Host and viral factors influencing disease progression and treatment response in chronic HBV infection

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ปัจจัยของมนุษย์และไวรัส ที่มีอิทธิพลต่อการดำเนินโรคและการตอบสนองต่อการรักษาในการติดเชื้อไวรัสตับอักเสบบีเรื้อรัง

น.ส.อุมาพร ลิโมทัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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การติดเชื้อไวรัสตับอักเสบบี เป็นสาเหตุสำคัญในการเกิดโรคตับอักเสบเรื้อรัง โรคตับแข็งและมะเร็งตับ ผลลัพธ์ทางคลินิกของการติดเชื้อไวรัสตับอักเสบบีขึ้นอยู่กับปฏิสัมพันธ์ที่ซับซ้อนระหว่างโฮสต์และไวรัส การศึกษานี้พบว่าระดับ HBV RNA ในซีรั่มเป็นตัวบ่งชี้ทางชีวภาพที่สำคัญของการติดเชื้อไวรัสตับอักเสบบี และอาจนำมาใช้เพื่อประเมินการตอบสนองต่อการรักษาด้วยยาต้านไวรัสและใช้ติดตามการดำเนินโรคได้ ระดับ HBV RNA ในซีรั่มมีความแตกต่างกันไปในแต่ละระยะของการติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง และมีความสัมพันธ์กับปริมาณ cccDNA ในเนื้อตับ ซึ่งแสดงให้เห็นว่าสามารถใช้ปริมาณ HBV RNA ในซีรั่ม เป็นตัวบ่งชี้ทางชีวภาพในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรังได้ นอกจากนี้การวัดระดับ HBV RNA ก่อนการรักษาด้วยยา กลุ่มเพกกิเลต อินเตอร์เฟียรอน และการตรวจวัดการเปลี่ยนแปลงของระดับ HBV RNA อย่างต่อเนื่องระหว่างการรักษา ช่วยในการทำนายผลการตอบสนองต่อการรักษาได้ นอกจากนั้น ปัจจัยทางพันธุกรรมของโฮสต์สามารถส่งผลต่อการติดเชื้อไวรัสตับอักเสบบีได้เช่นเดียวกัน งานวิจัยนี้ ได้ทำการการศึกษาอิทธิพลของความหลากหลายทางพันธุกรรม (single nucleotide polymorphisms หรือ SNPs) ในยีนที่เกี่ยวข้องกับวิตามินดี ได้แก่ *25-Hydroxyvitamin D-1 alpha-hydroxylase* (*CYP27B1*), *vitamin D binding protein* (*DBP*) และ *vitamin D receptor* (*VDR*) และความหลากหลายทางพันธุกรรมของยีน signal transducer and activator of transcription 4 (STAT4) ต่อผลลัพธ์ของการติดเชื้อไวรัสตับอักเสบบี ซึ่งพบว่าความหลากหลายทางพันธุกรรมของยีน *CYP27B1* ตำแหน่ง rs4646536 และยีน *STAT4* ตำแหน่ง rs7574865 มีความสัมพันธ์กับการเพิ่มความเสี่ยงในการเกิดมะเร็งตับอย่างมีนัยสำคัญ นอกจากนี้ SNPs ทั้งสองตำแหน่งนี้ ยังสามารถใช้ในการทำนายการตอบสนองต่อการรักษาด้วยกลุ่มเพกกิเลต อินเตอร์เฟียรอน ในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรังได้ ดังนั้น SNPs นี้อาจใช้เป็นตัวบ่งชี้ทางพันธุกรรมสำหรับการทำนายการตอบสนองต่อการรักษาและการเกิดมะเร็งตับในประชากรไทยได้

สาขาวิชา	วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต .....
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KEYWORD: Hepatitis B virus, chronic HBV infection, pegylated interferon, hepatocellular carcinoma, serum HBV RNA, CYP27B1, vitamin D binding protein, STAT4, vitamin D receptor, single nucleotide polymorphisms

Umaporn Limothai : Host and viral factors influencing disease progression and treatment response in chronic HBV infection. Advisor: Prof. Yong Poovorawan, M.D. Co-advisor: Prof. Pisit Tangkijvanich, M.D.

Hepatitis B virus (HBV) infection represents a major risk factor of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). The clinical outcome of HBV infection depends on complex of host and virus interactions. In this study, we found that serum HBV RNA levels has emerged as a potential viral marker for active HBV infection and may be used to evaluate response to antiviral therapy and disease progression. Serum HBV RNA levels varied among different phases of chronic HBV infection (CHB). There was a correlation between serum HBV RNA levels and amount of intrahepatic covalently closed circular DNA (cccDNA), suggesting that serum HBV RNA quantification represents a useful non-invasive marker for disease monitoring in patients with CHB. In addition, the measurement of serum HBV RNA prior to pegylated interferon (PEG-IFN)-based therapy could identify patients with high probability of sustained response (SR, HBV DNA <2,000 IU/mL). Moreover, early HBV RNA kinetics identified subgroup of patients who had a minimal chance of achieving favorable outcome, which could allow response guided therapy. Host genetic factors can also affect the outcome of HBV infection. We investigated the influence of single nucleotide polymorphisms (SNPs) in vitamin D-related genes including *25-Hydroxyvitamin D-1 alpha-hydroxylase* (*CYP27B1*), *vitamin D binding protein* (*DBP*) and *vitamin D receptor* (*VDR*) and genetic variation in *signal transducer and activator of transcription 4* (*STAT4*) on HBV infection outcomes. The results of this study showed that variations of both rs4646536 in the *CYP27B1* gene and rs7574865 in the *STAT4* gene might contribute to increased susceptibility to HBV-related HCC in the Thai population but did not correlate with HBV susceptibility and HBV natural clearance. In addition, both SNPs were predictive factors of response to PEG-IFN therapy in Thai patients with CHB. Thus, the determination of these SNPs could maximize cost-effectiveness of PEG-IFN in patients with CHB.

Field of Study:	Medical Sciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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Umaporn Limothai

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
CR	Combined response
CccDNA	Covalently closed circular DNA;
CHB	Chronic hepatitis B
CYP27B1	Cytochrome P450 family 27 subfamily B member 1
DBP	*Vitamin D binding protein*
ddPCR	Droplet digital *polymerase chain reaction*
ETV	Entecavir
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HBeAg	Hepatitis B e antigen
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
NAs	Nucleoside/nucleotide analogues
NPV	Negative predictive value
PEG-IFN	Pegylated Interferon
PPV	Positive predictive value
SNPs	Single nucleotide polymorphisms
SR	Sustained response
VDR	*Vitamin D receptor*
VR	Virological response

CHAPTER I: INTRODUCTION

Background and Rationale

Hepatitis B virus (HBV) is a major cause of chronic hepatitis and the leading cause of cirrhosis and hepatocellular carcinoma (1, 2). HBV is double-stranded DNA viruses which replicating their DNA genome through reverse transcription from pre-genomic RNA (pgRNA) (3). The mechanism involved in chronic hepatitis B virus (CHB) infection remains unclear but it is likely related to inadequate activation of immune response towards eliminating the virus. Moreover, the virus can persist in hepatocytes of patient as covalently closed circular DNA (cccDNA), which is difficult to eradicate (4). Therefore, the major goal of treatment is to prevent cirrhosis and HCC development.

The natural history of CHB infection is generally divided into four phases according to the level of HBV replication and the response of host immune system: immune tolerance; immune reactive HBeAg positive; inactive carrier and HBeAg negative chronic hepatitis (5). The degree of disease progression in each patient greatly varies. Thus, it is essential to identify factors correlated with the progression of HBV-induced liver disease.

The best available HBV antiviral treatment is pegylated interferon-α (PEG-IFN), which has an immunomodulatory effect and enhances the cell-mediated immune response. The PEG-IFN function through induction of Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway to induce expression of several

IFN stimulated genes (*ISGs*), which can inhibit HBV replication and/or stimulate natural killer cell-mediated elimination of infected hepatocytes (6-9). However, the response rates of PEG-IFN treatment is restricted by its potential side effects. In addition, its efficacy significantly differs among individuals and therefore, identification of favorable predictors prior to therapy is essential for maximizing a high likelihood of treatment response. The therapeutic outcome of PEG-IFN apparently depends on the interaction of several factors, such as viral factors, host genetic variations and immune response (10).

Previous study based on genome-wide association study (GWAS) found that single nucleotide polymorphism (SNP) in *signal transducer and activator of transcription 4* (*STAT4*) especially rs7574865 was correlated with both HBV infection and HBV-related HCC in the Chinese population (11, 12). Moreover, the risk allele G at rs7574865 has been demonstrated to have an impact on the lower mRNA levels of *STAT4* in both the HCC tissues and non-tumor tissue in HBV-related HCC patients (11). *STAT4* is a key component of the JAK-STAT pathway (6) and it is a member of the STAT protein family which is activated by various cytokines such as interleukin (IL)-12 (13, 14) and type I interferon (IFN-α or IFN-β) (15, 16). After activation, *STAT4* enters to the nucleus then binds to the *STAT4* response element and activates its target genes which lead to the production of inflammatory cytokines, like IFN-γ and IL-17 (17) which are major players in a pro-inflammatory immune response (18). Previous studies reported that *STAT4* genetic polymorphisms play an important role in HBV

infection and spontaneous HBV clearance in the Chinese population (19-21). In addition, recent studies demonstrate that *STAT4* rs7574865 associated with the response to IFN-**α** treatment in a Chinese cohort of HBeAg-positive CHB patients (22) but this association has not been replicated yet in any other population.

In recent years, it has been demonstrated that vitamin D, a potent immune-modulator, is linked to the pathogenesis of several chronic disorders, including chronic liver disease (23). Moreover, common SNPs in *vitamin D-related genes*, including *vitamin D receptor* (*VDR*), *vitamin D binding protein* (*DBP*) and enzymes that initiate vitamin D formation such as 1-**α**-hydroxylase (encoded by *CYP27B1*), are shown to be associated with clinical outcome and response to PEG-IFN-based therapy in patients with chronic hepatitis C virus (HCV) infection (24-28). Among HBV-infected individuals, recent studies have suggested a possible role of these SNPs in viral persistence and HCC development (29, 30). In addition, it has recently been demonstrated that a variant of *CYP27B1* is associated with high probability of response to PEG-IFN therapy in an Italian cohort of HBeAg-negative CHB (31). The significance of this polymorphism on clinical outcome and predicting treatment response in other populations is currently unknown.

Theoretically, intrahepatic cccDNA and intrahepatic HBV DNA quantification remain the best accurate method to observe viral reservoir (32). However, liver biopsy is an invasive procedure, with pain and major complications occurring. Therefore, serological markers which can reflect intrahepatic cccDNA activity are very

challenge. Serum HBV DNA levels and HBsAg titers are serological markers which have been used to predict the risk of cirrhosis and HCC. Nevertheless, there are studies found that cirrhosis and HCC can still occur in the majority of patients achieved undetectable HBV DNA (33) and HBsAg seroclearance (34, 35). Other favorable markers which can be the better predictor in these contexts are quite desired.

Recent studies identified that HBV RNA present in the serum of chronically HBV infected patients (36, 37). Furthermore, it is encapsidated and enveloped in virus-like particles (36). The amount of serum HBV RNA was appeared to increase after the reverse transcription process is blocked by NAs in vitro and in transgenic mice (36). The detectable serum HBV RNA is correlate with risk of HBV rebound after withdrawing of NA treatment in CHB patients (36). In addition, the circulating HBV RNA may reflect the amount of HBV RNA in the liver and transcriptionally active cccDNA in patients (38). Therefore, serum HBV RNA might be a potential predictive biomarker for disease monitoring, predicting treatment response and disease outcome of CHB. However, data on the level of HBV RNA in sera of patients with various stages of CHB infection and in CHB patients who were receiving PEG-IFN treatment are restricted.

Presently, the principal technique for monitoring serum HBV RNA is quantitative polymerase chain reaction (qPCR) also known as real-time PCR (36, 37, 39). Nonetheless, this method needs more than 10^2 copies of HBV RNA to produce a detectable signal (36). The requirement hardly achieved with serum samples

of HBeAg-negative CHB infection patients, particularly after anti-viral treatment. Therefore, a highly sensitive quantification methodology for serum HBV RNA in HBeAg-negative CHB infection patients and during anti-viral treatment of CHB patients is very desirous. Droplet Digital PCR (ddPCR) is a recent approach based on water-oil emulsion droplet technology that became commercially available. The ddPCR is created on the combination of traditional PCR and fluorescent-probe–based detection techniques to allow highly sensitive absolute quantification of nucleic acids without the requirement for standard curves. A sample is partitioned into thousands of droplets then PCR amplification occurs within each droplet. For this reason, ddPCR can improve the detection sensitivity and enable the detection of rare events in the samples (40, 41). Therefore, this technology may represent an ideal assay for monitoring serum HBV RNA both in HBeAg-negative CHB infection and during anti-viral treatment of CHB patients. However, data on its capability in monitoring HBeAg-negative CHB patients and receiving PEG-IFN therapy are rather limited.

This study we were investigated the association of *STAT4* and *vitamin D related genes* polymorphisms with clinical outcomes including disease activity and HCC development and treatment response to PEG-IFN. Moreover, we determined serum HBV RNA levels in patients with various stages of chronic HBV infection and evaluate the kinetics of serum HBV RNA levels in CHB patients treated with PEG-IFN. Another objective we compare the detection rate of serum HBV RNA between droplets digital PCR and real-time quantitative PCR.

Research questions

1. Do *vitamin D related genes* and *STAT4* polymorphisms associate with disease progression and treatment response in chronic HBV infection?
2. Do serum HBV RNA levels correlate with clinical outcomes and treatment response in chronic HBV infection?
3. Does droplet digital PCR increase the detection rate of serum HBV RNA as compared with quantitative real-time PCR?

Objectives

1. To investigate the association of *vitamin D related genes* and *STAT4* polymorphisms with disease progression and treatment response in chronic HBV infection.
2. To evaluate the association of serum HBV RNA levels with clinical outcomes and treatment response in chronic HBV infection.
3. To compare the detection rate of serum HBV RNA between droplet digital PCR and quantitative real-time PCR.

Hypothesis

1. *Vitamin D related genes and STAT4* polymorphisms associate with disease progression and treatment response in chronic HBV infection.
2. Serum HBV RNA levels correlate with clinical outcomes and treatment response in chronic HBV infection
3. The droplet digital PCR technique increases the detection rate of serum HBV RNA better than quantitative real-time PCR.

Keywords

Chronic hepatitis B virus (CHB), serum hepatitis B virus RNA (serum HBV RNA), droplet digital PCR (ddPCR), quantitative polymerase chain reaction (qPCR), single nucleotide polymorphisms (SNPs), *signal transducer and activator of transcription 4* (*STAT4*), vitamin D receptor (*VDR*), vitamin D binding protein (*DBP*), *1-α-hydroxylase* (*CYP27B1*), hepatocellular carcinoma (HCC), pegylated interferon (PEG-IFN)

Conceptual framework

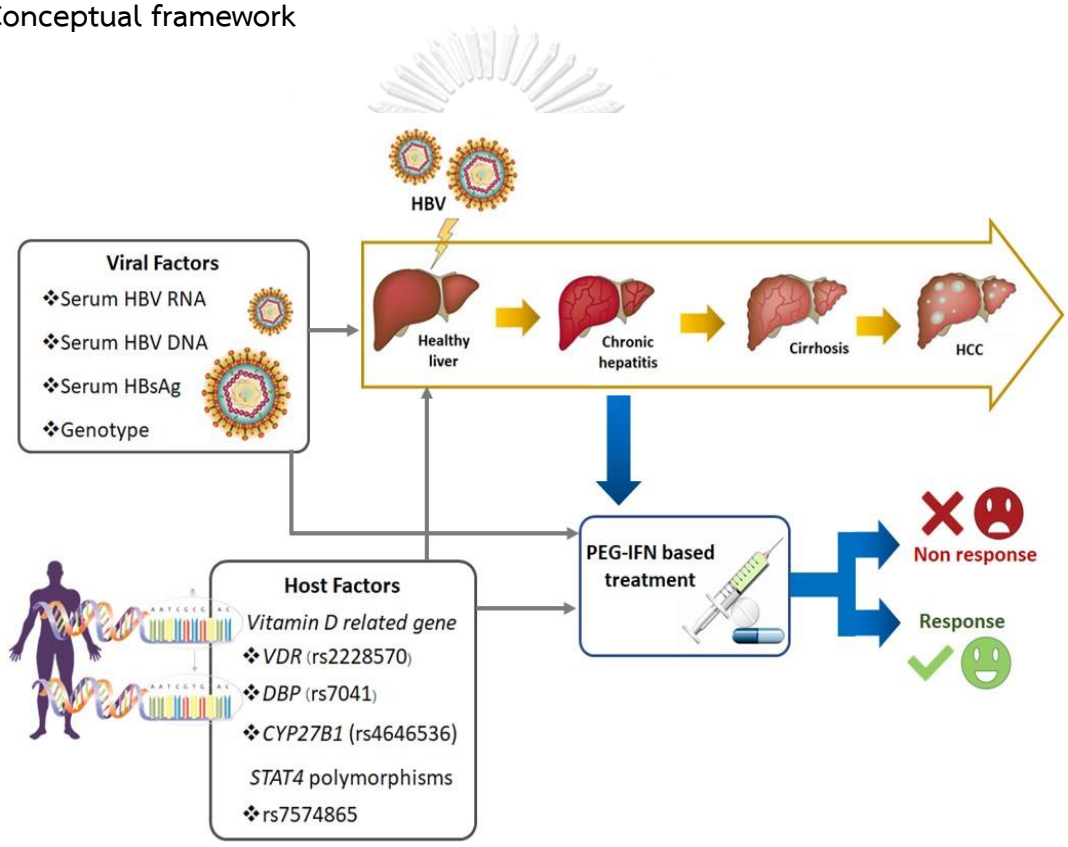


Figure 1. Conceptual framework

Research design

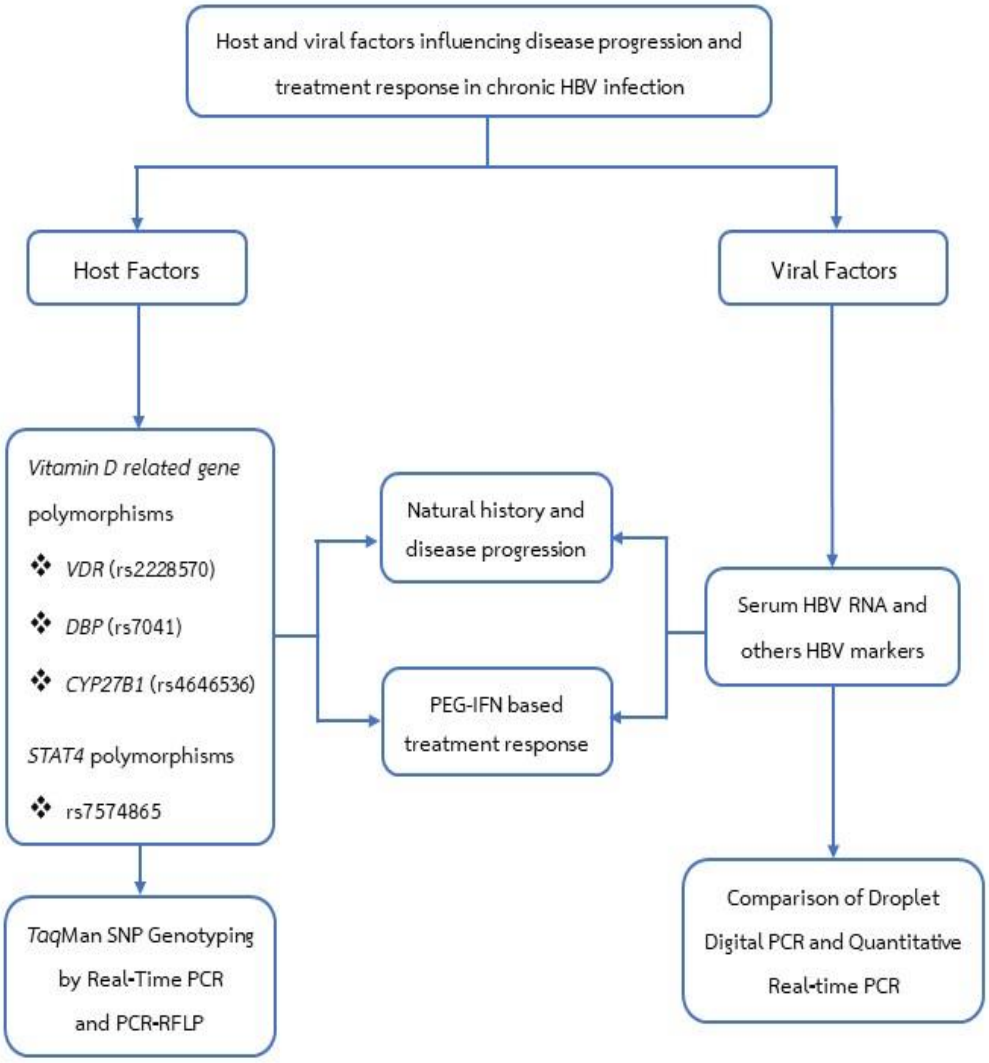


Figure 2. Research design

Expected benefit and application

The expect outcome is to find an important role of host and viral factors to predict treatment response and disease outcome in CHB patient. Any correlation found in this study may provide a potential predictive biomarker for disease monitoring, predicting treatment response and disease outcome of CHB. Moreover, the molecular diagnosis techniques based on quantitative real-time PCR and digital PCR can be applied for rapid, sensitive and specific detection of the HBV RNA to verify the clinical diagnosis of these viruses. In addition, the data of this research will be published in peer-reviewed international journals with high impact factor.

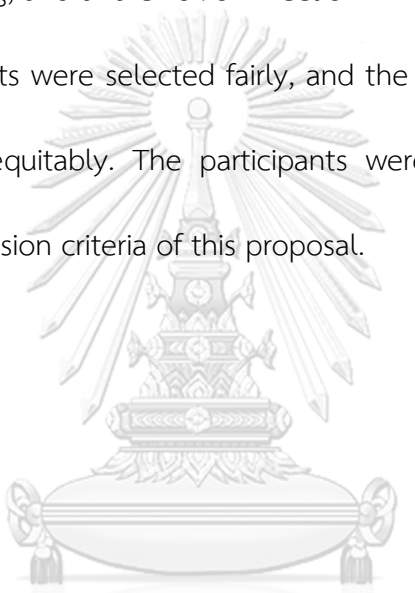
Limitations

The present study was limited by the sample size of CHB patients who were received PEG-IFN treatment. Moreover, pre- and post-treatment serum sample were not available in all patients enrolled.

Ethical Considerations

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (IRB No. 016/61). The Belmont Report identifies 3 basic ethical principles for any human subject research:

1. Respect for persons: All patients with CHB infection was informed of the study's purpose. Written informed consent was obtained from the patients prior entering into the study.
2. Beneficence: There is no benefit to the participants. The specimens of participants were used only for laboratory research. There is a small risk of bruising and fainting, and a rare risk of infection.
3. Justice: The subjects were selected fairly, and the risks and benefits of research were distributed equitably. The participants were recruited following to the inclusion and exclusion criteria of this proposal.



CHAPTER II: REVIEW OF RELATED LITERATURES

Hepatitis B virus (HBV)

HBV is a small enveloped DNA virus, which is a member of *Hepadnaviridae* family. Currently HBV is classified into 10 genotypes (A-J) (42). The viral genome contains partially double-stranded DNA or relaxed-circular DNA (rcDNA) with minus (-) and plus (+) strand. The HBV genome is approximately 3200 nucleotides long. The minus (-) strand consist of 4 overlapping open reading frames (ORFs) which serves as a template for synthesis the viral protein (43) (Figure 3) (32).

1. S-ORF encodes the surface envelope proteins (HBsAg)
2. C-ORF encodes the nucleocapsid core protein (HBcAg) and hepatitis B e antigen (HBeAg)
3. P-ORF encodes the DNA polymerase or reverse transcriptase
4. X-ORF encodes the x antigen (HBxAg)

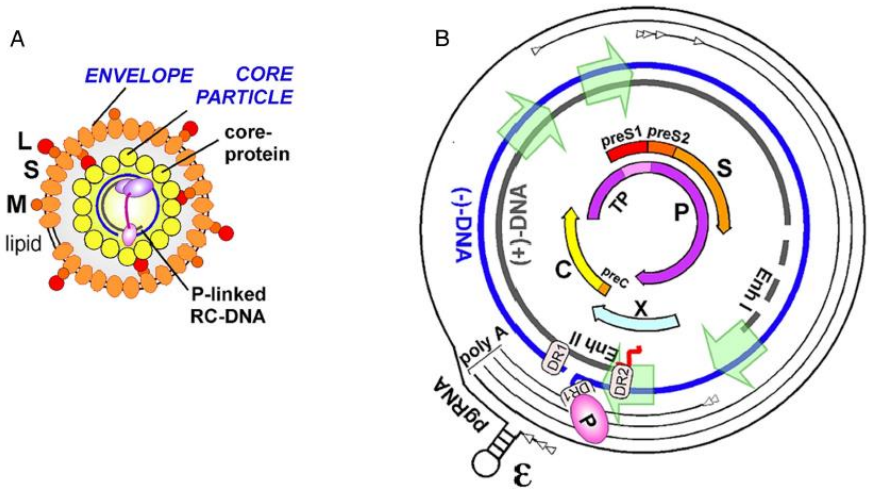


Figure 3. HBV virion structure (A) and genome organization (B)

The replication of Hepatitis B virus

HBV is a DNA virus that has 2 special mechanisms during their replication cycle

1. Once within the nucleus of hepatocyte, the HBV rcDNA is converted into a covalently closed circular DNA (cccDNA) molecule which serves as a template of HBV RNAs and subsequent generation of progeny virions.

2. During the HBV replication cycle, there is the conversion of viral DNA into RNA intermediate which also known as pregenomic RNA. The pregenomic RNA is used for generating the minus strain DNA by reverse transcriptase enzyme.

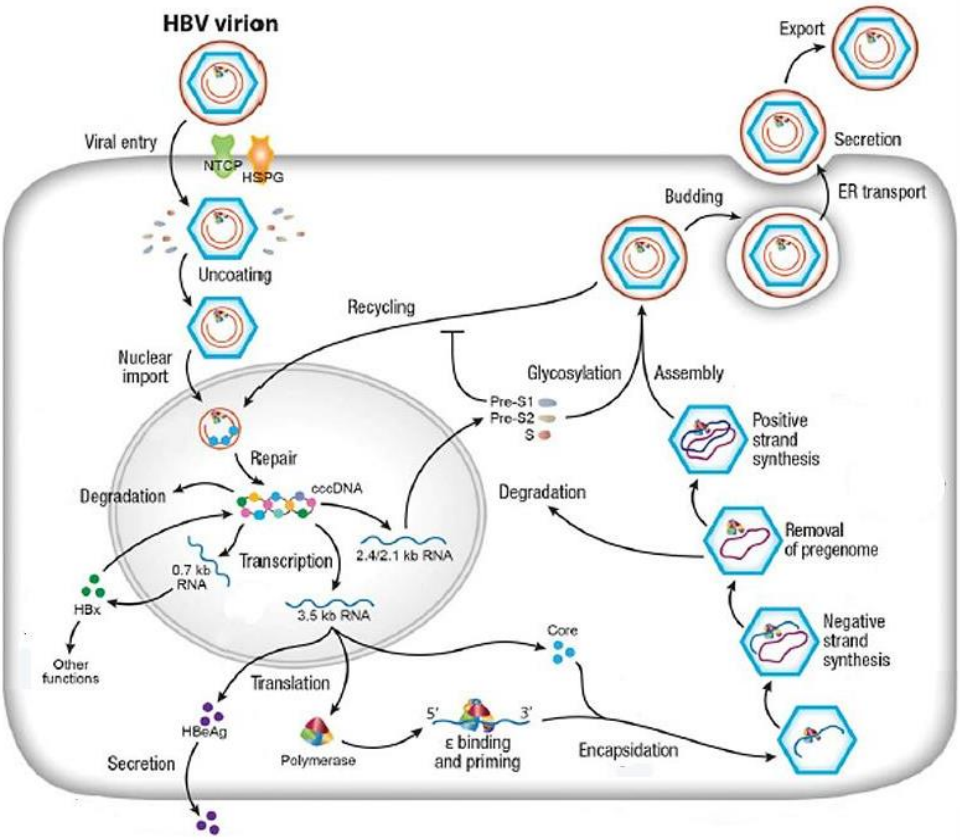


Figure 4. The HBV replication cycle

(44)

HBV enters into the hepatocyte by the binding of specific receptor sodium/taurocholate cotransporter (NTCP) in liver (45). The HBV genome with nucleocapsid is transported into the cytoplasm and then the nucleocapsid is degraded by uncoating process at nuclear pore (46). Then, only the rcDNA enters to the nucleus after that it is repaired to a full-length circular DNA, which is called covalently closed circular DNA (cccDNA). Afterward cccDNA is combined with host histone protein and various proteins into a minichromosome which function as a template for HBV transcription and translation (47) (Figure 4). The cccDNA is transcribed by hepatocyte RNA polymerase II to difference size of HBV RNAs (Figure 3):

1. 3,5 kb mRNA (precore and pregenomic RNA) coding for HBeAg, core protein and polymerase protein.
2. 2.4 kb mRNA and 2.1 kb mRNA coding for large, middle and major surface protein.
3. 0.7 kb mRNA coding for X protein.

The HBV pregenomic RNA has a 5' **ε**-stem loop which bound by polymerase. This binding trigger capsid formation; the pregenomic RNA and polymerase are encapsidated within core particles in the cytoplasm. Inside the core particle, minus strand DNA synthesis is initiated by reverse transcription process and subsequently degradation of the RNA template by RNaseH. After that, plus strand DNA synthesis is begun by short RNA from non-degraded pregenomic as a primer (48, 49). Lastly the newly generated nucleocapsid containing rcDNA can be either enveloped with

surface protein at endoplasmic reticulum (ER) to form infectious virion or recycled back to the nucleus for amplify cccDNA to maintain the hepatocyte cccDNA level (50, 51). Moreover, the complete virion and subviral particle like HBsAg and HBeAg can be found in the patient serum during active phase of HBV infection (52) (Figure 4).

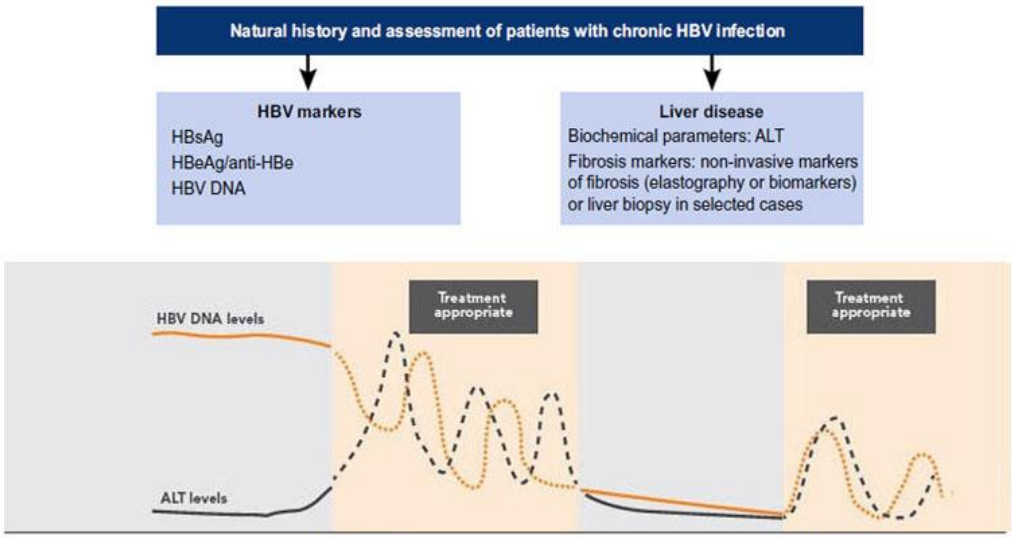
Natural history of chronic HBV infection

The clinical course of HBV infection is greatly complex and variable. An approximately 90% of infants born to HBeAg-positive mothers develop CHB whereas approximately 25%-50% of acute infections in children aged 1-5 years and <5% in older children and adults progress to CHB (53). The natural history of chronic HBV infection results from a complex interplay between the virus and the host. They are classified into 4 phases based on the HBeAg status, the HBV DNA and alanine aminotransferase (ALT) levels.

1. **HBeAg-positive chronic HBV infection:** the first phase of chronic HBV infection which mainly observed during two or three decades after perinatal infection. This phase is characterized by the presence of HBeAg in serum, very high level of HBV DNA and ALT levels persistently within the normal range. HBsAg level is persistently high in serum (54). There is minimal or no inflammation and fibrosis in the liver. In contrast, the integration of HBV DNA into the host genome and hepatocyte clonal expansion might occur indicating that HCC development could be already initiated in

this phase. The HBeAg spontaneous clearance rate is very low. Because of high viral load level, the patients could transmit the virus to others.

2. **HBeAg-positive chronic hepatitis B**: this phase is characterized by the presence of HBeAg in serum, high levels of HBV DNA and elevated ALT. In the liver, there is moderate or severe active inflammation and increased development of fibrosis which results from immune-mediated liver damage (55). The consequence of this phase in is quite variable. Most patients can accomplish HBV DNA suppression and HBeAg seroconversion and then enter to the low replication phase. Some patients cannot control the virus and become to the HBeAg-negative CHB phase (5).



	HBeAg positive		HBeAg negative	
	Chronic infection	Chronic hepatitis	Chronic infection	Chronic hepatitis
HBsAg	High	High/intermediate	Low	Intermediate
HBeAg	Positive	Positive	Negative	Negative
HBV DNA	$>10^7$ IU/ml	10^4-10^7 IU/ml	<2,000 IU/ml*°	>2,000 IU/ml
ALT	Normal	Elevated	Normal	Elevated*
Liver disease	None/minimal	Moderate/severe	None	Moderate/severe
Old terminology	Immune tolerant	Immune reactive HBeAg positive	Inactive carrier	HBeAg negative chronic hepatitis

Figure 5. Natural history and assessment of patients with chronic HBV infection based upon HBV and liver disease markers

(5).

3. **HBeAg-negative chronic HBV infection**: this phase is characterized by the absence of HBeAg and the presence of anti-HBe, ALT levels persistently within the normal range and HBV DNA level down below 2,000 IU/mL or undetectable. However, reliable identification of inactive carrier phase using the combined HBV DNA and ALT level is difficult because patients who seemingly in this phase may have HBV reactivation experience (56). In addition, ALT are not specific to virus-induced liver damage and their change may be resulted from other co-factors of liver damage (57). Some patients may have HBV DNA levels more than 2,000 IU/mL with fluctuations that can reach to 20,000 IU/mL coexisted with constantly normal ALT level and minimal inflammation and low fibrosis in the liver. If the patients remain in this phase, they have a low risk of cirrhosis progression or HCC development but the progression to HBeAg negative chronic hepatitis may occurs. HBsAg loss or HBsAg seroconversion may spontaneously occurs about 1-3% of cases per years(55). Generally, the patients may have serum HBsAg level less than 1,000 IU/mL (58).

4. **HBeAg-negative chronic hepatitis B**: this phase is characterized by the absence of HBeAg with detectable anti-HBe. The serum HBV DNA is persistent or fluctuating at moderate to high level, as well as ALT level is fluctuating or persistently elevated. There is necroinflammation and fibrosis in the liver (55). Most of the patients in this phase harbor genome mutation in the precore and/or the basal core promoter regions which lead to impairment or abolishment of HBeAg expression. The spontaneous clearance rate is low in this phase (55).

Factors affecting persistence and disease progression

Patients persistently infected with HBV progress to CHB, and about 10–15% of carriers progress to liver cirrhosis, liver failure, and HCC (59). Therefore, the degree of disease progression in each patient greatly varies. Thus, it is essential to identify factors correlated with the progression of HBV-induced liver disease. The clinical course and severity of CHB infection involved in complex interplay among three factors (60). First importance factor is HBV such as viral load (61), HBV genotype (62) and genetic mutation in HBV genome (63) which could affect to the host immune responses. The second is host factors such as gender, family history, age at infection, obesity, immune response and genetic variants which can change the outcomes of HBV infection. Finally, environmental factors including co-infection with other viruses, exposure to other liver toxins and carcinogens such as alcohol, aflatoxin (64). All these factors influence the development of cirrhosis or HCC in CHB patients.

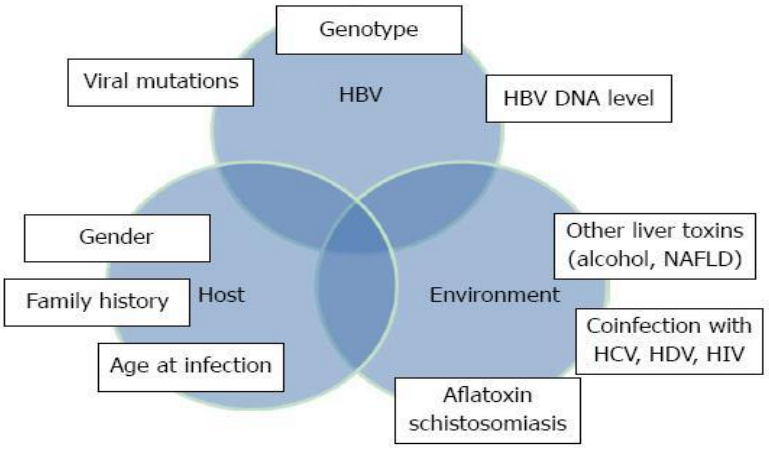


Figure 6. Factors affecting to the natural history of chronic hepatitis B infection

(65).

Biomarkers of HBV infection

Theoretically, intrahepatic cccDNA and intrahepatic HBV DNA quantification remain the best accurate method to observe viral reservoir (32). However, liver biopsy is an invasive procedure, with pain and major complications occurring. Therefore, routine liver biopsy has not been well accepted by patients. Moreover, there are no available commercial assays for cccDNA quantification at present. Therefore, serological markers which can reflect intrahepatic cccDNA activity are very challenge.

Noninvasive serological markers which can reflect an intrahepatic viral replicative activity is very much desired. Serum HBV DNA levels and HBsAg titres are serological markers which have been used to predict the risk of cirrhosis and HCC. Nevertheless, there are studies found that cirrhosis and HCC can still occur in the majority of patients achieved undetectable HBV DNA (33) and HBsAg seroclearanc (34, 35). Other favorable markers which can be the better predictor in these contexts are very challenge.

Serum HBV RNA

Previous studies indicated that HBV RNA in serum could be detected during lamivudine (66, 67) or entecavir therapy (68). Lamivudine and entecavir are nucleoside analogue which can inhibit reverse transcriptase activity, leading to HBV

RNA accumulation in hepatocytes. Nevertheless, it is still unclear that how the HBV RNA is released from hepatocytes into circulation and is not degraded.

Rokuhara A et al. demonstrated that HBV RNA is incorporated into viral particles. Moreover, it can be detected in the serum of CHB patients and did not decrease during lamivudine therapy. The author suggested that serum HBV RNA might serve as a novel marker which differs from HBV DNA in lamivudine therapy (66).

Hatakeyama T et al. showed that patients who were treated with lamivudine had high levels of HBV RNA in serum because reverse transcription are interrupted, and RNA replicative intermediates are unaltered by the drug. In addition, patients who develop viral resistant strains with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of reverse transcriptase had higher serum HBV RNA level than in those without mutants. Therefore, HBV RNA is a useful predictor of early occurrence of viral mutation during lamivudine therapy (67).

The data from Huang YW et al. determined the detectability of serum HBV RNA during consecutive combination treatment of interferon and lamivudine. They found that HBV RNA was not detectable in any patients before treatment, but it became detectable at the end of treatment or follow-up in patients treated with NA monotherapy. In contrast, interferon can inhibit serum HBV RNA induced by lamivudine therefore interferon might potentiate the HBV suppression (69).

Tsuge M et al. indicated that serum HBV DNA plus RNA levels may be a beneficial marker for predicting CHB re-activation after discontinuation of NA therapy (68).

Huang YW et al. investigated the predictive value of serum HBV RNA for initial virological response during NA treatment. They found that low serum HBV RNA level at week 12 of NA therapy can independently predict the virological response in CHB treated patients. Therefore, serum HBV RNA levels may be beneficial for optimizing treatment of CHB (70).

Van Bommel F et al. found an association between early decreases in serum HBV RNA levels and subsequent HBeAg seroconversion during treatment with NA. They proposed that serum HBV RNA kinetics could be used as a novel marker for predicting response to NA treatment in HBeAg-positive patients (39)

Jansen L et al. illustrated that HBV RNA can be detected in plasma specimens from both treated and untreated CHB patients. During long-term NA therapy, level of HBV RNA remained higher than HBV DNA. Although NA therapy strongly reduces HBV DNA levels, its influence on HBV RNA levels is limited. In HBeAg-positive patients, PEG-IFN based therapy induced a stronger decline in the HBV RNA load than NA monotherapy. In addition, its reduction during treatment with NA or PEG-IFN can predict HBeAg loss. In HBeAg-negative patients, a lower HBV RNA level at baseline was independently correlated with response to PEG-IFN based therapy.

Immunoprecipitation with antibodies to HBsAg and HBcAg indicated association of plasma HBV RNA and HBV virions.(37)

Jie Wang et al. investigated the nature, origin and underlying mechanisms of serum HBV RNA by performing a series of experiments. The results showed that serum HBV RNA is pregenomic RNA encapsidated and enveloped in virus-like particles. In addition, HBV RNA levels increase after the reverse transcription activity is blocked by NA *in vitro* and in transgenic mice. Therefore, the release of HBV RNA-containing virions seems to accompany that of HBV DNA-containing particles during normal conditions, but they may turn into the prominent type of virus-like particles when reverse transcription is blocked. The author hypothesized that circulating HBV RNA should reflect the amount of HBV RNA in the liver and transcriptionally active cccDNA in patients. Moreover, the detectable serum HBV RNA is correlate with risk of HBV rebound after withdrawing of NA treatment in CHB patients. The author concluded that HBV RNA might be a potential predictive biomarker to monitor safe discontinuation of NA treatment (36). Thereafter, Giersch K et al. found that serum HBV RNA level clearly correlated with HBV RNA in liver and significantly correlated with cccDNA in HBeAg positive HBV infected humanized uPA/ SCID/beige (USB) mouse model. For this reason, serum HBV RNA may reasonably serve as a useful marker for the persistence of active cccDNA in the liver (71). The data from Jie Wang et al. further supported that in treatment naïve cohort, serum HBV RNA correlated with intraphepatic cccDNA in HBeAg-positive patients, but not in HBeAg-negative

patients. These results indicated the possible effect of HBeAg status to the relationship between serum HBV RNA and cccDNA. HBsAg loss is currently considered as the functional cure in CHB antiviral therapies. However, the low-level of serum HBsAg might be originated from integrated HBV DNA so it might not be able to reflect the intrahepatic cccDNA activity. Interestingly, pregenomic RNA can only be generated from cccDNA so it could reflect the intrahepatic cccDNA status. For this reason, Jie Wang et al suggested that the loss of HBV RNA in serum could be defined as treatment para-functional cure (38).

On the contrary, study by Gao Y et al. examined whether serum HBV RNA could be a strong surrogate marker for reflecting cccDNA activity compared with serum HBV DNA, HBsAg, and HBeAg in HBeAg-positive CHB patients. They found that baseline cccDNA levels correlated better with serum HBV DNA levels than with serum HBV RNA levels. However, they did not find any correlations between baseline cccDNA levels and HBeAg or HBsAg levels. After 96 weeks of NA therapy, the decline of serum HBV RNA correlated with cccDNA levels, but it was showed a week correlation than that seen in the HBsAd decline (72)

Recently, the study by Wang J and co-workers pointed out that serum HBV RNA level correlated with intrahepatic HBV RNA level, the ratio of intrahepatic HBV-RNA to cccDNA and liver histopathology in patients receiving NAs treatment. The authors suggested that serum HBV could likely be used as a noninvasive diagnostic biomarker for liver disease progression in patients receiving NAs treatment (73).

Quantitative polymerase chain reaction

Clinical viral diagnostic approaches commonly rely on quantitative polymerase chain reaction (qPCR) as a gold standard technique for viral detection and quantification in patient samples. In qPCR experiment, DNA is amplified altogether with DNA-binding dyes or fluorescently labeled sequence specific probes that create a signal during the PCR reaction (74).

The qPCR focuses on the exponential phase of reaction which occur at all the reagents are fresh and available. Within this phase, the real-time PCR instrument calculates threshold cycle (Ct) which defined as the cycle number at which fluorescent intensity passes the fixed threshold. It can be used to verify the original amount of template in the reaction based on a standard curve (74).

The qPCR has driven major advances in clinical diagnostic, but this approach has significant limitations. First, quantification is depended on a standard curve, which needs careful calibration and consistent source material. Second, the PCR efficiency in unknown samples and in standard curve is assumed that it is exactly equal. However, this assumption may be the cause of variation especially when the concentration of target is low (75).

Droplet digital polymerase chain reaction

Digital polymerase chain reaction (dPCR) is a recent approach that became commercially available. The dPCR is created on the combination of traditional PCR

and fluorescent-probe–based detection techniques to allow highly sensitive absolute quantification of nucleic acids without the requirement for standard curves. Droplet Digital PCR (ddPCR) is a technique for performing dPCR based on water-oil emulsion droplet technology. The ddPCR uses the primers and probes as same as qPCR but this technique was developed to improve precision and absolute quantification of target DNA sequences (40, 41).

The improvements of ddPCR are accomplished by partitioning the PCR reaction into thousands of individual reactions before amplification process, based on water-in-oil droplet partitions. Therefore, it leads to forming isolated replicate PCRs and each droplet theoretically carries 1 or no copies of target DNA. At the end of reaction, the number of positive per negative droplets is counted for calculating the number of target DNA molecules in the initial sample based on Poisson algorithm. If the individual reactions contain more than one target molecules, the counting of positive droplets will underestimate the exact number of molecules (40, 41). This case can be corrected by the Poisson equation as following;

Number of copies per reaction = $-\ln(1 - p)$, which p represents the number of positive droplets

This equation calculates an average number of molecules per reaction from the observed proportion of positive droplets.

ddPCR provides several advantages for nucleic acid quantification. Firstly, the numerous samples partitioning by ddPCR lead to the accurate measurement of a

small quantity of target DNA. Secondly, ddPCR can reduce competition which comes from high concentration of DNA targets in reaction, as a result, rare targets are enriched, and signal-to-noise is increased. Thirdly, error rates can be reduced by getting rid of the amplification efficiency of PCR, allowing reliable quantification of targets DNA. Last, ddPCR is an end-point measurement technique which does not require a standard curve. Therefore, ddPCR offer more precise and reproducible data than qPCR (40, 41).

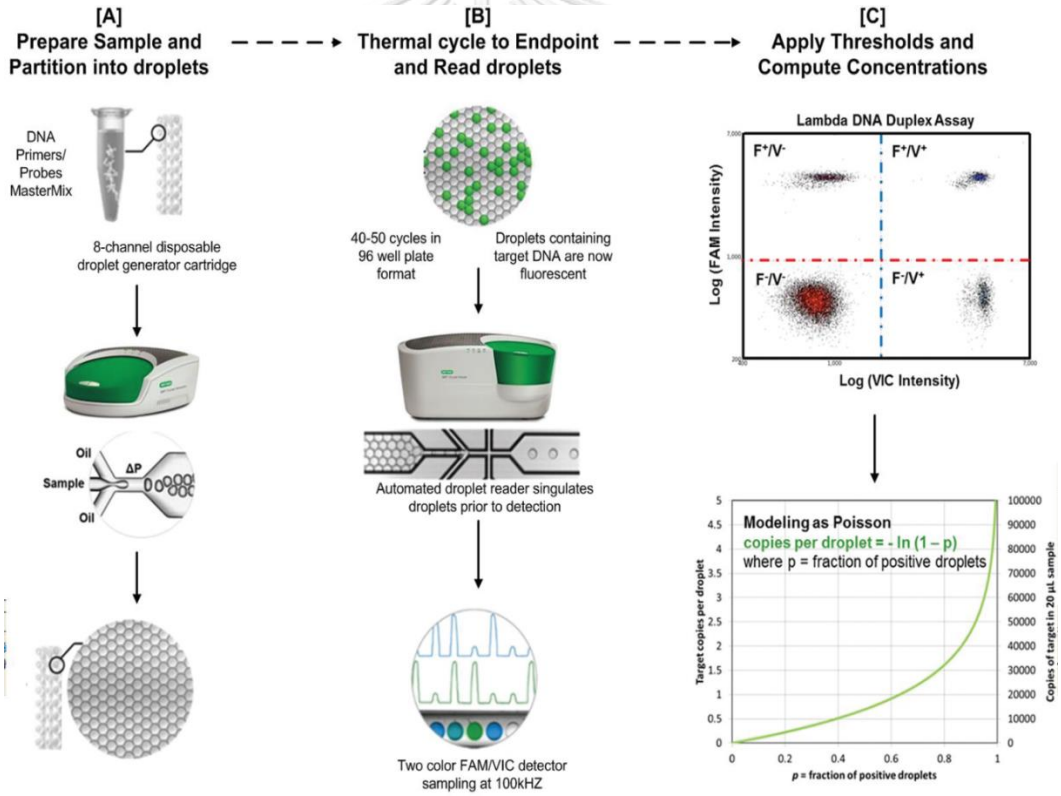


Figure 7. Schematic showing the droplet digital PCR workflow

(40).

For this reason, ddPCR offers broad-ranging applications for research and clinical diagnostic such as absolute quantification especially viral load quantification analysis, copy number variation and rare sequences detection.

Hepatitis B virus therapies

Currently, there are two approaches which available for antiviral treatment in CHB patients, including nucleot(s)ide analogs (NAs) and interferon alpha (IFN-α). (76). For NAs, they directly inhibit reverse transcriptase enzyme but have no direct effect to cccDNA. Because HBV relapse often occurs after discontinuation of NAs treatment therefore long-term administration is required. On the contrary, long-term NA use can induce drug resistance development. Therefore, presently PEG-IFN is the most effective for HBV therapy (77).

IFN-α or Pegylated Interferon (PEG-IFN) has an immunomodulatory effect and enhances the cell-mediated immune response. The PEG-IFN function through induction of Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway to induce expression of several IFN stimulated genes (ISGs), which can inhibit HBV replication and/or stimulate natural killer cell-mediated elimination of infected hepatocytes (6-9). It is effective with a definite treatment course (48-52 weeks) and related with HBsAg and HBeAg loss.

However, the response to PEG-IFN treatment is accomplished only in a minority of CHB patients. Moreover, it has unpleasant side effects such as fever,

fatigue, depression that lead to an early treatment termination. In addition, the therapeutic outcome of PEG-IFN apparently depends on the interaction of several factors, such as viral factors, host genetic variations and immune response (10). For example, C and D genotypes of HBV shown a lower treatment response rate than patients with A and B genotypes (78). Therefore, in clinical practice it is required selecting patients who would have the most probability to accomplish treatment response (79, 80).

Host genetic factors and hepatitis B infection

Increasing evidence has indicated that host genetic variations may play an essential role in natural history and therapeutic outcome of patients with chronic viral hepatitis (10). In patients with chronic HCV infection, SNPs in the *interferon-λ3 (IFNL3*) and *interferon-λ4* (*IFNL4*) genes have been shown to be predictors of spontaneous HCV clearance and response to PEG-IFN-based therapy (81-83). Nonetheless, the association of these SNPs with disease progression and response to PEG-IFN therapy in patients with CHB is uncertain (84) with conflicting data among published reports, which might be related to the heterogeneity of the study populations (10).

Signal transducer and activator of transcription 4 polymorphisms and hepatitis B infection

STAT4 is a key component of the JAK-STAT pathway (6) and it is a member of the STAT protein family which is activated by various cytokines such as interleukin (IL)-12 (13, 14) and type I interferon (IFN-α or IFN-β) (15, 16). After activation, *STAT4* enters to the nucleus then binds to the *STAT4* element and activates its target genes which lead to the production of inflammatory cytokines, like IFN-γ and IL-17 (17) which are major players in a pro-inflammatory immune response (18).

IFN-γ is a cytokine that has an important role in host defense mechanisms (85). Lower activation of IFN-γ through *STAT4* may decrease its antiviral and antitumor functions (86, 87). However, overexpression of IFN-γ may result in the development of autoimmune diseases (88). In liver, IFN-γ is a key cytokine which can induces hepatocyte apoptosis and inhibits hepatocyte cell cycle progression (87). The equilibrium of IFN-γ activation through *STAT4* can influence both antiviral and antitumor activity (86, 87).

Previous study based on genome-wide association study (GWAS) found that SNPs in signal transducer and activator of transcription 4 (*STAT4*) especially rs7574865 was correlated with both HBV infection and HBV-related HCC in the Chinese population (11, 12) probably through its interaction with endogenous IFN-α. Patients carrying GG genotype of rs7574865 are more likely to progress to CHB and HCC than those with the GT/TT genotype (11, 12, 89). These studies advocated the role of the JAK-STAT

signaling pathway which well-established in CHB and HCC. In addition, they suggested that *STAT4* plays a key role more than other genes in the pathway with regarding to clarifying individual diversities in the risk of CHB and HCC. Moreover, the risk allele G at rs7574865 has been demonstrated to have an impact on the lower mRNA levels of *STAT4* in both the HCC tissues and non-tumor tissue in HBV-related HCC patients (11). The authors suggested a possible explanation for this occurrence that lowered *STAT4* expression cannot effectively stimulate the expression of IFN-**γ**, leading to lowered antiviral and antitumor activity.

Recent studies reported that *STAT4* genetic polymorphisms play an important role in HBV infection and spontaneous HBV clearance in the Chinese population (19-21). Lu Y., et al. found that the minor alleles of the four SNPs in the *STAT4* gene (rs7574865, rs7582694, rs11889341, and rs8179673) associated with spontaneous HBV clearance, whereas the major alleles correlated with the progress of the HBV-related liver disease in a Chinese Han population (19). Haplotype-based association analysis reported by Jiang X., et al. demonstrated that the haplotype CTCTT, formed by the five SNPs in *STAT4* region (rs8179673, rs7574865, rs4274624, rs11889341, and rs10168266) was correlated with HBV infection and clearance in a Chinese Han population (21).

In addition, a study by Jiang DK and co-worker reported that *STAT4* rs7574865 associated with the response to IFN-**α** treatment in a Chinese cohort of HBeAg-positive CHB patients (22). They found that the GG genotype (a risk factor of CHB and

HBV-related HCC) was correlated with a reduced rate of sustained virologic response in both patients who received both IFN-**α** or PEG-IFN, compared to the rs7574865 GT/TT genotype. The results of this study demonstrated that CHB patients with the GG genotype are less possibly to achieve IFN-**α** treatment response and more likely to be susceptible to CHB and HCC. Nevertheless, this association has not been replicated yet in any other population.

Vitamin D-related genetic polymorphisms and hepatitis B infection

Vitamin D is the precursor of steroid hormone 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$ or calcitriol) with well-known effect on calcium homeostasis (90). Recently, there are many reports shown the significance of vitamin D in proliferation and differentiation process of the cell (91). Moreover, vitamin D has immunomodulatory and anti-inflammatory properties (92). In recent years, it has been demonstrated that vitamin D is linked to the pathogenesis of several chronic disorders, including chronic liver disease (23).

VDR is an intracellular hormone receptor which specifically binds the $1,25(OH)_2D_3$ and interacts with specific nucleotide response elements of target genes to produce a variety of biological effects. The *VDR* gene is located on chromosome 12 and is highly polymorphic (93). Many SNPs have been identified in this region. A previous study indicated that the *VDR* gene polymorphism affected the expressions and production of *VDR*. In addition, they found that in the human hepatic stellate

cell, this polymorphism involved in the fibrotic processes (94). Variations at Fok1 locus that is in the translation start site of *VDR* gene influenced to a shortening of the *VDR* protein by three amino acids. In addition, it is correlated with worse outcomes in cancer (95). The different structure of *VDR* protein may affect the biological functions of vitamin D and involved in carcinogenesis (96). The *VDR* gene variants have been reported to associate with increased risk of cancers including breast (97), prostate (98) and colorectal(99).

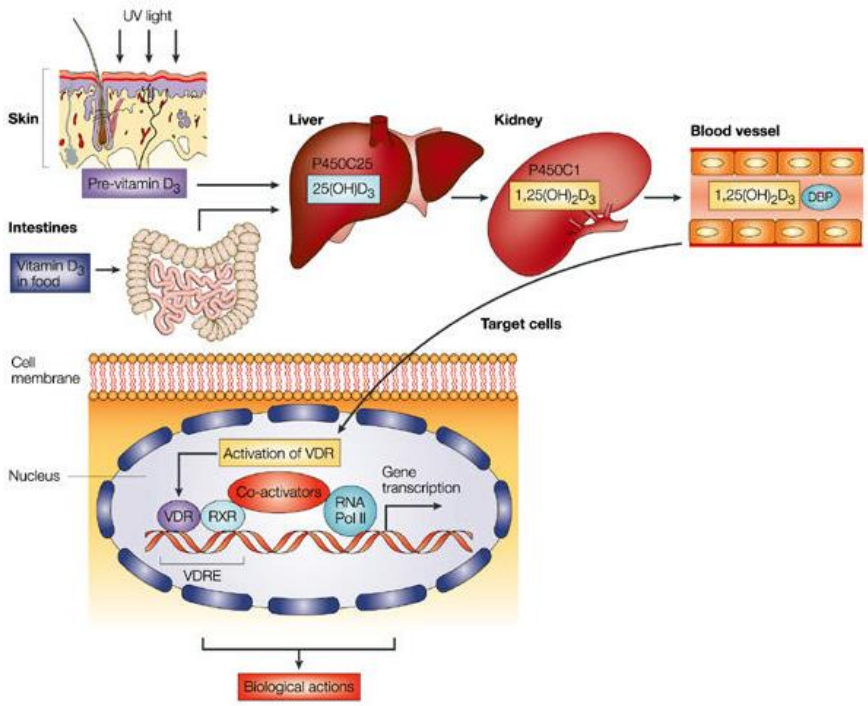


Figure 8. Vitamin D biosynthesis

(100)

DBP is a multifunctional protein which plays a role in the transport of vitamin D metabolites. Almost all the 25(OH) D_3 and active hormone 1, 25$(OH)_2D_3$ exist in the circulation bind to *DBP* (101). It was reported that *DBP* regulates the half-life of

25(OH) D_3 in the circulation through stabilization of the hormone and maintains serum vitamin D levels. In addition, *DBP* also has anti-inflammatory and immunoregulatory functions. It has been reported that *DBP* play a significant role in several chronic disease including cancers (102) . Polymorphisms in the *DBP* gene have been demonstrated that it influences plasma concentrations of 25(OH) D_3 (103). The rs7041 is one of the SNPs in the *DBP* gene which has been indicated conversely correlated with concentrations of 25(OH)D_3 in plasma. In addition, the *DBP* gene polymorphism have been involved in cancers such as breast (104) and prostate (105).

The *CYP27B1* is a gene on chromosome 12 encodes the 1α-hydroxylase. The enzyme responsible of conversion of 25(OH)D_3 into 1,25$(OH)_2D_3$ that evidenced the major biological effects on immune response (106). Moreover, polymorphisms within *CYP27B1* gene cause altered serum levels of 25(OH)D_3 and/or mRNA expression.

Common SNPs in *vitamin D-related genes*, including *VDR*, *DBP* and *CYP27B1* are shown to be associated with clinical outcome and response to PEG-IFN-based therapy in patients with chronic hepatitis C virus (HCV) infection (24-28). Among HBV-infected individuals, recent studies have suggested a possible role of these SNPs in viral persistence and HCC development (29, 30). The data from Peng Q. and co-worker found that both genotype and allele frequencies of the *VDR* rs2228570 and *DBP* rs7041 polymorphisms contributed to increased susceptibility to HBV-related HCC in the Chinese population (29).

In addition, it has recently been demonstrated that a variant of *CYP27B1* is associated with high probability of response to PEG-IFN therapy in an Italian cohort of HBeAg-negative CHB (31). However, the significance of this polymorphism on predicting treatment outcome in other populations is currently unknown.

CHAPTER III: METHODS AND RESULTS

Part 1: Association of vitamin D-related genes and *STAT4* Polymorphisms with HBV Infection Outcomes in Thai Population

(Manuscript in preparation)

Abstract

Background: Though *STAT4* is regarded to associate with chronic hepatitis B (CHB) susceptibility and HBV natural clearance, its role in hepatocellular carcinoma (HCC) development is obscure. The role of vitamin D-related genes including *25-Hydroxyvitamin D-1 alpha-hydroxylase (CYP27B1), vitamin D binding protein (DBP) and vitamin D receptor (VDR)* in CHB susceptibility and clearance as well as HCC development is still contentious. Therefore, we conducted this study, aiming to clarify these obscure relationships.

Methods: We recruited 1077 Thai subjects including HCC (n = 298), CHB (n=379), resolved HBV infection (n = 220) and HBV uninfected subjects (n = 180). The *CYP27B1* rs4646536, *DBP* rs7041, *VDR* rs2228570 and *STAT4* rs7574865 were genotyped.

Results: The results showed that *CYP27B1* rs4646536 associated with HCC development under an additive model (OR=1.60, 95%CI=1.05-2.43, *p*=0.026). In addition, we found that the *STAT4* rs7574865 GG genotypes were correlated with a significant increased HCC risk when compared with the TT or GT (OR=1.68, 95%CI=1.15-2.45, p=0.008). However, the experiment showed that *CYP27B1*

rs4646536, *DBP* rs7041, *VDR* rs2228570 and *STAT4* rs7574865 did not associate with CHB susceptibility and HBV natural clearance.

Conclusions: Vitamin D-related genes (*CYP27B1* rs464653) and *STAT4* rs7574865 polymorphisms may contribute to increased susceptibility to HBV-related HCC in the Thai population but did not correlate with HBV susceptibility and HBV natural clearance.

Background and Rationale

Hepatitis B virus (HBV) is a major cause of chronic hepatitis and the leading cause of cirrhosis and hepatocellular carcinoma (1, 2). Most HBV infections occurred in adults are often self-limited with natural clearance of virus from blood and liver. Less than 5% the infections in adults did not resolved and still developed into chronic infection (53). About 20% patients would develop to cirrhosis after chronic HBV infection and 5–10% would progress to HCC. The reasons why some adults could accomplish natural clearance, and some would progress to CHB and HCC, remain obscure.

Previous study based on genome-wide association study (GWAS) found that single nucleotide polymorphism (SNP) in *signal transducer and activator of transcription 4* (*STAT4*) especially rs7574865 was correlated with both HBV infection and HBV-related HCC in the Chinese population (11, 12). Moreover, the risk allele G at rs7574865 has been demonstrated to have an impact on the lower mRNA levels of *STAT4* in both

the HCC tissues and non-tumor tissue in HBV-related HCC patients (11). *STAT4* is a key component of the JAK-STAT pathway (6) and it is a member of the STAT protein family which is activated by various cytokines such as interleukin (IL)-12 (13, 14) and type I interferon (IFN-α or IFN-β) (15, 16). After activation, *STAT4* enters to the nucleus then binds to the *STAT4* response element and activates its target genes which lead to the production of inflammatory cytokines, like IFN-γ and IL-17 (17) which are major players in a pro-inflammatory immune response (18). Previous studies reported that *STAT4* genetic polymorphisms play an important role in HBV infection and spontaneous HBV clearance in the Chinese population (19-21). In addition, our previous data suggest that SNP rs7574865 in *STAT4* might contribute to progression to HCC in the Thai population (89), whether it correlates with spontaneous HBV clearance remains unknown.

Moreover, it has been demonstrated that vitamin D, a potent immune-modulator, is linked to the pathogenesis of several chronic disorders, including chronic liver disease(23). Moreover, common SNPs in *vitamin D-related genes*, including *vitamin D receptor* (*VDR*), *vitamin D binding protein* (*DBP*) and enzymes that initiate vitamin D formation such as 1-α-hydroxylase (encoded by *CYP27B1*), are shown to be associated with clinical outcome and response to PEG-IFN-based therapy in patients with chronic hepatitis C virus (HCV) infection (24-28). Among HBV-infected individuals, recent studies have suggested a possible role of these SNPs in viral persistence and HCC development (29, 30). In addition, it has recently demonstrated that a variant of

CYP27B1 is associated with high probability of response to PEG-IFN therapy in an Italian cohort of HBeAg-negative CHB (31). The significance of this polymorphism on clinical outcome in other populations is currently unknown.

Therefore, in this study, we aimed to investigate the association of *CYP27B1* rs4646536, *DBP* rs7041, *VDR* rs2228570 and *STAT4* rs7574865 with HCC development, HBV susceptibility and clearance in Thai population.

Materials and Methods

Patients

The present study recruited 1077 Thai subjects including HCC (n = 298), CHB (n=379), resolved HBV infection (n = 220) and HBV uninfected subjects (n = 180). All Thai patients with CHB and HCC were recruited at the King Chulalongkorn Memorial Hospital. CHB were defined as those who had histories of at least 6 months HBsAg positivity as well as no HBsAb (anti-HBs negative) existed in the serum. HCC were diagnosed based on typical imaging studies and/or histology (fine needle aspiration or surgical resection) in accordance with the guidelines of American Association for the Study of Liver Diseases (AASLD) (107). Whereas patients with resolved HBV and uninfected subjects were recruited from the Thai Red Cross Society (age>40 years) which had been screened by Immunoassay (Architect i2000SR, Abbott, USA.) for HBsAg, antibody to anti-HBs and antibody to hepatitis B core protein (anti-HBc). Subjects with resolved HBV infection were those who were negative for HBsAg, but

positive for both anti-HBs and anti-HBc. Those who were coinfected with hepatitis C virus or human immunodeficiency virus (HIV) were excluded from this study. This study was conducted following the Helsinki Declaration and Good Clinical Practice guidelines and all patients gave written informed consent and. The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Serological and virological assays

The levels of HBsAg, anti-HBsAg, HBeAg and Anti-HBe were determined by commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL).

Genomic DNA preparation and genotyping

Human genomic DNA was extracted from 200 µl of buffy coats sample by phenol-chloroform-isoamyl alcohol extraction method as previously described (108). Briefly, the cells were lysed with lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0 and 0.5% SDS) and proteinase K followed by phenol/ chloroform extraction and ethanol precipitation. The DNA pellet were dissolved in 30 uL sterile distilled water. The DNA quality were determined by spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA).

Polymorphisms in the *CYP27B1* gene (rs4646536) and *STAT4* gene (rs7574865) was determined by a commercial *Taq*Man SNP Genotyping Assays (Applied

Biosystems, Foster City, CA, USA) in ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously (109, 110). Fluorescent signals (FAM and VIC) were acquired at the end of each cycle. Positive and negative controls were included in each experiment to ensure an appropriate data interpretation. Allelic discrimination plot was analyzed using Applied Biosystems QuantStudio Real-Time PCR System Software.

SNP rs2228570 in the *VDR* gene was genotyped using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method as described previously, with some modifications (111). In brief, the gene fragments containing the polymorphic sites were amplified using forward primer 5'-TGG CAC TGA CTC TGG CTC TGA-3'and reverse primer 5'-CTC CCT TCA TGG AAA CAC CTT G-3'. The PCR cycles were as follows: 94 °C for 3 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 58°C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. Then, the PCR products were identified under ultraviolet light after electrophoresis in 2% agarose gel stained with ethidium bromide (EB). After that, the amplified products were digested with 2 U *FokI* (New England BioLabs, Newton, MA, USA) overnight at 37°C. Cleaved DNA fragments were identified by ultraviolet light after electrophoresis in 3% agarose gel stained by EB. The PCR products digested with *FokI* produced the following band profiles: 1 fragment of 266 bp in individuals with the CC genotype; 2 fragments of 205 bp and 61 bp in individuals with the TT genotype; 3 fragments of 266 bp, 205 bp and 61 bp in individuals with the TC genotype.

SNP rs7041 in the *DBP* gene was also genotyped using PCR–RFLP method as described previously, with some modifications (109). An amplification using forward primer 5'-TAC CAC AGG TAT AGA ATT TT-3'and reverse primer 5'-AGT GGA GGG TTA CAT TTT CCT-3'. The PCR cycles were as follows: 94 °C for 3 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 53°C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR products were digested with 2 U *HaeIII* (New England BioLabs, Newton, MA, USA) overnight at 37°C. The restriction pattern was analyzed under ultraviolet light after electrophoresis in 3% agarose gel stained with EB. The enzymatic digestion with *HaeIII*, produced a band profiles that allowed the distinction between the TT (304 bp), GT (304 bp, 183 bp, 121 bp) and GG (183 bp and 121 bp) genotypes.

Statistical analyses

Continuous variables were expressed as mean ± standard deviation (SD). Differences in demographic characteristics were compared among groups with one-way ANOVA (for continuous variables) and chi square test. (for categorical variables). The Hardy–Weinberg equilibrium (HWE) was evaluated was tested in all groups of study subjects using Pearson's Chi-square as carried out in online software (http://ihg.gsf.de/ihg/snps.html). P-values were in HWE when there were more than 0.01. Associations of different genetic models with HCC risk were assessed under additive, allelic, dominant and recessive models. Odds ratios (ORs) with

95% confidence intervals (CIs) were calculated by MedCalc Software (https://www.medcalc.org/calc/odds_ratio.php). P-values below 0.05 were considered statistical significance.

Results

Demographic data of the participants

The clinical characteristics of all participants were shown in Table 1. The data showed that the HCC group was older than the CHB, resolved and uninfected group. The proportion of male was highest in the HCC group (87.2%) whereas proportion of females was highest in uninfected group (41.7%).

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

Characteristics	HCC (n = 298)	CHB (n=379)	Resolved (n = 220)	Uninfected (n = 180)
Age (years)	58.3±0.7	41.9±0.6	50.0±0.4	46.3±0.4
Male	260 (87.2%)	245 (64.6%)	145 (65.9%)	105 (58.3%)
HBsAg positive	298 (100%)	379 (100%)	0	0

Genotype distribution and minor allele frequencies (MAF) of the SNPs

The SNP genotyping assays were successful in all samples. The genotype frequencies of each SNPs in the whole cohort were not deviated from Hardy–Weinberg equilibrium (P>0.01). Table 2 demonstrates genotype distribution and minor allele frequency (MAF) of the studied SNPs in controls and patients with HCC. The genotype distributions and MAF of these 4 SNPs were comparable among all studied groups.

Table 2. Genotype distribution and minor allele frequencies (MAF) of the SNPs

Polymorphisms	HCC (n = 298)	CHB (n=379)	Resolved (n = 220)	Uninfected (n = 180)	*P*
CYP27B1					0.263
rs4646536					
TT	54±18.1	82±21.6	49±22.3	35±19.4	
CT	151±50.7	175±46.2	105±47.7	73±40.6	
CC	93±31.2	122±32.2	66±30.0	72±40.0	
MAF (G allele) [a]	0.57	0.55	0.54	0.60	
HWEp	0.59	0.20	0.56	0.04	
VDR rs2228570					0.894
TT	67±22.5	75±19.8	48±21.8	41±22.8	
CT	141±47.3	192±50.7	111±50.5	82±45.6	
CC	90±30.2	112±29.6	61±27.7	57±31.7	
MAF (C allele) [a]	0.54	0.55	0.53	0.54	
HWEp	0.41	0.66	0.85	0.27	
***DBP* rs7041**					0.443
TT	139±46.6	179±47.2	111±50.5	76±42.2	
GT	129±43.3	153±40.4	92±41.8	85±47.2	
GG	30±10.1	47±12.4	17±7.7	19±10.6	
MAF (G allele) [a]	0.32	0.33	0.29	0.34	
HWEp	0.99	0.11	0.73	0.50	
STAT4					0.105
rs7574865					
TT	35±11.7	52±13.7	28±12.7	19±10.6	
GT	118±39.6	167±44.1	93±42.3	96±53.3	
GG	145±48.7	160±42.2	99±45.0	65±36.1	
MAF (G allele) [a]	0.68	0.64	0.66	0.63	
HWEp	0.15	0.43	0.40	0.06	

Data expressed as n (%), differences between groups were tested by Chi-square test,

*P< 0.05, MAF minor allele frequency, [a] Defined by using data from public database (NCBI).

Association analysis of vitamin D-related genes and *STAT4* polymorphisms with HBV clearance and susceptibility

To identify genetic variations associated with HBV susceptibility, we first compared genotype frequencies of vitamin D-related genes, *STAT4* between CHB

group and resolved group respectively. The minor allele frequencies of vitamin D-related genes and *STAT4* in both the two groups (CHB vs. resolved) were as follows: *CYP27B1* rs4646536, 0.55 VS 0.54; *VDR* rs2228570, 0.55 VS 0.53, *DBP* rs7041, 0.33 VS 0.29; *STAT4* rs7574865, 0.64 VS 0.66. The results showed that there was no significant difference in genotype distribution among the CHB group compared with the resolved group. Allele model further confirmed the similar trend in comparison with allele frequency (*CYP27B1* rs4646536, OR=0.94, 95%CI=0.75-1.20, *p*=0.636; *VDR* rs2228570 OR=0.93, 95%CI=0.73-1.17, *p*=0.519; *DBP* rs7041, OR=1.20, 95%CI =0.93-1.56, *p*=0.155; *STAT4* rs7574865, OR=0.92, 95%CI=0.72-1.18, *p*=0.509). These results provided evidence that *CYP27B1* rs4646536, *VDR* rs2228570, *DBP* rs7041 and *STAT4* rs7574865 might not be associated with HBV clearance in Thai population (Table 3).

Next, we appraised the correlation between CHB and uninfected group. We also did not observe any significant differences in genotype and allele frequencies of *CYP27B1* rs4646536, *VDR* rs2228570, *DBP* rs7041 and *STAT4* rs7574865 polymorphisms between the CHB patients and healthy controls (Table 3). These results demonstrated that these 4 SNPs might not be associated with HBV susceptibility in Thai population.

Table 3. Association of vitamin D-related genes and *STAT4* polymorphisms with CHB susceptibility, HBV natural clearance.

Genotype	CHB		Resolved		Uninfected		CHB VS Resolved			CHB VS Uninfected		
	n	%	n	%	n	%	OR	95% CI	*p*	OR	95% CI	*p*
***CYP27B1* rs4646536**												
CC	122	32.2	66	30.0	72	40.0	1.00			1.00		
CT	175	46.2	105	47.7	73	40.6	0.90	0.61-1.32	0.598	1.41	0.95-2.10	0.089
TT	82	21.6	49	22.3	35	19.4	0.91	0.57-1.44	0.674	1.38	0.85-2.26	0.196
Allelic model												
C	419	55.3	237	53.9	217	60.3	1.00			1.00		
T	339	44.7	203	46.1	143	39.7	0.94	0.75-1.20	0.636	1.23	0.95-1.58	0.115
Dominant model												
CC	122	32.2	66	30.0	72	40.0	1.00			1.00		
CT+TT	257	67.8	154	70.0	108	60.0	0.90	0.63-1.29	0.578	1.40	0.97-2.03	0.070
Recessive model												
CC+CT	297	78.4	171	77.7	145	80.6	1.00			1.00		
TT	82	21.6	49	22.3	35	19.4	0.96	0.64-1.44	0.856	1.14	0.73-1.78	0.552
***VDR* rs2228570**												
CC	112	29.6	61	27.7	57	31.7	1.00			1.00		
CT	192	50.7	111	50.5	82	45.6	0.94	0.64-1.39	0.764	1.19	0.79-1.80	0.403
TT	75	19.8	48	21.8	41	22.8	0.85	0.53-1.37	0.508	0.93	0.57-1.53	0.778
Allelic model												
C	416	54.9	233	53.0	196	54.4	1.00			1.00		
T	342	45.1	207	47.0	164	45.6	0.93	0.73-1.17	0.519	0.98	0.76-1.26	0.891
Dominant model												
CC	112	29.6	61	27.7	57	31.7	1.00			1.00		
CT+TT	267	70.4	159	72.3	123	68.3	0.91	0.63-1.32	0.635	1.10	0.75-1.62	0.611
Recessive model												
CC+CT	304	80.2	172	78.2	139	77.2	1.00			1.00		
TT	75	19.8	48	21.8	41	22.8	0.88	0.59-1.33	0.554	0.84	0.54-1.29	0.416
***DBP* rs7041**												
TT	179	47.2	111	50.5	76	42.2	1.00			1.00		
GT	153	40.4	92	41.8	85	47.2	1.03	0.73-1.46	0.863	0.76	0.52-1.11	0.162
GG	47	12.4	17	7.7	19	10.6	1.71	0.94-3.13	0.080	1.05	0.58-1.91	0.872
Allelic model												
T	511	67.4	314	71.4	237	65.8	1.00			1.00		
G	247	32.6	126	28.6	123	34.2	1.20	0.93-1.56	0.155	0.93	0.71-1.21	0.600
Dominant model												
TT	179	47.2	111	50.5	76	42.2	1.00			1.00		
GT+GG	200	52.8	109	49.5	104	57.8	1.14	0.82-1.59	0.447	0.82	0.57-1.17	0.267
Recessive model												
TT+GT	332	87.6	203	92.3	161	89.4	1.00			1.00		
GG	47	12.4	17	7.7	19	10.6	1.69	0.94-3.02	0.077	1.20	0.68-2.11	0.528
***STAT4* rs7574865**												
TT	52	13.7	28	12.7	19	10.6	1.00			1.00		
GT	167	44.1	93	42.3	96	53.3	0.97	0.57-1.63	0.900	0.64	0.36-1.13	0.127
GG	160	42.2	99	45.0	65	36.1	0.87	0.52-1.47	0.603	0.90	0.50-1.64	0.729
Allelic model												
T	271	35.8	149	33.9	134	37.2	1.00			1.00		
G	487	64.2	291	66.1	226	62.8	0.92	0.72-1.18	0.509	1.07	0.82-1.38	0.633
Dominant model												
TT	52	13.7	28	12.7	19	10.6	1.00			1.00		
GG+GT	327	86.3	192	87.3	161	89.4	0.92	0.56-1.50	0.731	0.74	0.42-1.30	0.295
Recessive model												
TT+GT	219	57.8	121	55.0	115	63.9	1.00			1.00		
GG	160	42.2	99	45.0	65	36.1	0.89	0.64-1.25	0.507	1.29	0.90-1.86	0.170

Association analysis of vitamin D-related genes and *STAT4* polymorphisms with HCC development

To identify the correlation of these genetic polymorphisms with HBV progression, we compared the genotype frequency between HCC group and CHB group. The minor allele frequencies in the two groups were shown as follows (HCC vs. CHB): *CYP27B1* rs4646536, 0.57 VS 0.55; *VDR* rs2228570, 0.54 VS 0.55; *DBP* rs7041, 0.32 VS 0.33; *STAT4* rs7574865, 0.68 VS 0.64. Univariate analysis showed that there were no significant differences in the genotype and allele frequencies of these 4 SNPs between the HCC group and CHB group (Table 4). These results implied that *CYP27B1* rs4646536, *VDR* rs2228570, *DBP* rs7041 and *STAT4* rs7574865 polymorphisms might not contribute to HCC development in patients with CHB infection.

To determine the association of these 4 SNPs with HCC susceptibility, we compared the genotype frequency between HCC group and uninfected group. The minor allele frequencies of the 4 SNPs in both the two groups (HCC vs. uninfected) were as follows: *CYP27B1* rs4646536, 0.57 VS 0.60; *VDR* rs2228570, 0.54 VS 0.54; *DBP* rs7041, 0.32 VS 0.34; *STAT4* rs7574865, 0.68 VS 0.63. Under additive model, the results showed that *CYP27B1* rs4646536, CT genotype was more frequently distributed in the HCC group than in uninfected group (OR=1.60, 95%CI=1.05-2.43, *P*=0.026) suggesting that the CT genotype might be associated with an increased risk of HCC. In addition, we found that the *STAT4* rs7574865 GG genotypes were

correlated with a significant increased HCC risk when compared with the TT or GT (OR=1.68, 95%CI=1.15-2.45, p=0.008) (Table 4). However, we did not find significant association between *VDR* rs2228570, *DBP* rs7041 and HCC susceptibility (*P*>0.05).

Table 4. Association of vitamin D-related genes and *STAT4* polymorphisms with HCC development

Genotype	HCC		CHB		Uninfected		HCC VS CHB			HCC VS Uninfected		
	n	%	n	%	n	%	OR	95% CI	*p*	OR	95% CI	*p*
***CYP27B1* rs4646536**												
CC	93	31.2	122	32.2	72	40.0	1.00			1.00		
CT	151	50.7	175	46.2	73	40.6	1.13	0.80-1.60	0.484	**1.60**	**1.05-2.43**	**0.026 ***
TT	54	18.1	82	21.6	35	19.4	0.86	0.56-1.34	0.512	1.19	0.71-2.01	0.507
Allelic model												
C	337	56.5	419	55.3	217	60.3	1.00			1.00		
T	259	43.5	339	44.7	143	39.7	0.95	0.77-1.18	0.641	1.17	0.89-1.52	0.257
Dominant model												
CC	93	31.2	122	32.2	72	40.0	1.00			1.00		
CT+TT	205	68.8	257	67.8	108	60.0	1.04	0.75-1.44	0.824	1.47	1.00-2.16	0.051
Recessive model												
CC+CT	244	81.9	297	78.4	145	80.6	1.00			1.00		
TT	54	18.1	82	21.6	35	19.4	0.80	0.55-1.18	0.258	0.92	0.57-1.47	0.719
***VDR* rs2228570**												
CC	90	30.2	112	29.6	57	31.7	1.00			1.00		
CT	141	47.3	192	50.7	82	45.6	0.91	0.64-1.30	0.617	1.09	0.71-1.67	0.697
TT	67	22.5	75	19.8	41	22.8	1.11	0.72-1.71	0.630	1.04	0.62-1.72	0.895
Allelic model												
C	321	53.9	416	54.9	196	54.4	1.00			1.00		
T	275	46.1	342	45.1	164	45.6	1.04	0.84-1.29	0.708	1.02	0.79-1.33	0.860
Dominant model												
CC	90	30.2	112	29.6	57	31.7	1.00			1.00		
CT+TT	208	69.8	267	70.4	123	68.3	0.97	0.70-1.35	0.854	1.07	0.72-1.60	0.737
Recessive model												
CC+CT	231	77.5	304	80.2	139	77.2	1.00			1.00		
TT	67	22.5	75	19.8	41	22.8	1.18	0.81-1.70	0.393	0.98	0.63-1.53	0.941
***DBP* rs7041**												
TT	139	46.6	179	47.2	76	42.2	1.00			1.00		
GT	129	43.3	153	40.4	85	47.2	1.09	0.79-1.50	0.617	0.83	0.56-1.23	0.350
GG	30	10.1	47	12.4	19	10.6	0.82	0.49-1.37	0.450	0.86	0.46-1.64	0.652
Allelic model												
T	407	68.3	511	67.4	237	65.8	1.00			1.00		
G	189	31.7	247	32.6	123	34.2	0.96	0.76-1.21	0.733	0.89	0.68-1.18	0.433
Dominant model												
TT	139	46.6	179	47.2	76	42.2	1.00			1.00		
GT+GG	159	53.4	200	52.8	104	57.8	1.02	0.76-1.39	0.880	0.84	0.58-1.21	0.347
Recessive model												
TT+GT	268	89.9	332	87.6	161	89.4	1.00			1.00		
GG	30	10.1	47	12.4	19	10.6	0.79	0.49-1.29	0.343	0.95	0.52-1.74	0.865
***STAT4* rs7574865**												
TT	35	11.7	52	13.7	19	10.6	1.00			1.00		
GT	118	39.6	167	44.1	96	53.3	1.05	0.64-1.71	0.846	0.67	0.36-1.24	0.201
GG	145	48.7	160	42.2	65	36.1	1.35	0.83-2.18	0.228	1.21	0.64-2.27	0.552
Allelic model												
T	188	31.5	271	35.8	134	37.2	1.00			1.00		
G	408	68.5	487	64.2	226	62.8	1.21	0.96-1.52	0.105	1.29	0.98-1.69	0.072
Dominant model												
TT	35	11.7	52	13.7	19	10.6	1.00			1.00		
GG+GT	263	88.3	327	86.3	161	89.4	1.19	0.76-1.89	0.446	0.89	0.49-1.60	0.691
Recessive model												
TT+GT	153	51.3	219	57.8	115	63.9	1.00			**1.00**		
GG	145	48.7	160	42.2	65	36.1	1.30	0.96-1.76	0.095	**1.68**	**1.15-2.45**	**0.008 ***

Part 2: Association of vitamin D-related genetic variations and treatment response to pegylated interferon in patients with chronic hepatitis B

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Abstract

Background: Vitamin D, a potent immune-modulator, has been linked to the pathogenesis of chronic hepatitis B (CHB). This study was aimed at investigating the association between single nucleotide polymorphisms (SNPs) in vitamin D-related genes and treatment response to pegylated interferon (PEG-IFN) in patients with CHB.

Method: A total 275 Thai patients (122 HBeAg-positive and 153 HBeAg-negative CHB) treated with 48-week PEG-IFN were recruited. Virological response (VR) at 48 weeks post treatment was defined as HBeAg seroconversion plus HBV DNA <2,000 IU/mL for HBeAg-positive CHB and HBV DNA <2,000 IU/mL for HBeAg-negative CHB. The SNPs *VDR* (rs2228570), *DBP* (rs7041) and *CYP27B1* (rs4646536) were analyzed.

Results: The distribution of TT, CT and CC genotypes of rs4646536 in this cohort was 21.8%, 46.2% and 32.0%, respectively. There was no different in its distribution according to HBeAg status. In HBeAg-positive CHB, patients with TT genotype, compared with non-TT genotype, achieved higher VR (53.3% vs. 31.5%; *P*=0.032) and HBsAg clearance (20.0% vs. 5.4%; *P*=0.016). In HBeAg-negative CHB, the corresponding figures were 60.0% vs. 30.9% (*P*=0.003) and 16.7% vs. 5.7% (*P*=0.045), respectively. Patients with TT-genotype had more rapid HBsAg decline than those with non-TT genotype. However, SNPs rs2228570 and rs7041were not associated with VR and HBsAg clearance. Logistic regression analysis demonstrated that SNP rs4646536 and baseline HBsAg level were independent predictors of VR in both HBeAg-positive and HBeAg-negative CHB.

Conclusions: Our data suggest that SNP rs4646536 in the *CYP27B1* gene is a predictive factor of response to PEG-IFN therapy in Thai patients with CHB.

Key words: Chronic hepatitis B, Single nucleotide polymorphisms, pegylated interferon, vitamin D, *CYP27B1*, HBsAg clearance

Introduction

Hepatitis B virus (HBV) infection is a main etiological factor of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (112). Pegylated interferon alfa (PEG-IFN), an immunomodulatory agent, is one of the approved therapy for chronic hepatitis B (CHB) (112). PEG-IFN treatment results in a sustained virological suppression in approximately 30-40% of patients with HBeAg-positive CHB and 20-30% of patients with HBeAg-negative CHB (113). In addition, the rates of HBsAg clearance could be achieved in approximately 3-7% of patients treated with PEG-IFN, which is higher than those receiving nucleoside/nucleotide analogues (NA) (113). However, the response rates of PEG-IFN treatment is restricted by its potential side effects. In addition, its efficacy significantly differs among individuals and therefore, identification of favorable predictors prior to therapy is essential for maximizing a high likelihood of treatment response.

The therapeutic outcome of PEG-IFN apparently depends on the interaction of several factors, such as viral factors, host genetic variations and immune response (10). In recent years, it has been demonstrated that vitamin D, a potent immune-modulator, is linked to the pathogenesis of several chronic disorders, including

chronic liver disease(23). Moreover, common single nucleotide polymorphisms (SNPs) in vitamin D-related genes, including *vitamin D receptor (VDR), vitamin D binding protein (DBP)* and enzymes that initiate vitamin D formation such as 1-**α**-hydroxylase (encoded by *CYP27B1*), are shown to be associated with clinical outcome and response to PEG-IFN-based therapy in patients with chronic hepatitis C virus (HCV) infection (24-28). Among HBV-infected individuals, recent studies have suggested a possible role of these SNPs in viral persistence and HCC development (29, 30). In addition, it has recently been demonstrated that a variant of *CYP27B1* is associated with high probability of response to PEG-IFN therapy in an Italian cohort of HBeAg-negative CHB (31). The significance of this polymorphism on predicting treatment outcome in other populations is currently unknown. Thus, the aim of this study was to clarify the impact of these vitamin D-related SNPs on the treatment outcome in Thai patients with HBeAg-positive and HBeAg-negative CHB receiving PEG-IFN therapy.

Materials and methods

Patients

Thai patients with CHB, who were treated with 48-week PEG-IFN therapy between January 2010 and May 2015 and had been follow-up for at least 12 months after therapy at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand were recruited. These patients were treated with either PEG-IFN-alfa2a (180 μg/week) or

PEG-IFN-alfa2b (1.5 μg/kg body weight/week). All patients had HBsAg positivity, elevated serum alanine aminotransferase (ALT) and serum HBV DNA levels for at least 6 months before therapy. Patients with HCV and/or human immunodeficiency virus co-infection were excluded. Virological response (VR) for HBeAg-positive CHB was defined as HBeAg seroconversion plus HBV DNA level <2,000 IU/mL at 48 weeks after complete treatment. VR for HBeAg-negative CHB was defined as HBV DNA level <2,000 IU/mL at 48 weeks post therapy. Combined virological and serological response (CR) was defined as VR plus HBsAg <100 IU/mL at 48 weeks post treatment.

Written informed consents were obtained from patients and the study was approved by the Institutional Review Board. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice.

Serological and virological assays

Qualitative HBsAg, HBeAg, anti-HBe and anti-HBs testing were conducted by commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). HBsAg quantification was performed by Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN). Serum HBV DNA levels were tested by Abbott RealTime HBV assay (Abbott Laboratories, Chicago, IL) with the lower limit of detection of 10 IU/mL. HBV genotypes were performed by direct sequencing, as described previously (114).

Assays of vitamin D-related SNPs

Genomic DNA was isolated from 100 µl of buffy coat samples by phenol/chloroform extraction method and dissolved in 30 µl of distilled water. SNP rs4646536 in the *CYP27B1* gene was determined by a commercial TaqMan PCR assay (Applied Biosystems, Foster City, CA, USA), as described previously (115).

SNP rs2228570 in the *VDR* gene was genotyped using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method as described previously, with some modifications (111). In brief, the gene fragments containing the polymorphic sites were amplified using forward primer 5'-TGG CAC TGA CTC TGG CTC TGA-3'and reverse primer 5'-CTC CCT TCA TGG AAA CAC CTT G-3'. The PCR cycles were as follows: 94 °C for 3 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 58°C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. Then, the PCR products were identified under ultraviolet light after electrophoresis in 2% agarose gel stained with ethidium bromide (EB). After that, the amplified products were digested with 2 U FokI (New England BioLabs, Newton, MA, USA) overnight at 37°C. Cleaved DNA fragments were identified by ultraviolet light after electrophoresis in 2% agarose gel stained by EB. The PCR products digested with FokI produced the following band profiles: 1 fragment of 266 bp in individuals with the CC genotype; 2 fragments of 205 bp and 61 bp in individuals with the TT genotype; 3 fragments of 266 bp, 205 bp and 61 bp in individuals with the TC genotype.

SNP rs7041 in the *DBP* gene was also genotyped using PCR–RFLP method as described previously, with some modifications(109). An amplification using forward primer 5'-TAC CAC AGG TAT AGA ATT TT-3'and reverse primer 5'-AGT GGA GGG TTA CAT TTT CCT-3'. The PCR cycles were as follows: 94 ℃ for 3 min, 35 cycles of denaturing at 94 ℃ for 30 s, annealing at 53℃ for 30 s, extension at 72 ℃ for 30 s, and a final extension at 72 ℃ for 7 min. The PCR products were digested with 2 U HaeIII (New England BioLabs, Newton, MA, USA) overnight at 37℃. The restriction pattern was analyzed under ultraviolet light after electrophoresis in 2% agarose gel stained with EB. The enzymatic digestion with HaeIII, produced a band profiles that allowed the distinction between the TT (304 bp), GT (304 bp, 183 bp, 121 bp) and GG (183 bp and 121 bp) genotypes.

Liver stiffness measurement (LSM)

Baseline LSM prior to PEG-IFN therapy was performed by transient elastography (FibroScan, Echosens, Paris, France) after fasting for at least 2 hours. Results as the median value of all measurements were reported in kilopascals (kPa), which were based on at least 10 validated measurements, the success rate > 60% and the interquartile range <30%(116).

Statistical Analysis

Statistical analysis was performed by the SPSS software for Windows 22.0 (SPSS Inc., Chicago, IL). Comparisons between groups were assessed 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. Logistic regression was applied for odd ratios of variables associated with treatment response. *P*-values<0.05 were considered statistically significant.

Results

Patient Characteristics

Overall, 275 patients who had complete a full course of PEG-IFN treatment and followed up were recruited in this study. Among them, there were 122 and 153 patients with HBeAg-positive and HBeAg-negative CHB, respectively. Table 5 summarizes demographic and clinical characteristics of the patients according to HBeAg status. Patients with HBeAg-positive CHB had lower mean age, but had higher baseline ALT, $\log_{10}$ HBV DNA and $\log_{10}$ HBsAg levels compared with HBeAg-negative CHB. There was no significant difference between groups in the distribution of patient's gender and HBV genotypes, as well as baseline LSM. In the HBeAg-positive CHB group, VR, CR and HBsAg clearance were achieved in 45 (36.9%), 25 (20.5%) and 11 (9.0%) patients, respectively. The corresponding figures in the HBeAg-negative CHB group were 56 (36.6%), 28 (18.3%) and 12 (7.8%), respectively.

For SNP rs2228570 (*VDR*), the frequency of CC, TC and TT genotypes in the entire cohort was 84(10.5%), 136(49.5%) and 55(20.0%), respectively. Regarding SNP rs7041 (*DBP*), the distribution of TT, GT and GG genotypes was 128(76.5%),

114(41.5%) and 33(12.0%), respectively. For SNP rs4646536 (*CYP27B1*), the distribution of TT, CT and CC genotypes was 60(21.8%), 127(46.2%) and 88(32.0%), respectively. There was no difference in the frequencies of these SNPs between the HBeAg-positive and HBeAg-negative CHB groups.

Table 5. Baseline characteristics and treatment response according to HBeAg status

Characteristics	HBeAg-positive CHB (n=121)	HBeAg-negative CHB (n=153)	*P* value
Age, year	35.1 ± 8.6	41.6 ± 9.6	<0.001
Sex, male	80 (65.6%)	84 (54.5%)	0.073
ALT, U/L	112.7 ± 64.7	75.0 ± 35.3	<0.001
Log_{10} HBV DNA, IU/ml	7.3 ± 0.9	5.4 ± 0.9	<0.001
Log_{10} HBsAg, IU/ml	4.0 ± 0.6	3.4 ± 0.5	<0.001
HBV genotypes			0.532
B	19 (15.6%)	30(19.6%)	
C	90 (73.8%)	116 (75.8%)	
Missing	13 (10.6%)	7 (4.6%)	
Liver stiffness, kPa	7.6 ± 3.3	8.1 ± 2.5	0.214
Virological response	45 (36.9%)	56 (36.6%)	0.961
Combined response	25 (20.5%)	28 (18.3%)	0.647
HBsAg clearance	11 (9.0%)	12 (7.8%)	0.727
SNPs rs2228570			0.116
CC	30 (24.6%)	54 (35.3%)	
TC	63 (51.6%)	73 (47.7%)	
TT	29 (23.8%)	26 (17.0%)	
SNP rs7041			0.986
TT	57 (46.7%)	71 (46.4%)	
GT	50 (41.0%)	64 (41.8%)	
GG	15 (12.3%)	18 (11.8%)	
SNP rs4646536			0.600
TT	30 (24.6%)	30 (19.6%)	
CT	55 (45.1%)	72 (47.1%)	
CC	37 (30.3%)	51 (33.3%)	

ALT, alanine aminotransferase; HBsAg, Hepatitis B surface antigen; SNPs, Single nucleotide polymorphisms; Data described as means ± SD or n (%)

Vitamin D-related SNPs and treatment response

Treatment response rates in relation to vitamin D-related SNPs in the whole cohort are shown in Figure 9. For SNP rs4646536, individuals harboring TT genotype had significantly higher rates of VR, CR and HBsAg clearance in comparison to those with CT and CC genotypes. However, there was no such difference in treatment response rates according to rs2228570 and rs7041genotypes.

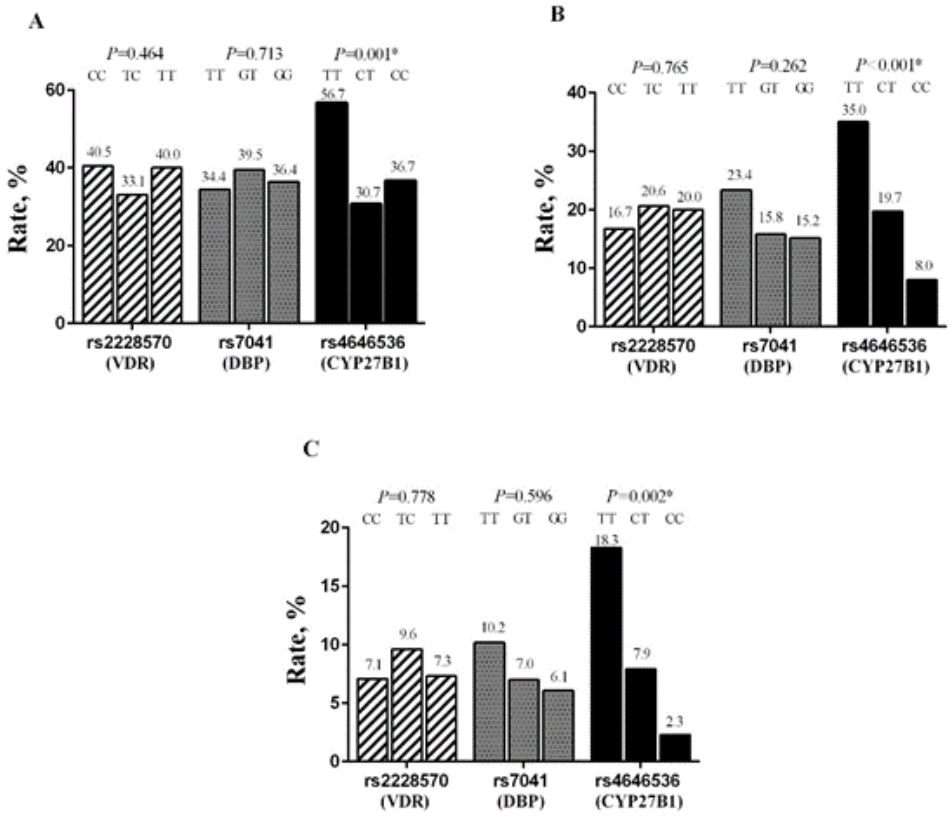


Figure 9. Treatment response in relation to vitamin D-related SNP genotypes (A) Virological response, (B) Combined response, (C) HBsAg clearance

Table 6 demonstrates baseline characteristics and treatment response in relation to rs4646536 genotypes. There was no difference between the TT and non-TT groups in terms of age, sex, baseline ALT, $\log_{10}$ HBV DNA, $\log_{10}$ HBsAg level and

LSM in patients with HBeAg-positive and HBeAg-negative CHB. However, the TT group had significantly higher response rates in comparison to those with the non-TT group.

Table 6. Baseline characteristics and treatment response according to rs4646536 genotypes

Characteristics	HBeAg-positive CHB		*P* value	HBeAg-negative CHB		*P* value
	TT (n=30)	non-TT (n=92)		TT (n=30)	non-TT (n=123)	
Age, year	34.0 ± 8.9	35.5 ± 8.9	0.432	39.6 ± 10.1	42.1± 9.4	0.208
Sex, male	19 (63.3%)	61 (66.3%)	0.766	16 (53.3%)	68 (55.3%)	0.847
ALT, U/L	113.4 ±70.0	112.4 ± 63.3	0.942	83.5 ± 43.3	72.9 ±33.0	0.140
Log_{10} HBV DNA, IU/ml	7.4 ± 0.9	7.2 ± 1.0	0.462	5.7 ± 1.0	5.4 ± 0.9	0.137
Log_{10} HBsAg, IU/ml	4.0 ± 0.5	4.0 ± 0.6	0.732	3.4 ± 0.4	3.5 ± 0.5	0.281
HBV genotype C	19 (63.3%)	61 (66.3%)	0.647	22 (73.3%)	95 (81.2%)	0.340
Liver stiffness, kPa	7.9 ± 4.1	7.5 ± 3.0	0.625	7.8 ± 1.6	8.1 ± 2.6	0.490
Virological response	16 (53.3%)	29 (31.5%)	0.032*	18 (60.0%)	38 (30.9%)	0.003*
Combined response	10 (33.3%)	15 (16.3%)	0.045*	11 (36.7%)	17 (13.8%)	0.004*
HBsAg clearance	6 (20.0%)	5(5.4%)	0.016*	5 (16.7%)	7 (5.7%)	0.045*

ALT, alanine aminotransferase; HBsAg, Hepatitis B surface antigen; Data described as means ± SD or n (%)

Serum HBsAg kinetics in relation to rs4646536 genotypes were also examined. In HBeAg-positive CHB, patients with TT genotype showed a more rapid decline of serum HBsAg levels from baseline compared with those with non-TT genotype: baseline (4.0 ± 0.5 vs. 4.0 ± 0.6 log_{10}IU/mL, *P*=0.732), week 4 (0.2 ± 0.4 vs. 0.1 ± 0.3

log_{10}IU/mL, *P*=0.115), week 12 (0.6 ± 1.0 vs. 0.3 ± 0.4 log_{10}IU/mL, *P*=0.012), week 24 (1.1 ± 1.3 vs. 0.6 ± 0.9 log_{10}IU/mL, *P*=0.026), week 48 (1.3 ± 1.4 vs. 0.9 ± 1.2 log_{10}IU/mL, *P*=0.084), week 72 (1.3 ± 1.6 vs. 0.7 ± 1.2 log_{10}IU/mL, *P*=0.022), and week 96 (1.4 ± 1.7 vs. 0.7 ± 1.3 log_{10}IU/mL, *P*=0.021) (Figure 10A).

Similarly, the decreases in HBsAg levels during and after therapy were also higher in patients with HBeAg-negative CHB harboring TT genotype than those with non-TT genotype: baseline (3.4 ± 0.4 vs. 3.5 ± 0.5 log_{10}IU/mL, *P*=0.281), week 4 (0.2 ± 0.3 vs. 0.1 ± 0.2 log_{10}IU/mL, *P*=0.020), week 12 (0.5 ± 0.6 vs. 0.4 ± 0.6 log_{10}IU/mL, *P*=0.233), week 24 (1.0 ± 1.0 vs. 0.6 ± 0.8 log_{10}IU/mL, *P*=0.007), week 48 (1.3 ± 1.2 vs. 0.8 ± 0.9 log_{10}IU/mL, *P*=0.008), week 72 (1.1± 1.2 vs. 0.5 ± 0.8 log_{10}IU/mL, *P*=0.001), and week 96 (1.2 ± 1.2 vs. 0.5 ± 0.8 log_{10}IU/mL, *P*<0.001) (Figure 10B).

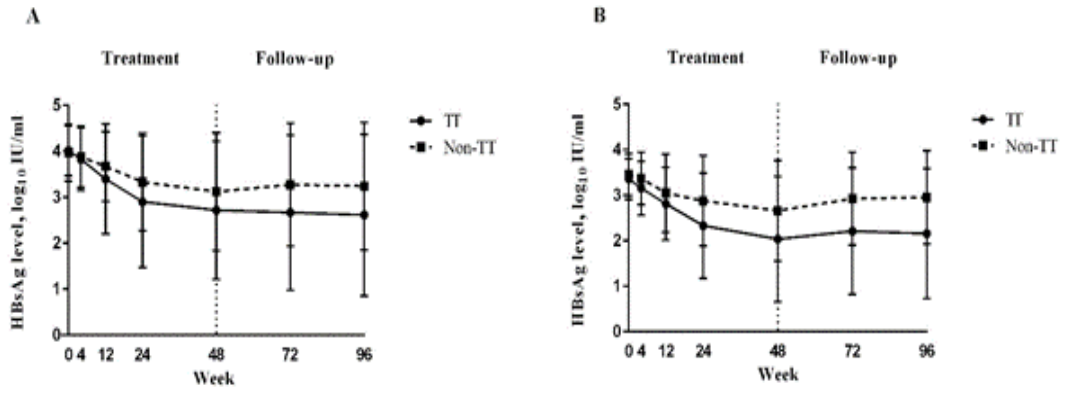


Figure 10. Serum HBsAg kinetics in relation to rs4646536 genotypes
(A) HBeAg-positive CHB, (B) HBeAg-negative CHB

Baseline predictors of treatment response

To identify factors associated VR, baseline characteristics including patient's age, gender, ALT, HBV DNA and HBsAg levels, HBV genotype, LSM, SNPs rs2228570, rs7041 and rs4646536 were evaluated by logistic regression analyses. In the HBeAg-positive CHB group, baseline ALT, HBsAg levels and SNP rs4646536 were associated with VR in univariate and multivariate analyses. In the HBeAg-negative CHB group, pretreatment HBsAg level and SNP rs4646536 were factors associated with VR in univariate and multivariate analyses (Table 7).

If baseline HBsAg and SNP rs4646536 were combined together, patients with HBeAg-positive CHB who carried TT genotype and had baseline HBsAg<4.0 $\log_{10}$IU/mL could achieve VR, CR and HBsAg clearance of 53.8% (7/13), 30.8% (4/13) and 23.1% (3/13), respectively. In the HBeAg-negative CHB group, patients carried TT genotype with baseline HBsAg<3.5 $\log_{10}$IU/mL achieved VR, CR and HBsAg clearance of 75.0% (15/20), 35% (7/20) and 25% (5/20), respectively.

Table 7. Logistic regression analysis of pretreatment factors to predict virological response (A) HBeAg-positive CHB (B) HBeAg-negative CHB

Characteristics	Category	Virological response (week 96)			
		Univariate analysis		Multivariate analysis	
		Odd ratio (95%CI)	*P*	Odd ratio (95%CI)	*P*
HBeAg-positive CHB					
Baseline					
Age (yr)	< 40 vs. ≥ 40	1.85 (0.66-5.17)	0.243		
Sex	Male vs. Female	1.47 (0.68-3.17)	0.323		
ALT (U/L)	< 100 vs. ≥ 100	2.68 (1.26-5.72)	0.011*	2.75 (1.24-6.10)	0.013*
Log_{10} HBV DNA (IU/mL)	< 7.0 vs. ≥ 7.0	1.66 (0.78-3.55)	0.187		
Log_{10} HBsAg (IU/mL)	< 4.0 vs. ≥ 4.0	2.48 (1.17-5.28)	0.018*	2.75 (1.23-6.16)	0.014*
HBV genotypes	B vs. C	2.50 (0.92-6.83)	0.072		
Liver stiffness (kPa)	<8.0 vs. ≥8.0	1.65 (0.69-3.94)	0.256		
SNPs					
rs2228570 (*VDR*)	CC vs. non CC	0.99 (0.42-2.32)	0.977		
rs7041 (*DBP*)	TT vs. non TT	0.87 (0.41-1.81)	0.700		
rs4646536 (*CYP27B1*)	TT vs. non TT	2.48 (1.07-5.76)	0.034*	2.99 (1.21-7.36)	0.017*
HBeAg-negative CHB					
Baseline					
Age (yr)	< 40 vs. ≥ 40	1.29 (0.66-2.50)	0.454		
Sex	Male vs. Female	1.10 (0.56-2.11)	0.802		
ALT (U/L)	< 100 vs. ≥ 100	0.78 (0.30-2.05)	0.615		
Log10 HBV DNA (IU/mL)	< 5.0 vs. ≥ 5.0	1.10 (0.56-2.16)	0.790		
Log10 HBsAg (IU/mL)	< 3.5 vs. ≥ 3.5	2.54 (1.27-5.10)	0.008*	2.39 (1.17-4.87)	0.017*
HBV genotypes	B vs. C	0.46 (0.18-1.17)	0.105		
Liver stiffness (kPa)	<8.0 vs. ≥8.0	1.23 (0.60-2.53)	0.576		
SNPs					
rs2228570 (*VDR*)	CC vs. non CC	1.48 (0.75-2.94)	0.257		
rs7041 (*DBP*)	TT vs. non TT	0.80 (0.41-1.55)	0.504		
rs4646536 (*CYP27B1*)	TT vs. non TT	3.36 (1.47-7.65)	0.004*	3.13 (1.35-7.26)	0.008*

ALT, alanine aminotransferase; HBsAg, Hepatitis B surface antigen; SNPs, Single nucleotide polymorphisms; *VDR*, *Vitamin D receptor*; *DBP*, *Vitamin D binding protein*; *CYP27B1*, *1-α-hydroxylase*

Part 3: Genetic variation in *STAT4* is associated with treatment response to pegylated interferon in patients with chronic hepatitis B

(Submitted in Asian Pacific Journal of Allergy and Immunology, April 2nd,2019)

Abstract

Background: Signaling pathways in the *STAT4* gene play an essential role in interferon-mediated antiviral effects.

Objective: This study was aimed at investigating the role of rs7574865, a single nucleotide polymorphism (SNP) in *STAT4*, in patients with chronic hepatitis B (CHB) treated with pegylated interferon (PEG-IFN).

Methods: A total 261 Thai patients (115 HBeAg-positive and 146 HBeAg-negative CHB) treated with 48-week PEG-IFN were recruited. Virological response (VR) at 48 weeks post treatment was defined as HBeAg seroconversion plus HBV DNA <2,000 IU/mL for HBeAg-positive CHB and HBV DNA <2,000 IU/mL for HBeAg-negative CHB. The SNP was analyzed by TaqMan PCR assay.

Results: The distribution of GG, GT and TT genotypes of rs7574865 was 41.8%, 42.9% and 15.3%, respectively. There was no different in its distribution according to HBeAg status. Overall, patients with TT genotype, compared with non-TT genotype, achieved higher VR (64.3% vs. 30.5%; *P*<0.001) and HBsAg clearance (23.8% vs. 5.0%; *P*<0.001). There was the same trend in the HBeAg-positive group (VR, 52.4% vs. 30.9%; *P*=0.077; HBsAg clearance, 23.8% vs. 6.4%; *P*=0.028) and in the HBeAg-negative

group (VR, 68.4% vs. 32.3%; *P*=0.004; HBsAg clearance, 21.1% vs. 4.7%; *P*=0.026). Multiple regression analysis demonstrated that low baseline HBsAg level and TT genotype were factors independently associated with VR and HBsAg clearance.

Conclusions: Our data support that SNP rs7574865 is associated with response to PEG-IFN therapy in Thai patients with CHB, regardless of baseline HBeAg status. Thus, the determination of this SNP could maximize cost-effectiveness of PEG-IFN in patients with CHB.

Introduction

Worldwide, hepatitis B virus (HBV) is a major etiological factor for the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).(117) Pegylated interferon alfa (PEG-IFN), acting through its dual immunomodulatory and antiviral effects, is an approved agent for treatment of chronic hepatitis B (CHB).(113) It has been shown that long-term therapeutic effect of PEG-IFN is sustained and responders have a reduced risk of cirrhosis and HCC.(118) However, the overall response rates to PEG-IFN in HBeAg-positive and HBeAg-negative CHB are limited with approximately 30-40% and 20-30%, respectively.(113) Moreover, PEG-IFN treatment has some potential side effects and its efficacy varies among individuals. Thus, in clinical practice it is essential to identify patients with a high likelihood of response prior to initiate PEG-IFN therapy.

The therapeutic effectiveness of PEG-IFN in patients with CHB is associated with dynamic interaction of viral and host factor.(10) Regarding host genetic variations, a recent genome-wide association study (GWAS) demonstrated that a single nucleotide polymorphism (SNP), rs7574865 located in the third intron of the *signal transducers and activators of transcription 4* (*STAT4)* gene, was linked to an increased risk of HBV-associated HCC in Chinese populations.(11) Indeed, *STAT4* is a member of the STAT protein family that could be activated by several cytokines including IFN-alfa in response to viral infections.(17) Subsequent studies also showed that the SNP was related to increased risk of advanced fibrosis or cirrhosis in Chinese and Caucasians patients with CHB.(12, 119) Recently, it was reported that patients with the minor T allele of rs7574865 was more likely to achieve spontaneous HBsAg clearance than those harboring the major G allele. (19, 21) Interestingly, a recent report also showed that rs7574865 represented one of the most important factors associated with sustained response in Chinese patients with HBeAg-positive CHB receiving IFN or PEG-IFN therapy.(22) Despite these observations, the association of the SNP with PEG-IFN response in other ethnical populations or in patients with HBeAg-negative CHB remains to be confirmed. Thus, this study was aimed at investigating the predictive role of this SNP in Thai patients with HBeAg-positive and HBeAg-negative CHB receiving PEG-IFN therapy.

Materials and methods

Patients

Thai patients with HBeAg-positive or HBeAg-negative CHB, who were treated and completed a full course of PEG-IFN therapy between January 2010 and May 2015 at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand were recruited in this study. These patients were treated with PEG-IFN-alfa2a (180 μg/week) or PEG-IFN-alfa2b (1.5 μg/kg body weight/week) for 48 weeks and followed up for at least 48 weeks after therapy. Patients who had previously received NA were eligible, but not within 6 months prior to PEG-IFN therapy. All these patients had serum HBsAg positivity for at least 6 months before therapy, with elevated serum alanine aminotransferase (ALT) and HBV DNA levels. Individuals co-infected with hepatitis C virus (HCV) and/or human immunodeficiency virus (HIV) were excluded. Virological response (VR) for HBeAg-positive CHB was defined as HBeAg seroconversion plus HBV DNA level <2,000 IU/mL at 48 weeks post treatment. For the HBeAg-negative CHB group, VR was defined as HBV DNA level <2,000 IU/mL at 48 weeks post treatment.(5)

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. Written informed consents were obtained from patients and the study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Serological and virological assays

Qualitative measurements of HBsAg, HBeAg, anti-HBe and anti-HBs were tested by commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). HBsAg quantification were performed by Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN). Serum HBV DNA quantification were performed by Abbott RealTime HBV assay (Abbott Laboratories, Chicago, IL). HBV genotypes were determined by direct sequencing, as previously described.(114)

Genotyping of rs7574865

Genomic DNA was isolated from 100 μL of buffy coat samples using phenol-chloroform extraction method. The SNP rs7574865 of *STAT4* was determined by a commercial TaqMan SNP genotyping assay (Applied Biosystems, CA, USA; Part number: C__29882391_10), as described previously.(120) The polymerase chain reaction (PCR) assay was performed according to the manufacturer's instructions. Fluorescent signals (FAM and VIC) were acquired at the end of each cycle. Allelic discrimination plot was analyzed using Applied Biosystems QuantStudio Real-Time PCR System Software (Applied Biosystems, CA, USA).

Liver stiffness measurement

Liver stiffness (LS) measurement was performed prior to PEG-IFN therapy using transient elastography (FibroScan, Echosens, Paris, France). Results were recorded in kilopascals (kPa) as the median value of all measurements. The

procedure was based on at least 10 validated measurements: the success rate was over 60% and the interquartile range was less than 30%.(116)

Statistical Analysis

Statistical analysis was performed using SPSS version 22 software (SPSS, Chicago, IL, USA). The Mann-Whitney U test or Student's test were used to compare continuous variables, and the $\chi 2$ test were used to compare categorical variables. Logistic regression was used to assess odd ratios of factors associated with VR and HBsAg clearance. *P*-values<0.05 were considered statistically significant.

Results

Patient Characteristics

Of the 302 consecutive patients with completed a full course of treatment and followed up, 261 patients had complete clinical data and available blood samples for genomic DNA isolation and thus were included in this study. Among these patients, there were 115 and 146 patients with HBeAg-positive and HBeAg-negative CHB, respectively. Table 8 summarizes demographic and baseline clinical characteristics of the patients in relation to HBeAg status. Briefly, the HBeAg-positive CHB group had a significantly lower mean age, but a higher proportion of male gender compared with the HBeAg-negative CHB group. In addition, the HBeAg-positive CHB group had higher baseline levels of ALT, $\log_{10}$ HBV DNA and $\log_{10}$ HBsAg compared with the HBeAg-negative CHB group. However, no significant difference

between groups was observed regarding HBV genotype distribution and baseline LS measurement. Among the HBeAg-positive CHB group, VR and HBsAg clearance were achieved in 40 (34.8%) and 11 (9.6%) patients, respectively. Among the HBeAg-negative CHB group, the corresponding figures were 54 (37.0%) and 10 (6.8%), respectively.

Table 8. Baseline characteristics and treatment response in relation to HBeAg status

Characteristics	HBeAg-positive CHB (n=115)	HBeAg-negative CHB (n=146)	*P* value
Age, year	35.1 ± 8.8	41.4 ± 9.6	<0.001*
Sex, male	79 (68.7%)	78 (53.4%)	0.015*
ALT (U/L)	114.1 ± 60.1	74.9 ± 35.7	<0.001*
Log_{10} HBV DNA (IU/ml)	7.2 ± 0.9	5.4 ± 0.9	<0.001*
Log_{10} HBsAg (IU/ml)	4.0 ± 0.6	3.4 ± 0.5	<0.001*
HBV genotypes			0.608
B	17 (14.8%)	28(19.2%)	
C	94 (81.7%)	112 (76.7%)	
Missing	4 (3.5%)	6 (4.1%)	
Liver stiffness (kPa)	7.6 ± 3.3	8.0 ± 2.4	0.369
Virological response	40 (34.8%)	54 (37.0%)	0.795
HBsAg clearance	11 (9.6%)	10 (6.8%)	0.494
SNP rs7574865			0.251
GG	42 (36.5%)	67 (45.9%)	
GT	52 (45.2%)	60 (41.1%)	
TT	21 (18.3%)	19 (13.0%)	

ALT, alanine aminotransferase; HBsAg, Hepatitis B surface antigen; SNP, Single nucleotide polymorphism; Data described as means ± SD or n (%), *<0.05

The frequency of rs7574865 genotypes and treatment response

The distribution of GG, GT and TT genotypes of rs7574865 in this cohort was 41.8%, 42.9% and 15.3%, respectively. The distribution of the corresponding genotypes in the HBeAg-positive CHB group were 42(36.5%), 52(45.2%) and 21(18.3%), respectively, and in the HBeAg-negative CHB group were 67(45.9%), 60(41.1%) and 19(13.0%), respectively, which was not significantly different between these two groups (*P*=0.251).

Overall, patients with TT genotype, compared with non-TT genotype, achieved higher VR (64.3% vs. 30.5%; *P*<0.001) and HBsAg clearance (23.8% vs. 5.0%; *P*<0.001). There was the same trend in the HBeAg-positive group (VR, 52.4% vs. 30.9%; *P*=0.077; HBsAg clearance, 23.8% vs. 6.4%; *P*=0.028) and in the HBeAg-negative group (VR, 68.4% vs. 32.3%; *P*=0.004; HBsAg clearance, 21.1% vs. 4.7%; *P*=0.026) (Figure 11).

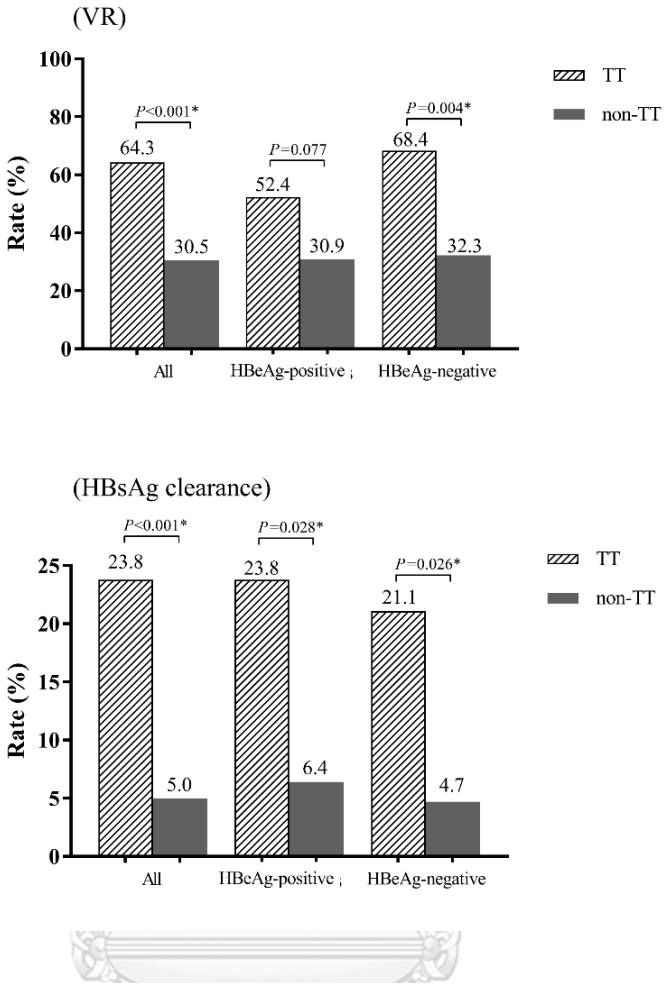


Figure 11. Treatment response in relation to rs7574865 genotypes

Serum HBsAg kinetics in associated with rs7574865 genotypes

Serum HBsAg kinetics in associated with rs7574865 genotypes were further investigated. Among HBeAg-positive CHB, patients with TT genotype showed a higher decline of serum HBsAg levels at the end of follow-up compared with those with non-TT genotype: baseline (4.0 ± 0.7 vs. 3.9 ± 0.6 $\log_{10}$IU/mL, *P*=0.924), week 4 (0.1 ± 0.3 vs. 0.1 ± 0.3 $\log_{10}$IU/mL, *P*=0.929), week 12 (0.2 ± 0.3 vs. 0.3 ± 0.7 $\log_{10}$IU/mL, *P*=0.257), week 24 (0.8 ± 1.0 vs. 0.7 ± 1.0 $\log_{10}$IU/mL, *P*=0.795), week 48 (1.2 ± 1.4 vs.

0.9 ± 1.1 log_{10}IU/mL, *P*=0.253), week 72 (1.4 ± 1.5 vs. 0.7 ± 1.1 log_{10}IU/mL, *P*=0.018), and week 96 (1.5 ± 1.5 vs. 0.7 ± 1.1 log_{10}IU/mL, *P*=0.013) (Figure 12A).

Likewise, decline HBsAg levels at the end of follow-up were higher in patients with HBeAg-negative CHB harboring TT genotype than those with non-TT genotype: baseline (3.4 ± 0.5 vs. 3.4 ± 0.5 log_{10}IU/mL, *P*=0.400), week 4 (0.2 ± 0.3 vs. 0.1 ± 0.2 log_{10}IU/mL, *P*=0.301), week 12 (0.6 ± 0.8 vs. 0.4 ± 0.6 log_{10}IU/mL, *P*=0.114), week 24 (0.9 ± 0.9 vs. 0.6 ± 0.8 log_{10}IU/mL, *P*=0.177), week 48 (1.2 ± 1.2 vs. 0.8 ± 0.9 log_{10}IU/mL, *P*=0.139), week 72 (1.0± 1.0 vs. 0.5 ± 0.8 log_{10}IU/mL, *P*=0.028), and week 96 (1.1 ± 1.0 vs. 0.5 ± 0.8 log_{10}IU/mL, *P*=0.008) (Figure 12B).

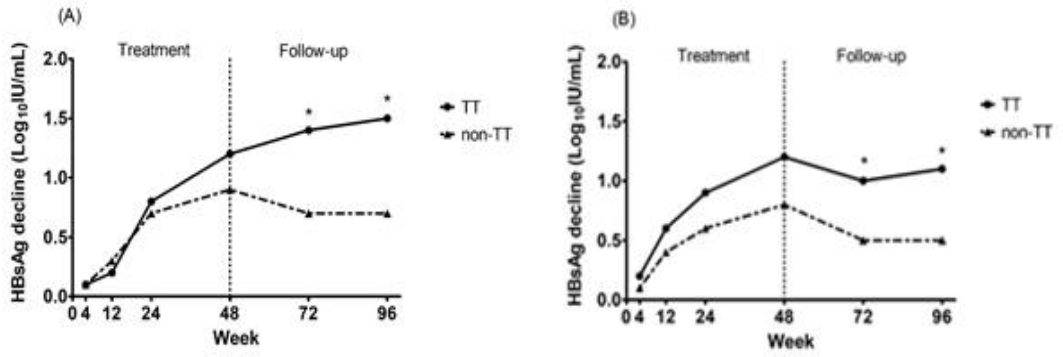


Figure 12. Serum HBsAg decline from baseline during and after therapy in relation to rs7574865 genotypes
(A) HBeAg-positive CHB (B) HBeAg-negative CHB

Factors associated with treatment response

To identify factors associated with VR and HBsAg clearance, baseline characteristics of patients were analyzed by logistic regression analyses. Potential predictors of virological response included sex, age, ALT level, HBV DNA level, HBsAg

level, LS value and SNP rs7574865. As shown in Table 9, low baseline HBsAg level and SNP rs7574865 were factors independent associated with VR and HBsAg clearance.

If pretreatment HBsAg and SNP rs7574865 were combined, patients carried TT genotype with baseline HBsAg < 3.5 $\log_{10}$ IU/mL achieved VR and HBsAg clearance of 81.3% (13/16) and 31.3% (5/16), respectively. Conversely, patients carried non-TT genotype with high baseline HBsAg ≥ 3.5 $\log_{10}$ IU/mL achieved VR and HBsAg clearance of 23.1% (31/103) and 3% (4/130), respectively.

Table 9. Logistic regression analysis of pretreatment factors to predict treatment response (A) Virological response (B) HBsAg clearance

(A)

Factors	Category	Virological response (week 96)			
		Univariate analysis		Multivariate analysis	
		Odd ratio (95%CI)	*P*	Odd ratio (95%CI)	*P*
Age (year)	≥ 40 vs. < 40	0.71 (0.42-1.19)	0.189		
Sex	Male vs. Female	1.19 (0.71-2.00)	0.503		
ALT (U/L)	≥ 100 vs. < 100	1.22 (0.70-2.15)	0.482		
Log_{10} HBV DNA (IU/mL)	< 7.0 vs. ≥ 7.0	1.36 (0.78-2.36)	0.282		
Log_{10} HBsAg (IU/mL)	< 3.5 vs. ≥ 3.5	2.82 (1.67-4.75)	<0.001*	2.96 (1.73-5.02)	<0.001*
HBV genotypes	B vs. C	1.02 (0.52-1.99)	0.963		
Liver stiffness (kPa)	≥8.0 vs. <8.0	0.86 (0.49-1.51)	0.589		
SNP rs7574865	TT vs. Non-TT	1.80 (1.27-2.54)	0.001*	1.87 (1.30-2.68)	0.001*

(B)

Factors	Category	HBsAg clearance (week 96)			
		Univariate analysis		Multivariate analysis	
		Odd ratio (95%CI)	*P*	Odd ratio (95%CI)	*P*
Age (year)	≥ 40 vs. < 40	0.96 (0.39-2.37)	0.937		
Sex	Male vs. Female	1.15 (0.46-2.82)	0.769		
ALT (U/L)	≥ 100 vs. < 100	1.08 (0.40-2.89)	0.883		
Log_{10} HBV DNA (IU/mL)	< 7.0 vs. ≥ 7.0	1.68 (0.68-4.17)	0.261		
Log_{10} HBsAg (IU/mL)	< 3.5 vs. ≥ 3.5	2.71 (1.08-6.79)	0.034*	2.85(1.10-7.33)	0.030*
HBV genotypes	B vs. C	0.86 (0.27-2.72)	0.801		
Liver stiffness (kPa)	≥8.0 vs. <8.0	0.65 (0.23-1.85)	0.420		
SNP rs7574865	TT vs. Non-TT	2.25 (1.40-3.60)	0.001*	2.30 (1.42-3.72)	0.001*

Part 4: Correlation of serum HBV RNA quantification with other viral markers in the natural history of chronic HBV infection

(Manuscript in preparation)

Abstract

Background: Hepatitis B virus (HBV) RNA quantification is a novel serum marker reflecting the activity of intrahepatic covalently closed circular DNA (cccDNA). However, methodology for sensitive quantification of HBV RNA has been a technical challenge and it is desired in the clinic. The aim of this study was to develop a sensitive method for quantification of HBV RNA in serum sample from patients and to examine the correlation between serum HBV RNA and other viral markers in various phases during the natural history of chronic HBV infection.

Methods: Serum HBV markers at initial presentation of 417 treatment-naïve patients were retrospectively analyzed. Patients were classified into 4 groups based on EASL 2017 guideline as following: phase 1; HBeAg-positive chronic infection (N=47), phase 2; HBeAg-positive chronic hepatitis (N=89), phase 3; HBeAg-negative chronic infection (N=156) and phase 4; HBeAg-negative chronic hepatitis (N=125). Serum HBV RNA was quantified by a droplet digital PCR amplification system.

Result: Serum levels of HBV DNA, HBV RNA and HBsAg were lowest in phase 3 and highest in phase 1. The mean levels of serum HBV RNA in the respective 4 phases were 6.70±0.68, 6.51±1.74, 2.33±0.52 and 3.52±1.38 (log10 copies/ml). Serum HBV RNA levels were positively correlated with serum HBV DNA in phase 1 (Pearson,

R=0.586, P<0.001), phase 2 (R=0.524, P<0.001) and phase 4 (R=0.780, P<0.001) but not in phase 3 (R=0.113, P=0.160). The correlations between serum HBV RNA and HBsAg were found only in phase 1 (R=0.536, P<0.001) and phase 2 (R=0.496, P<0.001). Among patients undergoing liver biopsy, the data showed that serum HBV RNA correlated well with intrahepatic cccDNA in phase 2 (n=20, R=0.501, P<0.05) and in phase 4 (n=70, R=0.339, P<0.01). However, serum HBsAg levels correlated with intrahepatic cccDNA only in phase 2 (R=0.456, P<0.05) but not in phase 4 (R=0.058, P=0.631).

Conclusion: Serum HBV RNA levels varied among different phases of chronic HBV infection. Compared with HBsAg, serum HBV RNA quantification exhibited a better surrogate marker reflecting the amount of intrahepatic cccDNA. These findings suggest that serum HBV RNA quantification represents a useful marker for disease monitoring in patients with chronic HBV infection.

Introduction

Hepatitis B virus (HBV) is a major cause of chronic hepatitis and the leading cause of cirrhosis and hepatocellular carcinoma (1, 2). HBV is double-stranded DNA viruses which replicate their DNA genome through reverse transcription from pre-genomic RNA (pgRNA) (3). The mechanism involved in chronic hepatitis B virus (CHB) infection remains unclear but it is likely related to inadequate activation of immune response towards eliminating the virus. Moreover, the virus can persist in

hepatocytes of patient as covalently closed circular DNA (cccDNA), which is difficult to eradicate (4). Therefore, the major goal of treatment is to prevent cirrhosis and HCC development.

The natural history of CHB infection is generally divided into four phases according to the level of HBV replication and the response of host immune system: HBeAg-positive chronic HBV infection; HBeAg-positive chronic hepatitis B; HBeAg-negative chronic HBV infection and HBeAg-negative chronic hepatitis B (5). The degree of disease progression in each patient greatly varies. Thus, it is essential to identify factors correlated with the progression of HBV-induced liver disease.

Theoretically, intrahepatic cccDNA and intrahepatic HBV DNA quantification remain the best accurate method to observe viral reservoir (32). However, liver biopsy is an invasive procedure, with pain and major complications occurring. Therefore, serological markers which can reflect intrahepatic cccDNA activity are very challenge. Serum HBV DNA levels and HBsAg titers are serological markers which have been used to predict the risk of cirrhosis and HCC. Nevertheless, there are studies found that cirrhosis and HCC can still occur in the majority of patients achieved undetectable HBV DNA (33) and HBsAg seroclearance (34, 35). Other favorable markers which can be the better predictor in these contexts are quite desired.

Recent studies identified that HBV RNA present in the serum of chronically HBV infected patients (36, 37). Furthermore, it is encapsidated and enveloped in

virus-like particles (36). The amount of serum HBV RNA was appeared to increase after the reverse transcription process is blocked by nucleos(t)ide analogs (NAs) in *vitro* and in transgenic mice (36). The detectable serum HBV RNA is correlated with risk of HBV rebound after withdrawing of NAs treatment in CHB patients (36). In addition, the circulating HBV RNA may reflect the amount of HBV RNA in the liver and transcriptionally active cccDNA in patients (38). Therefore, serum HBV RNA might be a potential predictive biomarker for disease monitoring, predicting treatment response and disease outcome of CHB. However, data on the level of HBV RNA in sera of patients with various stages of CHB infection is restricted.

Presently, the principal technique for monitoring serum HBV RNA is quantitative polymerase chain reaction (qPCR) also known as real-time PCR (36, 37, 39). Nonetheless, this method needs more than 10^2 copies of HBV RNA to produce a detectable signal (36). The requirement hardly achieved with serum samples of HBeAg-negative CHB infection patients, particularly after anti-viral treatment. Therefore, a highly sensitive quantification methodology for serum HBV RNA in HBeAg-negative CHB infection patients and during anti-viral treatment of CHB patients is very desirous. Droplet Digital PCR (ddPCR) is a recent approach based on water-oil emulsion droplet technology that became commercially available. The ddPCR is created on the combination of traditional PCR and fluorescent-probe–based detection techniques to allow highly sensitive absolute quantification of nucleic acids without the requirement for standard curves. A sample is partitioned into

thousands of droplets then PCR amplification occurs within each droplet. For this reason, ddPCR can improve the detection sensitivity and enable the detection of rare events in the samples (40, 41). Therefore, this technology may represent an ideal assay for monitoring serum HBV RNA in HBeAg-negative CHB infection.

The aim of this study was to develop a sensitive method for quantification of HBV RNA in serum sample from patients and to examine the correlation between serum HBV RNA and other viral markers in various phases during the natural history of chronic HBV infection.

Material and methods

Patients

In this study, 417 CHB patients naïve to antiviral treatment were retrospectively recruited from the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The study subjects could be divided into four phases according to the natural history of chronic HBV infection. Phase of HBV infection was determined using the presence of HBeAg, HBV DNA levels and ALT serum levels according to the current guidelines from the European Association for the Study of the Liver (EASL) 2017 (5) including HBeAg-positive chronic HBV infection (N=47), HBeAg-positive chronic hepatitis B (N=89), HBeAg-negative chronic HBV infection (N=156) and HBeAg-negative chronic hepatitis B (N=125). In brief, the HBeAg-positive chronic HBV infection phase was defined as presence of HBeAg in serum, very high level of HBV

DNA and ALT levels persistently within the normal range. The HBeAg-positive chronic hepatitis B was defined as presence of HBeAg in serum, high levels of HBV DNA and elevated ALT. The HBeAg-negative chronic HBV infection was defined as absence of HBeAg and presence of anti-HBe, ALT levels persistently within the normal range and HBV DNA level down below 2,000 IU/mL or undetectable. The HBeAg-negative chronic hepatitis B was defined as absence of HBeAg with detectable anti-HBe. The serum HBV DNA is persistent or fluctuating at moderate to high level, as well as ALT level is fluctuating or persistently elevated. This study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. Written informed consents were obtained from each patient and the study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Serological and virological assays

Serum samples were stored at −70°C until analysis. The levels of HBsAg, anti-HBsAg, HBeAg and Anti-HBe were determined by commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). Serum HBsAg quantifications were assessed by Elecsys HBsAgII Quant reagent kits (Roche Diagnostics, Indianapolis, IN) and HBV DNA levels were quantified by Abbott Real Time HBV assay (Abbott Laboratories). Intrahepatic cccDNA have been quantitatively

measured by quantitative real-time polymerase chain reaction (qPCR) in our previous study (121)

RNA extraction and cDNA synthesis

Total RNA was extracted from 200 µl of serum sample using guanidine thiocyanate (GTC) protocol as previously described (122). Briefly, the cells were lysed with denaturing solution (4 M GTC, 25 mM sodium citrate, pH 7.0, 0.5% (wt/vol) N-laurosylsarcosine and 0.1 M 2-mercaptoethanol) followed by phenol/ chloroform extraction and isopropanol precipitation. The RNA pellet was dissolved in 20 uL of DEPC-treated water. The RNA sample was subsequently treated with DNAse (DNase I, RNase-free, Thermo Scientific, USA) according to manufacturer's instructions, to get rid of any DNA present. Then 4 uL of RNA was used as input for reverse transcription in a 20 uL total reaction volume by ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. Each reaction was contained reaction buffer, MgCl2, dNTP mix, ribonuclease inhibitor, reverse transcriptase and HBV-specific RT primer as previously described (36). The sequence of HBV-specific RT primer is: 5'ATTCTCAGACCGTAGCACACGACACCGAGATTGAG-ATCTTCTGCGAC-3' in which the random sequence ATTCTCAGACCGTAGCACACGACAC is anchored at the 5' end of HBV specific sequence CGAGATTGAGATCTTCTGCGAC. The same cDNA preparations were used for both ddPCR and qPCR to ensure consistency.

Construction of plasmids as a positive control

Standard plasmids containing specific HBV RNA target fragments were used as template for optimization of the assays for serum HBV RNA quantification. Plasmid DNA were constructed by insertion of the core region of HBV into the pGEM-T Easy Vector (Promega, Madison, WI) by TA-cloning strategy. The resulting plasmid constructs were confirmed by PCR and DNA sequencing (First BASE Laboratories, Selangor Darul Ehsan, Malaysia).

TaqMan based quantitative Real-time PCR

The levels of HBV RNA were determined using methods reported by previous studies (36) and the procedures were as follows. The serum HBV RNA was quantified by qPCR in ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with TaqMan probe method. The sequence of primers and probe were as follows:

Forward primer 5'-AYAGACCATCAAATGCCC-3'

Reverse primer (Anchored sequence): 5'ATTCTCAGACCGTAGCACACGACAC-3'

Probe: 5' FAM-CTTATCAACACTTCCGGARACTACTGTTGTTA GAC-BHQ1-3'

as previously described (36). The qPCR reaction mixture (10uL) was contained 5 ul 2 x master mix (QPCR Probe Master Mix LRox, biotechrabbit, Germany), 0.4 uL forward primer (10 uM), 0.4 uL reverse primer (10 uM), 0.4 uL TaqMan probe (5 uM), 2 uL cDNA template and 1.3uL double distilled water (ddH2O). The reaction mixture was denatured at 95 ℃ for 3 min, followed by 40 cycles at 95 ℃ for 15 s, 60 ℃ for 30 s.

The level of HBV cDNA was quantified by comparing the signals to a standard curve using applied biosystems quantstudio real-time PCR system software. To ensure that no HBV DNA is measured, qPCR was performed in parallel on RNA samples without reverse transcription (no-RT controls).

Droplet Digital PCR

Serum HBV-RNA was quantified using droplet digital PCR (ddPCR; Bio-rad QX200, USA) with a 20 ul reaction mixture volume, which included 10 uL of ddPCR supermix (2x) (Bio-rad), 1.8 uL of primers (10 nM), 1.0 uL of TagMan Probe, 2 uL of the cDNA template, and 3.4 uL of ddH2O.

The sequence of primer and probe were the same as that of the qPCR system. For each ddPCR reaction mixture, 70 uL of droplet generation oil was added to the DG8 cartridge, and the droplets were produced by a droplet generator of the QX200 Droplet Digital PCR system (Bio-Rad, USA). The droplets, which contain the PCR reaction mixture and droplet generation oil, were then transferred to a 96-well PCR plate for amplification using the T100 Thermal Cycler (Bio-Rad, USA). The experiments were performed using the following protocol: one cycle at 95 ℃ for 10 min, 40 cycles at 94 ℃ for 30 s and 55 ℃ for 1 min and one cycle at 98 ℃ for 10 min. Subsequently, a droplet reader (Bio-Rad, USA) was used to calculate the number of both positive and negative droplet events from each PCR reaction mixture. A PCR reaction mixture with no DNA template was used as a reference

control for potential PCR contamination. The concentration of cDNA was calculated by QuantaSoft analysis software (Bio-Rad, USA). The ddPCR data and the concentration of positive droplets were determined by calculating the ratio of the positive droplets over the total droplets combined with Poisson distribution.

Data Analysis and Statistics

Statistical analysis was performed by SPSS statistics software for Windows 22.0 (SPSS Inc., Chicago, IL) and GraphPad Prism v5.0 (GraphPad Software, San Diego, CA). Comparisons between groups were assessed 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. Correlation between baseline parameters were examined by Pearson's correlation or Spearman's rank correlation test. *P*-values<0.05 was considered statistically significant.

Results

Optimization of the ddPCR assay

To determine the optimum annealing temperature for the ddPCR assay, temperature gradient was set at the following four temperatures: 55, 56.4, 60.5, and 62.0°C on the thermal cycler. The optimal range of annealing temperatures giving the largest difference in fluorescence between negative and positive droplets was 55°C (Figure 13). At lower annealing, there are non-specific targets amplified and

affected to amount of copy number. Therefore, an optimized annealing temperature of 55°C was chosen for the subsequent ddPCR tests.

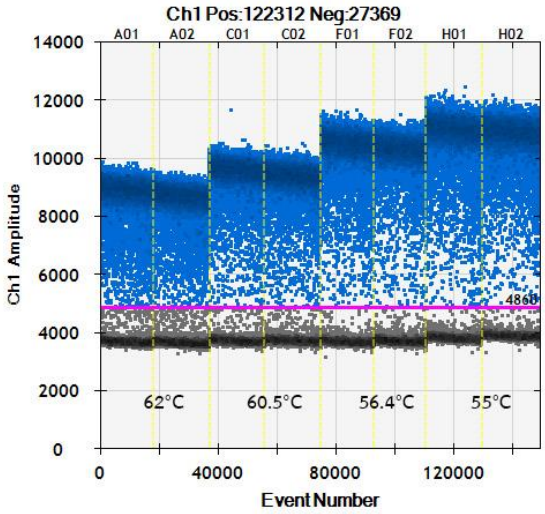


Figure 13. The optimum annealing temperature for the ddPCR assay

Comparison of analytical sensitivity, linearity and dynamic range between ddPCR and qPCR assays

Tenfold serial dilution series of positive plasmid DNA was used to compare the analytical sensitivity, linearity and dynamic range of the two assays. The qPCR assay exhibited good linearity (R^2 = 0.998) with dynamic ranges of detection between (10^8-10^3 copies/mL) **(**Figure 14A). The slope was −3.36, equivalent to a PCR efficiency of 98.3%. According to the standard curves, the detection limit of the qPCR assays was determined as 10^3 copies/mL. Quantitative linearity of ddPCR assay was also assessed by positive plasmid DNA. The log10-transformed copy numbers concentration measured by ddPCR were plotted against the corresponding log_{10}-

transformed predicted values of serially diluted plasmid DNA and fitted with a linear regression mode. The measurements of ddPCR assay exhibited good linearity (R^2 = 0.995, P<0.001) between the target input amounts and measured values in a dynamic range of 10^6-10^2 copies/mL (Figure 14B). However, when the input of DNA template was higher than 10^6 copies, the ddPCR droplets become completely saturated. In this study, the sensitivity of the ddPCR assay for plasmid DNA was down to 10^2 copies/mL, which is more sensitive than qPCR. Importantly, no nonspecific reactions were observed in negative controls in either assay.

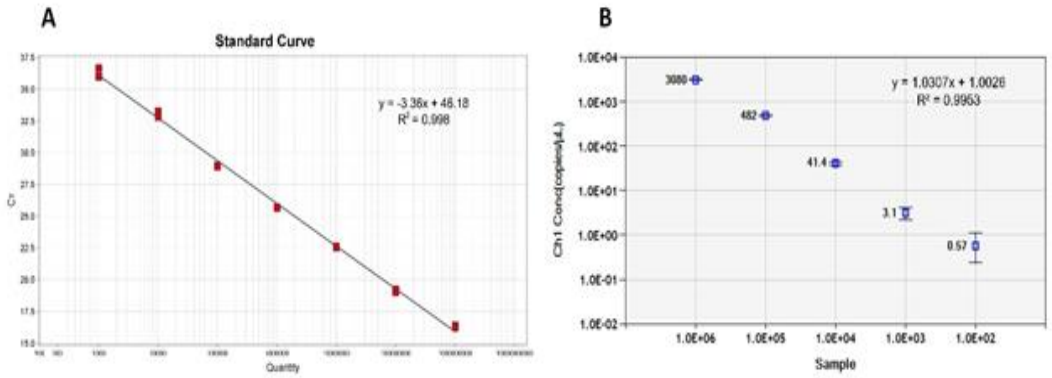


Figure 14. Quantification of serially diluted HBV plasmid DNA by ddPCR and qPCR

(A) qPCR standard curve from a 10-fold dilution series of plasmid DNA. The slope of the fitted line is −3.36, R^2 = 0.998.

(B) 1D Amplitude ddPCR analysis plot shows detection of plasmid as a function of dilution.

Evaluation using clinical samples

To further evaluate the sensitivity of ddPCR versus that of qPCR in the detection of serum HBV RNA, we serially diluted cDNA extracted from serum sample (HBV RNA has been quantified as approximately 3338 copies/ml by ddPCR) into moderate concentration, (approximately 668 copies/ml), low concentration (approximately 134 copies/ml), and very low concentration (approximately 27 copies/ml). Then, the diluted cDNA was subjected to both assays. Each assay was performed five times for each dilution.

The qPCR yielded 100% positive results for the low concentration sample and 60% positive results for the very low concentration sample (Table 10). Although, ddPCR yielded 100% positive results for the low concentration sample and 80% positive results for the very low concentration sample. Interestingly, ddPCR values were less variable than those for qPCR, at all concentrations. No nonspecific reactions were observed in negative controls in either assay.

Table 10. HBV RNA quantification in qPCR and ddPCR

Copies/ml	ddPCR				qPCR				P values
	No. positive/ Total	Copies/ml	Mean±SD	CV (%)	No. positive /Total	Copies/ml	Mean±SD	CV (%)	
High concentration (3338)	5/5	2963; 3300; 3375; 3675; 3338	3330±254	7.6	5/5	3937; 4340; 5118; 3075; 3819	4057.9±748.6	18.4	0.073
Moderate concentration (668)	5/5	750; 675; 551; 863; 713	710±113	15.9	5/5	739; 957; 1214; 927; 908	948.9±170.4	18.0	<0.05
Low concentration (134)	5/5	75; 26; 75; 49; 49	55±21	37.7	5/5	112; 116; 119; 255; 91	138.6±66.0	47.7	<0.05
Very low concentration (27)	4/5	0; 26; 23; 71; 26	29±26	88.5	3/5	35; 0; 0; 36; 29	20.1±18.5	92.1	0.539
Negative control	0/5	0; 0; 0; 0; 0	0	N/A	0/5	0; 0; 0; 0; 0	0	N/A	N/A

Comparison of diagnostic performance between the ddPCR and qPCR assays

417 samples from the CHB patients were tested by ddPCR and qPCR assays to determine whether ddPCR can replace the qPCR. We used the same cDNA templates for both assays. One important difference between our ddPCR and qPCR strategies was the number of technical replicates included. For ddPCR we did not setup a technical replicate in the assay, while for qPCR we used three technical replicates. This choice was made based on the relative cost of the two assays.

Serum HBV RNA was detected in 170/417 (40.8%) of CHB patients by qPCR, including 47/47 (100%) in phase 1, 85/89 (95.5%) in phase 2 and 38/125 (30.4%) in phase 4. However, the RNA was not detected in CHB patients in phase 3 by using qPCR. Interestingly, the ddPCR assay can be used to detect serum HBV RNA in 279/417 (66.9%) of CHB patients including 47/47 (100%) in phase 1, 89/89 (100%) in phase 2, 51/156 (32.7%) in phase 3 and 92/125 (73.6%) in phase 4 (Table 11). In fact, the HBV RNA detected in serum of CHB patients by ddPCR were not completely matched with those by qPCR (Table 12), the concordance between the two assays was 308 of 417 samples (73.9%).

Table 11. Performance of ddPCR and qPCR assay for detection of serum HBV RNA in difference phase of CHB infected patients

Phase (N)		qPCR				ddPCR			
		Positive (%)		Negative (%)		Positive (%)		Negative (%)	
1	47	47	100.0	0	0.0	47	100.0	0	0.0
2	89	85	95.5	4	4.5	89	100.0	0	0.0
3	156	0	0.0	156	100.0	51	32.7	105	67.3
4	125	38	30.4	87	69.6	92	73.6	33	26.4
All	417	170	40.8	247	59.2	279	66.9	138	33.1

Table 12. Correlation of serum HBV RNA between ddPCR and qPCR assay

Phase	qPCR	ddPCR	
		Positive	Negative
1	Positive	47	0
	Negative	0	0
2	Positive	85	0
	Negative	4	0
3	Positive	0	0
	Negative	51	105
4	Positive	38	0
	Negative	54	33
all	Positive	170	0
	Negative	109	138

The ddPCR assay showed higher positive predictive value compared to the qPCR assay, indicating that ddPCR is more robust method for detection of serum HBV RNA in the patients in phase 3 and 4 of CHB infection.

The 170 samples found positive with both tests were used to draw the correlation curve (Figure 15A), the Pearson r coefficient was 0.929 ($P < 0.001$). Bland-Altman plots (Figure 15B) comparing the mean quantitative values for ddPCR with those for qPCR, demonstrating close agreement between the two methods (mean difference is 0.24 log_{10} copies/mL; SD 0.57 log_{10}copies/mL).

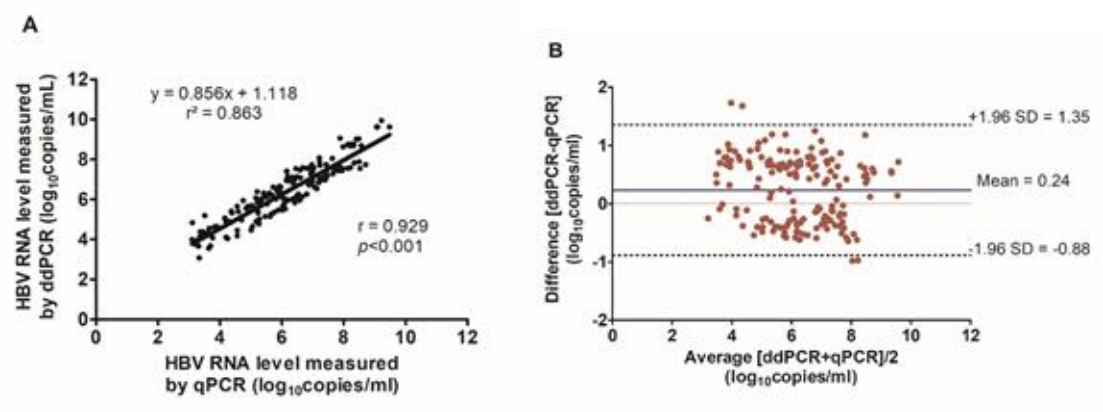


Figure 15. Correlation analysis using clinical samples

(A) Comparison of HBV RNA load by ddPCR and qPCR using cDNA samples (N=170). The results are expressed in log_{10} copies/mL.

(B) Difference in quantitation between ddPCR and qPCR. Each point represents the difference observed between the results of the 2 methods against their mean. The standard deviation (SD) is 0.571.

Baseline characteristics of enrolled patients

The baseline characteristics of enrolled patients are presented in Table 13. Based on the criteria described above, they were classified into four distinct groups: phase 1 (N=47), phase 2 (N=89), phase 3 (N=156) and phase 4 (N=125). As expected,

HBeAg negative patients (phase 3 and 4) were older than HBeAg positive patients (phase 1 and 2) ($p < 0.05$). The male-to-female ratios, ALT, HBV DNA and HBsAg levels in these four groups were generally different ($p < 0.05$).

Table 13. Baseline characteristics of enrolled patients

Characteristic	Phase 1 (n=47)	Phase 2 (n=89)	Phase 3 (n=156)	Phase 4 (n=125)	P-value
Age (years)	29± 8	39± 10	53± 12	44± 10	<0.05
Sex (M/F)	24/23	52/37	65/91	82/43	<0.05
ALT (U/L)	24± 7	119± 150	22± 11	54± 41	<0.05
HBV DNA (Log_{10} IU/mL)	8.48± 0.47	7.60± 1.39	2.84± 0.81	5.43± 1.17	<0.05
HBsAg (Log_{10} IU/mL)	4.66± 0.35	3.97± 0.74	2.26± 1.50	3.36± 0.59	<0.05

Data expressed as the mean±standard deviation

The level of HBV RNA and its relationship with other markers

Because ddPCR exhibited better performance than qPCR, we selected ddPCR for serum HBV RNA quantification. The levels of HBV RNA across four phases of diseases were examined (Figure 16). Highest level of HBV RNA was observed in phase 1 (6.70±0.68 copies/mL) and phase 2 (6.51±1.74 copies/mL) whereas lowest level was found in phase 3 (2.33±0.52 copies/mL), in which 105 of 156 patients tested negative for HBV RNA. The mean level of HBV RNA in phase 4 is 3.52±1.38 copies/mL with 33 of 125 tested negative. The general feature of HBV RNA distribution is like that of HBV DNA and HBsAg.

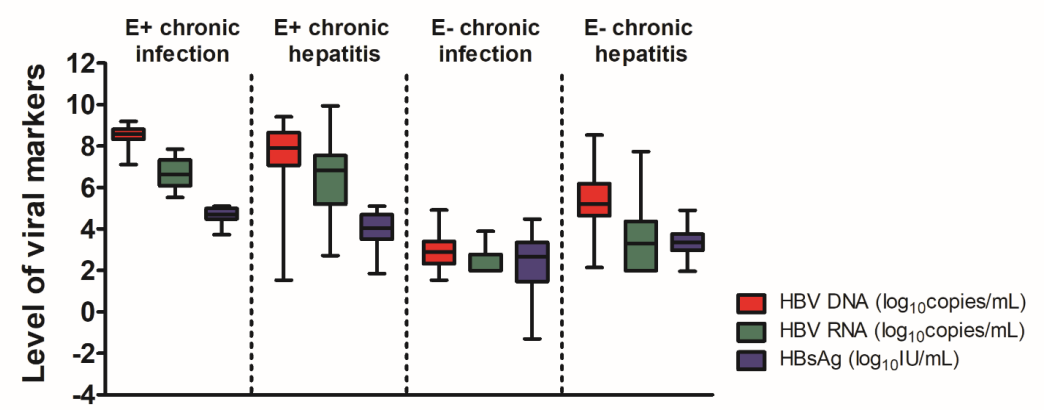


Figure 16. Distribution of HBV DNA, HBV RNA, and HBsAg throughout the natural history of CHB.

We next evaluated the correlation patterns among levels of major viral antigens and nucleic acid in a phase-specific manner. As shown in table 14, the HBV RNA level was correlated with ALT levels in phase 4. The HBV RNA and DNA levels exhibited high degree of correlation in phase 1 (r = 0.586), phase 2 (r = 0.524) and phase 4 (r = 0.780) but not in phase 3. However, we noticed that HBV RNA and HBsAg level showed correlation only in phase 1 (r = 0.536) and phase 2 (r = 0.496).

Table 14. The correlations between serum HBV RNA and clinical characteristics of chronic HBV patients

Characteristic	Serum HBV RNA				
	Phase 1	Phase 2	Phase 3	Phase 4	All
Gender	r = -0.134 *p* = 0.370	r = 0.034 *p* = 0.749	r = -0.058 *p* = 0.469	r = -0.205 *p* = 0.022	r = -0.113 *p* = 0.021
Age	r = -0.203 *p* = 0.187	r = -0.256 *p* = 0.015	r = 0.022 *p* = 0.789	r = -0.188 *p* = 0.192	r = -0.501 *p* < 0.001
ALT	r = 0.050 *p* = 0.736	r = -0.064 *p* = 0.549	r = -0.035 *p* = 0.662	r = 0.417 *p* < 0.001	r = 0.281 *p* < 0.001
HBsAg	r = 0.536 *p* < 0.001	r = 0.496 *p* < 0.001	r = 0.053 *p* = 0.514	r = 0.021 *p* = 0.817	r = 0.556 *p* < 0.001
HBV DNA	r = 0.586 *p* = 0.001	r = 0.524 *p* < 0.001	r = 0.113 *p* = 0.160	r = 0.780 *p* < 0.001	r = 0.863 *p* < 0.001

Among patients undergoing liver biopsy from whom the intrahepatic cccDNA have been quantitatively measured in our previous study. The data showed that serum HBV RNA correlated well with intrahepatic cccDNA in phase 2 (n=20, r=0.501, p<0.05) and in phase 4 (n=70, r=0.339, p<0.01) (Figure 17A and 17B). However, serum HBsAg levels correlated with intrahepatic cccDNA only in phase 2 (r=0.455, p<0.05) but not in phase 4 (r=0.058, p=0.63) (Figure 17C and 17D). Altogether, this suggests that the serum HBV RNA level shows superiority in reflecting amount of cccDNA in liver than HBsAg level.

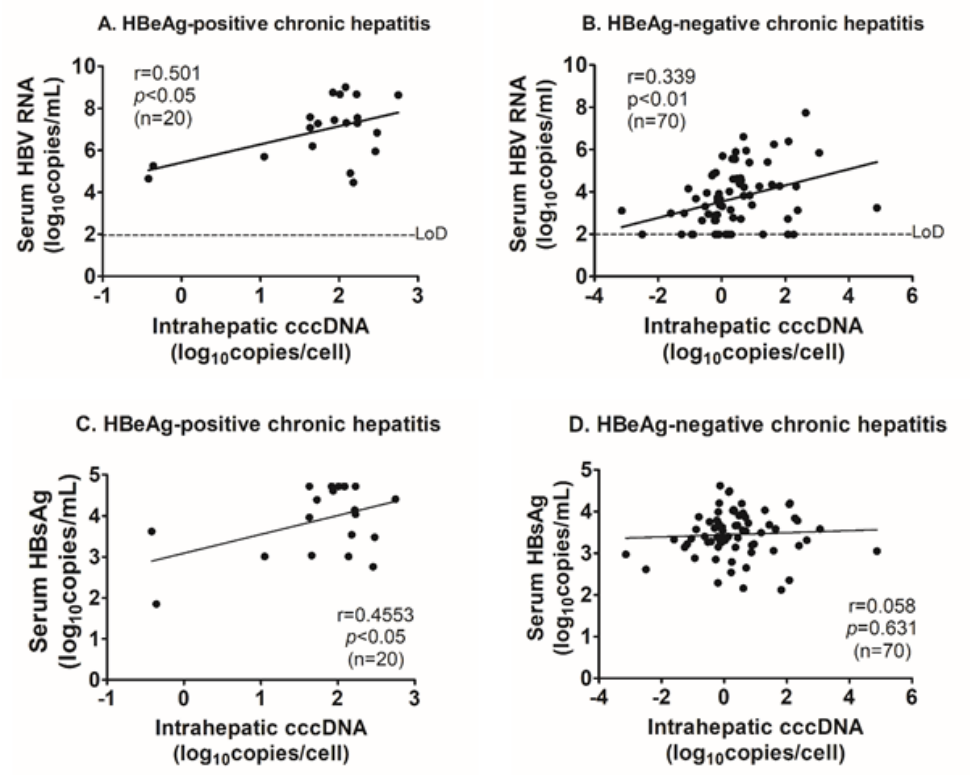


Figure 17. Correlations of serum HBV RNA, HBsAg and intrahepatic cccDNA levels in HBeAg-positive chronic hepatitis group (A, C) and HBeAg-negative chronic hepatitis group (B, D).

Part 5: Baseline and kinetics of serum HBV RNA predict response to pegylated interferon-based therapy in patients with HBeAg-negative chronic hepatitis B

(Manuscript submitted in Journal of Viral Hepatitis, March 18th, 2019)

Abstract

Serum hepatitis B virus (HBV) RNA has emerged as a novel biomarker of treatment response. This study was aimed at investigating the role of this marker in predicting long-term outcome of patients with HBeAg-negative chronic hepatitis B (CHB) receiving pegylated interferon (PEG-IFN)-based therapy. Serial serum samples from 91 patients with HBeAg-negative CHB previously treated with PEG-IFN alone or combined with entecavir in a randomized trial was retrospectively analyzed. HBV RNA quantification were examined by droplet digital PCR. At the end of 3 years post-treatment follow-up, sustained response (SR, HBV DNA <2,000 IU/mL), and HBsAg clearance were achieved in 37.4% (34/91) and 7.7% (7/91), respectively. Baseline serum HBV RNA concentrations correlated with HBV DNA and cccDNA but did not correlate with HBsAg levels. Multiple regression analysis showed that pretreatment HBV RNA and HBsAg were independently associated with SR and HBsAg clearance. Baseline HBV RNA (cut-off 2.0 $\log_{10}$ copies/mL) had a positive predictive value (PPV) and a negative predictive value (NPV) in predicting SR of 80.8% and 80.0%, respectively. At the same cut-off value, PPV and NPV for predicting HBsAg clearance were 30.8% and 95.4%, respectively. At week 12 during therapy, HBV RNA level ≥ 2

log_{10} copies/mL displayed high NPVs of achieving SR and HBsAg clearance (95% and 100%, respectively). In conclusion, the measurement of HBV RNA prior to PEG-IFN-based therapy could identify patients with high probability of SR. In addition, HBV RNA kinetics may serve as a promising 'stopping rule' in patients infected with HBV genotypes B or C.

Keywords: Chronic hepatitis B, pegylated interferon, entecavir, HBV RNA, HBsAg, cccDNA, stopping rule,

Introduction

Hepatitis B virus (HBV) infection represents a major risk factor of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (112). Among individuals with chronic HBV infection, hepatitis B e antigen (HBeAg)-negative chronic hepatitis B (CHB) is a difficult-to-treat population by currently available antiviral therapies (112, 117). Although nucleos(t)ide analogues (NAs) achieve profound viral suppression, this therapeutic option requires long-term treatment for patients with HBeAg-negative CHB. In contrast, a finite duration of pegylated interferon (PEG-IFN) could induce a sustained off-treatment virological response and, more importantly, hepatitis B surface antigen (HBsAg) clearance, which is considered as a 'functional cure'(112, 117). However, its uncertain response rate with potential side effects and lack of suitable baseline predictors could compromise the clinical utility of PEG-IFN.

As a result, it is crucial to identify reliable predictive biomarkers to maximize cost-effectiveness of PEG-IFN.

It is well-recognized that monitoring HBsAg quantification during therapy is helpful for predict response outcome to PEG-IFN (123, 124). Nonetheless, the measurement of this marker at baseline has restricted value in identification of responders. Recenty, serum HBV RNA quantification has emerged as a novel biomarker of HBV infection in terms of treatment response and prognosis(125). Previous data demonstrated that serum HBV RNA levels were well correlated with the transcriptional activity of intrahepatic covalently closed circular DNA (cccDNA)(71, 126), and exhibited unique patterns in different phases of chronic HBV infection(126). In addition, serum HBV RNA levels were shown to be predictive of treatment response to antiviral therapy, particularly NAs (36, 39, 70, 127). A recent report also demonstrated that HBV RNA quantification at baseline could predict response to combination of PEG-IFN and adefovir in HBeAg-negative CHB (37). However, additional studies with larger sample sizes are needed to evaluate the predictive roles of HBV RNA kinetics in this subgroup of patients with CHB. Thus, this study was aimed at investigating the clinical applicability of HBV RNA kinetics in Thai patients with HBeAg-negative CHB previously treated with PEG-IFN-based therapy in a randomized trial.

Materials and Methods

Patients

Serum HBV RNA levels were measured from stored samples of Thai patients with HBeAg-negative CHB, who were previously enrolled in a randomized trial conducted at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand between November 2010 and April 2014 (128). Briefly, these patients were treated with PEG-IFN alfa-2b (1.5 μg/kg/week) alone or combination with entecavir (ETV, 0.5 mg/day) for 48 weeks and had follow-up for additional 48 weeks. Inclusion criteria were HBsAg positive for ≥ 6 months, HBeAg negative and serum anti-HBe positive, elevated serum alanine aminotransferase (ALT), HBV DNA levels >2,000 IU/mL and no evidence of advanced liver disease. Exclusion criteria were co-infected with hepatitis C virus or human immunodeficiency virus, evidence of HCC by imaging studies or severe co-morbid disorders.

Patients were eligible for this retrospective study if they had available stored sera for HBV RNA quantification at weeks 0, 4, 12, 24, 48, 72 and 96. Moreover, these patients were followed at 6-month intervals for additional 2 years to determine the 3-year post-treatment response, which included sustained response (SR, defined as persistent HBV DNA <2,000 IU/mL) and HBsAg clearance (defined as HBsAg quantification <0.05 IU/mL). Patients achieving SR were defined as responders, while participants who had elevated viral load ≥ 2,000 IU/mL at least 2 consecutive

determinations during follow-up were defined as non-responders. All patients gave written informed consent and the study was conducted following the Helsinki Declaration and Good Clinical Practice guidelines. The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Serum HBV RNA quantification

HBV RNA was isolated from 200 µL stored serum samples (kept at -80 ^{o}C) using the guanidine thiocyanate method and treated with DNase I (Thermo Fisher Scientific, Waltham, MA). Isolated HBV RNA was reverse transcribed using ImProm-II Reverse Transcriptase (Promega, Madison, WI) with HBV specific RT primer. Serum HBV RNA was quantified using droplet digital PCR (ddPCR) (Bio-Rad, Hercules, CA) as previously described (129). Briefly, a 20 µL reaction mixture volume was included with 10 µL of ddPCR supermix for probes (2x) (Bio-Rad, Hercules, CA), 1.8 µL of primers (10 nM), 1 µL of TagMan Probe (5nM), 2 µL of the cDNA template, and 3.4 µL of ddH_2O. The sequence of primers and probe were designed as the followings:

HBV-specific RT primer: 5'-ATTCTCAGACCGTAGCACACGACACCGAGATTGAGATCTTCTGCGAC-3',

forward primer; 5'-AYAGACCATCAAATGCCC-3',

reverse primer: 5'-ATTCTCAGACCGTAGCACACGACAC-3'

probe: 5' FAM-CTTATCAACACTTCCGGARACTACTGTTGTTAGAC-BHQ1-3'

as previously described (36). The experiments were performed using the following protocol: one cycle at 95 ^{o}C for 10 min, 40 cycles at 94 ^{o}C for 30s and 55 ^{o}C for 1

min and one cycle at 98 ºC for 10 min. The concentration of serum HBV-RNA was calculated by QuantaSoft analysis software (Bio-Rad, Hercules, CA). The lower limit of detection (LLoD) was determined at 100 copies/mL (2.0 $\log_{10}$ copies/mL).

Testing for other HBV markers

Qualitative HBsAg, HBeAg and anti-HBe were analyzed by enzyme-linked immunosorbent assay kits (Abbott Laboratories, Chicago, IL). Serum HBV DNA levels were determined by Abbott RealTime HBV assay (Abbott Laboratories, Chicago, IL). Serum HBsAg quantification was measured by Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN). Pre-treatment intrahepatic cccDNA quantification was performed in liver biopsy specimens as previously described (121).

Statistical Analysis

Data were calculated with the SPSS version 22 software (SPSS, Chicago, IL, USA) and expressed as percentages and mean ± standard deviation (SD) as appropriate. The $\chi 2$ or Fisher's exact test was performed for categorical variables and the Mann–Whitney *U*-test or Student's *t*-test was used for quantitative variables. Correlations between baseline parameters were applied by the Pearson correlation coefficient. Areas under the receiver operating characteristic curve (ROC) were performed for diagnostic performance of baseline HBsAg and HBV RNA. Multiple regression analysis was used to calculate baseline parameters predicting SR and

HBsAg clearance. All tests were two-sided with *P* values <0.05 were statistically significant.

Results

Baseline characteristics

Among 126 patients enrolled in the above-mentioned randomized trial, 91 (72%) individuals had adequate blood samples available for HBV RNA quantification and were then included in this study. These patients were followed at 6-month intervals to determine the 3-year post-treatment response. Among these 91 patients, there were 48 and 43 individuals treated with PEG-IFN alone and PEG-IFN plus ETV combination therapy, respectively. At 3 years' post-treatment follow-up, SR rates of the corresponding groups were found in 20 (41.7%) and 14 (32.6%) (*P*=0.394), while HBsAg clearance was achieved in 7 (14.6%) and 4 (9.3%) (*P*=0.530), respectively. Baseline characteristics of patients regarding SR are demonstrated in Table 15. Briefly, responders had significantly lower mean HBsAg and HBV RNA levels than non-responders, while other baseline characteristics were comparable between groups.

Table 15. Baseline characteristics of patients according to sustained response (SR)

Baseline Characteristics	Responders (n=34)	Non-responders (n=57)	*P*
Gender (Males)	25 (73.5%)	42 (73.7%)	0.987
Age (years)	40.5±10.4	39.7±9.2	0.695
ALT (U/L)	75.4±39.1	76.7±41.1	0.880
Treatment			0.334
PEG-IFN monotherapy	20 (58.8%)	28 (49.1%)	
PEG-IFN plus entecavir	14 (41.2%)	29 (50.9%)	
HBV Genotypes			0.132
B	5 (14.7%)	17 (29.8%)	
C	29 (85.3%)	40 (70.2%)	
Log_{10} HBV DNA (IU/mL)	5.2±0.7	5.5±0.8	0.116
Log_{10} HBsAg (IU/mL)	3.3±0.4	3.5±0.5	0.001*
Log_{10} HBV RNA (copies/mL)	2.4±0.8	3.1±1.1	0.002*
Liver necroinflammation (A2-A3)	15 (45.5%)	18 (34.0%)	0.363
Liver fibrosis (F2-F4)	21 (63.6%)	28 (52.8%)	0.375

Data express as mean ± standard deviation or n (%); ALT, alanine aminotransferase; PEG-IFN, pegylated interferon; HBV DNA, hepatitis B virus DNA; HBsAg, hepatitis B surface antigen; HBV RNA, hepatitis B virus RNA, *, *P*-value<0.05

Correlation between parameters at baseline

Pretreatment log_{10} HBV RNA correlated with log_{10} HBV DNA (r=0.546, *P*<0.001), log_{10} cccDNA levels (r=0.476, *P*<0.001) and ALT levels (r=0.379, *P*<0.001), but did not have a correlation with log_{10} HBsAg (r=0.143, *P*=0.178). In contrast, baseline log_{10} HBsAg had a weak correlation with log_{10} HBV DNA (r=0.268, *P*=0.010) but did not

correlate with $\log_{10}$ cccDNA (r=0.122, *P*=0.361) and ALT levels (r=0.044, *P*=0.681). Baseline $\log_{10}$ HBV DNA also correlated with $\log_{10}$ cccDNA (r=0.294, *P*=0.025) and ALT levels (r=0.365, *P*<0.001). The correlations between baseline serum and intrahepatic viral markers are shown in Figure 18.

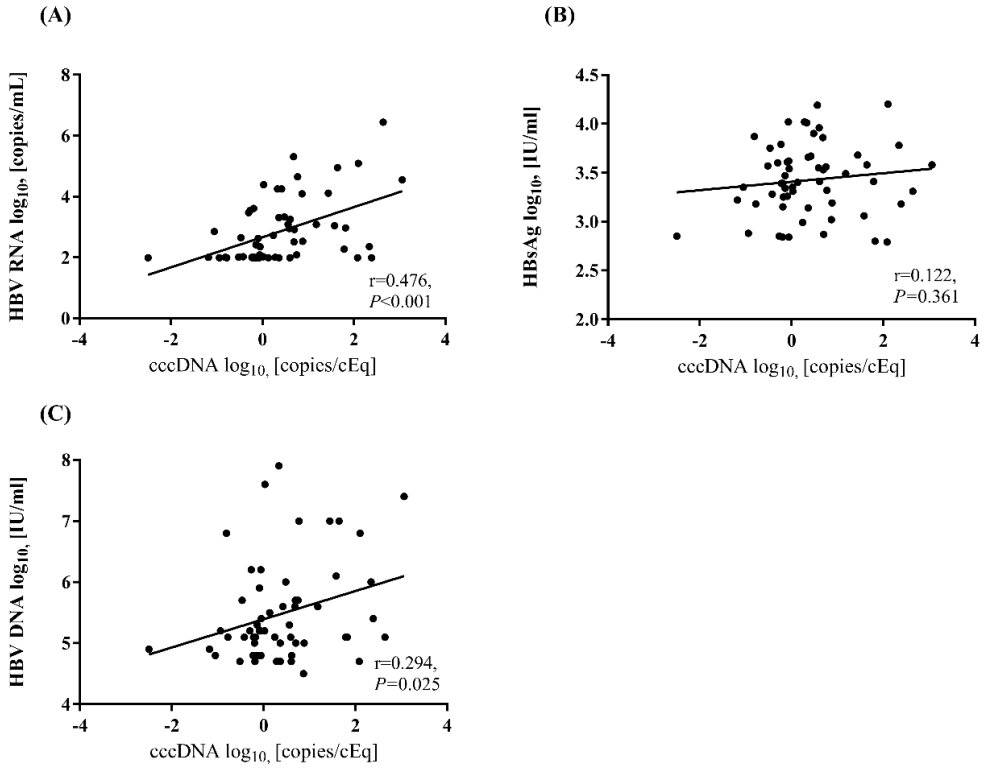


Figure 18. Correlations of serum viral markers and intrahepatic cccDNA levels at baseline (A) HBV RNA, (B) HBsAg, (C) HBV DNA

Baseline HBsAg and HBV RNA in predicting treatment response

The areas under ROC (AUROCs) of baseline $\log_{10}$ HBsAg and $\log_{10}$ HBV RNA levels in predicting SR were 0.713 (95% CI, 0.597-0.829; *P*=0.001) and 0.767 (0.656-0.877; *P*<0.001), respectively. The combined use of both markers had an AUROC of

0.775 (0.671-0.879; *P*<0.001) (Figure 19A). Similarly, the AUROCs of log_{10} HBsAg, log_{10} HBV RNA and combined both markers in predicting HBsAg clearance were 0.895 (0.794-0.996; *P*<0.001), 0.742 (0.582-0.902; *P*=0.010) and 0.901 (0.795-1.000; *P*<0.001), respectively (Figure 19B).

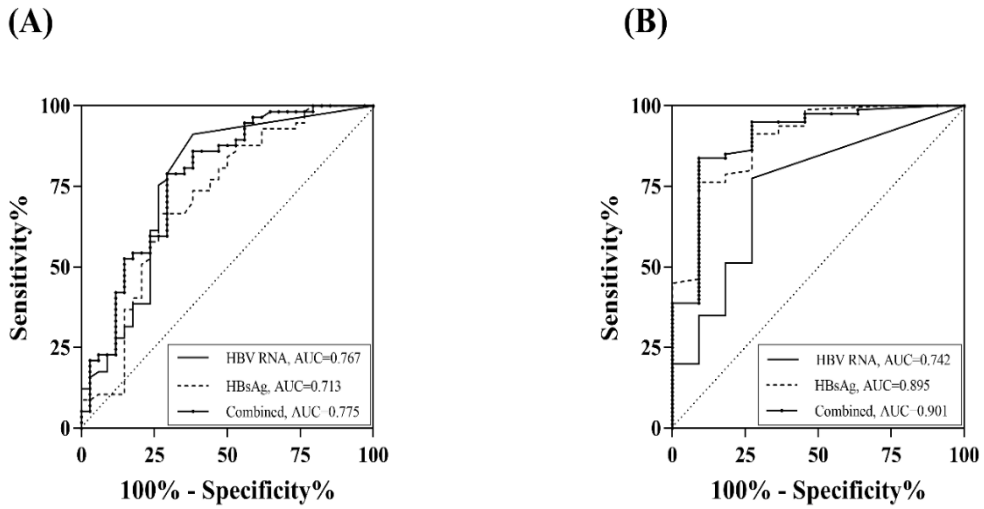


Figure 19. Receiver operating characteristic curve (ROC) in predicting response (A) Sustained response (SR), (B) HBsAg clearance

Based on the ROC, the optimal cut-off values for HBsAg and HBV RNA levels in predicting SR were 3.3 log_{10} IU/mL and 2.0 log_{10} copies/mL, respectively. Using these cut-off points, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) in predicting SR and HBsAg clearance are shown in Table 16. For SR, low HBsAg (<3.3 log_{10}IU/mL) and undetectable baseline HBV RNA (<2.0 log_{10} copies/mL) had PPVs of 56.8% and 80.8%, respectively, while unfavorable combined markers (HBsAg ≥3.3 log_{10}IU/mL plus HBV RNA ≥2.0 log_{10} copies/mL) displayed an NPV

of 88.4%. Regarding HBsAg clearance, high baseline HBsAg, detectable HBV RNA and combined both markers had NPVs of 98.1%, 95.4% and 97.7%, respectively.

Table 16. Baseline HBsAg and HBV RNA in predicting treatment response

Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Sustained response				
Log_{10} HBsAg (3.3 IU/mL)	61.8	71.9	56.8	75.9
Log_{10} HBV RNA (2.0 copies/mL)	61.8	91.2	80.8	80.0
Combination of both markers †	85.3	66.7	60.4	88.4
HBsAg clearance				
Log_{10} HBsAg (3.3 IU/mL)	90.9	66.3	27.0	98.1
Log_{10} HBV RNA (2.0 copies/mL)	72.7	77.5	30.8	95.4
Combination of both markers †	90.9	52.5	20.8	97.7

†Combined HBsAg (log_{10} 3.3 IU/mL) and HBV RNA (log_{10} 2.0 copies/mL); PPV: positive predictive value, NPV: negative predictive value

Multiple regression analysis of factors associated with treatment response

Based on multiple regression analysis, baseline parameters were further investigated to identify variables associated with SR and HBsAg clearance (Table 17). It was showed that low HBsAg and HBV RNA levels at baseline were selected as factors associated with SR. Moreover, older age, low HBsAg and HBV RNA at baseline and high liver necroinflammation scores were predictors of HBsAg clearance.

Table 17. Multiple regression analysis of baseline variables in predicting respons

Variables	Category	Sustained response		HBsAg clearance	
		OR (95%CI)	*P*	OR (95%CI)	*P*
Age (year)	< 40 vs. ≥ 40	0.80 (0.34-1.87)	0.607	0.18 (0.04-0.90)	0.036*
Sex	Male vs. Female	0.99 (0.38-2.60)	0.987	1.71 (0.34-8.53)	0.515
ALT (U/L)	≥ 60 vs. < 60	1.59 (0.68-3.75)	0.289	1.13 (0.32-4.00)	0.853
HBV genotypes	B vs. C	0.66 (0.27-1.59)	0.355	1.20 (0.29-5.00)	0.798
Treatment regimens	PEG-IFN vs. Combination	1.48 (0.63-3.49)	0.371	1.67 (0.45-6.14)	0.444
Log_{10} HBV DNA (IU/mL)	< 5.0 vs. ≥ 5.0	1.78 (0.75-4.24)	0.195	0.90 (0.24-3.34)	0.879
Log_{10} HBsAg (IU/mL)	< 3.3 vs. ≥ 3.3	4.14 (1.68-10.19)	0.002*	19.63 (2.37-161.50)	0.006*
Log_{10} HBV RNA (copies/mL)	< 2.0 vs. ≥ 2.0	2.20 (1.24-3.91)	0.007*	9.19 (2.21-38.26)	0.002*
Necroinflammatory activity	A2-A3 vs. A0-A1	1.62 (0.67-3.95)	0.288	5.33(1.30-21.87)	0.020*
Fibrosis stage	F0-F1 vs. F2-F4	1.56 (0.4-3.81)	0.326	3.94 (0.80-19.46)	0.093

Data express as odds ratio (OR) and 95% confidence intervals (CI); ALT, alanine aminotransferase; PEG-IFN, pegylated interferon, *, *P*-value<0.05

Kinetics of serum HBV RNA according to SR

At baseline, mean HBV RNA in patients achieving SR (responders) and non-responders were 2.4±0.8 vs. 3.1±1.1 log_{10} copies/mL, respectively (*P*=0.002). Mean HBV RNA levels in the corresponding groups are shown in Figure 20A: week 4 (2.1±0.5 vs. 2.4±0.8 log_{10} copies/mL, *P*=0.03), week 12 (2.1±0.3 vs. 2.2±0.7 log_{10} copies/mL, *P*=0.097), week 24 (2.0±0.1 vs. 2.2±0.6 log_{10} copies/mL, *P*=0.038), week 48 (2.0±0.0 vs. 2.1±0.4 log_{10} copies/mL, *P*=0.024), week 72 (2.0±0.1 vs. 2.7±1.1 log_{10} copies/mL, *P*<0.001) and week 96 (2.0±0.0 vs. 3.0±1.4 log_{10} copies/mL, *P*<0.001).

At baseline, HBV RNA levels were undetectable in 21/34 (61.8%) and 5/57 (8.8%) of responders and non-responders, respectively (*P*<0.001). During therapy, the

rates of undetectable HBV RNA in the corresponding groups were as the following: week 4 [27/34 (79.4%) vs. 31/57 (54.4%), *P*=0.024], week 12 [33/34 (97.1%) vs. 36/57 (63.2%), *P*<0.001] and week 24 [34/34 (100%) vs. 44/57 (77.2%), *P*=0.002]. Based on the results at week 12 during therapy, undetectable HBV RNA could yield PPV and NPV in predicting SR of 47.8% and 95.5%, respectively.

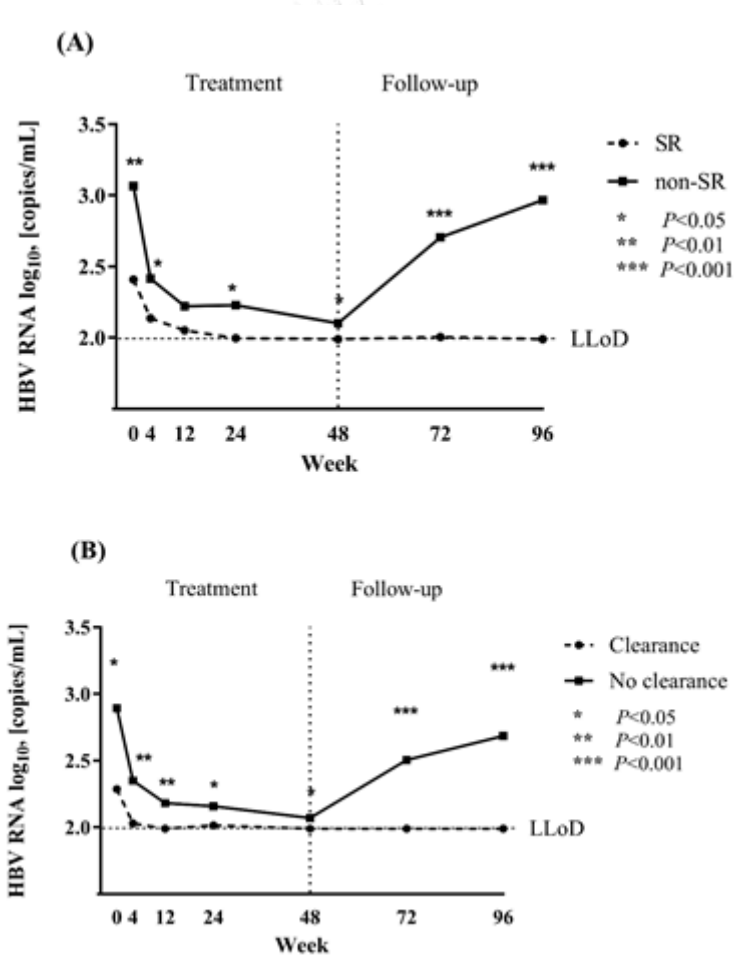


Figure 20. HBV RNA kinetics according to treatment response

(A) Sustained response (SR), (B) HBsAg clearance

Kinetics of serum HBV RNA according to HBsAg clearance

Patients achieving HBsAg clearance, compared with those without clearance, had significantly lower HBV RNA at baseline (2.3±0.6 vs. 2.9±1.1 $\log_{10}$ copies/mL, *P*=0.01), during therapy and follow-up: week 4 (2.0±0.1 vs. 2.4±0.7, $\log_{10}$ copies/mL *P*=0.001), week 12 (2.0±0.0 vs. 2.2±0.6 $\log_{10}$ copies/mL, *P*=0.005), week 24 (2.0±0.1 vs. 2.2±0.5 $\log_{10}$ copies/mL, *P*=0.032), week 48 (2.0±0.0 vs. 2.1±0.3 $\log_{10}$ copies/mL, *P*=0.024), week 72 (2.0±0.0 vs. 2.5±1.0 $\log_{10}$ copies/mL, *P*<0.001) and week 96 (2.0±0.0 vs. 2.7±1.2 $\log_{10}$ copies/mL, *P*<0.001) (Figure 20B).

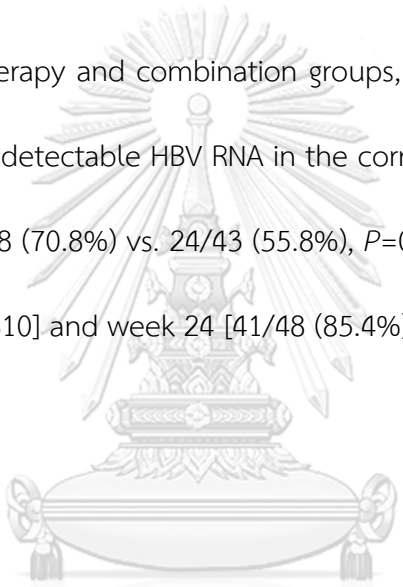
At baseline, HBV RNA levels were undetectable in 8/11 (72.7%) and 18/80 (22.5%) of patients with and without HBsAg clearance, respectively (*P*=0.002). During therapy, the rates of undetectable HBV RNA in the corresponding groups were as the following: week 4 [9/11 (81.8%) vs. 49/80 (61.3%), *P*=0.183], week 12 [11/11 (100%) vs. 57/80 (71.3%), *P*=0.040] and week 24 [11/11 (100%) vs. 67/80 (83.8%), *P*=0.149]. Based on these data, undetectable HBV RNA at week 12 yielded PPV and NPV in predicting HBsAg clearance of 15.9% and 100%, respectively.

Kinetics of serum HBV RNA according to treatment regimens

The mean HBV RNA levels at different time points according to treatment regimen were further investigated. Pretreatment HBV RNA levels were similar in the monotherapy and combination groups (2.9 ± 1.0 vs. 2.8 ± 1.1 $\log_{10}$ copies/mL, *P*=0.730). Also, there was no difference in HBV RNA levels between groups during

therapy and follow-up: week 4 (2.3±0.7 vs. 2.3±0.7, $\log_{10}$ copies/mL *P*=0.764), week 12 (2.2±0.7 vs. 2.1±0.4 $\log_{10}$ copies/mL, *P*=0.396), week 24 (2.2±0.6 vs. 2.1±0.4 $\log_{10}$ copies/mL, *P*=0.319), week 48 (2.1±0.3 vs. 2.0±0.2 $\log_{10}$ copies/mL, *P*=0.501), week 72 (2.5±1.0 vs. 2.4±0.9 $\log_{10}$ copies/mL, *P*=0.700) and week 96 (2.5±1.1 vs. 2.7±1.3 $\log_{10}$ copies/mL, *P*=0.534).

At baseline, HBV RNA levels were undetectable in 14/48 (29.2%) and 12/43 (27.9%) of the monotherapy and combination groups, respectively (*P*=0.894). During therapy, the rates of undetectable HBV RNA in the corresponding groups were as the following: week 4 [34/48 (70.8%) vs. 24/43 (55.8%), *P*=0.190], week 12 [35/48 (72.9%) vs. 33/43 (76.7%), *P*=0.810] and week 24 [41/48 (85.4%) vs. 37/43 (86.0%), *P*=0.932].



CHAPTER IV: DISCUSSION AND CONCLUSION

Part 1: Association of vitamin D-related genes and *STAT4* Polymorphisms with HBV Infection Outcomes in Thai Population

In this study, we investigated vitamin D-related genes and *STAT4* polymorphisms with CHB susceptibility, HBV clearance and HCC development in Thai population. Our study first pointed out that *CYP27B1* rs4646536 was correlate with HCC development, but they did not associate with CHB susceptibility as well as HBV natural clearance. Regarding *STAT4* rs7574865, it also plays a role in HCC development, but it has no impact on CHB susceptibility and viral clearance.

STAT4 is a transcription factor belonging to the STAT protein family which regulated immune response. Its transmitted signals from IL-12 and type 1 IFNs to induce IFN-γ production(17, 130). Polymorphisms of the *STAT4* gene have been demonstrated to be associated with rheumatoid arthritis and systemic lupus erythematosus (131). Previously, many studies investigated the association between *STAT4* polymorphism and the risk of HCC, but the results were inconsistent. In this study, we found that *STAT4* rs7574865 was significantly associated with HCC risk when comparing between HCC patients and healthy controls in recessive model (TT+GT vs GG, OR=1.68, 95%CI=1.15-2.45, $p<0.05$) which is consistent with previous reports (89, 132). The SNP rs7574865 located within the third intron of *STAT4* gene, it was correlated with both HBV infection and HBV-related HCC in the Chinese

population (11, 12). Patients carrying GG genotype of rs7574865 are more likely to progress to CHB and HCC than those with the GT/TT genotype (11, 12, 89). Moreover, the risk allele G at rs7574865 has been demonstrated to have an impact on the lower mRNA levels of *STAT4* in both the HCC tissues and non-tumor tissue in HBV-related HCC patients (11). A possible explanation for this occurrence that lowered *STAT4* expression cannot effectively stimulate the expression of IFN-**γ**, leading to lowered antiviral and antitumor activity.

Two previously published studies in Chinese Han populations reported that rs7574865 is related to HBV infection and HBV clearance (19, 21)**.** However, we did not find any significant association of *STAT4* polymorphisms with CHB susceptibility and natural clearance in Thai population. In agreement with previous studies in Chinese Han subjects (133), which indicates that *STAT4* may plays different roles in HBV seroclearance as well as HBV susceptibility. Moreover, a study in Tibetans and Uygurs populations in China, Liao et al. (20) found that *STAT4* rs7574865 seemed to be Tibetan specific in HBV natural clearance. Therefore, its role in spontaneous HBV clearance might be population specific and further studies are needed.

Vitamin D is the precursor of steroid hormone 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$ or calcitriol) with well-known effect on calcium homeostasis (90). It has been recognized that active form of vitamin D ($1,25(OH)_2D_3$), exerts anti-proliferative, pro-differentiating effects and anti-angiogenic in various of cancer cells (134). Moreover, vitamin D has immunomodulatory and anti-inflammatory properties (92).

In recent years, it has been demonstrated that vitamin D is linked to the pathogenesis of several chronic disorders, including chronic liver disease (23).

Common SNPs in *vitamin D-related genes*, including *VDR*, *DBP* and *CYP27B1* are shown to be associated with clinical outcome and response to PEG-IFN-based therapy in patients with chronic HCV infection (24-28). Among HBV-infected individuals, recent studies have suggested a possible role of these SNPs in viral persistence and HCC development (29, 30).

VDR is an intracellular hormone receptor which specifically binds the $1,25(OH)_2D_3$ and interacts with specific nucleotide response elements of target genes to produce a variety of biological effects. The *VDR* gene is located on chromosome 12 and is highly polymorphic (93). The rs2228570 variations in *VDR* gene influenced to a shortening of the *VDR* protein by three amino acids. In addition, it is correlated with worse outcomes in cancer (95). The *VDR* gene variants have been reported to associate with increased risk of cancers including breast (97), prostate (98) and colorectal(99).

DBP is a multifunctional protein which plays a role in the transport of vitamin D metabolites. It has been reported that *DBP* play a significant role in several chronic disease including cancers (102) . Polymorphisms in the *DBP* gene have been demonstrated that it influences plasma concentrations of 25(OH) D_3 (103). The rs7041 is one of the SNPs in the *DBP* gene which has been indicated conversely

correlated with concentrations of 25(OH)D_3 in plasma. In addition, the *DBP* gene polymorphism have been involved in cancers such as breast (104) and prostate (105).

Recently, Peng Q. and co-worker found that both genotype and allele frequencies of the *VDR* rs2228570 and *DBP* rs7041 polymorphisms contributed to increased susceptibility to HBV-related HCC in the Chinese population (29), which is inconsistent with our findings. We did not find any difference in both *VDR* rs2228570 and *DBP* rs7041 polymorphisms between HCC, CHB, natural HBV clearance, and healthy controls. This discrepancy might be caused by genetic heterogeneity that exists for *VDR* and *DBP* gene polymorphisms in divergent populations.

The *CYP27B1* gene on chromosome 12 encode 1α-hydroxylase, which responsible for conversion of 25(OH)D into biologically active form of vitamin D that plays an important role in immune system function (135). This is the first study about the impact of a *CYP27B1* rs4646536, on the HCC susceptibility. We revealed that rs4646536 was significantly associated with HCC risk when comparing between HCC patients and healthy controls (OR=1.60, 95%CI=1.05-2.43, $p<0.05$). This SNP has been previously related to type 1 diabetes (136), autoimmunity (137), multiple sclerosis (115) and cancer (138). Moreover, *CYP27B1* rs10877012 (C>A) and rs4646536 (C>T) polymorphisms which were in perfect linkage disequilibrium (LD) (136) altered *CYP27B1* mRNA expression level (139). There are few studies that have examined the association of *CYP27B1* polymorphisms with HBV infection. One study indicated that *CYP27B1*-1260 (rs10877012) promoter polymorphism is associated with the

persistence, but not susceptibility to HBV infection in Chinese HBV patients. It is hypothesized that *CYP27B1* (rs4646536) or other polymorphism in the LD with this SNP may affect to the level of 1α-hydroxylase and the conversion of 25(OH)D to $1,25(OH)_2D_3$ (136).

In conclusions, this study demonstrated that the vitamin D-related genes (*CYP27B1* rs464653) and *STAT4* rs7574865 polymorphisms might contribute to increased susceptibility to HBV-related HCC in the Thai population but did not correlate with HBV susceptibility and HBV natural clearance. However, further functional study is required to validate these predictions.

Part 2: Association of vitamin D-related genetic variations and treatment response to pegylated interferon in patients with chronic hepatitis B

Increasing evidence has indicated that host genetic variations may play an essential role in natural history and therapeutic outcome of patients with chronic viral hepatitis(10). In patients with chronic HCV infection, SNPs in the *IFNL3* and *IFNL4* genes have been shown to be predictors of spontaneous HCV clearance and response to PEG-IFN-based therapy (81, 82). Nonetheless, the association of these SNPs with response to PEG-IFN therapy in patients with CHB is uncertain with conflicting data among published reports, which might be related to the heterogeneity of the study populations(10). For vitamin D-related SNPs, recent studies have showed that the SNPs including *VDR*, *DBP* and *CYP27B1* polymorphisms are predictors of therapeutic outcome of PEG-IFN-based therapy in patients with

chronic HCV infection (24, 25, 28, 140). To date, there are limited data that specifically evaluate the association of the SNPs with treatment response to PEG-IFN therapy in patients with CHB.

In this report, we aimed at clarifying whether the SNPs in these genes control key steps in vitamin D metabolism and transport were associated with therapeutic outcome of PEG-IFN in Thai patients with CHB. The results of this cohort of individuals mainly infected with HBV genotype B and C showed that SNPs rs2228570 (*VDR*) and rs7041 (*DBP*) did not affect treatment response. In contrast, the data clearly showed that SNP rs4646536 (*CYP27B1*) was independently associated with an increased probability of VR regardless of HBeAg status. Specifically, patients with the TT genotype had approximately threefold increased VR rates in comparison with those harboring the non-TT genotype in both HBeAg-positive and HBeAg-negative CHB groups. In addition, this favorable genotype was associated with rapidly decrease in HBsAg levels during and after therapy, higher rates of achieving HBsAg clearance and low HBsAg level (<100 IU/mL) at the end of follow-up. Indeed, the level of HBsAg <100 IU/mL selected in this study is regarded as an optimal cut-off threshold highly predictive of spontaneous HBsAg clearance in longitudinal follow-up of Asian populations(141). These data indicate the potential role of host genetic variations in vitamin D-related genes in modulating outcome of patients with CHB treated with PEG-IFN.

Remarkably, our results agreed with previous data conducted in the Italian cohort(31). In that report of diverse ethnic populations with mainly infected with HBV genotypes A, D and E, it was showed that the TT (vs. CT/CC) genotype of rs4646536 was an independent predictor of treatment response to PEG-IFN in patients with HBeAg-negative CHB. Moreover, the favorable TT genotype was associated with an increased likelihood of treatment response across all HBV genotypes, including difficult-to-treat genotype E. Of noted, the distribution of TT genotype in our study was approximately 22%, which was like that reported in the Italian cohort (25%)(31). Given its consistence in genetic frequency and strong association with treatment response, it is speculated that the TT genotype of rs4646536 may serve as a useful genetic marker for predicting PEG-IFN treatment response regardless of HBV genotypes, HBeAg status and background ethnicity.

The underlying mechanism by which the *CYP27B1* variant affects therapeutic outcome of PEG-IFN in patients with CHB remains unclear but might be in part related to the synthesis or activation of vitamin D. In this context, several hydroxylation steps are involved in the complex process of vitamin D metabolism. Among these pathways, 1α-hydroxylase encoded by the *CYP27B1* gene that converts 25(OH)D to generate 1α,25-$(OH)_2$D, is considered to be a crucial step of vitamin D activation (23). The conversion of this active form, however, is not restricted to the kidney but can also take place in other cell types, in particular, immune cells such as dendritic cells, macrophages and T-lymphocytes (142). Each of these immune cell

types are therefore capable of locally producing active vitamin D as autocrine or paracrine mechanisms in the regulation of immune function (23, 142).

Increasing evidence has indicated that vitamin D status may be involved in the pathogenesis of liver diseases by modulating innate and adaptive immunity (143). Moreover, vitamin D insufficiency or deficiency is commonly found in variety of chronic liver diseases, including CHB (23, 144). Although the exact role of vitamin D in CHB has not been fully elucidated, a recent study showed a positive correlation between vitamin D deficiency and low ALT level, a surrogate marker of liver inflammation, which might reflect inadequate immune response against HBV infection (145). A long-term prospective report also demonstrated that vitamin D deficiency was related to adverse clinical manifestations, including complications of cirrhosis and HCC (146). Moreover, polymorphisms in vitamin D-related genes, including the *CYP27B1* variants, are known to influence vitamin D status and clinical outcome (147). For instance, a polymorphism within the *CYP27B1* gene (rs10877012), which is in linkage disequilibrium with rs4646536, could alter mRNA expression and circulating vitamin D in patients with type 1 diabetes (139). In addition, the *CYP27B1* polymorphisms had substantial impact on vitamin D concentrations and response rates to PEG-IFN-based therapy in patients with chronic HCV infection (24, 148). Taken together, it is reasonable to speculate that the *CYP27B1* variant (rs4646536) might affect vitamin D activity, thereby altering its immunological properties and modulating therapeutic response to PEG-IFN in patients with CHB. In light of this

association, functional roles of this SNP in the pathogenesis of CHB and PEG-IFN responsiveness need to be elucidated in further studies.

Besides the predictive role of rs4646536 polymorphism, our data also demonstrated that low pre-treatment HBsAg level was associated with treatment response in both HBeAg-positive and HBeAg-negative CHB, which was in line with most previous reports (149). A relatively low HBsAg quantification might represent strong immune control (150), resulting in greater rates of virological response and HBsAg clearance in this sub-group of patients. Interestingly, the response rates were noticeably high among individuals who carried favorable TT genotype of rs4646536 and had low baseline HBsAg level. These data suggest that simultaneous evaluation of both host and viral factors at baseline may improve predictive value and could help to maximize cost-effectiveness before initiating PEG-IFN treatment.

This study had some limitations including its retrospective design, which disallowed us to determine the possible effect of the studied SNPs on pre-treatment serum vitamin D concentrations. Nonetheless, it should be mentioned that the interpretation of vitamin D levels in patients with CHB must be taken in the context of multiple variations as potential confounding factors such as individual's age, HBV genotypes, calcium levels and seasonal variability (145). Despite the limitations, our data have suggested that SNP rs4646536 is an independent predictive marker of treatment response to PEG-IFN in Thai patients with CHB, which has confirmed and extended the observations of previous published data. Thus, the determination of

this SNP may serve as a useful genetic marker for assessing an increased likelihood of response to PEG-IFN in clinical practice. Additional studies are, however, warranted to verify this correlation of the *CYP27B1* polymorphism with treatment outcome in other racial-ethnic populations and geographical areas.

Part 3: Genetic variation in *STAT4* is associated with treatment response to pegylated interferon in patients with chronic hepatitis B

It has been well recognized that both viral- and host-related factors influence the natural history and treatment outcome of patients with HBV or HCV infection.(10) In the context of host genetic variation, a previous landmark GWAS identified SNPs in the *interferon lambda-3 (IFNL3)* gene on chromosome 19 (19q13.13) associated with clearance and response to combined PEG-IFN and ribavirin therapy in patients with HCV infection.(151, 152) Regarding HBV infection, a GWAS revealed that SNPs in the *human leucocyte antigen (HLA)* class II genes *(HLA-DP)* were related to spontaneous HBV clearance among Asian populations. Subsequent studies also validated that *HLA-DP* polymorphisms were association with response to PEG-IFN therapy.(128, 153) More recently, a GWAS demonstrated that SNP rs7574865 in the *STAT4* gene was linked to an increased risk of developing HCC in Chinese individuals with CHB.(11) In addition, another Chinese cohort showed that rs7574865 TT genotype predicted HBeAg seroconversion in patients with HBeAg-positive CHB receiving IFN-based therapy.(22)

In this report, we investigated the effect of rs7574865 on treatment outcome to PEG-IFN in Thai population. Our study, recruited subjects with both HBeAg-positive and HBeAg-negative CHB, clearly showed that the frequency of rs7574865 TT genotype was significantly associated with favorable outcome to PEG-IFN. Specifically, subjects harboring TT and non-TT genotypes had the probabilities in achieving VR of approximately 65% and 30%, respectively. Moreover, the corresponding figures for attaining HBsAg clearance were 24% and 5%, respectively. Indeed, HBsAg clearance or 'functional cure' represents a goal of antiviral therapy, which is associated with improved clinical consequences, such as reduced incidence of cirrhosis and HCC. Together, these results might indicate that rs7574865 TT genotype was linked to PEG-IFN responsiveness in Thai individuals and the determination of this SNP might improve cost-effectiveness of the therapy.

STAT4 is a vital transcription factor for T helper1 cell activation and differentiation that could be induced by several cytokines in response to viral infections to regulate inflammation and anti-viral activities.(17) Given that *STAT4* is a crucial signaling of the Janus kinase (JAK)-STAT pathway, through which IFN-alfa is modulated, variations in the *STAT4* gene might represent a good genetic predictor for immune-modulatory agents such as IFN-based therapy. As rs7574865 is in the third intron of the *STAT4* gene, its potential effects might be associated with the alteration of *STAT4* expression. For instance, a recent study demonstrated that the presence of the minor T allele of rs7574865 enhanced *STAT4* mRNA transcription and protein

expression in blood samples of healthy controls and patients with autoimmune disorders.(120, 154) In HBV-related HCC, a quantitative RT-PCR assay confirmed that the expression levels of *STAT4* mRNA were significantly lower in both tumor and adjacent tissues of patients harboring the risk G allele when compared to individuals with the T allele.(11) Another recent study in patents with CHB also showed that the G allele was associated with lower *STAT4* mRNA expression in liver and PBMCs, impaired *STAT4* phosphorylation and a reduction in IFN-gamma secretion following *ex vivo* stimulation.(119) Together, these results indicate that patients with CHB carrying the TT genotype are more likely to enhance STAT4 expression, which might result in a higher likelihood of responsiveness to PEG-IFN therapy.

Currently, baseline predictors of response to PEG-IFN therapy in patients with CHB are not well defined. In this report, the response rates of patients with CHB were not influenced by age, gender, baseline ALT, HBV DNA level and HBV genotype. Instead, rs7574865 and HBsAg level were selected as independent predictors of VR in multivariate analysis. The finding that low baseline HBsAg level was predictive of response was in line with previous data in patients with HBeAg-positive and HBeAg-negative CHB treated with IFN-based therapy.(155-157) In fact, low serum HBsAg quantification might represent strong immune control,(150) and this may in part explain a favorable treatment outcome including VR and HBsAg clearance. Interestingly, the response rates were particularly high among subgroups of patients carrying favorable TT genotype, who also had low baseline HBsAg quantification. For

example, patients harboring TT genotype with baseline HBsAg <3.5 $\log_{10}$ IU/mL had a high chance of achieving VR and HBsAg clearance of approximately 80% and 30%, respectively. Together, such findings provide insight into the virus–host interactions and indicate that combined use of these markers could further improve predictive value to some extent.

Although this is the first study that examines the association between rs7574865 genotypes and PEG-IFN responsiveness in patients with both HBeAg-positive and HBeAg-negative CHB, our report had some limitations as being a retrospective study with a relatively small sample size of patients achieving HBsAg clearance. Nonetheless, our data clearly demonstrated that the SNP was an independent predictor of treatment outcome to PEG-IFN. Thus, the determination of this polymorphism, together with HBsAg quantification, is useful for identification of patients with high probability of response and may help to individualize decision-making before initiating PEG-IFN therapy. Further prospective large-scale analyses are, however, needed to confirm these observations.

Part 4: Correlation of serum HBV RNA quantification with other viral markers in the natural history of chronic HBV infection

Earlier studies demonstrate that serum HBV RNA quantification may serve as a better surrogate marker for cccDNA activity (72, 126) and a useful marker for assessment of treatment efficacy(37, 39, 158). In recent years, various qPCR-based quantitative methods for detection of serum HBV RNA have been developed but the

sensitivity of qPCR is still unsatisfied. In this study, we demonstrated that ddPCR-based amplification system is more sensitive than that of the qPCR and both methods showed a high degree of linearity and quantitative correlation for clinical sample. Interestingly, the ddPCR can be used to detect serum HBV RNA especially in HBeAg-negative patients that yielded negative results by qPCR. Recently, Wang J., et al developed DNA binding dye (EvaGreen)-based ddPCR assay to determine HBV RNA in entecavir-treated patients, LoD of their method was 200 copies/mL (73). Conversely, TaqMan hydrolysis probe-based ddPCR assay was used in our study. The advantage of using hydrolysis probe to detect HBV RNA is not only high specificity but also a high signal to noise ratio. As shown above, the ddPCR could become a useful tool for detecting HBV RNA in samples with low copy numbers, thus facilitating accurate diagnosis of CHB infection.

Ours data show that serum HBV RNA levels varied among phases of CHB infection in untreated patients. The HBV RNA and DNA levels exhibited high degree of correlation in all phases except in phase 3. In fact, HBV-RNA remained undetectable in most of patients (67%) in phase 3 by ddPCR thus, we were not able to perform a reliable analysis in this phase. The correlation coefficient of serum HBV RNA and DNA is higher in HBeAg-negative patients than HBeAg-positive patients ($r = 0.721$ and $r = 0.516$, $P < 0.001$, respectively) which agreed with a previous report (159). Nevertheless, a previous report by van Campenhout et al. show a comparable correlation of full-length polyadenylated HBV RNA (flRNA) to DNA between HBeAg-

positive and HBeAg-negative patients (160). The inconsistency may be owing to the differences in HBV RNA quantitation assays, the number of subjects or patient characteristics.

The correlations between serum HBV RNA and HBsAg were found only in HBeAg-positive patients not in HBeAg-negative patients. This is in line with previous findings which were reported a weak or no correlation between the two markers in HBeAg-negative patients (126, 159, 160). A plausible reason for this event is that in HBeAg-negative patients, HBsAg is mostly derived from an integrated HBV genome rather than the cccDNA (161).

The relationship between serum HBV RNA and intrahepatic cccDNA levels has been controversial (72, 126, 159). Gao Y. et al.(72) and Huang H. et al.(159) reported that serum HBV RNA correlated with cccDNA in HBeAg-positive patients. In our study serum HBV RNA correlated with intrahepatic cccDNA both in HBeAg-positive patients ($r=0.501$, $P<0.05$) and in HBeAg-negative patients ($r=0.339$, $P<0.01$). The different of CHB stages and detection methods for cccDNA and serum HBV RNA might affect to their correlation. For this reason, the development of accurate and standardized systems for quantitation of cccDNA and serum HBV RNA is very necessary. However, serum HBsAg levels correlated with intrahepatic cccDNA only in HBeAg-positive patients ($r=0.455$, $P= P<0.05$) but not in HBeAg-negative patients ($r=0.058$, $P=0. 631$). Thus, it can be concluded that serum HBV RNA might be superiority non-invasive biomarker in reflecting amount of cccDNA in liver than HBSAg.

In summary, our data demonstrated that the ddPCR may enhance the rate of HBV RNA detection from samples with low viral loads, thereby improving diagnosis and management of CHB infected patients. This assay might also be useful in diagnosing other infections especially for samples with low viral loads. Serum HBV RNA levels varied among different phases of chronic HBV infection. Moeover, Compared with HBsAg, serum HBV RNA quantification can be a better surrogate marker reflecting the amount of intrahepatic cccDNA. Finally, these findings suggest that serum HBV RNA quantification represents a useful marker for disease monitoring in patients with chronic HBV infection.

Part 5: Baseline and kinetics of serum HBV RNA predict response to pegylated interferon-based therapy in patients with HBeAg-negative chronic hepatitis B

Sustained off-treatment viral suppression is a well-accepted clinical end point of antiviral therapy in patients with CHB. At present, baseline parameters have not been fully established to predict positive or negative outcomes in patients with HBeAg-negative CHB treated with PEG-IFN (162, 163). In this report, our cohort with longitudinal follow-up demonstrated that low baseline HBV RNA level was associated with response to PEG-IFN-based therapy. These data were in agreement with a recent report of patients with HBeAg-negative CHB treated with PEG-IFN and adefovir (37). At the cut-off 2.0 $\log_{10}$ copies/mL, our results showed that the PPV and NPV of baseline HBV RNA for SR were approximately 80%, which were superior to those of baseline HBsAg levels. Moreover, combined measurement of HBV RNA and HBsAg could

increase the NPV for SR as high as approximately 90%. These data indicate that HBV RNA could identify subgroups of patients with high and low likelihoods of achieving sustained viral suppression before the start of treatment.

A 'functional cure' defined by HBsAg clearance with or without anti-HBs appearance is considered to be the ultimate goal of antiviral therapy and is associated with favorable long-term clinical outcomes of patients with CHB (164). However, this end-point has been achieved in only a minority of patients receiving currently available treatment strategies. In this study, HBsAg clearance rate at 3-year post-treatment was approximately 10%, which was comparable to previously well-documented data of patients treated with PEG-IFN (165). Recent data demonstrated that low baseline HBsAg level was predictive of HBsAg clearance in patients with HBeAg-negative CHB treated with PEG-IFN (157). In this respect, our data further showed that patients achieving HBsAg clearance had significantly lower baseline HBsAg and HBV RNA levels than those without HBsAg clearance. Interestingly, it was demonstrated in this study that high HBV RNA or high HBsAg concentrations at baseline were predictive for identification of patients with low probability of subsequent HBsAg clearance (NPVs of 95% and 98%, respectively). Together, it appears that both markers could allow prediction of functional cure prior to PEG-IFN-based therapy with high NPVs. Further large-scaled studies are, however, required to validate these observations.

Early on-treatment kinetics of established viral markers including HBV DNA and HBsAg quantification have been proposed as predictors for classifying responders and non-responders (166). Indeed, monitoring these parameters would allow the optimization of PEG-IFN-based therapy to reduce unnecessary exposure to its side-effects. Of note, current treatment guidelines recommend using a combination of absence HBsAg decline plus HBV DNA<2 $\log_{10}$IU/mL as a "week-12 stopping rule" for patients infected with HBV genotype D (NPV of 100%) (5). So far, there are no convincing data regarding this stopping rule in other HBV genotypes. For example, NPVs for prediction of non-responders in patients with HBV genotypes B and C varied of 66-100% (124, 167). Thus, additional viral markers are required to optimize therapeutic outcome of PEG-IFN in patients infected with HBV genotype B or C.

In this study, sustained responders had significantly greater decline in HBV RNA levels compared with non-responders. Thus, on-treatment kinetics of HBV RNA could help individualize decision-making to discontinue PEG-IFN-based therapy. Specifically, it was shown in this report that detectable HBV RNA (≥ 2 $\log_{10}$ copies/mL) at week 12 exhibited a high NPV (95%) of achieving SR. For predicting HBsAg clearance, the NPV of detectable HBV RNA at week 12 was as high as 100%. Thus, it is anticipated that measuring HBV RNA alone might be applicable as a novel on-treatment stopping rule (NPV ≥ 95%) in patients with HBV genotypes B or C. Consistent with our observation; a recent study of patients with HBeAg-positive CHB (mostly HBV genotypes B or C) indicated that on-treatment serum HBV RNA levels

were predictive of PEG-IFN response. In that report, HBV RNA levels >5.5 log_{10} copies/mL at week 12 could identify non-responders with an NPV of >90% (158).

Ideally, the measurement of intrahepatic cccDNA, representing the major template of HBV replication, would be an optimal tool for monitoring treatment outcome (168). Nonetheless, its clinical applicability is restricted by the requirement of repeated liver biopsies. As a result, non- invasive surrogate markers of intrahepatic cccDNA are highly needed. Our findings showed that serum HBV-RNA quantification positively correlated with intrahepatic cccDNA levels at baseline. Thus, low or undetectable HBV RNA levels may represent estimating minimal levels of cccDNA in the liver and contribute to greater probability of achieving favorable outcome as demonstrated in this study. In agreement with our report, a positive correlation between serum HBV RNA levels and intrahepatic cccDNA during NA treatment was previously observed (72, 129). These data suggest the clinical application of quantitative HBV RNA as a non-invasive marker of intrahepatic viral replicative intermediates during antiviral treatment. In contrast, there was no correlation between baseline HBsAg and cccDNA levels, reflecting an alternative pathway such as viral integration might be responsible for HBsAg production (169).

Although our report provided a comprehensive description of HBV RNA kinetics in long-term follow-up of patients receiving PEG-IFN, this study had some limitations that need to be mentioned. Firstly, this was a retrospective report in which all patients from our previous randomized trial were not recruited in this study

due to unavailability of serum samples for HBV RNA testing. Secondly, a standard protocol for HBV RNA quantification has not been well established at present, which may influence its detection (125). Thirdly, serum HBV RNA quantification could be influenced by several viral factors including HBV genotypes (160). Thus, it is possible that the results obtained in this report might not be applicable to other HBV genotypes. Additional prospective studies, particularly among ethnically diverse populations and different HBV genotypes, are necessary to confirm these observations.

In summary, our data demonstrated that serum HBV RNA level was positively correlated with intrahepatic cccDNA in patients with HBeAg-negative CHB. The measurement of this marker at baseline was superior to HBsAg quantification in predicting subsequent sustained response to PEG-IFN-based therapy. In addition, on-treatment HBV RNA quantification could serve as a promising stopping rule in patients infected with HBV genotypes B or C. Together, this novel marker has the potential to allow individualized treatment outcome of PEG-IFN-based therapy and might also be applied to other emerging HBV therapies that aim at achieving a 'functional cure'.

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3: Limothai U, Chuaypen N, Khlaiphuengsin A, Posuwan N, Wasitthankasem R, Poovorawan Y, Tangkijvanich P. Association of interferon-gamma inducible protein 10 polymorphism with treatment response to pegylated interferon in HBeAg-positive chronic hepatitis B. Antivir Ther. 2016;21(2):97-106. doi: 10.3851/IMP2992. Epub 2015 Sep 17. PubMed PMID: 26376789.

4: Limothai U, Wasitthankasem R, Poovorawan Y, Tangkijvanich P. Single Nucleotide Polymorphism of Interferon Lambda-4 Gene is not Associated with Treatment Response to Pegylated Interferon in Thai Patients with Chronic Hepatitis B. Asian Pac J Cancer Prev. 2015;16(13):5515-9. PubMed PMID: 26225703.

AWARD RECEIVED

1. 2015 Outstanding Research Award; Ratchadapiseksomphot Endowment Foundation, Chulalongkorn University in the topic of "Associations of HLA-DPA1, IL28B, IFNL4 and IP-10 gene polymorphisms with PEG-interferon-based treatment in Thai patients with chronic hepatitis B"

2. 1st runner up for oral presentation award in the topic of "Correlation of serum HBV RNA quantification with other viral markers in the natural history of chronic HBV infection" in Thai Association for the Study of the Liver (THASL) 13th Annual Conference 2018 (8-10 March 2018) at Dusit Thani Pattaya, Chonburi, Thailand