รายงานวิจัยฉบับสมบูรณ์

โครงการอณูไวรัสวิทยาและความสำคัญทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสอื่นของไวรัสตับอักเสบบี: บทบาทของซีซีซีดีเอนเอ สายพันธุ์และการกลายพันธุ์

Molecular virology and clinical aspects of occult and co-infections of viral hepatitis B: roles of cccDNA, genotypes and mutations

โดย

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สิงหาคม 2552

สัญญาเลขที่ BRG5080025

รายงานวิจัยฉบับสมบูรณ์

โครงการอณูไวรัสวิทยาและความสำคัญทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสอื่นของไวรัสตับอักเสบบี: บทบาทของซีซีซีดีเอนเอ สายพันธุ์และการกลายพันธุ์

Molecular virology and clinical aspects of occult and co-infections of viral hepatitis B: roles of cccDNA, genotypes and mutations

คณะผู้วิจัย
1. รองศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์
ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย
สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "อณูไวรัสวิทยาและความสำคัญทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสอื่นของไวรัสตับอักเสบบี: บทบาทของซีซีซีดีเอนเอ สายพันธุ์และการกลายพันธุ์“ ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ BRG5080025 ระยะเวลาดำเนินการ 2 ปี ตั้งแต่ 31 กรกฎาคม 2550 ถึงวันที่ 30 กรกฎาคม 2552 ผู้รับทุนขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย ที่ให้การสนับสนุนอย่างเต็มที่จนทำให้โครงการวิจัยนี้สำเร็จลุล่วงด้วยดี ขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญยิ่งในความสำเร็จของโครงการ ได้แก่ ศาสตราจารย์ปิยะรัตน์ โตสุโขวงศ์ ศาสตราจารย์นายแพทย์อภิวัฒน์ มุทิรางกูร รองศาสตราจารย์แพทย์หญิงวโรชา มหาชัย รองศาสตราจารย์แพทย์หญิงณัฏฐิยา หิรัญกาญจน์ ผู้ช่วยศาสตราจารย์นายแพทย์ปิยะวัฒน์ โกมลมิศร์ ผู้ช่วยศาสตราจารย์แพทย์หญิงนฤมล วิเศษโอภาส (คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย) รองศาสตราจารย์ ดร. ปรัชญา คงทวีเลิศ (คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่) และผู้ช่วยวิจัยที่มีส่วนช่วยเหลือให้โครงการวิจัยสำเร็จลง ได้แก่ นางสาวอภิรดี เทียมบุญเลิศ และนางสาวภัทรธิดา สงวนหมู่ รวมทั้งภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในฐานะสถาบันต้นสังกัดที่ให้การสนับสนุนโดยอำนวยความสะดวกในโครงการเป็นอย่างดียิ่ง

สารบัญ

บทคัดย่อ

รหัสโครงการ: BRG5080025

ชื่อโครงการ: อณูไวรัสวิทยาและความสำคัญทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสอื่นของไวรัสตับอักเสบบี: บทบาทของซีซีซีดีเอนเอ สายพันธุ์และการกลายพันธุ์

ชื่อนักวิจัยและสถาบัน: รองศาสตราจารย์นายแพทย์พิสิฐ ตั้งกิจวานิชย์
ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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ระยะเวลาโครงการ: 31 กรกฎาคม 2550-30 กรกฎาคม 2552

โครงการวิจัย:

ไวรัสตับอักเสบบีเป็นเชื้อไวรัสที่เป็นสาเหตุสำคัญของเกิดโรคตับอักเสบชนิดเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับ ปัจจุบันเชื่อว่าปัจจัยต่างๆที่เกี่ยวข้องกับไวรัสเช่นความแตกต่างของสายพันธุ์ การเกิดสายพันธุ์ผสม การกลายพันธุ์ของยีนในบางตำแหน่ง รวมทั้งการเปลี่ยนแปลงของระดับของซีซีซีดีเอนเอในตับ มีความสำคัญต่อการดำเนินโรคที่แตกต่างกัน จุดมุ่งหมายของโครงการนี้เพื่อศึกษาความชุกและความสำคัญทางคลินิกของปัจจัยต่างๆดังกล่าวในกลุ่มประชากรที่มีการติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง ผลการศึกษาในเชิงระบาดวิทยาพบว่าเชื้อไวรัสสายพันธุ์ซี (genotype/subtype C/adr) เป็นสายพันธุ์ที่พบมากที่สุดในประเทศไทยและประเทศเพื่อนบ้าน การกลายพันธุ์ที่ตำแหน่ง 'a' determinant พบได้ค่อนข้างบ่อยในกลุ่มประชากรของประเทศเพื่อนบ้านเมื่อเทียบกับกลุ่มประชากรไทย ซึ่งการกลายพันธุ์นี้ไม่น่าจะเกี่ยวข้องกับการฉีดวัคซีนป้องกันการติดเชื้อไวรัสตับอักเสบบี นอกจากนี้ยังพบว่าการกลายพันธุ์บริเวณ pre-S โดยเฉพาะ pre-S2 deletions และ pre-S2 start codon mutation พบได้บ่อยในกลุ่มประชากรไทยและประเทศเพื่อนบ้าน การศึกษาแบบ case-control พบว่าการกลายพันธุ์แบบ A1762T/G1764A และ G1899A ในกลุ่มประชากรที่เป็นตับอักเสบแบบเรื้อรังมีส่วนสัมพันธ์กับความเสี่ยงของการเกิดมะเร็งตับ นอกจากนี้พบว่าในผู้ป่วยตับอักเสบแบบเรื้อรังที่มีการกลายพันธุ์แบบ A1762T/G1764A และบริเวณ pre-S มีการตอบสนองที่ดีต่อการรักษาด้วยยาฉีดต้านไวรัสชนิดเพคอินเตอร์เฟอรอน การวัดระดับของ HBsAg และ HBeAg ในเลือดมีความสัมพันธ์กับระดับของซีซีซีดีเอนเอในตับ ซึ่งสามารถทำนายผลการตอบสนองต่อการรักษาด้วยยาเพคอินเตอร์เฟอรอนได้เป็นอย่างดี ผลวิจัยจากโครงการนี้จะเป็นประโยชน์ทางระบาดวิทยาและช่วยอธิบายกลไกการดำเนินของโรค ตลอดจนการรักษาผู้ป่วยตับอักเสบแบบเรื้อรังจากเกิดจากไวรัสตับอักเสบบี ส่วนการศึกษาทางอณูไวรัสวิทยาและทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสเอชไอวีของโครงการนี้ กำลังอยู่ในระหว่างการดำเนินการ

ABSTRACT

Project Code: BRG5080025

Project Title: Molecular virology and clinical aspects of occult and co-infections of viral hepatitis B: roles of cccDNA, genotypes and mutations

Investigator: Associate Professor Pisit Tangkijvanich, M.D.

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Project Period: 31 July 2007-30 July 2009

Project Description:

Hepatitis B virus (HBV) infection is a major public health problem in Thailand and worldwide. Chronic HBV infection is associated with a high lifetime risk of developing cirrhosis and hepatocellular carcinoma (HCC). Several molecular virological factors appear to strongly influence outcome in chronic infection including HBV genotypes, intergenotype recombinations, specific viral mutations and intrahepatic covalently closed circular DNA (cccDNA) levels over time. This project was aimed at studying the prevalence and clinical significance of these viral factors in diverse groups of HBV-infected individuals. In epidemiological studies, our data showed that genotype/subtype C/adr was the predominant strain circulating in Thai populations and migrant workers originated from neighboring countries. We also showed that 'a' determinant variants were more common in migrant workers than in Thai HBV carriers, and might not be attributed to vaccine-induced mutation. In addition, natural occurring pre-S mutations, especially pre-S2 deletions and pre-S2 start codon mutations were rather common in Thai and neighboring populations. 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. Regarding antiviral therapy, our data suggested that A1762T/G1764A mutations and pre-S mutations were associated with a high rate of response to PEG-IFN treatment. In addition, quantitative HBsAg and HBeAg determination, which reflected cccDNA levels in the liver, were valuable viral markers for predicting and monitoring the response to PEG-IFN therapy. These data provide useful information regarding the epidemiology and clinical importance of HBV genetic variability in patients chronically infected with the virus. The molecular virological and clinical aspects of HBV in occult and co-infections with HIV are under active investigation.

หน้าสรุปโครงการ (Executive Summary)
ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา

1. ชื่อโครงการ อณูไวรัสวิทยาและความสำคัญทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสอื่นของไวรัสตับอักเสบบี: บทบาทของซีซีซีดีเอนเอ สายพันธุ์และการกลายพันธุ์

Molecular virology and clinical aspects of occult and co-infections of Viral hepatitis B: Roles of cccDNA, genotypes and mutations

2. ชื่อหัวหน้าโครงการ น.พ. พิสิฐ ตั้งกิจวานิชย์
รองศาสตราจารย์ ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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3. สาขาที่ทำการวิจัย อณูชีววิทยาของไวรัสตับอักเสบ

4. คำหลัก (Keyword) Viral hepatitis B, cccDNA, genotype, mutation, occult infection

6. ระยะเวลาดำเนินงาน 2 ปี

7. ปัญหาที่ทำการวิจัยและความสำคัญของปัญหา

Hepatitis B virus (HBV) infection is a major public health problem, with more than 400 million HBV carriers estimated worldwide. 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). The clinical outcome of HBV infection is complex and is influenced by viral factors including HBV genotypes, intergenotype recombinations and specific viral mutations. In addition, the crucial role of intrahepatic covalently closed circular DNA (cccDNA), which represents the template for viral replication and persistence, has been increasing recognized but scant data from patients have been reported so far. Current lines of evidence also suggest that occult or overt HBV infections are associated with the progression of liver disease in patients co-infected with human immunodeficiency virus (HIV). In addition, the molecular virological mechanism underlying occult HBV infection remains incompletely defined and is probably contributed to multifactor such as genotypic variation and

certain genomic mutations of the virus. As the genetic variability of HBV differs geographically and the data available in literatures are still limited, this study will provide essential information regarding the epidemiological, molecular virological and clinical aspects of chronic hepatitis B.

8. วัตถุประสงค์

The aims of this study were to

1) Compare molecular epidemiology of HBV in Thailand and neighboring countries
2) Determine viral genetic variability, including HBV genotypes and specific mutations, in Thai patients with HBV-associated HCC
3) Determine viral genetic factors of HBV in predicting virological response to PEG-IFN therapy
4) Determine intrahepatic cccDNA levels in patients with chronic hepatitis B and correlate their levels with other viral factors such as HBsAg, HBeAg and HBV DNA levels in the serum
5) Compare the prevalence and viral genetic variability, including genotypes, intergenotype recombinations and specific mutations, in patients with HBV infection and patients with HBV/HIV co-infection

9. ระเบียบวิธีวิจัย

1) Collect serum samples from diverse groups of HBV-infected individuals and stores at -80^0C until analyzed
2) Collect liver tissue samples obtained from liver biopsies of patients with chronic hepatitis, immediately frozen and stored at –80^0C until analyzed
3) Collect patients' clinical data
4) Determine serological and virological tests of HBV, including qualitative and quantitative HBsAg, HBeAg and HBV DNA level
5) Perform nested PCR and direct sequencing of HBV genome for studying genotypes and mutations in serum samples
6) Determine intrahepatic cccDNA and HBV DNA levels in liver tissue samples
7) Analyze the data

Project Description

1. Introduction and Rationale

Hepatitis B virus (HBV) infection is a major health problem worldwide, affecting approximately 400 million people.[1] The clinical outcome of HBV infection is complex and is influenced by many factors, including age at infection, viral factors (HBV genotype, intergenotype recombination, mutations, and level of HBV replication), host factors (gender, age, and immune status), and exogenous factors such as concurrent infection with other hepatotropic viruses (hepatitis C virus; HCV) or alcohol. The clinical spectrums of HBV infection range from acute self-limited infection to the most severe liver diseases including fulminant hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). In Thailand, chronic HBV infection is the most common risk factor for development of HCC, accounting for approximately 65% of cases.[2] Chronic HBV infection exerts its pro-oncogenic properties through both indirect and direct mechanisms.[3] 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.

HBV is transmitted parenterally, primarily from infected mothers to their newborn infants and horizontally between young children and, later in life, via sexual contact. Acute infections of immune-competent adults are cleared in more than 90% of cases, but perinatal transmission leads to neonatal infections that almost invariably become persistent; infections in early childhood also have a much higher probability of persistence than those in adults. Persistent (chronic) infections are associated with impaired immune responses to the virus, especially by cytotoxic T lymphocytes (CTLs), and individuals infected perinatally may exhibit almost complete immune tolerance. HBV is not cytopathic and such individuals initially may sustain very high levels of virus replication with little pathology. A soluble protein, hepatitis B e antigen (HBeAg) is secreted by the infected hepatocytes and may be detected in the serum along with hepatitis B virions. Over time, tolerance may break down and host immune responses lead to inflammatory activity in the liver, with lysis of infected hepatocytes. Such responses may lead to reduced virus replication and decreasing serum concentrations of HBV and of HBeAg. Ultimately,

HBeAg may become undetectable with seroconversion to antibody (anti-HBe), signaling a quiescent phase with undetectable or low levels of viremia and little inflammation. Antiviral therapies aim to clear virus replication, or at least to achieve progression to this quiescent phase. Without treatment, in late-stage disease high levels of virus replication may persist in the face of a vigorous immune response, leading to severe inflammation and fibrosis, and ultimately, cirrhosis, and HCC.[4, 5] Approximately 15 to 40% of people who develop chronic HBV infection are expected to progress to cirrhosis and HCC.[6]

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.[7] NA directly inhibit reverse transcriptase and thereby impair viral replication, whereas IFN has marked immunomodulatory, but less pronounced direct antiviral effects. 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.[8] Currently, peginterferon alfa (PEG-IFN), created by attaching a polyethylene glycol (PEG) molecule to IFN, significantly improves pharamacokinetics and results in more convenient dosing interval than conventional IFN. [9]

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The genome of HBV

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.[10] The human HBV genome comprises a partially double stranded 3.2 kb DNA organized into four open-reading frames (Fig 1). [1, 11]

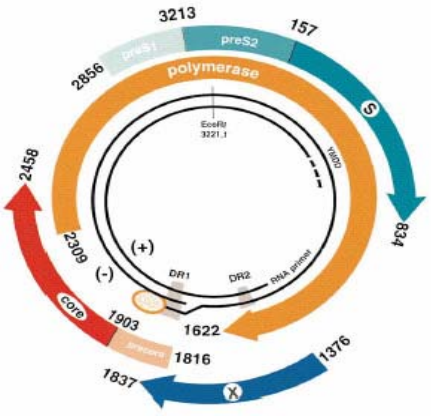

Fig. 1 Genome of hepatitis B virus

The pre-S/S ORF codes for the three surface proteins, 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 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 codes for the X protein plays an important regulatory role by acting principally as a transactivator of both viral and cellular genes. This protein has been implicated to be involved in HBV-related hepatocarcinogenesis. [1, 12]

The HBV lifecycle starts with virion attachment to an unknown specific receptor complex.[1] The viral envelope then fuses with the cell membrane, releasing the nucleocapsid into the cytoplasm. The virus is decapsidated, and the genomic HBV DNA and HBV DNA polymerase are transferred to the nucleus. One crucial step in the HBV life cycle is the formation of a covalently closed circular form of the viral genome through DNA repair of the relaxed circular replicative HBV DNA inside the nuclei of hepatocytes.[13] Covalently closed circular DNA (cccDNA) is transcribed by cellular RNA polymerases into messenger RNAs for viral protein synthesis, and into a pre-genomic RNA, which is subsequently encapsidated in the cell cytoplasm together with a molecule of HBV DNA polymerase. The latter has a reverse transcriptase (rt) function that catalyzes the synthesis of the negatively stranded genomic DNA, while the pre-genomic RNA 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 (Fig 2).[10, 14]

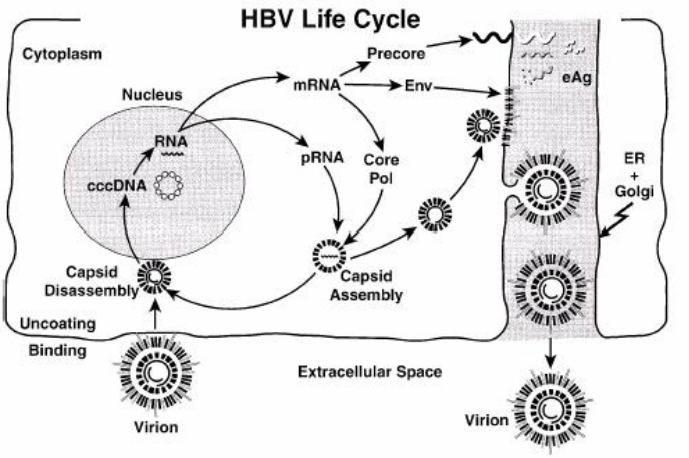

Fig. 2 Life cycle of hepatitis B virus

It is believed that chronic HBV infection is maintained by cccDNA in the hepatocytes. During chronic infection, HBV cccDNA accumulates in cell nuclei in which it persists as a stable episome and acts as a template for the transcription of viral genes. Previous data have shown that as the chronic hepatitis B disease progresses to the late stage with decreased viral replication, cccDNA becomes the predominant form of intrahepatic HBV DNA.[15] In animal models of HBV infection, hepadnaviral cccDNA appears to be relatively unaffected by nucleoside analogue therapy, despite the reduction of all other viral DNA forms in the liver, supporting that the limiting factor in eliminating HBV infection is the clearance of cccDNA reservoir from infected cells.[16] Despite the crucial role of cccDNA during persistent infection and the importance of understanding resolution mechanisms, scant data collected from patients have been reported. Recently, detection and quantification of cccDNA from liver biopsies were demonstrated using a selective real-time polymerase chain reaction (PCR) assay.[15, 17] However, a number of questions remain to be answered, including the following: 1) Are intrahepatic cccDNA levels in HBeAg-positive and HBeAg-negative chronic hepatitis different? 2) Does intrahepatic cccDNA levels correlate with serum HBsAg, HBeAg and HBV DNA levels? 3) Does intrahepatic cccDNA levels at the end of antiviral therapy (e.g. pegIFN therapy) predict sustained response to therapy?

HBV genotypes and intergenotype recombinations

HBV can be classified into eight major genotypes (A to H) based on nucleotide (nt) diversity of $\geq$8%.[18] 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.[19] Within each genotype, the four major subtypes based on the allelic determinants of the hepatitis B surface antigen (HBsAg) are adr, adw, ayr, and ayw. The adr and adw2 subtypes are the most common in Thailand.[20] Besides the differences in geographical distribution, there is growing evidence that the viral genotypes may influence the clinical outcomes of patients with chronic HBV infection. 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 compared to genotype B.[21-24] Genotype C is also associated with a lower rate of HBeAg seroconversion and a lower response rate to IFN therapy compared to genotype B.[25, 26] However, the association between HBV genotype and the risk of developing HCC is still controversial.[22, 27-29] In addition, the impact of HBV genotype on clinical features and prognosis of patients with HCC remains unclear.

It is increasingly accepted that, in addition to the currently classified genotypes of HBV, recombination between genotypes generates novel variants that contribute to the genetic diversity of HBV. HBV strains of certain genotypes in which a part of the open reading frame is replaced by the corresponding part of those of the other genotypes have been reported.[30, 31] Among these, recombinant forms between A and D, B and C, and C and D have been identified.[32] However, in many cases, recombinants may be separate instances of phylogenetically related viruses that have become widely distributed geographically. For example, a recombinant generated through the exchange of the core gene of genotype C into a genotype B variant, often described as genotype Ba (with Bj designating nonrecombinant genotype B), has become prevalent in mainland East Asia.[31, 33] Regarding clinical aspect, increasing data suggest the importance of recombination may influence the biological characteristics of virus and clinical outcome of patients. For instance, the genotype B with recombination might be involved in the severity of clinical disease.[33] Recently, we identified a novel recombinant of HBV genotypes G and C isolated from a Thai patient with HCC, though the possibility that such a recombination might contribute to the development of HCC in this case was unknown.[34] With a small number of recombinant strains available, it is not clear how frequently they occur and whether they might influence the evolution and pathogenicity of HBV in patients infected with the virus.

HBV mutations

Viral reverse transcriptases lack a proofreading function and are thus inherently error prone. As a result, HBV populations exist in the host as heterogeneous mixtures known as quasi-species. The frequency of HBV mutation has been estimated to be approximately 1.4 to 3.2 $x10^{-5}$ nt substitutions per site per year, around 10-fold higher than that of other DNA viruses.[35] 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 mutation rates, however, are lower than those of other retroviruses, mainly because of the constraints imposed by the overlapping reading frames.[36] 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. The other common HBV variants include double mutations in the basal core promotor (BCP) region (A1762T/G1764A) overlapping with the X gene ORF which result in a substantial decrease in HBeAg production.[37] These double mutants have been reported in up to 50-80% of patients with HBeAg-negative chronic hepatitis B in Europe and Asia[38], and have been implicated in HCC development.[39-42] 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.[42-45] 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.[46] The X protein is a multifunctional regulator that modulates host transcription, cell cycle progress, protein degradation, apoptosis and signal transduction pathways.[47] It has been shown that mutations in the X gene may contribute to the development of HCC in HBV-infected patients.[48, 49] However, current knowledge regarding the mutational patterns in the entire X region among patients with HCC is rather limited.

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.[50] The pre-S1 region is associated with a regulatory element with the nucleotide sequence CCAAT controlling transcription of the S gene, whose products are implicated in HBV attachment to the hepatocyte surface for initiating infection.[51] The pre-S2 region could bind to polymerized human serum albumin, although the significance of this binding is unknown.[52] Several lines of evidence indicate a role of pre-S deletion mutants in the clinical course of persistent HBV infection. It has been shown that naturally occurring pre-S deletion mutants tend to accumulate during a later stage of persistent HBV infection, including cirrhosis and HCC.[53] In these mutants, an immune epitope is deleted thus facilitating escape of the host immune surveillance and engendering loss of important functional sites, which may cause intracellular viral accumulation and contribute to more progressive liver injury and hepatocarcinogenesis.[54] In addition, mutations within the major hydrophilic region of the S gene, located from residue 120 to 160, have been selected during vaccination and after treatment of liver allograft recipients with hepatitis B immunoglobulin (HBIG).[55, 56] Most common variants in this "a" determinant region are a mutation from glycine to arginine at residue 145 (sG145R) and from aspartate to alanine at residue 144 (sD144A). The sG145R mutation has been showed to be associated with HBV vaccine failure.

Treatment of chronic HBV infection with NA has resulted in the outgrowth of otherwise minor quasi-species containing mutations in the HBV polymerase gene. Antiviral resistance to lamivudine has been mapped to the YMDD locus in the catalytic or C domain of HBV polymerase, whereas resistance to adefovir dipivoxil is associated with mutations in the D and B domains of the enzyme.[57-59] According to the new nomenclature, the mutations within the reverse transcriptase (rt) gene that were selected during lamivudine therapy are designated as rtM204I/V/S (domain C) ± rtL180M (domain B). The major mutations associated with adefovir-resistant HBV are designated as rtN236T (domain D) and rtA181T.[57, 59]

Occult HBV infection

Occult HBV infection is characterized by undetectable serum hepatitis B surface antigen (HBsAg) but detectable HBV-DNA in serum and/or in liver.[60] In many instances occult hepatitis B is associated with positivity for hepatitis B core antibody (anti-HBc) and/or hepatitis B surface antibody (anti-HBs). However, no anti-HBc or anti-HBs could be detected in a significant proportion of individuals.[60] Much evidence suggests that this so-called occult or cryptic HBV

infection is highly prevalent and may have a relevant clinical impact.[61] A previous study has shown that cirrhosis and HCC may occur in up to 30% of chronic HBV-infected patients several years after clearance of HBsAg.[62] Occult HBV infection has been demonstrated in serum and liver tissue of HBsAg-negative patients suffering from chronic hepatitis, cirrhosis and HCC.[60, 61] Residual HBV infection has also been shown in patients who had remission of hepatitis and clearance of HBsAg, either spontaneously or as a result of antiviral therapy.[63, 64] In areas where HBV infection is prevalent such as Thailand, a substantial number of HBV-related diseases might therefore be missed if HBsAg alone is used for diagnosing the infection.

The molecular/immunological mechanisms underlying occult HBV infection remain incompletely defined and are probably multifactorial.[60] The occult status is often mainly due to a strong suppression of viral replication and gene expression but failure to completely eradication of the virus.[61] This might be related to the long-term persistence in the nuclei of the hepatocytes of the HBV cccDNA. For instance, patients with occult HBV infection have low HBV DNA levels: $10^{2\text{-}3}$ copies/mL in the serum and 0.01–0.1 copy per liver cell.[65] Occult HBV is also associated in some cases with mutant viruses undetectable by HBsAg assays. For example, mutations at the 'a' determinant have been shown to reduce the binding of anti-HBs antibodies and potentially 'escape' the detection of immunoassays for HBsAg.[66] HBV 'a' escape mutants have been reported among "healthy" individuals with isolated anti-HBc, children who underwent anti-HBs seroconversion with remission of liver disease, and HBsAg negative patients with chronic active hepatitis.[67, 68] In addition, mutations in the X region have been known to effect the DNA polymerase region due to overlapping ORFs in the HBV genome; this may inhibit the replication efficiency of HBV, resulting in lower level of synthesis of HBsAg.[69] Finally, co-infection with other viruses, especially HCV, could down-regulate HBV replication result in persistence of occult HBV infection.

HBV and human immunodeficiency virus (HIV) co-infection

Co-infection with HBV and HIV is common, because both share major risk factors for transmission and both are endemic in Southeast Asia. It is estimated that approximately 10% of the HIV-infected population worldwide suffers from chronic hepatitis B.[70] However, the prevalence of co-infection with HBV and HIV varies according to geographic region and risk category. In western countries, chronic HBV infection is ten-fold more frequent among HIV-positive individuals than in the general population. Men who have sex with men show the highest

rates of co-infection (6–10%); rates of co-infection are slightly lower among intravenous drug users and much lower among people infected through heterosexual contacts.[71]

HIV infection has a deleterious effect on the course and natural history of HBV infection by impairing the quantity and quality of the innate and adaptive immune response. In contrast, the reciprocal relationship, the effect of HBV on HIV disease progression, is not clear.[72] HIV establishes a chronic and latent infection that induces extensive damage of the immune system through virus-related and indirect pathogenic mechanisms. Therefore, HIV-infected individuals show a quantitative depletion of CD4+ T cells but also an overall immune dysfunction that includes a complex dysregulation of the cytokine network, a decrease of the functional capability of CD8+ CTL and, at late stages, a significant reduction in their number, and an aberrant activation of cells of the immune system.[73] A prospective study of 5293 men, 326 of whom had chronic hepatitis B, demonstrated that mortality attributable to liver disease was significantly increased in those who were co-infected with HIV and HBV (14.2/1000 person-years) compared with those with HBV alone (0.8/1000 person-years).[74] It was found that individuals with lower CD4+ counts appeared to be at greatest risk. This finding also supports cross-sectional studies demonstrating increased cirrhosis in persons with HIV and HBV.[75] However, there are scarce data on the outcome of HBV infection in the era of highly active antiretroviral therapy (HAART).

There are several points in the natural history and molecular virology of chronic hepatitis B in HIV-infected patients that deserves future studies. For example, the natural history of chronic hepatitis B and cirrhosis in HIV-positive patients receiving HAART with or without drugs with dual activity is still unclear. Of interest, is the increasing detection of occult HBV infection in HIV-positive individuals with some studies finding that as many as 85% of HIV infected patients have 'anti-HBc alone' serological pattern. [76] No data are available on occult HBV infection in HIV infected patients from Southeast Asia. Furthermore, the role of occult HBV infection in the pathogenesis of liver injury in co-infected individuals is unknown. Since the viral interactions between HIV and HBV are complex, further studies are needed to investigate why there is frequent detection of HBsAg but low HBV DNA prevalence in HIV-negatives, compared to low HBsAg carriage and high HBV DNA prevalence in HIV-positives. Interestingly, the prevalence of HBV genotypes in HIV-infected patients seems to differ from that of the general population.[77] As the roles of HBV genotypes on clinical outcome have been mentioned previously, studies on the impact of HBV genotypes on the clinical course of HBV/HIV co-infection are needed. Finally, the great risk of some populations, especially intravenous drug

users, to be repeatedly exposed to different HIV and HBV strains, it is likely that mixed HBV infections or HBV recombination are more frequent than actually recognized.[78]

2. Objectives

The aims of this study were to

1) Compare molecular epidemiology of HBV in Thailand and neighboring countries
2) Determine viral genetic variability, including HBV genotypes and specific mutations, in Thai patients with HBV-associated HCC
3) Determine viral genetic factors of HBV in predicting virological response to PEG-IFN therapy
4) Determine intrahepatic cccDNA levels in patients with chronic hepatitis B and correlate their levels with other viral factors such as HBsAg, HBeAg and HBV DNA levels in the serum
5) Compare the prevalence and viral genetic variability, including genotypes, intergenotype recombinations and specific mutations, in patients with HBV infection and patients with HBV/HIV co-infection

3. Methodology

3.1 Subjects

To study the molecular molecular epidemiology of HBV in Thailand and neighboring countries, serum samples were obtained from 6213 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 conducted from May to October 2004.[79] In addition, 3009 serum samples collected from 1119 Cambodians, 787 Laotians and 1103 Myanmarese migrant workers in Thailand were included as representations of their respective countries.

Serum samples were also obtained from patients with chronic hepatitis, cirrhosis and HCC, who had attended at King Chulalongkorn Memorial Hospital, Bangkok between July 2002 and June 2006. 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.[80] 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 role of viral genetic factors associated with PEG-IFN therapy, 50 patients with chronic hepatitis B (39 men and 11 women) who had completed the treatment with PEG-IFN alpha-2b (PEG-IFN-α2b; Shering-Plough, Kenillworth, NJ) and had been followed-up between August 2005 and January 2008 at King Chulalongkorn Memorial Hospital were retrospectively investigated. The patients were age between 22 and 61 years. Paired liver biopsies were performed before and at the end of treatment for histology and intrahepatic viral DNA analysis. The liver histology was graded by the histological activity index (HAI) according to the criteria of Knodell et al.,[81] which comprise two major components namely necroinflammation (HAI-I) and fibrosis (HAI-F). In this study, 33 and 17 patients were classified as HBeAg-positive and HBeAg-negative chronic hepatitis, respectively.

PEG-IFN-α2b was administered subcutaneously at a dose of 1.5 μg/kg weekly for 48 weeks. All patients were followed-up for up to 48 weeks after treatment (week 96) to assess sustained virological response (SVR). In the HBeAg positive group, SVR was defined as HBeAg seroconversion and sustained inhibition of viral replication (HBV DNA level <2,000 IU/mL) until 12 months post treatment. In the HBeAg negative group, SVR was defined as sustained inhibition of viral replication (HBV DNA level <200 IU/mL) until 12 months post treatment.[82] Patients without SVR were defined as non-responders. According to these criteria, 12 (36.4%) and 6 (35.3%) of the HBeAg-positive and HBeAg-negative groups, respectively were classified as

responders. Serum samples were collected from each patient at baseline, during therapy (week 12, 24, 36 and 48) and during follow-up (week 72 and 96).

All serum samples were collected and stored at -80^0C. Liver biopsy samples were rapidly frozen 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 chronic hepatitis B in King Chulalongkorn Memorial Hospital had been approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University.

3.2 HBV DNA extraction, amplification and sequencing

One hundred microliter of HBsAg-positive sera was subjected to extract HBV DNA. The positive serum 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) following by phenol/chloroform/isoamyl alcohol extraction. HBV DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ), using primers from interest gene regions as appropriate. For example, the Pre-S1/Pre-S2/S region was amplified by 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 PC/C region was amplified by X101 (5'-TCT GTG CCT TCT CAT CTG-3': position 1552-1569) and CO2 (5'-GTG AGG TGA ACA ATG TTC CG-3': position 2053-2034). The X/BCP/PC regions (nucleotides (nt) 1287-2038) was amplified by using the primers Xi1: 5'AGCTTGTTTTGCTCGCAGC3' (forward primer, nt. 1287-1305), and Ci 1: 5' TTCCGGAGACTCTAAGGCC 3' (reverse primer, nt. 2020-2038).

The total 25 µl reaction volume consist of 10 µl of 2.5X 5 PRIME MasterMix solution (5 PRIME GmbH, Hamburg, Germany), 0.5 µl of 25 µM forward and reverse primer, 2 µl of DNA template and sterile distilled water. The thermocycler was programed to the HBV DNA amplification as following conditions: initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 1.30 minutes and a final extension step at 72°C for 7 minutes. The HBV DNA amplicons were isolated by electrophoresis method using 2% agarose gel, 100 volt, 60 minutes and stained with ethidium bromide. The size of PCR products were estimated by using the migration pattern of a 100-by DNA ladder under the UV light exposure. The expected products were cut and purified by the Perfectprep® Gel Cleanup kit (Eppendorf, Hamburg, Germany). The purified samples were

sent to commercial DNA sequencing company (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia) for base sequencing. Nucleotide sequences were edited by Chromas Lite program version 2.01 (Technelysium Pty Ltd., Queensland, Australia) and assembly by SeqMan (DNASTAR Lasergene software, Madison, WT).

3.3 Genotyping, subtyping and phylogenetic analysis

For phylogenetic analysis, nucleotide sequences were aligned with each available human genotype from GenBank database (National Center for Biotechnology Information, BesthesDa, MD) by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the alignments were constructed the phylogenetic trees using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ) for genotyping. Some sequences were genotyping by the Viral Genotyping Tool (National Center for Biotechnology Information, Besthesda, MD). 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 by using the translate tool in ExPASy Proteomics Server (available on: http://www.expasy.ch/tools/dna.html). The distribution of the HBV subtypes was deduced from amino acid sequences at positions 122 (Lys, Arg for d, y determinants), and 160 (Lys, Arg for w, r).[83]

3.4 Quantification of cccDNA and intrahepatic HBV DNA

Approximately 5-10 mg of liver biopsy specimens taken at baseline and end of treatment were studied. Liver tissue was incubated over night in lysis buffer with proteinase K followed by phenol/ chloroform/ iso-amyl alcohol extraction. Total intrahepatic HBV DNA was determined by real-time PCR using SYBR Green I fluorescent dye, applying the same conditions as specified for the measurement of serum HBV DNA.

HBV cccDNA was quantitatively determined as described previously, with some modifications.[84] Briefly, the reaction mixture comprised 1.0 µl of DNA sample, 6.0 µl of 2.5X MasterMix solution, 0.3 µl of 25 mM Magnesium solution (5 PRIME Mastermix, 5 PRIME GmbH, Hamburg, Germany), 0.75 µl of 25 µM forward primer, 0.75 µl of 25 µM reverse primer, 0.24 µl of 10X SYBR Green (QIAGEN, Hilden, Germany) and distilled water to a final volume of 15 µl. Real-time PCR amplification was carried out in a LightCyCler™ (Roach, Basel, Switzerland). After a pre-incubation step at 95°C for 10 min in order to activate the Taq

polymerase, amplification was performed during 40 cycles including denaturation (94°C, 15 s), annealing (59°C, 30s) and extension (72°C, 60s). A single fluorescent signal was obtained once per cycle at 80°C after extension step.

To standardize the extracted DNA from liver tissue in term of copies per genome equivalent, the amount of the β-globin gene was measured. Primer sequences for the β-globin gene were describe previously.[85] PCR was performed in 10 µl reaction volumes containing 1 µl of DNA, 5 µl of 2.5X Mastermix solution, 0.1 µl of 25 µM forward primer, 0.1 µl of 25 µM reverse primer, 0.25 µl of 10X SYBR Green and distilled water. Amplification was performed for 3 min at 94°C, 40 cycles of 10 s at 95°C for denaturation, 15 s at 60°C for annealing and 20 s at 72°C for extension. The fluorescence intensity of the PCR products was measured at 78°C. A standard curve was created the same as the quantification of HBV DNA by using pGemT-Easy Vector inserted with the amplicon.

3.5 Serological and virological assays

Qualitative HBsAg, HBeAg and anti-HBe measurements were carried out using a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL). The quantification of HBsAg was performed using the ARCHITECT *i*2000SR (Abbott Diagnostic, Abbott Park, Chicago, IL) according to the manufacturer's specifications.[86] The sensitivity of the assay ranged from 0.05 to 250 IU/mL. Samples with HBsAg titers beyond the upper range were diluted with phosphate buffered saline (PBS) into 1:10, 1:1000 and 1:10000 prior to further analysis.

The quantification of HBeAg was performed using the ARCHITECT *i*2000SR (Abbott Diagnostic, Abbott Park, IL), a two-step immunoassay based on the use of chemiluminescence microparticles (CIMA), according to the manufacturer's instructions.[87] Briefly, undiluted samples were mixed with paramagnetic beads coated with anti-HBe antibodies. HBeAg in the sample then attached to the magnetic beads through the presenting antibodies. After a washing step, a conjugate and reactant were added leading to the emission of light, which was proportional to the determined HBeAg concentration. The assay was calculated based on the ratio of the sample relative light unit (RLU) to the cutoff RLU (S/CO) for each specimen. Samples with S/CO values > 1.0 were considered positive results for HBeAg.

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 to 8.7 log genome equivalents/mL(LGE/mL).

3.6 Statistical Analysis

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 for categorical variables and by the Mann–Whitney *U*-test or Student's *t*-test for quantitative variables. Pearson correlation coefficient was tested for correlation between two variables. Area under the receiver operating characteristic (ROC) curve was calculated to assess the predictive values of variables. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated in accordance with standard methods. *P*-values <0.05 were considered statistically significant. Data were analyzed using the SPSS software for Windows 14.0 (SPSS Inc., Chicago, IL).

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*).

Table 1 The prevalence of HBV genotypes in different geographic regions of Thailand

	Genotype (%)			Total
	A	**B**	**C**	
Chiangrai (Northern)	1 (1.61)	10 (16.13)	51 (82.26)	62
Nakhon Si Thammarat (Southern)	1 (5.00)	-	19 (95.00)	20
Udon Thani (Northeast)	-	1 (2.17)	45 (97.83)	46
Chonburi (Central)	-	6 (31.58)	13 (68.42)	19
Total	2 (1.36)	17 (11.56)	128 (87.08)	147

Table 2 The prevalence of HBV subtypes in different geographic regions of Thailand

	Subtype (%)			Total
	adr	adw	ayw	
Chiangrai (Northern)	49 (79.03)	12 (19.35)	1 (1.61)	62
Nakhon Si Thammara (Southern)	19 (95.00)	1 (5.00)	-	20
Udon Thani (Northeast)	45 (97.83)	-	1 (2.17)	46
Chonburi (Central)	11 (57.89)	8 (42.11)	-	19
Total	124 (84.35)	21 (14.23)	2 (1.36)	147

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 were constructed (Fig 3A and 3B).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.

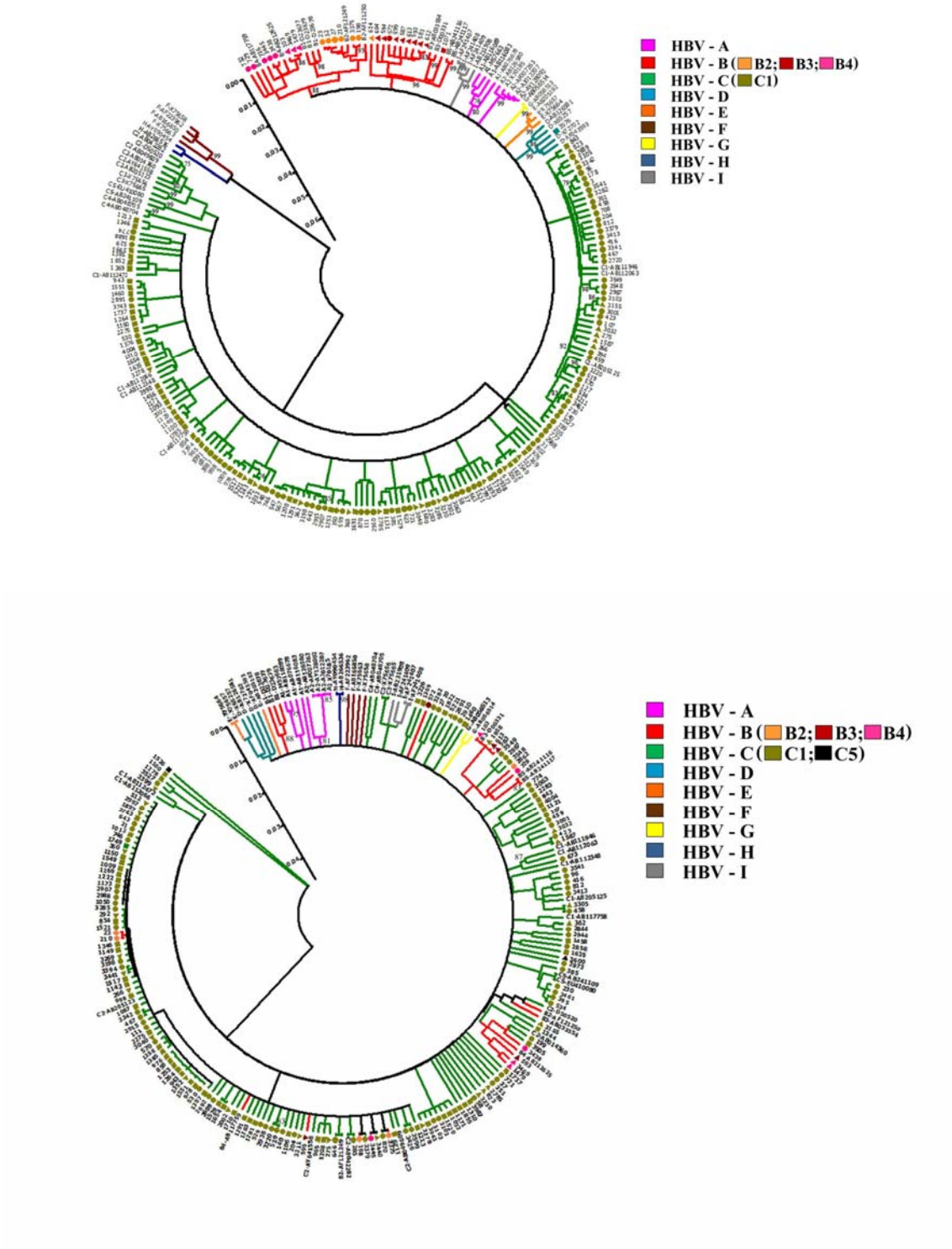

Fig. 3 Phylogram depicting the phylogenetic relationship of the sequences and representative sequences of human HBV strains from GenBank. Regions include in the comparison were: (A) the large S gene including preS1, preS2 and S gene; (B) the C gene, including nucleotide position 1814 – 2000. Percentage bootstrap values (>75%) were shown at the respective nodes. The scale bar at the bottom indicated the genetic distance.

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 3.

Table 3 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])	81:20:1	31:11:0	46:28:6	158:59:7
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 (%)

Although the entire genome sequence was not performed, three isolates with suspected intergenotypic 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 subgenotype B2 and C1, with its recombination breakpoints estimated at nucleotide 573 (Fig 4A). Isolate 3794 represented a recombinant of genotypes G/C1 with its recombination breakpoints between nucleotides 2006 and 157 (Fig 4B). Isolate 612 was classified to subgenotype B3 in pre-S/S gene but showed subgenotype C1 between nucleotides 1554 and 1974 (figure not shown).

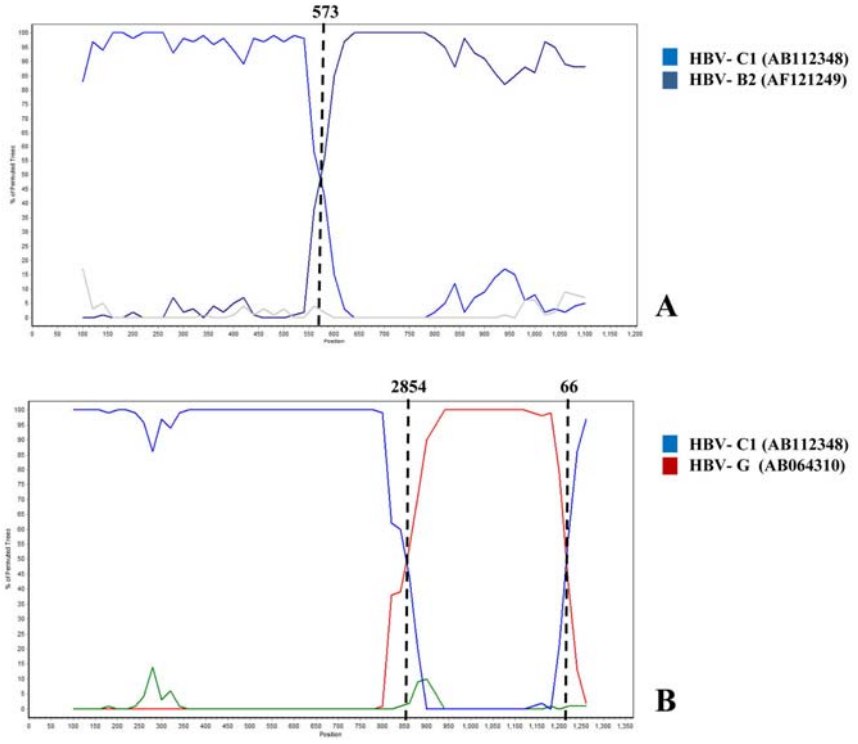

Fig.4 Bootscanning analysis of suspected recombinant isolates. (A) complete *S* gene of isolate 31 was compared with HBV-B2 (AF121249) and HBV-C1 (AB112348); (B) Nucleotide position 2006 – 157 of isolate 3794 was compared with HBV-C1 (AB112348) and HBV-G (AB064310). Dash line (s) showed the breaking 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.[88] 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,[89] and subgenotypes C1 and B4 were dominant strains in Cambodia.[90] 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.[22] Genotype C is also associated with a lower rate of HBeAg seroconversion and a lower response rate to IFN therapy compared to genotype B.[28] 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%).[79] 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,[33] whereas recombination of genotypes C/D has been detected in Tibet and China.[91, 92] In addition, recombinants between genotypes A/C and genotypes A/D have been documented in Vietnam[93] and India,[94] respectively. Recently, a novel genotype I, with a complex recombination involving genotypes C, A and G has been reported in Vietnam and Laos.[90, 95] 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.[33] 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.[34]

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 (Table 4). The alignment of amino acid sequences of the partial S region of these 35 samples is shown in Fig 5.

Table 4 Prevalence of 'a' determinant mutations in migrant workers

Mutations	Cambodia (n = 102)	Laos (n = 42)	Myanmar (n = 80)	Total (n = 224)
No. PCR positive	94 (92.2)	38 (90.5)	62 (77.5)	194 (86.6)
Ile126Ser/Asn	6 (6.4)	2 (5.3)	4 (6.5)	12 (6.2)
Pro127Arg	1 (1.1)	0	0	1(0.5)
Gly130Arg	0	1 (2.6)	0	1(0.5)
Thr131Asn/Pro	0	1 (2.6)	2 (3.2)	3 (1.5)
Met133Thr	2 (2.1)	0	0	2 (1.0)
Phe134Leu	1 (1.1)	0	0	1(0.5)
Thr140Ile	0	0	1(1.6)	1(0.5)
Pro142Leu	1 (1.1)	0	0	1(0.5)
Gly145Arg/Ala	3 (3.2)	1 (2.6)	0	4 (2.1)
Trp156Leu	0	0	1(1.6)	1(0.5)
Ala157Gly	0	0	1(1.6)	1(0.5)
Ala159Val	1 (1.1)	0	0	1(0.5)
Pro120Thr + Ala128Asp + Cys138Tyr + Phe158Leu	0	1 (2.6)	0	1(0.5)
Lys122Gln + Thr131Asn + Met133Thr	1 (1.1)	0	0	1(0.5)
Gly130Arg + Met133Thr	1 (1.1)	0	0	1(0.5)
Thr131Asn + Phe134Tyr	1 (1.1)	0	0	1(0.5)
Thr131Asn + Phe134Tyr + Asp144Glu	1 (1.1)	0	0	1(0.5)
Ala128Val + Phe134Tyr + Phe158Leu + Ala159Gly	0	0	1(1.6)	1(0.5)

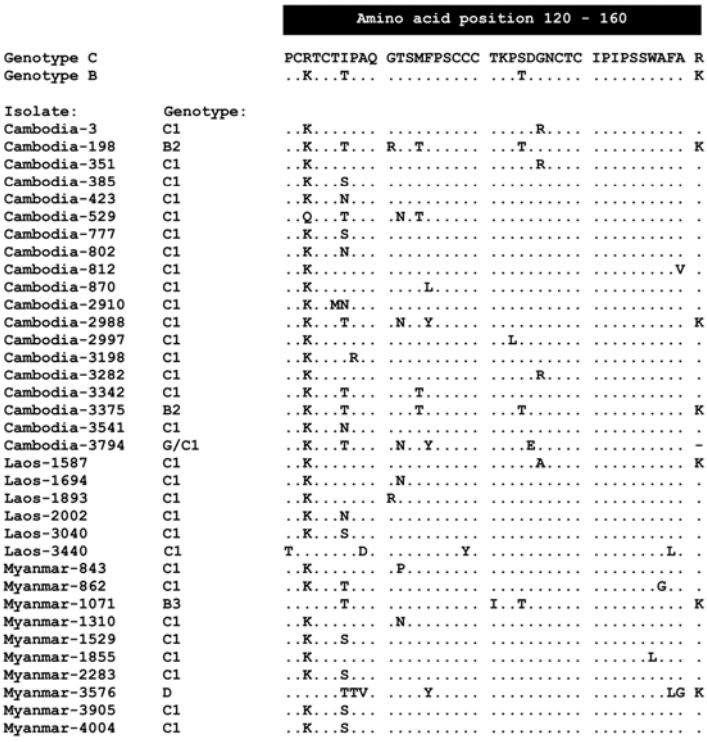

Fig. 5 The amino acid sequences alignment of the 'a' determinant region of 35 samples

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.[56] 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[56]. 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.[96] 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,[97] 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%).[98] 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.[99, 100] Thus, it is speculated that these mutants within the 'a' determinant region might have emerged through natural immunoselective pressure of the host, which in turn are infectious and have been circulated among individuals chronically infected with the virus.

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*). The alignment of amino acid sequences of the entire pre-S1/pre-S2 region of the 14 samples is shown in Fig 6.

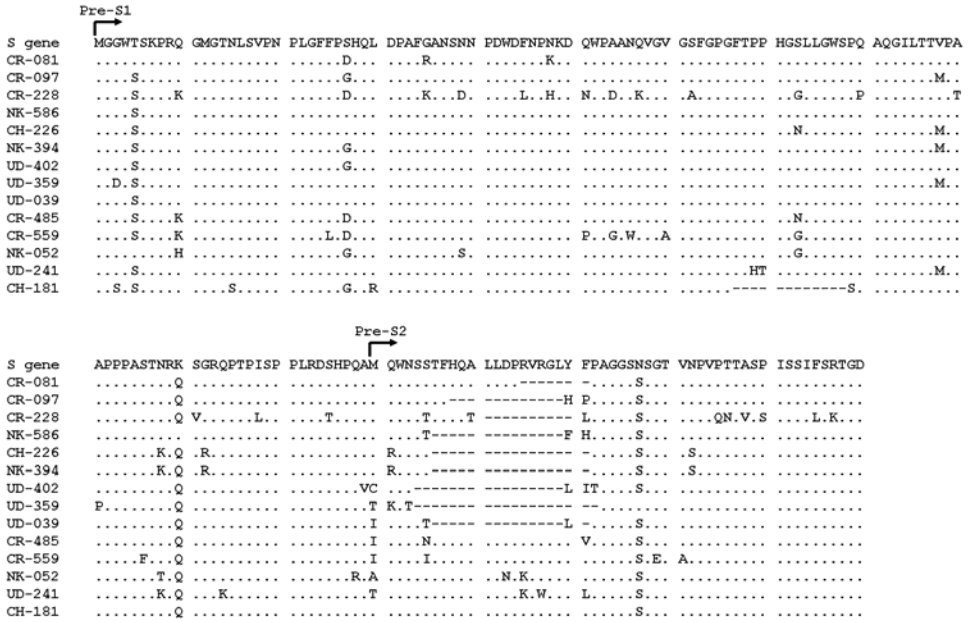

Fig. 6 The amino acid sequences alignment of the entire pre-S of 14 samples.

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 7.

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[50] 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.[101] 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.

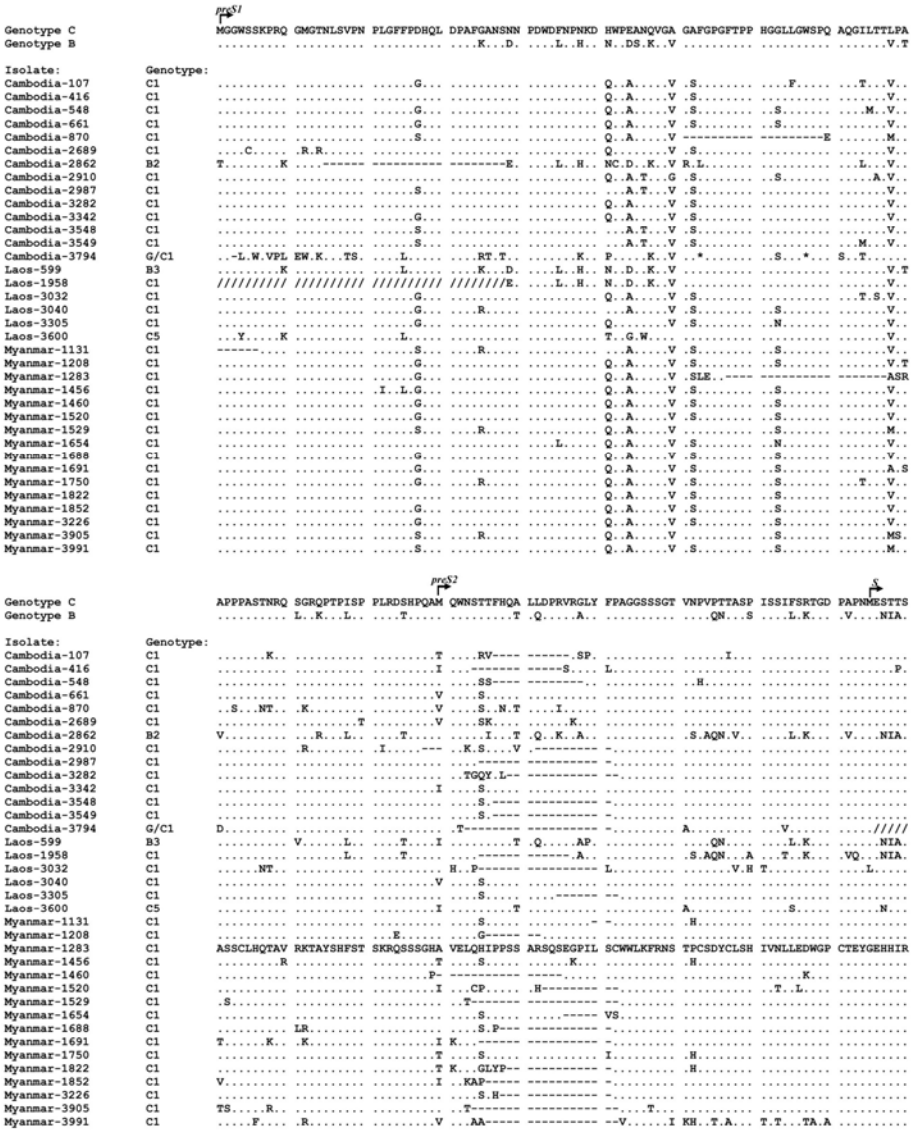

Fig.7 The amino acid sequences alignment of the entire pre-S1/pre-S2 region of 36 samples.

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[101, 102] 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.[53, 101, 102] The mutations found in our studies, along with similar observations in previous reports, could help elucidate the evolutionary pattern of mutations in the clinical course of persistent HBV infection.

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 5. 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 5).

Table 5 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 6). 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.

Table 6 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 (%)

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 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 6). 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.

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 7 (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 7).

Table 7 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 A1762T/G1764A and G1899A 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 8).

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 9. 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.

Table 8 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

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.[39, 45, 103, 104] 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.[105] 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.[40] As expected, our data also demonstrated that A1762T/G1764A mutations were genotype C related.

Table 9 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 (%)

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.[106] 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[41], 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.[107] 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.[39] In contrast, G1899A mutation was found at low prevalence with no clinical association in other previous reports.[108, 109] 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.

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[39, 53]. 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.[110]

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.[111] Furthermore, the presence of BCP double mutants may be associated with decreasing T-cell immune responses[112]. 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.[112] 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.[113]

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.[114] Previous studies also reported that other amino acid substitutions, such as A36T, P38S, A44L and H94Y were significantly associated with the risk of HCC.[44, 48, 115, 116] 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 Viral genetic factors of HBV in predicting response to PEG-IFN therapy

Demographic and baseline clinical characteristics of the patients are shown in Table 10. HBeAg-negative patients had significantly higher mean age, but lower baseline HBV DNA and HBsAg levels than patients with HBeAg-positive hepatitis. No significant difference between groups was observed in respect to sex, pretreatment ALT level, necroinflammatory and fibrosis scores.

Based on sequencing and phylogenetic analysis of the pre-S region, serum samples from 6 (12%) patients (all in the HBeAg-positive group) belonged to genotype B, and those of 44 (88%) (27 in the HBeAg-positive group) to genotype C. Based on the preC/C region results, all 50 cases belonged to genotype C and were determined as subgenotype Cs. Thus, all HBV strains of genotype B in this study were subgenotype B2, which is a recombinant with the preC/C region of genotype C. There was no significant difference in HBV genotype distribution between the HBeAg-positive and HBeAg-negative groups (Table 10).

Table 10 Demographic/clinical characteristics of HBeAg-positive and HBeAg-negative patients

	HBeAg-positive (n=33)	HBeAg-negative (n=17)	*P*
Age, yr	37.4 ±9.1	43.3 ±8.0	0.023
Sex, male	26 (78.8%)	13 (76.5%)	1
Pretreatment ALT level, U/L	92.6±84.4	102.6±56.1	0.62
Pretreatment HBV DNA, $\log_{10}$ IU/mL	6.2±0.8	5.7±0.8	0.04
Pretreatment HBsAg level, IU/mL	14189.03±19445.4	2668.5±5037.4	0.003
Pretreatment HBsAg level, $\log_{10}$ IU/mL	3.9±0.6	2.9±0.8	<0.001
Necroinflammatory score	4.0±1.6	4.3±1.8	0.62
Fibrosis score	1.3±0.9	2.0±1.4	0.11
Genotype			0.061
B	6 (18.2%)	0 (0%)	
C	27 (81.8%)	17 (100%)	
C1653T	1 (3.0%)	4 (23.5%)	0.04
T1753C/deletion	6 (18.2%)	10 (58.8%)	0.003
Double BCP mutation			0.004
A1762T/G1764A	13 (39.4%)	15 (88.2%)	
Deletion	4 (12.1%)	1 (5.9%)	
G1896A	3 (9.1%)	7 (41.2%)	0.021
Pre-S mutation			0.1
Pre-S2 start codon	2 (6.1%)	3 (17.6%)	
Deletion	1 (3.0%)	2 (11.8%)	
Antiviral response	12 (36.4%)	6 (35.3%)	1

Mutational hot spots in the EnhII/BCP/PC regions were found at nt 1753, 1762, 1764 and 1896. The C1653T and T1753C mutations were observed in 5 (10%) and 16 (32%) cases, respectively. The A1762T/G1764A mutations were found in 28 (56%) cases. In addition, five patients showed the following deletions at or around nt 1762-1764. One patient had deletions at nt 1762-1764 and 1773-1775, while another had deletions at nt 1762-1764 and 1771-1776. One additional patient had deletions at nt 1751-1764 and 1771-1777, while another one had a deletion at nt 1756-1770. All these cases with deletions belonged to the HBeAg-positive group. Furthermore, another patient in the HBeAg-negative group had a deletion at nt 1755-1763 and G1764A mutation (Figure 8A, 8B).

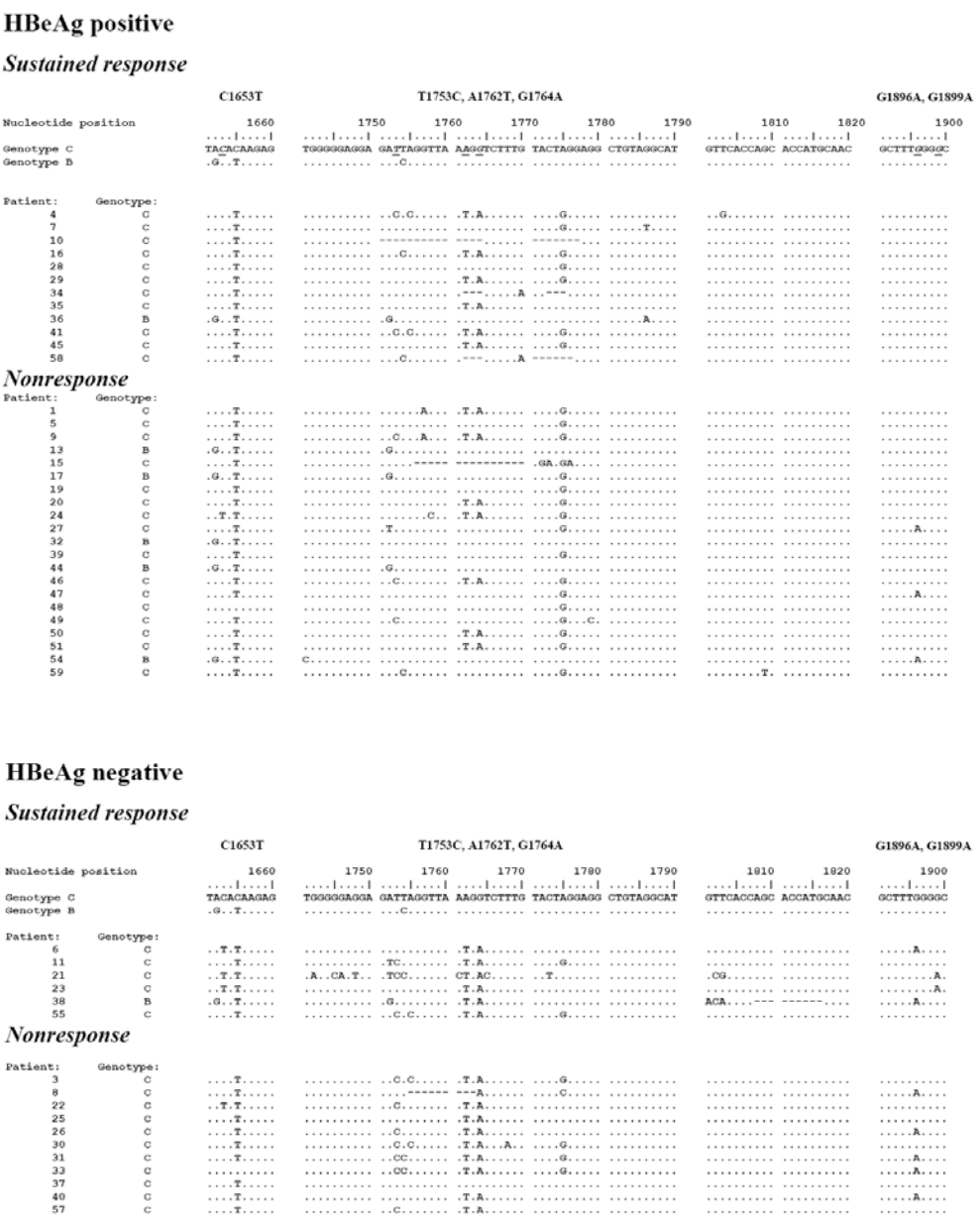

Fig. 8 The nucleotide sequences alignment of parts of the EhnII/ BCP/ PC regions. A) HBeAg-positive patients B) HBeAg-negative patients Nucleotide deletions are marked by a *hyphen* (-). Consensus sequence was established from HBV databank sequences.

Comparison between groups showed that the frequencies of C1653T, T1753C, double BCP and PC mutations were significantly higher in HBeAg-negative patients (Table 10). In HBeAg-positive group, patients harboring double BCP mutations exhibited HBV DNA levels comparable to those with wild-type populations (6.8±0.6 and 7.1±0.9 $\log_{10}$ copies/mL, respectively, P=0.482). Patients with HBV genotype C had higher frequencies of double BCP and T1753C mutations than those with HBV genotype B ($P<0.001$ and $P=0.023$, respectively).

However, the frequencies of PC and C1653T mutations were not significantly different between genotypes B and C.

Pre-S mutations were detected in 8 of 50 (16%) samples. All these samples belonged to genotype C, and were exclusively detected in viral populations harboring double BCP mutations. Among these, pre-S2 start codon mutation was the most common (10%), followed by pre-S2 deletion (4%) and pre-S1 deletion (2%) (Figure 9A, 9B). The prevalence of pre-S mutations was higher in the HBeAg-negative group but no significant difference was found (Table 10).

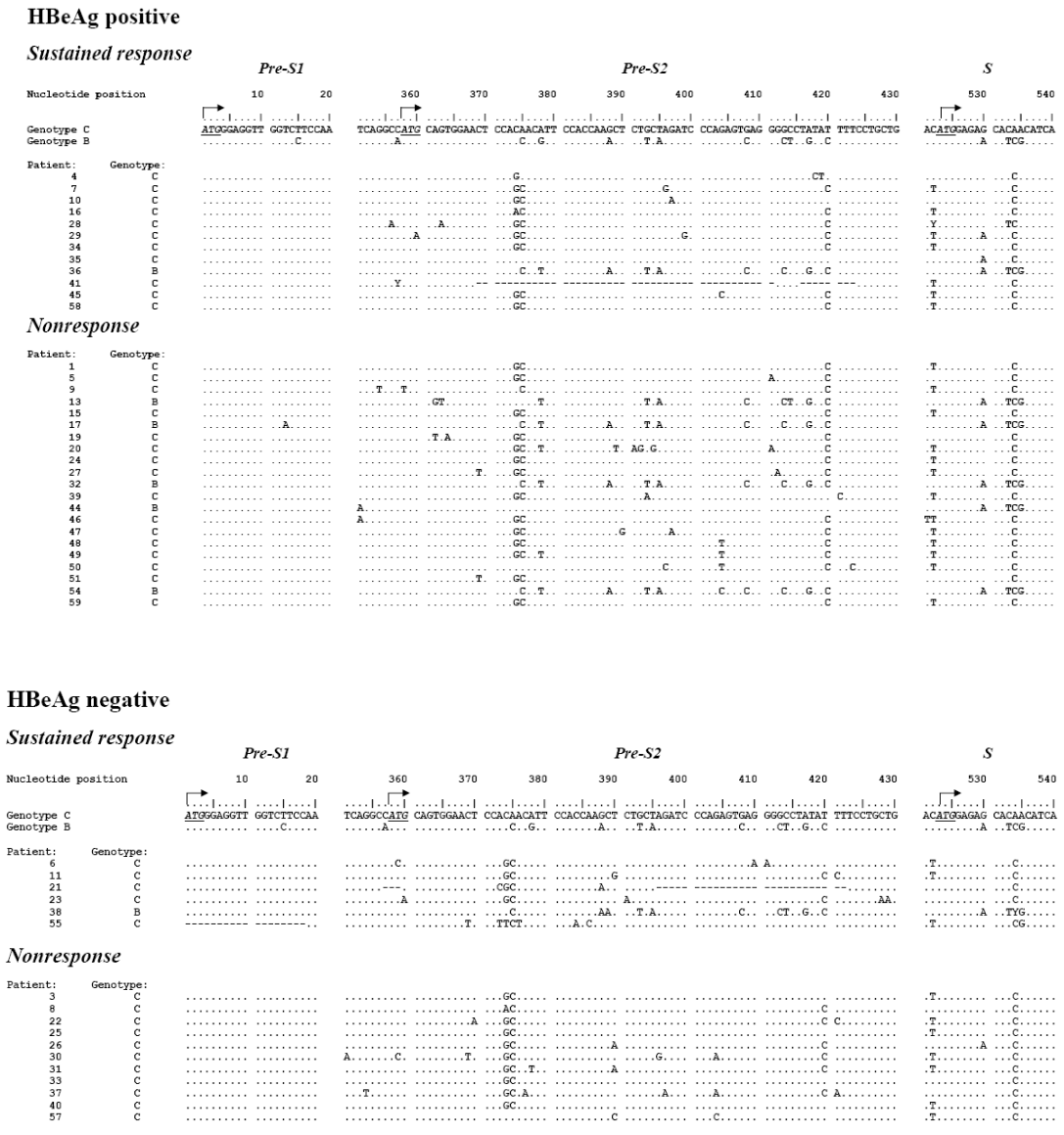

Fig. 9 The nucleotide sequences alignment of parts of the pre-S1/pre-S2 regions

A) HBeAg-positive patients B) HBeAg-negative patients Nucleotide deletions are marked by a *hyphen* (-). Consensus sequence was established from HBV databank sequences.

In the HBeAg-positive group, low pretreatment HBsAg level and the presence of double BCP mutations at entry were found to be significant predictors of response to PEG-IFN therapy in univariate analysis (Table 11). There was no correlation between the presence of C1653T, T1753C, G1896A, pre-S mutations and response to PEG-IFN treatment in this group.

Table 11 Baseline characteristics of HBeAg-positive patients with and without response to PEG-IFN therapy

	Responder (n=12)	Nonresponder (n=21)	*P*
Age, yr	38.9 ±10.8	36.5 ±8.2	0.51
Sex, male	8 (66.7%)	18 (85.7%)	0.19
Pretreatment ALT level, U/L	89.6±52.2	94.2±99.5	0.86
Pretreatment HBV DNA, $\log_{10}$ IU/mL	6.0±0.5	6.4±0.9	0.19
Pretreatment HBsAg level, IU/mL	4703.7±3859.5	19609.2±22626.9	0.007
Pretreatment HBsAg level, $\log_{10}$ IU/mL	3.5±0.4	4.1±0.6	0.005
Necroinflammatory score	3.6±1.4	4.3±1.7	0.23
Fibrosis score	1.3±0.9	1.3±1.0	0.81
Genotype			0.38
B	1 (8.3%)	5 (23.8%)	
C	11 (91.7%)	16 (76.2%)	
C1653T	0 (0%)	1 (4.8%)	1
T1753C/deletion	3 (25%)	3 (14.3%)	0.64
Double BCP mutation/deletion	9 (75%)	8 (38.1%)	0.041
G1896A	0 (0%)	3 (14.3%)	0.28
Pre-S mutation/deletion	2 (24%)	1 (4.8%)	0.54

In the HBeAg-negative group, low pretreatment HBsAg level and presence of pre-S mutation/deletions at entry were found to be significant predictors of response (Table 12). However, there was no correlation between the presence of C1653T, T1753C, A1762T/G1764A, G1896A, and response to PEG-IFN treatment in this group.

Table 12 Baseline characteristics of HBeAg-negative patients with and without response to PEG-IFN therapy

	Responder (n=6)	Nonresponder (n=11)	*P*
Age, yr	47.0 ±5.8	41.3 ±8.5	0.12
Sex, male	5 (83.3%)	8 (72.7%)	1
Pretreatment ALT level, U/L	89.5±44.1	109.7±62.5	0.45
Pretreatment HBV DNA, $\log_{10}$ IU/mL	5.9±0.8	5.6±0.9	0.56
Pretreatment HBsAg level, IU/mL	346.4±410.2	3935.1±5959.6	0.014
Pretreatment HBsAg level, $\log_{10}$ IU/mL	2.3±0.6	3.2±0.6	0.008
Necroinflammatory score	5.3±2.6	3.9±1.4	0.39
Fibrosis score	3.0±1.4	1.6±1.3	0.15
Genotype			1
B	0 (0%)	0 (0%)	
C	6 (100%)	11(100%)	
C1653T	3 (50%)	1 (9.1%)	0.09
T1753C/deletion	3 (50%)	7 (63.6%)	0.61
Double BCP mutation/deletion	6 (100%)	10 (90.9%)	1
G1896A	2 (33.3%)	5 (45.5%)	1
Pre-S mutation/deletion	4 (66.7%)	1 (9.1%)	0.028

Multivariate analysis with stepwise logistic regression identified low pretreatment HBsAg level as an independent factor associated with sustained response in HBeAg-positive (odds ratio (OR), 14.67; 95% confidence interval (CI), 1.59-135.30; *P*=0.018), and HBeAg-negative hepatitis (OR, 22.50; 95% CI, 1.61- 314.56; *P*=0.021).

The results of this study showed that pretreatment C1653T and T1753C mutants were not associated with adverse response to PEG-IFN therapy. However, the presence of A1762T/G1764A at entry was associated with a higher rate of PEG-IFN response in HBeAg-positive patients. This is consistent with previous reports that HBeAg-positive patients harboring

double BCP mutants responded better to conventional IFN therapy.[117-120] HBeAg, known to induce immunotolerance,[121] 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.[12] As a consequence, lower level of HBeAg could contribute to a decrease in immunotolerance and enhance host immune response, which in turn lead to viral clearance following PEG-IFN treatment. In contrast, we could not find any correlation between the presence of PC mutant and PEG-IFN responsiveness. Indeed, there is no conclusive evidence that PC mutant has an impact on the outcome of IFN-based therapy.[117, 118, 122, 123]

Although the role of pre-S mutants on the clinical course of chronic HBV infection has been recognized,[53] the role of these variants associated with PEG-IFN responsiveness has never been investigated. In HBeAg-negative patients, our data showed that pre-S mutation/deletions were observed more frequently in sustained responders, suggesting such variants may be associated with a higher response rate to PEG-IFN therapy. Although the reason is unclear, it is speculated that most of the mutation/deletion regions encompassed epitopes for T and B cells[124], which could lead to an alteration of the immune response and result in different outcome following PEG-IFN therapy. Interestingly, pre-S mutation/deletions were exclusively found in populations harboring double BCP mutations. Since both BCP and pre-S deletion mutants develop in the quasispecies during the course of persistent HBV infection, it is possible that the occurrence of these mutations are as a consequence of disease progression and may represent biomarkers for predicting the development of advanced liver disease.[125, 126]

In this study, a low level of serum HBV DNA before treatment was not associated with response to PEG-IFN therapy. In contrast, low level of pretreatment HBsAg was consistently a predictor of the antiviral response in both HBeAg-positive and HBeAg-negative groups. This finding is in agreement with previous data that low pretreatment HBsAg levels have been considered as a predictor of HBsAg seroconversion following conventional IFN treatment.[127] It has been shown that low pretreatment HBsAg is more reliable than serum HBV DNA levels in predicting good response to PEG-IFN and lamivudine treatment.[128] Thus, the present study provides additional evidence that quantitative HBsAg determination should be considered before administering IFN-based therapies.

The association between HBV genotypes and the response to PEG-IFN treatment remains controversial.[129, 130] Our data showed that genotypes B and C were comparable in term of PEG IFN responsiveness. This finding could be in part attributed to the observation that all strains

of genotype B were subgenotype Ba, which is a recombinant with the preC/C region of genotype C. Indeed, the epidemiologic study has demonstrated that all HBV strains in Thailand belong to subgenotype Ba.[131] Consequently, subgenotype Ba and genotype C, both of which share the sequences of the preC/C region, showed a similar response rate to PEG-IFN therapy. Whether there is any correlation between HBV genotypes/subgenotypes and the response to PEG-IFN therapy requires further investigation to address this interesting issue.

4.4 Intrahepatic cccDNA levels and kinetics of HBsAg, HBeAg and HBV DNA levels in predicting virological response to PEG-IFN therapy in HBeAg-positive chronic hepatitis B

Baseline characteristics of patients with HBeAg-positive chronic hepatitis are shown in Table 13. Responders had significantly lower baseline $\log_{10}$HBsAg, $\log_{10}$HBeAg, $\log_{10}$cccDNA and $\log_{10}$ total intrahepatic HBV DNA levels than non-responders. No significant difference between groups was observed in respect to age, sex, pretreatment ALT level, HBV genotype, necroinflammatory and fibrosis scores. Lower HBV DNA levels at baseline tended to predict SVR; however, the difference between groups did not reach statistical significance.

Table 13 Characteristics of responders and non-responders at baseline

	Responder (n=10)	Non-responder (n=20)	*P*
Age, yr	37.0 ±9.4	36.7 ±8.2	0.921
Sex, male	7(70%)	16 (80%)	0.657
ALT level, U/L	96.6±65.6	90.9±71.3	0.852
HBV genotype			0.333
B	1 (10%)	5 (25%)	
C	9 (90%)	15 (75%)	
Serum HBsAg level, $\log_{10}$ IU/mL	3.46±0.47	4.01±0.64	0.013*
Serum HBeAg level, $\log_{10}$ S/CO	2.22±0.52	2.73±0.54	0.023*
Serum HBV DNA level, $\log_{10}$ copies/mL	5.93±0.67	6.43±0.93	0.107
cccDNA level, $\log_{10}$ copies/genome equivalent	0.28±0.75	1.46±0.74	0.001*
Total intrahepatic HBV DNA level, $\log_{10}$ copies/genome equivalent	1.19±0.36	2.10±0.69	0.001*
Necroinflammatory score	3.8±1.6	4.2±1.8	0.563
Fibrosis score	1.1±0.8	1.2±0.9	0.719

Pretreatment log_{10} HBsAg showed positive correlation with both log_{10} HBeAg (r=0.516, *P*=0.003) and log_{10} (serum HBV DNA) (r=0.633, *P*<0.001), as well as with log_{10} cccDNA (r=0.517, *P*=0.008) and log_{10} (total intrahepatic HBV DNA) (r=0.635, *P*=0.001). Log_{10} HBeAg displayed correlation with log_{10} (serum HBV DNA) (r=0.513, *P*=0.004) and log_{10} cccDNA (r=0.507, *P*=0.010), but not with log_{10} (total intrahepatic HBV DNA) (r=0.362, *P*=0.076). Log_{10} (serum HBV DNA) was not significantly correlated with log_{10} cccDNA (r=0.295, *P*=0.152) and log_{10} (total intrahepatic HBV DNA) (r=0.347, *P*=0.089).

At end of treatment, responders had significantly lower serum HBsAg, HBeAg, HBV DNA, cccDNA and intrahepatic HBVDNA levels than non-responders. There was a tendency towards lower ALT, necroinflammatory and fibrosis scores in responders, but the difference between groups did not reach statistical significance (Table 14).

Table 14 Characteristics of responders and non-responders at end of treatment

	Responder (n=10)	Non-responder (n=20)	*P*
ALT level, U/L	50.9±36.0	70.0.0±44.7	0.221
Serum HBsAg level, log_{10} IU/mL	2.59±0.97	3.41±0.64	0.031*
Serum HBeAg level, log_{10} S/CO	0.28±1.08	2.41±0.94	<0.001*
Serum HBV DNA level, log_{10} copies/mL	3.18±1.57	5.59±177	0.001*
cccDNA level, log_{10} copies/genome equivalent	-1.13±1.06	0.66±0.99	<0.001*
Intrahepatic HBV DNA level, log_{10} copies/genome equivalent	-0.87±0.98	0.82±0.92	<0.001*
Necroinflammatory score	2.2±1.2	3.1±1.7	0.133
Fibrosis score	0.6±0.5	1.0±0.9	0.135

At end of treatment, log_{10} HBsAg was well correlated with log_{10} HBeAg (r=0.567, *P*=0.001) and log_{10} (serum HBV DNA) (r=0.591, *P*=0.001), as well as with log_{10} cccDNA (r=0.673, *P*<0.001) and log_{10} (total intrahepatic HBV DNA) (r=0.531, *P*=0.006). Log_{10} HBeAg had significant correlation with log_{10} (serum HBV DNA) (r=0.682, *P*<0.001), log_{10} cccDNA (r=0.678, *P*<0.001) and log_{10} (total intrahepatic HBV DNA) (r=0.753, *P*<0.001). Log_{10} (serum HBV DNA) was also significantly correlated with log_{10} cccDNA (r=0.614, *P*=0.001) and log_{10} (total intrahepatic HBV DNA) (r=0.684, *P*<0.001).

Reduction of HBsAg and HBeAg was correlated with reduction of $\log_{10}$ cccDNA (r=0.421, *P*=0.032, and r=0.570, *P*=0.002, respectively) and $\log_{10}$ (total intrahepatic HBV DNA) (r=0.420, *P*=0.033, and r=0.535, *P*=0.005, respectively), whereas reduction of $\log_{10}$ HBV DNA was not significantly correlated with reduction of $\log_{10}$ cccDNA (r=0.365, *P*=0.066) and $\log_{10}$ (total intrahepatic HBV DNA) (r=0.330, *P*=0.099).

During treatment and follow-up, patients who developed SVR showed a consistent decline in serum HBsAg, with a mean decrease of 0.41±0.36, 0.69±0.61, 0.82±0.67, 0.96±0.75, 1.05±0.84 and 1.15±0.79 $\log_{10}$ IU/mL at week 12, 24, 36, 48, 72 and 96, respectively. It should be noted that in this patient group serum HBsAg still decreased after cessation of treatment. Non-responders, however, showed a decrease in HBsAg levels at week 12 which leveled off during weeks 24 to 48, and had a tendency to rebound after cessation of treatment (Fig 10).

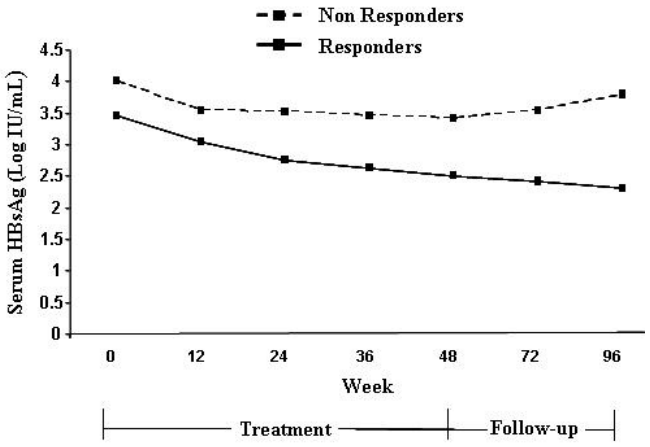

Fig. 10 Kinetics of serum HBsAg during PEG-IFN therapy and follow-up in responders (solid line) and non-responders (dashed line).

Patients who developed SVR showed a marked decrease in serum HBeAg, with a mean decrease of 0.65±0.81, 1.08±0.98, 1.57±1.00, 1.95±1.32, 2.05±1.22 and 2.07±1.21 $\log_{10}$ S/CO at week 12, 24, 36, 48, 72 and 96 respectively. However, serum HBeAg levels did not significantly decrease during treatment in non-responders (Fig 11).

Patients who developed SVR showed a marked decline in serum HBV DNA, with a mean decrease of 1.11±1.30, 1.96±1.55, 2.73±1.69, 2.76±1.69, 3.84±1.65 and 3.92±1.59 $\log_{10}$ copies/mL at week 12, 24, 36, 48, 72 and 96, respectively. Patients who did not achieve SVR demonstrated slightly decreased HBV DNA levels during therapy which had a tendency to rebound after cessation of treatment (Fig 12).

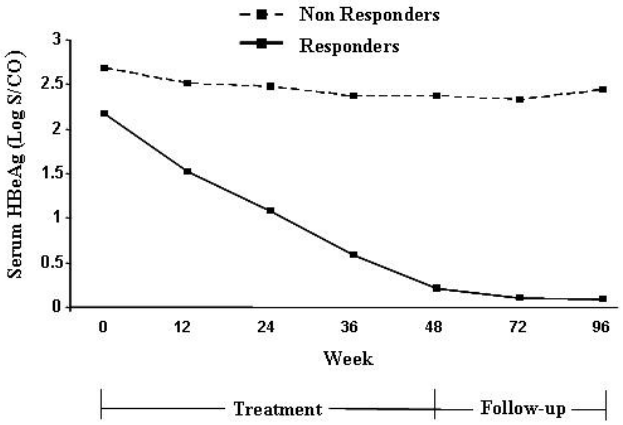

Fig. 11 Kinetics of serum HBeAg during PEG-IFN therapy and follow-up in responders (solid line) and non-responders (dashed line).

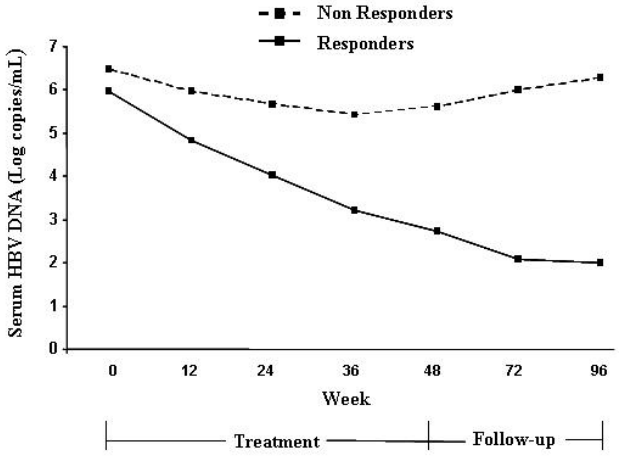

Fig. 12 Kinetics of serum HBV DNA during PEG-IFN therapy and follow-up in responders (solid line) and non-responders (dashed line).

At baseline, $\log_{10}$ HBeAg provided a prediction of SVR comparable to $\log_{10}$ HBsAg but better than $\log_{10}$ HBV DNA. The area under ROC of $\log_{10}$ HBeAg for SVR was 0.79 (95% confidence interval, 0.62-0.95; *P*=0.012), whereas the areas under ROC of $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA were 0.75 (95% CI, 0.57-0.92; *P*=0.029) and 0.64 (95% CI, 0.44-0.84; *P*=0.210), respectively.

At week 12 during therapy, $\log_{10}$ HBeAg provided a better prediction of SVR than $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA. The area under ROC of $\log_{10}$ HBeAg for SVR was 0.83 (95% CI,

0.68-0.98; *P*=0.004), whereas the areas under ROC of $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA were 0.73 (95% CI, 0.55-0.91; *P*=0.043) and 0.73 (95% CI, 0.53-0.92; *P*=0.041), respectively.

Similarly, quantitative HBeAg provided the best prediction of SVR at week 24. The area under ROC of $\log_{10}$ HBeAg for SVR was 0.91 (95% CI, 0.79-1.01; *P*=0.001), whereas the areas under ROC of $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA were 0.78 (95% CI, 0.62-0.95; *P*=0.013) and 0.80 (95% CI, 0.62-0.98; *P*=0.009), respectively.

Based on ROC curve analysis, a cut-off point for serum HBsAg, HBeAg and HBV DNA levels considered most accurate for predicting SVR was determined. At week 24 of treatment, HBeAg level below 2.0 $\log_{10}$ S/CO (100 S/CO) had a sensitivity of 85%, and NPV of 92.3% to predict SVR. At the same time point, HBV DNA concentrations below 4.6 $\log_{10}$ copies/mL (approximately 40,000 copies/mL) had sensitivity and NPV of 75% and 85.3%, respectively, while the sensitivity and NPV of HBsAg levels below 3.2 $\log_{10}$ IU/mL (approximately 1,600 IU/mL) were 65% and 82.1%, respectively (Table 15).

Table 15 HBsAg, HBeAg and HBV DNA levels to predict virological response at week 96

	Sensitivity	Specificity	PPV	NPV	Accuracy
Week 12					
Log_{10} HBsAg < 3.4 IU/mL	65.0	70.0	52.0	80.0	68.3
Log_{10} HBeAg < 2.4 S/CO	75.0	80.0	65.2	86.5	78.3
Log_{10} HBV DNA <5.0 copies/mL	70.0	70.0	53.9	82.4	70.0
Week 24					
Log_{10} HBsAg < 3.2 IU/mL	65.0	80.0	61.9	82.1	75.0
Log_{10} HBeAg < 2.0 S/CO	85.0	90.0	80.9	92.3	88.3
Log_{10} HBV DNA <4.6 copies/mL	75.0	75.0	57.7	85.3	73.3

PPV: positive predictive value, NPV: negative predictive value

In this study, HBsAg clearance occurred in one patient (3.3%), who also achieved SVR. The kinetics of serum HBsAg of this patient compared with those who developed SVR without HBsAg clearance are shown in Figure 13. The patient who cleared HBsAg had a relatively low pretreatment HBsAg level and a more rapid decline of the protein level during therapy, particularly after the first 12 weeks of PEG-IFN therapy than those who did not clear HBsAg. In the patient who cleared HBsAg, the decrease in serum HBsAg at week 12, 24, 36, 48, 72 and 96 was 0.60, 1.58, 1.95, 2.35, 2.70 and 2.70 $\log_{10}$ IU/mL, respectively, while the mean decrease in

serum HBsAg was 0.41±0.38, 0.60±0.55, 0.70±0.57, 0.81±0.60, 0.87±0.65 and 1.03±0.59 $\log_{10}$ IU/mL, respectively in patients who did not clear HBsAg.

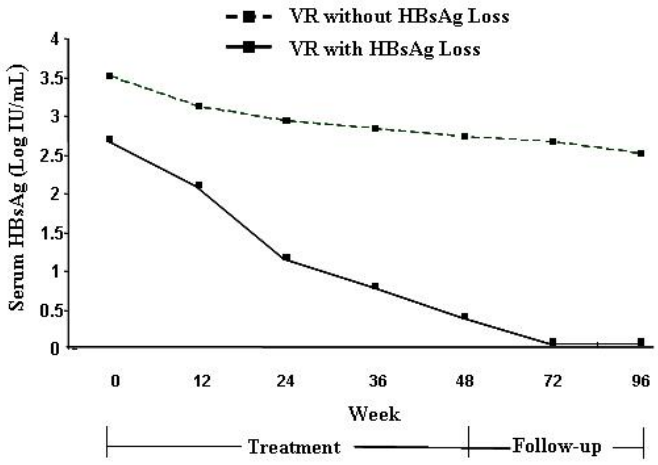

Fig. 13 Kinetics of serum HBsAg during PEG-IFN therapy and follow-up in a patient who achieved virological response (VR) and cleared HBsAg (solid line) and those who developed VR without HBsAg clearance (dashed line).

Although the elimination of HBsAg is the ultimate goal in the management of chronic hepatitis B, it is uncommon in clinical practice, accounting for less than 5% of cases associated with PEG-IFN therapy.[132] In this study, HBsAg clearance was achieved in approximately 3% of patients. Another more realistic endpoint in HBeAg-positive patients is HBeAg seroconversion, which has been considered a surrogate marker for long-term therapeutic response and improved clinical outcome of the patients.[132] In recent meta-analysis studies, patients treated with IFN-based therapies who achieved HBeAg seroconversion had significantly decreased HBV-related complications, including the development of cirrhosis and HCC.[133, 134] In this study, the rate of HBeAg seroconversion (approximately 33%) was comparable to previous reports involving a larger number of patients treated with PEG-IFN alone or in combination with lamivudine.[129, 130]

In current treatment recommendations, monitoring serum HBV DNA levels at baseline and during therapy has been practically considered to predict the response to IFN-based therapies.[135] However, measurement of serum HBV DNA level may not always accurately reflect the state of disease in the liver. In recent years, the development of methods for quantification of intrahepatic HBV replicative forms has provided additional molecular markers to predict antiviral treatment response.[15, 17, 136] It has recently been shown that cccDNA and total intrahepatic HBV

DNA levels, which represent the active replicative and transcriptional form of the virus, are superior to serum HBV DNA as predictors of SVR to antiviral therapy in patients receiving either lamivudine monotherapy or combination therapy of lamivudine and PEG-IFN.[137] Nonetheless, a major drawback in measuring cccDNA is the requirement for liver tissue. To avoid this obstacle, the validation of non-invasive surrogate markers such as the quantification of serum HBsAg and HBeAg is required in the clinical setting of antiviral therapy.[138]

In this study, we showed that baseline serum HBV DNA levels did not correlate with the amount of intrahepatic viral DNA and were not associated with SVR to PEG-IFN therapy. In contrast, baseline HBsAg and HBeAg levels were significantly lower in responders than in patients who did not show HBeAg seroconversion. These observations are not surprising as both serum HBsAg and HBeAg levels prior to treatment were well correlated with intrahepatic HBV replicative forms. Indeed, low levels of cccDNA and total intrahepatic HBV DNA at baseline represented good predictors of SVR, indicating that patients who will most likely benefit from the treatment are those who tend to have lower virus levels in the liver.[137, 139] Our findings are in agreement with previous data in that low baseline HBsAg is more reliable than serum HBV DNA levels for predicting good response to PEG-IFN and lamivudine treatment in HBeAg-positive patients.[128] In HBeAg-negative patients, low pretreatment HBsAg level was considered the only significant prognostic predictor of HBsAg seroconversion by multivariate analysis following conventional IFN treatment.[127] Likewise, a recent large study of PEG-IFN-α2a therapy demonstrated that HBeAg seroconversion was significantly associated with pretreatment HBeAg concentrations; as pretreatment level of HBeAg increased, the rate of subsequent HBeAg seroconversion diminished.[140]

It has been demonstrated that dynamic monitoring of quantitative HBeAg may be useful to predict a response to conventional IFN therapy.[141-143] Our data showed that, among responders, more rapid and consistent decline of HBeAg levels was observed during therapy and follow-up period. We demonstrated that serial measurement of HBeAg levels was superior to HBV DNA and HBsAg levels in predicting the likelihood of subsequent HBeAg seroconversion. At week 24 of treatment, HBeAg levels exceeding 2.0 $\log_{10}$ S/CO (100 S/CO) had an NPV for predicting SVR of approximately 92%, which surpassed that obtained by quantitative HBsAg (82%) and HBV DNA (85%) analysis. This high NPV of serum HBeAg at week 24 could allow the selection of patients who should cease therapy to reduce unnecessarily prolonged exposure to costly and unpleasant side-effects of PEG-IFN. In fact, such a “stopping rule” of quantitative HBeAg at

week 24 of therapy has been well addressed by recent data using PEG-IFN-α2a therapy,[140] which may be analogous to the current recommendation for patients with chronic hepatitis C treated with PEG-IFN and ribavirin .

The reason why dynamic monitoring of HBeAg levels offered a better prediction of SVR than that of serum HBV DNA levels could be explained by the pattern of HBV DNA decline induced by PEG-IFN. Based on the results of a recent study of PEG-IFN-α2b monotherapy, a proportion of patients with treatment response had a late decline of HBV DNA up to week 32 of therapy.[144] In our study, 30% of patients with HBeAg seroconversion had a late decline of HBV DNA after week 24 of treatment. Thus, SVR in PEG-IFN monotherapy could not be predicted sufficiently on the basis of HBV DNA decline at an early stage of treatment. As a result, the measurement of HBeAg levels as early as week 12 or 24 of PEG-IFN monotherapy could provide a better predictor than HBV DNA monitoring. It should be noted that an early and significantly more pronounced decline of HBV DNA levels was observed in patients who received the combination therapy of lamivudine and PEG-IFN than in those treated with PEG-IFN alone.[144] Consequently, it was shown that an early decrease in HBV DNA could possibly be a predictor of treatment response to the combination therapy.[145]

Unlike HBV DNA, the reductions of HBsAg and HBeAg paralleled with the reduction of cccDNA, suggest that the decline of HBV proteins in serum reflect a diminished cccDNA pool in the liver. Indeed, serum HBsAg and HBeAg are produced by transcription and translation of the surface and precore/core genes of the HBV genome, respectively.[146] These data further confirm that quantitative HBsAg, as well as HBeAg titers, may be useful surrogate markers for cccDNA and may have clinical applicability for predicting SVR to PEG-IFN therapy.[128, 139] In this study, although monitoring HBsAg levels in the whole study population appeared to be less indicative for VR than HBeAg levels, the reduction of HBsAg in individuals could predict HBsAg clearance. Previous data have demonstrated that mathematical modeling of HBsAg decline can predict HBsAg clearance for both IFN-based and NA-based therapies.[127] Interestingly, a recent study suggested that a rapid decline in HBsAg levels was associated with a high likelihood of HBsAg clearance in HBeAg-negative patients who had been treated with PEG-IFN.[87] Moreover, an HBsAg level below 10 IU/mL at week 48 and a decline exceeding 1 $\log_{10}$ IU/mL during treatment were significantly associated with HBsAg clearance during follow-up in HBeAg-negative patients treated with PEG-IFN.[147] In this study, our data showed that the patient who cleared HBsAg at week 72 exhibited a more rapid decline in serum HBsAg during therapy

than those who developed SVR without HBsAg clearance. Thus, our results confirmed that a significant decline in HBsAg concentration during therapy might be used to predict HBsAg clearance during the follow-up period. These data also highlight the effects of PEG-IFN therapy in modulating host immune response, resulting in sustained response after treatment and eventual HBsAg clearance.

4.5 Prevalence and molecular characterization of occult and co-infection of HBV in HIV-infected patients

This study aims at evaluating the prevalence of HBV genotype, intergenotype recombination and genomic mutations in patients who are co-infected with HIV. We will detect HBV DNA, sequence the whole HBV genome and perform phylogenetic analysis in approximately 30-40 cases of HBV-HIV co-infection, and compare to those without HIV infection. Active investigation of this proposed study is ongoing in our laboratory.

5. Conclusion and Future Prospect

Regarding the molecular epidemiological studies, 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 virological 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 virological factors of HBV in predicting response to PEG-IFN therapy, our data suggest that pretreatment quantitative HBsAg and HBeAg determination, which reflect the levels of intrahepatic cccDNA and intrahepatic HBV DNA, may be useful for predicting the response to PEG-IFN therapy and should be recommended in regular clinical practice. Double BCP mutations and pre-S mutations may also be associated with a high rate of antiviral response to PEG-IFN treatment in HBeAg-positive and HBeAg-negative hepatitis, respectively. Thus, analysis of these viral factors at baseline may lead to a better selection of patients for optimal therapy. In addition, quantitative measurement of HBeAg during therapy may provide better prediction of treatment response than HBV DNA and HBsAg levels. Also, serial monitoring of HBsAg levels during therapy may help predict HBsAg clearance after cessation of treatment. However, further studies on larger sample sizes will be required to confirm these observations.

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Output

Publications from the project

8 published articles, 2 manuscripts and 1 abstract

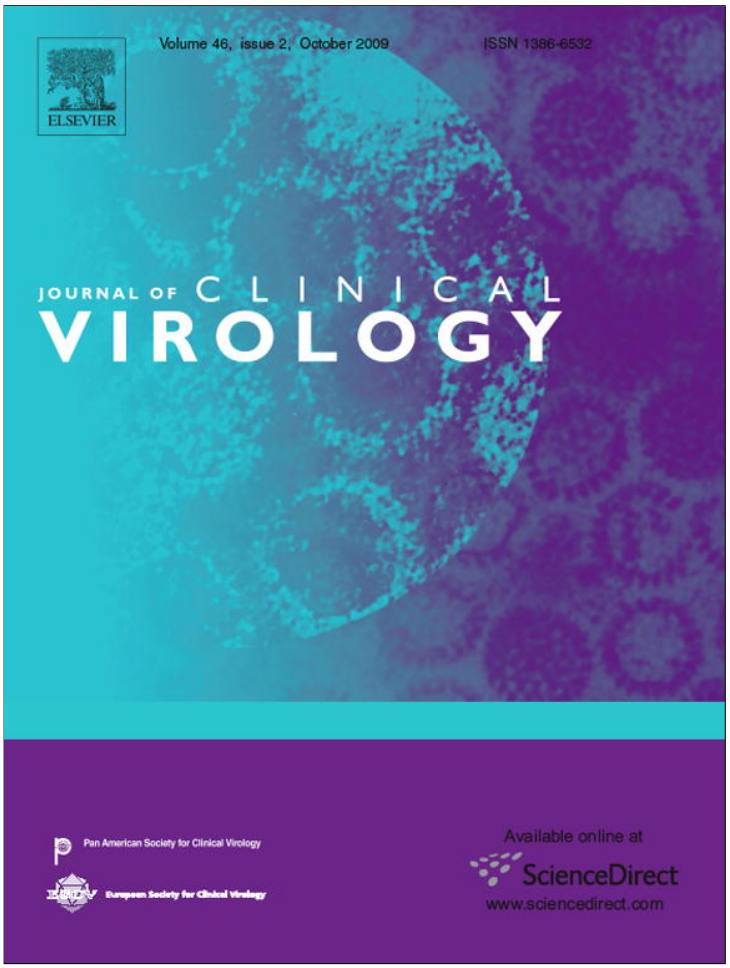
1. Low pretreatment serum HBsAg level and viral mutations as predictors of response to peg-interferon alpha-2b therapy in chronic hepatitis B. J Clin Virol 2009; 46: 117-23. (Impact factor; IF=3.320)
2. Comparison between quantitative HBsAg, HBeAg and HBV DNA levels for predicting virological response to peg-interferon alpha-2b therapy in HBeAg-positive chronic hepatitis B. Hepatol Res 2009 (in press). (IF=1.562)
3. The diagnostic role of serum glypican-3 in differentiating hepatocellular carcinoma from non-malignant chronic liver disease and other liver cancers. J Gastroenterol Hepatol 2009 (Epub ahead of print). (IF=2.275)
4. Prevalence, whole genome characterization and phylogenetic analysis of hepatitis B virus in captive orangutan and gibbon. J Med Primatol 2008; 37: 277-89. (IF=1.047)
5. Molecular epidemiological study of hepatitis B virus in Thailand based on the analysis of Pre-S and S genes. Hepatol Res 2008; 38: 244-51. (IF=1.562)
6. Dynamics of HBV DNA levels, HBV mutations and biochemical parameters during oral antiviral therapies. Asian Pac J Allergy Immunol 2007; 25: 183-8. (IF=0.569)
7. Serum LINE-1 hypomethylation level as a prognostic marker for hepatocellular carcinoma. Clin Chim Acta 2007; 379(1-2): 127-33. (IF=2.960)
8. Role of serum interleukin-18 as a prognostic factor in patients with hepatocellular carcinoma. World J Gastroenterol 2007; 13: 4345-9. (IF=2.081)
9. 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 (submitted)
10. Molecular epidemiological study of hepatitis B virus among migrant workers from Cambodia, Laos and Myanmar in Thailand (submitted)
11. High response to hepatitis B virus vaccination in HIV-1 infected subjects with isolated antibody to hepatitis B core antigen (abstract)

บทความสำหรับการเผยแพร่

ไวรัสตับอักเสบบีเป็นสาเหตุสำคัญของเกิดโรคตับอักเสบชนิดเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับ ในปัจจุบันมีประชากรทั่วโลกมากกว่า 400 ล้านคนที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง ซึ่งในแต่ละปีจะมีผู้เสียชีวิตเนื่องจากตับแข็งและมะเร็งตับไม่น้อยกว่า 1 ล้านคน ประเทศไทยเป็นประเทศที่มีความชุกของไวรัสตับอักเสบบีค่อนข้างสูงเช่นเดียวกับประเทศในภูมิภาคเอเชียตะวันออกเฉียงใต้ แม้ว่าอัตราการติดเชื้อในปัจจุบันจะมีแนวโน้มลดลงโดยเฉพาะในเด็กเนื่องจากความสำเร็จของการฉีดวัคซีนในการป้องกันโรคของประเทศ แต่อัตราการติดเชื้อในผู้ใหญ่ยังคงไม่ลดลงมากนัก ดังนั้นไวรัสตับอักเสบบีและโรคตับที่เกี่ยวข้องโดยเฉพาะมะเร็งตับจึงยังคงเป็นปัญหาสำคัญทางการแพทย์และสาธารณสุขของประเทศไทยในปัจจุบัน

ผู้ติดเชื้อไวรัสตับอักเสบบีแต่ละรายมีการดำเนินโรคแตกต่างกัน ทั้งนี้อาจขึ้นอยู่กับปัจจัยที่เกี่ยวข้องกับเชื้อไวรัสโดยที่มีส่วนทำให้ความรุนแรงของโรคในผู้ติดเชื้อแต่ละคนแตกต่างกัน องค์ความรู้ในด้านระบาดวิทยาระดับโมเลกุล อณูชีววิทยาของไวรัสตับอักเสบบี และข้อมูลที่เชื่อมโยงทางคลินิก จากการศึกษาวิจัยของโครงการเรื่อง "อณูไวรัสวิทยาและความสำคัญทางคลินิกของการติดเชื้อแบบแฝงและการติดเชื้อร่วมกับไวรัสอื่นของไวรัสตับอักเสบบี: บทบาทของซีซีซีดีเอนเอ สายพันธุ์และการกลายพันธุ์" ซึ่งได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัยเป็นเวลา 2 ปีตั้งแต่ 31 กรกฎาคม 2550 ถึงวันที่ 30 กรกฎาคม 2552 นี้ แสดงให้เห็นว่าเชื้อไวรัสสายพันธุ์ซีเป็นสายพันธุ์ที่พบบ่อยที่สุดในประเทศไทยและประเทศเพื่อนบ้าน ความแตกต่างของสายพันธุ์และการกลายพันธุ์เชื้อไวรัสในบางตำแหน่งมีผลต่อการดำเนินของโรคและอาจมีความสัมพันธ์กับการตอบสนองต่อการรักษาด้วยยาต้านไวรัส นอกจากนี้การกลายพันธุ์ของเชื้อไวรัสในบางตำแหน่งอาจเกี่ยวข้องกับการกลไกการเกิดโรคมะเร็งตับ

ผลของการศึกษาวิจัยจากโครงการดังกล่าวข้างต้นนี้นอกจากจะเป็นประโยชน์อย่างยิ่งทั้งในด้านระบาดวิทยาของประชากรไทย โดยใช้เป็นข้อมูลเปรียบเทียบกับกลุ่มประชากรต่างๆทั่วโลกแล้ว ยังก่อให้เกิดองค์ความรู้ใหม่ๆที่เกี่ยวกับกลไกการเกิดโรค การพยากรณ์ความรุนแรงของโรคและการรักษาผู้ป่วยตับอักเสบเรื้อรังที่เกิดจากการติดเชื้อไวรัสตับอักเสบบี รวมทั้งจะเป็นประโยชน์ในการป้องกันและรักษาโรคมะเร็งตับต่อไปในอนาคต



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Low pretreatment serum HBsAg level and viral mutations as predictors of response to PEG-interferon alpha-2b therapy in chronic hepatitis B☆

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ABSTRACT

Background: Viral genomic mutations have become increasingly recognized as being associated with the outcome of chronic HBV infection. However, the role of viral mutations as a predictor of response to pegylated-interferon (PEG-IFN) therapy has so far remained unclear.
Study design: Viral mutations in the enhancer II/basal core promoter (BCP)/precore and the pre-S regions were characterized by direct sequencing in pretreatment serum samples of 50 patients with chronic hepatitis B (33 HBeAg-positive and 17 HBeAg-negative), who were treated for 48 weeks with PEG-IFN alpha-2b.
Results: Sustained virological response at 48 weeks post treatment, defined as HBeAg seroconversion and HBV DNA < 2000 IU/mL for HBeAg-positive patients, and HBV DNA < 200 IU/mL for HBeAg-negative patients, was achieved in 12 (36.4%) and 6 (35.3%) of HBeAg-positive and HBeAg-negative patients, respectively. Response to PEG-IFN therapy correlated to low pretreatment HBsAg level but did not correlate with HBV genotype, pretreatment alanine transaminase and HBV DNA levels. In HBeAg-positive hepatitis, PEG-IFN response correlated with the appearance of double BCP mutations (A1762T/G1764A) at baseline ($P = 0.041$). In the HBeAg-negative group, response to PEG-IFN therapy was associated with the presence of pre-S mutation/deletions ($P = 0.028$). Multivariate analysis identified low pretreatment HBsAg level as an independent factor associated with SVR in both groups.
Conclusions: Pretreatment quantitative HBsAg determination is useful for predicting response to PEG-IFN therapy. 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 hepatitis, respectively.

1. Introduction

Chronic hepatitis B virus (HBV) infection and its sequelae are major global health problems.[1] The currently approved agents for chronic hepatitis B are nucleoside/nucleotide analogues and interferon alfa (IFN)-based therapies.[2] IFN acts mainly as an immunomodulator and augments the cell-mediated immune response in the process of clearing the virus. The efficacy of conventional IFN therapy has been improved by using its pegylated form (peginterferon, PEG-IFN), resulting in higher response rates in patients with chronic hepatitis B. However, the overall sustained response rate to PEG-IFN can be achieved in only approximately one-third of patients.[3] Considering the fact that PEG-IFN treatment is expensive and could have some adverse effects, it is crucial to identify those patients for whom PEG-IFN treatment will be beneficial.

The effect of HBV mutations on the outcome of PEG-IFN therapy is largely unknown. The most well-known HBV variants include a precore (PC) stop codon mutation (G1896A), which abolishes hepatitis B e antigen (HBeAg) production, and double mutations in the basal core promoter (BCP) region (A1762T/G1764A), which downregulate HBeAg production. These mutations have been reported to influence the response to conventional IFN treatment.[4,5] 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.[6,7] Furthermore, naturally occurring mutations or deletions in the pre-S1/pre-S2 gene of HBV genome

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have been shown to frequently accumulate in chronically infected patients.[8,9] Whether these variants affect the response to PEG-IFN has been poorly investigated. Thus, the aim of this study was to determine pretreatment clinical and molecular virologic factors associated with response to PEG-IFN therapy in patients with chronic hepatitis B.

2. Materials and methods

2.1. Patients

Fifty patients with chronic hepatitis B (39 men and 11 women) who had completed the treatment with PEG-IFN alpha-2b (PEG-IFN-α2b; Shering-Plough, Kenillworth, NJ) and had been followed-up between August 2005 and January 2008 at King Chulalongkorn Memorial Hospital, Bangkok, Thailand were retrospectively investigated. The patients were aged between 22 and 61 years and fulfilled the following criteria: (1) sero-positive for hepatitis B surface antigen (HBsAg), elevation of serum alanine aminotrasferase (ALT) for at least 6 months and detectable serum HBV DNA; (2) no evidence of hepatocellular carcinoma (HCC) based on the clinical criteria and ultrasound examination at baseline; (3) no evidence of hepatitis C virus (HCV), hepatitis D virus (HDV) and human immunodeficiency virus (HIV) infection. Liver biopsy was performed within 6 months of entry. The liver histology was graded by the histological activity index (HAI) according to the criteria of Knodell et al.[10], which comprise two major components namely necroinflammation (HAI-I) and fibrosis (HAI-F). In this study, 33 and 17 patients were classified as HBeAg-positive and HBeAg-negative chronic hepatitis, respectively. Among these patients, 3 cases in the HBeAg-negative group were histological diagnosed of cirrhosis.

PEG-IFN-α2b was administered subcutaneously at a dose of 1.5 μg/kg weekly for 48 weeks. All patients were followed-up for up to 48 weeks after treatment (week 96) to assess sustained virological response (SVR). In the HBeAg-positive group, SVR was defined as HBeAg seroconversion and sustained inhibition of viral replication (HBV DNA level < 2000 IU/mL) until 12 months post treatment. In the HBeAg-negative group, SVR was defined as sustained inhibition of viral replication (HBV DNA level < 200 IU/mL) until 12 months post treatment.[11] Patients without SVR were defined as non-responders. According to these criteria, 12 (36.4%) and 6 (35.3%) of the HBeAg-positive and HBeAg-negative groups, respectively, were classified as responders.

Serum samples were collected from each patient at entry and stored at −70 °C until further tests were performed. All patients had been informed as to the study's purpose and had given their written consent. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

2.2. HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 100 μL serum sample by incubation in lysis buffer (10 mM Tris–HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/mL proteinase K) and phenol-chloroform-isoamyl alcohol extraction. The DNA pellet was resuspended in 30 μL sterile distilled water and subjected to amplification of the pre-S {nucleotides (nt) 2814–475} and EnhII/BCP/PC (nt 1552–2053) regions by polymerase chain reaction (PCR). The reaction mixture comprised 2 μL resuspended DNA, 0.5 μL of 25 mmol of each primer (pre-S region: Pre-S1F+ 5′-GG GTCACCATATTCTTGGGAAC-3′ and R3 5′-ACAAACGGGCAACATACCTTG-3′; EnhII/BCP/PC: X101 5′-TCTGTGCCTTCTCATCTG-3′ and CO2 5′-GTGAG GTGAACAATGTTCCG-3′), 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™ BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with CLUSTAL_X (version 1.83) program.

The obtained sequences were submitted to GenBank under accession numbers EU841367–EU841816 and EU850826–EU850874. The sequences were further characterized for the genotyping assay by phylogenetic analysis as previously described.[12]

2.3. Biochemical, serological and virological assays

Serum ALT level was measured with a commercial assay using an automated analyzer (Hitachi 912). Quantification of hepatitis B s antigen (HBsAg) was tested in pretreatment serum samples by ARCHITECT *i*2000SR (Abbott Diagnostic, Wiesbaden, Germany) according to the manufacturer's specifications.[13] The detection of the assay ranged from 0.05 to 250 IU/mL. Samples with HBsAg titers beyond the upper range were diluted with phosphate buffered saline (PBS) into 1:10, 1:1000 and 1:10,000 prior to further analysis. HBeAg and anti-HBe antibodies were determined using commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). Serum HBV DNA level was quantified using a commercially available kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection of the assay ranged from 60 to 4×10^6 IU/mL

2.4. Statistical analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney *U*-test or Student's *t*-test for quantitative variables. Multivariate analysis using stepwise logistic regression was performed to identify independent factors associated with PEG-IFN response. *P*-values < 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 14.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

Demographic and baseline clinical characteristics of the patients are shown in Table 1. HBeAg-negative patients had significantly higher mean age, but lower baseline HBV DNA and HBsAg levels than patients with HBeAg-positive hepatitis. No significant difference between groups was observed in respect to sex, pretreatment ALT level, necroinflammatory and fibrosis scores.

3.2. HBV genotype

Based on sequencing and phylogenetic analysis of the pre-S region, serum samples from 6 (12%) patients (all in the HBeAg-positive group) belonged to genotype B, and those of 44 (88%) (27 in the HBeAg-positive group) to genotype C (Fig. 1). Based on the

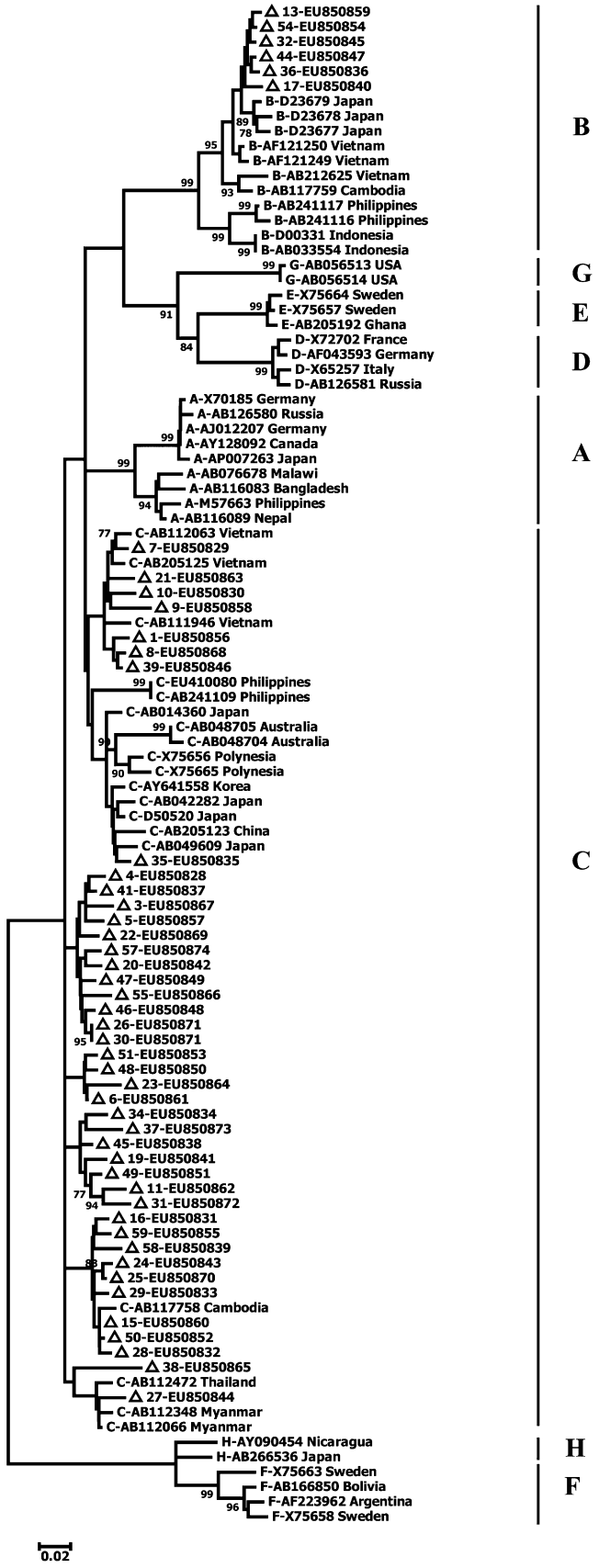

Fig. 1. Phylogenetic tree analysis of pre-S region obtained from sample sequences used in this study (EU841367–EU841816 and EU850826–EU850874).

Table 1
Demographic and clinical characteristics of HBeAg-positive and HBeAg-negative patients.

	HBeAg-positive ($n=33$)	HBeAg-negative ($n=17$)	P
Age, yr	37.4 ± 9.1	43.3 ± 8.0	0.023
Sex, male	26 (78.8%)	13 (76.5%)	1
Pretreatment ALT level, U/L	92.6 ± 84.4	102.6 ± 56.1	0.62
Pretreatment HBV DNA, $\log_{10}$ IU/mL	6.2 ± 0.8	5.7 ± 0.8	0.04
Pretreatment HBsAg level, IU/mL	14189.03 ± 19445.4	2668.5 ± 5037.4	0.003
Pretreatment HBsAg level, $\log_{10}$ IU/mL	3.9 ± 0.6	2.9 ± 0.8	<0.001
Necroinflammatory score	4.0 ± 1.6	4.3 ± 1.8	0.62
Fibrosis score	1.3 ± 0.9	2.0 ± 1.4	0.11
Genotype			0.061
B	6 (18.2%)	0 (0%)	
C	27 (81.8%)	17 (100%)	
C1653T	1 (3.0%)	4 (23.5%)	0.04
T1753C/deletion	6 (18.2%)	10 (58.8%)	0.003
Double BCP mutation			0.004
A1762T/G1764A	13 (39.4%)	15 (88.2%)	
Deletion	4 (12.1%)	1 (5.9%)	
G1896A	3 (9.1%)	7 (41.2%)	0.021
Pre-S mutation			0.1
Pre-S2 start codon	2 (6.1%)	3 (17.6%)	
Deletion	1 (3.0%)	2 (11.8%)	
Antiviral response	12 (36.4%)	6 (35.3%)	1

preC/C region results, all 50 cases belonged to genotype C and were determined as subgenotype Cs. Thus, all HBV strains of genotype B in this study were subgenotype B2, which is a recombinant with the preC/C region of genotype C. There was no significant difference in HBV genotype distribution between the HBeAg-positive and HBeAg-negative groups (Table 1).

3.3. HBV mutations in the EnhII/BCP/PC regions

Mutational hot spots in these regions were found at nt 1753, 1762, 1764 and 1896. The C1653T and T1753C mutations were observed in 5 (10%) and 16 (32%) cases, respectively. The A1762T/G1764A mutations were found in 28 (56%) cases. In addition, five patients showed the following deletions at or around nt 1762–1764. One patient had deletions at nt 1762–1764 and 1773–1775, while another had deletions at nt 1762–1764 and 1771–1776. One additional patient had deletions at nt 1751–1764 and 1771–1777, while another one had a deletion at nt 1756–1770. All these cases with deletions belonged to the HBeAg-positive group. Furthermore, another patient in the HBeAg-negative group had a deletion at nt 1755–1763 and G1764A mutation (Fig. 2A and B). Comparison between groups showed that the frequencies of C1653T, T1753C, double BCP and PC mutations were significantly higher in HBeAg-negative patients (Table 1). In HBeAg-positive group, patients harboring double BCP mutations exhibited HBV DNA levels comparable to those with wild-type populations (6.8 ± 0.6 and 7.1 ± 0.9 $\log_{10}$ copies/mL, respectively, $P = 0.482$). Patients with HBV genotype C had higher frequencies of double BCP and T1753C mutations than those with HBV genotype B ($P < 0.001$ and $P = 0.023$, respectively). However, the frequencies of PC and C1653T mutations were not significantly different between genotypes B and C.

3.4. HBV mutations in the pre-S region

Pre-S mutations were detected in 8 of 50 (16%) samples. All these samples belonged to HBV genotype C, and were exclusively detected in viral populations harboring double BCP mutations.

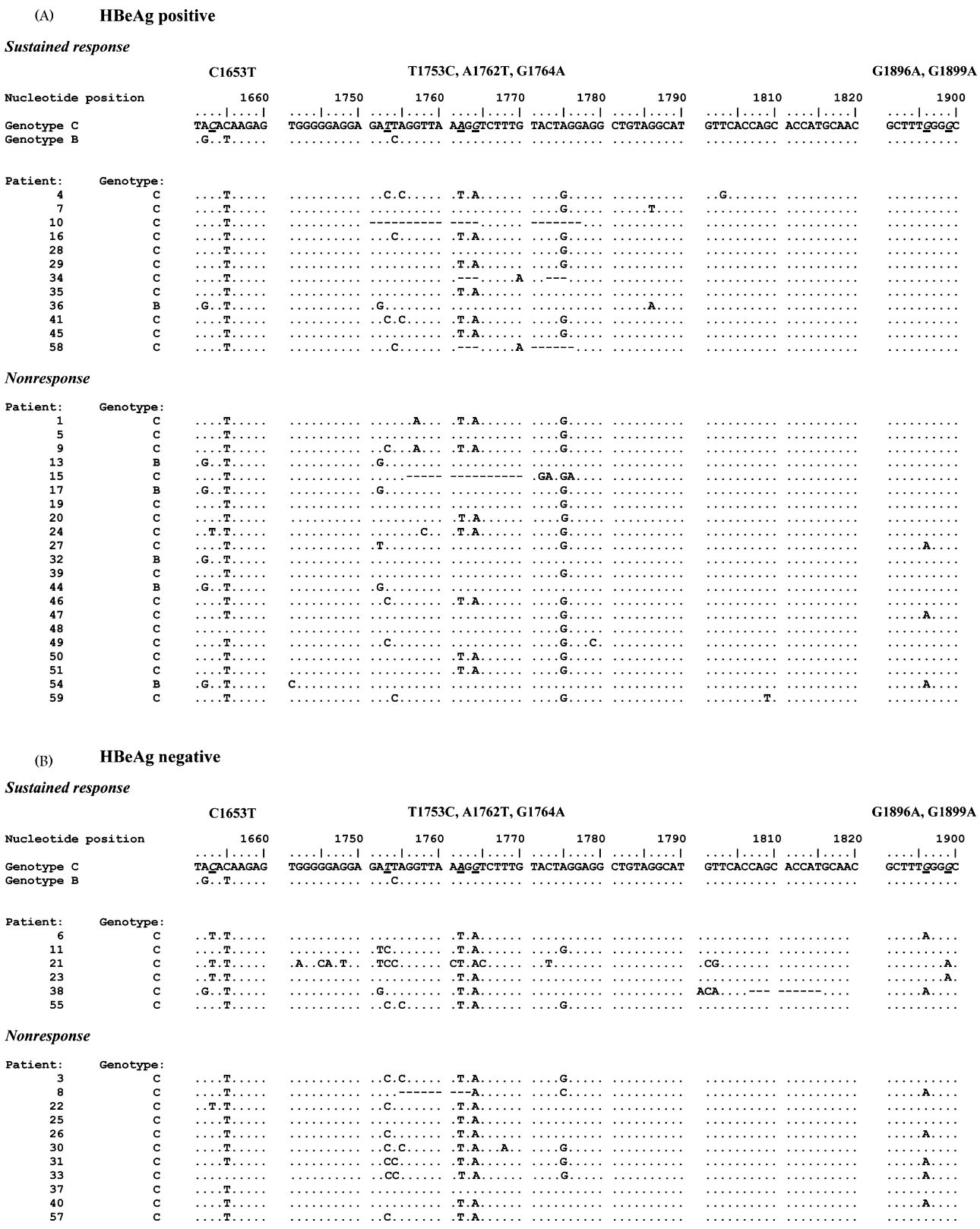

Fig. 2. The nucleotide sequences alignment of parts of the enhancer II/basal core promoter/precore regions. (A) HBeAg-positive patients and (B) HBeAg-negative patients. Nucleotide deletions are marked by a *hyphen* (-). Consensus sequence was established from HBV databank sequences.

Among these, pre-S2 start codon mutation was the most common (10%), followed by pre-S2 deletion (4%) and pre-S1 deletion (2%) (Fig. 3A and B). The prevalence of pre-S mutations was higher in the HBeAg-negative group but no significant difference was found (Table 1).

3.5. Factors associated with sustained response

In the HBeAg-positive group, low pretreatment HBsAg level and the presence of double BCP mutations at entry were found to be significant predictors of response to PEG-IFN therapy in

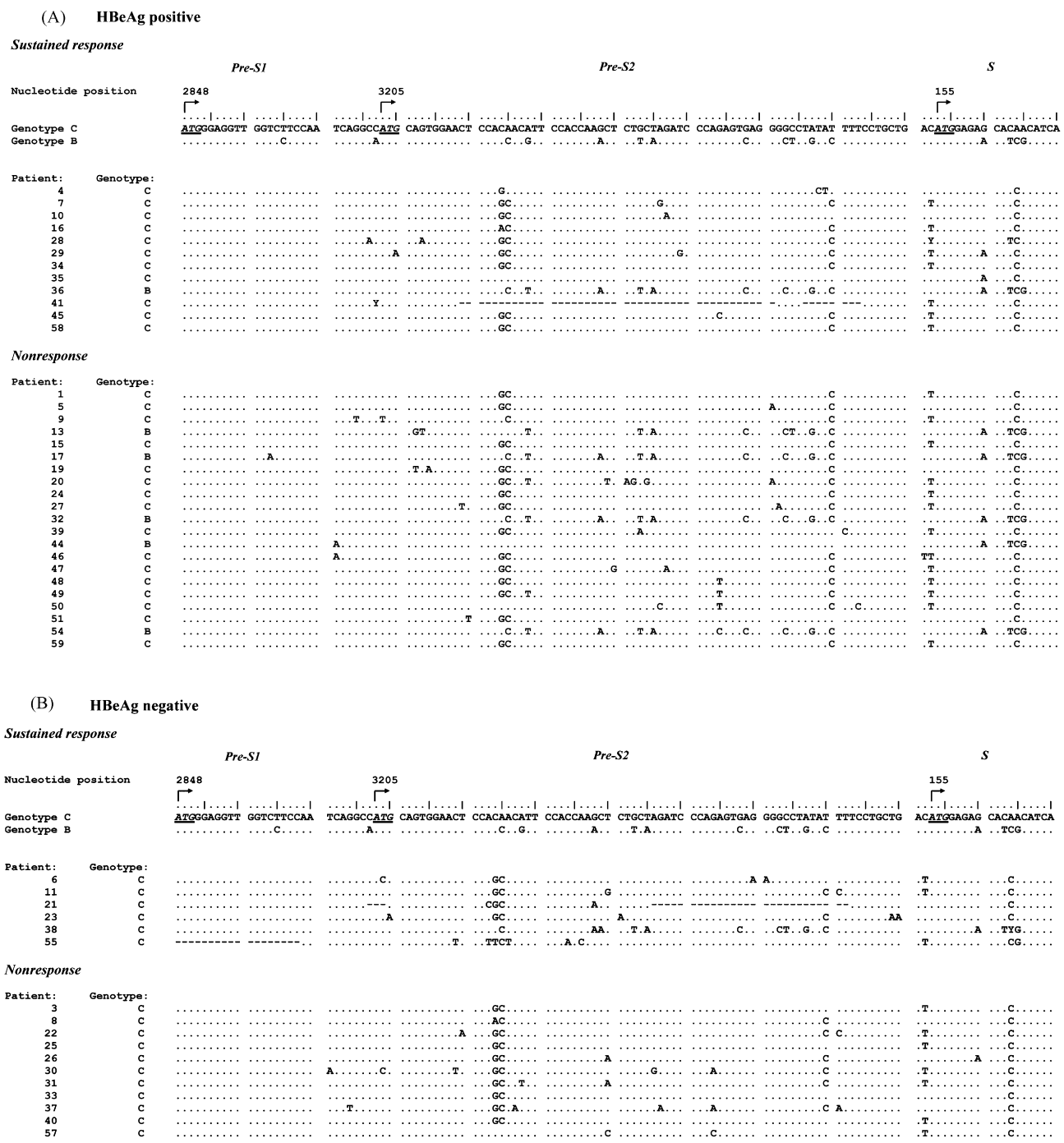

Fig. 3. The nucleotide sequences alignment of parts of the pre-S1/pre-S2 regions. (A) HBeAg-positive patients and (B) HBeAg-negative patients. Nucleotide deletions are marked by a *hyphen* (-). Consensus sequence was established from HBV databank sequences.

univariate analysis (Table 2). There was no correlation between the presence of C1653T, T1753C, G1896A, pre-S mutations and response to PEG-IFN treatment in this group. In the HBeAg-negative group, low pretreatment HBsAg level and presence of pre-S mutation/deletions at entry were found to be significant predictors of response (Table 3). However, there was no correlation between the presence of C1653T, T1753C, A1762T/G1764A, G1896A, and response to PEG-IFN treatment in this group. Multivariate analysis with stepwise logistic regression identified low pretreatment HBsAg level as an independent factor associated with sustained response in HBeAg-positive (odds ratio (OR), 14.67; 95% confidence interval (CI), 1.59–135.30; $P = 0.018$), and HBeAg-negative hepatitis (OR, 22.50; 95% CI, 1.61–314.56; $P = 0.021$).

4. Discussion

The results of this study showed that pretreatment C1653T and T1753C mutants were not associated with adverse response to PEG-IFN therapy. However, the presence of A1762T/G1764A at entry was associated with a higher rate of PEG-IFN response in HBeAg-positive patients. This is consistent with previous reports that HBeAg-positive patients harboring double BCP mutants responded better to conventional IFN therapy.[14–17] HBeAg, known to induce immunotolerance[18], 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.[19] As a consequence, lower level of HBeAg could

Table 2
Baseline characteristics of HBeAg-positive patients with and without response to PEG-IFN therapy.

	Responder ($n = 12$)	Non-responder ($n = 21$)	P
Age, yr	38.9 ± 10.8	36.5 ± 8.2	0.51
Sex, male	8 (66.7%)	18 (85.7%)	0.19
Pretreatment ALT level, U/L	89.6 ± 52.2	94.2 ± 99.5	0.86
Pretreatment HBV DNA, $\log_{10}$ IU/mL	6.0 ± 0.5	6.4 ± 0.9	0.19
Pretreatment HBsAg level, IU/mL	4703.7 ± 3859.5	19,609.2 ± 22,626.9	0.007
Pretreatment HBsAg level, $\log_{10}$ IU/mL	3.5 ± 0.4	4.1 ± 0.6	0.005
Necroinflammatory score	3.6 ± 1.4	4.3 ± 1.7	0.23
Fibrosis score	1.3 ± 0.9	1.3 ± 1.0	0.81
Genotype			0.38
B	1 (8.3%)	5 (23.8%)	
C	11 (91.7%)	16 (76.2%)	
C1653T	0 (0%)	1 (4.8%)	1
T1753C/deletion	3 (25%)	3 (14.3%)	0.64
Double BCP mutation/deletion	9 (75%)	8 (38.1%)	0.041
G1896A	0 (0%)	3 (14.3%)	0.28
Pre-S mutation/deletion	2 (24%)	1 (4.8%)	0.54

Table 3
Baseline characteristics of HBeAg-negative patients with and without response to PEG-IFN therapy.

	Responder ($n = 6$)	Non-responder ($n = 11$)	P
Age, yr	47.0 ± 5.8	41.3 ± 8.5	0.12
Sex, male	5 (83.3%)	8 (72.7%)	1
Pretreatment ALT level, U/L	89.5 ± 44.1	109.7 ± 62.5	0.45
Pretreatment HBV DNA, $\log_{10}$ IU/mL	5.9 ± 0.8	5.6 ± 0.9	0.56
Pretreatment HBsAg level, IU/mL	346.4 ± 410.2	3935.1 ± 5959.6	0.014
Pretreatment HBsAg level, $\log_{10}$ IU/mL	2.3 ± 0.6	3.2 ± 0.6	0.008
Necroinflammatory score	5.3 ± 2.6	3.9 ± 1.4	0.39
Fibrosis score	3.0 ± 1.4	1.6 ± 1.3	0.15
Genotype			1
B	0 (0%)	0 (0%)	–
C	6 (100%)	11(100%)	–
C1653T	3 (50%)	1 (9.1%)	0.09
T1753C/deletion	3 (50%)	7 (63.6%)	0.61
Double BCP mutation/deletion	6 (100%)	10 (90.9%)	1
G1896A	2 (33.3%)	5 (45.5%)	1
Pre-S mutation/deletion	4 (66.7%)	1 (9.1%)	0.028

contribute to a decrease in immunotolerance and enhance host immune response, which in turn lead to viral clearance following PEG-IFN treatment. In contrast, we could not find any correlation between the presence of PC mutant and PEG-IFN responsiveness. Indeed, there is no conclusive evidence that PC mutant has an impact on the outcome of IFN-based therapy.[14,15,20,21]

Although the role of pre-S mutants on the clinical course of chronic HBV infection has been recognized,[22] the role of these variants associated with PEG-IFN responsiveness has never been investigated. In HBeAg-negative patients, our data showed that pre-S mutation/deletions were observed more frequently in sustained responders, suggesting such variants may be associated with a higher response rate to PEG-IFN therapy. Although the reason is unclear, it is speculated that most of the mutation/deletion regions encompassed epitopes for T and B cells[23], which could lead to an alteration of the immune response and result in different outcome following PEG-IFN therapy. Interestingly, pre-S mutation/deletions were exclusively found in populations harboring double BCP mutations. Since both BCP and pre-S deletion mutants develop in the quasispecies during the course of persistent HBV infection, it is possible that the occurrence of these mutations are as a consequence of disease progression and may represent biomarkers for predicting the development of advanced liver disease.[8,24]

In this study, a low level of serum HBV DNA before treatment was not associated with response to PEG-IFN therapy. In contrast, low level of pretreatment HBsAg was consistently a predictor of the antiviral response in both HBeAg-positive and HBeAg-negative groups. This finding is in agreement with previous data that low pretreatment HBsAg levels have been considered as a predictor of HBsAg seroconversion following conventional IFN treatment.[25] It has been shown that low pretreatment HBsAg is more reliable than serum HBV DNA levels in predicting good response to PEG-IFN and lamivudine treatment.[26] Moreover, circulating HBsAg levels are strongly correlated with intrahepatic covalently closed circular DNA (cccDNA),[27] and cccDNA is superior to serum HBV DNA as a predictor of sustained response to antiviral therapy.[27,28] Thus, the present study provides additional evidence that quantitative HBsAg determination should be considered before administering IFN-based therapies.

The association between HBV genotypes and the response to PEG-IFN treatment remains controversial.[29,30] Our data showed that genotypes B and C were comparable in term of PEG IFN responsiveness. This finding could be in part attributed to the observation that all strains of genotype B were subgenotype Ba, which is a recombinant with the preC/C region of genotype C. Indeed, the epidemiologic study has demonstrated that all HBV strains in Thailand belong to subgenotype Ba.[31] Consequently, subgenotype Ba and genotype C, both of which share the sequences of the preC/C region, showed a similar response rate to PEG-IFN therapy. Whether there is any correlation between HBV genotypes/subgenotypes and the response to PEG-IFN therapy requires further investigation to address this interesting issue.

As this report has been one of the first studies to describe the molecular virological factors, particularly viral mutations and dele-

tions, that predict the response to PEG-IFN therapy, there have been some limitations. First, the number of patients included in this study was relatively small. Second, since HBV populations infecting patients are usually distributed as quasi-species,[32] 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 products 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. Nonetheless, such minor strains might be less predictive of the response to PEG-IFN therapy than when they present as the predominant viral populations.

In conclusion, our data suggest that pretreatment quantitative HBsAg determination may be useful for predicting the response to PEG-IFN therapy and should be recommended in regular clinical practice. Double BCP mutations and pre-S mutations may also be associated with a high rate of antiviral response to PEG-IFN treatment in HBeAg-positive and HBeAg-negative hepatitis, respectively. Thus, analysis of these viral factors at baseline may lead to a better selection of patients for optimal PEG-IFN therapy. Further studies with larger sample sizes are required to verify these observations.

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Original Article

Comparison between quantitative hepatitis B surface antigen, hepatitis B e-antigen and hepatitis B virus DNA levels for predicting virological response to pegylated interferon-α-2b therapy in hepatitis B e-antigen-positive chronic hepatitis B

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Aim: The aim of this study was to compare the clinical applicability of quantitative serum hepatitis B surface antigen (HBsAg), hepatitis B e-antigen (HBeAg) and hepatitis B virus (HBV) DNA for predicting virological response (VR) to pegylated interferon (PEG-IFN) therapy.

Methods: Thirty HBeAg-positive chronic hepatitis B patients who received PEG-IFN-α-2b for 48 weeks were enrolled. Quantitative HBsAg, HBeAg and HBV DNA were measured before, during and after the therapy. Paired liver biopsies were performed before and after treatment for covalently closed circular (ccc)DNA and intrahepatic HBV DNA analysis.

Results: VR at 48 weeks post-treatment, defined as HBeAg seroconversion and HBV DNA less than 10 000 copies/mL was achieved in 10 (33.3%) patients. Responders had significantly lower baseline HBsAg, HBeAg, cccDNA and intrahepatic HBV DNA levels than non-responders. Baseline and reduced levels of $\log_{10}$ HBsAg and $\log_{10}$ HBeAg correlated well with those of $\log_{10}$ cccDNA and $\log_{10}$ total intrahepatic HBV DNA. Responders showed consistent decrease in serum HBsAg, HBeAg and HBV DNA levels during therapy. HBeAg level of 2.0 $\log_{10}$ sample to cut-off ratio at week 24 on therapy provided the best prediction of sustained virological response, with sensitivity and negative predictive values of 85% and 92%, respectively. One patient (3.3%) who cleared HBsAg at follow up exhibited a more rapid decline in serum HBsAg during therapy than those who developed VR without HBsAg clearance.

Conclusion: Quantitative measurement of serum HBeAg during therapy may be superior to serum HBsAg and HBV DNA as a prediction of HBeAg seroconversion. Kinetics of HBsAg levels on therapy may help predict HBsAg clearance after treatment.

Key words: covalently closed circular DNA, hepatitis B e-antigen, hepatitis B surface antigen, hepatitis B virus DNA, quantification, pegylated interferon, virological response.

INTRODUCTION

CHRONIC HEPATITIS B virus (HBV) infection is a major global health problem.[1] Long-term follow-up studies have shown that sustained responders to antiviral therapy have a significant reduction in HBV-related complications including cirrhosis and hepatocellular carcinoma (HCC). The approved antiviral agents for chronic hepatitis B are nucleoside/nucleotide analogs (NA) and α-interferon (IFN-α)-based therapies.[2] IFN acts mainly as an immunomodulator 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.[2] Currently, pegylated interferon (PEG-IFN) has replaced its conventional form as it is more convenient and has better therapeutic efficacy. However, PEG-IFN therapy can result in only 30–40% hepatitis B e antigen (HBeAg) seroconversion, which is still far from satisfactory.[3]

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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 PEG-IFN.

Although quantification of serum HBV DNA remains the most widely used virological marker in the management of patients with chronic hepatitis B, its measurement is costly and may not always represent a reliable indicator of sustained response to antiviral therapy.[4] Increasing evidence has shown that quantitative assays for (HBsAg) and HBeAg may be valuable in identifying patients likely to respond to PEG-IFN therapy.[5–9] For example, baseline HBsAg titer, which is correlated with intrahepatic covalently closed circular (ccc)DNA, is considered a potential predictor of virological response (VR) to combined PEG-IFN and lamivudine treatment in HBeAg-positive hepatitis.[6] In addition, quantification of serum HBeAg before and during therapy may be a useful marker for predicting VR in HBeAg-positive patients treated with PEG-IFN.[7] These data provide essential clues on the importance of measuring HBsAg and HBeAg levels in HBeAg-positive hepatitis during treatment with PEG-IFN, which could offer a new paradigm for predicting treatment response and determining treatment cessation. However, direct comparison between quantitative measurements of HBsAg, HBeAg and HBV DNA for predicting VR to PEG-IFN therapy has never been performed. Thus, the aims of this study were to directly compare the clinical applicability of measuring quantitative HBsAg, HBeAg and HBV DNA before and during PEG-IFN therapy for prediction of VR in patients with HBeAg-positive chronic hepatitis B.

METHODS

Patients

THIRTY PATIENTS WITH HBeAg-positive chronic hepatitis B (23 men and seven women) who had completed the treatment with PEG-IFN-α-2b (Shering-Plough, Kenillworth, NJ, USA) and had been followed up between August 2005 and January 2008 at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, were retrospectively investigated. The patients were between 22 and 54 years of age and fulfilled the following criteria: seropositive for HBsAg and HBeAg, elevation of serum alanine aminotransferase (ALT) for at least 6 months and detectable serum HBV DNA. Paired liver biopsies were performed before and at the end of treatment for histology and intrahepatic viral DNA analysis. The liver histology was graded by the histological activity index (HAI) according to the criteria of Knodell *et al.*[10] which comprise two major components, namely, necroinflammation (HAI-I) and fibrosis (HAI-F).

Pegylated IFN-α-2b was administrated s.c. at a dose of 1.5 μg/kg weekly for 48 weeks. All patients were followed up for an additional 48 weeks after treatment (week 96) to assess VR. VR was defined as HBeAg seroconversion and sustained inhibition of viral replication (HBV DNA level <10 000 copies/mL) until 12 months post-treatment.[11]. Patients without VR were defined as non-responders. According to these criteria, 10 (33.3%) and 20 (66.7%) were classified as responders and non-responders, respectively. Among the responders, HBsAg clearance was achieved in one patient. Thus, the rate of HBsAg clearance in this study was 3.3%.

Serum samples were collected from each patient at baseline, during therapy (weeks 12, 24, 36 and 48) and during follow up (weeks 72 and 96) and stored at −70°C until further tests were performed. Pre- and post-treatment liver biopsy specimens were stored at −70°C until analysis. All patients had been informed as to the study's purpose and had given their written consent.

Serological and virological assays

Qualitative HBsAg, HBeAg and anti-HBe measurements were carried out using a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL, USA). The quantification of HBsAg was performed using the ARCHITECT *i*2000SR (Abbott Diagnostic, Chicago, IL, USA) according to the manufacturer's specifications.[12] The sensitivity of the assay ranged 0.05–250 IU/mL. Samples with HBsAg titers beyond the upper range were diluted with phosphate buffered saline (PBS) into 1:10, 1:1000 and 1:10 000 prior to further analysis.

The quantification of HBeAg was performed using the ARCHITECT *i*2000SR (Abbott Diagnostic), a two-step immunoassay based on the use of chemiluminescence microparticles (CIMA), according to the manufacturer's instructions.[13] Briefly, undiluted samples were mixed with paramagnetic beads coated with anti-HBe antibodies. HBeAg in the sample then attached to the magnetic beads through the presenting antibodies. After a washing step, a conjugate and reactant were added leading to the emission of light, which was proportional to the determined HBeAg concentration. The assay was calculated based on the ratio of the sample relative light unit (RLU) to the cut-off RLU (S/CO) for each specimen. Samples with S/CO values more than 1.0 were considered positive results for HBeAg.

Hepatitis B virus DNA was extracted from 100 µL serum with proteinase K in lysis buffer followed by phenol/chloroform/isoamyl alcohol extraction. The DNA pellet was dissolved in 30 µL sterile distilled water. Of the resuspended HBV DNA solution, 1 µL was subjected to quantification of HBV DNA by real-time polymerase chain reaction (PCR) using SYBR Green I fluorescent dye as previously described.[14] The HBV genotypes were determined by direct sequencing on the surface gene, as previously described.[14]

Quantification of cccDNA and intrahepatic HBV DNA

Approximately 5–10 mg of liver biopsy specimens taken at baseline and end of treatment were studied. Liver tissue was incubated overnight in lysis buffer with proteinase K followed by phenol/chloroform/iso-amyl alcohol extraction. Total intrahepatic HBV DNA was determined by real-time PCR using SYBR Green I fluorescent dye, applying the same conditions as specified for the measurement of serum HBV DNA.

Hepatitis B virus cccDNA was quantitatively determined as described previously, with some modifications.[15] Briefly, the reaction mixture comprised 1.0 µL of DNA sample, 6.0 µL 2.5X MasterMix solution, 0.3 µL of 25 mM magnesium solution (Mastermix; 5 PRIME, Hamburg, Germany), 0.75 µL of 25 µM forward primer, 0.75 µL of 25 µM reverse primer, 0.24 µL of 10X SYBR Green (QIAGEN, Hilden, Germany) and distilled water to a final volume of 15 µL. Real-time PCR amplification was carried out in a LightCyCler (Roach, Basel, Switzerland). After a pre-incubation step at 95°C for 10 min in order to activate the Taq polymerase, amplification was performed during 40 cycles including denaturation (94°C, 15 s), annealing (59°C, 30 s) and extension (72°C, 60 s). A single fluorescent signal was obtained once per cycle at 80°C after extension step.

To standardize the extracted DNA from liver tissue in term of copies per genome equivalent, the amount of the β-globin gene was measured. Primer sequences for the β-globin gene were describe previously.[16] PCR was performed in 10 µL reaction volumes containing 1 µL of DNA, 5 µL of 2.5X Mastermix solution, 0.1 µL of 25 µM forward primer, 0.1 µL of 25 µM reverse primer, 0.25 µL of 10X SYBR Green and distilled water. Amplification was performed for 3 min at 94°C, 40 cycles of 10 s at 95°C for denaturation, 15 s at 60°C for annealing and 20 s at 72°C for extension. The fluorescence intensity of the PCR products was measured at 78°C. A standard curve was created the same as the quantification of HBV DNA by using pGemT-Easy Vector inserted with the amplicon.

Ethical considerations

The study was in accordance with the principles of the 1975 Declaration of Helsinki and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. Comparisons between groups were analyzed by the c2 or Fisher's exact test for categorical variables and by the Mann–Whitney *U*-test or Student's *t*-test for quantitative variables. Pearson's correlation coefficient was tested for correlation between two variables. Area under the receiver–operator curve (ROC) was calculated to assess the predictive values of variables for VR. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated in accordance with standard methods. $P < 0.05$ were considered statistically significant. Data were analyzed using the SPSS software for Windows ver. 14.0.

RESULTS

Baseline characteristics

BASELINE CHARACTERISTICS OF the patients are shown in Table 1. Responders had significantly lower baseline $\log_{10}$ HBsAg, $\log_{10}$ HBeAg, $\log_{10}$ cccDNA and $\log_{10}$ total intrahepatic HBV DNA levels than non-responders. No significant difference between groups was observed in respect to age, sex, pretreatment ALT level, HBV genotype, necroinflammatory and fibrosis scores. Lower HBV DNA levels at baseline tended to predict VR; however, the difference between groups did not reach statistical significance.

Pretreatment $\log_{10}$ HBsAg showed positive correlation with both $\log_{10}$ HBeAg ($r = 0.516$, $P = 0.003$) and $\log_{10}$ (serum HBV DNA) ($r = 0.633$, $P < 0.001$), as well as with $\log_{10}$ cccDNA ($r = 0.517$, $P = 0.008$) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.635$, $P = 0.001$). $\log_{10}$ HBeAg displayed correlation with $\log_{10}$ (serum HBV DNA) ($r = 0.513$, $P = 0.004$) and $\log_{10}$ cccDNA ($r = 0.507$, $P = 0.010$), but not with $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.362$, $P = 0.076$). $\log_{10}$ (serum HBV DNA) was not significantly correlated with $\log_{10}$

Table 1 Characteristics of responders and non-responders at baseline

	Responder ($n = 10$)	Non-responder ($n = 20$)	P
Age, year	37.0 ± 9.4	36.7 ± 8.2	0.921
Sex, male	7 (70%)	16 (80%)	0.657
ALT level, U/L	96.6 ± 65.6	90.9 ± 71.3	0.852
HBV genotype			0.333
B	1 (10%)	5 (25%)	
C	9 (90%)	15 (75%)	
Serum HBsAg level, $\log_{10}$ IU/mL	3.46 ± 0.47	4.01 ± 0.64	0.013
Serum HBeAg level, $\log_{10}$ S/CO	2.22 ± 0.52	2.73 ± 0.54	0.023
Serum HBV DNA level, $\log_{10}$ copies/mL	5.93 ± 0.67	6.43 ± 0.93	0.107
cccDNA level, $\log_{10}$ copies/genome equivalent	0.28 ± 0.75	1.46 ± 0.74	0.001
Total intrahepatic HBV DNA level, $\log_{10}$ copies/genome equivalent	1.19 ± 0.36	2.10 ± 0.69	0.001
Necroinflammatory score	3.8 ± 1.6	4.2 ± 1.8	0.563
Fibrosis score	1.1 ± 0.8	1.2 ± 0.9	0.719

ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

cccDNA ($r = 0.295$, $P = 0.152$) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.347$, $P = 0.089$).

End of treatment characteristics

At end of treatment, responders had significantly lower serum HBsAg, HBeAg, HBV DNA, cccDNA and intrahepatic HBVDNA levels than non-responders. There was a tendency towards lower ALT, necroinflammatory and fibrosis scores in responders, but the difference between groups did not reach statistical significance (Table 2).

At end of treatment, $\log_{10}$ HBsAg was well correlated with $\log_{10}$ HBeAg ($r = 0.567$, $P = 0.001$) and $\log_{10}$ (serum HBV DNA) ($r = 0.591$, $P = 0.001$), as well as with $\log_{10}$ cccDNA ($r = 0.673$, $P < 0.001$) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.531$, $P = 0.006$). Log_{10} HBeAg had significant correlation with $\log_{10}$ (serum HBV DNA) ($r = 0.682$, $P < 0.001$), $\log_{10}$ cccDNA ($r = 0.678$, $P < 0.001$) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.753$, $P < 0.001$). Log_{10} (serum HBV DNA) was also significantly correlated with $\log_{10}$ cccDNA ($r = 0.614$, $P = 0.001$) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.684$, $P < 0.001$).

Reduction of HBsAg and HBeAg was correlated with reduction of $\log_{10}$ cccDNA ($r = 0.421$, $P = 0.032$, and $r = 0.570$, $P = 0.002$, respectively) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.420$, $P = 0.033$, and $r = 0.535$, $P = 0.005$, respectively), whereas reduction of $\log_{10}$ HBV DNA was not significantly correlated with reduction of $\log_{10}$ cccDNA ($r = 0.365$, $P = 0.066$) and $\log_{10}$ (total intrahepatic HBV DNA) ($r = 0.330$, $P = 0.099$).

Kinetics of serum HBsAg, HBeAg and HBV DNA levels

During treatment and follow up, patients who developed VR showed a consistent decline in serum HBsAg, with a mean decrease of 0.41 ± 0.36, 0.69 ± 0.61,

Table 2 Characteristics of responders and non-responders at end of treatment

	Responder ($n = 10$)	Non-responder ($n = 20$)	P
ALT level, U/L	50.9 ± 36.0	70.0.0 ± 44.7	0.221
Serum HBsAg level, $\log_{10}$ IU/mL	2.59 ± 0.97	3.41 ± 0.64	0.031
Serum HBeAg level, $\log_{10}$ S/CO	0.28 ± 1.08	2.41 ± 0.94	<0.001
Serum HBV DNA level, $\log_{10}$ copies/mL	3.18 ± 1.57	5.59 ± 177	0.001
cccDNA level, $\log_{10}$ copies/genome equivalent	−1.13 ± 1.06	0.66 ± 0.99	<0.001
Intrahepatic HBV DNA level, $\log_{10}$ copies/genome equivalent	−0.87 ± 0.98	0.82 ± 0.92	<0.001
Necroinflammatory score	2.2 ± 1.2	3.1 ± 1.7	0.133
Fibrosis score	0.6 ± 0.5	1.0 ± 0.9	0.135

ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

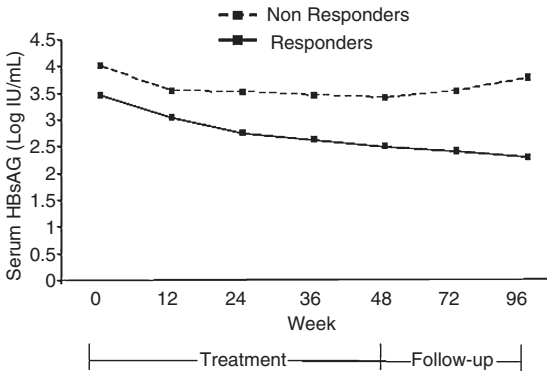

Figure 1 Kinetics of serum hepatitis B surface antigen (HBsAg) during pegylated interferon therapy and follow up in responders (solid line) and non-responders (dashed line).

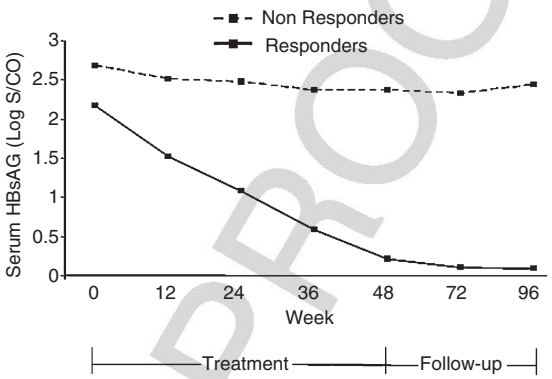

Figure 2 Kinetics of serum hepatitis B e-antigen (HBeAg) during pegylated interferon therapy and follow-up in responders (solid line) and non-responders (dashed line).

0.82 ± 0.67, 0.96 ± 0.75, 1.05 ± 0.84 and 1.15 ± 0.79 $\log_{10}$ IU/mL at weeks 12, 24, 36, 48, 72 and 96, respectively. It should be noted that in this patient group serum HBsAg still decreased after cessation of treatment. Non-responders, however, showed a decrease in HBsAg levels at week 12 which leveled off during weeks 24–48, and had a tendency to rebound after cessation of treatment (Fig. 1).

Patients who developed VR showed a marked decrease in serum HBeAg, with a mean decrease of 0.65 ± 0.81, 1.08 ± 0.98, 1.57 ± 1.00, 1.95 ± 1.32, 2.05 ± 1.22 and 2.07 ± 1.21 $\log_{10}$ S/CO at weeks 12, 24, 36, 48, 72 and 96, respectively. However, serum HBeAg levels did not significantly decrease during treatment in non-responders (Fig. 2).

Patients who developed VR showed a marked decline in serum HBV DNA, with a mean decrease of 1.11 ± 1.30, 1.96 ± 1.55, 2.73 ± 1.69, 2.76 ± 1.69, 3.84 ± 1.65 and 3.92 ± 1.59 $\log_{10}$ copies/mL at weeks 12, 24, 36, 48, 72 and 96, respectively. Patients who did not achieve VR demonstrated slightly decreased HBV DNA levels during therapy which had a tendency to rebound after cessation of treatment (Fig. 3).

Predictors of VR at baseline and during therapy

At baseline, $\log_{10}$ HBeAg provided a prediction of VR comparable to $\log_{10}$ HBsAg but better than $\log_{10}$ HBV DNA. The area under ROC of $\log_{10}$ HBeAg for VR was 0.79 (95% confidence interval [CI], 0.62–0.95; $P = 0.012$), whereas the areas under ROC of $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA were 0.75 (95% CI, 0.57–0.92; $P = 0.029$) and 0.64 (95% CI, 0.44–0.84; $P = 0.210$), respectively.

At week 12 during therapy, $\log_{10}$ HBeAg provided a better prediction of VR than $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA. The area under ROC of $\log_{10}$ HBeAg for VR was 0.83 (95% CI, 0.68–0.98; $P = 0.004$), whereas the areas under ROC of $\log_{10}$ HBsAg and $\log_{10}$ HBV DNA were 0.73 (95% CI, 0.55–0.91; $P = 0.043$) and 0.73 (95% CI, 0.53–0.92; $P = 0.041$), respectively.

Similarly, quantitative HBeAg provided the best prediction of VR at week 24. The area under ROC of $\log_{10}$ HBeAg for VR was 0.91 (95% CI, 0.79–1.01; $P = 0.001$),

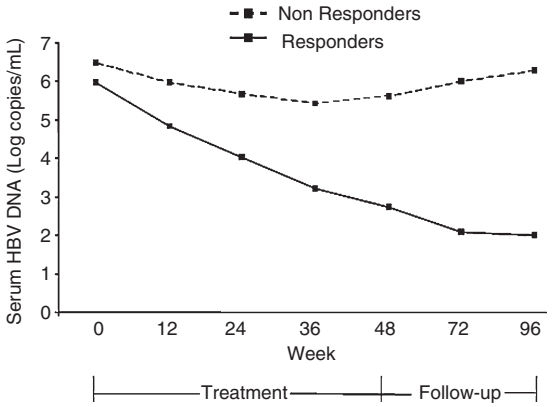

Figure 3 Kinetics of serum hepatitis B virus (HBV) DNA during pegylated interferon therapy and follow up in responders (solid line) and non-responders (dashed line).

Table 3 HBsAg, HBeAg and HBV DNA levels to predict virological response at week 96

	Sensitivity	Specificity	PPV	NPV	Accuracy
Week 12					
Log_{10} HBsAg <3.4 IU/mL	65.0	70.0	52.0	80.0	68.3
Log_{10} HBeAg <2.4 S/CO	75.0	80.0	65.2	86.5	78.3
Log_{10} HBV DNA <5.0 copies/mL	70.0	70.0	53.9	82.4	70.0
Week 24					
Log_{10} HBsAg <3.2 IU/mL	65.0	80.0	61.9	82.1	75.0
Log_{10} HBeAg <2.0 S/CO	85.0	90.0	80.9	92.3	88.3
Log_{10} HBV DNA <4.6 copies/mL	75.0	75.0	57.7	85.3	73.3

HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NPV, negative predictive value; PPV, positive predictive value; S/CO, sample to cut-off ratio.

whereas the areas under ROC of log_{10} HBsAg and log_{10} HBV DNA were 0.78 (95% CI, 0.62–0.95; $P = 0.013$) and 0.80 (95% CI, 0.62–0.98; $P = 0.009$), respectively.

Based on ROC curve analysis, a cut-off point for serum HBsAg, HBeAg and HBV DNA levels considered most accurate for predicting VR was determined. At week 24 of treatment, HBeAg level below 2.0 log_{10} S/CO (100 S/CO) had a sensitivity of 85%, and NPV of 92.3% to predict VR. At the same time point, HBV DNA concentrations below 4.6 log_{10} copies/mL (~40 000 copies/mL) had sensitivity and NPV of 75% and 85.3%, respectively, while the sensitivity and NPV of HBsAg levels below 3.2 log_{10} IU/mL (~1600 IU/mL) were 65% and 82.1%, respectively (Table 3).

HBsAg clearance

In this study, HBsAg clearance occurred in one patient (3.3%), who also achieved VR. The kinetics of serum HBsAg of this patient compared with those who developed VR without HBsAg clearance are shown in Figure 4. The patient who cleared HBsAg had a relatively low pretreatment HBsAg level and a more rapid decline of the protein level during therapy, particularly after the first 12 weeks of PEG-IFN therapy than those who did not clear HBsAg. In the patient who cleared HBsAg, the decrease in serum HBsAg at weeks 12, 24, 36, 48, 72 and 96 was 0.60, 1.58, 1.95, 2.35, 2.70 and 2.70 log_{10} IU/mL, respectively, while the mean decrease in serum HBsAg was 0.41 ± 0.38, 0.60 ± 0.55, 0.70 ± 0.57, 0.81 ± 0.60, 0.87 ± 0.65 and 1.03 ± 0.59 log_{10} IU/mL, respectively, in patients who did not clear HBsAg.

DISCUSSION

ALTHOUGH THE ELIMINATION of HBsAg is the ultimate goal in the management of chronic hepatitis B, it is uncommon in clinical practice, accounting for less than 5% of cases associated with PEG-IFN therapy.[17] In this study, HBsAg clearance was achieved in approximately 3% of patients. Another more realistic end-point in HBeAg-positive patients is HBeAg seroconversion, which has been considered a surrogate marker for long-term therapeutic response and improved clinical outcome of the patients.[17] In recent meta-analysis studies, patients treated with IFN-based therapies who achieved HBeAg seroconversion had significantly decreased HBV-related complications, including the development of cirrhosis and HCC.[18,19] In this study, the rate of HBeAg seroconversion (~33%) was comparable to previous reports involving a larger number of patients treated with PEG-IFN alone or in combination with lamivudine.[20,21]

In current treatment recommendations, monitoring serum HBV DNA levels at baseline and during therapy

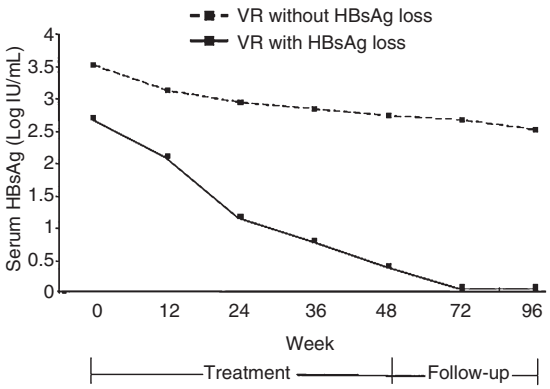

Figure 4 Kinetics of serum hepatitis B surface antigen (HBsAg) during pegylated interferon therapy and follow up in a patient who achieved virological response (VR) and cleared HBsAg (solid line) and those who developed VR without HBsAg clearance (dashed line).

has been practically considered to predict the response to IFN-based therapies.[4] However, measurement of serum HBV DNA level may not always accurately reflect the state of disease in the liver. In recent years, the development of methods for quantification of intrahepatic HBV replicative forms has provided additional molecular markers to predict antiviral treatment response.[22–24] It has recently been shown that cccDNA and total intrahepatic HBV DNA levels, which represent the active replicative and transcriptional form of the virus, are superior to serum HBV DNA as predictors of VR to antiviral therapy in patients receiving either lamivudine monotherapy or combination therapy of lamivudine and PEG-IFN.[25] Nonetheless, a major drawback in measuring cccDNA is the requirement for liver tissue. To avoid this obstacle, the validation of non-invasive surrogate markers such as the quantification of serum HBsAg and HBeAg is required in the clinical setting of antiviral therapy.[26]

In this study, we showed that baseline serum HBV DNA levels did not correlate with the amount of intrahepatic viral DNA and were not associated with VR to PEG-IFN therapy. In contrast, baseline HBsAg and HBeAg levels were significantly lower in responders than in patients who did not show HBeAg seroconversion. These observations are not surprising as both serum HBsAg and HBeAg levels prior to treatment were well correlated with intrahepatic HBV replicative forms. Indeed, low levels of cccDNA and total intrahepatic HBV DNA at baseline represented good predictors of VR, indicating that patients who will most likely benefit from the treatment are those who tend to have lower virus levels in the liver.[25,27] Our findings are in agreement with previous data in that low baseline HBsAg is more reliable than serum HBV DNA levels for predicting good response to PEG-IFN and lamivudine treatment in HBeAg-positive patients.[6] In HBeAg-negative patients, low pretreatment HBsAg level was considered the only significant prognostic predictor of HBsAg seroconversion by multivariate analysis following conventional IFN treatment.[28] Likewise, a recent large study of PEG-IFN-α-2a therapy demonstrated that HBeAg seroconversion was significantly associated with pretreatment HBeAg concentrations; as pretreatment level of HBeAg increased, the rate of subsequent HBeAg seroconversion diminished.[7]

It has been demonstrated that dynamic monitoring of quantitative HBeAg may be useful to predict a response to conventional IFN therapy.[29–31] Our data showed that, among responders, more rapid and consistent decline of HBeAg levels was observed during therapy and follow-up period. We demonstrated that serial measurement of HBeAg levels was superior to HBV DNA and HBsAg levels in predicting the likelihood of subsequent HBeAg seroconversion. At week 24 of treatment, HBeAg levels exceeding 2.0 $\log_{10}$ S/CO (100 S/CO) had an NPV for predicting sustained VR of approximately 92%, which surpassed that obtained by quantitative HBsAg (82%) and HBV DNA (85%) analysis. This high NPV of serum HBeAg at week 24 could allow the selection of patients who should cease therapy to reduce unnecessarily prolonged exposure to costly and unpleasant side-effects of PEG-IFN. In fact, such a "stopping rule" of quantitative HBeAg at week 24 of therapy has been well addressed by recent data using PEG-IFN-α-2a therapy,[7] which may be analogous to the current recommendation for patients with chronic hepatitis C treated with PEG-IFN and ribavirin.

The reason why dynamic monitoring of HBeAg levels offered a better prediction of VR than that of serum HBV DNA levels could be explained by the pattern of HBV DNA decline induced by PEG-IFN. Based on the results of a recent study of PEG-IFN-α-2b monotherapy, a proportion of patients with treatment response had a late decline of HBV DNA up to week 32 of therapy.[32] In our study, 30% of patients with HBeAg seroconversion had a late decline of HBV DNA after week 24 of treatment. Thus, VR in PEG-IFN monotherapy could not be predicted sufficiently on the basis of HBV DNA decline at an early stage of treatment. As a result, the measurement of HBeAg levels as early as week 12 or 24 of PEG-IFN monotherapy could provide a better predictor than HBV DNA monitoring. It should be noted that an early and significantly more pronounced decline of HBV DNA levels was observed in patients who received the combination therapy of lamivudine and PEG-IFN than in those treated with PEG-IFN alone.[32] Consequently, it was shown that an early decrease in HBV DNA could possibly be a predictor of treatment response to the combination therapy.[33]

Unlike HBV DNA, the reductions of HBsAg and HBeAg paralleled with the reduction of cccDNA, suggest that the decline of HBV proteins in serum reflect a diminished cccDNA pool in the liver. Indeed, serum HBsAg and HBeAg are produced by transcription and translation of the surface and precore/core genes of the HBV genome, respectively.[34] These data further confirm that quantitative HBsAg, as well as HBeAg titers, may be useful surrogate markers for cccDNA and may have clinical applicability for predicting VR to PEG-IFN therapy.[6,27] In this study, although monitoring HBsAg levels in the whole study population appeared to be less

indicative for VR than HBeAg levels, the reduction of HBsAg in individuals could predict HBsAg clearance. Previous data have demonstrated that mathematical modeling of HBsAg decline can predict HBsAg clearance for both IFN-based and NA-based therapies.[28] Interestingly, a recent study suggested that a rapid decline in HBsAg levels was associated with a high likelihood of HBsAg clearance in HBeAg-negative patients who had been treated with PEG-IFN.[13] Moreover, an HBsAg level below 10 IU/mL at week 48 and a decline exceeding 1 $\log_{10}$ IU/mL during treatment were significantly associated with HBsAg clearance during follow up in HBeAg-negative patients treated with PEG-IFN.[5] In this study, our data showed that the patient who cleared HBsAg at week 72 exhibited a more rapid decline in serum HBsAg during therapy than those who developed VR without HBsAg clearance. Thus, our results confirmed that a significant decline in HBsAg concentration during therapy might be used to predict HBsAg clearance during the follow-up period. These data also highlight the effects of PEG-IFN therapy in modulating host immune response, resulting in sustained response after treatment and eventual HBsAg clearance.

In conclusion, our data suggest that pretreatment quantitative HBsAg and HBeAg determination may be useful for predicting the response to PEG-IFN therapy. Quantitative measurement of HBeAg during therapy may provide better prediction of treatment response than HBV DNA and HBsAg levels. Also, serial monitoring of HBsAg levels during therapy may help predict HBsAg clearance after cessation of treatment. Further studies on larger sample sizes will be required to confirm these observations.

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HEPATOLOGY

Diagnostic role of serum glypican-3 in differentiating hepatocellular carcinoma from non-malignant chronic liver disease and other liver cancers

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Abstract

Background and Aims: The role of glypican-3 (GPC3), a novel serum marker, in differentiating hepatocellular carcinoma (HCC) from non-malignant chronic liver disease and other malignant space-occupying lesions in the liver is largely unknown. The aims of this study were to evaluate its diagnostic role and clinical correlations in patients with HCC.

Methods: Six groups were studied which included 40 healthy subjects, 50 patients with chronic hepatitis (CH), 50 patients with liver cirrhosis (LC), 100 patients with HCC, 50 patients with intrahepatic cholangiocarcinoma (ICC) and 50 patients with metastatic carcinoma (MCA). Serum GPC3 levels were measured by using a sandwich enzyme-linked immunosorbent assay method.

Results: Fifty-three percent of HCC patients had elevated serum GPC3 levels with values ranging 35.5–7826.6 ng/mL. The serum marker was undetectable in other groups except one patient (2%) with LC and another patient (2%) with MCA. In most cases of HCC, elevated GPC3 values did not correlate with α-fetoprotein (AFP) levels. Detectable GPC3 was significantly correlated with the presence of viral hepatitis markers but was not correlated with tumor size and stage of HCC. Serum GPC3 was superior to AFP in detecting small HCC (56.3% and 31.3%, respectively). A combination of serum GPC3 and AFP yielded an improved sensitivity for detecting small HCC to 75%.

Conclusion: Serum GPC3 is highly specific for detecting HCC. The combined use of serum GPC3 and AFP provides a potentially promising tool to better differentiate HCC from benign liver disorders, as well as from other liver cancers.

Introduction

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, particularly in Southeast Asia, where hepatitis B virus (HBV), and to a lesser extent, hepatitis C virus (HCV) infection are prevalent.[1] In Thailand, HCC is the most common malignant tumor, with an incidence of 6.8/100 000 in men and 2.3/100 000 in women per year.[2] Most patients with HCC are diagnosed at advanced stages and thus the prognosis is generally poor. The diagnosis of HCC could be achieved at an earlier stage by regular screening programs among high-risk populations by using imaging studies and serum tumor markers. Currently, serum α-fetoprotein (AFP), a fetal-specific glycoprotein, has undoubtedly been the most widely used tumor marker for the detection and monitoring of HCC. However, serum AFP is not always elevated to a diagnostic level in all patients, particularly in small HCC, and considerable numbers of patients with more advanced stages would be missed unless another diagnostic tool is used.[3,4] Moreover, its level may be elevated in non-malignant chronic liver diseases, including chronic hepatitis and cirrhosis, as well as in other primary and secondary liver cancers.[3,4] Therefore, the identification of alternative serum markers of HCC is needed.

Glypican-3 (GPC3) belongs to the glypican family of glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans, which plays an important role in cellular growth, differentiation and migration.[5] GPC3 has been reported to be increased in HCC in comparison with pre-neoplastic lesions and cirrhotic tissues at the mRNA and protein levels.[6–12] Interestingly, GPC3 mRNA levels are more frequently elevated than those of AFP, with the difference even greater in small HCC.[13] In contrast, GPC3 has been shown to be downregulated in various cancers, including breast cancer, ovarian cancer, lung adenocarcinoma and

cholangiocarcinoma.[14–17] Given the detection of circulating GPC3, it appears that this oncofetal protein can serve as a potential serum marker for the diagnosis of HCC.[6,9,18] However, data on its clinical correlations in patients with HCC are currently unknown. Furthermore, the role of this marker in differentiating HCC from other malignant space-occupying lesions in the liver has not yet been investigated. To address these issues, we examined whether serum GPC3 represented a useful diagnostic tool for differentiating HCC from benign chronic liver disease and from other liver cancers, including intrahepatic cholangiocarcinoma (ICC) and metastatic carcinoma (MCA). We also examined whether, in combination with AFP, serum GPC3 improved the diagnostic accuracy of HCC.

Methods

Patients and blood samples

Serum samples for the measurement of GPC3 and AFP levels were obtained from 100 consecutive patients (75 men, mean age 58.8 ± 12.7 years) who were diagnosed with HCC for the first time at King Chulalongkorn Memorial Hospital between October 2005 and August 2007. 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) guidelines.[19] 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 computed tomography (CT) or magnetic resonance imaging (MRI). In cases without typical imaging features, liver biopsy was performed to confirm the diagnosis of HCC. In this study, 39 patients were diagnosed with HCC based on histology and the remaining 61 cases were diagnosed by typical imaging patterns.

The clinicopathological data of the patients in this group at initial diagnosis were collected, which included sex, age, liver function tests, Child–Pugh score, tumor size, number of tumors, venous invasion, extrahepatic metastasis, and HCC staging classified by the CLIP score.[20] The CLIP score includes the variables of Child–Pugh classification, tumor morphology, AFP level and the presence of vascular invasion. In this study, 87 patients with HCC had underlying liver cirrhosis, 59 patients were positive for serum hepatitis B surface antigen (HBsAg), 11 patients were positive for HCV antibody (anti-HCV), and 17 patients were associated with alcohol-dependence but negative for HBsAg and anti-HCV. For the remaining 13 patients, the underlying etiology of the liver could not be determined. According to the CLIP score at initial presentation, there were 21 patients in the score 0 subgroup, 13 patients in score 1, 24 patients in score 2, 10 in score 3, 17 in score 4, nine in score 5 and six in score 6. Thirty-three patients had venous invasion, while extrahepatic metastasis was found in 16 patients.

The control group was comprised of 40 healthy volunteers with no apparent liver disease (26 men, mean age 32.8 ± 8.2 years). The non-malignant chronic liver disease (CLD) group included 50 patients with chronic hepatitis (CH) (34 men, mean age 43.3 ± 14.3 years), and 50 patients with liver cirrhosis (LC), who had no evidence of HCC (30 men, mean age 48.6 ± 9.1 years). The diagnosis of CH was based on persistent elevation of alanine transaminase (ALT) levels and confirmed by histopathology. The diagnosis of LC was based on histopathology and/or clinical features such as the presence of ascites, or esophageal varices. HBsAg was positive in 36 and 32 patients with CH and LC, respectively, while anti-HCV was positive in six and seven patients with CH and LC, respectively.

The other liver cancer groups comprised 50 patients with ICC (28 men, mean age 60.8 ± 11.4 years), and 50 patients with MCA (29 men, mean age 51.8 ± 14.9 years). The ICC was diagnosed based on liver tumor features detected by ultrasound/CT scan and confirmed by histology. The diagnosis of MCA was established by clinical settings and confirmed by histopathology. In this study, there were 15 patients with colorectal cancer, seven patients with gastric carcinoma, seven patients with ovarian cancer, 10 patients with lung cancer, three patients with pancreatic carcinoma, one each with lymphoma, renal cell carcinoma and gallbladder cancer, while the primary sites of five patients were unknown.

All subjects were informed about the objective of the study, and subsequently provided their consent. Blood was obtained during investigation at the initial presentation; sera were separated by centrifugation and stored at –70°C until tested for GPC3 level. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Measurement of serum GPC3 levels

Serum GPC3 levels were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA) method as described previously.[6] Briefly, Microtiter plates (Maxisorp; Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were coated at 4°C overnight with 1.6 mg/mL antihuman GPC3 (100 mL/well) in the coating buffer. The uncoated area was then blocked with 1% (w/v) bovine serum albumin (150 mL/well) for 60 min at 37°C. After washing, 100 mL of sample or standard (9.76–5000 ng/mL recombinant human glypican-3) were added. After incubation for 60 min at 37°C, plates were washed and added to the biotinylated anti-human glypican-3 (100 mL/well; 1:500) and incubated for 60 min at 37°C. After washing, the peroxidase-mouse monoclonal anti-biotin (100 mL/well; 1:2000) was added and incubated for 60 min at 37°C. The plates were washed again and then the peroxidase substrate (OPD; 100 mL/well) was added and incubated at 37°C for 15–20 min to allow the color to develop. The reaction was stopped by addition of 50 mL of 4 M H_2SO_4. The absorbance at 492 nm was measured using the Titertek Multiskan M340 multiplate reader.

Measurement of serum AFP levels

Serum AFP levels were determined using a commercially available ELISA kit (Cobus Core; Roche Diagnostics, Basel, Switzerland).

Statistical analysis

Data are expressed as percentage, mean and standard deviation. Comparisons between groups were analyzed by the χ^2-test or Fisher's exact test for categorical variables and by the Mann–Whitney test or Student's *t*-test when appropriate for quantitative variables. Receiver–operator curves (ROC) were constructed to evaluate the diagnostic performance of the serum markers in discriminating HCC from other groups. Sensitivity, specificity,

Table 1 Clinical characteristics and serum marker levels of the subjects

Group	*n*	Age† (years)	Sex (M/F)	HBsAg⁺/anti-HCV⁺	GPC3‡ (ng/mL)	AFP‡ (ng/mL)	AFP (ng/mL)		
							≤ 20	21–99	≥ 100
Controls	40	32.8 ± 8.2	26/14	0/0	0 (0–0)	6.1 (4.1–11.5)	40	0	0
CH	50	43.3 ± 14.3	34/16	36/6	0 (0–0)	8.0 (4.5–155)	43	5	2
LC	50	48.6 ± 9.1	30/20	32/7	0 (0–43.6)	11.2 (4.7–224)	34	13	3
HCC	100	58.8 ± 12.7	75/25	59/11	46.3 (0–7826.6)	105.5 (5.7–75000)	27	23	50
ICC	50	60.8 ± 11.4	28/22	0/0	0 (0–0)	10.0 (4.0–50.5)	45	5	0
MCA	50	55.9 ± 12.7	29/21	2/0	0 (0–202.5)	10.7 (4.7–155)	39	9	2

†Data express as mean ± standard deviation. ‡Data express as median (ranges). CH, chronic hepatitis; controls, healthy volunteers; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ICC, intrahepatic cholangiocarcinoma; LC, liver cirrhosis; MCA, metastatic carcinoma.

positive and negative predictive values and diagnostic accuracy were calculated in accordance with standard methods. Pearson's correlation coefficient was used to find the correlation between the serum levels of GPC3 and AFP. The logistic regression analysis was performed to evaluate the clinicopathological parameters associated with elevation of serum GPC3 and AFP levels. $P < 0.05$ for a two-tailed test were considered statistically significant. All statistical analyses were performed using the SPSS software for Windows ver. 14.0 (SPSS, Chicago, IL, USA).

Results

Clinical characteristics

Table 1 compares clinical characteristics of the subjects enrolled in this study. Patients with HCC, ICC and MCA were significantly older than those with CH, LC and healthy controls ($P < 0.001$). However, there was no significant difference in mean age between patients with HCC, ICC and MCA. Patients with LC and CH were significantly older than healthy controls ($P < 0.001$). Likewise, the mean age of patients with LC was significantly higher than that of patients with CH ($P = 0.028$). In this study, there was no difference in sex distribution between groups ($P = 0.155$). The prevalence of HBV and HCV infection was significantly higher in patients with HCC, LC and CH than that of patients with ICC, MCA and healthy controls ($P < 0.001$). However, there was no significant difference in viral hepatitis markers between patients with HCC, LC and CH.

Serum GPC3 and AFP concentrations

In this study, we found that 53 of 100 (53%) patients with HCC had elevated levels of serum GPC3 with values ranging 35.5–7826.6 ng/mL. GPC3 was undetectable in the other groups except one patient with LC (the concentration of GPC3 was 43.6 ng/mL) and another patient with squamous cell carcinoma of the lung (the concentration of GPC3 was 202.5 ng/mL). The level of serum GPC3 in patients with HCC was significantly higher than those of healthy controls, CH, LC, ICC and MCA ($P = 0.001$) (Table 1, Fig. 1a). There was no significant difference in the prevalence of detectable serum GPC3 between cirrhotic HCC (47 of 87 cases; 54.0%) and non-cirrhotic HCC (six of 13 cases; 46.2%) ($P = 0.767$). In addition, there was no significant difference in the median level of serum GPC3 between these two groups (189.0 and 117.5 ng/mL, respectively, $P = 0.581$).

Serum AFP levels were also measured in the same set of serum samples. The level of serum AFP in patients with HCC was significantly higher than those of healthy controls, CH, LC, ICC and MCA ($P = 0.002$) (Fig. 1b). Using the normal upper limit of AFP (20 ng/mL) as a reference, AFP was elevated in 73 (73%) patients with HCC. AFP values were within normal range in all healthy individuals, whereas values were elevated in seven (14.0%) patients with CH, 16 (32.0%) patients with LC, five (10%) patients with ICC and 11 (22%) patients with MCA. When using 100 ng/mL as a cut-off point, AFP concentration was elevated in 50 (50%) patients with HCC, two (4%) patients with CH, three (6%) patients with LC and two (4%) patients with MCA (Table 1).

Serum GPC3 and AFP as diagnostic markers

The ROC for GPC3 and AFP were generated on the same graph to compare the diagnostic accuracies of the two markers. As shown in Figure 2(a), the area under the curve of HCC and non-malignant chronic liver disease (CH and LC) was 0.767 (95% confidence interval [CI] 0.700–0.835] for GP3 and 0.855 (95% CI 0.804–0.906) for AFP. There was no significant difference between these two areas. Similarly, the area under the ROC of HCC and other liver cancers (ICC and MCA) was 0.765 (95% CI 0.697–0.833) for GP3 and 0.859 (95% CI 0.807–0.910) for AFP, which were not statistically different (Fig. 2b).

Based on the ROC analysis, the optimal cut-off points for AFP to differentiate HCC from non-malignant chronic liver disease and other liver cancers were 19 and 22 ng/mL, respectively. To simplify the analysis, we selected an AFP level of 20 ng/mL as the optimal cut-off point. At this concentration, the sensitivity, specificity and accuracy for differentiating HCC from non-malignant chronic liver disease were 73%, 77% and 75%, respectively. At a cut-off value of 100 ng/mL, the sensitivity, specificity and accuracy for differentiating HCC from non-malignant chronic liver disease were 50%, 95% and 72.5%, respectively. To differentiate HCC from other liver cancers, serum AFP at a cut-off value of 20 ng/mL exhibited a sensitivity, specificity and accuracy for diagnosing HCC of 73%, 84% and 78.5%, respectively. Similarly, at cut-off values of 100 ng/mL, the sensitivity, specificity and

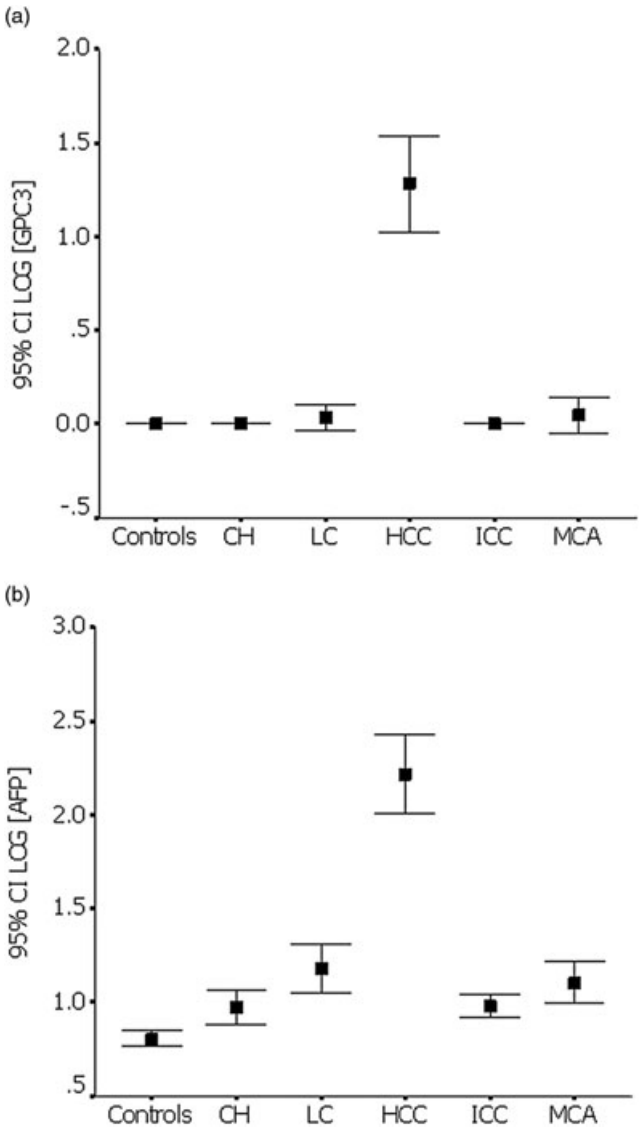

Figure 1 Serum glypican-3 (GPC3) and α-fetoprotein (AFP) levels in each group of patients. (a) 95% confidence interval of $\log_{10}$ (GPC3). (b) 95% confidence interval of $\log_{10}$ (AFP). CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; MCA, metastatic carcinoma.

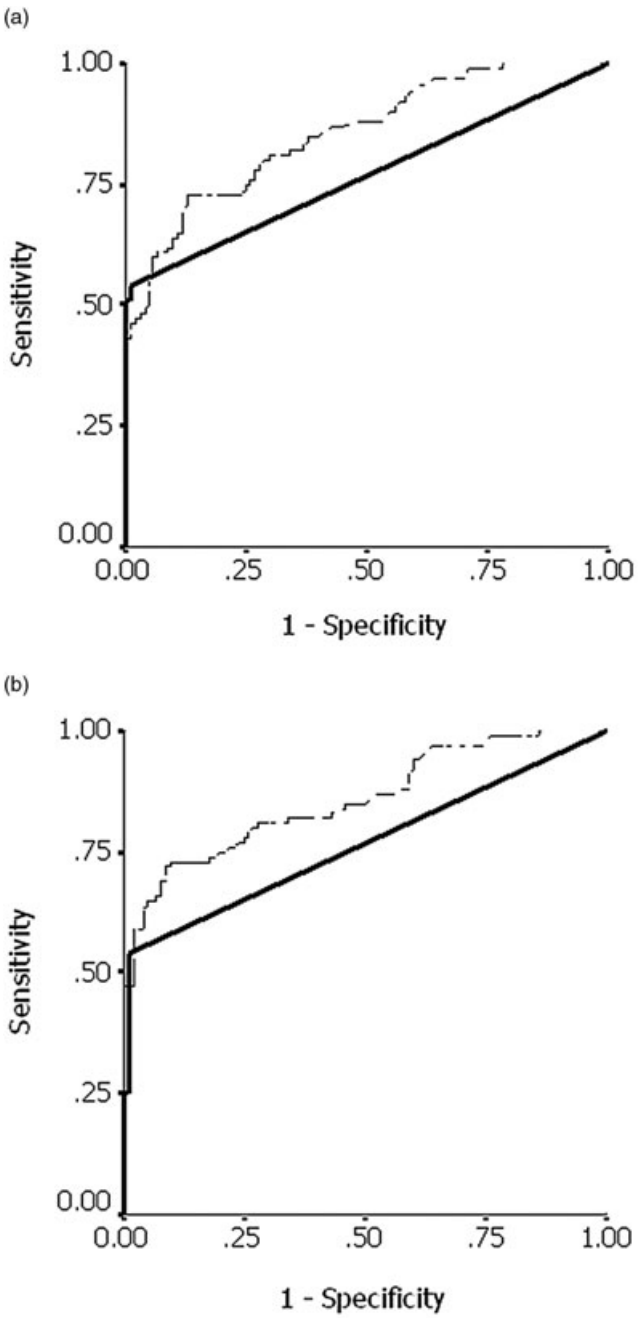

Figure 2 Receiver–operator curves of serum glypican-3 (GPC3) and α-fetoprotein (AFP) in differentiating hepatocellular carcinoma (HCC) from other groups. (a) HCC and non-malignant chronic liver disease (the areas under the curve for GPC3 and AFP were 0.767 and 0.855, respectively). (b) HCC and other liver cancers (the areas under the curve for GPC3 and AFP were 0.765 and 0.859, respectively). (a) — AFP, --- GPC3. (b) — AFP, --- GPC3.

accuracy of serum AFP for differentiating HCC from other liver cancers were 50%, 98% and 74%, respectively (Table 2).

In this study, there was no correlation between GPC3 and AFP values (Pearson's correlation coefficient for GPC3 and AFP values, –0.028; $P = 0.779$). As a result, the combined use of GPC3 and AFP significantly increased the sensitivity of the diagnosis of HCC. At a cut-off value of 20 ng/mL for serum AFP, 88 (88%) of the HCC patients had either elevated GPC3 or AFP levels, while the specificity were 76–84%. At a cut-off value of 100 ng/mL for serum AFP, the sensitivity and specificity of the combined tests were 78% and 94–97%, respectively. The sensitivity, specificity, positive and negative predictive values, and accuracy of GPC3, AFP and combined tests in differentiating HCC from the other groups are shown in Table 2.

Regarding small HCC (tumor size < 3 cm in diameter), we found that nine of 16 (56.3%) patients had elevated levels of

Table 2 Serum glypican-3 (GPC3), α-fetoprotein (AFP) and their combination for differentiation between hepatocellular carcinoma (HCC) and other groups

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HCC and CLD					
GPC3	53.0	99.0	98.1	67.8	76.0
AFP20	73.0	77.0	76.0	74.0	75.0
AFP100	50.0	95.0	90.9	65.5	72.5
GPC3 and AFP20	88.0	76.0	78.6	86.4	82.0
GPC3 and AFP100	78.0	94.0	92.9	81.0	86.0
HCC and other liver cancers					
GPC3	53.0	99.0	98.1	67.8	76.0
AFP20	73.0	84.0	82.0	75.7	78.5
AFP100	50.0	98.0	96.2	66.2	74.0
GPC3 and AFP20	88.0	84.0	84.6	87.5	86.0
GPC3 and AFP100	78.0	97.0	96.3	81.5	87.5

AFP100, AFP at a cut-off value of 100 ng/mL; AFP20, AFP at a cut-off value of 20 ng/mL; CLD, non-malignant chronic liver disease (chronic hepatitis and cirrhosis) Other liver cancers (intrahepatic cholangiocarcinoma and metastatic carcinoma); GPC3, detectable serum GPC3; NPV, negative predictive value; PPV, positive predictive value.

Table 3 Sensitivity of serum glypican-3 (GPC3), α-fetoprotein (AFP) and their combination in relation to size of hepatocellular carcinoma

Tumor size	GPC3	AFP20	AFP100	GPC3 and AFP20	GPC3 and AFP100
< 3 cm ($n = 16$)	9 (56.3)	5 (31.3)	5 (31.3)	12 (75.0)	11 (68.8)
3–5 cm ($n = 16$)	8 (50.0)	8 (50.0)	4 (25.0)	13 (81.3)	11 (68.8)
5–10 cm ($n = 33$)	17 (51.6)	24 (72.7)	18 (54.5)	31 (93.9)	27 (81.8)
> 10 cm ($n = 35$)	19 (54.3)	33 (94.3)	23 (65.7)	33 (94.3)	32 (91.4)

Data expressed as *n* (%). AFP100, AFP at a cut-off value of 100 ng/mL; AFP20, AFP at a cut-off value of 20 ng/mL; GPC3, detectable serum GPC3.

serum GPC3, whereas five (31.3%) patients had elevated serum AFP at cut-off values of 20 and 100 ng/mL, respectively. When both serum GPC3 and AFP (at cut-off values of 20 and 100 ng/mL, respectively) were determined in parallel, the sensitivity of the combined tests in detecting small HCC were 75% and 68.8%, respectively. The sensitivity of serum GPC3, AFP and their combination in relation with size of HCC is showed in Table 3.

Correlation of serum marker levels with disease characteristics

To evaluate the association between serum GPC3 levels and clinical features, the patients with HCC were divided into two groups based on the detection of the marker. Accordingly, there were 53 patients with detectable serum GPC3 and 47 patients with undetectable levels. The correlations between groups and various clinical parameters listed in Table 4 were analyzed. There was no significant correlation between serum GPC3 level and patient age, sex, Child–Pugh score, tumor size, tumor type, the presence of venous invasion, extrahepatic metastasis and CLIP score. However, detectable serum GPC3 level was significantly found in patients who had positive viral hepatitis markers (HBsAg and/or anti-HCV) ($P = 0.016$).

To investigate whether necroinflammatory activity of chronic hepatitis B and C might affect the detection of serum GPC3 in patients with HCC, we examined the histology activity index (HAI) of liver specimens according to the criteria of Knodell *et al.*[21] which comprise two major components, namely, necroinflammation (HAI-I) and fibrosis (HAI-F). In this respect, 28 liver specimens of patients with HCC were available for the analysis. There was no significant difference in HAI-I score between patients with detectable serum GPC3 (16 cases) and those without detectable serum protein (12 cases) (mean HAI-I scores were 6.6 ± 2.4 and 6.2 ± 2.3, respectively, $P = 0.656$). In addition, there was no correlation between serum GPC3 level and HAI-I score of the corresponding liver specimens (Pearson's correlation coefficient, –0.196; $P = 0.318$).

Similarly, the patients with HCC were divided into two groups based on the levels of serum AFP at cut-off values of 20 and 100 ng/mL, which represented the optimal point from the ROC analysis and median value in HCC patients, respectively. At a cut-off value of 20 ng/mL, there was no significant correlation between serum AFP level and patient age, sex, Child–Pugh score, etiology of liver disease, venous invasion and extrahepatic metastasis. However, high serum AFP levels were significantly associated with tumor type ($P = 0.005$), tumor size ($P < 0.001$) and CLIP score ($P < 0.001$) (Table 5.1). At a cut-off value of 100 ng/mL, there was no significant correlation between serum AFP level and patient age, sex, Child–Pugh score, etiology of liver disease, tumor type and extrahepatic metastasis. However, high serum AFP levels were significantly associated with tumor size ($P = 0.005$), the presence of venous invasion ($P = 0.010$) and CLIP score ($P = 0.001$) (Table 5.2).

Table 4 Relationship between serum glypican-3 (GPC3) levels and clinical features of patients with hepatocellular carcinoma

Variables	GPC3 Positive	GPC3 Negative	*P*
Age (yrs)			
≥ 60 (*n* = 48)	23	25	0.423
< 60 (*n* = 52)	30	22	
Sex			
Male (*n* = 75)	41	34	0.646
Female (*n* = 25)	12	13	
Underlying liver disease			
HBV- or HCV-related (*n* = 70)	43	27	0.016
Non-viral (*n* = 30)	10	20	
Child–Pugh score			
A (*n* = 45)	21	24	0.470
B (*n* = 40)	24	16	
C (*n* = 15)	8	7	
Tumor type			
Uninodular (*n* = 31)	15	16	0.799
Multinodular (*n* = 30)	16	14	
Massive (*n* = 39)	22	17	
Tumor size			
≤ 5 cm (*n* = 32)	17	15	1.000
> 5 cm (*n* = 68)	36	32	
Venous invasion			
Presence (*n* = 33)	17	16	1.000
Absence (*n* = 67)	36	31	
Extrahepatic metastasis			
Presence (*n* = 16)	10	6	0.430
Absence (*n* = 84)	43	41	
CLIP score			
Score 0–1 (*n* = 34)	18	16	0.605
Score 2–3 (*n* = 34)	16	18	
Score 4–6 (*n* = 32)	19	13	

HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 5.1 Relationship between serum α-fetoprotein (AFP) levels (cut-off value of 20 ng/mL) and clinical features of patients with hepatocellular carcinoma

Variables	AFP (≥ 20 ng/mL)	AFP (< 20 ng/mL)	*P*
Age (yrs)			
≥ 60 (*n* = 48)	39	9	0.074
< 60 (*n* = 52)	33	19	
Sex			
Male (*n* = 75)	55	20	0.615
Female (*n* = 25)	17	8	
Underlying liver disease			
HBV- or HCV-related (*n* = 70)	50	20	1.000
Non-viral (*n* = 30)	22	8	
Child–Pugh score			
A (*n* = 45)	30	15	0.326
B (*n* = 40)	29	11	
C (*n* = 15)	13	2	
Tumor type			
Uninodular (*n* = 31)	20	11	0.005
Multinodular (*n* = 30)	17	13	
Massive (*n* = 39)	35	4	
Tumor size			
≤ 5 cm (*n* = 32)	13	19	< 0.001
> 5 cm (*n* = 68)	59	9	
Venous invasion			
Presence (*n* = 33)	28	5	0.058
Absence (*n* = 67)	44	23	
Extrahepatic metastasis			
Presence (*n* = 16)	14	2	0.223
Absence (*n* = 84)	58	26	
CLIP score			
Score 0–1 (*n* = 34)	17	17	< 0.001
Score 2–3 (*n* = 34)	24	10	
Score 4–6 (*n* = 32)	31	1	

HBV, hepatitis B virus; HCV, hepatitis C virus.

Clinical parameters, including age, sex, etiology of liver disease, Child–Pugh score, tumor type, tumor size, venous invasion, extrahepatic metastasis and the CLIP score, were entered into the multivariate logistic regression analysis to evaluate the factors associated with elevation levels of the serum markers. The multivariate analysis revealed that presence of viral hepatitis marker(s) (serum HBsAg and/or anti-HCV positive) was the independent factor of predicting detectable serum GPC3 level. In contrast, large tumor size and high CLIP score represented the independent factors of predicting high serum AFP level in patients with HCC (Table 6).

Discussion

The progression of HCC is a multistage process with a large proportion of cases involving underlying cirrhosis.[1] In endemic areas of viral hepatitis like Thailand, infection rates of HBV have exceeded 50% of patients with chronic liver disease, which reflects a potential risk for the future development of HCC.[22] Currently, the measurement of serum AFP level has been the only marker routinely used for detecting and monitoring HCC. AFP is a glycoprotein expressed abundantly in fetal liver but not in normal adult liver, and it can be re-expressed by the tumor cells according to their differentiation.[23] Although AFP has high sensitivity in detecting HCC, it exhibits high false-positivity. For example, serum AFP at a cut-off value of 20 ng/mL shows a 60–80% sensitivity, although this sensitivity decreases to approximately 20–40% for the detection of small tumors.[24] In addition, a significant increase in serum AFP level (20–200 ng/mL) is detected in a considerable number of patients with chronic liver disease, including approximately 15–60% of patients with chronic hepatitis and approximately 10–50% with cirrhosis.[3,4] In the present study, the sensitivity and specificity of AFP for differentiating HCC from benign liver disease at a cut-off value of 20 ng/mL were 73% and 77%, respectively. Apart from AFP, other serological markers such as the *Lens culinaris* agglutinin-reactive α-fetoprotein (AFP-L3) and des-γ-carboxy-prothrombin (DCP) have been developed to improve the accuracy. However, these serum markers are not widely used in clinical practice.

In this study, we found that serum GPC3 levels were increased in 53% patients with HCC but was increased in only one patient with non-malignant chronic liver disease. Thus, the sensitivity and

the specificity of this serum maker for differentiating HCC from benign liver disease were 53% and 99%, respectively. These data were in agreement with previous reports from Canada and Japan,[6,9] where chronic HCV infection is the major etiological factor of HCC. Capurro *et al.*[6] demonstrated that GPC3 was undetectable in sera of healthy donors and patients with hepatitis, but its levels were significantly increased in 18 of 34 patients with HCC. In addition, only one of 20 patients with cirrhosis displayed elevated levels of serum GPC3. Therefore, the sensitivity and the specificity of GPC3 were 53% and 95%, respectively. Similarly, Nakatsura *et al.*[9] demonstrated that circulating GPC3 was found in sera of 40% (16/40) of HCC patients, but could not be detected in patients with benign liver diseases. As a result, the sensitivity and the specificity of GPC3 in that study were 40% and 100%, respectively. Collectively, previous data and our results confirm a very high specificity of GPC3 in differentiating HCC from non-malignant chronic liver disease.

Table 5.2 Relationship between serum α-fetoprotein (AFP) levels (cut-off value of 100 ng/mL) and clinical features of patients with hepatocellular carcinoma

Variables	AFP (≥ 100 ng/mL)	AFP (< 100 ng/mL)	*P*
Age (yrs)			
≥ 60 (*n* = 48)	24	24	1.000
< 60 (*n* = 52)	26	26	
Sex			
Male (*n* = 75)	35	40	0.356
Female (*n* = 25)	15	10	
Underlying liver disease			
HBV- or HCV-related (*n* = 70)	32	38	0.275
Non-viral (*n* = 30)	18	12	
Child–Pugh score			
A (*n* = 45)	18	27	0.076
B (*n* = 40)	21	19	
C (*n* = 15)	11	4	
Tumor type			
Uninodular (*n* = 31)	12	19	0.150
Multinodular (*n* = 30)	14	16	
Massive (*n* = 39)	24	15	
Tumor size			
≤ 5 cm (*n* = 32)	9	23	0.005
> 5 cm (*n* = 68)	41	27	
Venous invasion			
Presence (*n* = 33)	23	10	0.010
Absence (*n* = 67)	27	40	
Extrahepatic metastasis			
Presence (*n* = 16)	12	4	0.054
Absence (*n* = 84)	38	46	
CLIP score			
Score 0–1 (*n* = 34)	11	23	0.001
Score 2–3 (*n* = 34)	13	21	
Score 4–6 (*n* = 32)	26	6	

HBV, hepatitis B virus; HCV, hepatitis C virus.

In this study, it should be mentioned that detectable serum GPC3 level in patients with HCC was influenced by the presence of viral hepatitis markers (serum HBsAg and/or anti-HCV positive). These data suggest that serum GPC3 might be more sensitive for detecting HCC in patients with pre-existing chronic viral hepatitis infection than those without viral markers. Thus, it appears that measurement of serum GPC3 may be advantageous for the diagnosis of HCC, particularly in areas where HBV and HCV infections are prevalent. However, it remains in mind that the expression of GPC3 could be detected in benign liver tissue in chronic HCV infection with high-grade necroinflammatory activity, which might potentially lead to a misdiagnosis of HCC.[25] Despite these findings, the staining of GPC3 in liver tissue was invariably cytoplasmic and usually granular, never membranous as in many cases of HCC.[25] Based on our data, there was no correlation between serum GPC3 level and necroinflammatory activity of the liver, suggesting that active hepatitis may play a negligible if any role in the detection of serum GPC3 in patients with HCC.

In agreement with previous studies, our data also showed that the simultaneous measurement of GPC3 and AFP significantly increased the sensitivity and accuracy for HCC diagnosis.[6,9] These could be explained by the observation that there was no correlation between GPC3 and AFP values in most cases of HCC. Another important issue to be addressed is whether GPC3 would be a better marker for the detection of small HCC than AFP. Indeed, it has been demonstrated that the expression of GPC3 in small HCC was significantly greater than that of AFP.[6,13] Unlike AFP, our data showed that there was no correlation between serum concentration GPC3 and tumor size or tumor stage. In addition, we showed that a higher proportion of patients with HCC of less than 3 cm had positive GPC3 values compared with AFP at the cut-off 20 ng/mL

Table 6 Factors associated with elevation of serum glypican-3 (GPC3) and α-fetoprotein (AFP) levels in the multivariate logistic regression analysis

Variables	Regression coefficient	Standard error	Odds ratio	95% confidence interval	*P*
GPC3					
Viral marker positive†	1.414	0.525	4.111	1.470–11.497	0.007
AFP20					
Tumor size (> 5 cm)	2.926	0.797	18.652	3.910–88.988	< 0.001
High CLIP score (4–6)	4.213	1.446	27.580	3.974–149.192	0.004
AFP100					
Tumor size (> 5 cm)	1.527	0.621	4.605	1.363–15.560	0.014
High CLIP score (4–6)	2.406	0.841	11.085	2.132–57.624	0.004

†Serum hepatitis B surface antigen and/or anti-hepatitis C positive. AFP20, AFP at a cut-off value of 20 ng/mL; AFP100, AFP at a cut-off value of 100 ng/mL.

(56% and 31%, respectively), and the combination of these markers yielded an improved sensitivity for detecting small HCC to 75%. However, studies including a larger number of patients will certainly be required to confirm this observation.

The exact biological functions of GPC3 on hepatocarcinogenesis are still not well understood. GPC3 expression was, both on immunohistochemistry and by real-time reverse transcriptase polymerase chain reaction (RT–PCR), much higher in small HCC than in cirrhosis, indicating that the transition from premalignant lesions to small HCC is associated with a sharp increase of GPC3 expression in a majority of cases.[7] Recent data have shown that GPC3 promotes the growth of HCC by stimulating the autocrine/paracrine canonical Wnt signaling.[26] In fact, transcriptional profiles of GPC3 were increased in early and advanced HCC compared with normal tissue and dysplastic nodules.[8] In contrast, GPC3 may act as an inhibitor of cell proliferation and thus can induce apoptosis in certain types of tumors.[27] For instance, down-regulation of GPC3 has been reported in several types of malignancies, including mesotheliomas,[28] gallbladder cancer, breast cancer,[17] ovarian cancer[15] and lung adenocarcinoma.[14] It has been shown that GPC3 functions as a potential tumor suppressor in the lung[14] and inhibits invasion and metastasis in a breast cancer model.[29] Collectively, these data suggest that GPC3 can act as a negative regulator of growth in most cancer types. Thus, the observation that GPC3 protein was rarely found in the sera in patients with MCA was not surprising. In the current study, we found that 98% of MCA were negative for serum GPC3. The only case expressing GPC3 was metastatic squamous cell carcinoma of the lung. In fact, it has recently been shown that GPC3 is tended to be overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma of the lung.[30]

Another important finding of this study is that serum GPC3 is also useful for differentiating patients with HCC from those with ICC. Indeed, cholangiocarcinoma represents the second most common primary liver malignant tumor arising from cholangiocytes, which are the epithelial cells lining the bile duct apparatus.[31] Cholangiocarcinoma is an uncommon liver cancer in Western countries but is particularly common in certain parts of the world such as China and Southeast Asia, where liver flukes are endemic. Thus, in the geographic areas where both chronic viral hepatitis and liver flukes are highly prevalent, it remains an essential problem to differentiate HCC from cholangiocarcinoma, particularly ICC, because both tumors may at times present the same clinical characteristics. Although HCC is usually associated with underlying cirrhosis, while conversely, ICC develops in a non-cirrhotic liver, the occurrence of ICC in cirrhosis has been increasingly recognized.[32] Moreover, approximately 10% of patients with HCC presented with obstructive jaundice, a clinical feature that might imitate the hilar type of cholangiocarcinoma.[33] Accordingly, an accurate serum marker would be considered as a valuable adjunct to non-invasive imaging for differentiating these primary liver cancers. In this study, serum GPC3 exhibited a very high specificity (100%), because none of the ICC patients had detectable serum levels. This finding supports the difference of GPC3 expression pattern in the liver between HCC and ICC.[16] When positivity for either GPC3 or AFP ($\geq$ 20 ng/mL) was used, the combined assay provided additional diagnostic sensitivity to approximately 90%, suggesting the complementary role of the two markers in differentiation of HCC from ICC.

In conclusion, our study showed that serum GPC3 levels were significantly elevated in patients with early and advanced HCC, but were rarely detectable in patients with other liver cancers, patients with non-malignant chronic liver disease and healthy controls. There was no positive correlation of serum GPC3 levels with circulating AFP, indicative of the complementary role of the two markers. Thus, the combined assay of these two markers provides a potentially promising tool to better differentiate HCC from benign liver disorders, as well as from other malignant space-occupying lesions in the liver. Further studies in large scale are worthwhile to confirm these observations and to elucidate the clinical significance of serum GPC3 in patients with HCC.

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ORIGINAL ARTICLE

Prevalence, whole genome characterization and phylogenetic analysis of hepatitis B virus in captive orangutan and gibbon

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Abstract

Background Hepatitis B virus (HBV) is a public health problem worldwide and apart from infecting humans, HBV has been found in non-human primates.
Methods We subjected 93 non-human primates comprising 12 species to ELISA screening for the serological markers HBsAg, antiHBs and anti-HBc. Subsequently, we detected HBV DNA, sequenced the whole HBV genome and performed phylogenetic analysis.
Results HBV infection was detected in gibbon (4/15) and orangutan (7/53). HBV DNA isolates from two gibbons and seven orangutans were chosen for complete genome amplification. We aligned the *Pre-S*/*S*, *Pre-C*/*C* and entire genomes with HBV sequences and performed phylogenetic analysis. The gibbon and orangutan viruses clustered within their respective groups.
Conclusions Both geographic location and host species influence which HBV variants are found in gibbons and orangutans. Hence, HBV transmission between humans and non-human primates might be a distinct possibility and additional studies will be required to further investigate this potential risk.

Introduction

Human hepatitis B virus (HBV) is the prototype member of the family *Hepadnaviridae*. It is a spherical enveloped particle containing partially double stranded DNA and RNA dependent DNA polymerase. The majority of infections by this diminutive viral genome affect humans. Hence, various research projects have been aimed at accumulating information on human hepatitis B. In nature, HBV has been found in non-human primate species such as chimpanzees (*Pan troglodytes*) (ChHBV) [1], orangutans (*Pongo pygmaeus*) (OuHBV) [2], wild and captive gibbons (*Hylobates* sp. and *Nomascus* sp.) (GiHBV) [3], gorillas (*Gorilla gorilla*) (GoHBV) [4] and woolly monkeys (*Lagothrix lagothricha*) (WMHBV) [5]. However, information on epidemiology, genome and pathogenicity of non-human primate hepatitis B virus has remained rather limited and mainly been gleaned from captive animals. According to Deinhardt's

survey (1976), hepatitis B surface antigen (HBsAg) has been found in chimpanzees, gibbons and orangutans, whereas marmosets (*Callithrix jacchus*), squirrel monkeys (*Saimiri* sp.), baboons (*Papio* sp.), rhesus macaques (*Macaca mulatta*) and vervet monkeys (*Cercopithecus aethiops*) apparently are devoid of both HBsAg and antiHBs [6]. Up to now, there have been several studies on serological markers of HBV infection in *Cercopithecidae* monkeys [7, 8]. However, all studies showed negative results for serological HBV markers and no attempt at HBV amplification has been successful in this family [8]. Southeast Asia is an area endemic for HBV infection. Several studies have undertaken serological surveys on the families *Pongidae* and *Hylobatidae* to determine epidemiology, phylogenetic relationships and route of cross-species transmission. For example, Warren et al. have examined 195 orangutans from Borneo and Sumatra [2], Grethe et al. have investigated 12 gibbons from different parts of Thailand and one gibbon from Vietnam [4]. Noppornpanth et al. have performed studies on 101 captive gibbons from central Thailand [3]. Sall et al. have investigated the population of pileated gibbon and yellow-cheeked gibbon in the northern and south-western regions of Cambodia and east of the Mekong river [9]. From all these studies, a high prevalence (40–46%) of HBV infection in gibbons and orangutans in this region has become evident.

Upon characterization of the respective nucleotide sequences human hepatitis B virus was divided into distinct genetic groups. Accordingly, Okamoto et al. differentiated HBV into four genetic groups or genotypes (A, B, C, and D) based on nucleotide differences between sequences of 8% or above [10]. Subsequently, four additional genotypes of HBV (E, F, G and H) have been identified [11–16]. Genetic characterization is advantageous in that it reveals the relationship among these sequences as well as to the known primate HBV sequences. Many experiments have shown that the sequences of non-human primates are on a genetic branch separate from other known human HBV sequences [12, 13, 17–19]. Moreover, the phylogenetic clusters of this virus are directly related to geographic host distribution [4, 8, 9]. Cross–species transmission has been documented. GiHBV and OuHBV were grouped together because both species share the same habitat in Southeast Asia [20]. Interestingly, the primate viruses are closely related to the human HBV [21]. In addition, the structure of HBV virus found in humans and non-human primates is very similar. HBV transmission from humans to non-human primates or vice versa might be possible once their habitats overlap.

The study reported here has been aimed at elucidating epidemiology, pathogenicity and potential reservoirs of HBV infection in non-human primates. In addition, our group has sequenced the entire genomes of HBV isolated from the carriers. Furthermore, we have performed phylogenetic analysis on both virus isolates to investigate their genetic relatedness to the previously identified human and non-human primate strains of HBV.

Materials and methods

Study population

To investigate the potential reservoirs of HBV infection among captive non-human primates, 17 macaques (10 long-tailed macaques, *Macaca fascicularis*, four southern pig-tailed macaques, *Macaca nemestrina*, two stump-tailed macaques, *Macaca arctoides* and one rhesus macaque, *Macaca mulatta*), 15 gibbons (six white-cheeked gibbons, *Nomascus leucogenys*, one yellow-cheeked Gibbon, *Nomascus gabriellae,* six pileated gibbons, *Hylobates pileatus* and two white-handed gibbons, *Hylobates lar*), eight langurs (four silvered langurs, *Semnopithecus cristatus*, one Phayre's langur, *Semnopithecus phayrei*, and three dusky langurs, *Semnopithecus obscurus*) kept at Dusit zoo, Bangkok and 53 orangutans (*Pongo pygmaeus*) kept at Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand were subjected to this research project, which had been approved by the Faculty of Veterinary Science, Animal Care and Use Committee (FVS – ACUC), Mahidol University.

Sample collection

During the routine health check, all primates were anaesthetized. From each animal blood samples were collected by venipuncture and transferred to EDTA anticoagulant coated test tubes. Plasma was separated by centrifugation at 3000 rpm for 10 minutes and kept at −70°C until tested. The demographic data of the primates have been obtained from the records of the zoo and wildlife breeding center.

Serological method

Plasma samples were subjected to a biochemical analyzer (Hitachi 912, Roche Diagnostics, Mannheim, Germany) for biochemical analysis of alanine aminotransferase (ALT) and aspartate transaminase (AST). Plasma was assayed for HBsAg, antibodies to HBsAg (antiHBs), and antibodies to the HBV core antigen

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(antiHBc) by enzyme linked immunosorbent assay (ELISA) using the Murex HBsAg Version 3, Murex antiHBs and Murex antiHBc kit, respectively (Murex, Biotech Limited, Dartford, Kent, England).

HBV DNA extraction and detection

HBV DNA was extracted from 100-μl plasma samples using proteinase K in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation [22]. The DNA pellets were dissolved in 30 μl sterile distilled water. Subsequently, our team performed PCR and real-time PCR assays to determine the quantitative HBV DNA levels as previously described [23].

Whole genome amplification and sequencing

Two HBsAg positive samples of gibbons (*Nomascus leucogenys*) with high viral load and all seven samples of orangutans (*Pongo pygmaeus*) were subjected to complete HBV genome amplification by PCR using four primer sets selected from conserved regions so that the resulting amplicons overlapped contiguous fragments. The primer sequences of set one were PreS1F+ (5′-GGGTCACCATATTCTTGGGAAC-3′: position 2814 to 2835) and R5 (5′– AGCCCAAAAG-ACCCACAATTC–3′: position 1015 to 995); of set 2, F6 (5′– ATATGGATGATGTGGTATTGGG–3′: position 737 to 758) and X102 (5′– ACCTTTAACC-TAATCTCC-3′: position 1764 to 1748); of set 3, X101 (5′-TCTGTGCCTTCTCATCTG-3′: position 1552 to 1569) and CORE2 (5′-CCCACCTTATGAG-TCCAAGG-3′: position 2476 to 2457) and of set 4, CORE1 (5′-GAGTGTGGATTCGCACTCCTCC-3′: position 2268 to 2289) and R1 (5′-TGTAACACGAG-CAGGGGTCCTA-3′: position 201 to 180). The total 25-μl reaction mixture comprised 2 μl of a resuspended HBV viral DNA solution, 10 μl of 2.5X Eppendorf® MasterMix (Eppendorf, Hamburg, Germany), 0.5 μl of 25 μM primer, and sterile water. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 3 minutes followed by 35 cycles at 94°C for 30 s (denaturation), 55°C for 30 s (primer annealing), 72°C for 1.30 minutes (extension) and a final extension step at 72°C for 7 minutes. PCR-amplified products were examined by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light. Subsequently, the bands of interest were purified applying the Perfectprep®Gel Cleanup kit (Eppendorf, Hamburg, Germany). Cycle sequencing was performed using the AmpliTaq™ DNA Polymerase FS dye terminator cycle sequencing chemistry of the ABI PRISM™ BigDye™ Terminator CyCle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA). The reaction was performed according to the manufacturer's specifications. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and submitted to the GenBank database.

Phylogenetic analyses

The two sequences of captive gibbon HBV (G25 and G26) determined in the course of our previous study [3] in the Krabok Koo Wildlife Breeding Center, and the nine HBV sequences (two GiHBV and seven OuHBV sequences) obtained in the course of this study were aligned with each human genotype. Sequences were also compared with available complete genome sequences from chimpanzee, gibbon, orangutan, woolly monkey and gorilla. Our team performed phylogenetic analyses and genetic comparisons of HBV isolates applying the Clustal X (1.83) multiple alignment program. Subsequent analysis was performed by Molecular Evolutionary Genetics Analysis (MEGA) software version 3.1. Amino acid translations were accomplished using the ExPASy translation tool (available on http://www.expasy.ch/tools/dna.html).

Results

Seroprevalence of HBV in non-human primates

Ninety-three plasma samples of various non-human primates were tested for the presence of HBsAg and antiHBs and antiHBc antibodies. Sera positive for at least one marker of HBV infection were found only in gibbon (9/15; 60%) and orangutan (40/53; 75.47%). The results are shown in Table 1. Moreover, four gibbons and seven orangutans were identified as chronic carriers. To determine the liver pathology associated with this infection, ALT and AST levels were determined in the plasma of four HBsAg positive gibbons and 36 HBsAg negative animals. With one exception (GD14), the ALT and AST levels of infected individuals were within normal limits (0–40 U/l) upon comparison with HBsAg negative animals (Table 2). The mean ± SD of ALT and AST values in HBsAg negative animals were 33.64 ± 17.56 and 34.82 ± 15.15, respectively. Approximately 55.56% (5/9) of gibbons and 82.5% (33/40) of orangutans were non-carrier animals, as indicated by the presence of antiHBs and antiHBc antibodies.

Table 1 Seroprevalence of HBV and HBV DNA among captive non-human primates

Primate (n)	n	Positive HBV marker			
		HBsAg and antiHBc n (%)	antiHBs and antiHBc n (%)	antiHBc only n (%)	HBV DNA n (%)
1. Macaque (17)					
Macaca fascicularis	10	0 (0)	0 (0)	0 (0)	0 (0)
Macaca nemestrina	4	0 (0)	0 (0)	0 (0)	0 (0)
Macaca arctoides	2	0 (0)	0 (0)	0 (0)	0 (0)
Macaca mulatta	1	0 (0)	0 (0)	0 (0)	0 (0)
2. Langur (8)					
Semnopithecus cristatus	4	0 (0)	0 (0)	0 (0)	0 (0)
Semnopithecus phayrei	1	0 (0)	0 (0)	0 (0)	0 (0)
Semnopithecus obscurus	3	0 (0)	0 (0)	0 (0)	0 (0)
3. Gibbon (15)		4 (26.67)	5 (33.33)	0 (0)	4 (26.67)
Nomascus leucogenys	6	2 (33.33)	3 (50)	0 (0)	2 (33.33)
Nomascus gabriellae	1	1 (100)	0 (0)	0 (0)	1 (100)
Hylobates pileatus	6	1 (16.7)	1 (16.7)	0 (0)	1 (16.7)
Hylobates lar	2	0 (0)	1 (50)	0 (0)	0 (0)
4. Orangutan (53)					
Pongo pygmaeus	53	7 (13.21)	25 (47.17)	8 (15.09)	7 (13.21)
Total	93				

Table 2 Demographic data, ALT, AST, HBV serological markers and HBV viral load of captive non-human primates

No	Species	Sex	Age	Cage	Code	GenBank accession no.	ALT (U/l)	AST (U/l)	HBsAg	antiHBs	antiHBc	HBV DNA	HBV DNA (copies/μl)
1	*N. gabriellae*	M	8	–	GD13	–	17	14	+	–	+	+	2.830×10^6
2	*N. leucogenys*	F	21	–	GD14	EU155828	79	75	+	–	+	+	2.002×10^7
3	*N. leucogenys*	M	11	–	GD21	EU155829	18	14	+	–	+	+	3.085×10^7
4	*H. pileatus*	F	ND	–	GD22	–	16	9	+	–	+	+	5.26×10^6
5	*P. pygmaeus*	M	6–8	3/10	OS6	EU155821	ND	ND	+	–	+	+	3.660×10^6
6	*P. pygmaeus*	F	3–4	3/10	OS9	EU155822	ND	ND	+	–	+	+	1.875×10^6
7	*P. pygmaeus*	M	5	3/6	OS23	EU155823	ND	ND	+	–	+	+	4.680×10^5
8	*P. pygmaeus*	M	5	3/5	OS25	EU155824	ND	ND	+	–	+	+	7.070×10^6
9	*P. pygmaeus*	M	6–8	3/5	OS27	EU155825	ND	ND	+	–	+	+	1.210×10^7
10	*P. pygmaeus*	F	6–8	3/5	OS28	EU155826	ND	ND	+	–	+	+	5.440×10^6
11	*P. pygmaeus*	M	5	3/1	OS39	EU155827	ND	ND	+	–	+	+	8.250×10^5

ALT and AST normal range: 0–40 U/l. ALT and AST (Mean ± SD) values in animals with HBsAg negative were 33.64 ± 17.56 and 34.82 ± 15.15.
ND, no data.

Detection of HBV DNA in non-human primates

To quantify HBV DNA in non-human primates, we screened available plasma samples collected from various species by real-time PCR according to the previously described method [23]. The limit of this method is 100 copies per μl. The results from real-time PCR perfectly correlated with the results obtained by HBsAg screening. The levels of HBV DNA are shown in Table 2.

Gibbon and orangutan HBV nucleotide sequences

The complete HBV genomes from nine non-human primates, two from gibbon (*Nomascus leucogenys*) and seven from orangutan (*Pongo pygmaeus*), comprised 3182 nucleotides and showed genetic organization compatible with the human virus. The nucleotide sequences determined in this research study have been submitted to the GenBank database and assigned accession numbers EU155821–EU155827 (Orangutan), EU155828–EU155829 (Gibbon). All gibbon and orangutan HBV sequences were compared with the representative sequences in GenBank including the eight human HBV genotypes, orangutan, gibbon, chimpanzee, gorilla and woolly monkey sequences.

Pre-S/S gene

Our group aligned the nucleotides of the *Pre-S/S* region in order to establish nucleotide and amino acid

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differences between HBV isolates from gibbons, orangutans and human HBV genotypes B and C. Moreover, the GiHBV and OuHBV sequences were compared with other HBV strains. Based on the percentage of similarity, the highest percentage of similarity within each respective group, GiHBV and OuHBV displayed a higher percentage of similarity than to the human HBV (data not shown). The nucleotide sequences of two gibbons of this study, two gibbons from the previous study [3], seven orangutans and human HBV genotypes B and C were translated into amino acid sequences (Fig. 1A). Comparison of gibbon and orangutan *Pre-S/S* gene sequences with human HBV genotypes B and C, the genotypes endemic in Southeast Asia, revealed that HBV isolated from gibbons and orangutans had a deletion of 33 nucleotides representing 11 codons at the 5′end of the Pre-S1 region as had already been established (Fig. 1A). For G25, we discovered an insertion of Gln (Q) between Gly^{83} (G) and Ile^{84} (I). In addition, several variability were found in the Pre-S2 and S regions, but neither deletions nor insertions could be detected in all isolates.

On closer investigation, the only non-human primate (orangutan and gibbon) amino acids we found in the Pre-S1 region were Gln^{14} (Q), Glu^{27} (E), Leu^{33} (L), Thr^{56} (T), Val^{92} (V), in the Pre-S2 region, Val^{7} (V), and in the S region, Ala^{190} (A), Leu^{193} (L) and Ile^{213}(I).

Pre-C/C gene

The *Pre-C/C* gene sequences were less divergent than the *Pre-S/S* gene. Our team could not discern any mutations in either the core promoter region (nucleotide positions T1753C/A, A1762T and G1764A) or the *Pre-C* region (nucleotide positions T1858C, G1896A and G1899A). Yet, all our gibbon and orangutan sequences showed a G to T mutation at position 1896. This mutation induced an amino acid change from Try (W) to Leu (L) at amino acid residue 28 of the Pre-C region. The nucleotides at positions 2174 to 2413 of the core region were highly conserved; the resulting amino acid sequences were similar for all isolates. Alignment of the core protein's amino acids showed that the amino acids in this region are highly conserved among OuHBV, GiHBV and human HBV. The only non-human primate (orangutan and gibbon) amino acids are Leu^{28} in the Pre-C region and Val^{27} (V), Asn^{51} (N), Val^{59} (V), Thr^{67} (T), Ser^{70} (S), Asn^{74} (N), Pro^{179} (P) and Ala^{180} (A) in the C region (Fig. 1B).

Complete HBV genome

All sequences were analyzed by comparison with each of the human HBV genotypes A-H. The results showed that all isolates were 98–99% identical within the orangutan group and 93–98% within the gibbon group. Comparison between gibbon and orangutan sequences showed 90–91% identity (data not shown).

Phylogenetic analyses of gibbon and orangutan HBV

To determine the phylogenetic relationships, phylogenetic trees of the *Pre-S/S*, the *Pre-C/C* region and the complete nucleotide sequence were constructed.

Phylogenetic tree of Pre-S/S gene

This phylogenetic tree comprises the *Pre-S/S* nucleotide sequences of OuHBV and GiHBV isolates from the present project, representative of non-human primates and of each human HBV genotype from GenBank. All *Pre-S/S* sequences including gibbon sequences from our previous study were examined by neighbor joining analysis. The results are shown in Fig. 2A. Furthermore, the HBV isolates from the gibbons (GD14, GD21, G25 and G26) were found distantly (91–93%) related to the OuHBV sequences (OS6, OS9, OS23, OS25, OS27, OS28, and OS39). OuHBV in this study clustered with orangutan from Indonesia (Y17559) by 100% bootstrap value.

Phylogenetic tree of the Pre-C/C gene

The results of phylogenetic analysis of the *Pre-C/C* gene were similar to the Pre-S/S region in that the gibbon and orangutan. *Pre-C/C* gene was on separate branches from each human genotype (Fig. 2B). The GiHBV sequence determined from our earlier research branched most closely with AJ131574 for G25 and clustered with AY330914 and AJ131568 for G26. The bootstrap values were 49 and 98%, respectively. Whereas GD14 and GD21 were different from the GiHBV of the preceding study because they clustered with AJ131573 with a bootstrap value of 100%. Both AJ131573 and AJ131574 had been isolated from *H. concolor* kept in Dusit zoo, Thailand. All orangutan sequences related with AF193863, an orangutan virus from Kalimantan, Indonesia.

Phylogenetic tree of the complete HBV genome

The complete HBV sequences of non-human primates our team had arrived at were compared with sequences representative of each group of human HBV genotype, orangutan, gibbon, gorilla, chimpanzee, and woolly monkey HBV in the GenBank database (Fig. 2C). The woolly monkey sequences were used as an out group. The data support that each of the human genotypes clusters separately from non-human primates whereas all sequences obtained from gibbons and orangutans

(A) Pre-S/S protein

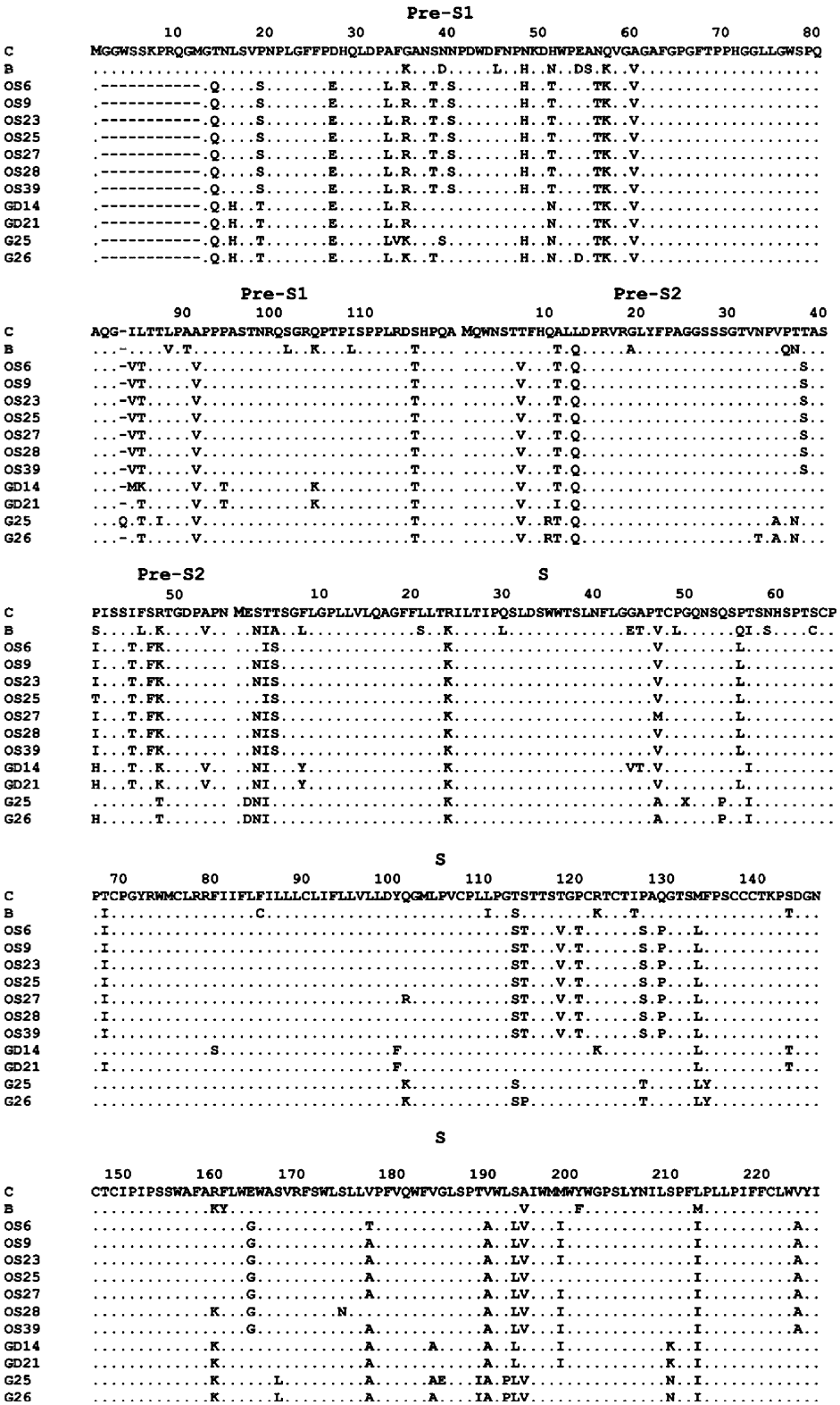

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Fig. 1 Alignment of amino acid sequences of the complete *S* gene (Pre-S1, Pre-S2 and S domain) (A) and complete *C* gene (Pre-C and Core domain) (B) from four gibbons (GD14, GD21, G25 and G26) and seven orangutans (OS6, OS9, OS23, OS25, OS27, OS28 and OS39) with human HBV genotypes B (accession no. D00331) and C (accession no. X04615). Dots indicated conserved amino acids. Dashes indicated deletion amino acids. Changing amino acids were indicated in letters. A master sequence based on the comparison was shown in the upper line. The percentages of similarity of the complete *S* genes were 99% within the orangutan group, 94% within the gibbon group, 89–91% between the orangutan and human HBV, 89–92% between the gibbon and human HBV and 91–93% between the orangutan and gibbon. While, *Pre-C/C* gene were 98–99% within the orangutan group, 94–98% between gibbon group, 90–92% between the orangutan and human HBV, 90–93% between the gibbon and human HBV and 92–94% similarity between the orangutan and gibbon.

(B) Pre-C/C protein

```
                Pre-C                                   C
                10        20                  10        20        30        40        50
C       MQLFHLCLIISCSCPTVQASKLCLGWLWG MDIDPYKEFGASVELLSFLPSDFFPSIRDLLDTASALYREALESPEHCSP
B       ............................. ..................................................
OS6     .........................L. ..........T.............V.....................
OS9     .........................L. ..........T.............V.....................
OS23    .........................L. ..........T.............V..V..................
OS25    .........................L. ......Y...............V.....................
OS27    .........................L. .........GT....F....E....V.....................
OS28    .........................L. ..........T.............V..R..................
OS39    .........................L. ..........T.............V.....................
GD14    .........................L. ..........TA.......P.....V.....................
GD21    .........................L. ..........T.............V.....................
G25     ................F........L. ..........T.............V.....................
G26     ................I........L. ..........T.............V.....................

                                        C
                60        70        80        90       100       110       120       130
C       HHTALRQAILCWGELMNLATWVGSNLEDPASRELVVSYVNVNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPP
B       ..............................................................................
OS6     N.......V.......T..S...N............N...N.....................................
OS9     N.......V.......T..S...N............N...N.....................................
OS23    N...F...V.......T..S...N............N...N.....................................
OS25    N.......V.......T..S...N............N...N.....................................
OS27    N.......V.......T..S...N............N...N.....................................
OS28    N......GV.......T..S...N............N...N.....................................
OS39    N.......V.......T..S...N............N...N.....................................
GD14    N.......V.......T..S...N............N...H.....................................
GD21    N.......V.......T..S...N............K.....................................
G25     N.......V.......T..S...N.............N.....................................
G26     N.......V.......T..S...N............N...N.....................................

                                C

                140       150       160       170       180
C       AYRPPNAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPGRRRSQSRESQC
B       ..........................................R...........
OS6     ..........................................R......PA...
OS9     ..........................................R......PA...
OS23    ..........................................R......PA...
OS25    ..........................................R......PA...
OS27    ..........................................R......PA...
OS28    ..........................................R......PA...
OS39    ..........................................R......PA...
GD14    ..........................................R......PA...
GD21    ..........................................R......PA...
G25     ..........................................R......PA...
G26     ..........................................R......PA...
```

Fig. 1 (Continued)

in this study can be grouped with previously published GiHBV and OuHBV sequences. The novel gibbon sequences clustered as a subgroup with the gibbon sequences previously obtained.

Discussion

Upon screening various non-human primate species for HBV infection, our group established that approximately 60% of gibbons and 75% of orangutans showed at least one marker of HBV. This rate in gibbons is higher than that found in our previous study (approximately 40%) [3]. In addition, the rates of active infection defined by detectable HBV DNA in gibbons (26.7%) and orangutans (13.2%) are similar to those of non-human primates in Central Africa and Southeast Asia [8]. However, the results in this study were obtained from wild-born primates kept in captivity, and the prevalence of HBV infection in wild gibbons and orangutans is unknown. In contrast, we

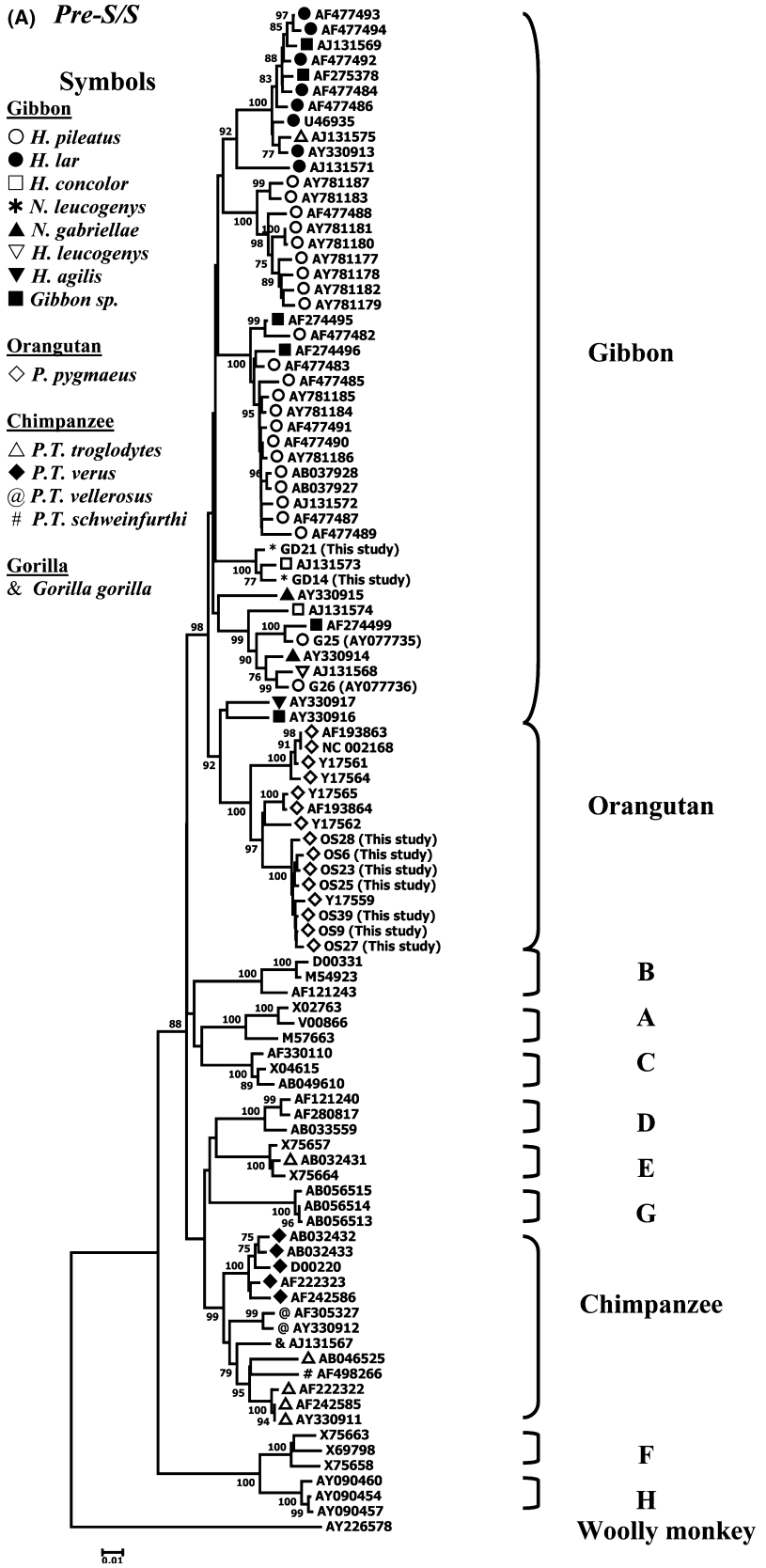

Fig. 2 Phylogram depicting the phylogenetic relationship of the sequence obtained from the present study and representative sequences of non-human HBV strains from GenBank. Regions included in the comparison were: (A) the large *S* gene including *Pre-S1*, *Pre-S2* and HBsAg gene; (B) the *C* gene, including Pre-C and Core region; (C) entire genome. Percentage bootstrap values (>75%) were shown at the respective nodes. The scale bar at the bottom indicated the genetic distance. The species origin of sequences obtained in this study and in previous studies was indicated by the symbol. [GenBank accession numbers: Gi-HBV – GD14 (EU155828); GD21 (EU155829), OuHBV – OS6 (EU155821); OS9 (EU155822); OS23 (EU155823); OS25 (EU155824); OS27 (EU155825); OS28 (EU155826); OS29 (EU155827)].

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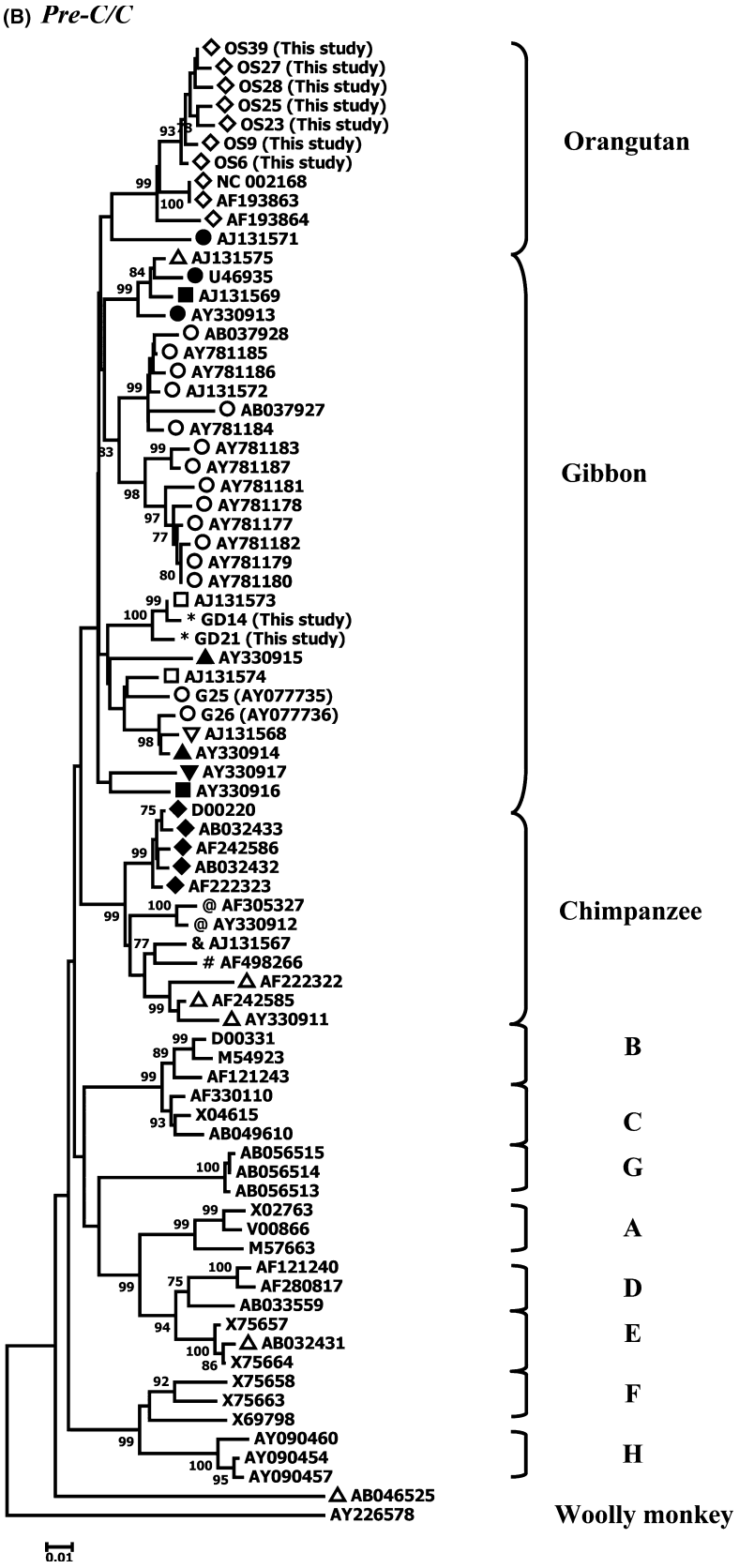

Fig. 2 (Continued)

(C) **Entire genome**

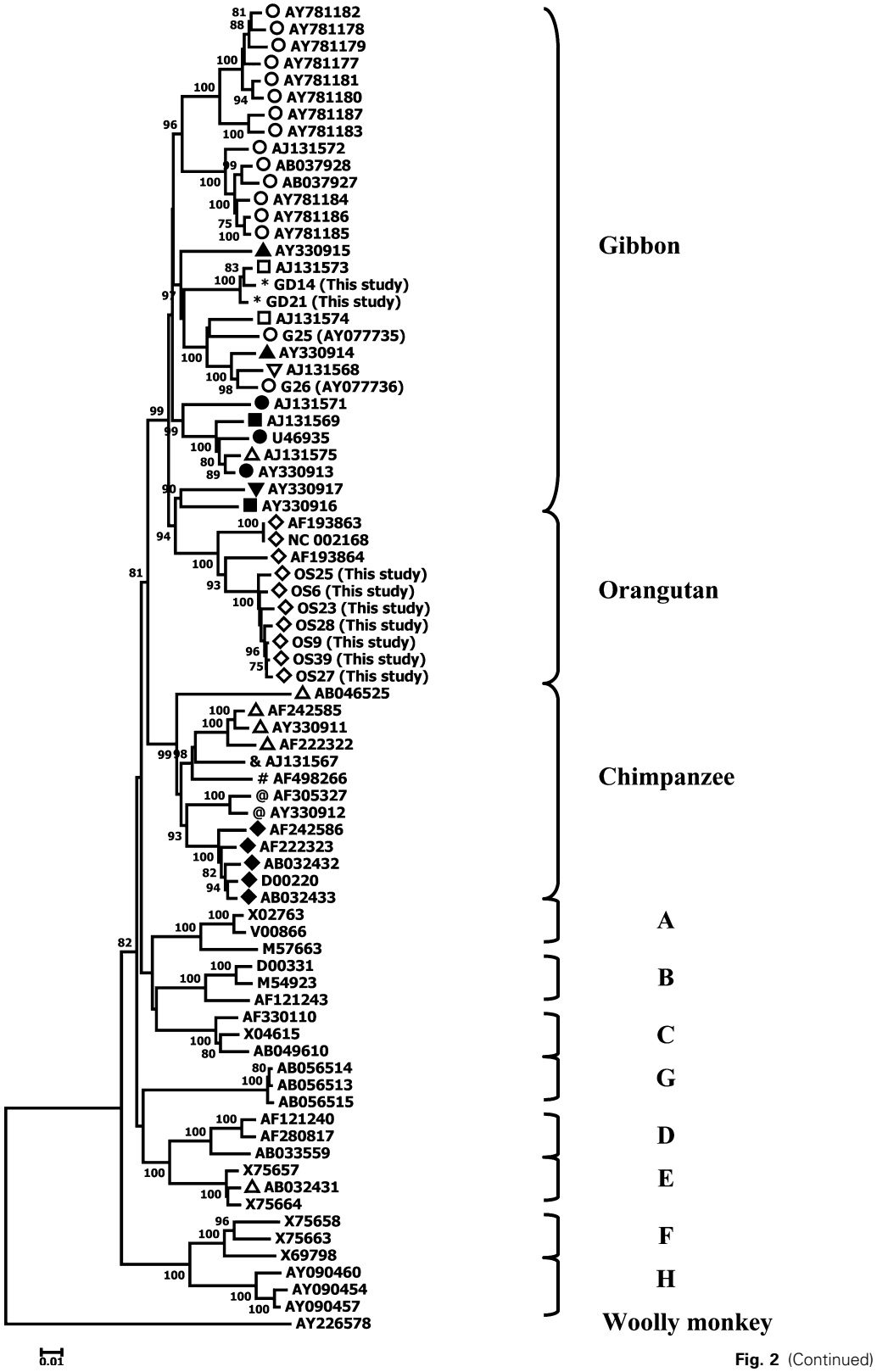

Fig. 2 (Continued)

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could neither detect any HBV marker nor HBV DNA by real-time PCR in either macaques or langurs. The finding that HBV can and does infect only the members of the families *Pongidae* and *Hylobatidae* strongly supports previous evidence for their rather narrow host range [6–8].

Our results regarding HBV serological markers showed that the majority of orangutans and gibbons had been infected with the virus but managed to resolve the infection, which became apparent by positive results for antiHBs, and negative results for HBsAg and HBV DNA. In contrast, some orangutans and gibbons became chronic carriers and displayed positive results for serum HBsAg and HBV DNA. Interestingly, approximately 15% of orangutans displayed only antiHBc without HBsAg or antiHBs usually accompanying this marker. Such atypical serology is likely attributable to resolved HBV infection since HBV DNA could not be detected in any of the orangutans' plasma. In humans, antiHBc-positive individuals lacking HBsAg are usually considered to have been previously exposed to HBV infection, but a proportion of these patients may have subclinical or occult HBV infection as HBV DNA can be detected in the liver. Occult HBV status is in some cases associated with mutant virus undetectable by commercial HBsAg assays, but more frequently results from a strong suppression of virus replication and gene expression [24]. Although the significance of isolated antiHBc in non-human primates is unclear, the serological markers of HBV infection in gibbons and orangutans may be similar to those described in humans.

In humans, several researchers have reported that patients with chronic HBV infection often present mutations in the basic core promoter region [24]. Accordingly, we aligned the Core regions to analyze nucleotide positions 1753, 1762 and 1764 including the *Pre-C* variant's nucleotide positions 1858, 1896 and 1899. We could not detect any mutations at those positions except for a G to T substitution at nucleotide position 1896. This substitution had occurred in all gibbon and orangutan HBV sequences described here, and was commonly found in non-human primate sequences. It has been proposed that the difference of RNA secondary structure between infected humans and non-human primates may be responsible for this discrepancy [25, 26].

Upon phylogenetic analysis, all seven complete genome sequences of HBV-infected orangutans obtained in this study grouped with those derived from previously published sequences. Indeed, they showed genetic relatedness to the HBV isolates from gibbons, particularly the *Hylobates* species that share geographical habitat ranges. The branches occupied by all orangutans analyzed clustered together, and displayed very close phylogenetic relatedness to an HBV isolate obtained from Indonesia (Y17559) [2]. This isolate showed a very high percentage of sequence similarity (approximately 98–99% identity of the *Pre-S* gene) to all isolates described here, suggesting that they had originated from a common source. Although the geographic origin of the orangutans described in this study was unknown, prior to their capture they probably inhabited Borneo and Sumatra, as wild living orangutans are generally restricted to these islands. Thus, the primary source of this HBV strain found in captive orangutans may have originated from the wild.

HBV isolates from gibbons in this study (*N. leucogenys*) were phylogenetically separated from gibbons (*H. pileatus and H.lar*) described in our preceding research, but were almost identical to a gibbon isolate (*H. concolor*) that was reported to have originated from a Thai zoo [4], suggesting several strains of HBV circulate in gibbons in Thailand. Previous data have shown that the HBV isolates from gibbons in different regions of Thailand and Vietnam could be classified into four phylogenetically distinct genomic groups [4]. Likewise, there appears to be a substantial difference in HBV strain distribution between gibbons from Thailand and those from Cambodia [9]. These observations can be explained by the different geographical location as well as different species (and sub-species) of non-human primates, which in turn may have determined the particular HBV strains infecting those animals in this geographic region.

In humans, a high percentage of individuals who become infected by horizontal transmission during adolescence or adulthood have a short duration of infectivity and clear the virus. In contrast, mother-to-child perinatal transmission generally leads to lifelong chronic HBV infection due to a prolonged stage of immunological tolerance and it is considered to be an essential mechanism for the persistence of HBV infection in human populations. Similar to HBV infection in humans, our previous study has documented that HBV in captive gibbons can be transmitted by vertical and horizontal routes [3]. Frequent vertical transmission in captive gibbons supports the assumption that this may be a main mechanism for the continuation of HBV infection in gibbons and potentially other ape species in the wild [8]. In addition, horizontal transmission also represents an important route for HBV distribution in the wild as well as in captivity. For instance, orangutans in the

wild are solitary apes with restricted contact with other individuals and thus, the possibility of horizontally acquired infection is limited. When captured and housed together, the probability of orangutans to be exposed to HBV appears to be increased. Indeed, the pronounced sequence similarity of HBV among infected orangutans described here strongly suggests that the transmission of HBV among these apes might have been relatively recent, possibly due to horizontal spread from an animal infected in the wild prior to its capture.

Despite the previous hypothesis of species-specific HBV infection, a geographical basis rather than species association accounting for the distribution of HBV variants has been increasingly recognized. For instance, HBV in orangutans consistently grouped within the gibbon clade in South-East Asia and similarly, a gorilla sequence (AJ131567) clustered with chimpanzee sequences in Central Africa. Lack of strict species-specificity of HBV variants was also reported for a chimpanzee sequence (AJ13575) grouped with a gibbon cluster, and another chimpanzee sequence (AB032431) grouped with human HBV genotype E. These observations support probable interspecies transmission which could be explained by sharing a common habitat in geographic regions with high prevalence of HBV infection, such as South East Asia and Central Africa. Thus, the more the regions both species inhabit overlap, the higher the probability of cross species transmission [8, 9]. This probability has currently been investigated by several researchers in order to elucidate the ultimate origin and evolution of HBV in humans and non-human primates.

Experimental transmission of human HBV to non-human primates by exposure to human saliva containing HBV has been reported [27, 28]. Hu et al. constructed a phylogenetic tree and found that the S gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from humans to chimpanzees [17]. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV [3]. Yet, non-human primate virus could probably be transmitted to humans as the respective HBV genomes are largely similar. Due to this similarity, HBV vaccine can be used to prevent cross-transmission between species. In fact, HBV isolated from our gibbons and orangutans contain glycine at position 145 of the '*a*' determinant indicating that HBV vaccines should be effective. However, the route of HBV transmission from non-human primates to humans ought to be elucidated.

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Original Article

Molecular epidemiological study of hepatitis B virus in Thailand based on the analysis of *pre-S* and *S* genes

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Aims: This study was undertaken to determine the prevalence and characteristics of hepatitis B virus (HBV) genotypes, antigen subtypes, "a" determinant variants and *pre-S* gene mutations circulating on a large scale in Thailand.

Methods: The sequences of the *Pre-S1*, *Pre-S2* and *S* regions were determined in serum samples of 147 HBsAg and HBV DNA-positive subjects who had been enrolled from the nationwide seroepidemiological survey conducted on 6213 individuals in 2004.

Results: The results showed that genotypes C, B and A accounted for 87.1%, 11.6% and 1.3%, respectively. The distribution of the HBV antigen subtypes was: adr (84.4%), adw (14.2%) and ayw (1.4%). Regarding the "a" determinant, 2/43 (4.65%) and 2/104 (1.92%) samples of vaccinated and non-vaccinated subjects, respectively, displayed mutations, all of which were Thr126Asn. Sequencing analysis showed the *pre-S* mutations in 14 (9.5%) samples, with *pre-S2* deletion as the most common mutant (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 *pre-S* mutations were associated with older age and higher mean serum HBsAg level.

Conclusion: This study demonstrated that HBV genotype/subtype C/adr and B/adw were the predominant strains circulating in Thailand. The "a" determinant variants seemed to be uncommon, and might not be attributed to vaccine-induced mutation.

Key words: genotype, hepatitis B virus, *pre-S* mutation, subtype, Thailand

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is associated with a diverse clinical spectrum of liver injury ranging from asymptomatic carriers to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).[1] HBV, a member of the *hepadnaviridae*, is a relaxed circular double-stranded DNA virus of approximately 3200 base pairs (bp) in length, with four overlapping open reading frames encoding the polymerase (P), core (C), envelope (pre-S1/pre-S2/S) and X proteins.[1] The virus shows remarkable genetic variability and is currently classified into eight genotypes, designated A to H, and four major serotypes, ayw, ayr, adw and adr, based on entire genome and S gene sequence analysis.[2,3] HBV genotype distribution appears to show varying geographic patterns.[4] For instance, genotypes A and D are predominant in Western countries and India, whereas genotypes B and C prevail in South-East Asia, China and Japan. Genotype E is restricted to Africa and genotype F is found in Central and South America. In Thailand, a highly endemic area of HBV infection, genotypes C and B are predominant in patients with chronic liver disease and in the general population, accounting for approximately 70–90% and 10–30%, respectively.[5–9]

Since 1992, HBV vaccination has been integrated into the expanded program on immunization (EPI) in Thailand. Based on our recent study, the subsequent coverage rates with the complete three-dose course of HBV

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vaccine were 82.3% and 97.3% in 1999 and 2004, respectively.[10,11] Hepatitis B surface antigen (HBsAg), used in current vaccines, contains an "a" determinant located between amino acids (aa) 121 and 149, which is believed to be the major target of polyclonal antibody against the antigen (antibody to hepatitis B surface antigen, anti-HBs). The emergence of "a" determinant variants has been observed in some vaccinated individuals and may remain undetected by current diagnostic assays.[12] Although the *S* gene mutants' potential significance for failure of immunization has been studied in some endemic countries,[13] it has so far remained unclear whether the "a" determinant variants play a role in vaccine failure in Thailand. In this study, using the serum samples from our recent large-scale survey,[10] we investigated the HBV genotypes and antigen subtypes circulating in four geographic areas of Thailand. We also determined the frequency of "a" determinant variants in these subjects, specifically those related to vaccine escape. Finally, we examined the prevalence and characteristics of the *pre-S* gene mutations predominant in these populations.

METHODS

Study population

FROM MAY TO October 2004, we conducted a nationwide seroepidemiological survey on 6213 healthy subjects from four provinces including Chiangrai, Udon Thani, Chonburi and Nakhon Si Thammarat, chosen as geographic representations of populations in the north, north-east, center and south of the country, respectively. The details of the study have been reported elsewhere.[10] In every province, approximately 1500 individual serum samples were collected and stored at −70°C until analysis. All serum samples were examined for HBsAg by using commercially available automated ELISA assays (AxSYM; Abbott Laboratory, North Chicago, IL, USA). Of these, 246 (4%) serum samples were seropositive for HBsAg. In the present study, 201 serum samples were available for hepatitis B e antigen (HBeAg) assay (Enzygnost HBe monoclonal; Dade Behring, Marburg, Germany) and molecular characterization of HBV. Table 1 shows the age distribution of the subjects regarding previous HBV vaccination. The project was approved by the Ministry of Public Health and the ethical committee of the Faculty of Medicine, Chulalongkorn University. The subjects or the parents of all participating children also consented to the study.

Table 1 Age distribution of the subjects regarding previous HBV vaccination

Age	No	Vaccine	Vaccine complete
(A)			
0–10	1932	1655	1617
10–20	1208	741	588
20–30	803	368	214
30–40	792	281	123
40–50	763	224	87
>50	715	159	31
Total	6213	3428	2660
(B)			
0–10	10	9	9
10–20	35	19	15
20–30	44	30	22
30–40	49	23	9
40–50	37	17	6
>50	26	8	1
Total	201	106	62

(A) 6213 healthy subjects; (B) 201 HBsAg-positive subjects.

HBV-DNA preparation, amplification and direct sequencing

Total DNA was extracted from 100 μL stored serum (−70°C) by using proteinase K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. DNA pellets were dissolved in 30 μL sterile water and subjected directly to polymerase chain reaction (PCR)-based amplification. PCR was performed by using primers to amplify the *Pre-S1*, *Pre-S2* and *S* genes. The primers consisted of a forward primer *Pre-S1* F (nt 2,817–2838: 5′-TCACCATATTCTTGGG AACAAGA-3′) and reverse primer R4 (nt 689–668: 5′-ATGGCACTAGTAACCTGAGCC-3′). The PCR conditions comprised initial predenaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1.30 min. Amplicons were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide and observation under UV light. For automated DNA sequencing, the PCR products 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, MA, USA). The sequencing products were subjected to a Perkin Elmer 310 Sequencer (Perkin-Elmer). The results were analyzed and HBV genotypes were determined by BLAST analysis. HBV-DNA sequences were also subjected to

Table 2 The prevalence of HBV genotypes in different geographic regions of Thailand

	Genotype (%)			Total
	A	B	C	
Chiangrai (northern)	1 (1.61)	10 (16.13)	51 (82.26)	62
Nakhon Si Thammarat (southern)	1 (5.00)	–	19 (95.00)	20
Udon Thani (north-east)	–	1 (2.17)	45 (97.83)	46
Chonburi (central)	–	6 (31.58)	13 (68.42)	19
Total	2 (1.36)	17 (11.56)	128 (87.08)	147

phylogenetic analysis. The distribution of the HBV subtypes was deduced from amino acid sequences at positions 122 (Lys, Arg for d, y determinants) and 160 (Lys, Arg for w, r determinants).[3]

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. The reference strain from GenBank accession numbers AB11946, AB112063 for genotype C subgenotype Cs; AB014360, D50520, X75665, AB042282 and AY641558 for genotype C subgenotype Ce; D23677, D23678 and D23679 for genotype B; AB116089 and AB116083 for genotype A subgenotype Aa; AJ012207, AY128092 and X70185 for genotype A.

Statistical analysis

Data were expressed as mean ± SD, and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney *U*-test or Student's *t*-test for quantitative variables. *P*-values below 0.05 were considered significant. All statistical analyses were performed using the SPSS software for Windows 10.0 (SPSS, Chicago, IL).

RESULTS

Distribution of HBV genotypes

OF THE 201 HBsAg-positive subjects, HBeAg was detected in 56 serum samples (27.9%). One hundred and forty-seven (73.1%) of the 201 HBsAg-positive subjects were positive for HBV-DNA in the sera, detected by PCR. The 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 and were determined as subgenotype Cs, 17 (11.6%) cases belonged to genotype B, and two (1.3%) cases to genotype A, with all of these being determined as subgenotype Ae. The distribution of the HBV antigen subtypes among these subjects was: adr (84.4%), adw (14.2%) and ayw (1.4%). In this study, all cases with genotype A belonged to subtype adw. For genotype B, 15 of them belonged to subtype adw and two to subtype ayw. For genotype C, 124 and four of them belonged to subtypes adr and adw, respectively. HBV genotype and subtype prevalence according to geographic distribution is shown in Tables 2 and 3, respectively. Although genotype C was the most common

Table 3 The prevalence of HBV subtypes in different geographic regions of Thailand

	Subtype (%)			Total
	adr	adw	ayw	
Chiangrai (northern)	49 (79.03)	12 (19.35)	1 (1.61)	62
Nakhon Si Thammara (southern)	19 (95.00)	1 (5.00)	–	20
Udon Thani (north-east)	45 (97.83)	–	1 (2.17)	46
Chonburi (central)	11 (57.89)	8 (42.11)	–	19
Total	124 (84.35)	21 (14.23)	2 (1.36)	147

Table 4 The clinical and virological data of the subjects with the "a" determinant mutations

	Age	Sex	Genotype	Subtype	Vaccine	HBsAg (S/N)
NK652	33	M	C	adr	–	324.54
NK052	58	F	C	adr	–	374.06
NK110	13	F	C	adr	+	389.18
UD767	8	M	C	adr	+	268.64

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 ($P = 0.001$).

The obtained sequences were submitted to GenBank under accession numbers DQ361314–DQ361535. The sequences were also aligned with those of the isolates of known genotype and subjected to phylogenetic analysis (Fig. 1).

Prevalence and characterization of the "a" determinant mutations

Subsequent sequencing revealed the prevalence and variation of the "a" determinant mutations among the populations 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 ($P = 0.355$). The clinical and virological data of these four subjects are shown in Table 4.

Prevalence and characterization of *pre-S* mutations

Based on direct sequencing, *pre-S* mutations were detected in 14 of 147 cases (9.5%). 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 ($378–8 \pm 64.4$ vs. 305.7 ± 111.0, $P = 0.017$). The alignment of amino acid sequences of the entire *pre-S1*/*pre-S2* region of the 14 samples is shown in Figure 2.

DISCUSSION

MOLECULAR EPIDEMIOLOGICAL STUDIES provide valuable information on understanding the prevalence and characteristics of HBV genotypes and mutations from different areas of the world. 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.[14] In this nationwide study, we confirmed the predominance of categories C/adr and B/adw among the HBV strains in Thailand, which accounted for more than 95% of cases. These findings are not surprising; they reflect the typical genotypes and subtypes circulating in Thailand and South-East 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.[15] Genotype C is also associated with a lower rate of hepatitis B e antigen (HBeAg) seroconversion and a lower response rate to alpha interferon therapy compared to genotype B.[16]

From the recent large-scale survey, we found that among subjects who had had complete vaccination, the HBsAg carrier rate was 21 of 2151 (0.98%).[10] The potential causes for these failures are unclear, but might include improper administration of the HBV vaccine, false-positive HBsAg results, intrauterine infection, low viral replication with or without integration of viral DNA into hepatocyte genomes and S gene mutation. The emergence of S gene variants, with mutations mainly occurring within the "a" determinant, has been observed in some vaccinees in several regions of the world.[12,17,18] Naturally occurring escape mutants have also been reported in chronic carriers after long-term

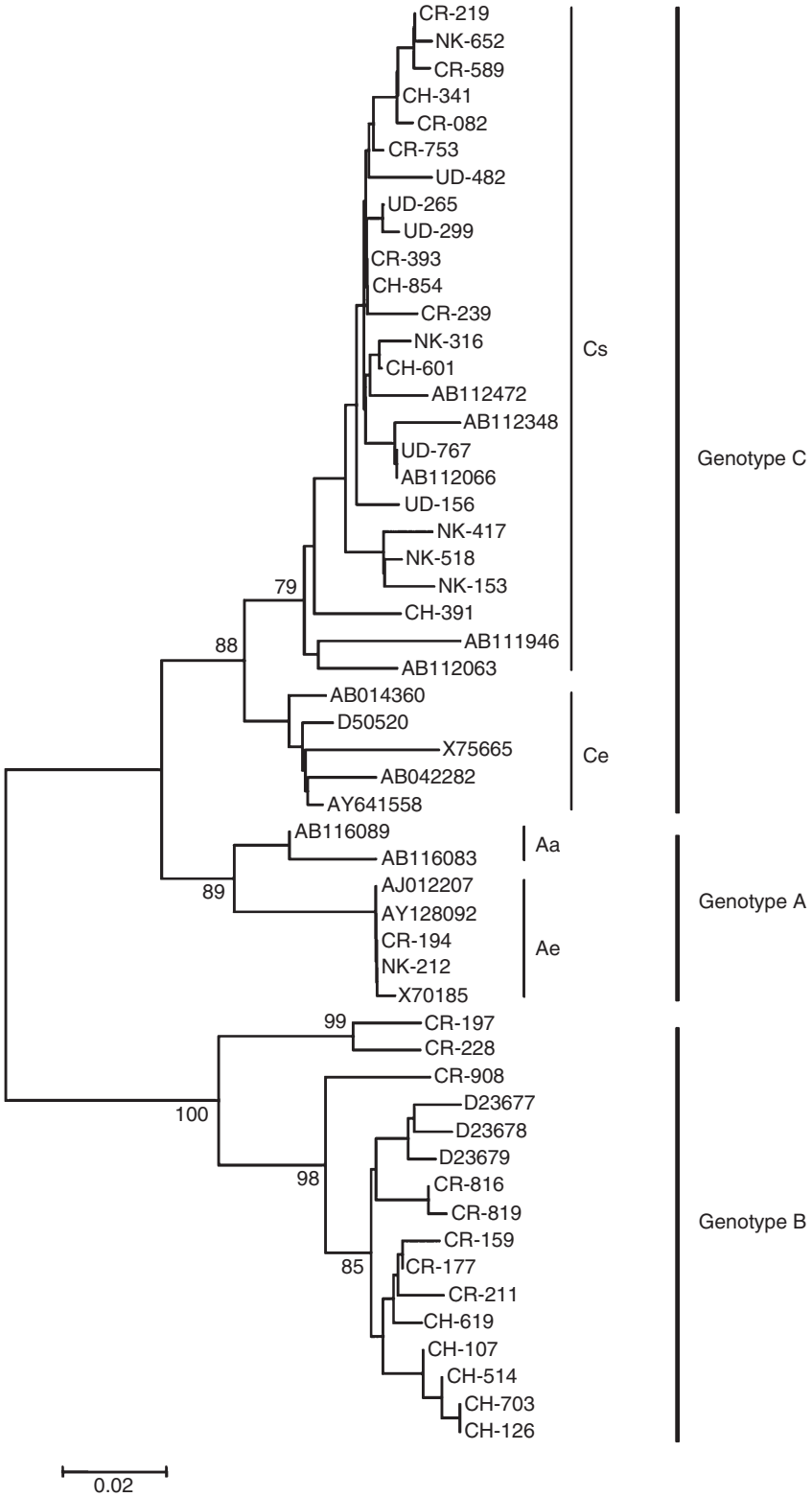

Figure 1 Phylogenetic analysis of the genotype of hepatitis B virus in different geographic regions of Thailand: Chiangrai (CR), Chonburi (CH), Nakhon Si Thammara (NK) and Udon Thani (UD).

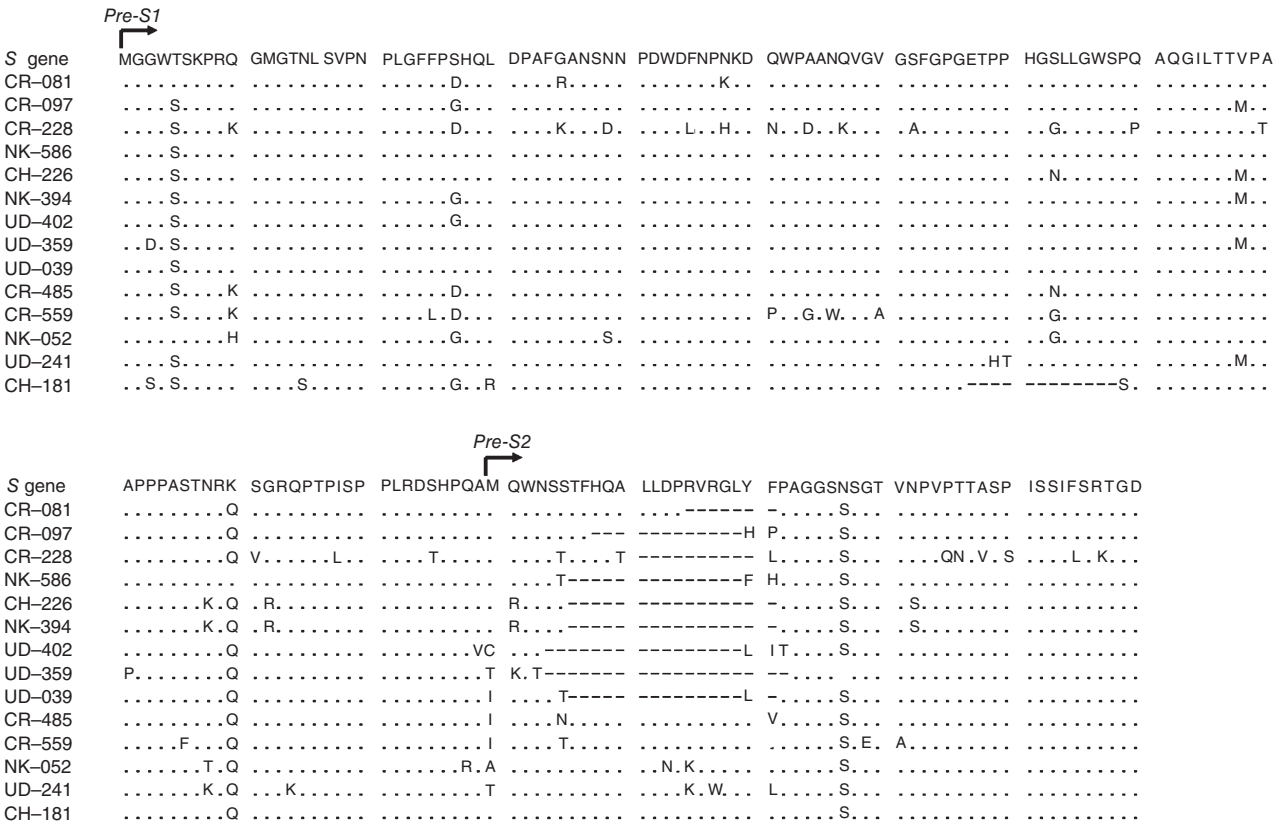

Figure 2 The amino acid sequences alignment of the entire *pre-S1*/*pre-S2* of 14 *pre-S* mutations samples. Chiangrai (CR), Chonburi (CH), Nakhon Si Thammara (NK) and Udon Thani (UD).

follow-up. 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.[19] The prevalence of the HBsAg mutants was also significantly higher among those fully vaccinated than among those not vaccinated. This finding suggests that vaccination might have increased a selection pressure on the emergence of surface mutants in relation to wild-type HBV. In this study, our data showed that only two vaccinated subjects and two non-vaccinated subjects had the same mutant-bearing virus affecting amino acid position 126. As a result, it seems that "a" determinant HBV mutants may 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,[20] 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 that observed in this study.

Interestingly, the characteristics of the "a" determinant mutations detected in the present study were that they occurred only at position 126. Indeed, the most common mutation causing vaccine escape involves the mutation at position 145, which is located in the second loop of the "a" determinant.[12] Subsequent studies conducted on vaccinated and non-vaccinated individuals have also demonstrated other variants affecting amino acid positions 120, 123, 124, 126, 129, 131, 141 and 144.[17,18] These changes in the "a" determinant may have emerged through host immunoselective pressure, as previously mentioned. Alternatively, it is also possible that the mutant is infectious and has been transmitted by

another individual. It should also be noted that the vast majority of studies on vaccine- or hyperimmune globulin-induced escape mutations have investigated subtype adw or ayw of HBV, in which position 126 is different from adr (threonine in subtypes adw and ayw [genotypes A, B and D] and isoleucine in subtype adr [genotype C]). Thus, differences in genotype may be another reason for the preferred mutations in the first loop, especially at position 126 in the present study investigating only subtype adr/genotype C.

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.[21] 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.[22] In that report, the prevalence of *pre-S* mutations among Thai patients amounted to 10.5%, which was consistent with the results of our study (9.5%). Regarding the site of mutations, our report showed that *pre-S2* deletion was the most common mutation type, followed by *pre-S2* start codon mutation, both *pre-S2* deletion and start codon mutation, and *pre-S1* deletion. 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 commonly prevailing.[22,23] Interestingly, our data showed a higher prevalence of *pre-S* mutations in 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 patients with *pre-S* mutations was significantly higher than that of those without the mutants. This observation also confirmed previous data suggesting that the prevalence of *pre-S* mutations tends to increase in direct relation to the patient's age.[22–24] The mutations found in this study, along with similar observations in previous reports, could help to elucidate the evolutionary pattern of mutations in the clinical course of persistent HBV infection.

In conclusion, our study demonstrated that HBV genotype/subtype C/adr and B/adw were the predominant strains circulating in Thailand. Furthermore, the "a" determinant variants seemed to be uncommon in HBV carriers, and might not be attributed to vaccine-induced mutation.

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CASE REPORT

Dynamics of HBV DNA Levels, HBV Mutations and Biochemical Parameters during Antiviral Therapy in a Patient with HBeAg-Negative Chronic Hepatitis B

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SUMMARY Chronic hepatitis B virus (HBV) infection leads to long-term sequelae such as cirrhosis and hepatocellular carcinoma. Antiviral therapy aims at controlling the viral replication and thus, decreasing the likelihood of such complications. In this study, we evaluated the dynamics of biochemical and virological parameters over 10 years of antiviral therapy in a Thai patient with chronic HBeAg-negative HBV infection, who had relapsed after two courses of interferon alfa treatment. Lamivudine administration initially led to a significant reduction in alanine aminotransferase (ALT) and HBV DNA levels, but a subsequent emergence of YIDD mutants caused an ALT flare and a virus breakthrough. A 4-log HBV DNA decrease and normalization of the ALT level were achieved within 3 months of adefovir monotherapy without any relapse during follow-up exceeding 20 months. Thus, careful monitoring during treatment and knowledge of cross-resistance to antiviral salvage therapy are crucial for the management of patients with chronic hepatitis B.

Treatment of chronic hepatitis B by antiviral therapy is aimed at driving viral replication to the lowest possible level, and thereby halting the progression of liver disease and preventing the onset of complications. The currently approved agents for treatment of chronic hepatitis B are standard or pegylated interferon alfa and nucleos(t)ide analogues such as lamivudine, adefovir and entecavir.[1] Compared with interferon alfa, the nucleos(t)ide analogues, which inhibit viral replication by targeting the HBV reverse transcriptase (RT) activity, have excellent safety and tolerability profiles that allow more patients to be treated for prolonged periods of time. These oral agents consistently result in improved liver histology, including reduced fibrosis, enhanced hepatitis B e antigen (HBeAg) seroconversion, normalization of alanine aminotransferase (ALT) levels, and clinical improvement in patients with advanced liver disease.[2] These agents, however, are also associated with the emergence of point mutations in the HBV

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polymerase gene (genotypic resistance) which can restore partial replication fitness to the mutant strains of HBV and lead to the redetection of previously suppressed HBV DNA and to a biochemical relapse (phenotypic resistance).[3]

Prolonged treatment with lamivudine results in the emergence of resistant viruses in 24% of patients following 1 year of therapy and 70% of patients following 4 years of therapy.[4] Mutations conferring resistance to lamivudine have been mapped in the conserved tyrosine, methionine, aspartate, aspartate (YMDD) motif within the C domain of the viral RT (rtM204I/V).[5,6] They are frequently associated with compensatory mutations in the conserved B domain (rtV173L, rtL180M) that partially restore the replicative capacity of YMDD mutant strains in vitro, and are associated with a 1000-fold reduction in the susceptibility to the drug.[6] Switch or add-on of other antiviral drugs such as adefovir is adequate to suppress lamivudine-resistant HBV and improves liver function in patients with chronic HBV infection.[6] Unlike lamivudine therapy, adefovir therapy is associated with delayed and infrequent selection of drug resistant viruses.[4]

In this report, we describe a patient who failed interferon alfa therapy and subsequently failed lamivudine therapy. The patient was successfully treated with adefovir. The aim of the study was to retrospectively investigate the dynamics of biochemical and virological parameters throughout the oral antiviral therapies administered to this patient.

CASE REPORT

The patient was a 24-year-old Thai man who had been diagnosed with HBeAg-negative chronic hepatitis B without hepatitis C virus (HCV) or human immunodeficiency virus (HIV) co-infections. In 1996, at the age of 13 years, the first diagnosis of chronic hepatitis B was documented because he developed jaundice and had persistently elevated ALT levels. Liver biopsy was not performed because of the patient's refusal. In October 1997 and November 1998, two 6-month courses of interferon-alfa (5 and 3 million units three times per week for the first and second course, respectively) were administered, with a relapse after an initial response. In January 2001, the patient was started on lamivudine (100 mg per day). In June 2005, after 52 months of uninterrupted lamivudine therapy, the treatment was switched to adefovir (10 mg/day) because of biochemical and virological relapse. Adefovir was then maintained for more than 20 months of follow-up (February, 2007) with a good response.

A total of 52 consecutive serum samples were obtained at various time points before and during oral antiviral therapies to monitor the biochemical and virological response, as well as HBV DNA dynamics. Serum samples were stored at -70°C and retrospectively examined as to genome sequence, amino acid substitutions at the YMDD position of DNA polymerase and HBV DNA viral load.

Prior to the onset of treatment and on each sample taken during anti-viral therapy, the serum ALT level, hepatitis B surface antigen (HBsAg), and hepatitis B e antigen (HBeAg) were determined by automated ELISA, in the routine laboratory, King Chulalongkorn Memorial Hospital, Bangkok, Thailand.

A 100-µl serum sample was incubated with proteinase K in lysis buffer. Following phenol-chloroform - isoamyl alcohol extraction and ethanol precipitation, the resulting DNA pellet was suspended in 30 µl of sterile water and stored at -20°C. HBV DNA levels and YMDD mutants were detected by real-time PCR, as previously described.[7,5] For YMDD detection in samples with a low viral load (< 100 copies/µl), these samples were first amplified using sense and anti-sense primers (primer sequences on request).

The dynamics of biochemical and virological parameters, including HBV DNA levels, are summarized in Fig. 1. Interferon therapy evoked an initial response, which was followed by a relapse once treatment was discontinued. Lamivudine administration induced a sharp decline in HBV DNA with a significant decrease in ALT levels. Yet, after 14 months of stably reduced HBV DNA viral loads, the evolution of a YIDD mutation led to an ALT flare and virological breakthrough characterized by a significant increase in HBV DNA levels exceeding 1.0 log above the nadir. A reduction of HBV DNA

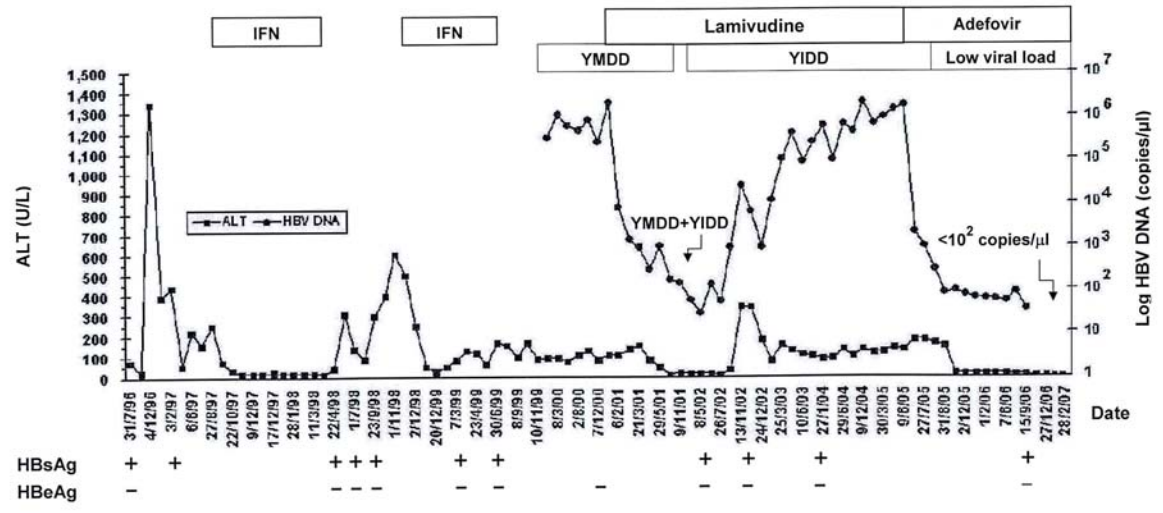

Fig. 1 Association between HBV DNA concentrations, HBV mutants and alanine aminotransferase (ALT) levels during and after anti-viral drug treatment

Table 1 HBV mutations during the course of treatment

Date (accession number)	Position of mutations									N.B.
	Polymerase gene					Core promoter		Precore		
	Adefovir resistance		Lamivudine resistance	Other mutation				Start codon	Stop codon	
	rtA181V/T	rtN236T	rtM204I/V	rtL82M	rtV84E	A1762T	G1764A	ATG	G1896A	
7/12/2000 (EF384200, EF384201)	A	N	M	L	V	T	A	ATG	G	2 years after IFN treatment
13/11/2002 (EF384206, EF394328)	A	N	I	M	E	T	A	ATG	G	2 years after Lamivudine treatment
9/2/2005 (EF384202, EF384203)	A	N	I	M	E	T	A	ATG	G	4 years after Lamivudine treatment
27/7/2005 (EF384204, EF384205)	A	N	I	M	E	T	A	ATG	G	1 month after Adefovir treatment

by 4.0 log IU/ml and ALT normalization was achieved within 3 months of adefovir monotherapy and repeatedly confirmed during the 20- month follow-up period.

To pinpoint the change in the YMDD motif, we first amplified the samples with low viral load by conventional PCR and subsequently detected the YIDD substitution (Fig. 1). We found the typical variant in the virus genome at about 9 months after the onset of lamivudine therapy and later. However, the YMDD substitution was not detectable in samples after treatment with adefovir as conventional PCR failed to amplify those samples due to their very low viral load.

Real-time PCR products representing four specific time points in the course of the antiviral therapy were subjected to nucleotide sequencing. The time points selected comprised 2 months before starting lamivudine therapy (7/12/2000), 2 years of lamivudine (13/11/2002), 4 years of lamivudine (9/2/2005) and 1 month of adefovir (27/7/2005) treatment (Fig. 1). This selection was based on potential resistance to the respective drug as a consequence of mutations in the polymerase gene motif, the core promoter and the precore gene. The details of the sequence method have been reported elsewhere.[5]

Direct sequencing showed the patient to be infected with HBV genotype C with core promoter mutations at positions 1762 (A to T) and 1764 (G to A), point mutations that may cause the absence of HBeAg. However, the precore region did not show any significant nucleotide change. Moreover, we found a mutation of the PreS2 start codon (G to A). Direct sequencing of the HBV genome also showed a G to A substitution at position 741 of the YMDD motif after 9 months of Lamivudine treatment resulting in an amino acid change from M to I (YIDD). The study conducted on this patient confirmed the real-time PCR result and the emergence of YMDD mutant viruses during long-term lamivudine treatment (Table 1). In addition to the above point mutation, we found C to A substitutions at positions 373 (rtL82M) and 400 (rtV84E).

As for adefovir resistance, we could not establish any adefovir-resistant mutation (rtA181V/T or rtN236T) in samples taken after starting adefovir treatment (data not shown) because of an undetectable viral load. All the nucleotide sequences in this study were submitted to Genbank under accession numbers EF384200-EF384206 and EF394328.

DISCUSSION

In this report, we have described the case of a patient chronically infected with HBV genotype C and serologically negative for HBeAg. The patient initially responded to standard interferon alfa therapy, but relapsed after cessation of treatment. Subsequent lamivudine therapy was also insufficient in controlling HBV replication as evidenced by a classical course of initial virological response followed by a viral breakthrough. After the emergence of lamivudine resistance, the patient was successfully treated with adefovir monotherapy.

Long-term administration of lamivudine results in the emergence of drug-resistant strains of HBV through the selection of mutants in the YMDD motif of the HBV polymerase. This results from a spontaneous error rate of the viral polymerase and thus, the accumulation of viral genome mutations during the natural history of infection. Consequently, the viral quasispecies may undergo significant changes under the selective pressure of antiviral therapy with subsequent selection of escape mutants.[3] Resistance should be suspected in patients with virological breakthrough accompanied by an increase in serum ALT levels, despite continuation of therapy. Therefore, as our data suggest, in the course of 14 months, HBV encoding the YIDD mutation had acquired superior replication fitness to the wild-type virus and the patient eventually progressed to virological resistance with ALT elevations. Treatment options for patients with lamivudine-resistant HBV have been limited to continuation or cessation of lamivudine therapy. Continuation of lamivudine therapy in patients with lamivudine-resistant HBV has been associated with deteriorating liver histology in some patients.[8] Cessation of therapy results in re-emergence of wild-type HBV, and patients remain at risk of developing progressive liver disease. Furthermore, re-introduction of lamivudine therapy leads to a more rapid reappearance of the mutant virus than in treatment-naive patients.[9]

Several studies have demonstrated the efficacy of adefovir in the treatment of lamivudine-resistant HBV. In one randomized study conducted on 59 patients with compensated lamivudine-resistant HBV infection, adefovir alone was as effective as adefovir/lamivudine combination therapy in suppressing HBV DNA and improving serum ALT after 1 year.[10] Another trial performed on 135 patients including 30% with decompensated disease showed that after 12 months on average, adefovir combined with lamivudine was superior to lamivudine alone in patients with lamivudine-resistant HBV.[11] Another study executed on 128 pre- and 196 post-liver transplantation patients with

lamivudine-resistant HBV demonstrated that adefovir salvage therapy improved Child-Pugh scores in more than 90% of patients in both groups after a median treatment of 19 and 56 months, respectively. Upon further follow-up, 52% of the 226 patients awaiting transplantation were removed from the waiting list because of improvement in liver function with long-term adefovir therapy. [12] In this study, the switch to adefovir accomplished rapid rescue from lamivudine resistance and thus confirms previously published data.

Whereas prolonged adefovir therapy is not associated with YMDD mutants, unique mutations in the RT region have recently been described. Adefovir resistance is associated with the selection of the rtN236T mutation within the D domain of the viral enzyme or with an rtA181V amino acid change in the B domain of the RT. The rtN236T mutation induces a 3- to 6-fold in vitro reduction in the susceptibility to adefovir compared to wild-type HBV, but retains some level of susceptibility to lamivudine.[13,14] More recent studies have also demonstrated that adefovir monotherapy continuously administered for one year may be associated with a high rate of adefovir resistance in patients with preexisting lamivudine-resistant HBV. For example, 18% of Korean patients with lamivudine-resistant HBV infection developed adefovir resistance after 1 year.[15] In another study carried out on HBeAg-negative lamivudine-resistant Italian patients, adefovir was added to lamivudine either at the time of phenotypic or genotypic resistance.[16] After 2 years of continuous combination therapy, no adefovir resistance was detected in either group. Yet, patients in whom adefovir was initiated at the time of genotypic resistance experienced more rapid viral suppression and ALT normalization than those in whom treatment was delayed. These studies suggest that adefovir should be added to lamivudine rather than substituted for it and that therapy should be started as soon as genotypic resistance to lamivudine is detected.[16] In addition, mutant strains of HBV resistant to both lamivudine and adefovir have been reported. A recent *in vitro* study performed on a viral construct containing both lamivudine- and adefovir-resistant point mutations (ie, rtL180M plus rtM204V plus rtN236T) demonstrated resistance to both lamivudine and adefovir but persistent susceptibility to entecavir, tenofovir, and interferon alfa.[14]

In conclusion, based on the results of this study as well as other reports, the switch to adefovir accomplished rescue from lamivudine-resistant HBV. However, drug resistance is eventually an expected consequence of long-term exposure to any oral antiviral agent because of the high rate of spontaneous HBV mutations and persistence of the cccDNA. Hence, careful monitoring during therapy and knowledge of cross-resistance to antiviral salvage therapy are essential to optimize the clinical outcomes of patients with chronic hepatitis B.

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Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma

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Abstract

Background: We investigated the clinical implications of global hypomethylation, one of the most consistent epigenetic changes in cancer, in the sera of patients with hepatocellular carcinoma (HCC).
Methods: Combined bisulfite restriction analysis PCR was used to assess the methylation status of LINE-1 repetitive sequences in genomic DNA derived from sera of 85 patients with HCC, 73 patients with cirrhosis, 20 healthy carriers of hepatitis B virus (HBV) and 30 healthy controls.
Results: Serum genome hypomethylation, the percentage of unmethylated LINE-1, was significantly increased in patients with HCC ($P<0.001$). The levels of serum LINE-1 hypomethylation at initial presentation correlated significantly with the presence of HBsAg, large tumor sizes, and advanced tumor stages classified by the CLIP score. Multivariate analyses showed that serum LINE-1 hypomethylation was a significant and independent prognostic factor of overall survival.
Conclusion: Serum LINE-1 hypomethylation may serve as a prognostic marker for patients with HCC.

Keywords: Global hypomethylation; Prognostic marker; Hepatocellular carcinoma; COBRA LINE-1; Hepatoma

1. Introduction

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, accounting for >500,000 new cases annually [1]. The prevalence of HCC is geographically variable, with the highest frequencies observed in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic. Despite remarkable improvements in surgical and ablative therapies, the overall prognosis of patients with HCC remains unsatisfactory because of its aggressiveness and high recurrence rates [2]. As a result, a reliable serum marker is needed and important for monitoring tumor progression, treatment responsiveness, and predicting the prognosis. Although several molecular biological factors related to HCC have been studied in recent years, a prognostic marker for this cancer in routine clinical practice is not yet available.

Recent advances in molecular biology have shown that genetic and epigenetic alterations accumulated through repeated destruction and regeneration of hepatocytes are responsible for multistage hepatocarcinogenesis [3]. Among these changes, widespread global DNA hypomethylation accompanied by region-specific hypermethylation is a common feature found in HCC. Global hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which are dispersed throughout the whole genome, in both noncoding repetitive sequences and genes [4]. Global losses of methylation in cancer may lead to alterations in the expression of proto-oncogenes critical to carcinogenesis and may facilitate chromosomal instability [5]. Previous studies have described the hypomethylation of genomic repetitive sequences, a marker of

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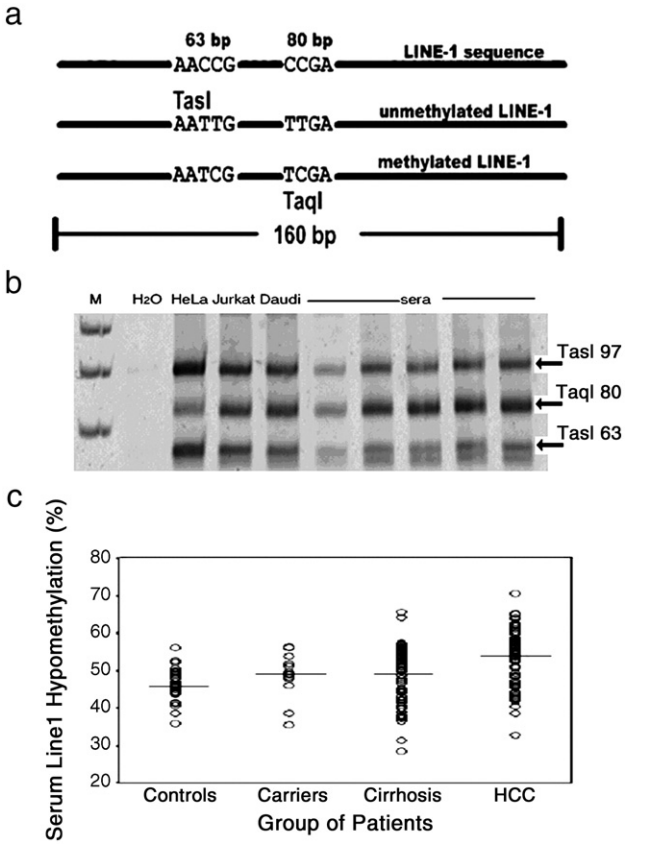

Fig. 1. Combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1) in sera. (a) Schematic illustration of COBRA LINE-1. The LINE-1 amplicon size is 160 bp. Methylated amplicons, *TaqI* positive, yielded two 80 bp DNA fragments, whereas unmethylated amplicons, *TasI* positive, yielded 63 and 97 bp fragments. (b) An example of COBRA LINE-1. M, 10-bp DNA size marker; H_2O, HeLa, Jurkat and Daudi are positive control and for adjusting inter-assay variation. Sera of HCC patients. *TasI* 63 and 97, unmethylated amplicons; *TaqI* 80, methylated amplicons. (c) Serum LINE-1 hypomethylation levels in each group of patients. The horizontal line indicated the mean levels of the patients' subgroups.

global genomic hypomethylation, in several malignancies including carcinoma of the breast, lung, head and neck, esophagus, stomach, urinary bladder, prostate, colon, and liver [6–12]. In addition, the extent of global hypomethylation appears to correlate with tumor progression and invasiveness in several cancer types, including HCC, indicative of its role in tumor development and progression [7,13–16].

Previous studies have demonstrated that tumor-derived nucleic acids can be detected in the sera of patients with cancers, and appear to be relevant surrogate markers for genetic alterations present in the primary tumors [17]. Based on these observations, circulating tumor-specific methylation has become an emerging serum or plasma marker for cancer diagnosis and prognosis [18]. Recently, we developed an improved quantitative combined bisulfite restriction analysis (COBRA) PCR protocol that efficiently evaluates the genome wide methylation status of LINE-1 repetitive sequences in genomic DNA derived from microdissected tissue and serum samples [7]. The LINE-1 methylation level measured by this technique has been shown to represent the genome wide methylation status by demonstrating linear correlation with conventional global hypomethylation assay by Southern blot and hybridization. Unlike conventional qualitative reports, COBRA LINE-1 not only confirmed that most cancers exhibited significantly increased levels of hypomethylation, compared with their normal tissue counterparts, but also normal tissues from different organs showed tissue-specific levels of methylated LINE-1. Furthermore, DNA derived from sera of patients with stomach cancer displayed significantly higher LINE-1 hypomethylation levels. These findings suggest the potential value of COBRA LINE-1 as a marker of various cancer types, including HCC. Nonetheless, whether serum LINE-1 hypomethylation level can serve as a molecular marker for HCC has not yet been investigated. To address this issue, we examined the methylation status of LINE-1 in serum samples of patients with HCC, and compared them with those of healthy individuals and patients with cirrhosis. We also determined the clinicopathological correlations and prognostic significance of this epigenetic alteration in sera of patients with HCC.

2. Materials and methods

2.1. Patients and blood samples

Eighty-five patients who were diagnosed with HCC in King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between April 1999 and December

Table 1
Relationship between serum LINE-1 hypomethylation levels and clinicopathological features in patients with HCC

Variables	Serum LINE-1 hypomethylation		*P*
	<53.17%	≥53.17%	
Age (y)			
≥60 (*n*=27)	13	14	NS
<60 (*n*=58)	23	35	
Gender			
Male (*n*=69)	29	40	NS
Female (*n*=16)	7	9	
Etiology of liver disease			
HBV positive (*n*=58)	19	39	0.011
HBV negative (*n*=27)	17	10	
Serum AFP level (ng/ml)			
≥400 (*n*=47)	18	29	NS
<400 (*n*=38)	18	20	
Tumor cell differentiation			
Well (*n*=13)	9	4	NS
Moderately and poorly (*n*=39)	17	22	
Tumor number			
Solitary (*n*=53)	24	29	NS
Multiple (*n*=31)	11	20	
Tumor size			
≤5 cm (*n*=15)	13	2	<0.001
>5 cm (*n*=70)	23	47	
Venous invasion			
Presence (*n*=30)	14	16	NS
Absence (*n*=55)	22	33	
Extrahepatic metastasis			
Presence (*n*=8)	1	7	NS
Absence (*n*=77)	35	42	
CLIP score			
Score 0–2 (*n*=41)	23	18	0.017
Score 3–5 (*n*=44)	13	31	

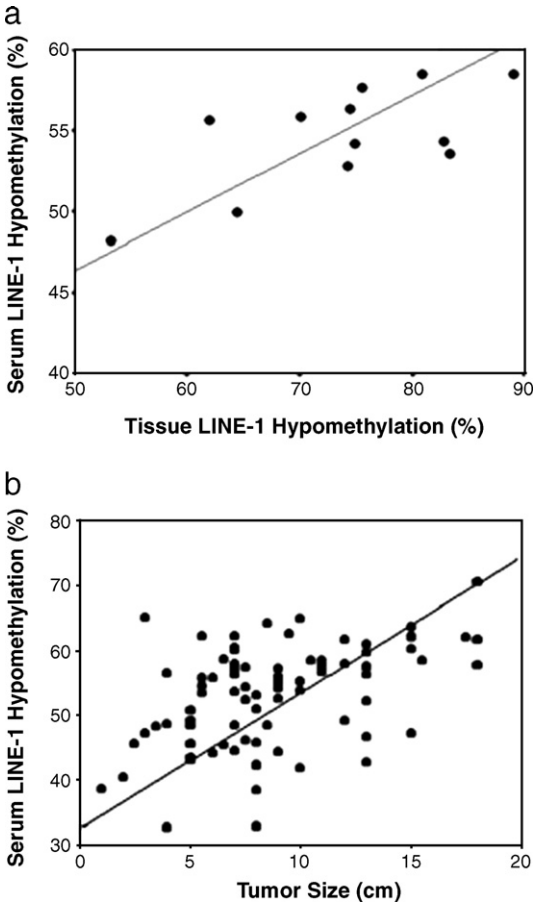

Fig. 2. Serum LINE-1 hypomethylation levels in patients with HCC exhibited a positive correlation with a) corresponding tumor tissue levels and b) tumor size.

2003 were recruited into the study. HCC was diagnosed based on liver tumor characteristics detected by imaging studies (ultrasonography or computed tomography), serum α-fetoprotein (AFP) >400 ng/ml, and/or histopathology. The clinicopathological data of the patients at initial diagnosis were collected, and included sex, age, liver function test, Child-Pugh classification, serum AFP concentration, tumor size, number of tumors, tumor cell differentiation, venous invasion, extrahepatic metastasis, and HCC staging classified by the CLIP score [19]. Overall survival time of the patients was defined as the period from initial presentation to the time of last follow-up (December 2004) or until they expired.

The patients with HCC consisted of 69 males and 16 females, with the mean age of 53.6±12.7 y. All patients had cirrhosis as underlying liver disease. In regard to predisposing etiologic factors, 58 patients (68.2%) were positive for serum hepatitis B surface antigen (HBsAg), 8 patients (9.5%) were positive for hepatitis C virus antibody (anti-HCV), and 12 patients (14.1%) were associated with alcohol-dependence. For the remaining 7 patients (8.2%), the etiology could not be determined. According to the CLIP score at initial presentation, there were 6 patients (7.1%) with score 0, 14 patients (16.5%) in the score 1 subgroup, 21 patients (24.7%) in score 2, 19 (22.4%) in score 3, 19 (22.4%) in score 4, and 6 (7.1%) in the score 5 subgroup. Thirty patients (35.3%) had venous invasion, while extrahepatic metastasis was found in 8 patients (9.4%). Twelve patients (14.1%) had undergone surgical resection, 25 patients (29.4%) had been treated with transarterial chemoembolization (TACE), and the remaining 48 patients (56.5%) had received no specific treatment because of an advanced tumor stage or refusal of the therapy.

The control groups comprised 30 healthy individuals (20 males, mean age 30.2±8.7 y), 20 healthy carriers of hepatitis B (14 males, mean age 31.5±10.3 y) and 73 patients with cirrhosis (55 males, mean age 50.3±11.7 y). Healthy carriers were defined as individuals who were positive for HBsAg, had persistent normal serum aminotransferase activities, and had normal abdominal ultrasonography. Cirrhosis was diagnosed based on histological examinations and/or imaging studies. With the patients' written consent, all serum samples were collected at the time of the diagnosis and stored at −70 °C until they were assayed.

2.2. DNA preparation

Peripheral blood (3 ml) was collected from each subject for the isolation of serum. Sera were centrifuged at 1600 ×*g*, transferred into plain polypropylene tubes and stored at −20 °C until further processing. DNA from 800 μl of serum samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol as recommended by the manufacturer. Twelve HCCs were microdissected from paraffin-embedded tissues.

After extraction, all DNA samples were treated with sodium bisulfite as previously described [20]. Briefly, genomic DNA was denatured in 0.22 mol/l NaOH at 37 °C for 10 min. Thirty microliters of 10 mmol/l hydroquinone and 520 μl of 3 mol/l sodium bisulfite were added for 16–20 h at 50 °C. The DNA was purified and incubated in 0.33 mol/l NaOH at 25 °C for 3 min, ethanol precipitated, then washed with 70% ethanol and resuspended in 20 μl of H_2O.

2.3. Measurement of serum LINE-1 hypomethylation

The COBRA LINE-1 assay of the serum was performed as described previously (13). We used 5′UTR of LINE-1.2 sequence from NCBI Accession Number M80343. DNA was isolated and extracted from serum, and treated with bisulfite. Two microliters of bisulfited DNA was subjected to 35 cycles of PCR with 2 primers, 5′-CCGTAAGGGGTTAGGGAGTTTTT-3′ and 5′-RTAAAACCCTCCRAACCAAATATAAA-3′, with an annealing temperature of 50 °C. The amplicons were digested in 10 μl reaction volumes with 2U of *TaqI* or 8U of *TasI* in 1× *TaqI* buffer (MBI Fermentas) at 65 °C overnight and

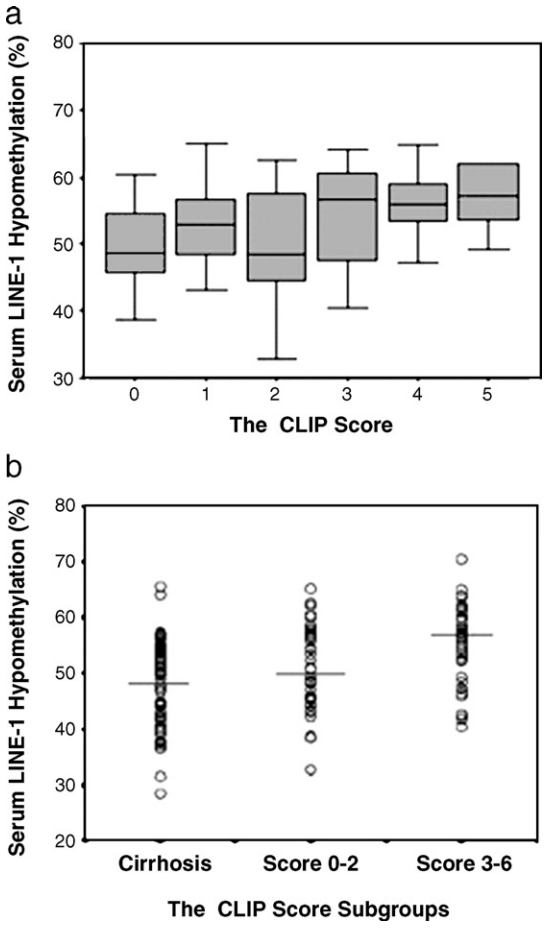

Fig. 3. Association between serum LINE-1 hypomethylation levels and HCC staging classified by the CLIP score. The horizontal line indicated the mean levels of the patients' subgroups.

then electrophoresed in 12% nondenaturing polyacrylamide gels. Intensities of DNA fragments were measured by PhosphorImager, using ImageQuant software (Molecular Dynamics). The LINE-1 amplicon size is 160 bp. Methylated amplicons, *TaqI* positive, yielded 2 80 bp DNA fragments, whereas unmethylated amplicons, *TasI* positive, yielded 63 and 97 bp fragments (Fig. 1). LINE-1 hypomethylation level was calculated as a percentage: the intensity of unmethylated LINE-1, digested by *TasI* divided by the sum of methylated LINE-1, digested by *TaqI*- and the *TasI*-positive amplicons. The same DNAs from HeLa, Daudi, and Jurkat cell lines were applied as positive controls in all COBRA LINE-1 experiments and to adjust for inter-assay variation.

2.4. Statistical analysis

All data are expressed as mean±S.D., and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney test or Student's *t*-test for quantitative variables. The analysis of overall survival was calculated by the Kaplan–Meier method and the differences in survival between the groups were compared using the log rank test. The Cox regression analysis was performed to identify which independent factors have a significant influence on overall survival. A $P<0.05$ was considered significant. All statistical analyses were performed using the SPSS software for Windows 10.0 (SPSS Inc., Chicago, IL).

3. Results

Serum LINE-1 hypomethylation in 85 patients with HCC obtained at the time of diagnosis ranged from 32.73% to 70.42%, with a mean of 53.17±7.74%. The average level of serum LINE-1 hypomethylation in these patients was significantly different from that of healthy controls (46.55±4.29%; range 35.94–56.01%), HBV carriers (48.07±6.20%; range 35.49–56.31%), and patients with cirrhosis (48.78±8.01%; range 28.51–65.75%) ($P<0.001$, $P=0.003$ and $P=0.001$, respectively). There was no significant difference in serum LINE-1 hypomethylation level between non-HCC individuals ($P>0.05$) (Fig. 1).

To evaluate the association between serum LINE-1 hypomethylation levels and clinicopathological features, the patients with HCC were divided into 2 groups based on the mean value (53.17%) of the whole HCC group. Accordingly, there were 36 patients with serum LINE-1 hypomethylation levels of <53.17% and 49 patients with serum levels of ≥53.17%. The correlations between hypomethylation in these 2 groups and various clinicopathological parameters listed in Table 1 were analyzed. There was no significant correlation between serum LINE-1 hypomethylation level and patient age, gender, AFP concentration, tumor cell differentiation, number of tumors, the presence of venous invasion, or extrahepatic metastasis. However, high serum LINE-1 hypomethylation levels were significantly associated with HBsAg positivity ($P=0.011$), tumor size >5 cm ($P<0.001$), and high CLIP score ($P=0.017$).

To determine the correlation between serum LINE-1 hypomethylation level and tissue expression, 12 cases of the

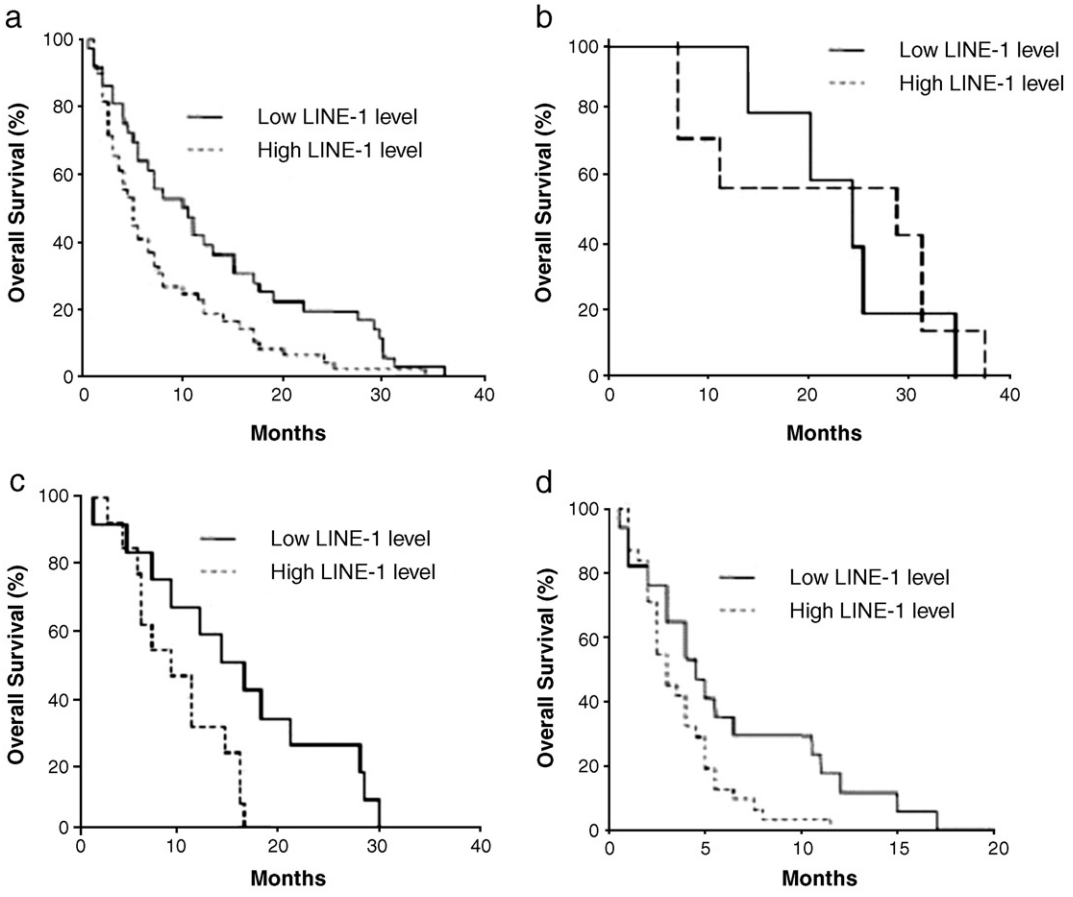

Fig. 4. Overall survival of HCC patients with serum LINE-1 hypomethylation less than or greater than 53.17% a) all patients, b) patients who were treated with surgery, c) patients who were treated with TACE and d) untreated patients. LINE-1 level, serum LINE-1 hypomethylation levels.

Table 2
Multivariate analysis of prognostic factors of survival with Cox's proportional hazards model in patients with HCC

Factors	Risk ratio	95% confidence interval	P
High serum LINE-1 hypomethylation level	1.742	1.087–2.793	0.021
Presence of vascular invasion	2.094	1.179–3.719	0.012
High CLIP score (score 3–5)	2.101	1.433–3.081	<0.001
No receiving therapy	5.712	3.116–10.471	<0.001

corresponding tumor tissue samples were analyzed. A positive correlation between serum and tumor LINE-1 hypomethylation was observed ($P=0.017$; Pearson r=0.670) (Fig. 2a). Serum LINE-1 hypomethylation levels also exhibited a positive correlation with tumor size ($P<0.001$; Pearson $r=0.439$) (Fig. 2b), and CLIP score ($P=0.005$; Pearson $r=0.304$) (Fig. 3a). In addition, serum LINE-1 hypomethylation levels in patients in the CLIP score 3–6 subgroup (55.56±6.83%) were significantly higher than those of patients in the score 0–2 subgroup (50.61±7.92%) and patients with cirrhosis (48.±8.01%) ($P=0.003$ and $P<0.001$, respectively). However, serum LINE-1 hypomethylation levels in patients in the score 0–2 subgroup were not significantly different from those with cirrhosis ($P=0.241$) (Fig. 3b).

We also examined the potential prognostic value of serum LINE-1 hypomethylation. The median overall survival of patients with LINE-1 hypomethylation levels of <53.17% was 10.5 months, which was significantly better than that of patients whose serum levels were ≥53.17% (median overall survival, 5.5 months; $P=0.012$ by log rank test) (Fig. 4a). Among patients who were treated with surgery, the median overall survival times for the low and high LINE-1 hypomethylation groups were 27.5 and 24.0 months, respectively ($P=0.670$) (Fig. 4b). Among patients who were treated with TACE, the median overall survival times for the low and high LINE-1 hypomethylation groups were 15.5 and 10.0 months, respectively ($P=0.024$) (Fig. 4c). In untreated cases, the median overall survival times for the low and high LINE-1 hypomethylation groups were 4.5 and 3.0 months, respectively ($P=0.030$) (Fig. 4d).

Serum LINE-1 hypomethylation level was entered into the multivariate analysis together with other variables that may influence prognosis. These factors included age, gender, AFP concentration, HBsAg status, Child-Pugh classification, tumor size, tumor number, venous invasion, extrahepatic metastasis, the CLIP score, and therapy for HCC. The multivariate analysis using the Cox proportional hazards model revealed that high serum LINE-1 hypomethylation level, presence of vascular invasion, high CLIP score, and absence of therapy for HCC were independent prognostic factors of overall survival (Table 2).

4. Discussion

An increasing number of studies have described the critical roles of epigenetic alterations in hepatocarcinogenesis, though most reports have focused on the function of DNA hypermethylation in silencing tumor suppressor genes [4,21]. Previous data have shown that CpG island-specific hypermethylation is already detectable in potentially precancerous lesions, including cirrhosis and dysplastic nodules, indicating its contribution to early stages of hepatocarcinogenesis [22]. Recently, the presence of cell-free-circulating DNA of hypermethylation of candidate tumor suppressor genes has been described in serum or plasma samples from patients with various types of cancers [21], including HCC [23,24]. Although the clinical significance of this epigenetic alteration in sera of patients with HCC is unclear, these data suggest the potential use of this molecular phenomenon in cancer diagnosis and monitoring. Conversely, relatively little is currently known concerning the status and clinical implications of global hypomethylation in sera of patients with HCC, as well as other cancer types.

In this study, we first demonstrated that serum LINE-1 hypomethylation levels were significantly higher in patients with HCC than in healthy individuals and patients with cirrhosis. These results were consistent with recent data, including our previous report, demonstrating that global hypomethylation is significantly increased in tumor tissues compared with cirrhotic and non-cirrhotic tissues [7,12]. Indeed, we have previously shown that though serum samples possess a wide distribution of LINE-1 methylation levels, there is a high correlation between the presence of a primary tumor and serum LINE-1 hypomethylation [7]. Thus, the increase in serum LINE-1 hypomethylation likely reflected the global methylation level in the tumor tissues. Notably, there was no significant difference in serum LINE-1 hypomethylation levels among non-HCC samples, suggesting that there might be no sequential increase of global hypomethylation between normal controls and cirrhosis. These results confirmed previous data that indicated that the levels of hypomethylation in normal liver tissues are comparable with those detected in chronic liver disease [12].

In addition to the tumor global methylation level, the increase in serum LINE-1 hypomethylation level likely reflected the malignancy status. While global hypomethylation is one signature of most cancer types, it often displays considerable specificity with regard to tumor type, stage, and the sequences affected [25]. Furthermore, the COBRA LINE-1 quantitative assay demonstrated that instead of being abruptly altered, genome wide hypomethylation in the tumor evolves progressively in multistage carcinogenesis [7]. In addition, several studies have reported that the amount of serum tumor DNA correlates directly with the advancement of the cancer; for example, serum or plasma Epstein Barr viral DNA level correlates with tumor size in nasopharyngeal carcinoma [1], and plasma human papilloma viral DNA level correlates with cervical cancer metastasis [26]. Nonetheless, in contrast to most tissue samples, a relatively wide range of LINE-1 hypomethylation levels in the sera could be anticipated, even within the same group of patients or tumor stages. One explanation for this observation could be the distinct LINE-1 methylation levels seen in different tissues and the high variability between individuals in tissues such as esophagus and thyroid [7].

Moreover, little is currently known about the mechanisms determining the amount of cellular DNA released into the circulation and whether there is eradication bias between methylated and unmethylated DNA [17]. With better understanding of such mechanisms, the specificity and sensitivity of serum LINE-1 hypomethylation as an indicator will improve.

General consequences of global hypomethylation are genomic instability and possible upregulate genes [4]. There are 3 possible specific biological roles of LINE-1 retrotransposons and consequently their methylation. First, LINE-1s are linked to genomic instability. Not only some LINE-1s are still retrotransposable [27] but also upregulation of LINE-1s creates DNA double-strand breaks [28]. Second, promoter hypomethylation of LINE-1 retrotransposable element activates antisense transcription as previously demonstrated in c-met gene of chronic myeloid leukemia [29]. Finally, reverse transcriptase of LINE-1s was demonstrated to possess a significant role in controlling cancer cells proliferation and differentiation [30].

In this study, analysis of serum LINE-1 hypomethylation in relation to clinicopathological features showed that a high level of LINE-1 hypomethylation was significantly correlated with more aggressive tumor behavior in patients with HCC. Specifically, a high serum LINE-1 hypomethylation level was observed more frequently in patients with tumors >5 cm in diameter and in advanced disease stages (CLIP score 3–6 subgroup). This is in accordance with previous reports demonstrating that there is a positive correlation between the expression levels of global hypomethylation in liver tissues and advanced stages of disease [16]. Similar findings of global hypomethylation in tissue specimens in association with tumor progression have been observed in a variety of cancers [14,15]. These data suggest an important and active role of global hypomethylation in the progression of HCC and other tumor types. As shown in this study, measurement of COBRA LINE-1 in the serum has several advantages over tissue-based techniques, including its rapidity, reproducibility, and noninvasiveness. Thus, in clinical settings, the quantitative serum LINE-1 hypomethylation assay may be more useful and feasible for predicting tumor progression than the measurement of hypomethylation in tumor specimens.

Several clinicopathological factors related to the prognosis of HCC have been reported recently. Of these, the CLIP score is an important prognosticator among the conventional pathological features of HCC [19]. In our study, a high serum LINE-1 hypomethylation level at initial diagnosis was a significant prognostic factor in terms of overall survival in patients who were treated with TACE, as well as in untreated cases. Furthermore, multivariate analyses revealed that this molecular marker was an independent, unfavorable predictor of long-term survival in patients with HCC. These findings strongly suggest that the prognosis of HCC is influenced by the extent of global hypomethylation in the cancer. Although the mechanisms remain to be elucidated, we speculate that progressive loss of genomic methylation might provide incremental survival advantages for tumor cells, and thus enhance tumor progression and aggressiveness. If our results are confirmed in large-scale prospective studies, the detection of serum LINE-1 hypomethylation may serve as a promising prognostic marker for patients with HCC.

In conclusion, we demonstrated the clinical implications of serum LINE-1 hypomethylation in HCC. A high level of LINE-1 hypomethylation was significantly associated with tumor progression and invasiveness. Moreover, a high serum LINE-1 hypomethylation level at initial presentation was an unfavorable prognostic marker for HCC. Apart from its prognostic role, it will be interesting to determine the usefulness of serum LINE-1 hypomethylation in monitoring tumor progression or treatment response in patients with HCC.

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RAPID COMMUNICATION

Role of serum interleukin-18 as a prognostic factor in patients with hepatocellular carcinoma

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Abstract

AIM: To determine whether serum interleukin-18 (IL-18) levels correlated with clinicopathologic features and prognosis in patients with hepatocellular carcinoma (HCC).

METHODS: Serum IL-18, IL-6 and IL-12 levels were measured by enzyme-linked immunosorbent assay (ELISA) from 70 patients with HCC and 10 healthy controls.

RESULTS: Serum IL-18, IL-6 and IL-12 levels of patients with HCC were significantly higher that those of the controls. The levels of IL-18 correlated significantly with the presence of venous invasion and advanced tumor stages classified by Okuda's criteria. Patients with high serum IL-18 levels ($\geqslant 10^5$ pg/mL) had a poorer survival than those with low serum IL-18 levels ($< 10^5$ pg/mL) (4 and 11 mo, respectively, $P = 0.015$). Multivariate analyses showed that serum IL-18 level, but not IL-6 and IL-12 levels, was a significant and independent prognostic factor of survival.

CONCLUSION: These findings demonstrate that serum IL-8 may a useful biological marker of tumor invasiveness and an independent prognostic factor of survival for patients with HCC. Thus, the detailed mechanisms of IL-18 involving in tumor progression should be further investigated.

Key words: Hepatocellular carcinoma; Interleukin-18; Serum marker; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide with a particularly high prevalence in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are common[1]. Although recent advances in the detection and treatment of HCC have improved the survival, the prognosis of most patients is somewhat unsatisfactory due to rapid clinical deterioration after the initial diagnosis and high incidence of recurrence after surgical resection[2]. In general, the natural history of HCC depends on the severity of the underlying liver disease, tumor characteristics and the efficacy of treatment interventions[3]. Besides these features, a number of biological markers including cytokines and growth factors have been demonstrated to be increased in the sera of patients with HCC and may be associated with a poor prognosis.

Interleukin-18 (IL-18), originally known as interferon-γ (IFN-γ)-inducing factor (IGIF), is a cytokine that shares structural and functional properties with interleukin-1 (IL-1)[4,5]. This cytokine is mainly produced by activated macrophages, but may also be expressed by Kupffer cells, T cells, B cells, keratinocytes, astrocytes, and osteoblasts[6]. Like IL-1, IL-18 is synthesized as an inactive precursor (pro-IL-18, 24 kDa), which is cleaved by interleukin-1 β-converting enzyme (ICE or caspase-1) into an active 18 kDa mature form[6-8]. IL-18 has multiple biological activities via its capacity to stimulate innate immunity and both Th1 and Th2 mediated responses[6,8]. It also exerts anti-tumor effects that are mediated by enhancement of NK cell activity, reduction of tumorigenesis, induction of apoptosis and inhibition of angiogenesis in tumor cells[9,10]. In addition, recent data have been suggested that inappropriate production of

IL-18 contributes to the pathogenesis of cancers and may influence the clinical outcome of patients[11]. Specifically, it has been demonstrated that serum IL-18 level may have prognostic significance in some types of cancer including colonic carcinoma, gastric carcinoma, esophageal carcinoma, breast cancer, and hematologic malignancies[12-16]. However, the prognostic role of serum IL-18 level in patients with HCC has never been investigated. Therefore, in this study, we determined whether serum IL-18 level correlated with clinicopathologic features and prognosis of patients with HCC.

MATERIALS AND METHODS

Patients and blood samples

For the purpose of this study, 70 patients with HCC were randomly selected from a pool of patients with chronic liver disease who were seen and followed at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between August 1997 and September 2003. The control group comprised 10 healthy adults from the blood bank. Serum samples were collected from each subject at the time of their clinical evaluation and stored at -70℃ until further tested. The study was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University. Informed consent was obtained according to the regulations of the committee.

The diagnosis of HCC was based on histopathology and/or a combination of mass lesions in the liver on hepatic imaging and serum alpha-fetoprotein (AFP) levels above 400 ng/mL. All demographic and clinical data were extracted from patients' files. The authors collected the data including sex and age, as well as clinical data such as liver function tests, severity of liver disease graded as the Child-Pugh status, Okuda staging, etiologic factors (HBsAg, Anti-HCV or alcohol abuse), serum AFP levels at the time of diagnosis, and presence of venous invasion diagnosed by CT scan.

Hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (anti-HCV) and AFP level were determined by enzyme-linked immunosorbent assays (ELISA) using commercially available kits (Auszyme Ⅱ, Abbott Laboratories, IL for HBsAg; ELISA Ⅱ, Ortho Diagnostic Systems, Chiron Corp., CA for anti-HCV; and Cobus® Core, Roche Diagnostics, Basel, Switzerland for AFP). Biochemical liver function tests were determined by automated chemical analyzer (Hitachi 911) at the central laboratory of the hospital.

Measurement of serum IL-6, IL-12 and IL-18 levels

Serum IL-6, IL-12 and IL-18 were determined by using ELISA kits (R&D systems, Inc., Minneapolis, MN). ELISA was performed according to the manufacturer' instructions.

Statistical analysis

Data are expressed 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. Survival curves were constructed using the Kaplan-Meier method and difference between curves was testing by the log-rank test. The Cox regression analysis was performed to identify which independent factors have a significant influence on the overall survival. *P* values below 0.05 for a two-tailed test were considered statistically significant. All statistical analyses were performed using the SPSS software for windows 10.0 (SPSS Inc., Chicago, IL).

Table 1 Clinical and demographic data of patients with HCC at the time of the diagnosis

Clinical features	mean ± SD or percentage (%)
Age (yr)	55.0 ± 13.6 (range, 26-89)
Sex (male:female)	60:10
Etiology	
Alcohol dependence	9/70 (12.8)
HBsAg-positive	39/70 (55.7)
Anti-HCV-positive	7/70 (10)
HBsAg- and anti-HCV-positive	2/70 (2.9)
Unknown	13/70 (18.6)
Liver function test	
Total bilirubin (mg%)	2.3 ± 3.5
Albumin (g/dL)	3.5 ± 0.7
AST (IU/L)	150.9 ± 130.4
ALT (IU/L)	82.9 ± 84.1
Alkaline phosphatase (IU/L)	512.0 ± 317.9
Prothrombin time (s)	14.4 ± 4.2
Child-Pugh classification	
A	43/70 (61.4)
B	23/70 (32.9)
C	4/70 (5.7)
Okuda staging system	
1	19/70 (27.2)
2	46/70 (65.7)
3	5/70 (7.1)
Venous invasion	17/70 (24.3)
Extrahepatic metastasis	12/70 (17.1)
AFP (≥ 400 ng/mL)	27/70 (38.6)

RESULTS

The clinical data of patients with HCC

The clinical and demographic data of patients with HCC in this study are shown in Table 1. Among the 70 recruited patients, 60 were men and 10 were women. The average age of the patients was 55.0 ± 13.6 years (ranged 26-89 years). All patients had underlying cirrhosis. Seventeen patients (24.3%) had venous invasion. Extrahepatic metastasis was found in 12 patients (17.1%). The mean total bilirubin (TB), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), albumin, alkaline phosphatase (AP), and prothrombin time (PT) were 2.3 ± 3.4 mg/dL, 150.9 ± 130.4 IU/L, 82.9 ± 84.1 IU/L, 3.5 ± 0.7 g/dL, 512.6 ± 317.9 IU/L, and 14.4 ± 4.2 sec, respectively. Twenty-seven patients (38.6%) had serum AFP higher than 400 ng/mL. According to Okuda staging system, there were 19 patients (27.2%) in stage 1, 46 patients (65.7%) in stage 2, and 5 patients (7.1%) in stage 3.

For predisposing etiologic factors, 9 patients (12.8%) were associated with alcohol-dependent. Thirty-nine patients (55.7%) were associated with HBsAg-positive, and 7 patients (10%) were associated with anti-HCV-positive.

Table 2 Serum levels of interleukins in patients with HCC and in healthy controls

	Healthy controls (n = 10)	HCC patients (n = 70)	P
IL-6 (pg/mL)	2.9 ± 13.4	31.2 ± 52.2	0.01
IL-12 (pg/mL)	1.5 ± 0.9	6.2 ± 9.6	0.03
IL-18 (pg/mL)	38.5 ± 22.4	104.6 ± 65.8	0.002

Two patients (2.9%) had both HBsAg-positive and anti-HCV-positive. The predisposing factors could not be determined in 13 patients (18.6%).

Seven patients (10%) had undergone surgical resection, 19 patients (27.1%) had been treated with transarterial chemoembolization (TACE), and the remaining 44 patients (62.9%) had received no specific treatment because of their advanced tumor stage or refusal to therapy.

Serum IL-18 levels of patients with HCC and the survival

As shown in Table 2, serum IL-18 levels in patients with HCC were significantly elevated compared with those of the controls (104.6 ± 65.8 *vs* 38.5 ± 22.4 pg/mL, $P = 0.002$). Similarly, the levels of serum IL-6 and IL-12 in patients with HCC were significantly increased compared with healthy subjects (31.2 ± 52.2 *vs* 2.9 ± 13.4 pg/mL, $P = 0.01$, and 6.2 ± 9.6 *vs* 1.5±0.9 pg/mL, $P = 0.02$, respectively). Serum IL-18 levels also exhibited a positive correlation with serum IL-6 and IL-12 levels ($P = 0.021$; Pearson $r = 0.276$ and $P = 0.002$; Pearson $r = 0.369$, respectively).

In order to evaluate the association between serum IL-18 and the survival, the patients with HCC were further categorized into two groups according to their serum IL-18 levels. In this respect, the cut point of 10^5 pg/mL, which represented the mean serum IL-18 level in the whole group, was used. There were 41 patients with serum IL-18 < 10^5 pg/mL and 29 patients with serum IL-18 ≥ 10^5 pg/mL. There was no statistically significant difference in age, gender, serum ALT, AST, AFP, PT and extrahepatic metastasis between these two groups (Table 3). However, patients with high serum IL-18 levels had significantly lower mean serum albumin level ($P = 0.01$), but had significantly higher mean total bilirubin ($P = 0.03$), serum AP levels ($P = 0.04$), exhibited more advanced tumor stages classified by Okuda's criteria ($P = 0.03$), and had higher percentage of venous invasion ($P = 0.02$) than patients with low serum IL-18 levels.

Kaplan-Meier survival curves revealed that the median survival of patients with low serum IL-18 and the other were 10.5 and 5.0 mo, respectively (Figure 1A). By using log-rank test, there was a statistically significant difference in the median survival between these two groups ($P = 0.007$). Among patients who were treated with surgery or TACE, the medial overall survival for the low and high serum IL-18 groups were 18.5 and 10.0 mo, respectively ($P = 0.021$) (Figure 1B). In untreated cases, the medial overall survival for the low and high serum IL-18 groups were 4.5 and 2.7 mo, respectively ($P = 0.043$) (Figure 1C).

Serum IL-18, IL-6 and IL-12 levels were entered into a Cox regression analysis together with other variables that may influence prognosis. These included age, gender, serum AFP level, HBsAg status, tumor size, tumor number, venous invasion, extrahepatic metastasis, Child-Pugh classification, Okuda staging, and therapy of HCC. Multivariate analyses revealed that independent prognostic factors of overall survival included high serum IL-18 level, venous invasion and no receiving therapy for HCC (Table 4).

Table 3 Comparison of clinical data of patients with HCC according to serum IL-18 levels

Clinical features	IL-18 < 10^5 pg/mL (n = 41)	IL-18 ≥ 10^5 pg/mL (n = 29)	P
Age (yr)	54.9 ± 15.3	55.2 ± 11.1	NS
Sex (male:female)	34:7	26:3	NS
Liver function test			
Total bilirubin (mg%)	1.4 ± 0.7	3.6 ± 4.9	0.03
Albumin (g/dL)	3.8 ± 0.7	3.3 ± 0.7	0.01
AST (IU/L)	136.8 ± 116.4	170.8 ± 148.3	NS
ALT (IU/L)	66.5 ± 39.2	106.2 ± 119.7	NS
Alkaline phosphatase (IU/L)	436.9 ± 275.2	612.1 ± 348.2	0.04
Prothrombin time (s)	13.9 ± 2.4	14.9 ± 5.8	NS
Okuda staging (1:2:3)	15:25:1	4:21:4	0.03
Venous invasion (+:-)	6:35	11:18	0.02
Extrahepatic metastasis (+:-)	6:35	6:29	NS
AFP (< 400 ng/mL: ≥ 400 ng/mL)	25:16	18:11	NS

DISCUSSION

Enhanced expression of proinflammatory, hematopoietic and angiogenic cytokines has been demonstrated in several human tumors[17]. Some of these cytokines may act as autocrine or paracrine tumor cell growth factors, inhibitors of apoptosis, attractors of immune cells, and promoters of angiogenesis[18,19]. Accordingly, it is likely that the deregulation of these cytokines may contribute to the development or progression of the malignant process. Currently, serum levels of several cytokines have been found to be increased in patients with HCC and may be correlated with clinical outcomes. For instance, higher level IL-10 was observed in patients with HCC[20], and increased IL-10 values were associated with a poor prognosis in patients undergoing surgical resection[20], as well as in patients with unresectable tumor[21] Similarly, serum IL-8 was shown to be a useful biological marker of tumor invasiveness and an independent prognostic factor of survival for patients with HCC[22].

To the best of our knowledge, this is the first study demonstrating that the levels of serum IL-18 were markedly elevated in patients with HCC compared with healthy controls. In addition, our data showed that a high-serum IL-18 level was significantly correlated with advanced tumor stage classified by Okuda's criteria. Furthermore, serum IL-18 levels were significantly correlated with venous invasion, a pathobiological feature indicative of tumor aggressiveness. These data suggest that serum IL-18 might be useful in the clinical setting to predict tumor invasiveness and stage. A high-serum IL-18 level was also a significant prognostic factor in terms of overall survival, as demonstrated by multivariate analysis. These results were in agreement with previous data suggesting that serum IL-18 levels are related to the prognosis of patients with various

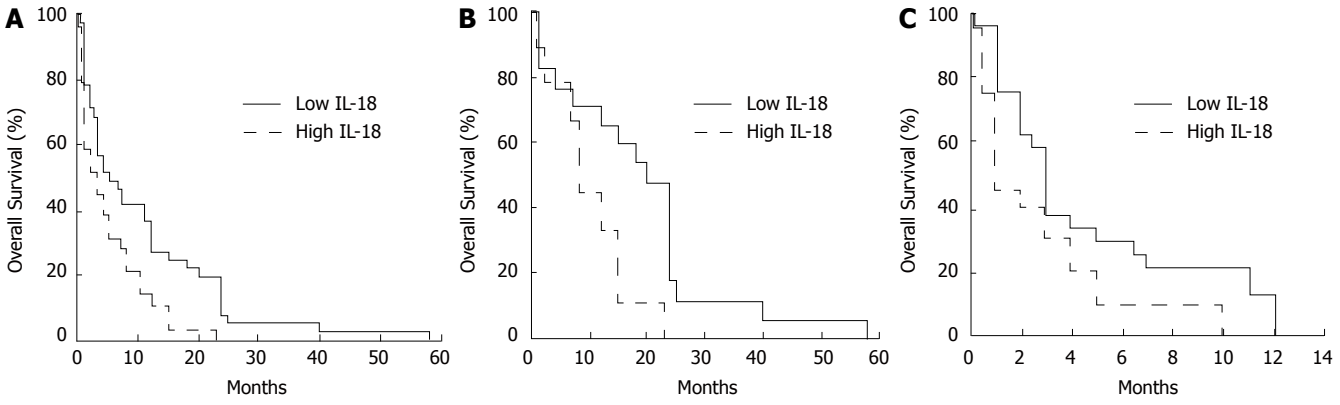

Figure 1 Overall survival of HCC patients with low serum IL-18 level (< 10^5 pg/mL) or high IL-18 level (≥ 10^5 pg/mL). **A**: All patients; **B**: Patients who were treated with surgery or TOCE; **C**: Untreated patients.

Table 4 Multivariate analysis of prognostic factors of survival with Cox's proportional hazards model

Factors	Risk ratio (95% CI)	*P*
High serum IL-18 level	1.86 (1.11-3.11)	0.019
Presence of venous invasion	2.09 (1.19-3.67)	0.010
No receiving therapy	5.01 (2.49-10.06)	< 0.001

malignant diseases in the gastrointestinal tract, including colonic, gastric and esophageal carcinoma[12,13,16]. In agreement with our results, it has been shown recently that the expression of IL-18 receptor in tumor tissues was found to be a significant predictor of a poor outcome in HCV-associated HCC patients[23].

The precise mechanisms underlying the positive correlation between serum IL-18 levels and advanced tumor stages are unclear. As previously mentioned, IL-18 exerts anti-tumor activity *via* several mechanisms including enhancement of NK cell function, induction of apoptosis *via* Fas/Fas ligand interaction and inhibition of angiogenesis[9,10]. Indeed, recent data have demonstrated that positive IL-18 immunoreactivity is significantly higher in the surrounding hepatocytes compared with the tumor portion from the same individual[24]. It has been shown that decreasing IL-18 production in tumor cells may be related to the down regulation of ICE gene expression, as demonstrated in colon and ovarian carcinoma[13,25]. In contrast, IL-18 and ICE transcripts have been detected in the corresponding normal colon and ovarian epithelium suggesting the bioactive IL-18 is most likely produced by the adjacent normal cells[13,25]. Taken together, it is speculated that IL-18 production by the normal adjacent hepatocytes may reflect the degree of defense mechanisms against tumor growth and dissemination of HCC.

IL-12, also known as NK cell stimulatory factor or cytotoxic lymphocyte maturation factor, is a multifunctional cytokine produced primarily by antigen-presenting cells (APC), such as monocytes and NK cells[26]. This cytokine augments proliferation, cytokine production and the development of Th1. Furthermore, IL-12, in combination with IL-18, induces anti-tumor effects against a variety of tumor cells via the activity of IFN-γ[27,28]. It has been shown that serum IL-12 levels are significantly higher in patients with gastric and esophageal cancers compared with healthy controls[12,29]. In patients with esophageal carcinoma, increasing serum IL-12 and IL-18 levels correlate with tumor growth and progression[12]. In contrast, serum IL-12 levels in patients with far-advanced gastric cancer are significantly lower that those with less-advanced stages[29]. In this study, we demonstrated that serum IL-12 levels were significantly higher in patients with HCC than in healthy controls. Furthermore, we found that its levels were correlation with IL-18 levels, suggesting that these cytokines may act synergistically in the anti-tumor activity. However, unlike IL-18, IL-12 levels were not confirmed as a prognostic factor in multivariate analysis.

IL-6 is a pleiotropic cytokine that was originally identified as a T cell-derived lymphokine inducing final maturation of B cells into antibody-producing cells[30]. This cytokine plays an important role in hematopoiesis, acute-phase responses and host defense mechanisms[31]. In addition, IL-6 has also shown to act as an autocrine growth factor in malignancy[30]. Increased serum levels of IL-6 have been demonstrated in patients with a variety of cancers and may be associated with a poor outcome[32-34]. However, the clinical significance of serum IL-6 levels in patients with HCC remains to be established[20,35,36]. In this study, we found that though levels of IL-6 were significantly higher in patients with HCC than in healthy subjects, its levels were not an independent prognostic factor in multivariate analysis. Thus, our results were in agreement with the reports conducted by Chau *et al* and Parasole *et al*[20,36].

In summary, our data demonstrated that serum IL-18 levels in patients with HCC correlated with advanced tumor stage classified by Okuda's criteria and the presence of venous invasion. Serum IL-18 level also exhibited an independent predictor of prognosis in patients with HCC. These data suggest that IL-18 contributes an important role in the pathogenesis and disease progression of HCC. If confirmed in additional longitudinal studies, the immuno-modulation of this cytokine may have therapeutic potential in the future.

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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: Sixty 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 in respect to G1613A, C1653T, C1766T/T1768A, A1846T/C, T1858C and G1896A mutations. By multiple logistic regression analysis, the present 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.

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.

HBV, a member of the family *Hepadnaviridae*, is a partially double-stranded DNA virus that contains four overlapping open reading frames (ORFs) encoding the surface, core, polymerase and X genes. The virus shows remarkable genetic variability and is currently classified into 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 of 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 a 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]. 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.

Material 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. Fifty-two were males and 8 were females, with the mean age (±SD) was 55.7 ± 9.8 years. Eighteen 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 -70^0C until use. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Biochemical, serological and virological assays

Serum alanine aminotrasferase (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). 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 to 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 resuspended in 30 µl sterile distilled water and subjected to amplification of the the X/BCP/PC regions (nucleotides (nt) 1287-2038) by polymerase chain reaction (PCR) using the primers Xi1: 5'AGCTTGTTTTGCTCGCAGC3' (forward primer, nt. 1287-1305), and Ci 1: 5' TTCCGGAGACTCTAAGGCC 3' (reverse primer, nt. 2020-2038). The obtained sequences span the region which included the entire X-protein ORF (nt. 1374-1836), the EnhII region (nt. 1685-1773), the basal core promoter (BCP) (nt. 1742-1849), direct repeat 1 (DR1) (nt. 1824-1834), direct repeat 2 (DR2) (nt.1590-1600), the precore (nt. 1814-1901), and a part of the core region (nt. 1901-2038).

Briefly, the reaction mixture comprised 2 µl resuspended DNA, 0.5 µl of 25 mmol of each primer, 10 µl of 2.5X MasterMix® (Eppendorf, 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™ BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with 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).

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. (Table1)

Comparisons of nucleotide sequences of EnhII/BCP/PC genes between patients with and without HCC

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 2)

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.

Comparisons of amino acids sequences of the X genes between patients with and without HCC

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 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 2)

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.

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 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 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 4)

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

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 5. 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.

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 compare 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 3-4-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[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 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[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 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[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 merits 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 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[7, 27]. 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[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[30]. 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, 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 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.

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 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 confirm these observations.

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Table 1 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 (%)

Table 2 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 (%)

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

Table 4 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

Table 5 Comparison of clinical and virological 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 (%)

Molecular Epidemiological Study of Hepatitis B Virus among Migrant Workers from Cambodia, Laos and Myanmar in Thailand

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Running head: Hepatitis B virus in migrant workers in Thailand

Abstract

Background/Aim: Although hepatitis B virus (HBV) infection is endemic in Southeast Asia, the molecular epidemiological data of HBV circulating in some countries are currently limited. The aims of this study were to evaluate HBV seroprevalence and its genetic variability present among migrant workers from Cambodia, Laos and Myanmar in Thailand.

Materials and Methods: Sera collected from 1119 Cambodians, 787 Laotians and 1103 Myanmareses workers were tested for HBsAg. HBV DNA was amplified and sequenced in the *preS/S* and *preC/C* regions for genotyping and genetic mutation analysis.

Results: The prevalence of HBsAg among migrant workers from Cambodia, Laos and Myanmar was 10.8%, 6.9% and 9.7%, respectively. Of those positive for HBV DNA, 86% were classified as genotype C (99% was subgenotypes C1) and 11.6% were genotype B (30.8%, 34.6% and 30.8% were subgenotypes 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 17.2% of samples had pre-S mutations, with pre-S2 deletion as the most common mutant (6.7%) followed by pre-S2 start codon mutation (3.8%) and both pre-S2 deletion and start codon mutation (3.3%). The prevalence of C1653T, T1753C/A/G, A1762T/G1764A and G1896A mutations in this study was 4.3%, 24.1%, 43.3% and 24.6%, respectively.

Conclusion: 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. Our data also demonstrated that HBV subgenotype C1 was the predominant strain and various naturally occurring mutations of HBV were not uncommon among these populations.

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 diseases ranging from chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV, belonging to family *Hepadnaviridae*, is a relaxed-circular double stranded DNA virus of approximately 3200 base pairs 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[1]. HBV shows remarkable genetic variability and is currently classified into at least eight genotypes, designated A to H and four major serotypes, including ayw, ayr, adw and adr[2, 3]. In each genotype, it can be divided into subgenotype base on 4-8 % divergence of the viral genome. HBV genotype and subgenotype distribution appear to show varying geographic patterns[4, 5]. For instance, 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 distributed in Japan, while subgenotypes B2–5 prevail in other countries. Subgenotype C1 is prevalent mainly in Southeast Asia, whereas subgenotypes C2 are commonly found throughout Far East Asia such as Japan, China and Korea[4, 5].

Chronic HBV infection and its related-hepatic complications is particularly important in Southeast Asian countries where the prevalence of the infection is relatively high, varying from 3-5% in Singapore and Malaysia to approximately 8-19% in Indonesia, Myanmar, Laos, Cambodia and Vietnam[6-12]. In Thailand, the prevalence of HBV infection has declined following implementation of national HBV vaccination program, with present prevalence of approximately 4%[13]. Despite the high prevalence of HBV infection in Southeast Asia, data on its molecular epidemiology in this region are scanty, particular in some countries such as Cambodia, Laos and Myanmar. 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. Growing influx of migrant populations may influence the prevalence of HBV infection and its illness burden in Thailand. The present study 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 that left from routine health check-up were stored at -70°C until analysis. In this study, 3009 serum samples collected from 1119 Cambodians (353 female; 763 male and 3 unidentified), 787 Laotians (413 female, 364 male and 10 unidentified) and 1103 Myanmarese (582 female, 423 male and 98 unidentified) were included. All serum samples were examined for Hepatitis B s antigen (HBsAg) by using commercially available automated ELISA assays (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 Ministry of Public Health and the ethical committee of the Faculty of Medicine, Chulalongkorn University.

HBV DNA extraction, amplification and sequencing

One hundred microliter of HBsAg-positive sera was subjected to extract HBV DNA. The positive serum 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) following by phenol/chloroform/isoamyl alcohol extraction. The Pre-S1/Pre-S2/S region was amplified by 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) and the PC/C region was amplified by X101 (5'-TCT GTG CCT TCT CAT CTG-3': position 1552-1569) and CO2 (5'-GTG AGG TGA ACA ATG TTC CG-3': position 2053-2034). The total 25 µl reaction volume consist of 10 µl of 2.5X 5 PRIME MasterMix solution (5 PRIME GmbH, Hamburg, Germany), 0.5 µl of 25 µM forward and reverse primer, 2 µl of DNA template and sterile distilled water. The thermocycler was programed to the HBV DNA amplification as following conditions: initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 1.30 minutes and a final extension step at 72°C for 7 minutes. The HBV DNA amplicons were isolated by electrophoresis method using 2% agarose gel, 100 volt, 60 minutes and stained with ethidium bromide. The size of PCR products were estimated by using the migration pattern of a 100-by DNA ladder under the UV light exposure. The expected products were cut and purified by the Perfectprep® Gel Cleanup kit (Eppendorf,

Hamburg, Germany). The purified samples were sent to commercial DNA sequencing company (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia) for base sequencing. Nucleotide sequences were edited by Chromas Lite program version 2.01 (Technelysium Pty Ltd., Queensland, Australia) and assembly by SeqMan (DNASTAR Lasergene software, Madison, WT).

Genotyping, subtyping and phylogenetic analysis

The sequences of each sample from both regions (pre-S1/pre-S2/S and preC/C) were aligned with each available human genotype from GenBank database (National Center for Biotechnology Information, BesthesDa, MD) by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the alignments were constructed the phylogenetic trees using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ) for genotyping. Some sequences were genotyping by the Viral Genotyping Tool (National Center for Biotechnology Information, Besthesda, MD). 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 by using the translate tool in ExPASy Proteomics Server (available on: http://www.expasy.ch/tools/dna.html). Subsequently, subtype was identified by using amino acids at the position 122 and 160 of the S protein.

HBV mutation analysis

HBV sequences were evaluated for mutations and deletions in the enhancer II (EnhII)/ basal core promotor (BCP)/ PC and the pre-S1/pre-S2 regions. The ‘a’ determinant mutations were analyzed from amino acids 120 and 160 of the S protein.

Statistic analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. All statistical analyses were performed using the SPSS software for Windows 17.0 (SPSS Inc., Chicago, IL).

Results

HBsAg detection

HBsAg positive sera were found in 282 of 3009 (9.4%) samples. In this group, there were 121 Cambodian (10.8%), 54 Laotian (6.9%) and 107 Myanmareses (9.7%). Among these subjects, HBV DNA was detected in 102 Cambodian (84.3%), 42 Laotian (77.8%) and 80 Myanmareses (74.8%) (Table 1).

Distributions of HBV genotypes and serotypes

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 were constructed (Fig 1 A and B). Of those positive for HBV DNA, 193 of 224 (86%) cases were determined as genotype C (99% and 1% were subgenotypes C1 and C5, respectively), 26 (11.5%) cases belonged to genotype B (30.8%, 34.6% and 34.6% were subgenotypes B2, B3 and B4, respectively), 1 (0.5%) cases to genotype A (subgenotype A2) 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 genotype and subtype according to individual's country is shown in Table 1.

Although the entire genome sequence was not performed in this study, three isolates with suspected intergenotypic 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 subgenotype B2 and C1, with its recombination breakpoints estimated at nucleotide 573 (Fig 2A). Isolate 3794 represented a recombinant of genotypes G/C1 with its recombination breakpoints between nucleotides 2006 and 157 (Fig 2B). Isolate 612 was classified to subgenotype B3 in pre-S/S gene but showed subgenotype 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 94 (18.0%) HBV isolates. Among these, 19/94 (20.2%) of Cambodian samples, 6/38 (15.8%) of Laotian samples and 10/62 (16.1%) of Myanmareses samples were found to have mutations. The most frequent mutation in

Cambodian, Laotian and Myanmareses isolates was Ile126Ser/Asn. In addition, multiple point mutations in the ‘a’ determinant region were detected in 6 isolates (Table 2). The alignment of amino acid sequences of the partial S region of these 35 isolates is shown in Fig 3.

Prevalence and characterization of pre-S/S mutations

Based on direct sequencing, pre-S mutations were detected in 36 of 209 cases (17.2%). In this study, 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%) (Table 3). The alignment of amino acid sequences of the entire pre-S1/pre-S2 region of the 36 samples is shown in Fig 4.

Prevalence and characterization of EnhII/BCP/PC mutations

Mutational hot spots in these regions were found at nucleotides 1653, 1753, 1762, 1764, 1896 and 1899. The C1653T mutation was observed in 8 out of 187 (4.3%) samples, of which 4/85 (4.7%), 3/37 (8.1%) and 1/65 (1.5%) belonged to Cambodian, Laotian and Myanmareses samples, respectively. The T1753C/A/G mutations were observed in 45 out of 187 (24.1%) samples, of which 23 (27.1%), 3 (8.1%) and 19 (29.2%) belonged to Cambodian, Laotian and Myanmareses isolates, respectively. The double A1762T/G1764A mutations were found in 81 out of 187 (43.3%) samples. Among these, 40 (47.1%), 10 (27.0%) and 31 (47.7%) belonged to Cambodian, Laotian and Myanmareses samples, respectively. In PC region, the G1896A mutation was found in 46 out of 187 (24.6%) samples, of which 19 (22.4%), 9 (24.3%) and 18 (27.7%) belonged to Cambodian, Laotian and Myanmareses samples, respectively. The G1899A mutation was found in 3 (3.5%) of Cambodia samples, 2 (5.4%) of Laotian samples and 2 (3.1%) of Myanmareses samples (Table 3). The alignment of nucleotide sequences of the EnhII/BCP/PC region is shown in Fig 5.

Discussion

Although chronic HBV infection prevails in Southeast Asia, the data on its molecular epidemiology in some countries in this part of the world are still limited. To our knowledge, this study is the first report that has compared the molecular characterization of HBV circulating in Cambodia, Laos and Myanmar. Our study, which included both the identification of the viral genotypes and subtypes in a significant number of HBV carriers from these countries, demonstrated that the predominant HBV strains belonging to the categories C1/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[10], and subgenotypes C1 and B4 were dominant strains in Cambodia[14]. 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%, which was similar to previous reports on seroprevalence in these countries but was higher than a recent nationwide survey in Thailand (4%)[13]. This difference in seroprevalence among populations 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[15], whereas recombination of genotypes C/D has been detected in Tibet and China[16, 17]. In addition, recombinants between genotypes A/C and genotypes A/D have been documented in Vietnam[18] and India[19], respectively. Recently, a novel genotype I, with a complex recombination involving genotypes C, A and G has been reported in Vietnam and Laos[14, 20]. Although the whole genome sequence was not performed in this study, we identified three HBV isolates with suspected intergenotypic recombinants. Of note, a hybrid subgenotypes B3/C1 in this study 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[15]. 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[21].

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[22]. 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[22]. In this study, however, the most common amino-acid substitution found in Cambodian, Laotian and Myanmareses 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 from recent data (6-12%)[23]. 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[22, 24, 25]. 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[26]. 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[6, 12]. Thus, it is speculated that these mutants within the ‘a’ determinant region might have emerged through natural immunoselective pressure of the host, which in turn are infectious and have been circulated among individuals chronically infected with the virus.

Naturally occurring HBV pre-S mutations/deletions has been frequently reported 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[27]. In fact, the prevalence of these mutations/deletions is rather variable and considerably different among diverse geographic areas ranged from 0% to 36%[28]. In this study, 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 higher than the results of our previous study in Thai populations (9.5%)[13]. Regarding 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 of recent reports from Japan, Korea and Thailand, according to which deletion in pre-S2 regions and pre-S2 start codon mutations were among the most prevailing [13, 28, 29].

The PC stop codon mutation (G1896A) and double mutations in the BCP region (A1762T/G1764A) are well-known naturally occurring HBV variants involving with hepatitis B e antigen (HBeAg) expression[30]. Apart from these variants, other mutations such as T1753C/A/G in the BCP region and C1653T in the EnhII region have been recognized as being associated with the outcome of chronic HBV infection[31]. In this study, the prevalence of T1753C/A/G and A1762T/G1764A in Cambodian and Myanmareses samples were rather comparable (nearly 30% and 50%, respectively) but higher than that found in Laotian samples (approximately 8% and 27%, respectively). In contrast, the prevalence of G1896A was relatively similar among groups (approximately 22-28%). The explanation of the discrepancy in the prevalence of A1762T/G1764A among groups is unclear, because the seroprevalence of HBeAg was not determined in these subjects.

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. We also demonstrated that HBV genotype/subtype C1/adr was the predominant strain circulating in these migrant workers. In addition, the 'a' determinant variants were frequently found 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, as well as double A1762T/G1764A and G1896A mutations were not uncommon among these populations.

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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])	81:20:1	31:11:0	46:28:6	158:59:7
Age	29.2±8.6	26.2±7.4	28.3±6.1	28.3±7.6
Genotype				
A2[b]	1 (1.0)	0 (0)	0 (0)	1 (0.4)
B	14 (13.7)	11 (26.2)	1 (1.3)	26 (11.6)
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)	79 (98.7)	194 (86.6)
C1	86 (84.3)	29 (69.0)	77 (96.3)	192 (85.7)
C5	0 (0)	1 (2.4)	1 (1.3)	2 (0.9)
D[b]	0 (0)	0 (0)	1 (1.3)	1 (0.4)
Suspected recombination				
B2/C1	1(1.0)	0 (0)	0 (0)	1 (0.4)
B3/C1	0 (0)	1(2.4)	0 (0)	1 (0.4)
G/C1	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 (%)

[a] Data not available; [b] *PreC* gene could not be amplified

Table 2 Prevalence of ‘a’ determinant mutations in migrant workers

Amino acid substitution	Cambodia (n = 102)	Laos (n = 42)	Myanmar (n = 80)	Total (n = 224)
No. PCR positive	94 (92.2)	38 (90.5)	62 (77.5)	194 (86.6)
Ile126Ser/Asn	6 (6.4)	2 (5.3)	4 (6.5)	12 (6.2)
Pro127Arg	1 (1.1)	0	0	1(0.5)
Gly130Arg	0	1 (2.6)	0	1(0.5)
Thr131Asn/Pro	0	1 (2.6)	2 (3.2)	3 (1.5)
Met133Thr	2 (2.1)	0	0	2 (1.0)
Phe134Leu	1 (1.1)	0	0	1(0.5)
Thr140Ile	0	0	1(1.6)	1(0.5)
Pro142Leu	1 (1.1)	0	0	1(0.5)
Gly145Arg/Ala	3 (3.2)	1 (2.6)	0	4 (2.1)
Trp156Leu	0	0	1(1.6)	1(0.5)
Ala157Gly	0	0	1(1.6)	1(0.5)
Ala159Val	1 (1.1)	0	0	1(0.5)
Pro120Thr + Ala128Asp + Cys138Tyr + Phe158Leu	0	1 (2.6)	0	1(0.5)
Lys122Gln + Thr131Asn + Met133Thr	1 (1.1)	0	0	1(0.5)
Gly130Arg + Met133Thr	1 (1.1)	0	0	1(0.5)
Thr131Asn + Phe134Tyr	1 (1.1)	0	0	1(0.5)
Thr131Asn + Phe134Tyr + Asp144Glu	1 (1.1)	0	0	1(0.5)
Ala128Val + Phe134Tyr + Phe158Leu + Ala159Gly	0	0	1(1.6)	1(0.5)

Data were expressed as no (%)

Table 3 Prevalence of pre-S and preC/C mutations in migrant workers

Gene	Mutation/deletions	Cambodia (n = 102)	Laos (n = 42)	Myanmar (n = 80)	Total (n = 224)
Pre-S	No. PCR positive	98 (96.1)	40 (95.2)	71 (88.8)	209 (93.3)
	Pre-S1 start codon mutaion + pre-S1 deletion	1 (1.0)	0	0	1 (0.5)
	Pre-S1 start codon deletion + pre-S2 deletion	0	0	1 (1.4)	1 (0.5)
	Pre-S1 deletion	0	0	1 (1.4)	1 (0.5)
	Pre-S1 deletion + pre-S2 deletion	1 (1.0)	0	0	1 (0.5)
	Pre-S2 start codon mutaion	3 (3.1)	3 (7.5)	2 (2.8)	8 (3.8)
	Pre-S2 start codon mutation + pre-S2 deletion	2 (2.0)	0	5 (7.0)	7 (3.3)
	Pre-S2 start codon deletion + pre-S2 deletion	1 (1.0)	0	1 (1.4)	2 (1.0)
	Pre-S2 start codon mutation + pre-S1 deletion	1 (1.0)	0	0	1 (0.5)
	Pre-S2 deletion	5 (5.1)	3 (7.5)	6 (8.5)	14 (6.7)
PreC/C	No. PCR positive	85 (83.3)	37 (88.1)	65 (81.3)	187 (83.5)
	C1653T	4(4.7)	3 (8.1)	1 (1.5)	8 (4.3)
	T1753C/A/G	23 (27.1)	3 (8.1)	19 (29.2)	45 (24.1)
	A1762T /deletion	41 (48.2)	13 (35.1)	32 (49.2)	86 (46.0)
	G1764A/deletion	44 (51.8)	10 (27.0)	31 (47.7)	85 (45.5)
	Double BCP mutations	40 (47.1)	10 (27.0)	31 (47.7)	81 (43.3)
	G1896A	19 (22.4)	9 (24.3)	18 (27.7)	46 (24.6)
	G1899A	3 (3.5)	2 (5.4)	2 (3.1)	7 (3.7)

Data were expressed as no (%)

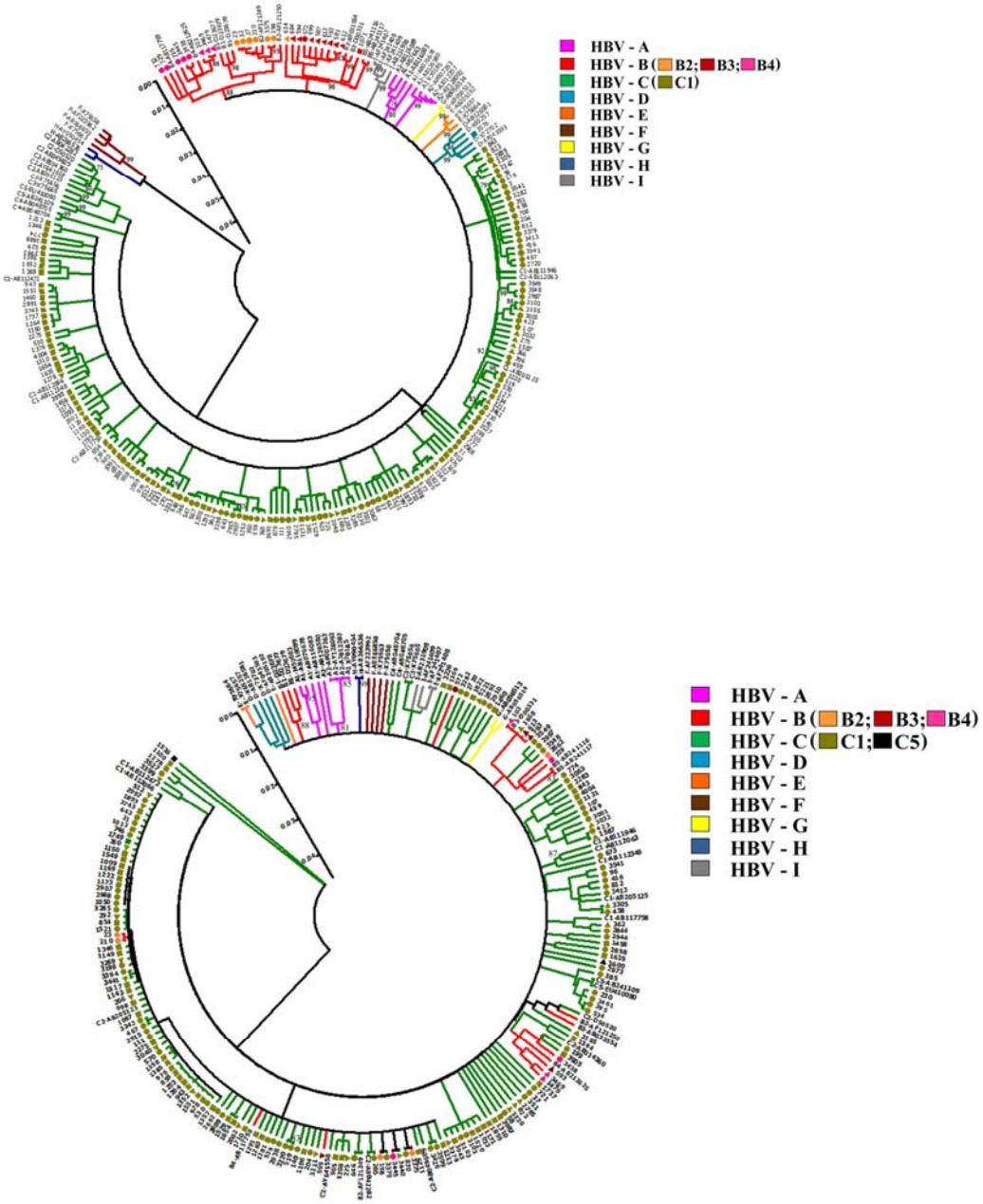

Fig. 1 Phylogram depicting the phylogenetic relationship of the sequence obtained from the present study and representative sequences of human HBV strains from GenBank. Regions include in the comparison were: (A) the large S gene including *preS1*, *preS2* and HBsAg gene; (B) the *C* gene, including nucleotide position 1814 – 2000. Percentage bootstrap values (>75%) were shown at the respective nodes. The scale bar at the bottom indicated the genetic distance. The country origin of migrant workers was indicated by the symbol (● - Cambodia, ▲- Laos and ■ - Myanmar**)**

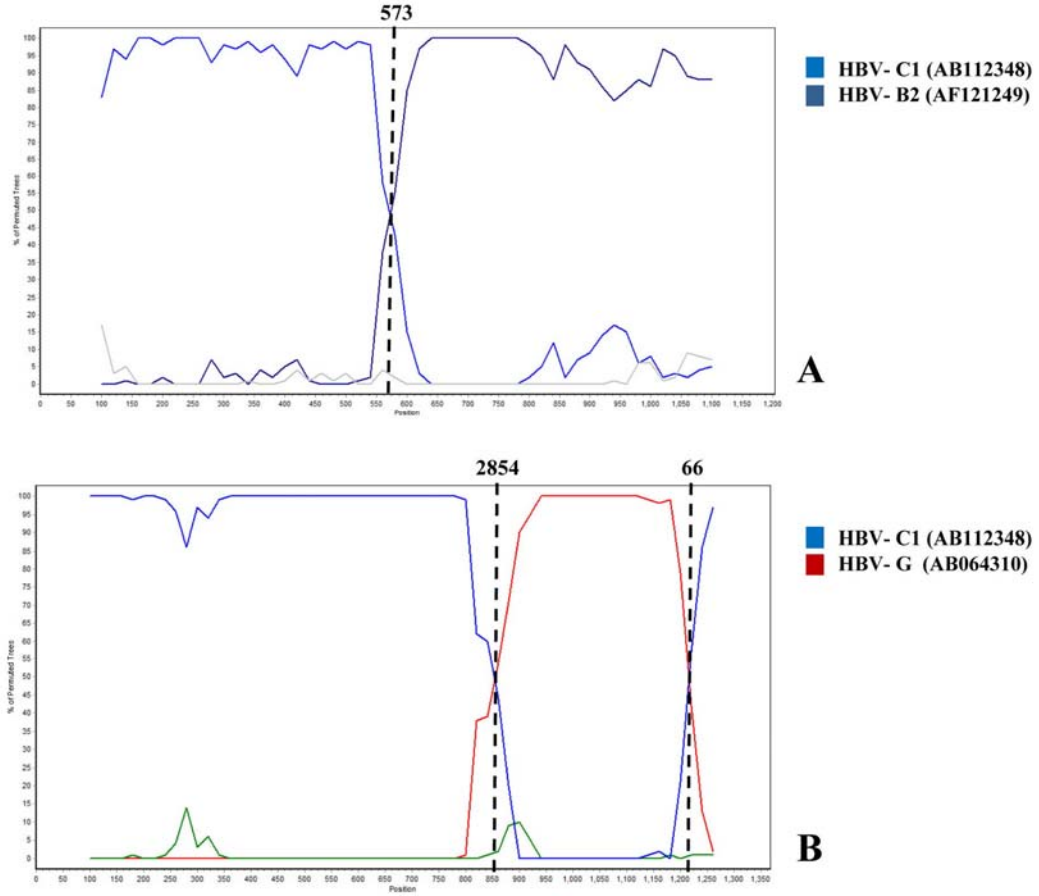

Fig.2 Bootscanning analysis of suspected recombinant isolates. (A) complete *S* gene of isolate 31 was compared with HBV-B2 (AF121249) and HBV-C1 (AB112348); (B) Nucleotide position 2006 – 157 of isolate 3794 was compared with HBV-C1 (AB112348) and HBV-G (AB064310). Dash line (s) showed the breaking point (s) of recombination. The number over the dash line showed the nucleotide position of each isolate compare with the reference strain (NC_003977)

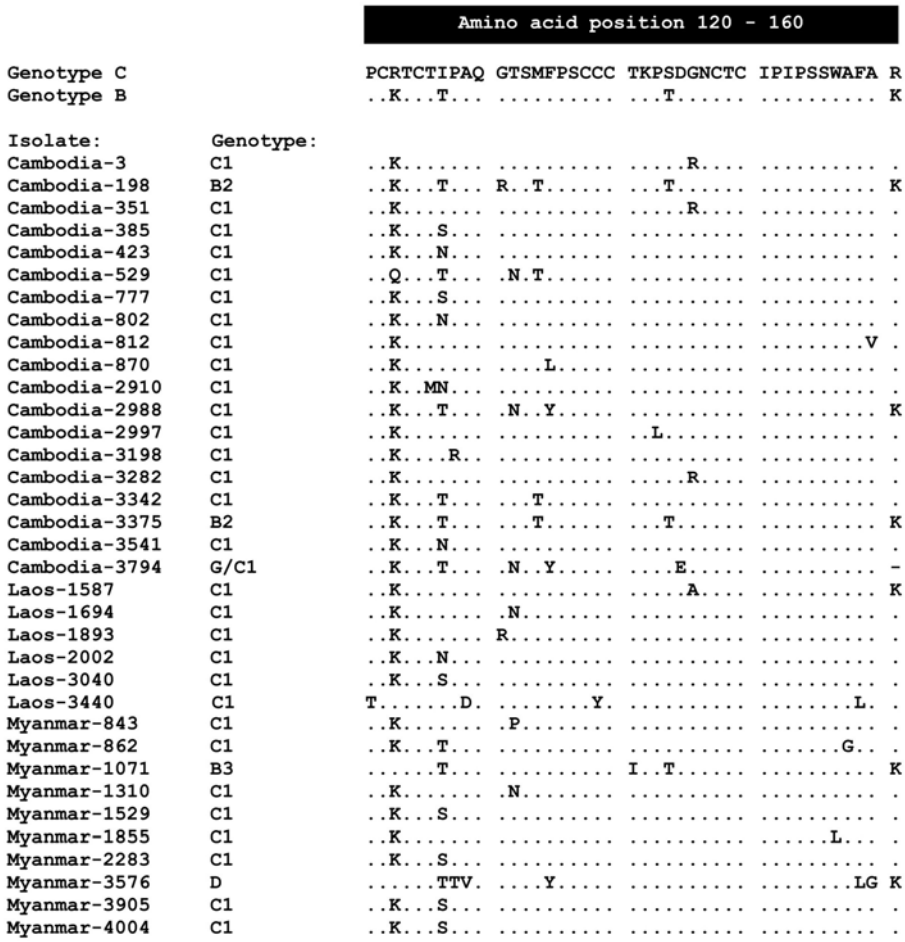

Fig.3 The amino acid sequences alignment of the ‘a’ determinant region of 35 samples.

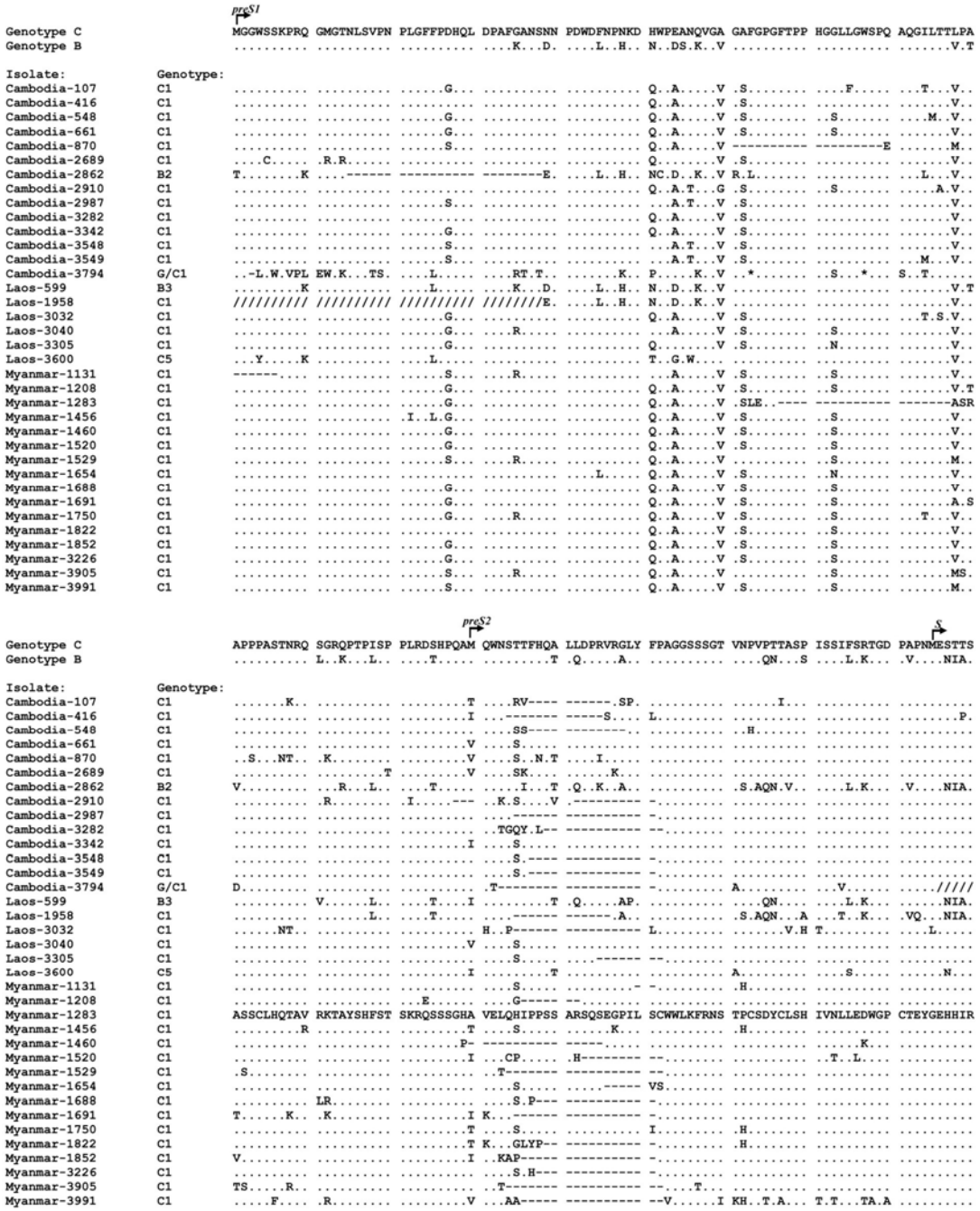

Fig.4 The amino acid sequences alignment of the entire pre-S1/pre-S2 region of 36 samples.

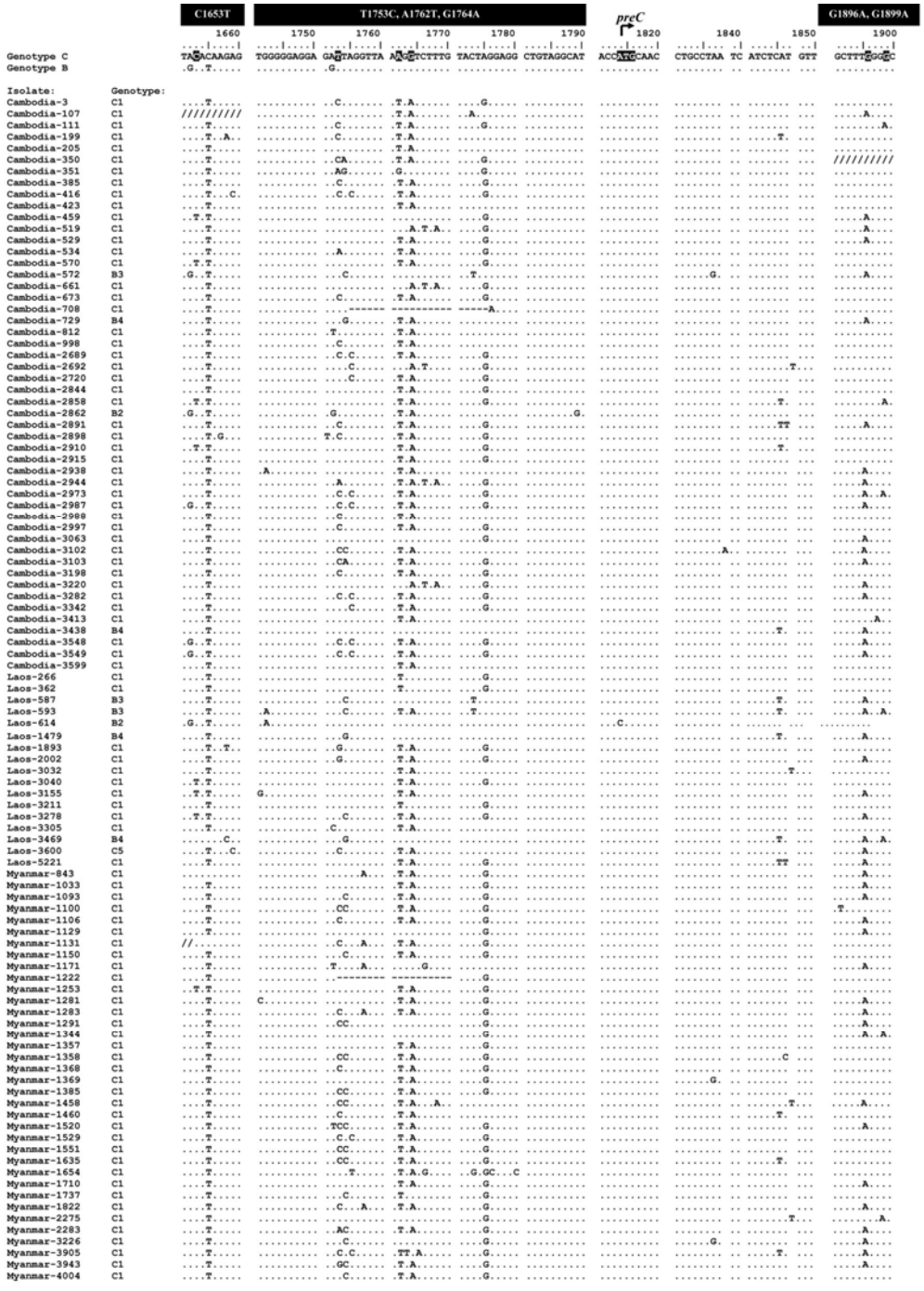

Fig.5 The nucleotide sequences alignment of the EnhII/BCP/PC region of samples in this study.

High response to hepatitis B virus vaccination in HIV-1 infected subjects with isolated antibody to hepatitis B core antigen

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Objective: In hyperendemic area of hepatitis B virus (HBV) infection, whether HIV-1 infected subjects who are positive for isolated antibody to hepatitis B core antigen (anti-HBc) should be vaccinated with hepatitis B vaccine is uncertain. We aimed at evaluating the anamnestic response to hepatitis B vaccine in this population.

Methods: 34 HIV-1 positive subjects and 7 HIV negative subjects who tested positive for anti-HBc but negative for hepatitis B surface antigen (HBsAg) and antibody to HBsAg (anti-HBs) received standard regimen of hepatitis B vaccine at 0,1, 6 months. An anamnestic response was defined as an anti-HBs titer of $\geq$ 10 IU/L. Anti-HBs was checked at 1-3 months after 3rd dose of vaccination.

Results: Of 985 HIV-infected subjects, 117 (11.8%) were HBsAg positive and 89 (9%) had isolated anti-HBc. 34 HIV positive subjects (21 Male) with isolated anti-HBc were included in the analysis. The median (IQR) CD4 was 487 (352 - 753) cells/mm^3; 91% had VL < 50 copies/mL, 10% had HCV co-infection. 24% were taking lamivudine containing regimens and 18% were taking tenofovir/emtricitabine based regimens for a median (IQR) duration of 12 (8 – 50) months prior to vaccination, all had HBV DNA <200 copies/mL. The overall anamnestic response rate was 68% in HIV positive subjects and 71% in HIV negative subjects. In the HIV positive subjects, 3 (9%) subjects had an anti-HBs titer>1000 IU/L and the median (IQR) titer in the other 20 subjects with positive anamnestic response was 44 (28 –138) IU/L. In the HIV negative subjects, no subjects had an anti-HBs titer>1000IU/L; the median response in the 5 subjects with an anamnestic response was 47 (14 – 49) IU/L.

Conclusion: After hepatitis B vaccination, the anamnestic response and anti-HBs seroconversion rate in HIV with isolated anti-HBc were high. Suppressed HBV DNA level during HBV-based HAART therapy might be an explanation for this finding.