทย์วิชัยยุทธ กรุงเทพฯ
4. วิทยากรบรรยายเรื่อง "Management of small HCC" ในงานประชุมวิชาการประจำปีสมาคมแพทย์ระบบทางเดินอาหารแห่งประเทศไทย ในวันที่ 20 พฤศจิกายน 2553 ณ โรงแรมรอยัล คลิฟ บีช รีสอร์ท พัทยา จังหวัดชลบุรี
5. วิทยากรบรรยายเรื่อง "Prevalence and treatment of hepatitis C virus genotype 6" ในงานประชุมวิชาการ 21st Conference of the Asian Pacific Association for the Study of the Liver (APSL 2011) ในวันที่ 20 กุมภาพันธ์2554 ณ ศูนย์ประชุมแห่งชาติสิริกิตต์ กรุงเทพฯ
6. วิทยากรบรรยายเรื่อง "ไวรัสตับอักเสบซี" ในงานประชุมวิชาการสำหรับประชาชน ในวันที่ 23 มีนาคม 2554 ณ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
7. วิทยากรบรรยายเรื่อง "ไวรัสตับอักเสบบีและซี" Pre-Post Donation Counseling Workshop ในงานประชุมวิชาการงานบริการโลหิตระดับชาติ ครั้งที่ 19 ประจำปี 2554 ในวันที่ 23 มีนาคม 2554 ณ โรงแรมริชมอนด์ จังหวัดนนทบุรี
8. วิทยากรบรรยายเรื่อง "Viral hepatitis C" ในงานประชุมวิชาการร่วมคณะแพทยศาสตร์สามสถาบัน พ.ศ. 2554: จุฬา-รามา-ศิริราช (Joint Conference in Medical Sciences 2011: Chula-Rama-Siriraj) ในวันที่ 15 มิถุนายน 2554 ณ ศูนย์ประชุมอิมแพคเมืองทองธานี จังหวัดนนทบุรี
9. วิทยากรบรรยายเรื่อง "Evidence-Based Occult Hepatitis B in Thai" ในงานประชุมวิชาการร่วมคณะแพทยศาสตร์สามสถาบัน พ.ศ. 2554: จุฬา-รามา-ศิริราช (Joint Conference in Medical Sciences 2011: Chula-Rama-Siriraj) ในวันที่ 16 มิถุนายน 2554 ณ ศูนย์ประชุมอิมแพคเมืองทองธานี จังหวัดนนทบุรี

10. วิทยากรบรรยายเรื่อง “มะเร็งตับ” ในงานประชุมวิชาการโรงพยาบาลนครปฐม ในวันที่ 13 กรกฎาคม 2554 ณ โรงพยาบาลนครปฐม จังหวัดนครปฐม

11. วิทยากรบรรยายเรื่อง “Hepatic fibrosis, regenerative and senecense process” ในการอบรม Basic science ของสมาคมแพทย์ระบบทางเดินอาหารแห่งประเทศไทย ในวันที่ 10 กันยายน 2554 ณ โรงแรมเดอะทวินทาวเวอร์ รองเมือง กรุงเทพฯ

12. วิทยากรบรรยายเรื่อง “Protecting the future for patients with chronic hepatitis B” ในงานประชุมวิชาการ Liver society in association with BMS ในวันที่ 8 ตุลาคม 2554 ณ โรงแรม Cape Nidhra Hotel หัวหิน จังหวัดประจวบคีรีขันธ์

13. วิทยากรบรรยายเรื่อง “การติดเชื้อไวรัสตับอักเสบบีร่วมกับการติดเชื้อเอชไอวี: ธรรมชาติของการเกิดโรคและการวินิจฉัย” ในงานประชุมวิชาการ HIV-NAT Symposium Series: HIV and Hepatic disease workshop ในวันที่ 27 กุมภาพันธ์ 2555 ณ ห้องประชุม ดำรงแพทยาคุณ อาคาร อปร.โรงพยาบาลจุฬาลงกรณ์

14. วิทยากรบรรยายเรื่อง “การแปลผลการคตรวจไวรัสตับอักเสบบีด้วยวิธี serology” ในงานประชุมวิชาการ HIV-NAT Symposium Series: HIV and Hepatic disease workshop ในวันที่ 27 กุมภาพันธ์ 2555 ณ ห้องประชุม ดำรงแพทยาคุณ อาคาร อปร.โรงพยาบาลจุฬาลงกรณ์

15. วิทยากรบรรยายเรื่อง “Efficacy and safety of tenofovir treatment for chronic HBV mono-infection” ในงานประชุมวิชาการ HIV-NAT Symposium Series: HIV and Hepatic disease workshop ในวันที่ 27 กุมภาพันธ์ 2555 ณ ห้องประชุม ดำรงแพทยาคุณ อาคาร อปร.โรงพยาบาลจุฬาลงกรณ์

16. วิทยากรบรรยายเรื่อง “Hepatology Highlight 2011” ในงานประชุมวิชาการประจำปีของสมาคมโรคตับแห่งประเทศไทย ในวันที่ 17 มีนาคม 2555 ณ โรงแรมเชอราตัน หัวหิน รีสอร์ท แอนด์ สปา จังหวัดประจวบคีรีขันธ์

17. วิทยากรบรรยายเรื่อง “Viral Hepatitis HBV; viruses, route of transmission, pathogenesis, diagnosis natural course and treatment” ในงานประชุมวิชาการ HIVNAT/Gilead Medical Education Program in Asia Course: HIV and Hepatic Disease ในวันที่ 26 มีนาคม 2555 ณ ห้องประชุมชั้น 7 คลินิกนิรนาม กรุงเทพฯ

18. วิทยากรบรรยายเรื่อง “การรักษาไวรัสตับอักเสบบี” ในโครงการเสวนาเรื่องรู้ทันโรคตับ ตอน “ไวรัสตับอักเสบบี ไขมันตับ ก้อนในตับ” ในวันที่ 25 เมษายน 2555 ณ ห้องประชุมเฉลิม พรมมาส คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

19. วิทยากรบรรยายเรื่อง “Clinical implication of HBsAg levels in the management of chronic hepatitis B ในงานประชุมวิชาการประจำปี 2555 ครั้งที่ 6 สถาบันโรคระบบทางเดินอาหารและตับ นันทนา–เกรียงไกร โชติวัฒนะพันธุ์ ในวันที่ 5 พฤษภาคม 2555 ณ โรงแรม Le Meridien Khao Lak and Spa Resort จังหวัดพังงา

20. วิทยากรบรรยายเรื่อง “Medical value of HBsAg quantification in chronic hepatitis B” ในงานประชุมวิชาการ one-day education of infectious diseases ของสภาเทคนิคการแพทย์ ในวันที่ 29 มิถุนายน 2555 ณ โรงแรมสยามซิตี้ กรุงเทพฯ

21.วิทยากรบรรยายเรื่อง “Novel diagnosis and treatment in viral hepatitis C” ในการประชุมวิชาการประจำปี ครั้งที่ 51 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในวันที่ 17 กรกฎาคม 2555 ณ อาคาร อปร คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

22.วิทยากรบรรยายเรื่อง “Multidisciplinary approach of hepatocellular carcinoma” ในการประชุมวิชาการประจำปี ครั้งที่ 51 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในวันที่ 18 กรกฎาคม 2555 ณ อาคาร อปร คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

23. วิทยากรบรรยายเรื่อง “ไวรัสตับอักเสบบี ภัยร้ายสู่มะเร็งตับ”ในการประชุมวิชาการของวิทยาลัยพยาบาลกองทัพบก ร่วมกับสมาคมโรคตับแห่งประเทศไทยและมูลนิธิโรคตับ ในวันที่ 20 กรกฎาคม 2555 ณ ห้องประชุมโรงพยาบาลชลบุรี จังหวัดชลบุรี

24.วิทยากรรับเชิญในรายการ “คุยกับหมอ” เรื่อง “ไวรัสตับอักเสบบี” ออกอากาศทางสถานีโทรทัศน์ Thai News Network2 (TNN2) ในวันที่ 21 กรกฎาคม 2555

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, Robert W. Storr Chair of Hepatic Medicine, University of Sydney, Australia

บทความสำหรับการเผยแพร่

ไวรัสตับอักเสบบีและไวรัสตับอักเสบซีเป็นสาเหตุสำคัญของเกิดโรคตับอักเสบแบบเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับตามลำดับ ในปัจจุบันประชากรทั่วโลกมีการติดเชื้อไวรัสตับอักเสบบีและไวรัสตับอักเสบซีมากกว่า 400 และ 170 ล้านคนตามลำดับ ส่วนในประเทศไทยมีความชุกของการติดเชื้อไวรัสตับอักเสบบีและไวรัสตับอักเสบซีประมาณร้อยละ 4 และ 2 ตามลำดับ ภาวะแทรกซ้อนต่างๆของโรคตับที่เกิดจากการติดเชื้อไวรัสตับอักเสบแบบเรื้อรัง เป็นปัญหาที่สำคัญอย่างยิ่งทางสาธารณสุขของประเทศ เพราะก่อให้เกิดความสูญเสียทางด้านเศรษฐกิจและสังคมเป็นอย่างมาก ดังนั้นองค์ความรู้พื้นฐานต่างๆของไวรัสตับอักเสบบีและไวรัสตับอักเสบซี จึงมีความสำคัญต่อความเข้าใจธรรมชาติของโรคอย่างถูกต้อง ตลอดจนนำไปสู่การวินิจฉัย การรักษาและการป้องกันโรคที่มีประสิทธิภาพ

ผู้ติดเชื้อไวรัสตับอักเสบบีและไวรัสตับอักเสบซีแต่ละคนมีการดำเนินของโรคแตกต่างกัน ทั้งนี้อาจขึ้นอยู่กับปัจจัยของเชื้อไวรัสและของผู้ป่วย องค์ความรู้ต่างๆในด้านระบาดวิทยาระดับโมเลกุลและอณูชีววิทยาของไวรัสตับอักเสบบีและไวรัสตับอักเสบซี รวมทั้งข้อมูลที่เชื่อมโยงทางคลินิกที่เกี่ยวข้องการเกิดมะเร็งตับและการรักษาด้วยยาต้านไวรัส ที่ได้จากการศึกษาวิจัยของโครงการเรื่อง "อณูไวรัสวิทยาของไวรัสตับอักเสบบีและซีที่สัมพันธ์กับการเกิดมะเร็งตับและการตอบสนองต่อการรักษาด้วยยาต้านไวรัส” ซึ่งได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัยเป็นเวลา 2 ปี ตั้งแต่ 31 พฤษภาคม 2553 ถึงวันที่ 30 พฤษภาคม 2555 นี้ แสดงให้เห็นว่าไวรัสตับอักเสบบีสายพันธุ์ซีเป็นสายพันธุ์ที่พบบ่อยที่สุดในประเทศไทยและประเทศเพื่อนบ้าน นอกจากนี้ยังพบว่าการกลายพันธุ์ของเชื้อไวรัสในบางตำแหน่งอาจเกี่ยวข้องกับการเกิดมะเร็งตับในประชากรไทย ส่วนระบาดวิทยาของไวรัสตับอักเสบซีพบว่าสายพันธุ์ที่พบได้บ่อยที่สุดในประชากรไทยได้แก่สายพันธุ์ 3, 1 และ 6 ตามลำดับ โดยสายพันธุ์ 6 ซึ่งเป็นสายพันธุ์เฉพาะของเอเชียตะวันออกเฉียงใต้ มีการกระจายของสายพันธุ์ย่อยในประชากรไทยแตกต่างจากที่พบในประชากรของประเทศเพื่อนบ้าน นอกจากนี้ยังพบว่าไวรัสตับอักเสบซีสายพันธุ์ 6 มีอัตราการตอบสนองต่อการรักษาด้วยยาต้านไวรัสแตกต่างจากสายพันธุ์ 1 และ 3

ผลของการศึกษาวิจัยจากโครงการดังกล่าวข้างต้น นอกจากจะเป็นประโยชน์อย่างยิ่งในด้านระบาดวิทยาระดับโมเลกุลของประชากรไทยโดยใช้เป็นข้อมูลเปรียบเทียบกับประชากรต่างๆทั่วโลกแล้ว ยังก่อให้เกิดองค์ความรู้ใหม่ๆที่เกี่ยวกับกลไกการเกิดโรค การพยากรณ์ความรุนแรงของโรคและการรักษาผู้ป่วยตับอักเสบเรื้อรัง รวมทั้งจะเป็นประโยชน์ในการป้องกันและโรคมะเร็งตับต่อไปในอนาคต

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ORIGINAL ARTICLE

A case–control study on sequence variations in the enhancer II/core promoter/precore and X genes of hepatitis B virus in patients with hepatocellular carcinoma

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Abstract
Purpose To evaluate the sequence variations in the enhancer II (EnhII)/basal core promotor (BCP)/precore (PC) and X genes of hepatitis B virus (HBV) in Thai patients with hepatocellular carcinoma (HCC) by conducting a cross-sectional case–control study.
Methods As much as 60 patients with HCC and 60 patients without HCC, who were matched for sex, age, hepatitis B e antigen (HBeAg) status, and HBV genotype, were included. Viral mutations in the EnhII/BCP/PC and X regions were characterized by direct sequencing in serum samples.
Results The prevalence of T1753C/A, A1762T/G1764A and G1899A mutations were significantly higher in the HCC group compared to the non-HCC group (43.3 vs. 23.3%, $P = 0.02$; 88.3 vs. 53.0%, $P < 0.001$; and 35.0 vs. 8.3%, $P = 0.001$, respectively). No significant difference between groups was found with respect to G1613A, C1653T, C1766T/T1768A, A1846T/C, T1858C, and G1896A mutations. By multiple logistic regression analysis, the presence of cirrhosis, A1762T/G1764A and G1899A mutations were independently associated with the risk of HCC.
Conclusion These data suggested that A1762T/G1764A and G1899A mutations were associated with the development of HCC in Thai patients.

Keywords Hepatitis B virus · Enhancer II · Core promoter · Precore · X genes · Hepatocellular carcinoma

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Introduction

Hepatitis B virus (HBV) infection is a major public health problem, with more than 350 million HBV carriers estimated worldwide [1]. Chronic HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carrier status, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). In HBV endemic areas, such as Southeast Asia, more than 60% of HCC cases are attributable to chronic infection with the virus [2]. Although the association between chronic HBV infection and HCC is well established, the virological factors, particularly HBV mutations, contributing to tumor development remain uncertain.

Hepatitis B virus, a member of the family *Hepadnaviridae*, is a partially double-stranded DNA virus that contains four overlapping open reading frames (ORFs) encoding the surface, precore/core, polymerase, and X genes. The virus shows remarkable genetic variability and is currently classified into eight genotypes, designated A to H based on genomic sequence analysis [3]. HBV has a high mutation rate compared with other DNA viruses because it lacks proofreading capacity during the replication via reverse transcription of its pregenomic RNA [4]. The well-known naturally occurring HBV variants include the precore (PC) stop codon mutation (G1896A), which abolishes hepatitis B e antigen (HBeAg)

production. The other common HBV variants include double mutations in the basal core promotor (BCP) region (A1762T/G1764A), which overlap with the ORF of the X gene and result in substantial decreases in HBeAg production [5]. These dual mutants have been reported in up to 50–80% of patients with HBeAg-negative chronic hepatitis B in Europe and Asia [6], and have been implicated in HCC development [7–9]. 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 [10–12].

The X-ORF encodes a 154 amino acid protein called hepatitis B virus X protein. This protein plays an important role in the regulation of viral genome expression, and has also been implicated in hepatocarcinogenesis [13]. The X protein is a multifunctional regulator that modulates host transcription, cell cycle progress, protein degradation, apoptosis, and signal transduction pathways [14]. It has been shown that mutations in the X gene may contribute to the development of HCC in HBV-infected patients [15, 16]. 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 [5]. However, current knowledge regarding the mutational patterns in the entire X region among patients with HCC is rather limited. Therefore, the aim of the current study was to evaluate the association between the mutations within the EnhII/BCP/PC and X genes and the risk of HCC by conducting a case–control study among Thai patients.

Materials and methods

Patients

Serum samples obtained from 60 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 July 2002 and June 2006. 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 [17]. Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyperattenuation at the arterial phase, 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.

Among these, 55 patients had cirrhosis as underlying liver disease. As much as 52 were males and 8 were females, with the mean age (±SD) of 55.7 ± 9.8 years. A total of 18 patients were positive and 42 were negative for HBeAg.

To examine the role of molecular virological factors in the development of HCC, 60 hepatitis B s antigen (HBsAg)-positive patients, who matched for age (±5 years), gender, HBeAg status, and HBV genotype with the patients with HCC, were selected as control patients. These patients visited our clinic every 4–6 months during the same period of recruitment of the present study and none had HCC development during follow-up. Of these control patients, 32 cases had cirrhosis diagnosed based on clinical features and/or histological examination.

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.

Biochemical, serological, and virological assays

Serum alanine aminotransferase (ALT), total bilirubin (TB), and albumin levels were measured with a commercial assay using an automated analyzer (Hitachi 912). Sera tested for HBsAg and HBeAg were determined using commercially available ELISA tests (Abbott Laboratories, Chicago, IL, USA). Serum HBV DNA level was quantified using a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection range of this assay was 2.7–8.7 log copies/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 re-suspended in 30 μL sterile distilled water and subjected to amplification of the X/BCP/PC regions (nucleotides (nt) 1,287–2,038) by polymerase chain reaction (PCR) using the primers Xi1: 5′-AGCTTGTTTTGC TCGCAGC-3′ (forward primer, nt. 1,287–1,305), and Ci1: 5′-TTCCGGAGACTCTAAGGCC-3′ (reverse primer, nt. 2,020–2,038). The obtained sequences span the region which included the entire X protein ORF (nt. 1,374–1,836), the EnhII region (nt. 1,685–1,773), the basal core promoter

(BCP) (nt. 1,742–1,849), direct repeat 1 (DR1) (nt. 1,824–1,834), direct repeat 2 (DR2) (nt. 1,590–1,600), the precore (nt. 1,814–1,901), and a part of the core region (nt. 1,901–2,038).

Briefly, the reaction mixture comprised 2 μL re-suspended DNA, 0.5 μL of 25 mmol of each primer, 10 μL of 2.5× MasterMix® (Eppendorf, Germany) and sterile distilled water to a final volume of 25 μL reaction. The reaction was performed in a PCR thermocycler (Eppendorf AG, 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, Hanburg, 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, USA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with CLUSTAL_X (version 1.83) program as previously described [18].

HBV genotyping

HBV genotypes were determined from serum samples, using PCR-restriction fragment length polymorphism (PCR–RFLP) genotyping based on analysis of the surface gene, as previously described [19].

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 clinical or viral 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 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of patients with and without HCC

The clinical features of patients with HCC and controls are showed in Table 1. Compared with the control group, patients with HCC had higher frequency of cirrhosis. In addition, patients with HCC had significantly poorer liver biochemical parameters (TB and albumin) compared to controls. However, there was no significant difference between groups in respect to ALT and HBV DNA levels (Table 1).

Comparisons of sequences in the EnhII/BCP/PC and X regions between the HCC and control groups

Based on direct sequencing of EnhII/BCP/PC regions, the mutations were found at nt. 1,613, 1,653, 1,753, 1,762, 1,764, 1,766, 1,768, 1,846, 1,858, 1,896, and 1,899. Compared with the controls, the HCC group had higher frequencies of T1753C/A, A1762T/G1764A, and G1899A mutations. However, no significant difference between groups was found with respect to G1613A, C1653T, C1766T/T1768A, A1846T/C, T1858C, and G1896A mutations (Table 2).

Single codon mutations were present in the X region, but with a generally scattered distribution, and without significant difference between the HCC and control groups. However, three mutational patterns including I127T/N, K130M, and V131I, corresponding to T1753C/A and double A1762T/G1764A mutations in the BCP region were found with significantly higher frequencies in the HCC group than in the controls. In contrast, no significant difference between groups was found with respect to A36T, P38S, A44L, and H94L mutations (Table 2).

In addition, four HBV variants in the HCC group showed the following deletions at or around nt. 1,762–1,764. One HBV variant had deletions at nt. 1,757–1,777, while another had deletions at nt. 1,756–1,764. One additional case had long deletions at nt. 1,594–1,827, while another had a deletion at nt. 1,762–1,776. Interestingly, one HBV variant in the HCC group had a 24-base insertion between nt. 1,674 and 1,675. All these cases belonged to the HBeAg-negative group.

Multivariate analysis of factors associated with HCC

To determine the independent contribution of clinical and virological features to the development of HCC, multiple logistic regression analysis was performed by using the significant factors identified in the univariate analysis. These factors included TB and albumin levels, the presence of cirrhosis, and nucleotide sequence variants list in Table 2 (C1653T, T1753C/A, A1762T/G1764A, and G1899A mutations). The significant factors associated with HCC development were A1762T/G1764A and G1899A mutations and the presence of cirrhosis (Table 3).

The cumulative effect of the mutations at A1762T/G1764A and/or G1899A, which were the significant factors

Table 1 Demographic and clinical characteristics of patients with or without HCC

Characteristics	Control group ($n = 60$)	HCC group ($n = 60$)	P
Age (years)	52.9 ± 8.6	55.7 ± 9.8	0.096
Sex			1
Male	52 (86.7)	52 (86.7)	
Female	8 (13.3)	8 (13.3)	
Total bilirubin (mg/dL)	1.5 ± 1.2	2.1 ± 1.4	0.014
Albumin (g/L)	3.6 ± 0.6	3.3 ± 0.6	0.005
ALT (U/L)	139.7 ± 101.4	161.1 ± 116.9	0.285
Cirrhosis	32 (53.3)	55 (91.7)	<0.001
HBeAg positivity	18 (30.0)	18 (30.0)	1
HBV genotype			1
B	16 (26.7)	16 (26.7)	
C	44 (73.3)	44 (73.3)	
HBV DNA level (log copies/mL)	6.1 ± 1.3	5.9 ± 1.4	0.451

Data were expressed as mean ± SD, no (%)

Table 2 Virological characteristics of HBV in the HCC and control groups

Characteristics	Control group ($n = 60$)	HCC group ($n = 60$)	P
Nucleotide sequences of EnhII/BCP/PC genes			
G1613A	18 (30.0)	24 (40.0)	0.339
C1653T	7 (11.7)	16 (26.7)	0.062
T1753C/A	14 (23.3)	26 (43.3)	0.02
A1762T/G1764A	33 (55.0)	53 (88.3)	<0.001
C1766T/T1768A	3 (5.0)	10 (16.7)	0.075
A1846T/C	14 (23.3)	16 (26.7)	0.833
T1858C	1 (1.7)	3 (5.0)	0.619
G1896A	17 (28.3)	26 (43.3)	0.127
G1899A	5 (8.3)	21 (35.0)	0.001
Amino acid sequences of X gene			
A36T	42 (70.0)	41 (68.3)	0.843
P38S	2 (3.3)	0 (0)	0.496
A44L	14 (23.3)	20 (33.3)	0.311
H94Y	7 (11.7)	16 (26.7)	0.062
I127T/N	18 (30.0)	39 (65.0)	<0.001
K130M	33 (55.0)	51 (85.0)	<0.001
V131I	33 (55.0)	52 (86.7)	<0.001

Data were expressed as mean ± SD, no (%)

Table 3 Multivariate analysis of factors associated with HCC

Factor	Odds ratio (95% CI)	P
A1762T/G1764A mutations	3.56 (1.16–10.89)	0.026
G1899A mutation	3.54 (1.09–11.47)	0.034
Presence of cirrhosis	8.44 (2.65–26.84)	<0.001

CI confidence interval, *OR* odds ratio

in multivariate analysis, was further examined. The odd ratio (OR) of HCC with A1762T/G1764A mutations was 6.19, while the OR with G1899A mutation was 5.92. With the presence of both A1762T/G1764A and G1899A mutations, the OR of HCC increased to 10.23. In setting of cirrhosis, the presence of A1762T/G1764A mutations substantially increased the OR of HCC to 15.00, while the presence of both A1762T/G1764A and G1899A mutations increased the OR to 13.44 (Table 4).

Comparison of clinical and virological features according to A1762T/G1764A mutations

The clinical and virological characteristics according to A1762T/G1764A mutations, which were the strongest mutations associated with HCC development, are shown in Table 5. Patients with HBV harboring A1762T/G1764A mutations had higher rates of cirrhosis and HBV genotype C

Table 4 Cumulative effect of factors on the risk of HCC

Characteristics	Control group ($n = 60$)	HCC group ($n = 60$)	Odds ratio (95% CI)	*P*
A1762T/G1764A	33 (55.0)	53 (88.3)	6.19 (2.43–15.83)	<0.001
G1899A	5 (8.3)	21 (35.0)	5.92 (2.06–17.06)	0.001
Cirrhosis	32 (53.3)	55 (91.7)	9.63 (3.38–27.41)	<0.001
A1762T/G1764A and G1899A	3 (5.0)	21 (35.0)	10.23 (2.86–36.67)	<0.001
Cirrhosis and G1899A	4 (6.7)	19 (31.7)	6.49 (2.05–20.51)	0.001
Cirrhosis and A1762T/G1764A	15 (25.0)	50 (83.3)	15.00 (6.12–36.74)	<0.001
Cirrhosis and A1762/G1764 and G1899A	2 (3.3)	19 (31.7)	13.44 (2.97–60.89)	<0.001

Data were expressed as no (%)

CI confidence interval, *OR* odds ratio

than those without such variants. In addition, the virus with A1762T/G1764A mutations had higher frequencies of T1753C/A, C1766T/T1768A, and G1899A mutations than the wild-type virus. However, no differences between groups were found with regard to other virological factors, including HBeAg positivity, HBV DNA level, C1653T, G1613A, A1846T/C, T1858C, and G1896A mutations.

Discussion

Identification of host and viral factors leading to the development of HCC may have important clinical implications in the management of patients with chronic HBV infection. There are now increasing data suggesting that HBV genotypes, HBeAg status, viral load, and emergence of genomic mutations may play an important role in causing different disease profiles in chronic HBV infection. This case–control study was aimed specifically to study the role of HBV mutations in EnhII/BCP/PC and X regions by excluding the confounding effects of viral factors, such as the status of HBeAg, HBV genotype, and viral load. This study also excluded the possibility of cohort effect that patients with chronic HBV infection are prone to have the evolution of viral mutations in advanced age. Thus, these results are more reliable than those of previous case series in which their confounding consequences from selection bias could not be avoided. Because host factors may vary among different populations, data from various ethnic groups and countries are needed to be compared before conclusions can be drawn. To our knowledge, the current case–control study is the first to reveal the association between HBV mutations and the development of HCC among Thai patients.

In this study, we found that double A1762T/G1764A mutations were an independent risk factor for the development of HCC, which was consistent with recent case–control studies conducted in China, Taiwan, and Korea [7, 12, 20, 21]. Also, the magnitude of the OR of HCC associated with the presence of the BCP double mutants in this study was approximately 3 to 4-fold, which was similar with reports of other studies. In fact, a prospective cohort of approximately 1,600 high-risk individuals in Qidong, China, showed that A1762T/G1764A mutations were detected in approximately 50% of HCC cases before cancer development, suggesting that these variants would indicate a high potential risk for hepatocarcinogenesis [22]. It has been reported that the development of A1762T/G1764A mutations is associated with HBV genotype and their prevalence is higher in genotype C than genotype B [8]. As expected, our data also demonstrated that A1762T/G1764A mutations were genotype C related. We also showed that the prevalence of T1753C/A mutation was significantly higher among the HCC group than the controls, although such mutant was not an independent risk factor of HCC in multivariate analysis. In this study, it should be noted that T1753C/A mutation always existed along with the presence of A1762T/G1764A mutations. Interestingly, previous data also demonstrated that T1753C/A mutation occurred later than A1762T/G1764A mutations in the course of chronic HBV infection [23]. These results suggested that A1762T/G1764A mutations might be the main HBV variants associated with the development of HCC, and T1753C/A mutation might also play an important, albeit lesser, role in hepatocarcinogenesis.

The association between the well-known G1896A mutation in the PC region and the risk of HCC development remains controversial. For instance, a Taiwanese study showed that the presence of the PC mutation significantly increased the risk for HCC [9], while another community-based cohort study with long-term follow-up conducted in the same country demonstrated that this mutant was associated with a decreased risk of HCC development [24]. In this study, our data showed that this common variant might not account for the increased risk of HCC among Thai populations. In contrast, point mutation at nt. 1,899 was an independent viral factor of HCC development. Our results were well matched with a recent study performed in Taiwan, which demonstrated that the prevalence of G1899A and not G1896A mutation was significantly higher among patients with HCC than those

Table 5 Comparison of clinical and virological characteristics according to A1762T/G1764A mutations

Characteristics	No A1762T/G1764A mutations ($n = 32$)	A1762T/G1764A mutations ($n = 88$)	P
Age (years)	52.9 ± 9.0	54.9 ± 9.4	0.268
Sex			0.385
Male	28 (87.5)	76 (86.4)	
Female	6 (12.5)	10 (13.6)	
Total bilirubin (mg/dL)	1.6 ± 1.2	1.9 ± 1.4	0.215
Albumin (g/L)	3.6 ± 0.6	3.4 ± 0.6	0.065
ALT (U/L)	145.2 ± 105.5	152.5 ± 111.5	0.74
Cirrhosis	20 (62.5)	67 (76.1)	0.043
HBeAg positivity	9 (28.1)	27 (30.7)	0.663
HBV genotype			<0.001
B	19 (59.4)	13 (14.8)	
C	15 (40.6)	73 (85.2)	
HBV DNA level (log copies/mL)	5.9 ± 1.5	6.1 ± 1.2	0.325
Mutations			
G1613A	8 (25.0)	34 (38.6)	0.137
C1653T	4 (12.5)	19 (21.6)	0.303
T1753C/A	0 (0)	40 (45.5)	<0.001
C1766T/T1768A	0 (0)	13 (14.8)	0.019
A1846T/C	8 (25.0)	22 (25.0)	0.815
T1858C	2 (6.3)	2 (2.3)	0.318
G1896A	14 (43.8)	3 (3.4)	0.527
G1899A	2 (6.3)	24 (27.3)	0.007

Data were expressed as mean ± SD, no (%)

without HCC [7]. In contrast, G1899A mutation was found at low prevalence with no clinical association in other previous reports [25, 26]. The reasons for these discrepancies among reports remain unclear and merit further studies to clarify the role of G1896A or G1899A mutant in HBV-related hepatocarcinogenesis.

Whether there are any additive or synergistic effects on the risk of HCC development with combinations of HBV mutations remain to be established. Recent studies demonstrated that certain complex HBV mutational patterns might be associated with the development of advanced liver diseases, including HCC [7, 27]. In this respect, our study showed that the risk of HCC was significantly increased in patients infected with HBV encoding both A1762T/G1764A and G1899A mutations. Of note, the risk of HCC was further increased among cirrhotic patients who were infected with HBV harboring A1762T/G1764A mutations or A1762T/G1764A and G1899A mutations in combination. These results suggest that these HBV mutations may serve as helpful virological markers for predicting the development of HCC, particularly in patients who already had cirrhosis. In agreement with our data, a recent prospective study demonstrated that A1762T/G1764A mutations were useful biomarkers for identifying a subset of male patients who were at increased risk of HCC [28].

Although the precise mechanism of A1762T/G1764A mutations in hepatocarcinogenesis remains uncertain, several hypotheses have been proposed. For instance, it has been shown that A1762T/G1764A mutants may enhance viral replication either by creating a hepatocyte nuclear factor 1 transcription factor-binding site or modulating the relative levels of precore and core RNAs [29]. Furthermore, the presence of BCP double mutants may be associated with decreasing T-cell immune responses [30]. 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 [5]. Thus, genomic variation in these regions could modify the oncogenic potential of the X protein and induce inactivation of p53-mediated apoptosis or impairment of DNA repair [31].

In this study, the rate of mutations affecting codons 130 (K130M) and 131(V131I) in the X protein, corresponding to double A1762T/G1764A mutations, significantly differed between patients with or without HCC. In addition, HBV with I127T/N mutation in the X protein, which corresponds to T1753C/A mutation was observed more frequently in patients with HCC than in the control group. These 'hot-spot' mutations are located in the carboxy functional region, and thus might be associated with the transactivating function of the X protein [32]. Previous

studies also reported that other amino acid substitutions, such as A36T, P38S, A44L, and H94Y were significantly associated with the risk of HCC [11, 15, 33, 34]. However, the prevalence of these mutations, except A36T, was found to be relatively low in our study and there was no significant difference in their prevalence between the HCC and non-HCC group. Thus, our data suggested that the emergence of these mutants might not lead to development of HCC in Thai patients. Instead, these mutants might occur during a long-standing inflammatory process of vertically transmitted chronic HBV infection among Thai populations.

In conclusion, our case–control study showed that A1762T/G1764A and G1899A mutations were independent virological factors associated with the risk of HCC. Identification of A1762T/G1764A and G1899A mutants may be valuable for predicting the development of HBV-associated HCC. Further large-scale prospective studies, which offer advantages over cross-sectional investigations, are needed to confirm these observations.

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Molecular Epidemiological Study of Hepatitis B Virus Among Migrant Workers From Cambodia, Laos, and Myanmar to Thailand

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Although hepatitis B virus (HBV) infection is endemic in Southeast Asia, molecular epidemiological data on HBV circulating in some countries are limited. The aims of this study were to evaluate the seroprevalence of HBV and its genetic variability among migrant workers from Cambodia, Laos, and Myanmar in Thailand. Sera collected from 1,119 Cambodian, 787 Laotian, and 1,103 Myanmarese workers were tested for HBsAg. HBV DNA was amplified and the pre-S/S region was sequenced for genotyping and genetic mutation analysis. HBsAg was detected in 282 (9.4%). The prevalence of HBsAg among migrant workers from Cambodia, Laos, and Myanmar was 10.8%, 6.9%, and 9.7%, respectively. Of 224 subjects positive for HBV DNA, 86% were classified as genotype C (99% were sub-genotype C1) and 11.6% were genotype B (30.8%, 34.6%, and 30.8% were sub-genotypes B2, B3, and B4, respectively). Various point mutations in the ''a'' determinant region were detected in approximately 18% of these samples, of which Ile126Ser/Asn was the most frequent variant. Sequencing analysis showed that 19.1% of samples had pre-S mutations, with pre-S2 deletion as the most common mutant (7.7%) followed by pre-S2 start codon mutation (3.8%) and both pre-S2 deletion and start codon mutation (3.3%). High prevalence of HBV infection (approximately 7–11%) was found among migrant workers from Cambodia, Laos, and Myanmar, which may reflect the current seroprevalence in their respective countries. The data also demonstrated that HBV sub-genotype C1 was the predominant strain and various mutations of HBV occurring naturally were not uncommon among these populations. ***J. Med. Virol. 82:1341–1349, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; seroprevalence; genotype; mutation; Southeast Asia

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major causes of chronic liver disease ranging from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) [Ganem and Prince, 2004]. HBV, a member of the family *Hepadnaviridae*, is a relaxed-circular double-stranded DNA virus of approximately 3,200 bp in length, with four overlapping open-reading frames encoding the polymerase (P), precore (PC)/core (C), envelope (pre-S1/pre-S2/S), and X proteins [Ganem and Prince, 2004]. HBV shows remarkable genetic variability and is

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classified currently into at least eight genotypes, designated A to H and four major serotypes, including *ayw*, *ayr*, *adw*, and *adr* [Norder et al., 1992; Kramvis et al., 2005]. Each genotype can be divided further into subgenotypes based on 4–8% divergence of the viral genome. HBV genotype and sub-genotype distribution appears to show varying geographic patterns [Allain, 2006; McMahon, 2009]. For example, genotypes A and D are predominant in Western countries and India, whereas genotypes B and C are common in Southeast Asia, China, and Japan. Genotype E is restricted to Africa, while genotypes F and H are found in indigenous populations in Alaska and Central and South America. In Asia, subgenotype B1 is predominant in Japan, while subgenotypes B2–5 prevail in other countries. Sub-genotype C1 is prevalent mainly in Southeast Asia, whereas subgenotype C2 is found commonly throughout the Far East as, for example, in Japan, China, and Korea [Allain, 2006; McMahon, 2009].

Chronic HBV infection and its related hepatic complications are important particularly in Southeast Asian countries where the prevalence of the infection is relatively high, varying from 3% to 6% in Singapore, Malaysia, and Brunei to approximately 6–12% in Indonesia, Philippines, Myanmar, Laos, Cambodia, and Vietnam [Lingao et al., 1989; Alexander et al., 1990; Sebastian et al., 1990; Amirudin et al., 1991; Budihusodo et al., 1991; Thüring et al., 1993; Lansang, 1996; Merican et al., 2000; James et al., 2001; Nakai et al., 2001; Caruana et al., 2005; Thuy et al., 2005; Srey et al., 2006; Jutavijittum et al., 2007; Duong et al., 2009; Utama et al., 2009]. In Thailand, the prevalence of HBV infection has declined upon implementation of the national HBV vaccination program, with present prevalence of approximately 4% [Theamboonlers et al., 1999; Luksamijarulkul et al., 2002; Chongsrisawat et al., 2006; Suwannakarn et al., 2008]. The predominant HBV genotypes in this region are genotypes C and B (Fig. 1). At present, a large number of migrant workers, originating from these countries, are employed in various sectors of Thai industries located in Bangkok and neighboring provinces. In 2006, registered and non-registered foreign workers in Thailand were approximately 1.8 million migrants [Martin, 2007]. Growing influx of migrant populations may influence the prevalence of HBV infection and the resulting disease burden in Thailand. The present study has been aimed at evaluating the HBV seroprevalence and its genetic variability, including genotypes, antigenic subtypes, and mutations present among these migrant workers. In addition, the phylogenetic relatedness of HBV strains isolated from these subjects was investigated.

MATERIALS AND METHODS

Study Populations

The serum samples of migrant workers collected for a routine health check-up were stored at −70°C until

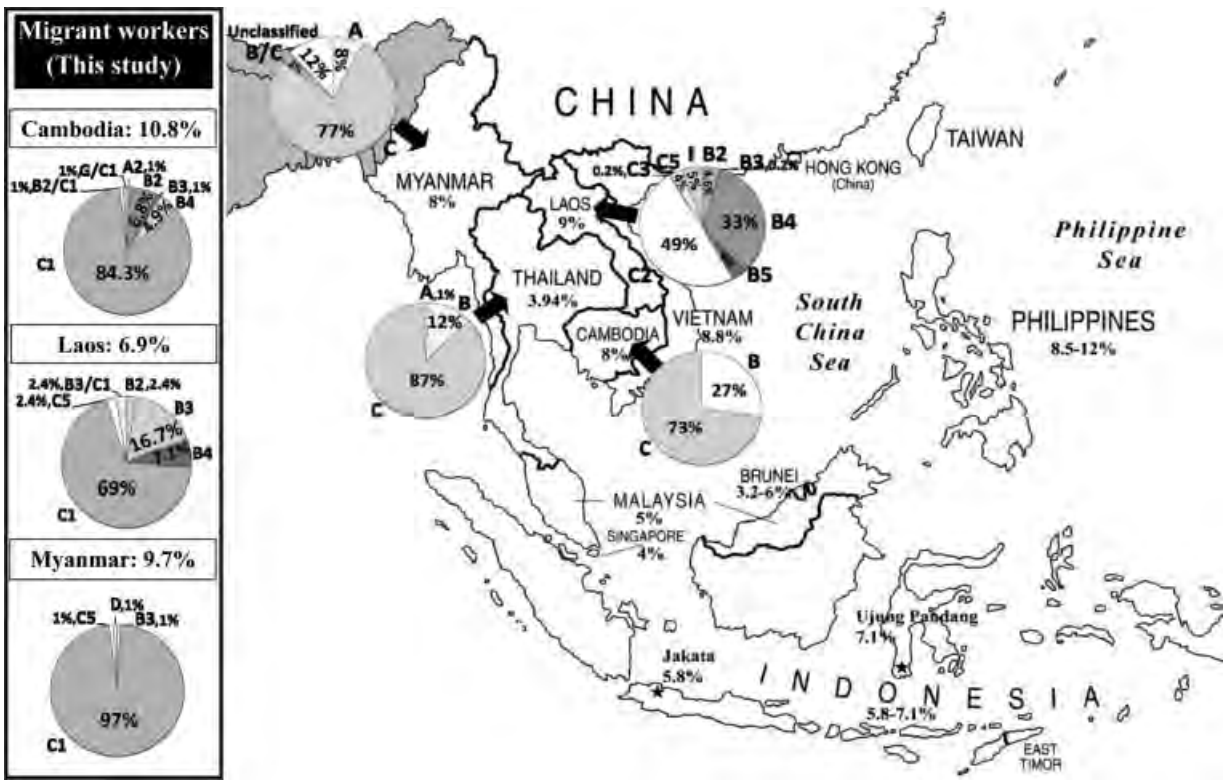

Fig. 1. The prevalence and genotypes of HBV infection in Southeast Asia countries derived from previous reports. Charts in the left corner demonstrate the prevalence and subgenotypes among migrant workers from Cambodia, Myanmar, and Laos in this study.

further analysis. In this study, 3,009 serum samples collected from 1,119 Cambodians (353 females, 763 males, and 3 unidentified), 787 Laotians (413 females, 364 males, and 10 unidentified), and 1,103 Myanmarese (582 females, 423 males, and 98 unidentified) were tested for hepatitis B surface antigen (HBsAg) by using a commercial automated ELISA (Murex Biotech Limited, Dartford, Kent, England). Samples positive for HBsAg were subjected to further analysis aimed at molecular characterization of HBV. The project had been approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University.

HBV DNA Extraction, Amplification, and Sequencing

HBV DNA was extracted from 100 μm each of HBsAg-positive sera. The respective serum samples were 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) at 50°C for 60 min followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. DNA pellet was re-suspended in 30 μl of distilled water. The pre-S1/pre-S2/S region was amplified using primers Pre-S1F+ (5′-GGG TCA CCA TAT TCT TGG GAA C-3′: position 2814–2835) and R5 (5′-AGC CCA AAA GAC CCA CAA TTC-3′: position 1015–995) The total 25-μl reaction volume consisted of 10 μl of 2.5× 5 PRIME MasterMix solution (5 PRIME GmbH, Hamburg, Germany), 0.5 μl of 25 μM forward and reverse primers, 2 μl of DNA template and sterile distilled water. The thermocycler was programmed for HBV DNA amplification as follows: initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1.30 min and a final extension step at 72°C for 7 min. The HBV DNA amplicons were separated by 2% agarose gel electrophoresis at 100 V for 60 min and stained with ethidium bromide. PCR product size was estimated in comparison with a 100-bp DNA ladder under UV light. The expected products were excised from the gel and purified using the Perfectprep® Gel Cleanup Kit (Eppendorf, Hamburg, Germany). The purified samples were sent to a commercial DNA sequencing company (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia) for sequencing. Nucleotide sequences were edited by Chromas Lite program version 2.01 (Technelysium Pty Ltd, Queensland, Australia) and assembled by SeqMan (DNASTAR Lasergene Software, Madison, WT).

Genotyping, Subtyping, and Phylogenetic Analysis

Each sequence was 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) for genotyping. The neighbor-joining method by Tamura-3 parameter was used for constructing phylogenetic trees. Uncompleted 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). HBV nucleotides were translated into amino acid sequences using the translation tool in ExPASy Proteomics Server (available on: http://www.expasy.ch/tools/dna.html). Subsequently, subtypes were identified based on the amino acids at positions 122 and 160 of the S protein.

HBV Mutation Analysis

HBV sequences were evaluated for mutations and deletions in the pre-S1/pre-S2 regions. The amino acids at positions 120 and 160 of the S protein were indicative for "a" determinant mutations.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. Comparisons among groups were analyzed by the Pearson χ^2 or Fisher's exact test for categorical variables and by one-way ANOVA Bonferroni adjustment for quantitative variables. *P*-values below 0.05 were considered significant. All statistical analyses were performed using the SPSS software for Windows 17.0 (SPSS, Inc., Chicago, IL).

RESULTS

HBsAg Detection

HBsAg was detected in 282 of 3,009 (9.4%) samples. This group comprised 121 Cambodians (10.8%), 54 Laotians (6.9%), and 107 Myanmarese (9.7%). Among these subjects, HBV DNA was detected in 102 Cambodians (84.3%), 42 Laotians (77.8%), and 80 Myanmarese (74.8%) (Table I).

Distribution of HBV Genotypes and Serotypes

All sequences obtained from this study were submitted to the GenBank database (accession nos. GQ855313–GQ85570 and GQ856585). Phylogenetic analysis was performed based on the pre-S1/pre-S2/S genes. Of those positive for HBV DNA, 194 of 224 (86.6%) cases were determined as genotype C (99% and 1% were sub-genotypes C1 and C5, respectively), 25 (11.2%) cases were identified as genotype B (32%, 36%, and 32% were sub-genotypes B2, B3, and B4, respectively), 1 (0.44%) case as genotype A (sub-genotype A2), and 1 (0.44%) case as genotype D. As for antigenic subtype distribution, *adr* was the most common (68.3%), followed by *ayw* (8.9%), *adw* (6.7%), and *ayr* (0.9%). The prevalence of HBV genotype and subtype with respect to geographic location is shown in

TABLE I. Prevalence of HBV Genotypes and Subtypes in Migrant Workers

	Cambodia (n = 1,119)	Laos (n = 787)	Myanmar (n = 1,103)	Total (n = 3,009)	*P*-value
No. HBsAg pos.	121 (10.8)	54 (6.9)	107 (9.7)	282 (9.4)	0.013*
No. HBV DNA pos.	102 (84.3)	42 (77.8)	80 (74.8)	224 (79.4)	0.008*
Gender (M/F/ND[a])	81 (79.4):20 (19.6):1 (1)	31 (73.8):11 (26.2):0	46 (57.5):28 (35):6 (7.5)	158 (70.5):59 (26.3):7 (3.2)	0.030*
Age (years; mean ± SD)	29.2 ± 8.6	26.2 ± 7.4	28.3 ± 6.1	28.3 ± 7.6	NS
15–20 (M/F/ND/total)	14:0:0	8:3:0	7:3:0	29 (18.4):6 (10.2):0:35 (15.6)	
21–30	37:13:0	18:6:0	19:16:6	74 (46.8):35 (59.3):6 (85.7):115 (51.3)	
31–40	19:4:0	3:1:0	18:9:0	40 (25.3):14 (23.7):0:54 (24.1)	
41–50	10:3:1	2:1:0	2:0:0	14 (8.9):4 (6.8):1 (14.3):19 (8.5)	
ND	1:0:0	0:0:0	0:0:0	1 (0.6):0:0:1 (0.5)	
Genotype					
A2[b]	1 (1.0)	0 (0)	0 (0)	1 (0.44)	NS
B	13 (12.7)	11 (26.2)	1 (1.25)	25 (11.2)	0.000*
B2	7 (6.9)	1 (2.4)	0 (0)	8 (3.6)	
B3	1 (1.0)	7 (16.7)	1 (1.3)	9 (4.0)	
B4	5 (4.9)	3 (7.1)	0 (0)	8 (3.6)	
C	86 (84.3)	30 (71.4)	78 (97.5)	194 (86.6)	0.000*
C1	86 (84.3)	29 (69.0)	77 (96.3)	192 (85.7)	
C5	0 (0)	1 (2.4)	1 (1.25)	2 (0.9)	
D[b]	0 (0)	0 (0)	1 (1.25)	1 (0.44)	NS
Suspected recombination					NS
B2/C1	1 (1.0)	0 (0)	0 (0)	1 (0.44)	
B3/C1	0 (0)	1 (2.4)	0 (0)	1 (0.44)	
G/C1	1 (1.0)	0 (0)	0 (0)	1 (0.44)	
Subtype					
adr	76 (74.5)	20 (47.6)	57 (71.25)	153 (68.3)	0.000*
adw	9 (8.8)	5 (11.9)	1 (1.25)	15 (6.7)	NS
ayr	1 (1.0)	1 (2.4)	0 (0)	2 (0.9)	NS
ayw	6 (5.9)	12 (28.6)	2 (2.5)	20 (8.9)	0.000*
Could not be identified	10 (9.8)	4 (9.5)	20 (25.0)	34 (15.2)	

NS, no statistical significance.
Data were expressed as mean ± SD, n (%).
[a]Data not available.
[b]*Pre-C* gene could not be amplified.
*P-values <0.05.

Table I. There were significant differences in genotype and serotype distribution among groups. Briefly, Cambodians and Laotians had significantly higher prevalence of genotype B but had significantly lower prevalence of genotype C than those of Myanmarese ($P < 0.05$). In addition, Laotians had significantly higher prevalence of serotype *ayw* but had significantly lower prevalence of serotype *adr* than those of Cambodians and Myanmarese ($P < 0.05$).

Although the entire genome were not sequenced in this study, three isolates with suspected inter-genotype recombinants were identified (isolate 31 with genotype B2/C1, accession no. GQ855407; isolate 612 with genotype B3/C1, accession nos. GQ855454 and GQ855560; and isolate 3794 with genotype G/C1, accession no. GQ856585). Isolate 31 proved to be a recombinant of sub-genotypes B2 and C1, with its recombination breakpoint estimated at nucleotide 573 (Fig. 2A). Isolate 3794 represented a recombinant of genotypes G/C1 with its recombination breakpoints between nucleotides 2854 and 56 (Fig. 2B). Isolate 612 was classified as sub-genotype B3 in the pre-S/S gene but showed sub-genotype C1 between nucleotides 1554 and 1974 (figure not shown).

Prevalence and Characterization of the "a" Determinant Mutations

In this study, various point mutations in the "a" determinant region were detected in 35 out of 194 (18.0%) HBV isolates. Mutations were found in 19/94 (20.2%) of Cambodian samples, 6/38 (15.8%) of Laotian samples, and 10/62 (16.1%) of Myanmarese samples. The most frequent mutation in Cambodian, Laotian, and Myanmarese isolates was Ile126Ser/Asn. In addition, multiple point mutations in the "a" determinant region were detected in six isolates (Supplement 1). Amino acid sequence alignment of the partial S region of these 35 isolates is shown in Figure 3.

Prevalence and Characterization of Pre-S/S Mutations

Sequencing of the amplicons showed that pre-S mutations were detected in 40 of 209 cases (19.1%). In this study, the prevalence of pre-S mutations/deletions among Cambodian, Laotian, and Myanmarese migrant workers was 18.4%, 15.0%, and 22.5%, respectively. As for the prevalence of site-specific pre-S/S mutations, pre-S2 deletion was the most common (7.7%), followed

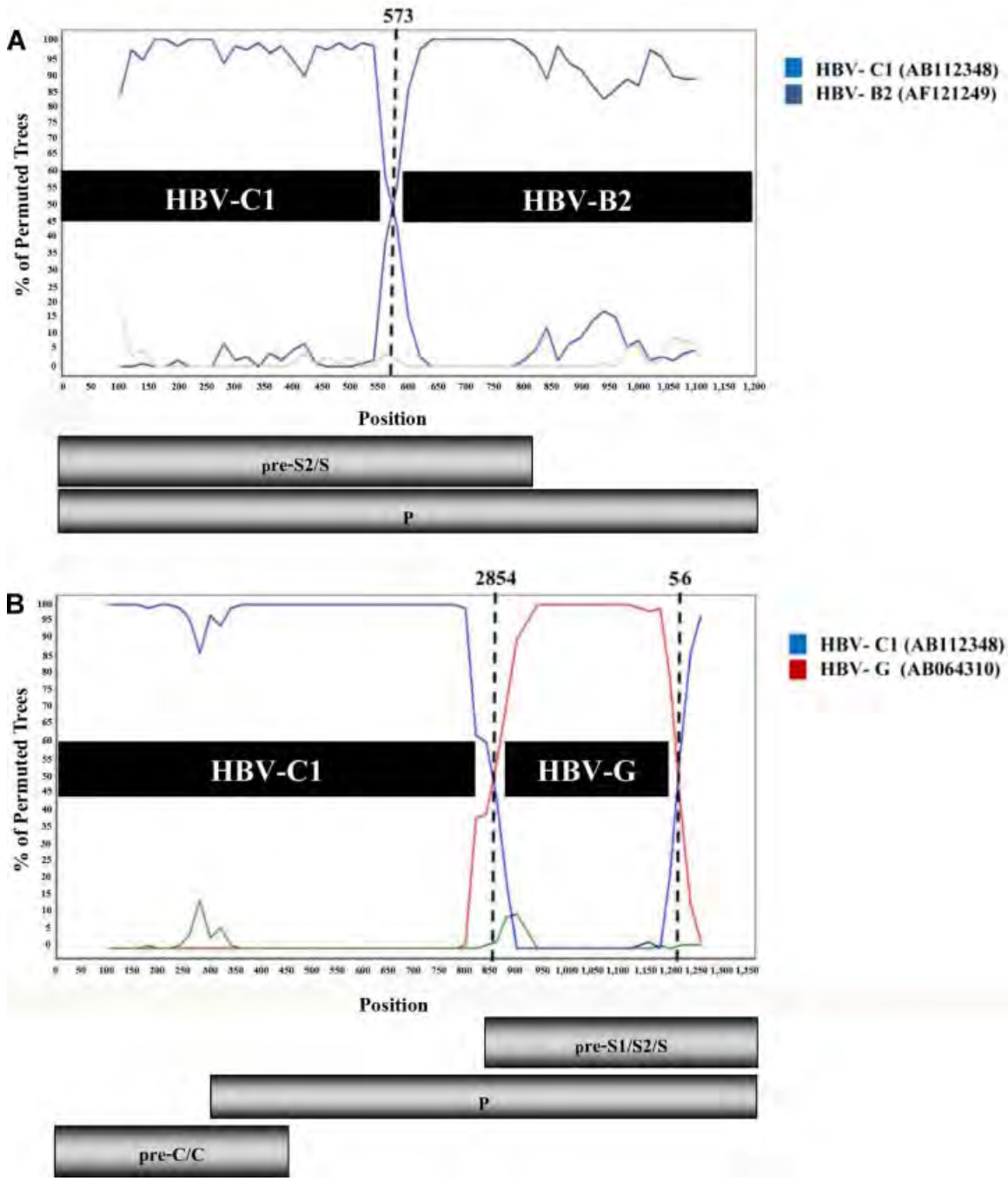

Fig. 2. Bootscanning analysis of suspected recombinant isolates. **A**: Complete S gene of isolate 31 was compared with HBV-B2 (AF121249) and HBV-C1 (AB112348); (**B**) isolate 3794, nucleotide positions 2006–157, was compared with HBV-C1 (AB112348) and HBV-G (AB064310). Dashed line(s) indicate(s) the breaking point(s) of recombination. The number above the dashed line indicates the nucleotide position of each isolate compared with the reference strain (NC_003977).

by pre-S2 start codon mutation (3.8%); both pre-S2 deletion and start codon mutation (3.3%); pre-S1 deletion (1.4%); pre-S2 start codon deletion and pre-S2 deletion (1.0%); pre-S1 start codon mutation and pre-S1 deletion (0.5%); pre-S1 start codon deletion and pre-S2 deletion (0.5%) and pre-S2 start codon mutation and pre-S2 deletion (0.5%). Amino acid sequence alignment of the entire pre-S1/pre-S2 region of the 40 samples is shown in Figure 4.

DISCUSSION

Although chronic HBV infection prevails in Southeast Asia, the data on its molecular epidemiology in some

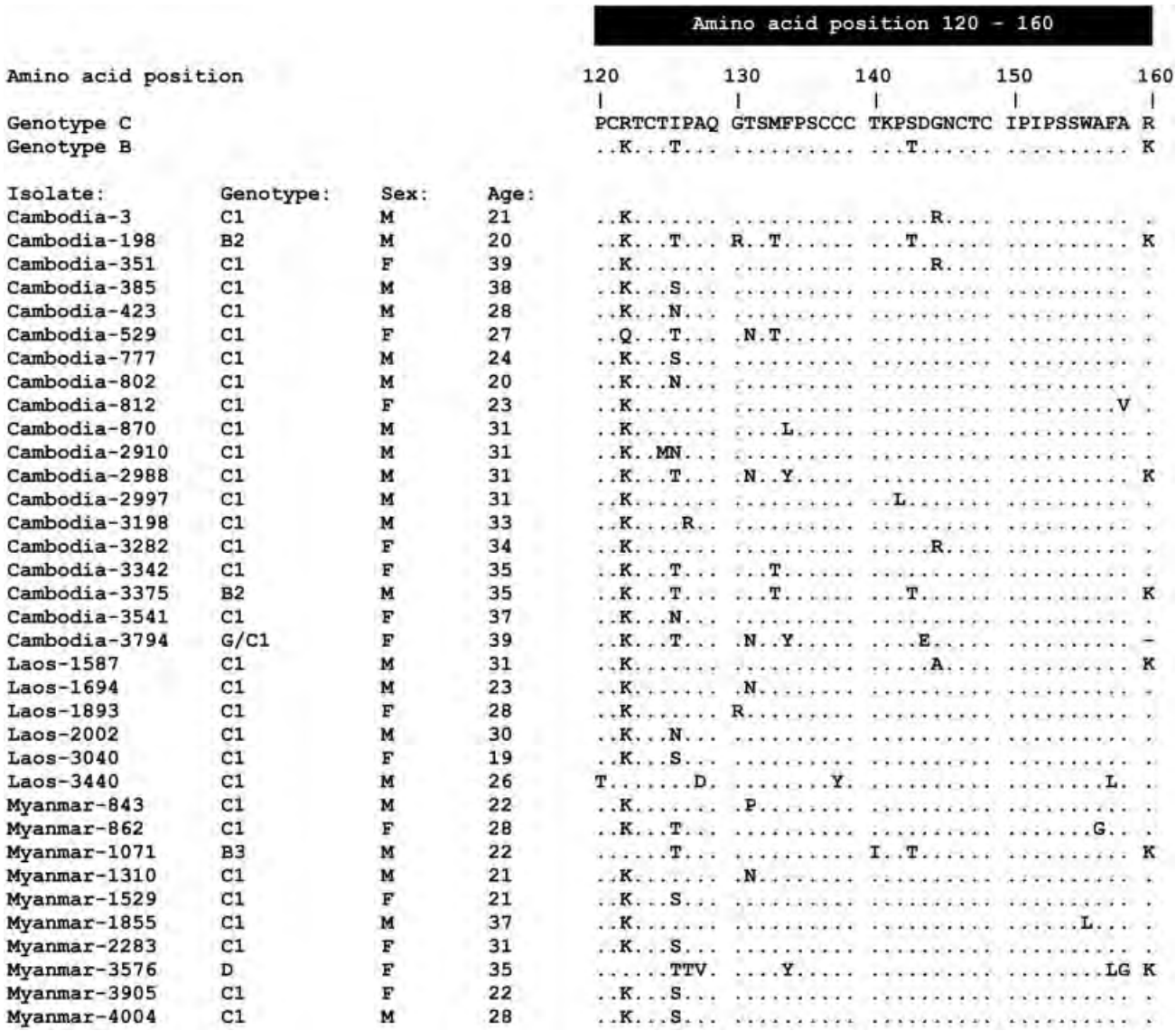

Fig. 3. Amino acid sequence alignment of the "a" determinant region of 35 samples.

countries in this part of the world are still limited. This has been the first comparative study on molecular characterization of HBV circulating in Cambodia, Laos, and Myanmar. This study, which included identification of both viral genotypes and subtypes in a significant number of HBV carriers from these countries, demonstrated that the predominant HBV strains belong to categories C1/*adr*, which accounted for more than 85% of cases. In this study, the most dominant strains of HBV found in migrant workers from the both countries (Myanmar and Laos) were HBV genotypes C1 and B3. In contrast to other previous studies, the most dominant strains of HBV in Laos were sub-genotype C2 and B4 [Olinger et al., 2008], and in Myanmar genotypes C and A [Nakai et al., 2001]. The difference may be the results of different population bring studied sampling. In this study, most of the migrant workers came from the boundaries between Thailand and Laos or Myanmar, whereas other reports studied in the population from the capital cities. These findings are not surprising but reflect the typical genotypes and subtypes circulating in Southeast Asia. The seroprevalence of HBsAg in these migrant workers was approximately 7–11%, similar to previous reports on seroprevalence in these countries but higher than a recent nationwide survey in Thailand (4%) [Theamboonlers et al., 1999; Luksamijarulkul et al., 2002]. This difference in seroprevalence among populations reflects a steady and remarkable decrease in chronic HBV carrier rate among Thai populations after the 1992 implementation of universal HBV vaccination.

HBV strains resulting from genomic recombination between different genotypes have been increasingly recognized in various parts of the world. In Asia, recombination of genotypes B/C has been reported in China, Hong Kong, Indonesia, Taiwan, Thailand, and

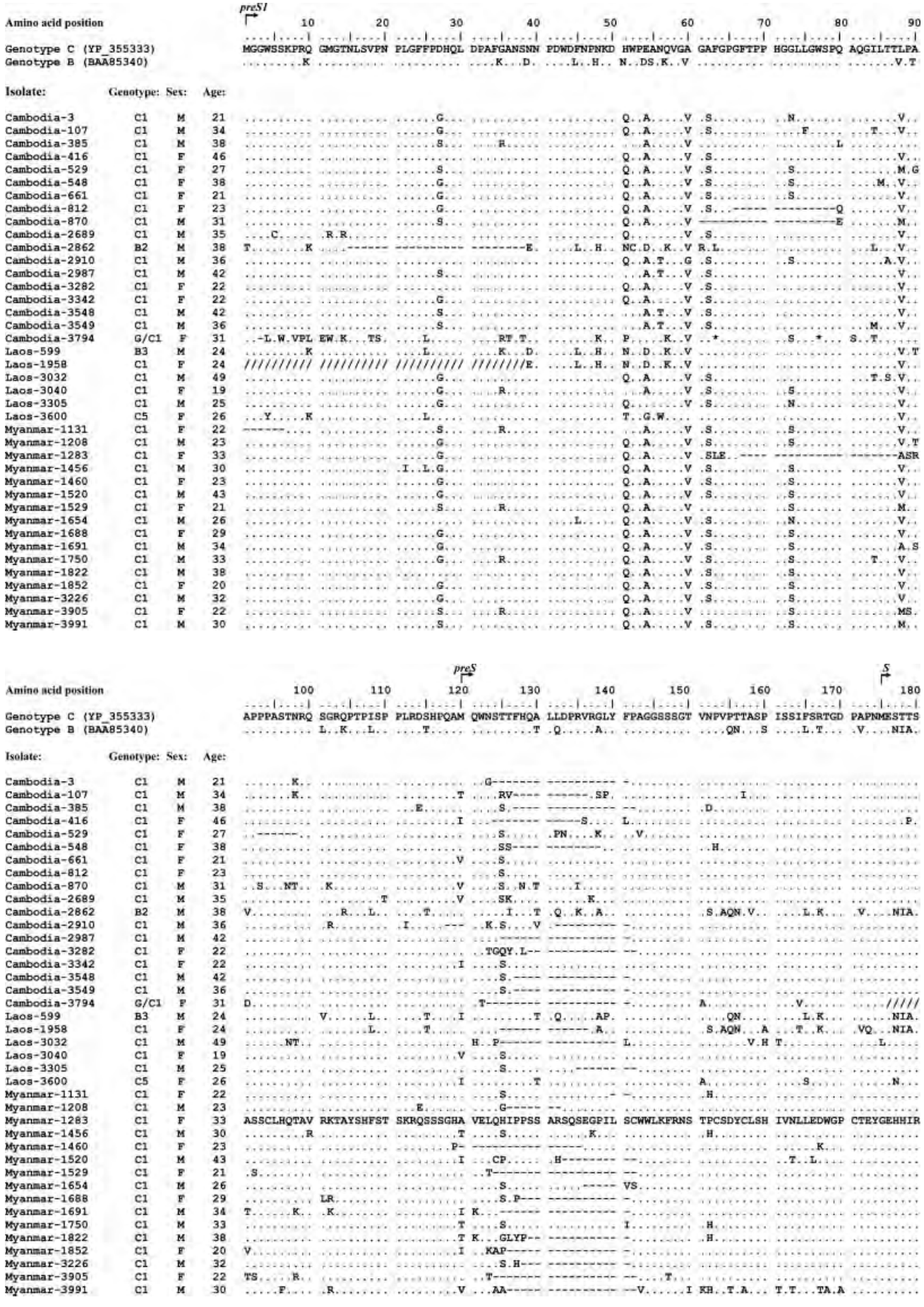

Fig. 4. Amino acid sequence alignment of the entire pre-S1/pre-S2 region of 40 samples.

Vietnam [Sugauchi et al., 2002], whereas recombination of genotypes C/D has been detected in Tibet and China [Cui et al., 2002; Wang et al., 2005]. In addition, recombinants between genotypes A/C and genotypes A/D have been documented in Vietnam [Hannoun et al., 2000] and India [Chauhan et al., 2008], respectively. Recently, a novel genotype I, with a complex recombination involving genotypes C, A, and G has been reported in Vietnam and Laos [Huy et al., 2008; Olinger et al., 2008]. Although the entire genome sequence was not determined in this study, three HBV isolates with suspected inter-genotype recombinants were identified. It is of note that a hybrid of genotypes B3/C1 in this study displayed recombination breakpoints in the vicinity of the pre-C/C region, which is the most common site of inter-genotype recombination as previously described [Sugauchi et al., 2002]. Another recombinant of genotypes G/C with its recombination breakpoints between nucleotides 2006 and 157 was also demonstrated in this study. Interestingly, the site of breakpoints in this recombinant was different from that found in a hybrid of genotypes G/C identified previously in a Thai patient with HCC [Suwannakarn et al., 2008].

Amino acid substitutions within the "a" determinant domain could lead to conformational changes which may interfere with active and passive immunization against HBV infection [Carman et al., 1990]. The most common vaccine escape mutant resulted from the mutation at position 145 (Gly145Arg), which is located in the second loop of the "a" determinant [Carman et al., 1990]. In this study, however, the most common amino acid substitution found in Cambodian, Laotian, and Myanmarese samples was located at position 126. In addition, the prevalence of "a" determinant mutants among chronic carriers from these countries was approximately 15–20%, which was slightly higher than the prevalence among random chronic carriers recently reported (6–12%) [Echevarria and Avellón, 2006]. It has been proposed that vaccination might have increased a selection pressure on the emergence of surface mutants in relation to wild-type HBV, as has been observed in several regions of the world [Carman et al., 1990; Cooreman et al., 2001; Coleman, 2006]. For example, a previous study in Taiwan demonstrated an increase in the prevalence of "a" determinant mutants in children from 7.8% before to 23.1% 15 years after the introduction of universal vaccination against HBV [Hsu et al., 2004]. High prevalence of the variants among migrant workers in this study, however, might not be associated with previous vaccination because the coverage rates of HBV vaccine administration in their countries are generally low [Caruana et al., 2005; Soeung et al., 2009]. Thus, it is speculated that these mutants within the "a" determinant region might have emerged in response to natural immunoselective pressure of the host. These infectious mutants have been circulating among individuals chronically infected with the virus.

HBV pre-S mutations/deletions occurring naturally have been reported frequently in chronic HBV carriers. 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]. In fact, the prevalence of these mutations/deletions is rather variable and different, ranging from 0% to 36%, between diverse geographic areas [Huy et al., 2003]. In this study, the prevalence of pre-S mutations/deletions among Cambodian, Laotian, and Myanmarese migrant workers amounted to 18.4%, 15.0%, and 22.5%, respectively, which was higher than that determined by the previous study conducted on Thai populations (9.5%) [Suwannakarn et al., 2008]. As for the site of mutations, this study showed that pre-S2 deletion was the most common mutation type, followed by pre-S2 start codon mutation and the combined pre-S2 deletion and start codon mutation. These results were in agreement with those reported recently from Japan, Korea, and Thailand, according to which deletion in pre-S2 regions and pre-S2 start codon mutations was among the most prevailing [Huy et al., 2003; Choi et al., 2007; Suwannakarn et al., 2008].

In conclusion, high seroprevalence of HBsAg (approximately 7–11%) was found among migrant workers from Cambodia, Laos, and Myanmar, which may reflect the present prevalence of HBV infection in their respective countries. HBV sub-genotype/subtype C1/*adr* was the predominant strain circulating in these migrant workers. In addition, the "a" determinant variants were found frequently in these populations, and might not be attributed to vaccine-induced mutation. Finally, pre-S mutations, especially pre-S2 deletions and pre-S2 start codon mutations were not uncommon among these populations.

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Geographic Distribution of Hepatitis C Virus Genotype 6 Subtypes in Thailand

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The nucleotide sequence of hepatitis C virus (HCV) genotype 6 found mostly in south China and south-east Asia, displays profound genetic diversity. The aim of this study to determine the genetic variability of HCV genotype 6 (HCV-6) in Thailand and locate the subtype distribution of genotype 6 in various geographic areas. Four hundred nineteen anti-HCV positive serum samples were collected from patients residing in - the central part of the country. HCV RNA positive samples based on reverse transcriptase-polymerase chain reaction (RT-PCR) of the 5′UTR were amplified with primers specific for the core and NS5B regions. Nucleotide sequences of both regions were analyzed for the genotype by phylogenetic analysis. To determine geographic distribution of HCV-6 subtypes, a search of the international database on subtype distribution in the respective countries was conducted. Among 375 HCV RNA positive samples, 71 had HCV-6 based on phylogenetic analysis of partial core and NS5B regions. The subtype distribution in order of predominance was 6f (56%), 6n (22%), 6i (11%), 6j (10%), and 6e (1%). Among the 13 countries with different subtypes of HCV-6, most sequences have been reported from Vietnam. Subtype 6f was found exclusively in Thailand where five distinct HCV-6 subtypes are circulating. HCV-6, which is endemic in south China and south-east Asia, displays profound genetic diversity and may have evolved over a considerable period of time. ***J. Med. Virol. 82:257–262, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; HCV genotype 6; geographic distribution; phylogenetic analysis

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INTRODUCTION

Hepatitis C virus infection is a worldwide public health problem, with an estimated 170 million chronic infections globally. Although acute presentation of HCV infection is generally mild and asymptomatic, about 80% of infected individuals develop chronic infection and progress to cirrhosis and hepatocellular carcinoma [Hoofnagle, 2002]. HCV is a single-stranded positive-sense RNA virus of the family Flaviviridae. The viral genome is approximately 9.6 kb in length, flanked by 5′ and 3′UTR, and encodes a polyprotein precursor of about 3,000 amino acids. The precursor is cleaved into at least 10 different proteins comprising the structural proteins, core, E1, E2, and p7 as well as the non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [Choo et al., 1991; Moradpour et al., 2007].

HCV has been classified into six major genotypes and numerous subtypes [Simmonds et al., 1993, 2005]. A new HCV genotype 7a isolate has also been described [Murphy et al., 2007]. Direct sequencing of the NS5B, the core, and the envelope regions has proven a reliable method for classification of HCV genotypes [Simmonds et al., 2005]. Some genotypes (genotypes 1, 2, and 3) are distributed globally, while others are endemic in different geographically restricted areas [Simmonds, 2004]. In south-east Asia, genotype 3a is the most common genotype followed by 1b and 6 variants [Mellor et al., 1996; Kanistanon et al., 1997; Sunanchaikarn et al., 2007].

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HCV genotype 6 (HCV-6) distributed primarily in south China and south-east Asia displays pronounced genetic diversity. Novel subtypes of genotype 6 have been discovered continuously and now comprise 22 subtypes, 6a–6v [Lu et al., 2008; Noppornpanth et al., 2008; Xia et al., 2008a,b; Wang et al., 2009]. These may represent the evolution of this genotype for a considerable time span that exceeds probably 1,000 years [Pybus et al., 2009]. 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 [Lwin et al., 2007; Sunanchaikarn et al., 2007].

In Thailand, approximately 2.15% of the general population has been infected chronically with HCV. The common genotypes are 3a, 1b, and 6 variants [Sunanchaikarn et al., 2007]. Epidemiological data on the subtypes of HCV-6 are still limited. To determine the genetic variability of these variants, Thai patients infected with HCV have been recruited and investigated emphasis on the diversity of genotype 6. The geographic distribution of the predominant subtypes in south China and south-east Asia has been described in the cohort study.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. The anti-HCV positive samples were chosen from blood specimens obtained for screening or investigation and treatment. The specimens were treated as anonymous with a coding number. In addition, all specimens were used exclusively for academic research.

Sample Collection

Serum samples were collected from Thai individuals resident mainly in the central part of the country. Each specimen was subjected to enzyme-linked immunosorbent assay (ELISA) for detection of anti-HCV using a commercial ELISA (Murex anti-HCV version 4.0; Abbott, North Chicago, IL) according to the manufacturer's instructions. HCV antibody positive samples were selected for further testing. All specimens were stored at −70°C until used.

RT-PCR Amplification

Total RNA was extracted from 419 anti-HCV positive sera by the guanidinium method [Theamboonlers et al., 2002] and reverse transcribed into cDNA using random primers (Promega, Madison, WI) and M-MLV reverse transcriptase (RT; Promega). For HCV RNA detection, the DNA fragment of the 5′UTR was amplified. Viral RNA positive samples were selected for amplification of core and NS5B regions. Nested polymerase chain reaction (PCR) amplification of the 5′UTR and the core region was performed as described previously [Mellor et al., 1995; Theamboonlers et al., 2002; Sunanchaikarn et al., 2007]. The NS5B region was amplified by nested PCR resulting in a 471-bp fragment, with outer primers, NS5B_F1 (CAATWSMMACBACCATCATGGC, positions 7999–8020), NS5B_R1 (CCAGGARTTRACTGGAGTGTG, positions 8805–8825); and inner primers, NS5B_F2 (GATGGGHHSBKCMTAYGGATTCC, positions 8159–8181), NS5B_R2 (CATAGCNTCCGTGAANGCTC, positions 8611–8630) (nucleotide numbering is 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 Mg^{2+}, and 1.25 U of *Taq* 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.

Sequencing

After gel purification (Perfectprep Gel Cleanup Kit, Eppendorf, Hamburg, Germany), the amplicons from both core and NS5B were subjected to sequencing as described elsewhere [Sunanchaikarn et al., 2007]. 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 the BLASTN program (http://www.ncbi.nlm.nih.gov).

Genotyping and Phylogenetic Analysis

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 alignments were performed with ClustalX (v.1.83). Phylogenetic trees of HCV-6 based on both core and NS5B sequences were constructed. Neighbor-joining trees were constructed with the 6-Parameter method and bootstrapped 1,000 times to confirm the reliability of the phylogenetic tree (Shin-I et al., 2008).

The reference sequences of HCV-6 variants were retrieved from GenBank, EMBL, and DDBJ as follows: genotype 1a (M62321), 2a (D00944), 3a (D17763), 4a (Y11604), 5a (Y13184), 6a (EU246930, Y12083), 6b (D37841, D84262), 6c (EF424629), 6d (D84263), 6e (DQ314805), 6f (D37846, D37858, D37859, D37860, D37861, D38078, D38079, DQ835760, DQ835764), 6g

(D63822, DQ314806), 6h (D84265, D88466), 6i (DQ835762, DQ835770), 6j (DQ835761, DQ835769), 6k (D84264), 6l (EF424628), 6m (DQ835763, DQ835765, DQ835766, DQ835767), 6n (D14180, DQ278894, DQ 640364, DQ640365, DQ640371, DQ835768, EU246938), 6o (EF424627), 6p (EF424626), 6q (EF424625), 6r (EF116174), 6s (EF116169, AY434111), 6t (EF632069, EF632070, EF632071), 6u (EU246940), 6v (DQ179117), and 6 (AY878650, AY878651, DQ278891, DQ278892, DQ278893).

Geographic Distribution

All HCV-6 sequences were retrieved from GenBank, EMBL, and DDBJ nucleotide sequence database. To avoid redundancy of the same viral strain, this study analyzed the designated subtype based on the name given to the isolate. The genotype 6 isolates from China, Hong Kong, Singapore, Indonesia, Vietnam, Laos, Cambodia, Thailand, Taiwan, Myanmar, and India stored at the databases have been included. This genotype has been identified in western countries, such as Canada, France, and the United States. The geographic distribution of HCV-6 in different countries was investigated in this study.

RESULTS

HCV RNA Detection and Sequencing

Of the 419 anti-HCV positive samples, 375 were positive for viral RNA by RT-PCR amplification of the 5′UTR. These samples were subjected to further amplification of core and NS5B regions followed by nucleotide sequencing. The viral genotypes were investigated employing a viral genotyping tool (http://www.ncbi.nlm.nih.gov). Seventy-one samples determined as genotype 6 were subjected subsequently to phylogenetic analysis. The nucleotide sequences determined in this study were submitted to the GenBank database under designated accession numbers FJ859193–FJ859334.

Phylogenetic Analysis of Core and NS5B Regions

All HCV sequences determined as genotype 6 were confirmed by phylogenetic analysis based on core and NS5B alignments and classified into five specific subtypes. Neighbor-joining trees of the core and NS5B sequences were constructed (Fig. 1) and the sequences clustering closely with the same clade of reference strains were classified as the corresponding genotypes. According to these results, subtypes 6f and 6n are predominant in Thailand, followed by 6i, 6j, and 6e (Table I).

Worldwide Distribution of HCV-6

All HCV-6 strains stored at the GenBank, EMBL, and DDBJ were included in this study. A total of 820 sequences of genotype 6 reported from 13 countries were examined for distribution of subtypes of HCV-6. Most of the reported sequences were from Vietnam (n = 231), followed by China (n = 205) and Thailand (n = 141) (Fig. 2). HCV-6 has not only been isolated in south China and south-east Asia but also in western countries, such as Canada (n = 64), the United States (n = 5), and France (n = 1). According to these databases, Canada shows the highest diversity of genotype 6 with at least 11 subtypes identified and classified as 6a, 6c, 6e, 6f, 6h, 6l, 6o, 6p, 6q, 6r, and 6s.

Recently, a novel subtype, 6v has been identified in Kunming, China [Wang et al., 2009]. HCV-6 exhibits extreme diversity with 22 subtypes identified so far (Fig. 2). Of these 22 subtypes, 6a is found mainly in China and Vietnam. Subtypes 6b and 6c are from Thailand. Subtypes 6d and 6t are found only in Vietnam. Subtypes 6e, 6o, and 6p are reported frequently from Vietnam, but also found in Canada and China. Subtypes 6h and 6l are found mainly in Vietnam. Subtype 6g was reported from China and Indonesia. Subtypes 6f, 6i, and 6j are most predominant in Thailand while subtype 6k circulates in China and Vietnam. Subtypes 6m and 6n circulate in Thailand and Myanmar, and on occasion in China. Subtype 6q has been reported from Cambodia, Laos, and can be found in Canada. Subtypes 6r and 6s have been isolated exclusively from Asian immigrants to Canada (Fig. 2), whereas subtype 6u and the novel 6v have appeared only in China.

DISCUSSION

Phylogenetic analysis based on full-length genome sequences serves as the "gold standard" for HCV genotype or subtype identification. Although this constitutes the most accurate method for virus classification, due to cost and time factors, it is not practical as a routine clinical investigation. Hence, DNA sequencing of subgenomic regions such as the core, the envelope, and NS5B regions is preferred [Simmonds et al., 1993, 2005; Robertson et al., 1998]. Since the 5′UTR is conserved and thus unsuitable for genotype determination, this region has been employed for viral RNA detection. As recombination among HCV strains has emerged [Kalinina et al., 2002; Kageyama et al., 2006; Moreau et al., 2006; Noppornpanth et al., 2006; Legrand-Abravanel et al., 2007; Kurbanov et al., 2008], genotyping based on one region may not be effective. To improve accuracy, this study examined the viral genotype by phylogenetic analysis based on the core and NS5B sequences.

Data were collected from the GenBank, EMBL, and DDBJ nucleotide sequence databases revealed that there are at least seven subtypes of HCV-6 circulating in Thailand (Fig. 2). In the course of this project, only five subtypes, 6e, 6f, 6i, 6j, and 6n could be identified. Subtypes 6b, 6c, and 6m have also been reported from Thailand, but they were not detected in the cohort study. According to previous studies, the target groups were blood donors and intravenous drug users from the northern part of Thailand [Apichartpiyakul et al., 1999; Thaikruea et al., 2004]. Since the geographic

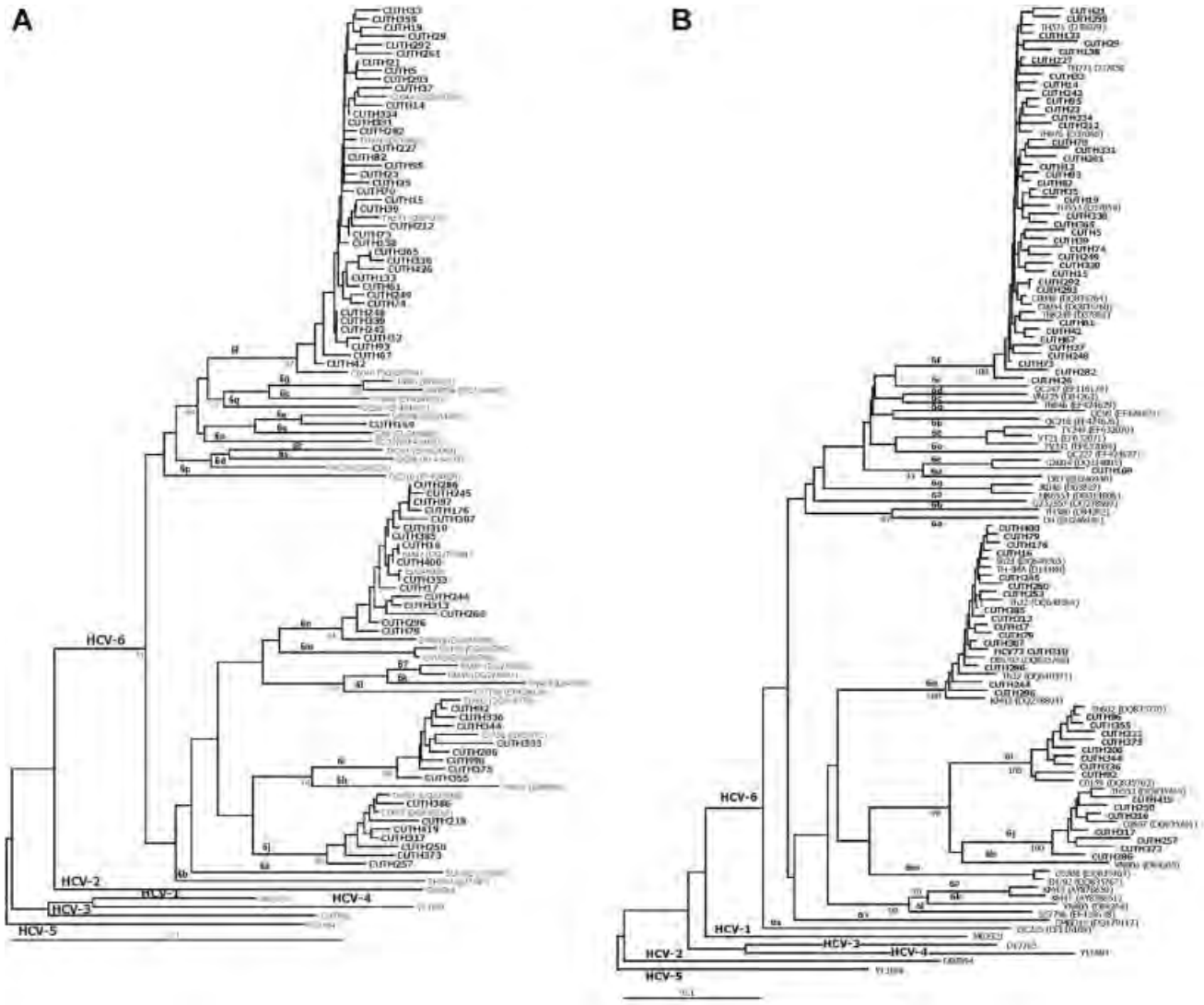

Fig. 1. Phylogenetic tree of HCV genotype 6 in Thailand constructed from (**A**) core and (**B**) NS5B nucleotide sequences. Boot strap values are shown in the tree root. (Reference strains are represented in bold characters.)

location of the respective studies may have an influence on the genotype 6 subtype distributions, samples for this project were collected from Thai people residing in the central area. In correlation with the database, the predominant subtype of genotype 6 was 6f, followed by 6n. Information relating to the southern part of the country is still limited and reports on genotype 6 strains have been very rare, in contrast to the data on HCV genotype distribution in the north and center of the country [Sunanchaikarn et al., 2007]. Similarly, genotype 6 has as yet not been reported from Malaysia, the country neighboring Thailand to the south [Mellor et al., 1996]. On the other hand, circulation of genotype 6 has been detected in western countries such as Canada. It appears that distribution of genotype 6 is not restricted to south-east Asia, but tends to spread to other regions of the world. Canada also has the highest diversity of HCV-6. Accumulation of Asian immigrants from endemic areas genotype 6 may have contributed to this phenomenon [Murphy et al., 2007].

TABLE I. Hepatitis C Virus Genotype 6 Subtypes Determined by Phylogenetic Analysis Based on Core and NS5B Regions

	HCV genotype 6 (%)					
Regions	6e	6f	6i	6j	6n	Total
Core	1 (1)	39 (56)	8 (11)	7 (10)	16 (22)	71
NS5B	1 (1)	39 (56)	8 (11)	7 (10)	16 (22)	71

This study constitutes the first report on the diversity of HCV-6 and its geographic distribution in Asia. One limitation of this study is that it does not represent the whole population of Thailand. In endemic areas, subtypes of HCV-6 are not distributed evenly, but each

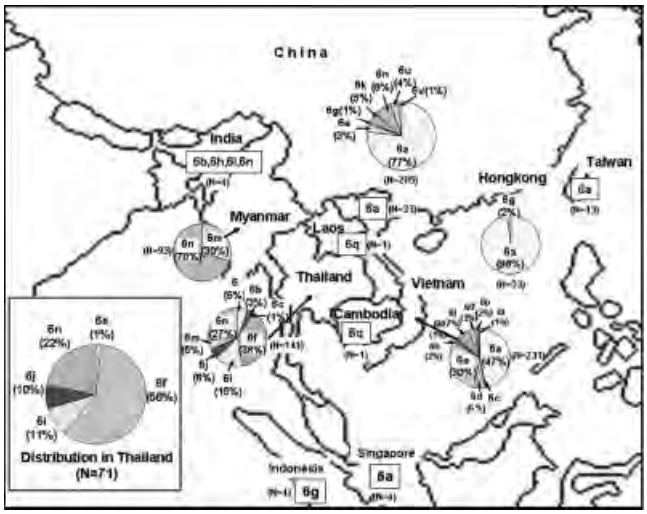

Fig. 2. Geographic distribution of HCV genotype 6 in south China and south-east Asia. Pie charts indicate the proportion of all HCV subtype 6 presents in each country. Pie chart at left bottom shows all HCV subtype 6 of Thailand detected in this study. The number of samples included in this study is indicated. The data have been obtained from GenBank, EMBL, and DDBJ nucleotide sequence databases.

subtype is found as a cluster restricted to the neighboring countries. Subtype 6a originates mainly from China, Hong Kong, Taiwan, Vietnam as well as Singapore (Fig. 2). All these countries either harbor one or more lineages or have experienced long-term migration from China, which may have resulted in the circulation of HCV carriers and may therefore be associated with similar transmission routes. HCV RNA positive blood donors (70 samples) were investigated in North Vietnam, and it was found that HCV-6 was highly prevalent (33 samples, 47%) and subtype 6a was predominant (26 of the 33 samples were genotype 6) [Duc et al., 2009]. However, as shown in Figure 2, Vietnam has experienced the highest variation in subtypes of HCV-6 in that at least 10 subtypes have been identified. As this country borders on several countries such as China, Cambodia, and Laos, migration may have contributed to the high diversity of this genotype. On the other hand, some subtypes are endemic in geographically restricted areas and are detected at low frequency (e g., 6d in Vietnam, 6f in Thailand). The specific prevalence of the respective genotype may have resulted from different patterns of historic transmission routes at each individual location, which in turn may have influenced, and thus, be reflected in the present genotype distribution.

Among six genotypes of HCV, genotype 6 shows extreme variation and sequence diversity. Up to now, 21 subtypes have been classified and a novel subtype has been designated as 6v [Wang et al., 2009]. Wang et al. [2009] have suggested that CMBD 14 and 86 which are located in north Thailand cluster in the same group with the new subtype. Hence, a novel or unclassified subtype may exist in the country. The 6v strains were isolated in the Kunming Province which borders on Myanmar. Thailand does not have a border with China but it does with Myanmar, with considerable migration among these countries. The increased number of genotype 6 subtypes reported from China may have resulted from trans-migration between the countries near the Mae Khong River and The Golden Triangle [Lu et al., 2006; Xia et al., 2008b; Wang et al., 2009]. A high prevalence of intravenous drug abuse has been reported from this area, which may play a role in HCV transmission [Xia et al., 2008a]. The data on HCV-6 in Myanmar, Laos, and Cambodia are still not well advanced.

The transmission process, factors that contribute to the considerable diversity and the manner of the viral spread have been puzzling. The most recent study has reported various subtypes of HCV-6 from Laos [Pybus et al., 2009]. The results of the current study have also confirmed that this genotype is highly variable and endemic in south-east Asia. There is strong evidence that HCV strains from the same country have clustered on the same branch of the phylogenetic tree. Even though HCV has been isolated from other regions, phylogenetic analysis has demonstrated that the subtypes of genotype 6 originated from Asian lineage. Based on the molecular clock and coalescence theory, Pybus et al. [2009] demonstrated that HCV-6 has evolved for more than 1,000 years, and its epidemic proliferation has been established during the early 20th century. This study has investigated genotype 6 from several countries, which may have been subjected to differences in evolutionary rate. Each individual country has experienced a particular historic pattern of transmission, such as civil war, the onset of intravenous drug abuse, the use of unsterile medical service, and healthcare infrastructure, which may be reflected in the different epidemic behavior of HCV infection [Tanaka et al., 2006].

It is concluded that five subtypes of HCV-6 are circulating in Central Thailand while subtype 6f was found exclusively in Thailand. HCV-6 is endemic in south China and south-east Asia displays profound genetic diversity and may have evolved over a considerable period of time, while the mechanism underlying the endemic process and the spread of the virus remain to be elucidated.

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Seroprevalence and Genotype of Hepatitis C Virus among Immigrant Workers from Cambodia and Myanmar in Thailand

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Key Words
Hepatitis C virus · Seroprevalence · Genotype · Immigrant workers · Cambodia · Myanmar

Abstract
Objective: There is a large number of immigrant workers from Cambodia and Myanmar in Thailand. The aim of our study was to determine seroprevalence and genotypes of hepatitis C virus (HCV) in this group. ***Methods:*** Immigrants aged between 15 and 60 years (1,431 Cambodians and 1,594 Myanmarese) were recruited into this study. Each sample was screened for anti-HCV by ELISA. RNA was extracted from seropositive samples and RT-PCR was performed in order to amplify the HCV core region. Each sample was subsequently sequenced, and the genotype was determined by phylogenetic analysis. ***Results:*** The prevalence of HCV infection in immigrant workers from Cambodia and Myanmar was 33 (2.3%) and 27 (1.69%) samples, respectively. Of the anti-HCV-positive individuals, 25 (75.8%) from Cambodia and 15 (55.6%) from Myanmar harbored viral RNA. Phylogenetic analysis showed that the predominant HCV genotypes in this group were 1a, 1b, 3a, 3b and 6 (6e, 6f, 6m, 6p and 6r). Most HCV isolates can be found in Thailand, though some subtypes of HCV-6 are uncommon. ***Conclusions:*** This study shows the HCV seroprevalence and genotypes among immigrant Cambodians and Myanmarese which may reflect the prevalence in each country and closely relate to the prevalence in the guest country.
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Introduction

A very high degree of genetic diversity of hepatitis C virus (HCV) has led to persistent infections. Currently, approximately 170 million people around the world are infected with this agent [1]. Chronic HCV carriers are at a significantly increased risk of liver cirrhosis and progression to hepatocellular carcinoma [2].

A high prevalence of HCV has been found in Southeast Asia. HCV epidemiology is well documented in Vietnam and Thailand. The prevalence of HCV infection was 1–2% in Vietnam [3–5]. Seroprevalence of HCV in Thailand is approximately 2.2% in the whole population [6]. However, HCV prevalence in Cambodia and Myanmar has not been well studied. Cambodia has a high prevalence of HCV infection. In 1991, a community-based study reported that

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6.5% of the population had developed antibodies to HCV [7]. Another report indicated that 10.4% of jaundice patients had antibodies to HCV [8]. In Myanmar, the first study conducted showed a high prevalence of HCV in thalassemia and liver disease patients [9]. Prevalence varies from approximately 2 to 11.6%, though most studies were performed based on a small sample size [10–12]. This variation may result from differences in geographical sampling area and target population.

HCV is a single-stranded RNA virus of positive polarity and the only member of the genus *Hepacivirus* in the Flaviviridae family. This virus shows an extremely high degree of genetic variation, and has been classified into six genotypes (1–6), which comprise various subtypes, assigned letters in alphabetical order [13]. A newly discovered seventh genotype has been documented [14]. Novel subtypes of HCV genotype 6 have been continuously identified in Southeast Asia [10, 12, 15, 16]. Thus, as yet unknown genotypes and subtypes remain to be elucidated in this part of the world.

Immigrant workers, especially from Myanmar and Cambodia have concentrated in Thailand. These groups may harbor some infectious diseases. New agents may be introduced into the indigenous population and impact public health. Therefore, it is essential to investigate and monitor some infectious agents, especially viral hepatitis C. This project has determined seroprevalence and genotypes of HCV among these groups and demonstrated that HCV prevalence of the migrant workers was closely related to the native population.

Materials and Methods

All study protocols were approved by the Ethics Committee of the Hospital and Faculty of Medicine, Chulalongkorn University. The anti-HCV positive blood samples were chosen from the specimens obtained during the routine annual check-up compulsory for immigrant workers. All the studied specimens were anonymous with a coding number for analysis, and permission was granted by the director of the hospital. In addition, all specimens were used exclusively for academic research and the patients were not remunerated.

Sample Collection

Serum samples were collected from immigrant workers in Thailand. Immigrants from Cambodia and Myanmar aged between 15 and 60 years who attended Bangpakok 9 International Hospital for their annual health check up were recruited. Sera collected from Cambodia and Myanmar workers amounted to 1,431 and 1,594 samples, respectively. Serum samples were collected from August 2007 to January 2009. Individuals of general good health were included. Immigrants resident in Thailand for more than 5 years were excluded as prolonged residence in the guest country might increase the potential for de novo HCV infection, and thus HCV prevalence detected would not be indicative of the country of origin. Also, individuals receiving immunosuppressive drugs, infected with HIV or displaying signs of immunodeficiency were excluded. This protocol was approved by the Ethics Committee, Ministry of Public Health and Faculty of Medicine, Chulalongkorn University, Bangkok. The specimens were labeled as anonymous with a coding number. Sera were collected and kept at –70° until further analysis.

Serological Tests and RT-PCR Amplification

All samples were subjected to enzyme-linked immunosorbent assay for anti-HCV detection using a commercially available kit (Murex anti-HCV v.4.0; Abbott Laboratory, North Chicago, Ill., USA). RNA was extracted from anti-HCV-positive serum samples applying the guanidine thiocyanate method [17]. Reverse transcription was performed using random primers and M-MLV reverse transcriptase (Promega, Madison, Wisc., USA). Viral RNA was detected by cDNA amplification of the 5′ noncoding region as previously described [6]. Amplification of the noncoding region was performed with 2.5 μl cDNA and the outer primer pair OC1 (GCCGACACTCCACCATGAAT, position: 18–37) and OC2 (CATGGTGCACGGTCTACGAG, position: 325–344). The PCR reaction mixture contained 5 pmol of each primer, 200 μM dNTP, 1.5 mM Mg^{2+}, 1.25 units of Taq DNA polymerase adjusted to a final volume of 25 μl with distilled water. The amplification conditions consisted of a preincubation step at 95° for 3 min followed by 35 cycles of denaturation at 94° for 1 min, annealing at 49° for 1 min and extension at 72° for 1.30 min, and concluded by a final extension step at 72° for 7 min. For nested PCR, 1.0 μl of PCR product was amplified under the same conditions using primers IC3 (GGAACTACTGTCTTCACGCAG, position: 51–71) and IC4 (TCGCAAGCACCCTATCAGGCA, position: 290–310). Nucleotide positions in this study refer to GenBank accession number M62321. The DNA fragment of the core region was amplified by nested PCR using specific primers (954 and 410 for the first round of amplification, and 953 and 951 for nested PCR) as described elsewhere [6, 18]. Some samples which showed ambiguous genotypes were subjected to further amplification of the NS5B region using specific primer pairs [19].

Sequencing and Phylogenetic Analysis

After gel electrophoresis, the PCR product of the core region was purified (HiYield Gel/PCR DNA Fragments Extraction Kit; RBC Bioscience, Taiwan) and subjected to sequencing. The sequences were edited manually using Chromas LITE (v2.01), BioEdit (v5.0.9; Ibis Therapeutics, Carlsbad, Calif., USA) and SeqMan (DNASTAR, Madison, Wisc., USA). All sequence results and reference strains of the core coding region were aligned using CLUSTALW v1.83. Neighbor-joining trees were generated using the Gojobori-Ishi-Nei six-parameter method. Confidence values were calculated based on bootstrap resampling tests multiplied by 1,000 (http://clustalw.ddbj.nig.ac.jp). The reference sequences were retrieved from GenBank, DDBJ and EMBL DNA database.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of HCV from Cambodia and Myanmar have been submitted to the GenBank database under accession numbers GU186925-GU186964.

Results

Seroprevalence of HCV among Immigrant Workers

In total, 1,431 and 1,594 serum samples were collected from Cambodia and Myanmar immigrant workers in Thailand, respectively. Male gender predominated among immigrants from Cambodia, in contrast to those from Myanmar (table 1). All subjects were between 15 and 57 years old, with a mean age of 27.13–27.77 years (table 1). The majority of the subjects in the present study were 24–26 years old. Samples retrieved from Cambodian workers showed 33 (2.3%) positive for HCV antibody by ELISA, as well as 25 (75.8%) samples positive for viral RNA upon RT-PCR of the 5′UTR. Participants aged between 21 and 35 years showed a high rate of HCV infection (table 2). Among the samples obtained from Myanmar workers, the most numerous immigrants to Thailand, 27 (1.69%) were positive for HCV antibody. The 21–35 years age group showed high infection rate, whereas none from the 36–40 years age group was anti-HCV positive (table 2). Fifteen samples proved positive for viral RNA. All RNA-positive samples were subjected to further analysis of the core region and subsequently to direct sequencing.

Phylogenetic Analysis of HCV Genotypes

HCV genotype of all sequences was determined by phylogenetic analysis based on the core region. HCV-6

Table 1. Prevalence of HCV infection with age and sex among immigrant workers in Thailand

	Cambodia (n = 1,431)	Myanmar (n = 1,594)
Sex		
Male	959 (67.02%)	631 (39.59%)
Female	469 (32.77%)	865 (54.27%)
No data	3 (0.21%)	98 (6.15%)
Age, years	27.77 ± 8.14	27.13 ± 6.19
Anti-HCV positive	33 (2.31%)	27 (1.69%)
RT-PCR positive	25 (75.76%)	15 (55.56%)

Values for age are expressed as mean ± SD.

Table 2. Distribution of anti-HCV-positive samples and genotypes among different age groups of Cambodian and Myanmar immigrant workers

Age group years	Anti-HCV			Genotype										
	male	female	total[3]	1a	1b	3a	3b	6e	6f	6m	6p	6r	6	Total
Cambodia (n = 1,431)														
21–25	5	0	5 (15.2)	0	2	0	0	0	1	0	0	0	0	3
26–30	3	3	6 (18.2)	0	0	1	0	1	0	0	0	1	1	4
31–35	5	2	7 (21.2)	0	2	1	1	0	1	0	0	0	0	5
36–40	2	2	4 (12.1)	0	1	0	0	0	0	0	0	2	0	3
41–45	1	3	4 (12.1)	0	1	0	0	3	0	0	0	0	0	4
46–50	3	1	4 (12.1)	0	0	0	2	0	0	0	1	1	0	3
>50	3	0	3 (9.1)	0	0	0	0	2	0	0	0	2	0	4
Total	22 (2.3)[1]	11 (2.3)[1]	33 (2.3)[1]	0 (0)[2]	6 (24)[2]	4 (16)[2]	1 (4)[2]	5 (20)[2]	2 (8)[2]	0 (0)[2]	1 (4)[2]	5 (20)[2]	1 (4)[2]	25 (75.8)[3]
Myanmar (n = 1,594)														
15–20	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
21–25	4	4	8 (29.6)	0	0	1	4	0	0	1	0	0	0	6
26–30	2	3	5 (18.5)	1	0	1	0	0	1	0	0	0	0	3
31–35	2	5	7 (25.9)	0	1	1	0	0	0	1	0	0	0	3
36–40	0	0	0 (0)	0	0	0	0	0	0	0	0	0	0	0
41–45	1	3	4 (14.8)	0	0	1	1	0	1	0	0	0	0	3
46–50	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
>50	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
Total	9 (33.3)[1]	18 (66.7)[1]	27 (1.7)[1]	1 (6.7)[2]	1 (6.7)[2]	4 (26.7)[2]	5 (33.3)[2]	0 (0)[2]	2 (13.3)[2]	2 (13.3)[2]	0 (0)[2]	0 (0)[2]	0 (0)[2]	15 (55.6)[3]

Figures in parentheses indicate percentages.
[1] Percent calculated with respect to all samples of each country.
[2] Percent calculated with respect to total RNA-positive samples.
[3] Percent calculated with respect to total anti-HCV-positive samples.

was predominant in Cambodian workers (56%), followed by 1b (24%), 3a (16%) and 3b (4%). This group showed at least four clusters of HCV-6, -6e, -6f, -6p and -6r (table 2). One sequence, CBD3571, did not cluster with any of the reference sequences but was grouped close to the clade of 6e and 6u (fig. 1). Subtype 6e from Vietnam and China was grouped with the Cambodian cluster (fig. 1). It seemed that subtype 6e was transmitted from Cambodia. Based on the cohort study and previous report, subtypes 6p and 6r were found mainly in Cambodia (fig. 1) [14].

To analyze the ambiguous isolates, the highly divergent strains, CBD3571 was further subjected to amplification and sequencing of the NS5B region using specific primer sets [19]. Phylogenetic analysis of the neighbor-joining tree generated by the 6-parameter model showed that the CBD3751 strain clustered most closely with subtype 6u (61% of 1,000 bootstrap resampling tests, data not shown). The respective strain occupied a distinct branch of both core and NS5B phylogenetic trees.

Phylogenetic analysis (table 2) showed that samples from Myanmar were mainly genotype 3b (33.2%), the most prevalent genotype in this study. The remaining strains were 3a (26.7%), 6 (26.7%), 1a (6.7%) and 1b (6.7%). Subtypes 6f and 6m were identified in this group. Subtype 6f was grouped with Cambodian and Thai strains (fig. 1). Subtype 6m is generally detected in Myanmar and Thailand. There was no specific cluster of subtype 1b, 3a, 3b and 6m isolates in this study. Subtype 6f from Cambodia and Myanmar has likely migrated from Thailand (fig. 1).

Discussion

Information on HCV infection in some Southeast Asian countries is quite limited, especially Cambodia and Myanmar. This study was carried out to determine the epidemiology of HCV among foreign immigrant workers from Cambodia and Myanmar. Anti-HCV seroprevalence of Cambodian workers was 2.3%, which was quite similar to the 2.2% determined for Thailand [6]. Myanmar immigrants showed low prevalence of anti-HCV at 1.69%. The subjects from the two countries recruited into the study were mainly young people with a mean age of 26–27 years, which may account for the low prevalence of anti-HCV in this survey, while older age groups tend to show a higher prevalence of HCV infection [6].

A high level of HCV infection (6.5%) has been detected mainly in adult males from Cambodia. Intravenous injection of various drugs, a popular habit in the Takeo province, may constitute the major source of infection [7]. Another report from rural Cambodia has shown that even in young age groups, HCV prevalence was very high (10.4%) [8]. In 2002, a community-based survey suggested that intravenous drug abuse was common and administered at excess rate among the general population. The population knew about HIV transmission associated with dirty needles, but only half were concerned that hepatitis virus could be transmitted by the same route [20]. In contrast to previous studies, the present study demonstrated a lower level of HCV infection (2.3%) mainly representative for healthy male Cambodians. Place of residence in their home country could not be identified. In the meantime, the Cambodian government has made an effort to discourage intravenous drug injection and improve public health [20]. Hence, the decrease in HCV infection rate observed with the samples tested could imply that the health care infrastructure of Cambodia has improved.

Viral RNA was detected in 75.8 and 55.6% of the anti-HCV seropositive samples from Cambodia and Myanmar, respectively. In agreement with various reports, the percentage of anti-HCV positive samples ranged from 50 to 90% [17, 21]. Some individuals who have naturally cleared the virus may remain seropositive without exhibiting viremia. However, owing to low viral load, HCV RNA could not be detected in some infected individuals. This study has provided information on various HCV genotypes detected in immigrants from Cambodia and Myanmar. The primer sets in this study can be used to detect various genotypes of the virus, especially the divergent HCV genotype 6 [18, 19, 22]. HCV genotypes and subtypes can potentially be determined based on the nucleotide sequence of the core region [23].

Various HCV genotypes were detected among Cambodian immigrants in this survey. Some genotypes are common in Thailand (1b, 3a, 3b, 6e and 6f), while some subtypes of HCV-6 are not found in the native population (6p, 6r and 6u; fig. 1) [19]. Subtype 6e is likely transmitted from Cambodia to other countries such as China and Vietnam (fig. 1). Subtype 6r seemed to originate from Cambodia in correlation with a previous study (fig. 2) [14].

There is a large influx of immigrants from Myanmar and Cambodia to Thailand. In 2007, the annual report from the Office of foreign worker administration Thailand showed that 498,091 and 26,096 people had immigrated from Myanmar and Cambodia, respectively (http://115.31.137.7/workpermit/main/Stat/syear.asp, reported in Thai). As a large sample size was available,

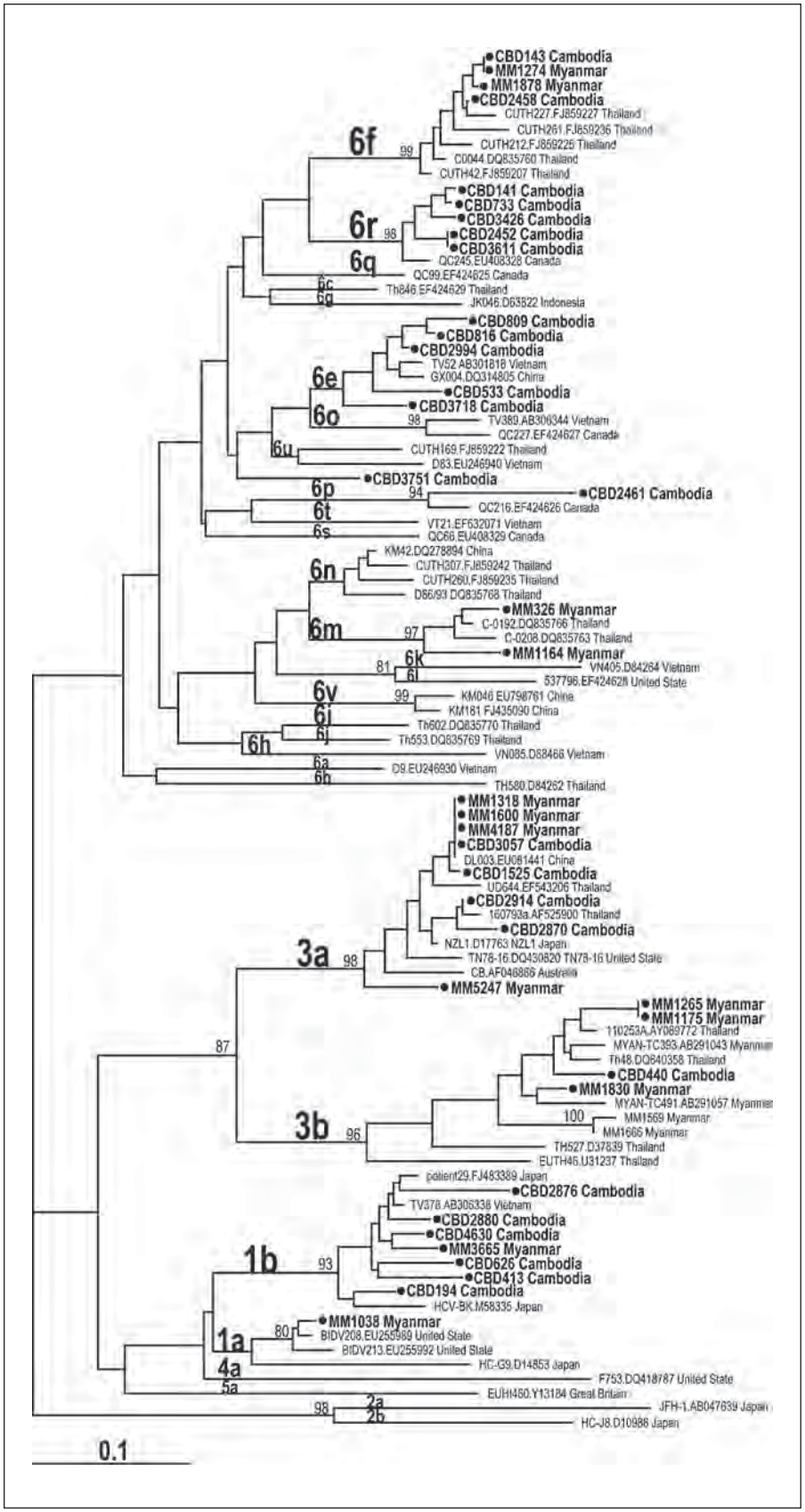

Fig. 1. Phylogenetic tree constructed on partial core coding sequences. Sequences determined in this study are in bold. HCV genotypes are indicated on the branch of the individual cluster. Reference sequences were obtained from GenBank database. Bootstrap values >80% were indicated at each node.

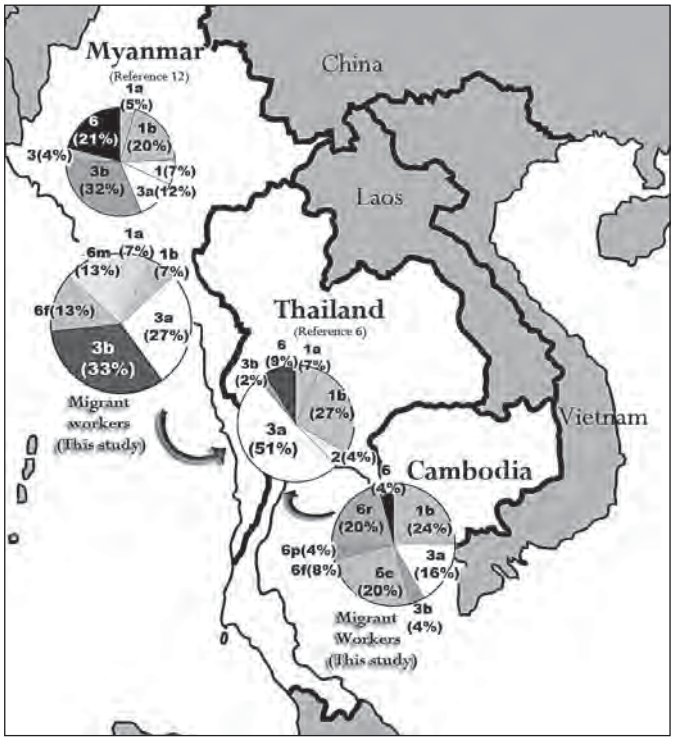

Fig. 2. Comparison of HCV genotypes in this study with those reported in previous studies in Thailand [6] and Myanmar [12].

healthy workers were included in this study and they may have migrated from different parts of the country. Based on the results of this study, the trend of HCV infection could be extrapolated to the general population. Even though the HCV infection rate was lower than expected [10, 11], the predominance of genotype 3 (3a; 26.7% and 3b; 33.5%) of Myanmar immigrant workers in this survey was similar to previous studies [11, 12]. HCV-3 is also the predominant genotype in Thailand followed by genotype 1b and genotype 6 (fig. 2) [6]. However, we have no data based on the previous study of HCV genotypes in Cambodia for comparison. Subtype 3a from Cambodia and Myanmar had mingled with subtypes from other countries (fig. 1). Genotype 3a is globally prevalent in injection drug users [24, 25], as well as common in some Asian countries [6, 12, 18, 26]. Therefore, unsafe needle sharing or drug abuse may introduce this genotype to the general population. Furthermore, these two countries are connected by trade, travel and migration all of which may contribute to similar patterns of virus transmission and genotype distribution.

HCV-6 is known as the genotype exclusive to Southeast Asia and as the most diverse genotype [19, 22, 27]. HCV-6 was predominant and subtype 6a was most prevalent in North Vietnam [28]. A previous report based on GenBank, EMBL and BBDJ database study suggested that subtype 6f was most prevalent and seemed to originate in Thailand [19]. The present study showed that this subtype is also circulating in Myanmar and Cambodia which may be due to the close connection and dynamic movement of migrating people among these countries. However, some subtypes are restricted to a specific geographical area. Thus, subtype 6r is specific for Cambodia, subtype 6p is found in Cambodia and Vietnam. Subtype 6m appeared to have migrated from Myanmar and mingled with the subtype prevalent in Thailand (fig. 1). It could be speculated that novel unassigned genotypes or subtypes may have accumulated in this area.

As immigrants can easily find employment in Thailand, their numbers are steadily increasing. Their respective original residence in their home countries could not be ascertained in this study. Despite the low incidence of HCV infection in these foreign workers, infectious diseases such as HIV, HAV and HBV may affect these groups. Hence, additional studies ought to be performed.

The prevalence of HCV infection in Cambodia and Myanmar immigrant workers determined in this study is similar to Thailand. Participants were mainly of a young age, which may provide an explanation for lower infection levels than previously reported. Various and as yet unclassified subtypes of HCV-6 may have accumulated in Southeast Asia. Further research should be focusing on HCV genotype distribution, novel subtypes of HCV-6, the evolution of the virus and incidence of HCV-related HCC in Southeast Asian countries.

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Response-guided therapy for patients with hepatitis C virus genotype 6 infection: a pilot study

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SUMMARY. The optimal duration of treatment with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in patients with hepatitis C virus (HCV) genotype 6 is unknown. This study was aimed at determining treatment response on the basis of rapid virological response (RVR) of HCV genotype 6 in comparison with genotypes 1 and 3. Sixty-six treatment naïve patients were treated with PEG-IFN-α2a (180 μg/week) plus weight-based RBV (1000–1200 mg/day). Patients with genotype 1 ($n = 16$) and genotype 3 ($n = 16$) were treated for a fixed duration of 48 and 24 weeks, respectively. Patients with genotype 6 ($n = 34$) who achieved RVR were treated for 24 weeks (response-guided therapy) and the remaining patients were treated for 48 weeks (standard therapy). The mean baseline HCV RNA levels were not statistically different between groups (6.4 ± 0.8, 6.0 ± 1.0 and 6.5 ± 0.8 Log_{10} IU/mL for genotypes 1, 3 and 6, respectively). Patients with genotypes 1, 3 and 6 achieved RVR in 43.8%, 87.5% and 73.5% of cases, respectively. One patient with genotype 1 and 3 with genotype 6 were considered nonresponders and discontinued therapy. Sustained virological response (SVR) was achieved in 62.5%, 81.3% and 76.5% of patients with genotypes 1, 3 and 6, respectively. The SVR rate in patients with genotype 6 who underwent response-guided therapy was 88%. This pilot study suggested that the SVR rate of HCV genotype 6 was at an intermediate level between those of genotypes 3 and 1. Treatment with PEG-IFN plus RBV for 24 weeks may be sufficient for patients with genotype 6 who achieve RVR. Prospective randomized trials are required to evaluate this response-guided strategy in a larger number of patients with genotype 6.

Keywords: hepatitis C, genotype 6, treatment duration, virological response, RVR.

INTRODUCTION

Hepatitis C virus (HCV) infection is a worldwide public health problem, with an estimated 170 million people infected with the virus [1]. HCV has been classified into six major genotypes and numerous subtypes, which display unique patterns of geographic distribution [2,3]. HCV genotypes 1–3 are distributed globally and account for the majority of HCV infections worldwide. HCV genotype 4 is predominantly found in the Middle East and North Africa, while genotype 5 is limited to South Africa. HCV genotype 6 is distributed primarily in south China and South-East Asia and displays pronounced genetic diversity [4]. In Thailand, approximately 2% of the general population have been chronically infected with HCV and the common genotypes are genotypes 3, 1 and 6, respectively [5,6].

The current standard therapy for patients with chronic HCV infection is a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) for 48 weeks for those with genotypes 1 and 4 and 24 weeks for those with genotypes 2 and 3 [7]. Prior limited studies have suggested that the response rate of HCV genotype 6 may be at an intermediate level between those of genotypes 3 and 1 [8–11]. However, the optimal treatment duration (24 *vs* 48 weeks) for genotype 6 is currently unknown. Previous retrospective studies showed that 48 weeks of combination therapy might be the optimal duration of treatment for genotype 6 [8,12]. However, recent prospective data demonstrated that a 24-week course of PEG-IFN plus RBV was sufficient for patients with

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; cEVR, complete early virological response; EVR, early virological response; HBV, hepatitis B virus; HCV, hepatitis C virus; pEVR, partial early virological response; RBV, ribavirin; RGT, response-guided therapy; RVR, rapid virological response; SVR, sustained virological response.

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genotype 6 and might not be statistically inferior to a 48-week duration of therapy [13].

Virological response kinetics during therapy have emerged as important prognostic factors of treatment outcome in patients with chronic HCV infection [7,14]. Absence of an early virological response (EVR) at week 12 during therapy is the best negative predictor for nonresponse to treatment. In contrast, rapid virological response (RVR; defined as undetectable HCV RNA at week 4) is regarded as the most important predictor for sustained virological response (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. 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 a shortened treatment duration of 24 weeks [15–17]. Taken together, these data suggest the feasibility of a response-guided therapy for patients with HCV genotype 6 based on the early viral kinetics. In this study, we conducted a pilot trial to individualize the duration of treatment (24 *vs* 48 weeks) in patients with HCV genotype 6 on the basis of RVR and compared SVR rates to those of patients with genotypes 1 and 3.

PATIENTS AND METHODS

Patients

Male and female patients aged 18–70 years with HCV genotypes 1, 3 and 6 infection who had not received antiviral therapy were eligible for enrolment and had to fulfil the following entry criteria: HCV RNA level more than 10 000 IU/mL; increased serum alanine aminotransferase (ALT) levels at screening and liver biopsy performed within 12 months preceding study enrolment confirming chronic hepatitis. Exclusion criteria were as follows: decompensated liver disease; hepatitis B virus (HBV) or human immunodeficiency virus (HIV) co-infection; other causes of liver disease; active injection drug use or alcohol dependence (self-reported intake, ≥40 g/day in women and ≥60 g/day in men); pregnancy or breast-feeding; serum creatinine level ≥1.5 mg/dL; haemoglobin concentration, <11 g/dL in women or <12 g/dL in men; neutrophil count, <1500 cells/mm^3; platelet count, <80 000 platelets/mm^3; a major psychiatric illness; seizure disorder; serious co-morbid conditions and evidence of malignant neoplastic diseases.

Study design

This pilot prospective study was conducted in a single tertiary hospital (King Chulalongkorn Memorial Hospital) in Bangkok, Thailand from May 2009 to April 2011. The protocol of the study had been approved by the Institutional Review Board, and all participants had provided written informed consent. The study followed the Helsinki Declaration and Good Clinical Practice guidelines. To compare the response rate of HCV genotype 6 with those of genotypes 1 and 3, patients infected with genotypes 1, 3 and 6 were enrolled on a 1:1:2 basis. All patients received PEG-IFN-α2a (Pegasys; Roche Pharmaceuticals, Bangkok, Thailand) 180 μg/week plus weight-based RBV (Copegus; Roche Pharmaceuticals) according to the following body weights: ≤75 kg, 1000 mg/day; and >75 kg, 1200 mg/day. Regarding treatment duration, patients infected with HCV genotype 1 (group 1) and HCV genotype 3 (group 3) were treated for a fixed duration of 48 and 24 weeks, respectively. Patients infected with HCV genotype 6 (group 6) who achieved RVR were assigned to treatment for 24 weeks [response-guided therapy (RGT)-group 6] and the remaining patients were treated for 48 weeks [standard therapy (ST)-group 6].

Patients with undetectable HCV RNA at week 12 were defined as having a complete early virological response (cEVR), whereas those with a minimum 2-log$_{10}$ decrease from the baseline in HCV RNA at week 12 were defined as having a partial early virological response (pEVR). Patients with no or minimal change in HCV RNA levels (<2-log$_{10}$ decrease from the baseline at week 12) were defined as nonresponders and therapy was discontinued. All patients who completed the treatment were followed up for an additional 24 weeks after the end of therapy to assess SVR.

Laboratory tests

HCV genotype was determined by nucleotide sequencing of the core and NS5B regions followed by phylogenetic analysis as described previously [5]. The levels of serum HCV RNA were assessed at baseline; at weeks 4, 12, 24, end-of-treatment and at 24 weeks of follow-up by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) (COBAS TaqMan HCV assay; Roche Diagnostics, Basel, Switzerland), in accordance with the manufacturer's instructions.

Assessment of efficacy

The primary efficacy end point was to achieve SVR, defined as undetectable HCV RNA 24 weeks after the end-of-treatment. Treatment failures were defined as follows: breakthrough (reappearance of HCV RNA during antiviral treatment period), relapse (reappearance of HCV RNA during follow-up period in patients with an end-of-treatment virological response), and nonresponse (a decrease in the HCV RNA level <2 log$_{10}$ after 12 weeks of treatment or detectable viral load at week 24), or discontinuation (treatment withdrawal for any reason). Secondary end points were to study the variables

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associated with SVR and to investigate the efficacy of week 4 virological response to predict treatment outcome.

Assessment of safety

Safety was assessed through the monitoring of adverse events and laboratory tests at weeks 2, 4, 6 and 8 then monthly thereafter during treatment and at weeks 12 and 24 after therapy discontinuation. Any life-threatening adverse event prompted treatment withdrawal. Stepwise reduction of RBV dosage of 200 mg/day and reductions of PEG-IFN-α2a dose to 135 and 90 μg/week were permitted to manage adverse events or laboratory abnormalities. Hematopoietic growth factors for the management of significant haematological toxicity were not used in this study.

Statistical analysis

The rates of SVR in each group of patients were calculated on an intention-to-treat basis. The Mann–Whitney's *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. Univariate and multivariate logistic regression analysis was used to assess odd ratios relating pretreatment variables and viral kinetics associated with SVR. $P < 0.05$ for a two-tailed test was considered statistically significant. All statistical analyses were performed using the SPSS software for Windows version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

A total of 66 patients were included in this pilot study. There were 16 patients in group 1, 16 patients in group 3 and 34 patients in group 6. Table 1 summarizes demographic and baseline characteristics of the patients by HCV genotypes. There were no significant differences in the baseline characteristics between each group in terms of gender distribution, mean age, body mass index (BMI), previous blood transfusion, ALT level, HCV RNA level and the degree of liver fibrosis assessed by histology. However, patients in group 6 had a significantly higher proportion of previous history of intravenous drug use compared with the other groups.

Virological response and treatment outcome

Of group 1, 14 (87.5%) patients completed the 48-week treatment and follow-up. One patient in this group was lost to follow up by week 24 during therapy. One patient showed minimal changes in HCV RNA levels at week 12 and therapy was discontinued because of nonresponse. Of group 3, all patients completed the 24-week treatment and follow-up. Of group 6, 25 (73.5%) patients who achieved RVR were assigned to treatment for 24 weeks (RGT-group 6) and the remaining 9 (26.5%) patients were assigned to treatment for 48 weeks (ST-group 6). All patients in RGT-group 6 completed the 24-week treatment and follow-up. Of ST-group 6, three patients were nonresponders and therapy was discontinued and the remaining 6 (66.7%) patients completed the 48-week treatment and follow-up (Fig. 1).

Figure 2 compares the virological response to the combined therapy within each group. RVR were achieved in 14 of 16 (87.5%) patients in group 3 and 25 of 34 (73.5%) patients in group 6, which was not of statistical significance ($P = 0.277$), but statistically more significant than that in group 1 (7 of 16 patients; 43.8%) ($P = 0.016$ and $P = 0.045$, respectively). The rates of cEVR were comparable between group 3 (15 of 16, 93.8%) and group 6 (30 of 34, 88.2%) ($P = 0.551$) and were higher than in group 1 (12 of 16 patients, 75%), although there was no significant difference ($P = 0.174$ and $P = 0.243$, respectively). The overall rate of SVR in group 3 (13 of 16 patients, 81.3%) was similar to that of group 6 (26 of 34 patients; 76.5%)

Table 1 Demographic and clinical baseline characteristics of the patients according to hepatitis C virus genotypes

Baseline characteristics	Genotype 1 ($n = 16$)	Genotype 3 ($n = 16$)	Genotype 6 ($n = 34$)	*P*
Age (yr)	46.4 ± 12.5	42.8 ± 8.2	41.2 ± 8.4	NS
Sex (male)	56.3%	81.3%	67.6%	NS
Body mass index (BMI) (kg/m^2)	23.4 ± 13.1	21.3 ± 5.7	23.7 ± 3.7	NS
Previous blood transfusion	50.0%	53.8%	33.3%	NS
Previous intravenous drug users	15.4%	23.1%	40.7%	0.041
ALT (U/L)	82.7 ± 57.5	82.6 ± 51.9	62.6 ± 54.5	NS
Log_{10} HCV RNA (IU/ml)	6.4 ± 0.8	6.0 ± 0.8	6.5 ± 0.8	NS
Liver fibrosis score				NS
Score 0–2	69.2%	66.7%	71.4%	
Score 3–4	30.8%	33.3%	28.6%	

ALT, alanine aminotransferase; Data described as means ± SD or proportions (%).

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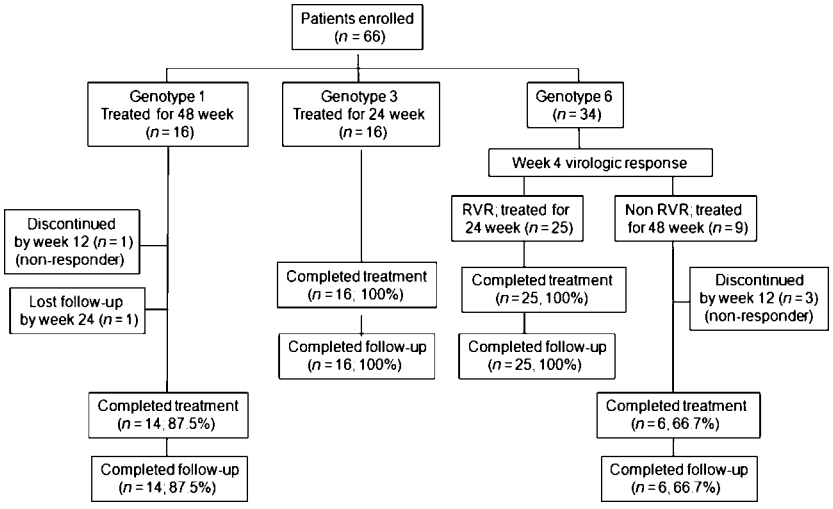

Fig. 1 Flow diagram of the patients enrolled in the study. RVR, rapid virological response (undetectable hepatitis C virus RNA at week 4).

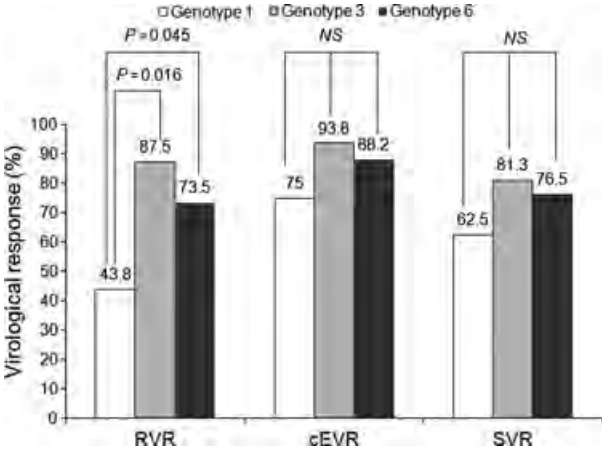

Fig. 2 Rates of virological response according to hepatitis C virus genotypes by intention-to-treat analysis. RVR, rapid virological response; cEVR, complete early virological response; SVR, sustained virological response; NS, no statistical significance.

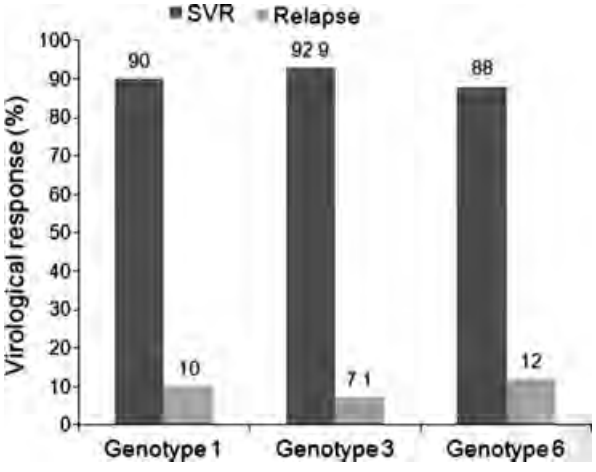

Fig. 3 Rates of sustained virological response (SVR) and relapse in patients achieving rapid virological response (RVR) according to hepatitis C virus genotypes.

($P = 0.704$) and was higher than in group 1 (10 of 16 patients; 62.5%), although there was no significant difference ($P = 0.245$ and $P = 0.309$, respectively).

Among patients who attained RVR, SVR was achieved in 9 of 10 (90%) patients in group 1, 13 of 14 (92.9%) patients in group 3 and 22 of 25 (88%) patients in RGT-group 6. The relapse rates among rapid responders in groups 1, 3 and RGT-group 6 were 10%, 7.1% and 12%, respectively. There was no statistical difference in terms of SVR and relapse rates among these groups (Fig. 3).

Among patients in RGT-group 6, the rates of cEVR and SVR were 100% and 88%, respectively. In this group, 10 of 10 (100%) patients with pretreatment viral load < 800 000 IU/mL and 12 of 15 (80%) patients with pretreatment viral load ≥ 800 000 IU/mL achieved SVR. For those in ST-group 6, the rates of cEVR and SVR were 55.5% and 44.4%, respectively, which were significantly lower than those in RGT-group 6 ($P = 0.035$ and $P = 0.025$, respectively) (Fig. 4). Patients in RGT-group 6 and ST-group 6 who achieved cEVR were likely to achieve SVR (88%, and 80%, respectively) Patients who did not achieve cEVR did not achieve SVR.

Predictors of SVR

To identify factors associated with SVR, baseline characteristics of patients and early viral kinetics during therapy were analysed by univariate and multivariate logistic regression

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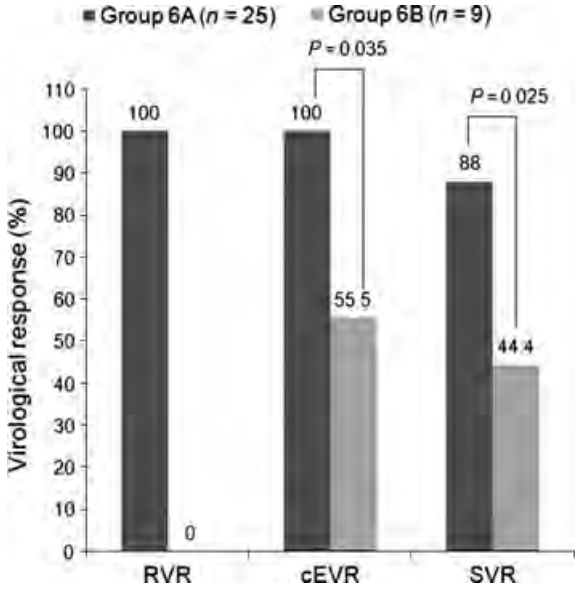

Fig. 4 Rates of virological response in groups 6A and 6B. RVR, rapid virological response; cEVR, complete early virological response; SVR, sustained virological response.

analyses. Potential predictors of SVR included sex, age, BMI, ALT level, liver fibrosis score, HCV genotype, HCV RNA level and achievement of RVR. A low HCV RNA level < 800 000 IU/mL (or $<\log_{10}$ 5.9 IU/mL) and achievement of RVR were factors predictive of SVR in univariate analysis. These factors were also independent predictors of SVR in multivariate analysis (Table 2).

Treatment adherence and safety assessment

Treatment adherence (defined as completion of at least 75–80% of intended dosage of PEG-IFN-α2a and at least 75–80% of intended dosage of RBV for at least 75–80% intended duration) was achieved in 81.3%, 75% and 82.5% of patients in group 1, 3 and 6, respectively (P = 0.825). A dose reduction of PEG-IFN-α2a was required in 12.5%, 18.8% and 14.7% of patients in group 1, 3 and 6, respectively (P = 0.881). A dose reduction of RBV was required in 25%, 31.3% and 26.5% of patients in group 1, 3 and 6, respectively (P = 0.914). None of the patients in this study discontinued the therapy because of serious adverse side effects.

DISCUSSION

Currently, the treatment outcome of patients with genotype 6 has not been adequately studied because of the limited number of cases in western countries. However, the optimal treatment duration of HCV genotype 6 is a particularly important consideration in south China and many southeast Asian countries in which this genotype is prevalent [18,19]. Most prior studies of HCV genotype 6 included patients treated for 48–52 weeks [12,20,21]. Recently, a small study of Asian American patients comparing a 48-week to a shortened 24-week regimen showed that a significantly higher SVR rate was achieved in those treated by the 48-week course (75% *vs* 49%) [22]. However, the limitation of the study was its retrospective design and the results were not analysed with regard to an intention-to-treat method. A retrospective study conducted in China showed that the rate of SVR in 22 patients with genotype 6 treated for 24 weeks was comparable with that of genotypes 2/3 (82% and 83%, respectively) [10]. In that study, the positive predictive values of RVR and EVR for HCV genotype 6 were comparable with those for genotypes 2/3 (87% *vs* 91% and 86% *vs* 87%, respectively). More recently, a randomized controlled trial of 60 patients with genotype 6 demonstrated that there was no significant difference in SVR rates in patients treated with 48-week and 24-week regimens (79% and 70%, respectively) [13]. In that study, RVR was a significant predictor of SVR in the 48-week group and tending towards significance in the 24-week group, although a sizeable number of patients did not have RVR measurement performed. These data indicate that 24 weeks of PEG-IFN plus RBV could effectively treat a subset of patients with genotype 6. However, the feasibility of a response-guided therapy by individualizing the duration of treatment according to viral kinetics in patients with genotype 6 has never been investigated.

To our knowledge, the present report is the first study directly examining the optimal duration of therapy based on RVR in patients with genotype 6. In this study, more than 70% of patients with genotype 6 achieved RVR and received an abbreviated 24-week regimen. Among these patients, the rate of relapse was approximately 10%, and nearly 90% of them eradicated the virus. These data are consistent with observations regarding treatment of HCV genotypes 1, 2, 3 and 4 [23],which suggest that monitoring RVR might be useful to guide treatment duration for patients with genotype 6. In particular, therapy might be shortened to 24 weeks in patients with genotype 6 achieving RVR, whereas a 48-week course was appropriate for those who cleared the virus after week 4. Thus, the integration of RVR into treatment decisions might identify patients with genotype 6 for whom an abbreviated course of therapy has proven to be satisfactory. The abbreviated regimen could offer advantages by reducing unnecessary medication exposure, which may make the treatment of HCV genotype 6 more affordable and maximize the cost effectiveness of therapy.

Several prospective trials of PEG-IFN and RBV have examined the use of RVR to select patients with HCV genotype 1 and non-1 genotypes for abbreviated therapy [15,24–26]. These studies have shown that a subset of patients with genotypes 2/3 and genotypes 1/4 who achieve RVR may be able to shorten therapy to 12–16 weeks, and 24 weeks, respectively, if certain pretreatment conditions are fulfilled. In recent meta-analyses of randomized

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Table 2 Univariate and multivariate logistic regression analysis of pretreatment characteristics and on-treatment viral kinetics parameters to predict sustained virological response (SVR) in patients with genotype 6

			Univariate analysis	Multivariate analysis	
Factors	*N*	SVR (%)	*P*	Odd ratio (95% CI)	*P*
Age (year)					
<45	22	68.2	0.21	–	
≥45	12	91.7			
Sex					
Male	23	65.6	0.227	–	
Female	11	90.9			
BMI (kg/m^2)					
<25	25	80.0	0.649	–	
≥25	9	66.7			
ALT (U/L)					
<80	26	80.8	0.355	–	
≥80	8	62.5			
Liver fibrosis score					
Score 0–2	26	76.9	0.444	–	
Score 3–4	8	62.5			
Log HCV RNA (IU/mL)					
<5.9	12	100	0.030	2.40 (0.30–4.50)	0.029
≥5.9	22	63.6			
RVR					
RVR	25	88.0	0.017	4.51 (1.22–8.79)	0.013
Non-RVR	9	44.4			

ALT, alanine aminotransferase; BMI, body mass index; RVR, rapid virological response; cEVR, complete early virological response; CI, confidence interval.
Factors with a *P* value <0.05 by univariate analysis were entered in multivariate logistic regression analysis.

controlled trials, it has been demonstrated that abbreviated therapies do not significantly compromise the likelihood of SVR among rapid responders with most favourable characteristics for SVR, including genotype 1 or 2 with low viral load and genotype 3 with a weight-adjusted RBV regimen [27]. On multivariate analysis, the independent factors associated with SVR among patients with genotype 6 in this study were RVR and low pretreatment viral load. In fact, all rapid responders with low pretreatment viral load eventually eradicated HCV infection after completing 24 weeks of therapy, whereas the relapse rate was relatively high (20%) in rapid responders with high pretreatment viral load. These data suggested that abbreviated therapy for HCV genotype 6 might be particularly effective for rapid responders who had low pretreatment viral load. However, owing to the small sample sizes analysed, the ability to draw conclusions was rather limited, and further studies would be required before an abbreviated course could be generally recommended.

In this study, we found that the rate of RVR in patients with genotype 6 was slightly lower than that of genotype 3 (74% and 88%, respectively), but was significantly higher than that of genotype 1 (44%). These results might reflect a predictive indicator of the subsequent SVR rate of patients with genotype 6, which was at an intermediate level between those of genotypes 3 and 1, as demonstrated in previous reports [8–11]. It should be mentioned that patients with genotype 2 have higher SVR rates than patients with genotype 3 [28]. Thus, it is speculated that patients with genotype 2 should have a higher probability of achieving SVR than patients with genotype 6. Also of interest was the observation that, although the proportion of patients achieving RVR varied by genotype, the probability of achieving SVR was consistently high (88–93%) across all genotypes among patients who achieved RVR. This result is consistent with previous data that patients who achieve RVR have the highest rates of SVR (80–90%), regardless of HCV genotype [23].

Although PEG-IFN represents the backbone of treatment, combination with RBV has been shown to directly influence the outcome of therapy in that it prevents 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/day) is recommended for treating patients with genotype 3 [7]. However, a weight-adjusted dose of RBV might be useful to enhance the response rate in patients with genotype 3 who

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do not achieve RVR and in those with RVR undergoing abbreviated therapy [29,30]. Currently, the optimal dose of RBV for the treatment of patients with genotype 6 is unknown. In previous studies, daily weight-based or fixed doses of RBV had been used, rendering comparisons rather complicated. Nonetheless, a recent prospective trial has adopted a weight-based dosage of RBV for abbreviated treatment (24 weeks), which might result in achieving SVR equivalent to that obtained with longer treatment duration (48 weeks) [13]. In our study, all patients, regardless of HCV genotypes, received a weight-adjusted dose of RBV (1000–1200 mg/day). Taken together, these data might reflect the need of a weight-based dosage of RBV in patients with genotype 6 undergoing abbreviated therapy.

Regarding risk factors for HCV acquisition, our data showed that patients with genotype 6 had a significantly higher proportion of previous history of intravenous drug users compared with patients infected with other genotypes. This discrepancy might be due to the small sample size of the study because barely statistical significance was observed. In this respect, a recent larger study of Southeast Asian Americans did not find significant differences in terms of risk factors among patients with HCV genotypes 1, 2/3 and 6 [31]. In contrast, another study conducted in Hong Kong showed that patients with genotype 1 were mainly infected through blood transfusion, while a statistically larger proportion of patients with genotype 6 were infected through intravenous drug injection [11].

In conclusion, the results of this pilot study suggest that the overall response rate of HCV genotype 6 is slightly lower than that of genotype 3 but higher than that of genotype 1. Although the small sample size that might limit conclusions on utility of both RVR and EVR, our data suggest that a response-guided therapy based on viral kinetics may be useful to optimize treatment in patients with HCV genotype 6. In particular, shortened treatment duration of 24 weeks could be sufficient in patients with low pretreatment viral load who achieve RVR. Further prospective randomized trials are required to evaluate this response-guided strategy in a larger number of patients with genotype 6.

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CONFLICT OF INTEREST STATEMENTS

The authors who have taken part in this study declare that they do not have anything to disclose regarding conflict of interest with respect to the manuscript.

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Molecular Analysis of Hepatitis B Virus Associated With Vaccine Failure in Infants and Mothers: A Case–Control Study in Thailand

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Perinatal transmission of hepatitis B virus (HBV) has been controlled incompletely despite adequate immunoprophylaxis in infants. The aim of this study was to characterize virological factors of HBV associated with vaccine failure in Thailand. Sera of 14 infected infants (13 HBeAg-positive and one HBeAg-negative) with vaccine failure and their respective mothers (group M1) were tested quantitatively for HBV DNA by real-time PCR, HBV genotypes and mutations were characterized by direct sequencing. Sera collected from 15 HBeAg-positive (group M2) and 15 HBeAg-negative (group M3) mothers whose infants had been vaccinated successfully served as controls. The results showed that group M1 and group M2 mothers had equal titers of HBV DNA but higher titers than group M3. All infected infants and their respective mothers had the same HBeAg status and HBV genotypes. DNA analysis in a pair of HBeAg-negative infant and mother revealed that both were infected with an HBV precore mutant (G1896A). Escape mutants in the ''a'' determinant region (residues 144 and 145) were detected in two (14%) infected infants. The prevalence of BCP mutations/deletions in groups M2 and M3 was higher significantly than in group M1 ($P = 0.022$ and $P < 0.001$, respectively). In conclusion, instead of the HBeAg status, a high titer of HBV DNA in mothers was the major contributor to perinatal transmission of HBV. Escape mutants might be associated with vaccine failure in some infants. BCP mutations/deletions in mothers might contribute to the prevention of mother-to-infant transmission of HBV. ***J. Med. Virol. 84:1177–1185, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis B virus; perinatal transmission; immunoprophylaxis; vaccine failure

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is associated with a diverse clinical spectrum of liver injury ranging from asymptomatic carriers to chronic hepatitis, cirrhosis, and liver cancer [Ganem and Prince, 2004]. Mother-to-infant or perinatal transmission is the predominant route of viral spread in areas of high and intermediate disease prevalence. Without immunoprophylaxis, the risk of perinatal transmission from mothers positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) to their infants is approximately 70–90% [Broderick and Jonas, 2003]. Yet, despite adequate passive and active immunization, newborns may still become infected

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with the virus, which might be attributed to several factors including intrauterine transmission or non-response to vaccination.

Several studies have demonstrated that failure of neonatal vaccination is associated directly with HBV in maternal sera, such as the presence of HBeAg and high HBV DNA level [Buchanan and Tran, 2010; Ni, 2010]. In addition, the emergence of *S* gene variants, with mutations occurring mainly in the "a" determinant might be attributed to vaccine failure, as has been observed in several regions of the world [Carman et al., 1990; Cooreman et al., 2001; Coleman, 2006]. Few studies, however, have examined the relationship between viral mutations at other genomic regions and an increased risk of HBV infection in infants. Thus, this study aimed at investigating whether genetic heterogeneity with regard to HBV genotypes and mutations in mothers infected chronically influences the efficiency of vaccination strategies in preventing perinatal transmission.

MATERIALS AND METHODS

Study Population

From June to October 2006, a seroepidemiological survey was conducted on 997 infants, who were born to mothers with chronic HBV infection in Chiangrai, a province in the North of Thailand. The details of the study have been described elsewhere [Tharmaphornpilas et al., 2009]. Of 517 infants, whose sera were available, 15 (2.9%) were progressed to chronic HBV infection. Among these infected infants, there were 14 were positive for HBeAg and the remaining one infant was negative for HBeAg. All these infants received a complete course of hepatitis B vaccination. One of the 14 HBeAg-positive infants was excluded because his mother could not be located. The mothers of the remaining 14 infected infants were recruited as cases in this study. To examine the role of molecular virological factors associated with perinatal transmission, an additional 30 HBsAg-positive mothers (15 HBeAg-positive and 15 HBeAg-negative) whose infants were not infected with the virus and received a complete course of hepatitis B vaccination were randomized as controls. The serum samples collected from participants were stored at -70°C until further testing. This study was approved by the Ethical Review Committee for Research in Human Subjects, Department of Disease Control, Ministry of Public Health, Thailand. Written informed consent was obtained from all participants.

Biochemical and Serological Tests

Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) were measured by routine method (Hitachi 912, Roche Diagnostics, Mannheim, Germany). HBsAg, antibodies to HBsAg (anti-HBs), HBeAg, antibodies to HBeAg (anti-HBe), and antibodies to HBcAg (anti-HBc) were examined by enzyme linked immunosorbent assay (ELISA) using the Murex HBsAg Version 3, Murex anti-HBs, Murex HBeAg/anti-HBe, and Murex anti-HBc kit, respectively (Murex, Biotech Limited, Dartford, Kent, England).

HBsAg concentration was quantitatively determined by chemiluminescent microparticle immunoassay (CMIA; ARCHITECT *i*2000SR, Abbott Laboratories, Chicago, IL). The sensitivity range of the assay was 0.05–250 IU/ml. Samples with HBsAg titers exceeding 250 IU/ml were diluted with phosphate buffered saline (PBS) variable at 1:10, 1:1,000, and 1:10,000 upon each sample. The assay was performed according to the manufacturer's protocol.

DNA Extraction and PCR Amplification

DNA was extracted from 100 μl serum with proteinase K 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) followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The DNA pellet was re-suspended in 30 μl sterile distilled water. The complete *S* and *C* genes were amplified by PCR using two sets of primers. The overlapping fragments generated by two sets of primers ascertained complete amplification of each gene (Table I).

The PCR reaction mixture consisted of 2 μl of a re-suspended HBV viral DNA solution, 10 μl of 2.5×

TABLE I. Primer Sets for Each Gene

Gene	Primer (5′ → 3′)	Position (nt)	Product size (bp)
S			
Set A			
PreS1F+	GGGTCACCATATTCTTGGGAAC	2814–2835	876
R3	ACAAACGGGCAACATACCTTG	475–455	
Set B			
F4	GTCCTCCAATTTGTCCTGG	348–366	667
R5	AGCCCAAAAGACCCACAATTC	1015–995	
C			
Set C			
X101	TCTGTGCCTTCTCATCTG	1552–1569	501
CO2	GTGAGGTGAACAATGTTCCG	2053–2034	
Set D			
FPC1	GCCTTCTGACTTCTTTC	1957–1973	519
CORE2	CCCACCTTATGAGTCCAAGG	2476–2457	

Eppendorf® MasterMix (Eppendorf, Hamburg, Germany), 0.5 μl of 25 μM forward and reverse primers, and sterile distilled water. PCR amplification was performed as follows: 94°C for 3 min; 94°C for 30 sec; 55°C for 30 sec; 72°C for 1.30 min for 35 cycles; and a final extension step at 72°C for 7 min.

Sequencing and Analysis Method

PCR-amplified product was subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. The PCR product band was extracted with the Perfectprep® Gel Cleanup kit (Eppendorf). Direct sequencing was performed by commercial sequencing company (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia). Nucleotide sequences were edited by Chromas Lite (Version 2.01, Technelysium Pty Ltd., Helensvale, Australia), assembled by using SEQMAN (LASERGENE program package, DNASTAR), aligned with Clustal X (Version 2.0.10, European Bioinformatics Institute, Cambridge, UK) and BioEdit Sequence Alignment Editor (Version 7.0.9.0, Ibis Biosciences, Carlsbad, CA). Amino acid sequences were determined by ExPaSy program (http://www.expasy.ch/tools/dna.html).

HBV Genotyping Methods

The complete *S* gene of each sequence was analyzed using the genotyping tool available at the National Library of Medicine's National Center for Biotechnology Information—NCBI (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

HBV DNA Quantitation

To determine HBV DNA concentration, 1 μl of the re-suspended HBV viral DNA solution was subjected to quantitative analysis by real-time PCR. Reaction mixture and condition have been documented elsewhere [Payungporn et al., 2004].

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 ANOVA with Bonferroni post-test as appropriate for quantitative variables. P values below 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 13.0 (SPSS Inc., Chicago, IL).

RESULTS

Demographic and Clinical Characteristics

The demographic and clinical characteristics of 14 infected infants (13 HBeAg-positive and 1 HBeAg-negative) and their respective mothers (group M1), and two control groups (group M2; HBeAg-positive mothers of non-carrier infants and group M3; HBeAg-negative mothers of non-carrier infants) are shown in Table II. The mean age was different significantly between groups M1 (22.9 ± 3.7 years) and M3 (27.6 ± 5.4 years; $P = 0.042$). No significant difference in AST levels was observed between groups M1, M2, and M3. However, ALT levels showed significant difference statistically between groups M2 (52.0 ± 45.7 U/L) and M3 (21.7 ± 10.8 U/L; $P = 0.018$). Moreover, Group M3 had a lower significantly mean HBsAg level (log 3.3 ± 0.9 IU/ml) than groups M1 (log 4.4 ± 0.4 IU/ml) and M2 (log 4.3 ± 0.5 IU/ml; $P < 0.001$, respectively). In addition, group M3 had a lower significantly mean HBV DNA level (log 3.4 ± 2.6 copies/ml) than groups M1 (log 7.6 ± 4.2 copies/ml) and M2 (log 8.0 ± 4.2 copies/ml; $P < 0.001$, respectively). There was no significant difference in HBsAg and HBV DNA levels between groups M1 and M2. The viral load of the HBeAg-negative mother in group M1 was log 9.6 copies/ml, while the viral load of her infant was log 4.4 copies/ml.

The mean ages of the infected infants, non-infected infants born to mothers with HBeAg-positive and non-infected infants born to mothers with HBeAg-negative were 15.4 ± 4.7, 17.7 ± 8.5, and 15.0 ± 6.6 months, respectively. There was no significant difference statistically in mean ages among infected infants, non-infected infants born to mothers with HBeAg-positive and negative groups ($P = 0.525$). In addition, the mean anti-HBs titer of infected infants was compared with anti-HBs titers of non-infected infants born to mothers with HBsAg-positive and negative. Anti-HBs titers were not detected in all of infected infants. The mean anti-HBs titers of non-infected infants born to HBsAg-positive and negative mothers were 170.5 ± 89.0 and 160.5 ± 112.1 mIU/ml, respectively. There was no statistical significance of anti-HBs titers between non-infected infants born to mothers with HBsAg-positive and negative ($P = 0.790$).

Distribution of HBV Genotype

Based on complete *S* gene sequences and the genotyping tool at the NCBI database, carrier infants and their respective mothers were infected with identical genotypes at the following distribution: five infants and their mothers were infected with genotype B (three belonged to subtype *adw* and two belonged to subtype *ayw*), nine infants and their mothers were infected with genotype C (all belonged to subtype *adr*; Fig. 1). In group M2, four cases were infected with genotype B (three belonged to subtype *adw* and one belonged to subtype *ayw*) and 11 were infected with genotype C (all belonged to subtype *adr*). In group M3, four cases were infected with genotype B (two belonged to subtype *adw*, one belonged to subtype *adr*, and one belonged to subtype *ayw*), nine cases were infected with genotype C (six belonged to subtype *adr* and three belonged to subtype *adw*), The HBV genotype(s) could not be determined in the sera of the two remaining cases. The distribution of HBV genotype was not different significantly among the mother groups (Table II).

TABLE II. Comparison of Demographic, Clinical, and Virological Data Between Infant and Mother Groups

Characteristic	HBV carrier infants (n = 14)	Mother of non-carrier infants			P-value (among mother groups)
		Respective mothers (M1; n = 14)	HBeAg positive (M2; n = 15)	HBeAg negative (M3; n = 15)	
Age	15.4 ± 4.7 months	22.9 ± 3.7 years	26.4 ± 4.9 years	27.6 ± 5.4 years	0.038*
Sex (males:females)	8:6	0:14	0:15	0:15	
ALT (U/L), mean ± SD	57.5 ± 81.8	31.2 ± 15.2	52.0 ± 45.7	21.7 ± 10.8	0.019*
AST (U/L), mean ± SD	71.6 ± 101.7	36.7 ± 26.3	46.9 ± 42.2	22.4 ± 6.5	0.079
log HBsAg conc. (IU/ml)	4.0 ± 0.6	4.4 ± 0.4	4.3 ± 0.5	3.3 ± 0.9	<0.001*
log HBV DNA conc. (copies/ml)	9.1 ± 1.5	7.6 ± 4.2	8.0 ± 4.2	3.4 ± 2.6	<0.001*
Genotype B:C (total)	5:9 (14)	5:9 (14)	4:11 (15)	4:9 (13)	0.870
Escape mutations (amino acid 144, 145)	2/14 (14.3%)	0/14	0/15	0/13	
PreS mutations/deletions	0/14	0/13	2/14 (14.3%)	2/13 (15.4%)	0.341
Basic core promoter mutations/deletions	2/14 (14.3%)	0/13	5/15 (33.3%)	7/12 (58.3%)	0.006*
PreC mutation	1/14 (7.1%)	1/13 (7.7%)	1/15 (6.7%)	5/12 (41.7%)	0.031*

*Statistic difference ($P < 0.05$); mean age M1 < M3 ($P = 0.042$); mean ALT M2 > M3 ($P = 0.018$); mean log HBsAg M1 > M3 ($P < 0.001$), M2 > M3 ($P < 0.001$); mean log HBV DNA M1 > M3 ($P < 0.001$), M2 > M3 ($P < 0.001$); Basic core promoter mutations/deletions M1 < M2 ($P = 0.022$), M1 < M3 ($P < 0.001$); *PreC* mutation M1 < M3 ($P = 0.047$), M2 < M3 ($P = 0.030$).

Comparison of *S* Gene Region

To compare HBV gene sequences among 14 mother–infant pairs in the control group, the entire *S* gene was amplified by performing nested PCR and direct sequencing. Thirteen pairs exhibited 99–100% sequence homology of the *S* gene, while one pair could not be compared due to inability to amplify the entire *S* gene sequence in the mother's serum.

Subsequent sequencing revealed the prevalence and variation of the "a" determinant mutations between amino acids 107 and 160 among the populations studied. Two out of 14 (14.3%) samples of the infected infants were found to have mutations at amino acid 144 (D144G) and amino acid 145 (G145K), respectively. There were no mutations detected in groups M1, M2, and M3 (Table II). The alignment data of the "a" determinant mutations are shown in Fig. 1.

As for mutations/deletions in the *PreS* region, no such mutation was detected in infants and their respective mothers' sera. *PreS1* deletion was observed in one sample of group M2, while *preS2* mutations/deletions were found in both mother group M2 and M3. *PreS2* start codon mutation was found in one sample of group M2 and one sample of group M3. *PreS2* deletion was found in one sample of groups M3. There was no significant difference in the prevalence of *PreS* mutations/deletions among the mother groups (Table II). The alignment data of *PreS* mutations/deletions are shown in Figure 2A.

Comparison of Basal Core Promoter (BCP)/Precore (PC) Regions

To compare HBV gene sequences among 14 mother–infant pairs in the control group, the BCP/PC regions were amplified by performing nested PCR and direct sequencing. Thirteen pairs exhibited 98–100% sequence homology of the *C* gene, while one pair could not be compared due to inability to amplify the sequence in the mother's serum.

Based on direct sequencing of the BCP region, mutation spots and deletions were found only at nucleotides 1753, 1762, and 1764. Double A1762T/G1764A mutations were found in two infected infants born to HBeAg-positive mothers, while such mutations were not detected in their mothers. In the control groups, one mother in group M2 and six mothers in group M3 had double A1762T/G1764A mutations. In addition, four cases in group M2 and an additional case in group M3 had deletions at or around nucleotides 1762–1764 (Fig. 2B). No significant difference between groups M2 and M3 was found with respect to the prevalence of BCP mutations/deletions. However, the prevalence of BCP mutations/deletions in group M2 and M3 were higher significantly than in group M1 ($P = 0.022$ and $P < 0.001$, respectively).

There was no significant difference between the HBV DNA levels in mothers with and without BCP mutations/deletions within group (M2; $P = 0.893$: M3; $P = 0.085$) and among M1, M2, and M3 groups

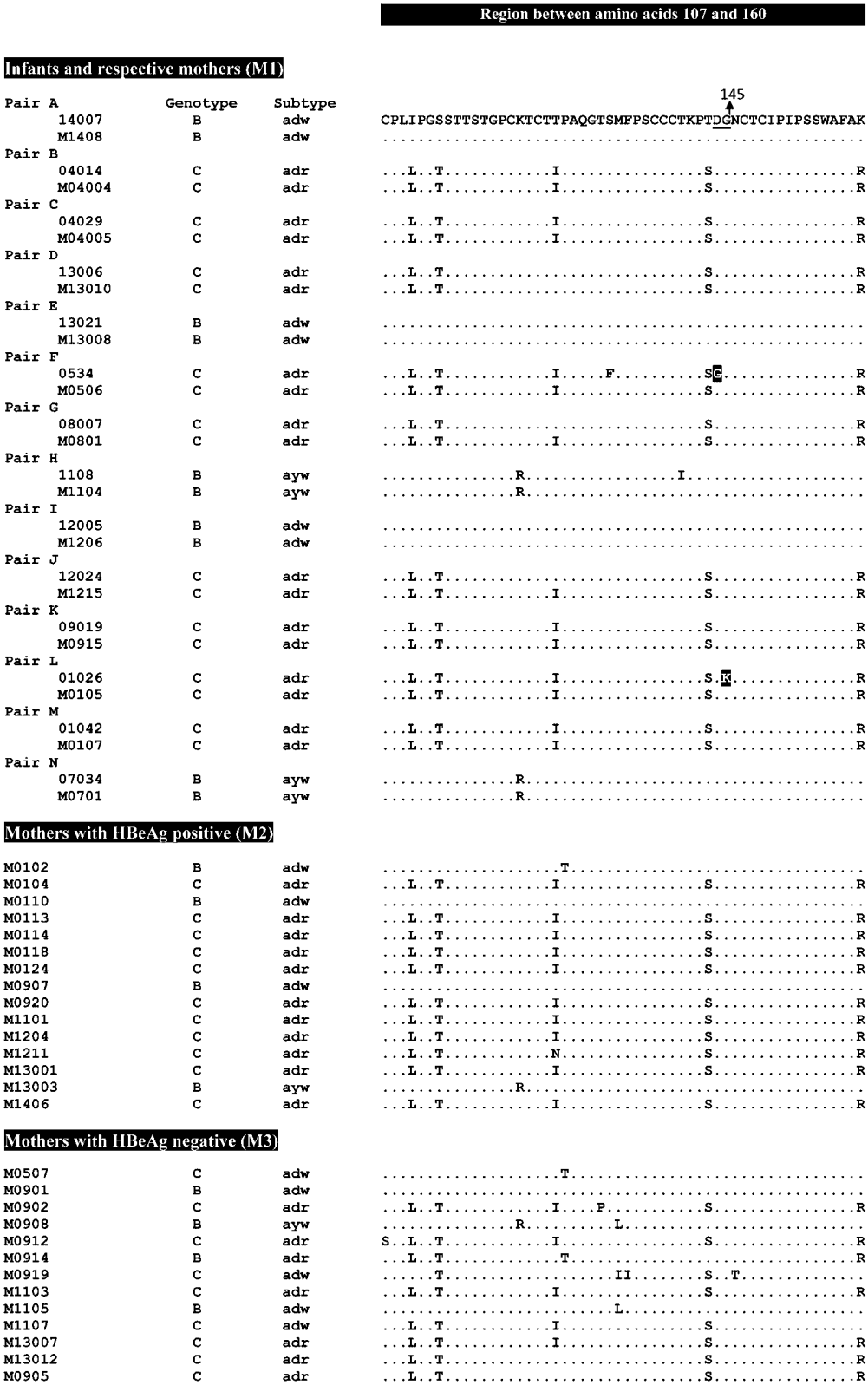

Fig. 1. Region comparison between amino acids 107 and 160. Dots indicate conserved amino acids. Amino acids 144 and 145 are underlined. Changing amino acids are indicated as letters. Solid squares indicate escape mutations.

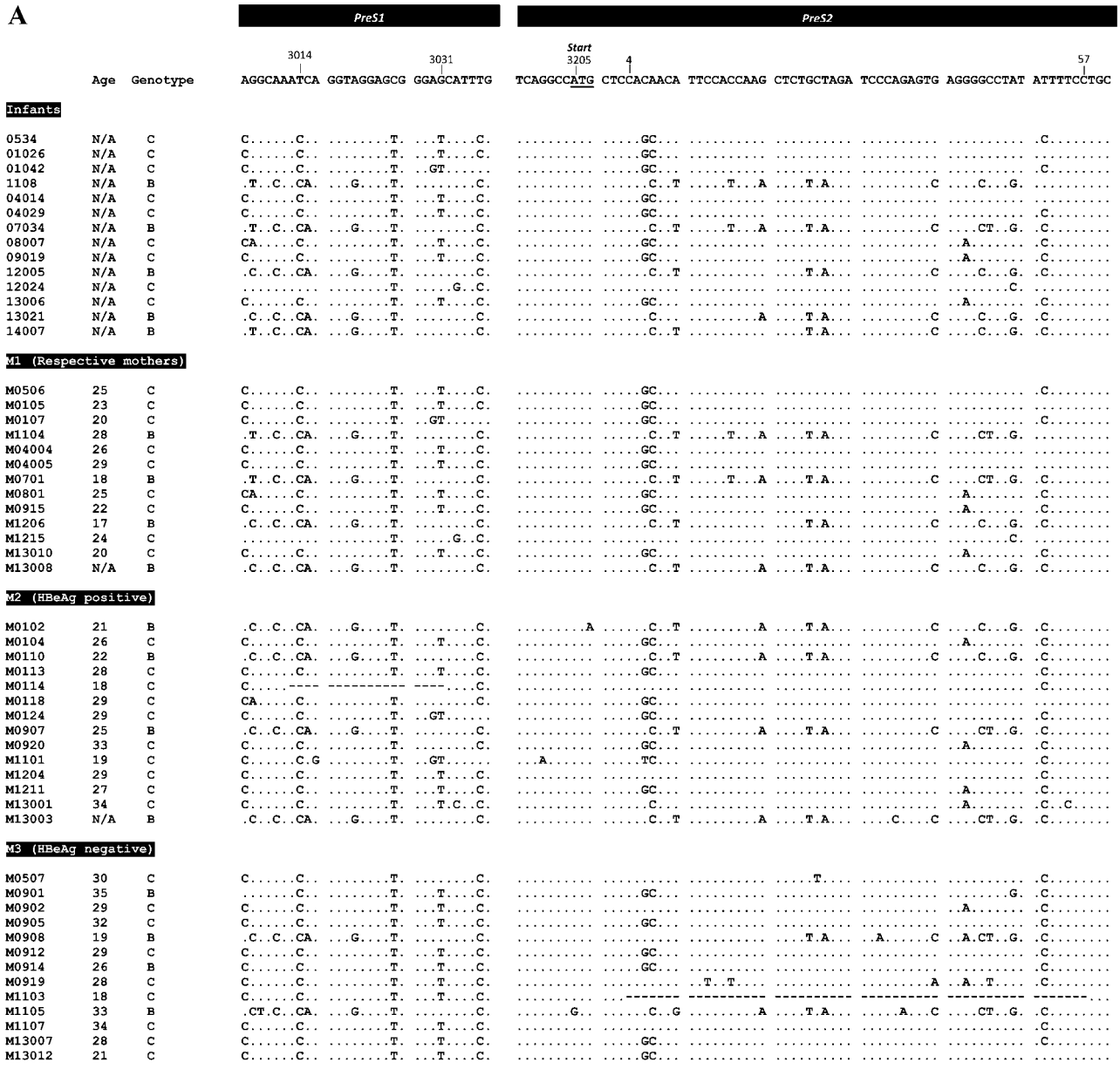

Fig. 2. Alignment comparison of nucleotides in (**A**) *PreS gene* and (**B**) basic core promoter and precore gene. Dots indicate conserved nucleotides. Dash indicates deleted nucleotides. Changing nucleotides are indicated as letters. Arrows with numbers indicate nucleotide positions. The start codon is underlined. Dashed squares indicate mutations found in the mother–infant pair whose serum was negative for HBeAg. N/A is data not available.

(mutations/deletions; $P = 0.125$: non-mutations/deletions; $P = 0.134$).

Regarding PC mutation, a G1896A substitution was found in the mother–infant pair whose serum was negative for HBeAg. In the control groups, one mother in group M2 and four mothers in group M3 had this mutation. A G1899A substitution was found in one mother of group M3. The prevalence of PC mutations in group M3 was higher significantly than in groups M1 and M2 ($P = 0.047$ and 0.030, respectively). There was no significant difference in the prevalence of PC mutations between groups M1 and M2 (Table II).

DISCUSSION

Upon conclusion of the previous large-scale survey in Thailand, the study was established that among subjects with complete vaccination, the HBV carrier rate was approximately 1% [Chongsrisawat et al., 2006], which was lower slightly than the data collected

B

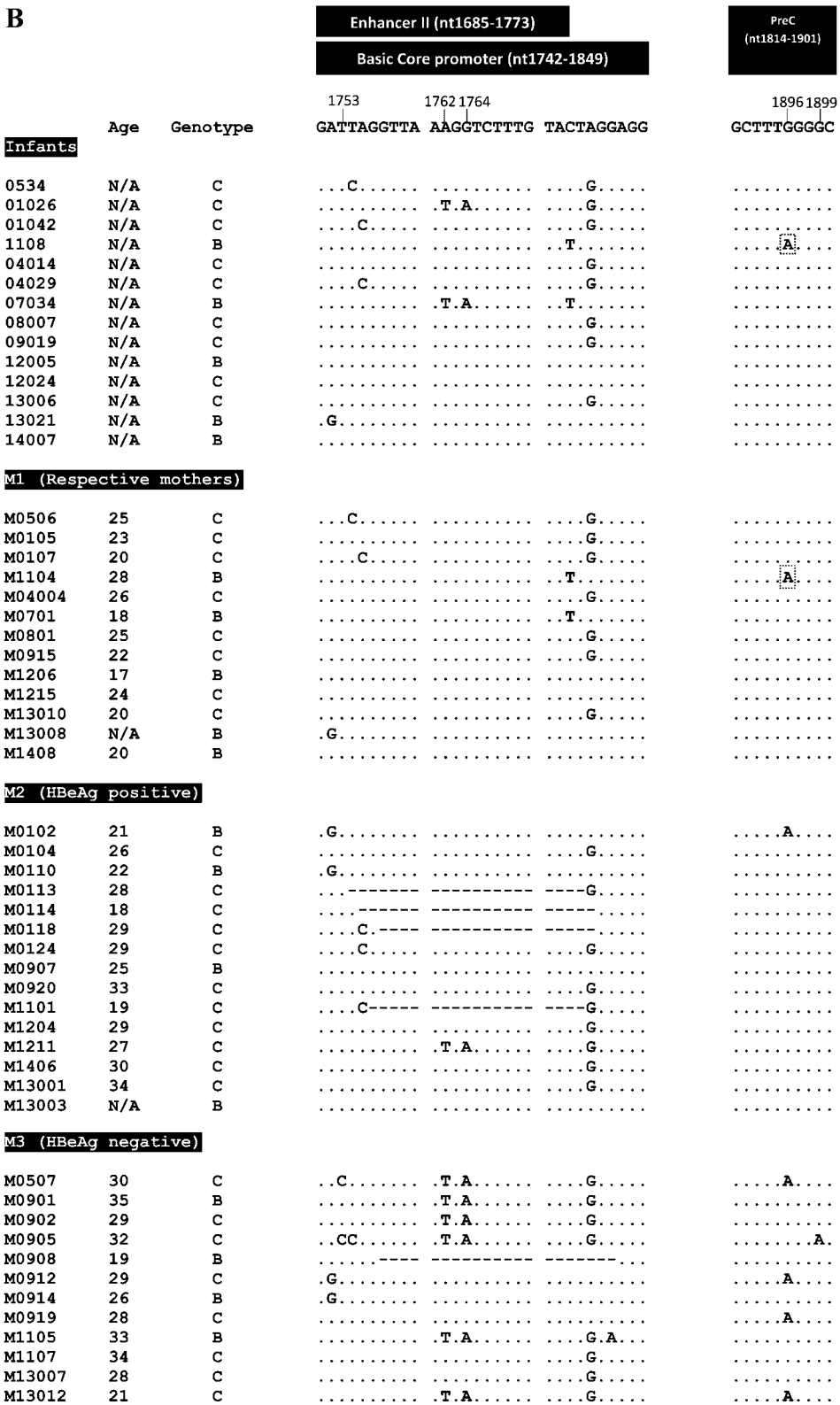

Fig. 2. (*Continued*)

from Chiangrai province [Tharmaphornpilas et al., 2009], as already mentioned in this study. The potential causes for the failure of HBV immunoprophylaxis have remained unclear but might be attributed to several factors including intrauterine infection or non-response to vaccination. In general, the risk of perinatal transmission from mothers negative for HBeAg is considered much lower than that from mothers who are positive for HBeAg [Aggarwal and Ranjan, 2004]. In fact, HBeAg-positive mothers are more likely to have higher HBV DNA titers than HBeAg-negative mothers. Furthermore, the infants of HBeAg-negative mothers are often able to clear the virus without evidence of chronic infection. For example, in a recent study conducted in Greece, neonatal viremia was detected in approximately 24% of infants born to HBeAg-negative mothers but none developed chronic HBV infection [Papaevangelou et al., 2011]. In this study, the results showed that most HBV-infected infants (approximately 93%) were born to HBeAg-positive mothers with high viral load. In addition, the only infant positive for HBsAg and negative for HBeAg was born to an HBeAg-negative mother with high viral load. These data confirm previous observations [Shao et al., 2011] on the more pronounced impact of HBV DNA level in comparison to HBeAg status on perinatal transmission of HBV and vaccine failure in infants.

Despite a maternal high viral load being the marker for HBV transmission, the data has also indicated that other factors might contribute to vaccine failure because the mean HBV DNA levels of HBeAg-positive mothers did not differ between cases and controls. Thus, the factors associated with vaccine failure in infants born to HBeAg-positive mothers were further analyzed. To that end, the study was focused on the influence of genetic heterogeneity including HBV genotypes and mutations on immunoprophylaxis failure.

HBV mutants should be considered as factors associated with immunoprophylaxis failure. The emergence of *S* gene variants, with mutations occurring mainly within the "a" determinant has been observed commonly in persons who had been vaccinated in several regions of the world [Carman et al., 1990; Cooreman et al., 2001; Coleman, 2006]. A study in Taiwan demonstrated an increase in the prevalence of such mutants in infants from 7.8% before to 23.1% 15 years after the introduction of universal vaccination against HBV [Hsu et al., 2004]. The prevalence of the "a" determinant mutants was also higher significantly among those vaccinated fully than in unvaccinated persons. Indeed, the reported frequency of "a" determinant mutations in cases of perinatal transmission following vaccination is approximately 12–40% [Lee et al., 2006]. These data suggest that vaccination might have increased selection pressure on the emergence of these mutants in relation to wild-type HBV. In the current study, two infants (approximately 14%) had the mutant-bearing virus affecting amino acid positions 144 and 145. In contrast, the isolates from their mothers did not exhibit such mutations, suggesting that the mutants might emerge or be selected for under the immune pressure during infection of the infants. As a consequence, the mutants might lead to immune escape and cause eventually failure of immunization in these infants.

Apart from the "a" determinant mutants, well-known occurring naturally HBV variants include PC stop codon mutation (G1896A), which abolishes HBeAg production and double mutations in the BCP region (A1762T/G1764A), which result in diminishing HBeAg production [Wai and Fontana, 2004]. These mutants are more common generally in HBeAg-negative chronic hepatitis B, but could also be detected in HBeAg-positive carriers. Moreover, some previous data have shown that these mutants might be related to the development of neonatal fulminant hepatitis, while some studies have not confirmed these finding [Friedt et al., 1999; Cacciola et al., 2002]. In this study, the occurrence of PC mutations was limited to HBeAg-negative carriers and was unlikely to be responsible for vaccine failure. In contrast, double A1762T/G1764A mutations were found in two infected infants born to HBeAg-positive mothers, while such mutations were not found in their mothers' sera, These findings suggest that viruses harboring the double mutations might emerge in the course of infection in the infants. These results were in agreement with a previous report on double BCP mutations detected in a group of infected perinatally infants who developed chronic infection, acute hepatitis or fulminant liver failure [Cacciola et al., 2002].

Interestingly, it should be noted that the occurrence of BCP mutations/deletions was more common significantly among HBeAg-positive mothers in the control group than that found in HBeAg-positive mothers whose infants were infected with the virus. These results might implicate that the presence of BCP mutations/deletions in the mothers could contribute to the prevention of mother-to-infant transmission of HBV once the mothers were in the phase of HBeAg-positive chronic hepatitis. Although the reason is unknown, it has been speculated that HBeAg, known to induce immunotolerance [Milich and Liang, 2003], has been used as a marker of infectivity and active viral replication. It has been shown that double BCP mutations downregulate precore mRNA transcription and decrease HBeAg production [Locarnini, 2004]. As a consequence, lower titers of HBeAg could contribute to a decrease in immunotolerance and enhance host immune response, which in turn would lead to viral clearance following perinatal transmission. Whether these mutants are associated with prevention of mother-to-infant transmission of HBV in HBeAg-positive mothers remains to be determined.

When HBV genotype is taken into consideration, carriers infected with genotype C, compared to those infected with genotype B, are more HBeAg positive frequently and exhibit higher HBV DNA titers that may contribute to accelerated progression of liver

disease [Kao et al., 2002]. Therefore, it has been speculated that genotype C may be responsible for the majority of perinatal transmissions in Asian populations. However, although approximately 55% of mothers in this study were infected with genotype C, the distribution of HBV genotypes did not differ between cases and controls. These data suggest that the HBV genotype of infected mothers has not had any influence on perinatal transmission, which is in agreement with previous observations [Liu et al., 2009; Singh et al., 2011].

This study might have had some limitations that should be considered in any interpretation of the results. First, the number of patients included in this study was small relatively. In fact, the small sample size was resulted directly from a low prevalent rate of mother-to-infant transmission found in the survey. Second, the maternal viral load at the time of collecting blood samples could be different from that during delivery. However, maternal HBV DNA levels in this study were expected to remain stable relatively over time because they were in stages of chronic HBV infection. Finally, since HBV populations infecting patients are distributed usually as quasi-species [Sheldon et al., 2006], variants are expected to coexist with wild-type strains in most cases. As viral mutations in this study were identified by direct sequencing of the PCR product without cloning, quantitative analysis for the relative amount of mutant or wild-type virus in mixed infection was not possible. As a result, data obtained in this study represented only predominant strains of HBV in the sera and minor viral variants could have escaped identification.

In conclusion, instead of HBeAg status, high HBV DNA level in mothers was the major contributor to perinatal transmission of HBV in this study. The precore mutant with high viral load could be transmitted from mothers to their offspring. Escape mutants might be associated with vaccine failure in some infants. In addition, BCP mutations/deletion in mothers might contribute to the prevention of mother-to-infant transmission of HBV in a selected group of HBeAg-positive mothers. Further large-scale studies will be required to confirm these observations.

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Molecular epidemiology and genetic history of hepatitis C virus 3a infection in Thailand

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Figure:	2	figures,
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Abstract

Objective: Among all hepatitis C virus infection, subtype 3a (HCV-3a) is the most common genotype in Thailand. This study has investigated the molecular epidemiology and epidemic history of HCV-3a in Thailand. **Methods**: Three hundred and fifty six serum samples of HCV infected Thai patients were recruited. HCV genotype was classified by phylogenetic analysis based on Core and NS5B regions. Molecular evolutionary analysis of HCV-3a was calculated by the BEAST v.1.5.4. **Results**: Based on phylogenetic analyses of 356 sequences in the Core and NS5B regions, subtype 3a (38.5%) was the most predominant, followed by 1a (21%), 1b (13.8%), and genotype 6 (19.9%) which comprised of subtypes 6e (0.3%), 6f (11%), 6i (1.9%), 6j (1.9%) and 6n (4.8%) and 3b (5.6%). The phylogenetic trees indicated the existence of a specific cluster of HCV-3a strains in the Thai population. Molecular evolutionary analysis dated the most recent common ancestor (tMRCA) of HCV in Thailand 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 strain 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**. Conclusion**: 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.

Introduction

Hepatitis C virus is a major public health problem affecting approximately 170 million people worldwide [1]. Acute hepatitis C usually establishes persistent infection which, in some individuals, can eventually culminate in hepatocellular carcinoma (HCC). Serious hepatitis C complications, such as cirrhosis and HCC, often occur without clinical symptoms after two or three decades of incubation [2,3]. More than 20 years of epidemiological surveillance have indicated that HCV related HCC is obviously increasing in developed countries [4]. In Europe and the United States, the annual HCC incidence has amounted to 3.7 percent of patients with HCV related cirrhosis while 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 6 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, United States and Japan. Subtypes 2a and 2b are predominant in Japan, North America and Europe while subtype 3a seems to be prevalent in the Indian subcontinent and Thailand [11-13]. The less common genotypes, genotypes 4 to 6, are restricted to certain geographic areas. Genotype 4 seems to be prevalent in the Middle East, genotype 5 in South Africa and genotype 6 in South China and Southeast Asia [13-16].

Mode of transmission appears to be associated with genotype distribution. Subtypes 1a and 3a are prevalent in young individuals and particularly in injecting drug users (IDUs) [17]. Previous studies have shown that HCV-3a is common among IDUs in the United States, United Kingdom, Uzbekistan, Russia and Thailand [10,18-20]. Evolutionary analysis has suggested that HCV-1a maybe spread via blood transfusion and unsafe medical practice [21]. Past and present HCV prevalence is important to predict the future burden of HCV related liver diseases. Due to asymptomatic and long-term complications of HCV infection, it is difficult to estimate the past prevalence of HCV from medical records. Applying coalescence theory, HCV population dynamics were estimated by molecular clock analysis, projecting to a point in time prior to virus discovery [9,22]. Previous studies have demonstrated that the initial spread time of HCV infection was associated with prevailing socioeconomic conditions, reflecting the present incidence as well as predicting the future development of HCC [11,22-25].

A population-based survey has shown that HCV prevalence was 2.2% in Thailand [12]. However, HCC incidence caused by HCV infection is of low prevalence in this country. Because HCC is associated directly with the incubation period of HCV infection and HCV-3a is the predominant genotype, the initial spread of HCV-3a among the population may occur later than in other countries [11,25]. Thus, the objective of this study is to estimate the time of HCV-3a introduction in the Thai population. This estimation maybe useful for predicting the rate of HCV related HCC incidence in the country. The suspected mode of transmission history of HCV infection was also described here.

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 Hepatitis C virus infected patients who attended at Chulalongkorn Hospital, Bangkok, the hospitals in Petchabun Province (Petchabun General Hospital, Lomsak General Hospital and Lomkao Crown Prince Hospital) as well as the National Blood Center, Thailand. Coded anonymous samples were collected from 2003 to 2009. All specimens were stored at -70ºC until tested. The demographic data of patients such as age, sex, native, habitat and risk factors were also collected.

DNA extraction and sequencing

Viral RNA was extracted from 356 serum specimens by using guanidine thiocyanate extraction [26]. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Medison, WI). 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 [14]. The resulting cDNA fragments were purified (HiYield Gel/PCR DNA Fragment Extraction Kit, RBC Bioscience, Taiwan) and subjected to direct sequencing (First BASE Laboratories, Malaysia). Nucleotide sequences were edited and assembled using Chromas LITE (v.2.0.1), BioEdit (v.5.09, Ibis Therapeutics, Carlsbad, CA) and SeqMan (DNASTAR, Madison, WI).

HCV genotyping

The genotype of each sample was determined by phylogenetic analysis of both Core and NS5B sequences. All sequences were aligned with reference genotypes retrieved from the Los Alamos HCV database [27] using CLUSTALW v1.83. Neighbor-joining trees of partial Core and NS5B sequences were constructed using the Gojobori-Ishi-Nei six-parameter method with bootstrap resampling tests using 1000 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 [14].

Phylogenetic analysis of HCV subtype 3a

In total, Core sequences and 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 [27]. 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 v.1.83 and the phylogenic relationships of Core and NS5B were constructed using the neighbor-joining method based on 6-parameter model with bootstrap test performed on 1000 replicates (http://clustalw.ddbj.nig.ac.jp).

Evolutionary analysis of HCV subtype 3a

The sequences of partial Core and NS5B regions originating from the same sample, which grouped in the Thai cluster, were further analyzed. The most appropriate nucleotide substitution model for the NS5B sequences dataset (325 base pairs) was determined using the jModeTest 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 model under the substitution model mentioned above. The Bayesian Skyline plot approach which implemented in BEAST v.1.5.4 (http://evolve.zoo.ox.ac.uk/beast/) was used to calculate the epidemic history. The previously estimated substitution rate of 5.8×10^{-4} substitutions per site per year obtained from NS5B gene was applied to generate an evolutionary timescale of years [28]. Each Markov Chain Monte Carlo (MCMC) analysis was run for 50,000,000 states and sampling every 5000 states. MCMC chain convergence, effective sample size and MCMC tree were investigated using Tracer v1.5 and annotated phylogenetic trees were displayed by FigTree v1.2.2, (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 (Supplement fig. 1); 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, 6w: DQ278892.

Results

Clinical Data and Patients' background

A total of 356 serum samples collected from hepatitis C infected patients with detectable HCV-RNA were subjected to RT-PCR amplification and sequencing of the Core and NS5B regions. The age of all subjects ranged between 17 and 73 years, the mean age was 43.01±10.9 years. Of 187 patients with clinical reports, 92% were diagnosed as having chronic liver disease, 5.3% had cirrhosis and 2.7% had HCC. The mean age of patients in the hepatocellular carcinoma group was higher than in the chronic hepatitis and cirrhosis group (57, 43.7 and 46.3 years old, respectively). Male gender was predominant in this study and among patients in the chronic hepatitis group; the male to female ratio was 2:1 (Supplement table 1). Most patients lived in the central area of Thailand (Supplement table 3). Only 130 subjects had known risk factors. The risk factors included blood transfusion (33.1%) followed by tattooing (27.7%), IVDU (26.9%), unsafe medical injection (23.8%) and having shaving performed by a barber (23.1%). Prostitution and needle stick injury has been documented in this study at relatively low frequency (13% and 11%, respectively). Spouses with HIV/HCV and HIV co-infection were reported by 2 patients (1.5%) of each suspected transmission route. Among all risk factors, IVDU incurred the greatest diversity of HCV genotypes identified (genotype 1, 2, 3 and 6) (Supplement table 2). 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.

Hepatitis C virus genotype

Phylogenetic analysis based on partial Core and NS5B sequences (n = 356 sequences) showed that most Thai patients carried subtype 3a (137, 38.5%), followed by subtype 1a (75, 21.1%), 1b (49, 13.8%), genotype 6 (71, 19.9%) and subtype 3b (20, 5.6%). Genotype 6 was classified as 6e (1, 0.3%), 6f (39, 11%), 6i (7, 2.0 %), 6j (7, 2.0%) and 6n (17, 4.8%) (Supplement fig. 1). The rare genotype 2 was detected in only two cases. Two samples showed discordant subtypes based on Core and NS5B in that one sample was 1b/1a and the other was 3a/3b (table 1). HCV genotype 3 could be detected in all regions of the country. HCV genotype 2 was identified in two patients residing in the central area. However, genotypes 1 and 6 were not found in the southern and western regions, respectively. This may be due to most of the patients in this study were from Chulalongkorn Hospital which is

located in the central area. HCV genotype 3 and genotype 1 can be found in all stages of liver disease. Genotype 2 was found only in chronic hepatitis. Genotype 6 was not detected in HCC (Supplement table 1).

Phylogenetic analysis of HCV subtype 3a

HCV subtype 3a isolated in this study was analyzed in comparison with reference sequences available at the Los Alamos HCV database. Figure 1 shows the neighbor-joining tree estimated from partial Core and NS5B sequences. In total, 136 of Core and 132 of NS5B sequences were clustered and classified as subtype 3a. In the subtype 3a clade, its sub-branches exhibited a phylogeographic structure that samples from the same origin tend to group together (fig. 1a and 1b).

The phylogenetic tree of partial Core sequences showed that the 92 isolates were in the Thai cluster while those of NS5B sequences showed 86 isolates (fig. 1a and b). To define these Thai clusters more clearly, the 74 samples with both Core and NS5B sequences grouped in the cluster were selected for further evolutionary analysis.

Evolutionary analysis of HCV subtype 3a

In general, the mutation rate of the Core gene is much slower than that of NS5B [29]. Thus, the NS5B dataset was analyzed using BEAST under a strict molecular clock and coalescence Bayesian Skyline plot model (BSP). Using the data from NS5B sequences, the origin of the most recent common ancestor (tMRCA) of the HCV-3a population in Thailand was estimated to the year 1914 (95% credible interval, 1874-1950).

Figure. 2 shows the BSP estimated from the NS5B dataset. The effective population size was relatively constant from the early 20^{th} century before changing to exponential growth in the 1960s. From the late 1970s to the present, the rate has been on a relatively slow decline.

Discussion

In this present study, we have described genotypic and evolutionary analysis of HCV-infected patients in Thailand, with the majority living in the central part of the country. The average age of the patients was likely to increase along with the severity of liver disease even though the number of cirrhotic and HCC cases remained small. Previous studies have found that the mean age of patients with HCC was significantly higher than that of chronic hepatitis patients suggesting that protracted infection may increase the likelihood of severe complications [13,15,25].

In Thailand, HCV genotype 3 was predominant and had subtype 3a as the most common subtype. Genotype 1 and 6 variants were also prevalent while subtype 3b showed low frequency followed by the rare subtypes 2a and 2c [12,30]**.** Genotypic distribution of HCV found in this study showed similar results to previous reports. However, these results do not represent the genotypic distribution of the country because most of the samples examined were obtained from patients living in the central region. Of all samples, there were two samples that showed discordant between subtypes from Core and NS5B regions, 1b/1a and 3a/3b (table 1). Even though, intra-genotypic recombination of HCV has been reported, additional analysis should be performed to identify the recombination break-point of these discrepant samples to confirm the recombination event [31].

Some HCV genotypes show the phylogeographic that the samples collected from the same sampling areas tend to group together, genotype 2 in Africa and genotype 6 in East Asia [24,32]. Phylogenetic analysis indicated that there was an HCV subtype 3a specific cluster for Thai strains, which may be attributable to a specific route of transmission in Thailand. According to an estimation derived from molecular evolutionary analysis, HCV-3a has been presented in Thailand for approximately 100 years. The time of presentation was in conjunction with the introduction of the western medicine into the country, such as small pox vaccination [33]. Consequently, the HCV-3a maybe spread by malpractice medical procedures during that time. Furthermore, phylogenetic reconstruction showed the close proximity of the Thai and Indian (or Pakistani) clusters supporting the argument of the close relationship between the HCV-3a viruses circulating in those countries. Historically, the Indian culture has had a profound influence on Thailand including culture, language, ceremony and food as well as migration of traders between both countries which may have contributed to the transmission of HCV for a long period of time. Previous research has

estimated that the virus had presented in India before Thailand, these suggested that HCV subtype 3a might originate from the Indian subcontinent [11].

As shown by the molecular clock estimation, HCV-3a has changed an expansion of its effective population from the mid 1960s to the 1970s. This expansion may be consistent with the likelihood of HCV transmission among a number of intravenous drug users during the US army deployment in Thailand in the course of the Vietnam War (1955-1975) (http://www.britannica.com/EBchecked/topic/628478/Vietnam-War, retrieved 11 November, 2011). At that time, the US army had set up camp at several locations of the country (north and northeast) and the use of stimulants via intravenous was widespread [34].

Although we propose that needle sharing maybe implicate for the major route of transmission of subtype 3a in Thailand, however, risk factor profile of the patients showed that blood transfusion represented the most profound risk factor in this study (Supplement table 2). This may be due to the incomplete data of the suspected risk factors collected from the patients that some patients could not recall all of the suspected historic routes of transmission.

In the past, blood donation was not popular in Thai people who traditionally preferred to receive blood from their friends or relatives [35]. Until 1985, the transfusion by using donated blood from voluntary donors was gradually replaced the old tradition. In 1990, the universal screening for HCV has been implemented by National Blood Bank, Thai Red Cross Society. Donors with positive anti-HCV antibodies was quite prevalent and accounted for almost 2% among first-time blood donors but gradually declined to 0.5% by 2009 [36]. On the other hand, even though the rate of HCV infection among this group was low this due to the blood screening policy of new voluntary blood donors prior to donation, the prevalence of HCV was still as high as 2.2% in the Thai population, including in the high-risk groups [12,20].

In 2007, the Bureau of Epidemiology reported that HCV was endemic in Petchaboon province and suspected route of transmission was inappropriate medical injection procedure [37]. This may be consistent with injections given by inadequately trained medical personnel who negligent of using disposable syringes or properly sterilized equipments [38]. The infection rate amounting to 70% in IVDU was extremely high especially when compared with 0.98% in new blood donors [20]. Thus initially needle sharing and later together with receiving contaminated blood products escalated the risks of HCV transmission at the early

HCV epidemic, however transfusion-related HCV infection has been under control after the implementation of the screening program in 1990.

Generally, HCV is the major cause of hepatocellular carcinoma cases in developed countries [13] but, in Thailand, the number of HCV infected patients has remained relatively low and most of HCC cases are associated with HBV [39]. This makes the HCV related HCC incidence has not been well documented in this population. In 2009, the Thailand National Cancer Institute reported that liver and bile duct cancer was the third common predominant in new cancer patients of Thailand. Liver cancer ranked as the third predominant in male (12.4%) and as the fifth predominant in female population (4%) [40]. Unfortunately, this report has not identified the cause of the disease whether it is virus-, alcohol- or parasite-related. The report from Bureau of Epidemiology in 2009 has shown that most hepatitis cases were the result of the infection with HBV (~51.9%) followed by HCV (~11.8%) and, the lesser extent, by hepatitis A virus, hepatitis D virus, hepatitis E virus and others [41]. Therefore, in comparison with HCV, HBV is the most predominant cause of HCC, especially in the older age group [39]. Due to the course of disease progression, liver disease resulted from HCV infection may take 20-30 years before developing to liver cirrhosis or hepatocellular carcinoma [2,3,22]. From this knowledge, the spreading of HCV in Thailand is on the way to meet the time for disease progression. Theorically, the HCV-related HCC incidence in Thailand may be rising in the next few decades.

This study shows that HCV-3a has been existent in Thailand for a century. This genotype may be firstly spread to Thailand via inadequate medical operations since the introduction of the western medicine. According to the estimation, HCV infection in the population has increased exponentially during the 1960s to 1970s. This suggests that the cirrhosis and HCC incidence of this population may increase over the next few decades.

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Legends

Fig. 1 Phylogenetic constructed in Core (a) and NS5B (b) region of HCV-3a. Thai isolates which reported in this study were labeled as pink color. Isolates with the same origin were cluster together and their origins were shown. (Country abbreviation: 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; Netherland, PK; Pakistan, RU; Russia, TH; Thailand, TJ; Tajikistan, TN; Tunisia, TW; Taiwan, US; United States and UZ; Uzbekistan).

Fig. 2 The Bayesian Skyline plot for HCV-3a of Thai cluster. The black line is the NS5B gene estimates median of the effective number of infections and the dot lines represent 95% highest posterior density (HPD) confidence interval.

Table 1.

Genotype of Hepatitis C virus determined in this study. The genotypes were classified based on Core and NS5B sequence.

Supplementary fig. 1. Phylogenetic trees are constructed based on partial (a) Core and (b) NS5B sequences of all samples isolated in this study. Reference sequences are indicated in bold letters.

Supplementary table 1

Clinical characteristics and genotype of HCV infected patients in Thailand. Of total, 187 patients have complete clinical data.

Supplementary table 2

Historic risk factors of patient classified according to genotype. Only 130 patients could identify suspected risk factors.

Supplementary table 3

Genotype distribution classified according to geographical region**.**

Table 1.

Genotype of Hepatitis C virus determined in this study. The genotypes were classified based on Core and NS5B sequence.

NS5B genotype												
Core genotype	**1a**	**1b**	**2a**	**3a**	**3b**	**6e**	**6f**	**6i**	**6j**	**6n**	**ND**	**Total(%)**
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)
6**n**										17		17(4.8)
ND		1		2	2							5(1.4)
Total(%)	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)

Supplementary table 1

Clinical characteristics and genotype of HCV infected patients in Thailand. Of total, 187 patients have complete clinical data.

	Clinical Feature			
	Chronic hepatitis (N=172)	**Cirrhosis (N=10)**	**HCC (N=5)**	**Total (N=187)**
Age (Mean±SD)	43.7±10.7	46.3±7.5	57±11.7	44.2±10.8
Sex (M/F)	115/56	5/5	3/2	123/63
Genotype (%)[a]				
HCV-1	47(85.5)	5(9.1)	3(5.1)	55(29.4)[b]
HCV-2	1(100)	0(0.0)	0(0.0)	1(0.5)[b]
HCV-3	72(93.5)	3(3.9)	2(2.6)	77(41.2)[b]
HCV-6	52(96.3)	2(3.7)	0(0.0)	54(28.9)[b]

[a] Percentage calculated with respected to each genotype

[b] Percentage calculated with respected to a total of 187 samples with clinical data

Supplement table 2

Historic risk factors of patient classified according to genotype. Only 130 patients could identify suspected risk factors.

	Genotype(%)				
Risk factor (n=130)	**HCV-1**	**HCV-2**	**HCV-3**	**HCV-6**	**Total(%)[a]**
IVDU[b]	8(22.9)	1(2.9)	12(34.3)	14(40.0)	35(26.9)
Tatoo	8(22.2)	0(0.0)	16(44.4)	12(33.3)	36(27.7)
Commercial sex	4(23.5)	0(0.0)	6(35.3)	7(41.2)	17(13.1)
Shaving barber	10(33.3)	0(0.0)	9(30.0)	11(36.7)	30(23.1)
Medical injection	9(29.0)	0(0.0)	9(29.0)	13(42.0)	31(23.8)
Blood transfusion	15(34.8)	0(0.0)	14(32.6)	14(32.6)	43(33.1)
Needle stick	1(6.7)	0(0.0)	13(86.7)	1(6.7)	15(11.5)
HIV co-infection	0(0.0)	0(0.0)	2(100)	0(0.0)	2(1.5)
HIV/HCV husband	0(0.0)	0(0.0)	1(50.0)	1(50.0)	2(1.5)

[a] Percentage calculated with respect to each of risk factor

[b] Intravenous drug use

Supplement table 3

Genotype distribution classified according to geographical region**.**

Region(%)	Genotype(%)				
	HCV-1	HCV-2	HCV-3	HCV-6	Total(%)[a]
Central	99(34.6)	2(0.7)	122(42.7)	63(22.0)	286(92.0)
Eastern	4(66.6)	0(0.0)	1(16.7)	1(16.7)	6(1.9)
Northern	1(20.0)	0(0.0)	3(60.0)	1(20.0)	5(1.6)
Northeastern	3(33.3)	0(0.0)	3(33.3)	3(33.3)	9(2.3)
Southern	0(0.0)	0(0.0)	2(1.5)	1(1.4)	3(1.0)
Western	1(50.0)	0(0.0)	1(50.0)	0(0.0)	2(0.6)
Total	108(34.7)	2(0.6)	132(42.4)	69(22.2)	311(87.4)[b]

[a] Percentage calculated with respect to the total of 311 samples with regional data

[b] Percentage calculated with respect to the total of 356 samples in the study

Title page

Type of manuscript: Original article

Title: 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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Abstract

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

Methods: Patients with chronic hepatitis B who had undergone liver biopsy were enrolled. The predictive accuracy was analyzed by comparing the areas under the receiver-operating characteristic curves (AUROCs).

Results: One hundred and fifty-six Thai patients were included, comprising 112 (71.8%) menand 44 (28.2%) women. The mean age of the patients was 40.1±12.2 years. 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 x 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.

Conclusions: 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: Liver stiffness, transient elastography, hyaluronic acid, hepatitis B, cirrhosis, ALT level, diagnostic accuracy, AUROC

Introduction

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 decisionsfor patients with chronic hepatitis B. Although percutaneous liver biopsy has been a gold standard to assess liver histopathology, this procedure has some limitations due to its invasive technique and risks 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 non-invasive methods in the assessment of liver fibrosis.

Liver stiffness measurement (LSM) using transient elastography has recently been introduced as a new, non-invasive tool for assessing liver fibrosis with high reproducibility[4]. This ultrasound-based technique allows an assessment of approximately 1/500 of the liver's total mass, thus ensuring a reduction in the sampling error compared to 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 on using LSM in assessing 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 pro-fibrogenic cytokines such as transforming growth factorβ1 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 thenon-invasive 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 an alcohol induced liver disease[11]. 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].The accuracy of HA for predicting liver fibrosis and cirrhosis in conjunction with ALT levels, however,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 non-invasive tests.

Patients and methods

Patients

This cross-sectional study included consecutive patients with chronic hepatitis B who had undergone 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 atleast 6 months and detectable serum HBVDNA.

Patients with the following conditions were excluded fromthe study: presence of HCV-co-infection or other cause of liver disease, sero-positive 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 kit (Abbott Laboratories, Chicago, IL, USA).Serum HBV DNA level was quantifiedusing 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 methodusing an HA-test kit (Allswell Singapore Pte., Ltd.) according to the manufacturer's specifications as

previously described.[15] Briefly, microtiter plates (Maxisorp, Nunc) were coated with umbilical cord HA (100 µl/ well) in the coating bufferat 4°C overnight. Wells were blocked with 150 µl of 1% (w/v) BSA in phosphate-buffered saline (PBS) for 60 minutes at room temperature. After washing, 100µl 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 µl/well; 1:2,000) was added and incubated for 60 minutes at room temperature. The plates were washed again and the peroxidase substrate (100µl/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 50µl of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

Liver stiffness measurement

LSMvalues wereobtained from each patient using transient elastography (FibroScan, Echosens, Paris, France) according to themanufacturer'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 allmeasurements. The procedure was based on atleast 10 validated measurements: the success rate (ratiobetween numbers of validated and total measurements) was over 60% and interquartile range was less than 30%.

Histopathology assessment

Liver biopsy specimens wereobtained using 16 G disposable needles (Hepafix; B. Braun, Melsungen, Germany) applying an ultrasound-guided technique. The specimens were fixed in formalin and embedded in paraffin blocks and stained with hematoxylin & eosin (H&E) and Masson's trichrome. Histopathology assessment 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 (≥F2), while advanced liver fibrosis was defined as METAVIR fibrosis stage of at least F3 (≥F3).

Statistical analyses

Data were expressed as mean ± standard deviation (SD) and percentages as appropriate. Comparisons between groups were analyzed by the $\chi 2$ or Fisher's exact test forcategorical variables and by the Mann–Whitney test or Student's t test when appropriatefor quantitative variables.Pearson's correlation coefficient was used. Univariate and multiple regression analysis were usedto 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% confidenceintervals (CI) were used as indicesof accuracy, with values close to 1.0 indicating high diagnosticaccuracy. Optimal cut-off values for each test werechosen tomaximize bothsensitivity and specificity.A two-sided probability value of $P < 0.05$ was considered statisticallysignificant. Data were analyzed using the SPSS softwarefor Windows ver. 17.0 (SPSS Inc., Chicago, IL).

Results

Characteristics of the patients

A total of 156 patients were enrolled in this study, comprising 112 (71.8%) men and 44 (28.2%) women. The mean age of the patients was 40.1±12.2 years. 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 to patients with absent (F0) and mild fibrosis (F1), patients with significant liver fibrosis (≥F2) had higher mean age. No significant difference between groupswas observed in respect to sex, body mass index (BMI), AST, ALT, TB, AP, albumin, platelet count, HBeAg positivity and HBV DNA level (Table 1).

LSM and HAaccording 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 LSMbetween F0-F1 and F2-F4 fibrosis stages (5.7±1.9 kPa *vs* 9.9±5.7 kPa, p<0.001), between F0-F2 and F3-F4 (6.2±2.2kPa*vs* 13.1±6.5kPa,p<0.001) and between F0-F3 and F4 (6.9±3.1kPa*vs* 15.7±7.1kPa, p<0.001).

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=0.0.009, p=0.015 and p=0.031, respectively) (Figure 1).

The optimalcut-off values of LSM for predicting stages of ≥F2, ≥F3, and F4 were 6.8kPa, 8.5 kPa and 10.0kPa, respectively. The optimalcut-off values of HA for predicting stages of ≥F2, ≥F3, and F4 were65ng/mL, 95 ng/mL and 110ng/mL, respectively. The cut-off 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), whileHA 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 patientswith serum ALT levels ≤80 IU/L (2 x 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 inthe subgroup of F2 patients (6.5±1.5 kPa *vs* 7.5±3.1 kPa, p=0.155), F3 patients (8.5±2.2kPa *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).

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

The AUROCs of LSM for predicting fibrosis stageof ≥F2 were significantly higher in patients with serum ALT levels ≤ 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 ≥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]. In contrast, the AUROCs of HA values for predicting fibrosis stages of ≥F2,≥F3 and F4 were not significantly different between patients with serum ALT levels ≤ 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 ≤ 80 IU/L, the AUROCs of LSM for predicting fibrosis stages of ≥F2, ≥F3 and F4 were significantly higher than those of HA values with the same fibrosis stages. In contrast, in patients with serum ALT levels >80 IU/L, the AUROCs for predicting fibrosis stages of ≥F2, ≥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 to decide therapeutic options, monitor disease progression and determine prognosis of the patients. Our study demonstrated that LSM was an accurate non-invasive 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 were consistent with recent studies conducted on Asian and Caucasian patients with chronic hepatitis B[6-8, 17-21]. In a multicenter French 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 cut-off values of LSM for ≥F2 (6.8 kPa), ≥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 cut-off 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 cut-off of 11.0 kPa were 93 and 87 %, respectively[6]. However, it should be mentioned that the cut-off 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 different populations studied and different study design methodology.

Previous studies have demonstrated that acute severe flares of hepatitis, as defined by ALT>10 x 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 x 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 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 due to their high ALT levels.

In order 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 cut-off 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 cut-offs 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]. Until now, however, 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 affect by ALT elevation. In this study, we chose to comparethe accuracy of LSM against serum HA in conjunction with ALT levelsfor 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 non-invasive 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 were very similar to 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 were 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 reducedin 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 methodin assessing liver fibrosis in patients with ALT elevation.

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Tables

Characteristics	All patients (n=156)	Patients with F0-F1 (n=69)	Patients with F2-F4 (n=87)	P value
Age (year)	40.1±12.2	37.3±10.4	42.4±13.1	0.009
Sex				NS
Male	112 (71.8)	49 (71.0)	63 (72.4)	
Female	44 (28.2)	20 (28.9)	24 (27.6)	
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 level (IU/L)	46.8±33.2	44.7±36.9	48.5±30.1	NS
ALT level (IU/L)	75.4±55.4	69.8±59.5	79.8±51.9	NS
Allkaline 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 status				NS
Positive	56 (35.9)	23 (33.3)	33 (37.9)	
Negative	100 (64.1)	46 (66.7)	54 (62.1)	
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, no statistical significance

Table 1 Demographic and clinical characteristics of the patients

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

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

		LSM	HA
≥F2	AUROCs (95% CI)	0.820 (0.752-0.888)	0.727 (0.649-0.804)
	Cut-off values	6.8kPa	65ng/mL
	Sensitivity (%)	73.6	64.4
	Specificity (%)	85.5	71.0
≥F3	AUROCs (95% CI)	0.910 (0.851-0.968)	0.848 (0.780-0.917)
	Cut-off values	8.5kPa	95ng/mL
	Sensitivity (%)	88.1	71.4
	Specificity (%)	87.7	82.5
F4	AUROCs (95% CI)	0.938 (0.896-0.980)	0.876 (0.806-0.947)
	Cut-off values	10.0kPa110ng/mL	
	Sensitivity (%)	90.5	81.0
	Specificity (%)	88.180.7	

LSM, liver stiffness measurement; HA, hyaluronic acid

PPV, positive predictive value; NPV, negative predictive value

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

Parameter	LSM		HA	
	r	P value	r	P value
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

Table 4 Parameters correlated with LSM and HA

		LSM	HA	P value
ALT ≤ 80 IU/L	≥F2	0.864 (0.788-0.941)	0.757 (0.663-0.851)	0.009
	≥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	≥F2	0.733 (0.602-0.864)	0.696 (0.558-0.823)	0.626
	≥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)

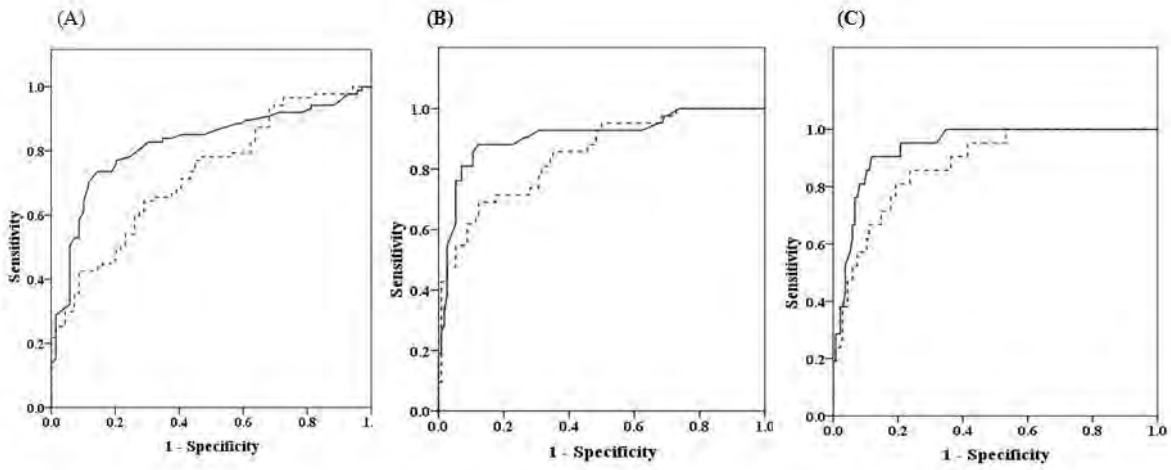
LSM, liver stiffness measurement; HA, hyaluronic acid

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

Figures

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

Figure 1.



PP12-42

Genotype of Hepatitis C Virus among Blood Donor in Udon Thani, the Northeast Thailand

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Background/aim; Data on HCV epidemiology and prevailing genotype in rural areas of Thailand are scarce. Udon Thani is a rural province in the north-east of Thailand, bordering directly on Laos. Both countries share a history of cultural connection and transmigration. Thus, this study was aimed at determining HCV genotype distribution among Thai blood donors in Udon Thani.

Methods: One hundred and ninety six anti-HCV positive serum samples of Thai blood donors were obtained from Udon Thani Hospital RNA extraction and RT-PCR were performed in order to amplify the HCV 5'UTR. HCV RNA positive samples were further subjected to amplification of the NS5B region, direct sequencing and genotype determination by phylogenetic analysis.

Results: Of the anti-HCV positive sera, 125 were positive for HCV RNA. Phylogenetic analysis based on the NS5B sequence revealed HCV genotypes 1a (22.1%), 1b (10.6%), 3a (37.5%), 3b (3.9%) and 6 (25.9%); subtype 6i (10.6%), 6f (9.6%), 6n (4.8%) and 6v (0.9%).

Conclusion: HCV-3a was highly prevalent in this region. Genotype 6 showed rather profound diversity and high frequency. In addition, one sample of subtype 6v was found in this study suggesting that this genotype may have been accumulating in this area and subsequently undergone variations. This study may be useful for surveillance and monitoring of the incidence of hepatitis and related diseases in this region of Thailand.

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Prevalence of aflatoxin induced p53 mutation at codon 249 (R249S) in hepatocellular carcinoma with or without HBsAg in Thailand

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Background: Missense hot spot mutation of p53 tumor suppressor gene on codon 249 of exon 7 (R249S) has been associated with aflatoxin B1 (AFB1) exposure, which is believed to have synergistic effect on hepatitis B virus (HBV)-induced hepatocarcinogenesis. However results of several studies comparing its prevalence among patients with hepatocellular carcinoma (HCC) are conflicting. This study was aimed at determining its prevalence among Thai patients, according to HBsAg status.

Methods: Paraffin embedded liver tissues obtained from 124 HCC patients who underwent liver resection and liver biopsy in King Chulalongkorn Memorial Hospital, were included. Restriction fragment length polymorphism (RFLP) was used to detect R249S mutation. Positive results were confirmed by direct sequencing.

Results: Sixty four (51.6 %) and 18 (14.5%) patients had serum HBsAg and anti-HCV positivity, respectively. R249S mutation was found in 9.4 % of HBsAg-positive HCC versus 6.7 % of HBsAg-negative HCC (P=0.58). Patients with R249S mutation were tended to be younger (55 ± 10 vs 60 ±13 years) and had more advanced Edmonson-Steiner grade of HCC (grade 3/4 in 80 % vs 75 %), though the differences did not reach statistical significance.

Conclusion: Compare to previous studies, our data showed relatively low prevalence of AFB1-related p53 mutation in HCC specimens. In addition, the prevalence of R249S did not differ between HBsAg-positive and HBsAg-negative HCC.

IL28B polymorphism is associated with treatment response in Thai patients with hepatitis C genotype 1, but not with genotype 3 and 6

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Background: Recent studies have shown an association between single nucleotide polymorphisms (SNPs) near *interleukin (IL)-28B* gene and sustained virological response (SVR) with pegylated-interferon (PEG-IFN) and ribavirin (RBV) in 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) infected patients is less clear.

Methods: A total of 133 Thai patients with chronic HCV infection treated with PEG-IFN/RBV were included (mean age, 46.6 years; 69.2% were male). Among these, 40 (30.1%), 56 (42.1%) and 37 (27.8%) patients were infected with HCV-1, HCV-3 and HCV-6, respectively. DNA extracted from serum samples was analyzed by direct sequencing of the SNP rs12979860.

Results: In this study, the SVR rates for HCV-1, HCV-3 and HCV-6 were 62.5%, 85.7% and 75.7%, respectively. The distribution of rs12979860 genotype in all patients was as follows: CC, 107 (80.5%); CT, 20 (15%); and TT, 6 (4.5%). There was significant difference in the distribution of CC genotype between HCV-1-infected patients (60%) and those infected with HCV-3 and HCV-6 (94.6% and 81.1%, respectively, $p<0.001$). In HCV-1, the SVR rate of CC genotype was significantly higher than that of non-CC genotype (79.2% and 37.5%, respectively, $p=0.018$). However, there was no such difference regarding the SVR rates in HCV-3 (84.9% and 100% for CC and non-CC genotypes, respectively) and HCV-6 (73.3% and 85.3% for CC and non-CC genotypes, respectively).

Conclusions: The SNP rs12979860 was strongly associated with SVR in patients infected with HCV-1, but not with HCV-3 and HCV-6. Thus, analysis of *IL28B* genotype might not be useful to guide treatment for Thai patients infected with HCV-3 and HCV-6.

Characterization of HBV mutations in untreated HIV-HBV co-infected patients based on complete genomic sequencing

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Abstract

Background: Co-infection 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 HBV mono-infection. However, it is unclear whether HBV mutational patterns might differ between co-infected and mono-infected individuals.

Methods: This study included 24 treatment-naïve co-infected and 31 treatment-naïve mono-infected Thai patients. HBV mutations were characterized by whole genome sequencing in serum samples. The clinical features and frequency of known clinically significant mutations were compared between the two groups.

Results: No significant difference between groups was found with respect to sex, age and HBeAg status. However, HBV DNA levels were significantly higher in co-infected patients. The distribution of HBV genotypes were comparable between groups and were mostly restricted 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 (BCP)/precore and the pre-S regions between the two groups.

Conclusions: Dual infections trended towards an increased HBV DNA level. There was no major difference in the frequencies of common HBV mutations between co-infected and mono-infected patients. Thus, HBV mutations might not be contributing to disease pathogenesis in Thai patients with co-infection.