การศึกษาตัวบ่งชี้ทางชีวภาพของไวรัสตับอักเสบบีในชิ้นเนื้อตับและในเลือดของผู้ป่วย เพื่อใช้
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นางสาวณัฐธยาน์ ช่วยเพ็ญ

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INTRAHEPATIC AND SERUM MARKERS OF HEPATITIS B VIRUS AS
PREDICTORS OF RESPONSE TO PEGYLATED INTERFERON THERAPY

Miss Natthaya Chuaypen

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A Dissertation Submitted in Partial Fulfillment of the Requirements
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เลือดของผู้ป่วย เพื่อใช้ทำนายผลการรักษาด้วยยาต้านไวรัสเพคอินเตอร์เฟียรอน
(INTRAHEPATIC AND SERUM MARKERS OF HEPATITIS B VIRUS AS
PREDICTORS OF RESPONSE TO PEGYLATED INTERFERON THERAPY) อ.
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สัญชัย พยุงภร, 116 หน้า.

ปัจจุบันตัวบ่งชี้ทางชีวภาพที่ใช้ทำนายผลการตอบสนองต่อยาต้านไวรัสเพคอินเตอร์เฟียรอน
ในผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรังยังไม่มีประสิทธิภาพที่ดี ดังนั้นงานวิจัยนี้จึงได้ศึกษาบทบาทของตัว
บ่งชี้ของไวรัสในเนื้อเยื่อตับและในเลือดเพื่อใช้ในการทำนายผลการรักษาของผู้ป่วยกลุ่ม HBeAg-
positive (กลุ่มที่1) และกลุ่ม HBeAg-negative (กลุ่มที่2) ที่ได้รับการรักษาด้วยเพคอินเตอร์เฟียรอน
เป็นเวลานาน 48 สัปดาห์และติดตามผลการรักษาอีกอย่างน้อย 24 สัปดาห์ ตัวบ่งชี้ในเนื้อเยื่อตับ
ประกอบด้วยการตรวจวัดปริมาณ cccDNA และการแสดงออกของแอนติเจนของไวรัสได้แก่ HBsAg
และ HBcAg ทั้งก่อนและหลังการรักษา ส่วนตัวบ่งชี้ในเลือดที่ศึกษาได้แก่การตรวจวัดการเปลี่ยนแปลง
ของระดับ HBsAg และ HBcrAg อย่างต่อเนื่องระหว่างการรักษา นอกจากนี้ยังได้ศึกษารูปแบบการ
กลายพันธุ์ของไวรัสบริเวณยีน EnhII/BCP/PC และ Pre-S/S ในตัวอย่างเลือดก่อนรักษาด้วยวิธี Sanger
sequencing และ Next Generation Sequencing (NGS) เพื่อหาความสัมพันธ์กับการตอบสนองต่อ
การรักษา ผลการศึกษาพบว่าผู้ป่วยกลุ่มที่ 1 มีระดับตัวบ่งชี้ของไวรัสในเลือดและในเนื้อเยื่อตับก่อนการ
รักษาสูงกว่ากลุ่มที่ 2 อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบว่าระดับของ HBcrAg ในเลือดก่อนการ
รักษาสัมพันธ์กับปริมาณ cccDNA ในเนื้อเยื่อตับในผู้ป่วยทั้งสองกลุ่ม แต่ระดับของ HBsAg ในเลือด
สัมพันธ์กับปริมาณของ cccDNA เฉพาะในผู้ป่วยกลุ่มที่ 1 เท่านั้น เมื่อศึกษาการเปลี่ยนแปลงในระหว่าง
การรักษาพบว่าการลดลงของระดับ HBcrAg และ HBsAg ในเลือดสัมพันธ์กับการลดลงของ cccDNA
อย่างมีนัยสำคัญทางสถิติ ผู้ป่วยที่ตอบสนองต่อการรักษา มีการลดลงของ HBcrAg และ HBsAg ใน
เลือดมากกว่ากลุ่มที่ไม่ตอบสนอง ผู้ป่วยกลุ่มที่ 1 ที่ติดเชื้อไวรัสที่มีการกลายพันธุ์บริเวณยีน
EnhII/BCP/PC มีการตอบสนองต่อการรักษาต่ำกว่าผู้ป่วยที่ติดเชื้อไวรัสที่ไม่มีการกลายพันธุ์ นอกจากนี้
ยังพบว่าการลดลงของการแสดงออกของ HBsAg ในเนื้อเยื่อตับสัมพันธ์กับผลการรักษา จากงานวิจัยนี้
สรุปได้ว่าระดับ HBcrAg ในเลือดก่อนการรักษาเป็นตัวบ่งชี้ปริมาณ cccDNA ในเนื้อเยื่อตับที่ดีกว่า
ระดับ HBsAg อย่างไรก็ตามการตรวจการเปลี่ยนแปลงของระดับ HBcrAg และ HBsAg ในระหว่าง
การรักษาจะช่วยในการทำนายผลการตอบสนองต่อการรักษา นอกจากนี้ยังพบว่าการตรวจด้วยเทคนิค
NGS สามารถให้ข้อมูลการกลายพันธุ์ของไวรัสที่สัมพันธ์กับผลการรักษาได้ละเอียดกว่าวิธีมาตรฐาน

ภาควิชา ชีวเคมี	ลายมือชื่อนิสิต	....
สาขาวิชา ชีวเคมีทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	....
ปีการศึกษา 2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม	....

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KEYWORDS: HEPATITIS B VIRUS, CCCDNA, HBSAG, HBCRAG, PEGYLATED-INTERFERON, TREATMENT RESPONSE / BIOMARKERS

NATTHAYA CHUAYPEN: INTRAHEPATIC AND SERUM MARKERS OF HEPATITIS B VIRUS AS PREDICTORS OF RESPONSE TO PEGYLATED INTERFERON THERAPY. ADVISOR: PROF. PISIT TANGKIJVANICH, M.D., CO-ADVISOR: ASST. PROF. SUNCHAI PAYUNGPORN, Ph.D., 116 pp.

Effective predictors of treatment response to pegylated interferon (PEG-IFN) in patients with chronic hepatitis B (CHB) are currently limited. This research was aimed at studying the predictive roles of intrahepatic and serum markers of hepatitis B virus (HBV) in patients with HBeAg-positive (group 1) and HBeAg-negative CHB (group 2) receiving standard course of 48-week PEG-IFN therapy and followed up for at least 24 weeks. Intrahepatic cccDNA and intrahepatic viral antigens, including HBsAg and HBcAg were assessed in paired pre- and post-treatment liver specimens. Kinetics of quantitative serum markers, including HBsAg and HBcrAg in response to therapy was also examined. In addition, pre-existing HBV mutations in the EnhII/BCP/PC and Pre-S/S regions at baseline were determined by Sanger sequencing and Next Generation Sequencing (NGS). The results showed that group 1 had significantly higher baseline intrahepatic and serum markers compared with group 2. Baseline quantitative HBcrAg correlated with cccDNA levels in both groups. In contrast, quantitative HBsAg correlated with cccDNA levels only in group 1 but not in group 2. However, changes in HBsAg and HBcrAg levels during therapy were correlated with the reduction of cccDNA in both groups. Generally, responders had more rapid decline of both serum markers during therapy compared with non-responders. In group 1, patients infected with HBV mutants in the EnhII/BCP/PC region had significantly lower response rates compared with those infected with wild-type strains. The decline in the expression of intrahepatic HBsAg, but not HBcAg, was associated with treatment response. In conclusion, serum HBcrAg represented a better surrogate marker of intrahepatic cccDNA compared with serum HBsAg. Monitoring both serum markers during PEG-IFN therapy may help identify patients with high and low probability of achieving response. NGS could accurately identify pre-treatment viral mutants that might be associated with treatment outcome.

Department: Biochemistry   Student's Signature ⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽

Field of Study: Medical Biochemistry   Advisor's Signature ⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽

Academic Year: 2015   Co-Advisor's Signature ⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽⎽

ACKNOWLEDGEMENTS

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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CHAPTER I

INTRODUCTION

1. BACKGROUND AND RATIONALE

Chronic infection with hepatitis B virus (HBV) is a global health problem and responsible for the majority of patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). In general, the therapeutic outcome for HBV mainly depends on both host and viral factors. The current antiviral agents for chronic hepatitis B (CHB) include nucleoside/nucleotide analogues (NA) and pegylated interferon (PEG-IFN) (2). PEG-IFN therapy results in a higher proportion of sustained off-treatment response than NA, but the overall response rates are still low with approximately 20-40% of patients could achieve favorable outcome (3). Moreover, PEG-IFN therapy may have some potential side-effects that lead to an early termination of therapy (4). Thus, it is necessary in clinical practice to appropriately select patients who would have the highest probability of achieving treatment response.

HBV, a member of the *hepadnaviridae* family, is a partially double-stranded DNA virus that replicates via covalently closed circular DNA (cccDNA) formation. Intrahepatic cccDNA, organized as a mini-chromosome in the nucleus of the hepatocytes, functions as the main template for the production of all messenger RNAs (mRNAs), including pre-genomic RNA (pgRNA) that is reverse transcribed for the synthesis of HBV genome (5). Increasing data have demonstrated that cccDNA is the major barrier for HBV eradication and is responsible for the chronicity of infection (6). Recent evidence has indicated that interferon (IFN) could accelerate cccDNA degradation and decrease its transcription (7, 8). Given that cccDNA is essential for

HBV replication, direct measurement of intrahepatic cccDNA levels could represent a strong predictor of response to antiviral therapy (9). However, the clinical application of quantitative cccDNA is restricted by the necessity of liver biopsy, which is an invasive procedure. Moreover, currently there is no commercially available assay for cccDNA quantification. As a consequence, identification and validation of non-invasive serum markers for cccDNA is highly needed.

Recently, serum hepatitis B surface antigen (HBsAg) quantification has been used as a potential marker of intrahepatic cccDNA, particularly in hepatitis B e antigen (HBeAg)-positive CHB (10). As a result, several studies have validated the clinical utility of this serum maker in patients receiving PEG-IFN (10). It is shown that serum HBsAg decline during therapy parallels the reduction of intrahepatic cccDNA (11, 12). In addition, PEG-IFN therapy could significantly decrease the intrahepatic HBsAg together with the decline of serum HBsAg levels (12). Thus, serial monitoring of HBsAg levels may help predict treatment outcome during PEG-IFN therapy in patients with HBeAg-positive CHB, although its utility in HBeAg-negative CHB remains unclear (9, 13).

More recently, hepatitis B core-related antigen (HBcrAg) has emerged as a novel serum marker of HBV. HBcrAg represents the combination of hepatitis B core antigen (HBcAg), HBeAg and p22 proteins that share common epitopes and could be simultaneously measured by enzyme immunoassay. Serum HBcrAg levels are strongly correlated with intrahepatic cccDNA (14, 15). In addition, HBcrAg level could be used to discriminate the different phases of chronic HBV infection and might also play an important role in predicting the occurrence of HCC (16-20). Among patients treated with NA, HBcrAg levels are associated with treatment response and could be a

predictive marker of viral relapse after stopping NA (21, 22). However, data on its applicability in monitoring patients receiving PEG-IFN therapy are rather limited.

HBV has heterogeneity leading to quasispecies with high mutation rates due to the lack of proofreading activity of polymerase during its replication. (23). The common mutations include G to A mutation in the pre-core (PC) region (G1896A) and double mutations in basal core promoter (BCP) (A1762T and G1764A), which lead to impair HBeAg production (24-27). Furthermore, mutations/deletions in the pre-S/S region could cause HBsAg reduction (28, 29). Previous study reported that the BCP double mutations were associated with treatment outcome of PEG-IFN (30), suggesting that certain HBV mutations at baseline might affect therapeutic outcome. Despite these findings, several minor mutations of less than 20% prevalence are usually undetected by conventional gene sequencing methods (31).

Next-Generation Sequencing (NGS) has recently overcome this technical difficulty and established reliable quantitative assays to verify all minor mutations. For instance, NGS studies have showed that the distribution of substitution frequencies in quasispecies, such as the pattern of Pre-S, X, and core regions deletion, have different patterns among several phases of CHB and certain novel substitutions are associated with disease progression (32). Among patients undergoing NA therapy, it has been shown that responders have higher pretreatment genetic complexity and diversity in the polymerase region than non-responders, indicating that HBV heterogeneity could be used as a predictor for treatment outcome (33). However, data on the characterization of full-length genomes of HBV in sera of patients with CHB treated with PEG-IFN are restricted.

This study was aimed at investigating clinical applicability of intrahepatic and serum viral markers in patients with HBeAg-positive and HBeAg-negative CHB receiving PEG-IFN therapy. We also characterized HBV genome at baseline in sera of patients treated with PEG-IFN by NGS technology.

1.1 Research questions

1.1.1 Do intrahepatic and serum markers represent good predictors of treatment

response to PEG-IFN?

1.1.2 Are certain mutations of HBV genome at baseline associated with

treatment response to PEG-IFN?

1.2 Objectives

1.2.1 To investigate the correlation between intrahepatic and serum markers at

baseline and during PEG-IFN treatment.

1.2.2 To investigate whether intrahepatic and serum markers of HBV could

predict treatment response to PEG-IFN.

1.2.3 To investigate the relation between baseline mutational patterns of HBV

genome detected by NGS and PEG-IFN treatment response

1.3 Hypothesis

1.3.1 Serum markers correlate with intrahepatic replication markers in response

to PEG-IFN treatment.

1.3.2 Kinetics of intrahepatic and serum markers are different between

responders and non-responders in patients receiving PEG-IFN therapy,

which could be used as predictors of treatment response.

1.3.3 The heterogeneity of HBV genome is associated with treatment response.

1.4 Keywords

- Hepatitis B virus, cccDNA, HBsAg, HBcrAg, pegylated interferon, treatment

response, mutations, biomarkers

1.5 Conceptual framework
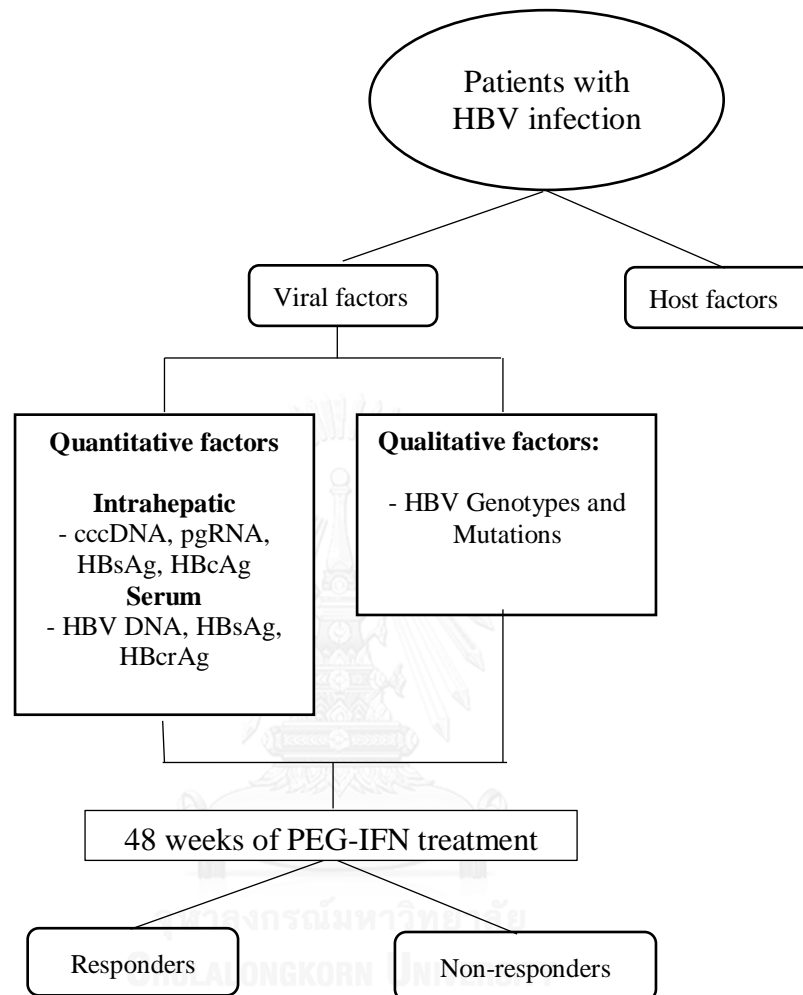
Figure 1. Conceptual framework

1.6 Research workflow
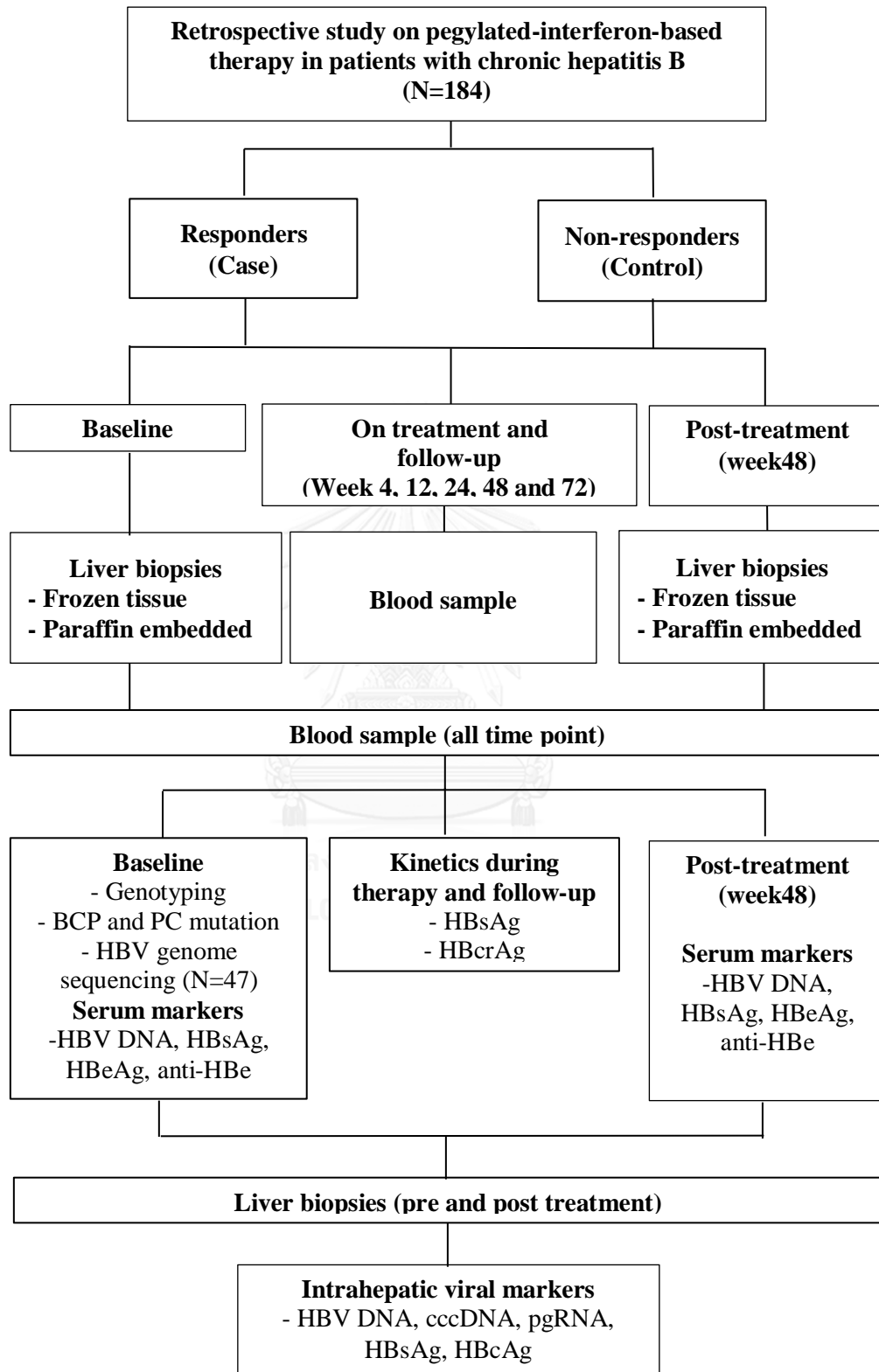
Figure 2. Research workflow

1.7 Expected benefit and application

1. Identify intrahepatic replication and serum markers as markers for predicting treatment response to PEG-IFN in patients with CHB.

2. Identify the heterogeneity of HBV genome in relation to PEG-IFN treatment response.

3. This research would provide decision making in selecting patients who might have high probability of treatment response.

4. Data from this study would be published as original articles in international peer-reviewed journals (Q1 and Q2) with high impact factor base on the ISI Web of Science (SCI).

1.8 Limitations

This study focused only on the roles of intrahepatic and serum viral markers in predicting treatment response to PEG-IFN. However, other factors, including host and environmental-related factors, might be in part contributed to treatment outcome. There were some limitations in the assessment of intrahepatic cccDNA and the expression of viral antigens, which required repeated liver biopsies. Thus, pre- and post-treatment liver tissue specimens were not available in all patients enrolled.

1.9 Ethical considerations

This study were approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University follow by the Belmont Report identifies three fundamental ethical principles for any human subject research:

1. Respect for persons: Written informed consent was obtained from the participants prior to subjects entering 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 hurting, 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 of this proposal.
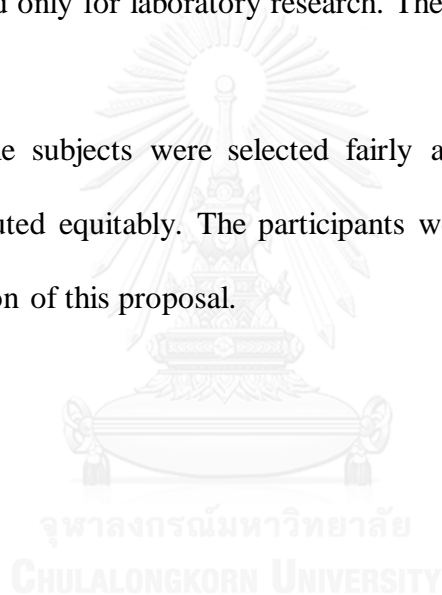
CHAPTER II

LITERATURE REVIEW

2. HEPATITIS B VIRUS

2.1 Structure of hepatitis B virus

Hepatitis B virus (HBV) is a major etiologic cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). HBV was firstly discovered in an Australian aborigine male in 1965 by Professor Baruch Blumberg and colleagues (34). HBV is an enveloped virus with a diameter of 42 nm and about 3.2 kb of double-stranded DNA genome, which belongs to the hepadnaviridae family. The minus strand is an incomplete closed circle and has a gap near the 5´ end of the plus strand. The polymerase is bound to the 5´ end of minus strand. The variable of gap region around 20-80% can be filled in by endogenous viral polymerase (23). The HBV genome contains four overlapping open reading frames (ORFs) including S, C, P and X.

The Pre-S/S gene consists of Pre-S1, Pre-S2 (nucleotide at position 2848 to 155) and S regions (nucleotide at position 155 to 835), which encode three surface antigens that are translated into small (S), medium (M) and large (L) surface proteins. The viral polymerase/reverse transcriptase (RT) region is encoded by the P genes (nucleotide at position 2307 to 1620). HBeAg and HBcAg are encoded by the precore and core genes (Pre-C/C; nucleotide at position 1814 to 2450). The enhancer II (EnhII; nucleotide at position 1685 to 1773) and basal core promoter (BCP; nucleotide at position 1742 to 1849) regions are overlapped with the X gene, which is translated into a multifunctional nonstructural protein called X protein (Figure 3) (35).
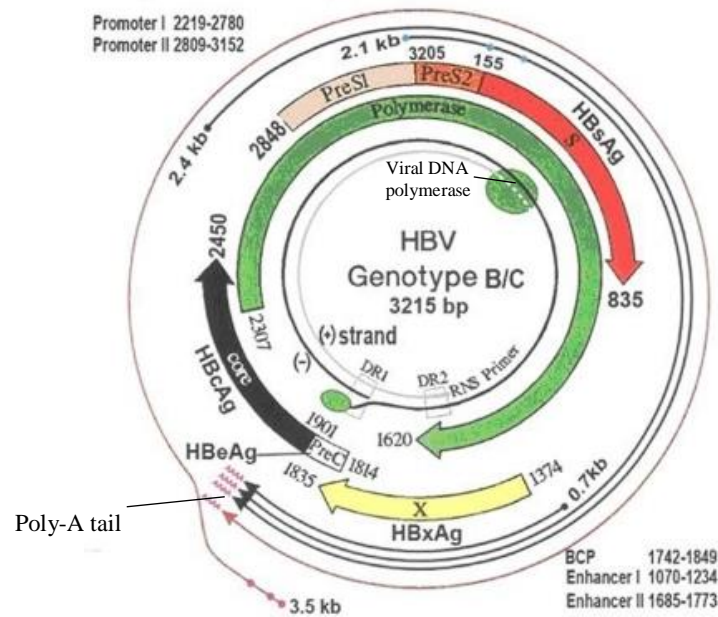
Figure 3. Structure of hepatitis B virus genome (36),
DR1: direct repeat 1, DR2: direct repeat 2

2.2 Hepatitis B virus replication

After binding to its receptor (sodium taurocholate cotransporting polypeptide; NTCP or SLC10A1), HBV enters into the hepatocytes. The viral DNA is then transferred to the nucleus and the relaxed circular DNA (rcDNA) is converted to a covalently closed circular DNA (cccDNA). Being organized in the form of mini-chromosome, cccDNA acts as the template for viral replication, which is subsequently transcribed into pregenomic RNA (pgRNA) and several messenger RNAs for viral protein synthesis, including surface (S), core (C) and X proteins. Following this process, pgRNA is then encapsidated in the cytoplasm together with HBV DNA polymerase and is reverse transcribed for the synthesis of the negatively stranded genomic DNA. A positive DNA 12 strand is subsequently produced by the polymerase, using the negative-strand as the template (37). Finally, newly generated nucleocapsids are encapsulated by HBsAg at the endoplasmic reticulum to form mature virions and

are released out of the cells by exocytosis. In addition, newly generated nucleocapsids can be recycled into the nucleus to maintain the pool of cccDNA. In addition to the formation of complete virions, subviral particles including HBsAg and HBeAg can be generated and can be found in the serum during active phases of the infection (35).
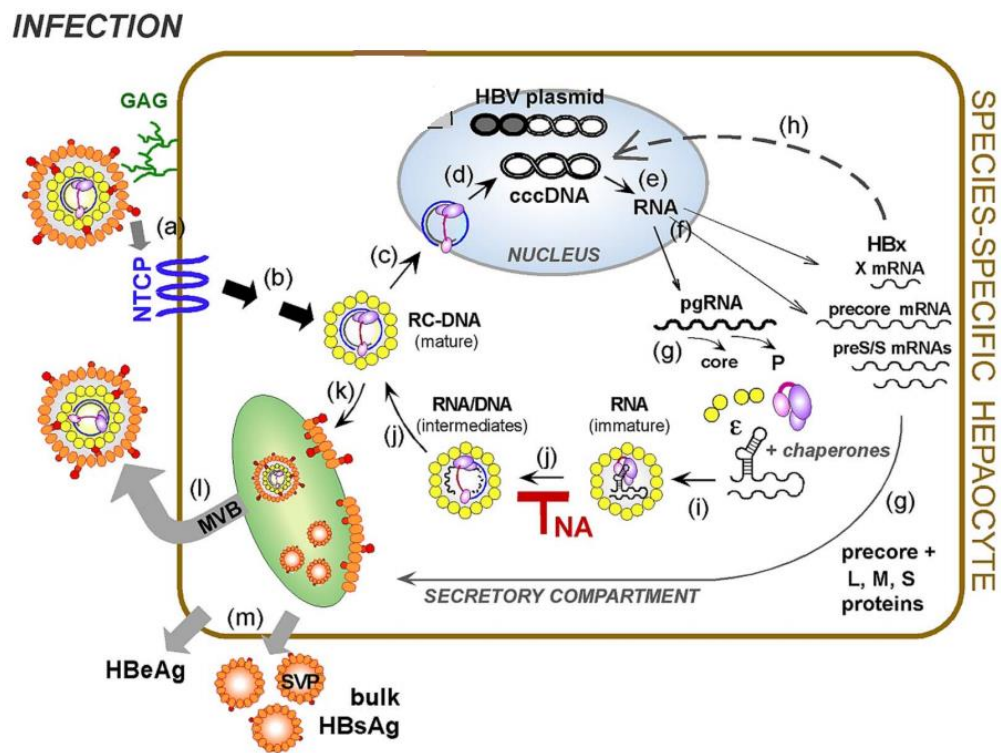
Figure 4. Mechanism of the HBV infections cycle (35).

(A) Attachment and entry. (B) Release of the nucleocapsid into the cytoplasm. (C) Nucleocapsid-mediated nuclear transport and release of P-linked relaxed circular (RC)-DNA at the nuclear pore into the nucleus. (D) RC-DNA to cccDNA conversion. (E) Transcription of viral RNAs. (F) RNA nuclear export. (G) Translation. (H) Prevention of cccDNA transcriptional silencing by HBV X protein (HBx). (I) Co-packaging of P and pgRNA into newly forming nucleocapsids. (J) First-strand DNA synthesis (inhibited by NAs), pgRNA degradation and second strand DNA synthesis leading to new RC-DNA. (K) Envelopment of mature RC-DNA containing nucleocapsids. (L) Secretion of new virions assisted by components of the multivesicular body (MVB) machinery. (M) Secretion of HBeAg and excess sub viral particles (SVP) constituting the bulk of HBsAg. GAG, glycosaminoglycans; L, large; M, middle; NTCP, sodium taurocholate cotransporting polypeptide; S, small.

2.3 Genotype of Hepatitis B virus

Currently, HBV has been classified into 10 genotypes, which are designated as A to J based on genomic sequence diversity of greater than 8%. The distributions of these 13 genotypes vary depended on geographical regions of the world (Figure 5). Genotype A is commonly found in north of Europe, north of America and south of Africa. Genotype B and C are frequently found in East Asia and South East Asia, while Genotype D has predominant at the Mediterranean area. Genotype E is restricted in Africa, while genotype F is predominant in South America and genotype H is mostly found in the central of America (38). In Thailand, HBV genotypes C and B are the most common genotypes, accounting for approximately 75-80% and 20%, respectively (39).
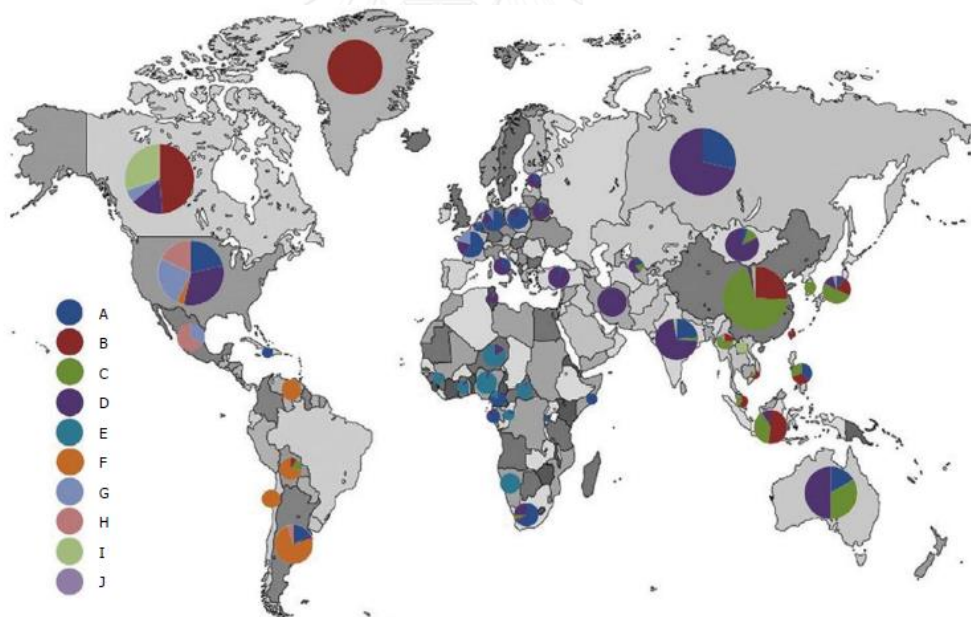
Figure 5. Geographic distribution of hepatitis B virus genotype (40).

2.4 Hepatitis B Virus common mutations and clinical relevance

HBV has heterogeneity leading to quasispecies with high mutation rates due to the lack of proofreading activity of polymerase during viral replication The mutation

rates are approximately $1.4\text{-}3.2 \times 10\text{-}5$ nucleotide substitutions per site per year, which are around 100-fold higher than other DNA viruses (41).

In fact, the rate and magnitude of virus replication are also essential for viral mutations. The high viral loads and turnover 14 rates of replication could influence the complexity of quasispecies. The mutant strains can become dominant overtime if they have replication efficiency and may responsible for viral persistence due to their capacity in escaping from host immune. The well-known naturally occurring mutants are within the precore (PC), basal core promotor (BCP) regions and mutations/deletions in the pre-S/S region.

2.4.1 Mutations in the EnhII/BCP/PC region

The mutations in the precore/core region (between nt1814 and 2450) are clinically significant because they could inhibit HBeAg synthesis and increase viral activity. The most common variant is single base substitution at nt 1896 (G to A; G1896A), which is stop codon at codon 28 (TGG to TAG) in the PC gene. In addition, the double mutations in the BCP gene (A1762T/G1764A) could lead to decrease in production of HBeAg and increase the activity of viral replication (9) (Figure 6). These mutations are strongly association with HBV genotypes, and have been found in up to 50-80% of patients with HBeAg-negative CHB (42, 43). Both double BCP and PC mutations are found commonly in patients with HBV reactivation in immunosuppressed conditions (44, 45). Early detection of BCP and/or PC mutant populations in HBV quasispecies could be a potential predictor of HBeAg seroconversion to anti-HBe as well as the evolution of the disease (46, 47).

Moreover, it has been shown that, based on pyrosequencing, high percentage of BCP mutation (>45%) is associated with higher risk for developing cirrhosis than those

with a lower mutant percentage (48). Certain PC variants, such as G1899A, frequently found in the PC region, is associated with HCC progression in HBeAgpositive CHB, while G1896A might play a similar role in HBeAg-negative CHB (49). In addition, other mutations including T1753C/A/G in the BCP region and C1653T located in the enhancer II region (EnhII) have become recognized as being associated with the prognosis of chronic HBV infection, including HCC development (Figure 6) (50).
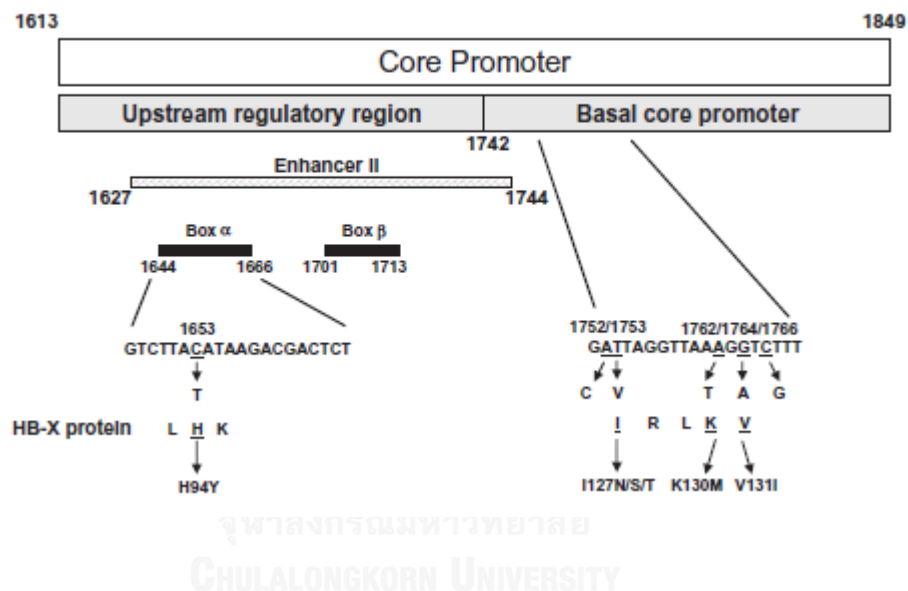
Figure 6. The variation of enhancer II and basal core promoter regions (51). Double mutation at nucleotide 1762 and 1764 in BCP region disturbing codons 130 and 131 of the HBx gene (K130M,V131I).

2.4.2 Mutations in the X gene

HBx is a non-structural protein with multifunctional activity that acts as signal transducer and regulates transcription, cell cycle checkpoint controls, cell proliferation and apoptosis (52). HBx can activate many host genes leading to genetic instability (53). Variation in X gene could play an important role in viral replication and has also been implicated in hepatocarcinogenesis (53). Some point mutations have been demonstrated to affect the functions of HBx and HCC development as well. HBx with K130M/V131I point mutation has been shown to induce hypoxia-inducible factor 1α, a cytokine that play a role in the development of HCC under hypoxic environment conditions. A novel HBx associated mutation at amino acids L30F/S144A was recorded in 13 out of 44 HCC tissue samples (54).

2.4.3 Mutations in the P gene

The P gene encodes the DNA polymerase and partially overlaps the core, S and X genes. It plays an important role as DNA primer, reverse transcriptase, and RNaseH 16 activity. Long-term treatment with NA has been found to be associated with mutations in the P gene. For example, antiviral resistance to lamivudine has been emerged in the Y (tyrosine) M (methionine) D (aspartic acid) D (aspartic acid) locus (YMDD) in the domain C, which is the center of polymerase enzyme activity. In addition, resistance to adefovir and telbivudine is associated with mutations located outside the YMDD motif (55, 56).

2.4.4 Mutations is Pre-S/S region

Mutations in the Pre-S/S region are common found in the natural selection, as well as therapeutic-induced phenomenon. The Pre-S region shows high degree of HBV heterogeneity with several variants detected including

point mutations, deletions or insertions (Figure 7) (28). In addition, HBV vaccination or HBV immuoglobulin (HBIg) can cause amino acid substitution within the major hydrophilic region, located from residue 99 to 170 ('a' determinant) of the S gene, particularly, glycine to arginine at position 145 (sG145R) or aspartate to alanine at residue 144 (sD144A). In addition, the common mutations in the Pre-S/S gene comprise of point mutation in the start codon of Pre-S1 and Pre-S2 leading to inhibit M protein expression or alter the epitope of B and T cells (57). Other mutations is deletion in the Pre-S1 and Pre-S2 region, these mutations have been occur in relation to immune escape, acute and chronic phase, severity of liver diseases and virus expression (29, 58-60). Additionally, the accumulation of mutated envelope protein in endoplasmic reticulum (ER) of hepatocytes could lead to ER stress, genomic instability, oxidative DNA damage, liver damage and HCC development (61-63). The majority of HBV mutations with their clinical association have been summarized in Table 1.
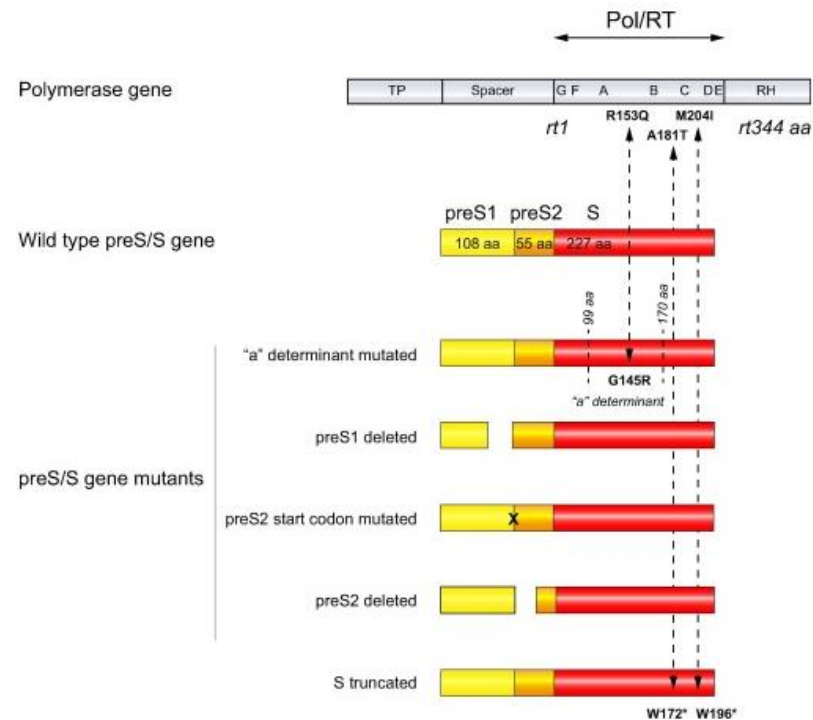
Figure 7. The overlap between polymerase and the envelope open reading frames, types and mutations of PreS/S region (28).

Table 1. The majority of hepatitis B virus mutations and clinical importance

HBV genomic region	Mutations	Clinical association	Ref.
Pre-core	G1896A	HBeAg negative mutants	(9)
BCP and enhancer II region	C1653T	Rapid disease progression	(45, 48, 52)
	A1753T	Rapid disease progression	
	A1762T/G1764A	HBeAg negative mutants,	
		Rapid disease progression and HCC	
X	K130M/V131I	HCC development	(54)
Pre-S	Deletions	HCC development	(28, 29, 57)
S	'*a*' determinant	Vaccine escape mutants	
Polymerase	in domains B,C and D	Resistance to nucleos(t)ide analogues	

2.5 Phases of Hepatitis B Virus infection

On the basis of viral and host interactions, the natural history of patients with chronic HBV infection can be divided into four phases, including immune tolerant phase, immune active phase (HBeAg-positive CHB), inactive carrier phase, and reactivation phase (HBeAg-negative CHB), as shown in figure 8 (2, 64, 65).

The immune tolerant phase is defined by HBeAg-positive, high serum HBV DNA with normal alanine aminotransferase (ALT) levels, and minimal histological activity/ fibrosis. After this phase, immune system attacks on the infected hepatocytes, resulting in the immune clearance phase, which is expressed by fluctuating HBV DNA levels (>20,000 IU/mL), increased level of ALT and active liver histology. In the third inactive phase that occurs after seroconversion of HBeAg to anti-HBe or HBeAg-negative, there are low levels of HBV DNA (2,000 IU/mL), moderate or severe necroinflammation with variable fibrosis. This phase represents the occurrence of PC or BCP mutations, which are unable to express HBeAg.

Serum HBsAg levels are different through these phases of chronic HBV infection. In fact, the immune tolerant phase shows the highest level of serum HBsAg. A decline in HBsAg level is observed in the immune clearance phase, with the lowest levels found in patients achieving HBeAg seroconversion (66). It has been estimated that approximately 15 to 40% of people who develop chronic HBV infection are expected to progress to cirrhosis and HCC (67).
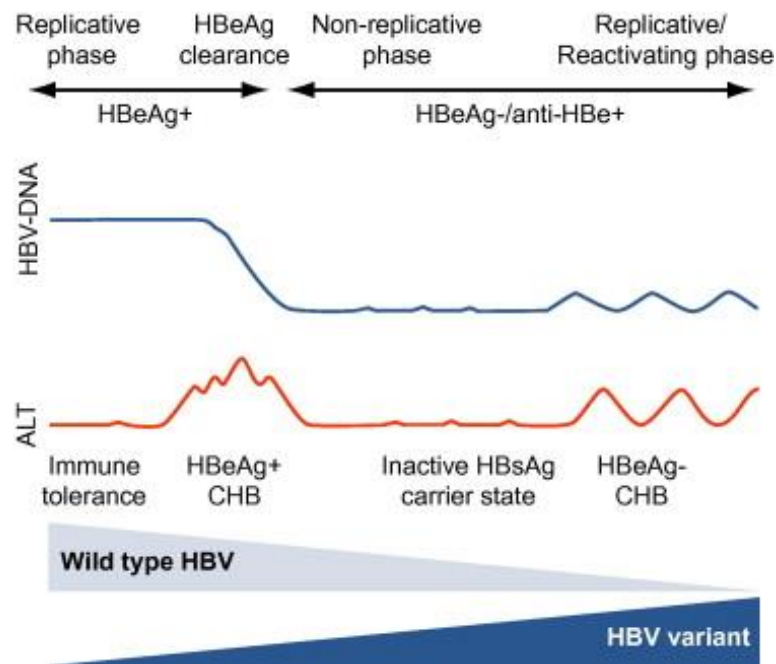
Figure 8. Natural history of HBV infection (68).

2.6 Treatment of chronic hepatitis B

The currently approved antiviral drugs for treatment of CHB are pegylated interferon alfa (PEG-IFN) and nucleoside/nucleotide analogues (NA) (69). PEG-IFN is effective with a definite course of treatment (12 months) and, unlike NA, has not been associated with drug resistance. In contrast, NA are highly potent inhibitors of HBV with low toxicity and side effects. However, even the most potent NA such as entecavir and tenofovir, long-term administration is required to maintain viral suppression because HBV relapse occurs often after NA cessation (70, 71). The goals of therapy are to maintain viral suppression, prevent disease progression, and improve clinical outcome. With current therapy, however, complete eradication of HBV cannot be achieved in most cases due to the persistence of cccDNA in the hepatocytes.

2.7 The mechanism and response rates of PEG-IFN therapy

PEG-IFN is an immunomodulatory agent that enhances the cell-mediated immune response in the process of clearing the virus. Its effect is related to Type I IFN that binds to the receptors (IFNα receptors) in the hepatocytes, and then activates Janus family of tyrosine kinases (JAK) and non-receptor tyrosine-protein kinase (TYK2) enzymes. This activation cause the phosphorylation of the signal transducer and activator of transcription (STAT) family proteins, which bind with IRF-9 to form IFN-stimulated gene factor 3 (or ISGF-3) complex. The signals then translocate into the nucleus to activate the transcription of IFN-stimulated genes and several antiviral proteins that have antiviral effects (70).

Another mechanism of IFN is the stimulation histone deacetylation and DNA methylation, causing cccDNA-bound chromatin to become "closed" chromatin configuration, which results in reduction of pgRNA transcription, HBsAg synthesis, and HBV replication.

In patients with HBeAg-positive CHB, treated with PEG-IFN could result in 30- 40% of HBeAg seroconversion. This event, which is usually defined as virological response (VR), is associated with decline in HBV DNA levels (71, 72). It is also related to the reduction of mortality rates and decreased HCC development (65, 73, 74). In patients with HBeAg-negative CHB, treatment response to PEG-IFN (20-30%) is defined by low levels of HBV DNA. However, HBV relapse can be occurred after 6-12 months post treatment. As PEG-IFN therapy is associated with low rates of VR, it is necessary to identify pre-treatment and on-treatment parameters for predicting response and non-response in patients treated with this agent.

2.8 Next Generation Sequencing

NGS technology is a massively parallel sequencing, which provides a number of sequence data in short time and generates a large volume of gene expression from genome analysis, identification of somatic copy number variation (CNVs), somatic single nucleotide variants (SNVs), fusion gene transcripts, and epigenetic modification. Recently, development of NGS could apply in clinical laboratory testing, include detection of germline variation in inherited disease, somatic variation in cancers, identification of drug-resistant and minor mutation in infection disease, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and HBV, which is associated with pathogenesis of disease, immune escape or resistance to antiviral therapies (75). Moreover, this technique can be used to identify new biomarkers for outcome of various diseases, a recurrence of disease, and treatment response.

Recently, a number of new NGS platforms have been released. For example, the 454 pyrosequencing method is a sequencing-by-synthesis method that includes a mixture of both emulsion PCR and pyrosequencing that relies on light generation after nucleotides are incorporated in a growing chain of DNA. The advantage of this approach is no requirement of gels, fluorescent dyes, or ddNTPs. Another platform is the Applied Biosystems Sequencing by Oligonucleotide Ligation and Detection (SOLiD) system, which is based on sequencing-by-ligation technology. In the SOLiD approach, sequencing is obtained by measuring serial ligation of an oligonucleotide to the sequencing primer by a DNA ligase enzyme (76). Up until now, the most common system is the Illumina Genome Analyzer MiSeq that utilizes fluorescent-labeled reversible-terminator nucleotides, sequencing primer and DNA polymerase. Fluorescent signal is recorded after incorporation by a four-channel fluorescent scanner

(77). Cheval et al. has reported that the Illumina platform could provide the higher number of reads and better detection of the viruses per run than the detection of Roche-454 pyrosequencing (78). In this study, the Illumina platform NGS was selected to perform ultra-deep pyrosequencing in order to detect the whole HBV quasispecies. The amplification of DNA sequencing by the Illumina Genome Analyzer as shown in figure 9, first step is ligation of Illumina adaptor to the ends of DNA fragments. The second step, the double strand DNA will be denatured into single strand DNA (ssDNA), ssDNA contain Illumina-specific adapter will bind to the specific oligonucleotides amplification primers on the surface of a glass flow cell, and is performed by an automatic instrument called a Cluster Station. ssDNA amplified by DNA polymerase as bridge amplification to generate clusters of DNA, Each cluster provide around one million copies of the original DNA fragment, which is appropriate for sequencing process. DNA sequencing by-synthesis use modified dNTPs containing a reversible terminator fluorescent and the 3-OH group, which blocks further incorporation so only a single base can be incorporated by a DNA polymerase to yield a DNA copy strand. The florescent can be detected, by Illumina Genome Analyzer. The fluorescent imaging step, which includes only a single fluorescent color (from four dNTPs), is used in each cycle and imaging. Following the addition of the four dNTPs to the templates, the images are recorded then the unincorporated nucleotide and terminators are removed to allow incorporation of the next base. This series of steps continues for 150-250 cycles, generate hundreds of clusters are sequenced in a massively parallel process and provide high throughput data. The enormous amount of raw data requiring a high quality bioinformatics software to analyses the information (79).
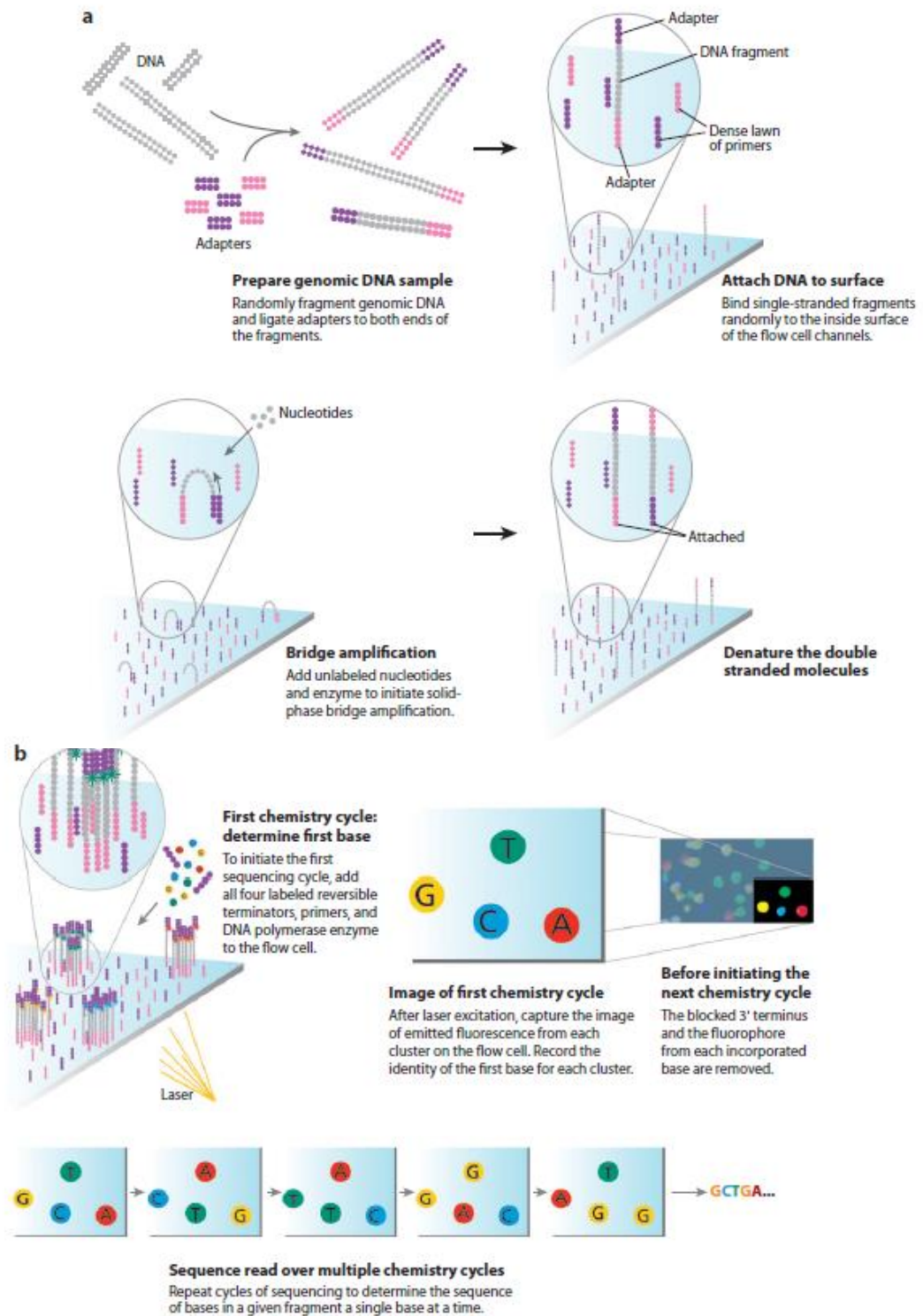
Figure 9. Overviews of NGS based on Illumina Genome Analyzer (79).

2.9 Hepatitis B whole genome and quasispecies

Whole genome sequencing (WGS) is the powerful technique for investigating the genome and identifying somatic mutations, single nucleotide polymorphism (SNPs), insertion, deletion, and copy number variations. In the past few years and using NGS, genome-wide association studies have provided the data, which showed the correlation between genetics, and diseases and was also involved in the pathogenesis of complex human disorders (80). WGS is helpful in inherited disorders detection, characterizing mutations involved with cancer progression, and monitoring disease outbreaks. Interestingly, whole-genome sequencing by NGS techniques might be applied in clinical practice and used to identify the genetic variation for patients or discover genetic variants of patients with rare disease or detect the minor mutations at a lower cost than the traditional techniques. Previous study have been investigated the quasispecies in HIV (81), HBV (82, 83), and HCV by NGS techniques (84, 85). The analysis of viral quasispecies by NGS (454 platform), showing high sensitivity for detecting drug-resistant variants in HIV (86, 87), genetic heterogeneity of HCV (84), and recently publication in HBV quasisepecies (32, 82, 88, 89). The high mutation rate of HBV is approximately 10^{-5}-10^{-3} substitutions/site/year as a result of the lack of proofreading activity of HBV reverse transcriptase during the replication of pgRNA, leading to the rapid replication rates (90-92). Accordingly, a large number of genetic variation, the high genetic diversity, a complex replication and mutation rates cause a heterogeneous of HBV population that are closely related but not identical, which is called as viral quasispecies (93, 94). HBV quasispecies is a significant factor with respect to the clinical manifestation, drug-resistance to NA antiviral treatment, and may effect on the outcomes and treatment response.

CHAPTER III

METHODOLOGY

3. MATERIALS AND METHODS

3.1 Materials

1. Automatic pipette: (0.1-2.0 µl), (0.5-10.0 µl), (2.0-20.0 µl), (20.0-200.0 µl) and (100-1,000 µl) (Eppendorf, Germany)

2. Hand homoginizer grinder (Lab valley, Thailand)

3. Microcentrifuge tube: 1.5 ml (ExtraGene, USA)

4. Parafilm (Plastic Packaging, USA)

5. PCR tube: 0.2 ml and 0.5 ml (Bioline, USA)

6. Pipette tip: 10 µl, 20 µl, 200 µl and 1,000 µl

7. Polypropylene conical tube 15 ml and 50 ml (ExtraGene, USA)

8. Real-Time PCR tube (Kisker Biotech, Netherlands)

3.2 Equipments

1. Autoclave (Hiramaya, USA)

2. Balance (Precisa, UK)

3. BenchMark ULTRA Automated IHC/ISH slide staining system (Ventana, US)

4. Centrifuge Universal 320r (Hettich Centrifuge, UK)

5. Electrophoresis chamber set (Major Science, USA)

6. Freezer -20º C (Sanyo, Japan)

7. Freezer -80 °C (Panasonic, Japan)

8. Heating block (Bioer Technology, USA)

9. Microwave oven (Sharp, Japan)

10. Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA)

11. Next Generation Sequencing (Illumina platform, USA)

12. PCR Mastercycler Gradient (Eppendorf, Germany)

13. Power supply model 250 (Major Science, USA)

14. StepOnePlus Real-Time PCR (Applied Biosystem, USA)

15. Vacuum (Schott Duran, Germany)

3.3 Reagents

Reagents for DNA extraction

1. Proteinase K (Life Technologies, USA)

2. Sodium dodecyl sulfate (SDS) (AMRESCO, USA)

3. Tris-HCl (Sigma, Singapore)

4. Ethylenediaminetetraacetic acid (EDTA) (Bio Basic, Canad)

5. Phenol (AMRESCO, USA)

6. Chloroform (RCI Labscan, Thailand)

7. Isoamyl alcohol (Carlo Erba Reagenti, Italy)

8. Glycogen (USB, Hongkong)

9. Sodium acetate (Sigma, Singapore)

10. Absolute ethanol (Merck, Germany)

11. Nuclease free water

cDNA revere transcription kit

1. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific)

Reagents for gel electrophoresis

1. Agarose molecular grade (AMRESCO, USA)

2. Boric acid (Bio Basic, Canada)

3. EDTA Tetrasodium Dihydrate (USB, Hongkong)

4. Ethidium bromide (AMRESCO, USA)

5. RedSafe Nucleic Acid Staining Solution (INtRON, USA)

6. Tris Base (Bio Basic, Canada)

7. 100 base pair DNA ladder (Thermo Scientific, USA)

Gel extraction kit

1. HiYield^{TM} Gel/PCR DNA Fragment Extraction Kit (RBC

    Bioscience)

2. QIAquick$^®$ Gel Extraction Kit (Qiagen, Netherland)

TA cloning vector

1. 10X Ligation buffer (Thermo Scientific, USA)

2. RBC TA cloning vector kit (RBC Bioscience, Taiwan)

3. T4 DNA ligase (Thermo Scientific, USA)

Reagents for transformation

1. Agar (Becton, Dickinson and Company (BD), USA)

2. Ampicillin (M&H Manufacturing, Thailand)

3. Glucose (Ajax Finechem, New Zealand)

4. Isopropyl-beta-D-thiogalactopyranoside (IPTG) (AMRESCO, USA)

5. Potassium chloride (KCl) (VWR international, USA)

6. Sodium chloride (NaCl) (Merck, Germany)

7. Tryptone (Becton, Dickinson and Company (BD), USA)

8. X-gal (AMRESCO, USA)

9. Yeast extract (Becton, Dickinson and Company (BD), USA)

Plasmid extraction kit

1. RBC Real Genomics HiYield™ Plasmid Mini Kit (RBC Bioscience, Taiwan)

Reagents for PCR amplification

1. 10X Dream*Taq* buffer (biotechrabbit, Germany)

2. dNTPs: dATP, dTTP, dCTP and dGTP (Thermo Scientific, USA)

3. Dream*Taq* polymerase (biotechrabbit, Germany)

4. Specific primers (Macrogen, Korea)

Reagents for Real-Time PCR

1. 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA)

2. Specific primers (Macrogen, Korea)

Human genomic Control Kit DNA

1. The LightCycler® Control Kit DNA (Roche Diagnostics, Switzerland)

Reagents for Next Generation Sequencing (NGS)

1. 5X Phusion HF Buffer (Thermo Scientific, USA)

2. High-Fidelity DNA polymerase (Thermo Scientific, USA)

3. NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (BioLabs, US)

4. AMPure XP Beads (Beckman Coulter, US)

Reagents for Immunohistochemistry

1. Polyclonal rabbit anti-Hepatitis B Virus Core Antigen (HBcAg) (DAKO, Denmark).

2. Monoclonal mouse antibody anti-HBsAg were diluted 1:50 in diluent (DAKO, Denmark).

3. UltraView Universal DAB Detection Kit (Ventana, USA)

4. Xylene and mounted with Consul-Mount (Thermo scientific)

Bioinformatics program

1. BioEdit Sequence Alignment Editor (Version 7.0.9)

2. Oligos Microsoft Visual Studio (Version 6.0 SP5, 2001)

3. CLC Genomics Workbench (Version 8.0.1)

Statistical analysis software

1. SPSS software for Windows version 20 (SPSS, Chicago, IL)

2. GraphPad Prism 6 (GraphPad Software, USA)

3.4 Study population

Thai patients (aged 18–65 years) with HBeAg-positive and HBeAg-negative CHB, who were treated with a full course of PEG-IFN-based therapy between January 2010 and November 2014 in the King Chulalongkorn Memorial Hospital, Bangkok, Thailand were retrospectively enrolled. The therapeutic regimens were included PEG-IFN-alfa2a (180 µg/week) or PEG-IFN-alfa2b (1.5 µg/kg body weight/week) for 48 weeks and followed up for a minimum of 24 weeks after therapy. The inclusion criteria were HBsAg positivity for at least 6 months, elevated serum alanine aminotransferase (ALT) greater than the upper limit of normal and elevated serum HBV DNA levels. Exclusion criteria were as follows: co-infection with HCV and/or human

immunodeficiency virus (HIV); other etiologic causes of liver disease; alcohol dependence or active injection drug use; pregnancy or breast-feeding; serious co-morbid conditions and malignancies; evidence of liver decompensation and HCC. Based on standard practice guidelines, the definition of virological response (VR) for HBeAg-positive CHB was HBeAg clearance plus HBV DNA level <2,000 IU/mL assessed at 24 weeks post treatment. For HBeAg-negative CHB, VR was defined as HBV DNA level <2,000 IU/mL at 24 weeks post therapy (95). The protocol of the study was conducted following the Helsinki Declaration and Good Clinical Practice guidelines with the approval of the Institutional Review Board. All participants in this study gave written informed consent.

- **Sample size:** Case-control study

$$N = \frac{[Z_\alpha\sqrt{2p(1-p)} + Z_\beta\sqrt{p_1(1-p_1) + p_2(1-p_2)}]^2}{(p_1 - p_2)^2}$$

N: required sample size

P2:   estimated proportion of study outcome in the unexposed group (0.3)

OR: odd ratio (2.89)

P1:   estimated proportion of study outcome in the unexposed group (0.553)

P: (P2+P1)/2 = 0.425

Zα: the desired level of statistical significance (Zα/2 = 1.96 for α = 0.05)

Zβ: the desired power (Zβ = 1.28)

$$N = \frac{\left[1.96\sqrt{(2\times0.425)(1-0.425)} + 1.28\sqrt{0.553(1-0.553) + 0.3(1-0.3)}\right]^2}{(0.553-0.3)^2} = \text{Total number} \geq 78$$

The appropriate sample size for this study would be at least 80 cases

3.5 Specimen collection

Pre- and post-treatment paired liver specimens obtained by ultrasound-guided liver biopsy were immediately stored in liquid nitrogen for the analysis of intrahepatic viral markers. In addition, liver tissues fixed and embedded in 10% formalin were sent for histopathological evaluation (96). Serum samples obtained from each patient were collected at baseline, during and after therapy (week 0, 4, 12, 24, 48 and 72) and were stored at -70 $^{\circ}$C until further tests.

3.6 Methods

3.6.1. Serological and Virological Assays

Qualitative serological markers of HBV, including HBsAg, HBeAg and anti-HBe were measured by commercially available enzyme-linked immunosorbent assay kits (Abbott Laboratories, Chicago, IL). Quantitative HBsAg were assessed by the Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN). Serum HBV DNA quantification were analyzed by the Abbott Real Time HBV assay (Abbott Laboratories, Chicago, IL).

3.6.2 Serum extraction for DNA

DNA samples were extracted from sera by phenol-chloroform-isoamyl alcohol method. Briefly, 200 µL of serum was incubated with 400 µL of lysis buffer [10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0 and 0.5% SDS] and 10 µL of proteinase K [20 mg/ml] (Life Technologies, USA) at 50 °C for 1 hour. Then, 250 µL of phenol and 250 µL of chloroform: isoamyl alcohol (49:1) were added, centrifuged at 13,500 rpm, 4°C for 40 minutes. After centrifugation, apply the aqueous phase to new micro centrifuge tube, added 4 µL of glycogen, 800 µL of absolute ethanol and 40 µL of 2M Sodium Oxaloacetate (NaOAc),

mix well by inversion and incubated at -70 °C for 30 minutes. Next, the samples were centrifuged at 13,500 rpm, 4°C for 30 minutes followed by discard the supernatant, then washed the pellet and incubated with 1 mL of 70% ethanol at room temperature for 5 minutes. After that, the samples were centrifuged at high speed (14,000 rpm), 4°C for 5 minutes and discarded the supernatant and dried for 15 minutes at room temperature. Finally, 30 µL of sterile distilled water was added to dissolve a pellet of DNA and immediately stored at -20°C until further analysis.

3.6.3 Liver tissues extraction for DNA, RNA and protein

Paired tissue specimens obtained from pre and post-treatment were homogenized by tissue grinder and then extracted for DNA, RNA and protein by Trizol reagent (Invitrogen, Carlsbad, California). Firstly, 200 µL of chloroform was added and the homogenized samples were then incubated in 1 mL of Trizol reagent for 5 min at room temperature and centrifuged at 12,000 xg for 15 min at 4 °C. After centrifugation, the samples were separated into 3 phases including aqueous phase, interphase and organic phase that represented RNA, DNA, and protein, respectively. Next, each phase of the samples was carefully removed and proceeded for the isolation of RNA, DNA and protein according to the manufacture's procedure.

3.6.4 HBV genotyping and mutations

HBV genotypes and mutations in the PC/BCP regions were determined by direct sequencing, as described previously (97). For HBV genotyping, DNA was amplified for 50 cycles using specific Pre-S primers (HBV_S_F and

HBV_S_R) as shown in Table 2. The reagents and thermo cycling condition for

HBV genotyping were demonstrated in Table 3 and Table 4, respectively.

Table 2. HBV primers

No	Name	Sequence (5'-3')	Nucleotide positions
1	HBV_S_F	TCCTCCAAYTTGTCCTGGTYATC	350–372
	HBV_S_R	AGATGAGGCATAGCAGCAGGAT	432–410
2	HBV_1583F	ACTTCGCTTCACCTCTGCACG	1583-1603
	HBV_C2291R	CDGGWGGAGTGCGRATCCACA	2291-2311

Table 3. Reagents for PCR Master Mix

PCR Reagents	Volume (µL)
10x Dream*Taq* Buffer	2.5
10 µM dNTPs	0.5
10 µM forward primer	0.5
10 µM reverse primer	0.5
Dream*Taq* DNA polymerase [5 units/µL]	0.12
Distilled water	4.4
DNA or cDNA template	1.0
Total volume	25.0

Table 4. PCR thermo-cycling condition for Pre-S region

Thermo-cycling condition			
PCR condition	**Temperature**	**Time**	**Cycles**
Holding	95 °C	3 minutes	
Denaturation	95 °C	15 seconds	
Annealing	55 °C	20 seconds	50 cycles
Extension	72 °C	30 seconds	
Final extension	74 °C	30 seconds	

For detection of BCP and PC mutations, DNA was amplified by a pair of primers (HBV_1583F and HBV_C2291R shown in Table 2). PCR products were processed on 2% of agarose gel, the specific band was cut and purified by gel extraction kit (HiYield™ Gel/PCR DNA Extraction Kit, Banqiao City, Taipei). The sequencing was performed following the manufacturer´s procedure. The obtained sequence was blasted and aligned with the reference sequence of Pre-S and BCP/PC regions of 10 HBV genotypes available in National Center for Biotechnology Information (NCBI) GenBank. Alignment was carried out using CLUSTAL W in Bioedit Software. The results of sequence analysis were classified as wildtype (WT) or mutant (detectable PC and/or BCP mutants).

Table 5. PCR thermo-cycling condition for BCP/PC regions

Thermo-cycling condition			
PCR condition	**Temperature**	**Time**	**Cycles**
Holding	95 °C	3 minutes	
Denaturation	95 °C	15 seconds	
Annealing	60 °C	1 minute	40 cycles
Extension	72 °C	30 seconds	
Final extension	72 °C	7 minutes	

3.6.5 Quantification of HBcrAg

The quantification of HBcrAg was performed using fully Automated Lumipulse G1200 chemiluminescence enzyme immunoassay (CLEIA) analyzer (Fujirebio Inc., Tokyo, Japan), as described elsewhere (98). In short, 150 µL of serum was incubated with 150 µL of pretreatment containing 15%

sodium dodecyl sulfate at 70°C for 10 min, cooled down at room temperature for 5 min and then incubated with three monoclonal antibodies (HB44, HB61, and HB114) against denatured HBcAg and HBeAg. Subsequently, it was washed and incubated with two alkaline phosphatase-labeled monoclonal antibodies as secondary antibodies (HB91 andHB110). Then, added 200 μL of substrate solution [AMPPD; 3-(2-spiroadamantan)–4-methoxy-4-(3-phosphoryloxy) phenyl–1, 2-dioxetane disodium salt], and incubated for 5 min at 37°C. The concentrations of HBcrAg were determined by relative chemiluminescence intensity and compared with standard curve. The results were firstly reported as kU/mL and then converted into U/mL. The lower limit of detection is 100 U/mL (2 $\log_{10}$ U/mL), and samples containing HBcrAg concentrations above the assay range (7 $\log_{10}$ U/mL) were diluted as 1:100 or 1:1,000 using the specific Specimen Diluent.

3.6.6 Quantification of intrahepatic markers

To identify intrahepatic cccDNA, pgRNA and HBV DNA levels, approximately 5-10 mg of liver tissue specimens obtained at baseline and end of treatment were used. Intrahepatic cccDNA, pgRNA and HBV DNA were quantitatively determined as described previously, with some modifications (99). In brief, total DNA and RNA were extracted by Trizol reagent following the manufacturer's protocol. The RNA was reversely transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and was measured for cccDNA and pgRNA by real time PCR using Maxima SYBR green/ROX qPCR Master Mix (Thermo Scientific, Waltham,

Massachusetts, USA) with specific primers as shown in Table 6 (99). PCR and

thermo cycling condition were performed and followed this table below.

Table 6. cccDNA, pgRNA and beta-globin primers

No	Name	Sequence (5'-3')	Nucleotide positions
1	cccDNA_F	CCGTGTGCACTTCGCTTCA	1575-1593
	cccDNA_R	GCACAGCTTGGAGGCTTGA	1882-1864
2	pgRNA_F	GGTCCCCTAGAAGAAGAACTCCCT	2367–2390
	pgRNA_R	CATTGAGATTCCCGAGATTGAGAT	2454–2431
3	Beta-globin_F	GTGCACCTGACTCCTGAGGAGA	
	Beta-globin_R	CCTTGATACCAACCTGCCCAG	

Table 7. Reagents for PCR Maxima SYBR green (Master Mix)

PCR Reagents	Volume (µL)
2x Maxima SYBR green	6.3
10 µM forward primer	0.4
10 µM reverse primer	0.4
Distilled water	4.4
DNA or cDNA template	1.0
Total volume	12.5

Table 8. PCR thermo-cycling condition for cccDNA and pgRNA

Thermo-cycling condition			
PCR condition	**Temperature**	**Time**	**Cycles**
Holding	95 °C	3 minutes	
Denaturation	95 °C	15 seconds	
Annealing	60 °C	1 minute	50 cycles
Extension	72 °C	10 seconds	

Beta-globin gene was used as internal control and stabilized for human genomic DNA in term of $\log_{10}$ copies/cell equivalence (cEq). DNA was amplified for beta-globin gene using the specific primers (Table 6). Quantification of beta-globin was examined by a commercially available human genomic DNA kit (The LightCycler® Control Kit DNA, Roche Diagnostics, Basel, Switzerland) (24). Total intrahepatic HBV DNA levels were detected by real-time PCR using the specific primers shown in table 2 (Forward and reverse primers; HBV_S_F and HBV_S_R) and Maxima SYBR green/ROX qPCR Master Mix (Thermo Scientific, Waltham, Massachusetts, USA). The number of intrahepatic total HBV DNA levels was expressed as copies/cEq.

The standard curve for HBV DNA was produced by a serial dilution of plasmid HBV genome (pUC19-pHBV, provided by Department of Virology and Liver unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan) with the concentration of 10^2-10^9 copies/μL.

3.6.7 Design primers for Whole HBV genome

The eleven primers located in the conserved regions of HBV genome (Genotype B and C) were designed to amplify HBV genome. Each product size was overlap around 100 bp (Table 9).

Table 9. HBV genome sequencing primers

NO	Name and nucleotide positions	Sequence	No. of sequence	Tm
1	HBV_F_677-697	TTACTAGTGCCATTTGTTCAG	21	58
	HBV_R_1094-1113	CTTGTAAGTTGGCGAGAAAG	20	58
2	HBV_F_979-1000	TGGAAAGTATGTCAAMGAATTG	22	58
	HBV_R_1379-1396	TGGCAGCACASCCKAGCA	18	58
3	HBV_F_1263-1281	GATCCATACTGCGGAACTC	19	58
	HBV_R_1663-1682	CATTGCTGARAGTCCAAGAG	20	58
4	HBV_F_1551-1569	GTCTGTGCCTTCTCATCTG	19	58
	HBV_R_1980-1998	GTGTCGAGGAGATCTCGAA	19	58
5	HBV_F_1893-1912	TTTRGGGCATGGACATTGAC	20	58
	HBV_R_2304-2324	TTGATAAGATAGGGGCATTTG	21	58
6	HBV_F_2197-2218	ACTATTGTGGTTTCACATTTCC	22	60
	HBV_R_2626-2648	CCTAGCAGGCATAATTAATTTTA	23	60
7	HBV_F_2549-2569	CATTCATTTRCAGGAGGACAT	21	58
	HBV_R_2955-2976	CCAATCTGGATTKTYTGAGTTG	22	60
8	HBV_F_2816-2836	GTCACCATATTCTTGGGAACA	21	60
	HBV_R_3192-3210	CTGCATGGCCTGAGGATGA	19	60
9	HBV_F_3148-3166	TCAGGAAGRCAGCCTACTC	19	58
	HBV_R_337-355	TGGAGGACAASAGGTTGGT	19	58
10	HBV_F_251-269	GACTCGTGGTGGACTTCTC	19	60
	HBV_R_673-694	AACAAATGGCACTAGTAAACTG	22	60
11	HBV_F_537-557	CTCAAGGAACCTCTAYGTTTC	21	60
	HBV_R_959-980	CAATCAATAGGYCTRTTTACAG	22	58

3.6.8 Whole HBV genome analysis by Next Generation Sequencing

Baseline serum samples collected from each patient were extracted for DNA by phenol/chloroform/isoamyl alcohol extraction then dissolved in 30 µL of distilled water. HBV genome was amplified by Phusion™ High-Fidelity DNA Polymerase; F-530 (Thermo Scientific, Waltham, Massachusetts, USA) using the primers shown in Table 9 and reagents and condition shown in Table 10.

Table 10. Reagents for PCR (Phusion) Master Mix

PCR Reagents	Volume (µL)
5x Phusion HF Buffer	4.0
dNTPs	0.4
10 µM forward primer	0.5
10 µM reverse primer	0.5
Phusion DNA polymerase	0.12
Distilled water	13.4
DNA template	1.0
Total volume	20.0

Table 11. PCR thermo-cycling condition for HBV genome

Thermo-cycling condition			
PCR condition	**Temperature**	**Time**	**Cycles**
Holding	98 °C	30 seconds	
Denaturation	98 °C	10 seconds	
Annealing	51 °C	30 seconds	35 cycles
Extension	72 °C	30 seconds	
Final extension	72 °C	7 minutes	

The PCR products were processed on 2% of agarose gel and purified by Hiyield™ Gel/PCR DNA fragments extraction kit (RBC Bioscience, New Taipei City, Taiwan). The concentration of DNA was measured by Qubit® Fluorometer and reported in term of ng/μL.

DNA library preparation was applied by NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (NEB). Briefly, the first step was to purify DNA fragment. The second step was the process of end-repair and 5' phosphorylation and dA tailing to create blunt-ended fragments following by DNA fragment ligation with adaptor. Finally, index was incorporated to DNA and amplified by high-fidelity polymerase (Figure 10).
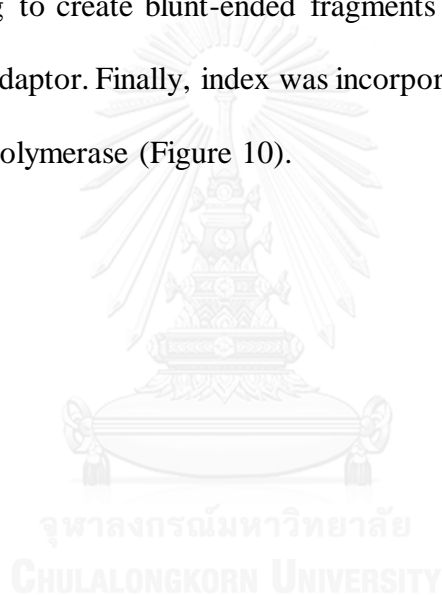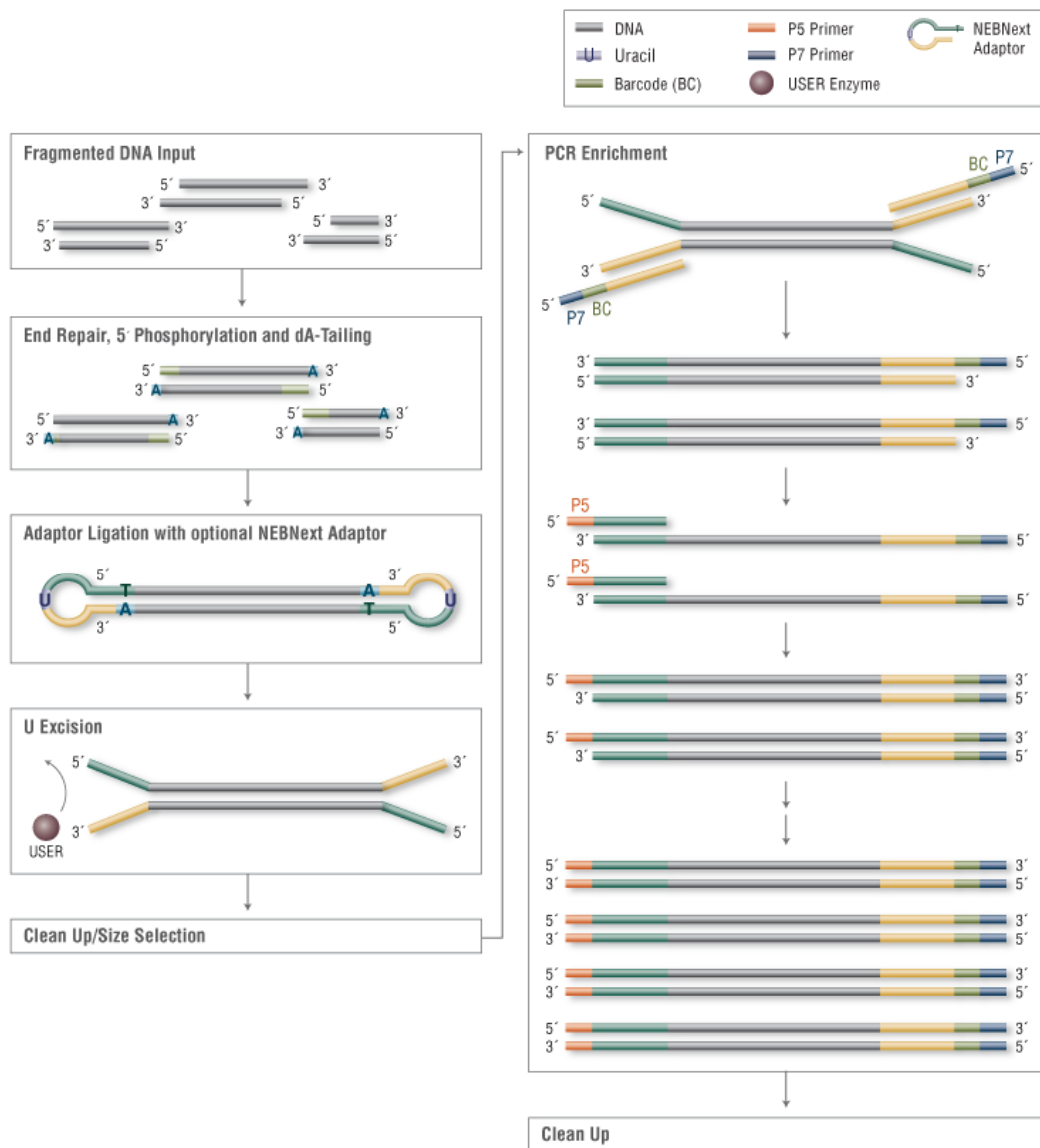
Figure 10. DNA Library Preparation Workflow (NEBNext® Ultra^{TM} DNA Library Prep Kit for Illumina®)

3.6.9 Protocol for DNA Library Preparation

The 5ng-1µg concentration of Fragmented DNA was used.

1. NEBNext End Prep (Table 12)

Table 12. The preparation of NEBNext End Prep Mix

Components	Volume
End Prep Enzyme Mix	3.0 µl
End Repair Reaction Buffer (10X)	6.5 µl
Fragmented DNA	55.5 µl
Total volume	65 µl

2. Mixed well by pipette followed by a quick spin.

3. Place in a thermocycler and process with the following program:

   - Incubated at 20 °C for 30 minutes then at 65 °C for 30 minutes and

   then hold at 4 °C.

4. Adaptor Ligation (Table 13)

Table 13. Components of adaptor ligation

Components	Volume
Blunt/TA Ligase Master Mix	15 µl
NEBNext Adaptor for Illumina	2.5 µl
Ligation Enhancer	1 µl
Master Mix from step 3	65 µl
Total volume	83.5 µl

5. Mix well by pipette followed by a quick spin.

6. Incubate at 20 °C for 15 minutes.

7. Add 3 µl of USERenzyme to the ligation mixture from step 6.

8. Mix well and incubate at 37°C for 15 minutes

9. Size selection of Adaptor-ligated DNA

   9.1 First bead selection

      9.1.1 Vortex 35 µl of AMPure XP beads to resuspend.

      9.1.2 Add 13.5 µl of distilled water to the ligation reaction for a
100 µl total volume.

      9.1.3 Add 55 µl of resuspended AMPure XP beads to the 100 µl
ligation reaction and mix by pipette up and down at least
10 times.

      9.1.4 Incubated at room temperature for 5 minutes.

      9.1.5 Quickly spin the tube and place the tube on magnetic stand.
After the solution is clear (5 minutes), carefully transfer the
supernatant containing DNA to a new tube.

   9.2 Second bead selection

      9.2.1 Add 15 µl resuspended AMPure XP beads to the
supernatant, mix well and incubated at room temperature
for 5 minutes.

      9.2.2 Quickly spin the tube and place the tube on magnetic stand.
After the solution is clear (5 minutes), carefully remove
and discard the supernatant (unwanted DNA).

10. Add 200 µl of 80% freshly prepared ethanol to the tube while in the
magnetic stand. Incubated at room temperature for 30 seconds, and
then carefully remove and discard the supernatant.

11. Repeat step 10 twice for a total of three washes.

12. Air the dry beads for 10 minutes while the tube is on the magnetic stand with the cap open.

13. Elute the DNA target from the beads into 28 µl of TE buffer. Mix well on a vortex mixer. Quickly spin the tube and place the tube on magnetic stand. After the solution is clear (5 minutes), transfer 23 µl to a new PCR tube for amplification.

14. PCR Amplification using reagents and thermos-cycling shown in Table 14 and 15.

Table 14. Components of PCR amplification

Components	Volume
Adaptor Ligated DNA fragments	23 µl
NEBNext High Fidelity 2X PCR Master Mix	25 µl
Index Primer	1.0 µl
Universal PCR Primer	1.0 µl
Total volume	50 µl

Table 15. PCR thermo-cycling condition for PCR amplification

PCR condition	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	
Denaturation	98 °C	10 seconds	
Annealing	65 °C	30 seconds	15 cycles
Extension	72 °C	30 seconds	
Final extension	72 °C	7 minutes	
Hold	4 °C	-	

15. Cleanup of PCR Amplification

    15.1 Vortex AMPure XP beads to resuspend.

    15.2 Add 50 µl of resuspended AMPure XP beads to the PCR

        reactions, mix by pipette up and down at least 10 times.

    15.3 Incubate at room temperature for 5 minutes.

    15.4 Add 200 µl of 80% ethanol to the tube while in the magnetic

        stand. Incubate at room temperature for 30 seconds, and

        then carefully remove and discard the supernatant.

    15.5 Repeat Step 15.4 once.

    15.6 Air the dry beads for 10 minutes while the tube is on the

        magnetic stand with the cap open.

    15.7 Elute the DNA target from the beads into 33 µl of TE buffer.

        Mix well by pipette up and down 10 times. Quickly spin the

        tube and place the tube on magnetic stand. After the

        solution is clear, transfer 28 µl supernatant to a new tube.

    15.8 Check the concentration by Qubit fluorimeter.

16. Pool samples with the concentration of 2 nM then take 230 µl of pool

    samples, add 10 µl of denature reagent and 10 µl of NaOH.

    Incubate at room temperature for 5 minutes then add 980 µl of

    HT1 [20 pmoles].

17. Add 180 of µl PhiX (30%) into the DNA mixture (420 µl from

    step 16), and 400 µl of HT1 [12 pmoles].

18. Load all sample mix into flow cells and process by NGS.

For second analysis, HBV genome sequencing was performed by Miseq Illumina Next Generation Sequencing and secondly analyzed by CLC genomic workbench 8.0.1 program (Figure 11A). In short, the overlapping pair reads were aligned and merged then the eleven primers of HBV genome and the adaptor sequence were trimmed. The obtained reads were mapped to reference HBV genome (NC_003977.2) in NCBI database (http://www.ncbi.nlm.nih.gov/) (Figure 11B) then selected a read mapping to extract consensus sequence (Figure 11C). The mutation rates were quantified with cut-off 1.0% mutation frequency by basic variance detection (6). The results were reported in term of percent of mutation rate, deletion, frequencies of substitution rate and variation of quasispecies.
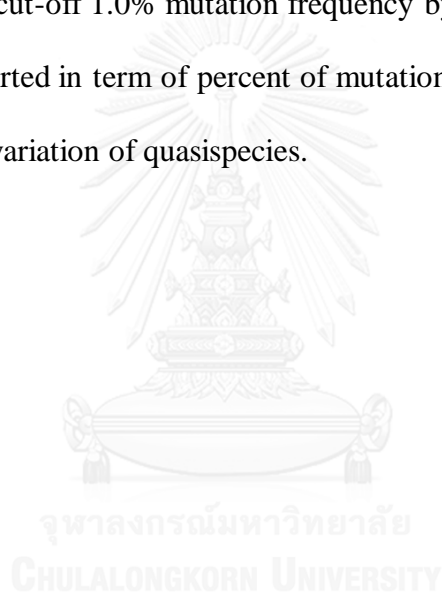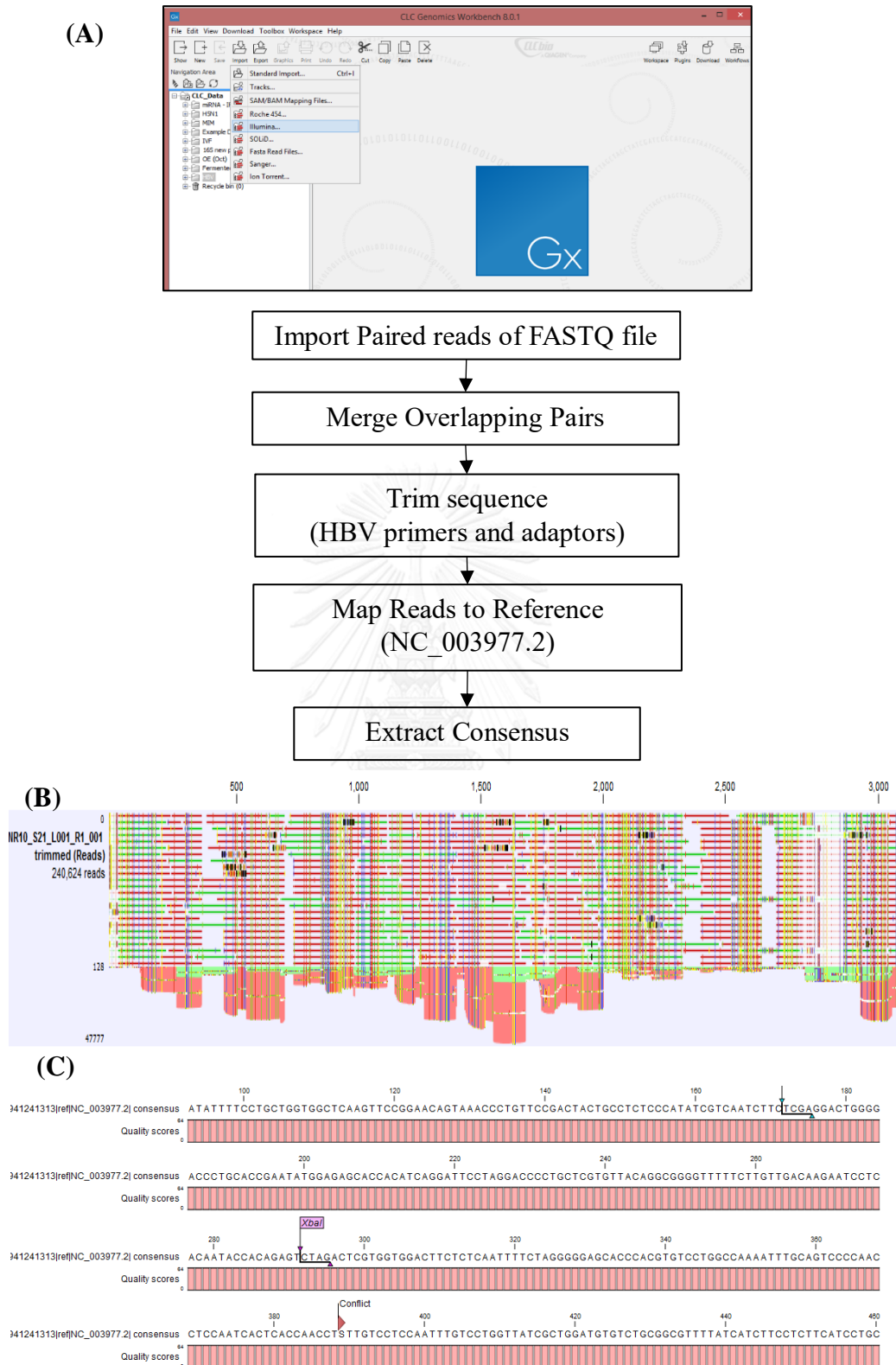
Figure 11. The CLC workbench 8.0.1 program and secondary analysis flowchart (A), the output of HBV genome (B) and consensus sequence (C)

3.6.10 Immunohistochemistry for HBsAg and HBcAg

Liver sections were cut into 4 μm size using Microm HM 355 S, and dried at 60 °C overnight. The polyclonal rabbit anti-Hepatitis B Virus Core Antigen (HBcAg) and monoclonal mouse antibody anti-HBsAg were diluted 1:50 in diluent (DAKO, Denmark). The Ventana staining procedure includes pretreatment with Cell Conditioner 2 (pH 6.0) for 60 min (standard), followed by incubation with 1:50 diluted antibody at 37 °C for 90 min. Secondary antibody incubation was performed by Ventana standard signal amplification, ultraWash, counter-staining with one drop of Hematoxylin for 4 min and one drop of bluing reagent for 4 min. The chromogenic detection was visualized by ultraView Universal DAB Detection Kit (Ventana, USA). After dehydration, sections were cleared with xylene and mounted with Consul-Mount (Thermo scientific, USA). The levels of HBcAg and HBsAg expression were scored by a double-blinded method. The degree of expression ranked on a scale of 0–5 (0%, 1–10%, 11–25%, 26–50%, 51–75% and >75%, respectively) by counting of HBsAg and HBcAg positive cells (100).

3.6.11 Statistical Analysis

Statistical analysis was applied by SPSS version 20 (SPSS Inc., Chicago, Illinois, U.S.A.). Data were expressed as mean ± standard deviation (SD) or percentages as appropriate. One-way ANOVA was used to compare the analysis of more than two groups. Tukey Post-hoc test was then used at the second stage of ANOVA test. Comparisons within groups (pre- and post-treatment) were analyzed by paired samples t-test for quantitative variables. Comparisons between groups (responders and non-responders) were analyzed by independent sample t-test for quantitative variables. Pearson's correlation coefficient was tested for correlation between two variables. To validate the best cut-off values, areas under the receiver operating characteristic curve (ROC) at specific time-points were used to assess the predictive values of variables in relation to VR. For predict the probability of VR, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated in accordance with standard methods. Logistic regression (Univariate and Multivariate analysis) was used to assess odd ratios relating variables associated with VR. *P*-values <0.05 were considered statistically significant.

CHAPTER IV

RESULTS

4.1 Characteristics of patients enrolled according to HBeAg status

4.1.1 Characteristics at baseline and rates of response

This study included 184 patients with CHB, who were treated with PEG-IFN for 48 weeks and had at least 24 weeks of follow-up. Base on HBeAg status, there were 63 and 121 patients with HBeAg-positive and HBeAg-negative CHB, respectively. The comparison of demographic and clinical characteristics data between these two groups are shown in Table 16.

At baseline, patients with HBeAg-positive CHB had significantly lower mean age but higher serum levels of ALT, HBV DNA, HBsAg and HBcrAg compared with patients with HBeAg-negative CHB. The HBeAg-positive group also had significantly higher intrahepatic HBV DNA, cccDNA and pgRNA levels than the other group. There was no different between groups regarding gender distribution and the severity of liver fibrosis assessed by transient elastography (FibroScan). All patients in this study were infected with HBV genotype B or C. In the HBeAg-positive group, the proportion of genotype C was significantly higher compared to that of the HBeAg-negative group.

At end of follow-up, 21 (33.3%) patients with HBeAg-positive CHB achieved VR (responders), while 42 (66.7%) patients did not achieve VR (non-responders). The corresponding figures for patients with HBeAg-negative CHB were 48 (39.7%) and 73 (60.3%), respectively. Regarding the rate of HBsAg

clearance, this end-point was achieved in 4 (6.3%) of patients with HBeAg-positive CHB and 9 (7.4%) of patients with HBeAg-negative CHB.

Table 16. Baseline characteristics of participants based on HBeAg status

Characteristics	HBeAg-positive (n = 63)	HBeAg-negative (n = 121)	*P* value
Age, years	33.2±9.1	40.1±9.5	<0.001*
Gender (% Males)	43 (68.3%)	85 (70.2%)	0.780
ALT, U/L	96.6±68.3	73.5±36.4	0.015*
HBV Genotypes			0.003*
B	2 (3.2%)	23 (19.0%)	
C	61 (96.8%)	98 (81.0%)	
Serum			
Log$_{10}$ HBV DNA, IU/ml	7.3±1.0	5.4±0.8	<0.001*
Log$_{10}$ HBsAg, IU/ml	3.9±0.9	3.5±0.5	<0.001*
Log$_{10}$ HBcrAg, U/ml	8.2±1.0	4.3±1.2	<0.001*
Intrahepatic			
Log$_{10}$ intrahepatic HBV DNA, copies/cEq	4.0±1.1	2.4±2.2	<0.001*
Log$_{10}$ intrahepatic cccDNA, copies/cEq	1.6±0.9	0.3±1.3	<0.001*
Log$_{10}$ intrahepatic pgRNA, copies/cEq	4.4±1.0	1.4±1.8	<0.001*
Liver fibrosis (kPa)	7.3±3.9	7.8±2.8	0.362
Virological response (VR)	21 (33.3%)	48 (39.7%)	0.400
HBsAg clearance	4 (6.3%)	9 (7.4%)	0.784

Data expressed as mean ± standard deviation or n (%); ALT, alanine aminotransferase, *P<0.05

4.1.2 Viral markers at end of treatment

Table 17 demonstrates serum and intrahepatic levels of viral markers at the end of treatment in patients with HBeAg-positive and HBeAg-negative CHB. Responders had significantly lower serum HBV DNA, HBsAg and HBcrAg levels than non-responders in both groups. Responders in the HBeAg-positive group showed lower levels of intrahepatic HBV DNA, cccDNA and pgRNA than non-responders, but these differences were not found significantly in the HBeAg-negative group. The same trends regarding the reduction of serum and intrahepatic markers from baseline in responders compared with non-

responders were found only in the HBeAg-positive group, but not in the HBeAg-negative group.

Table 17. Comparison of viral markers between responders and non-responders

End of treatment	HBeAg-positive			HBeAg-negative		
	Responders (n = 21)	Non-responders (n = 42)	*P*	Responders (n =4 8)	Non-responders (n=73)	*P*
Serum						
Log_{10} HBV DNA, IU/ml	0.8±1.2	5.1±2.3	<0.001*	0.3±0.6	1.2±1.6	<0.001*
Log_{10} HBsAg, IU/ml	1.4±2.0	3.3±1.2	<0.001*	1.6±1.2	3.1±0.7	<0.001*
Log_{10} HBcrAg, U/ml	5.5±1.0	7.2±1.2	<0.001*	3.1±1.0	3.5±0.9	0.012*
Intrahepatic						
Log_{10} HBV DNA, copies/cEq	1.9±0.9	2.6±1.0	0.043*	1.9±2.4	2.3±1.9	0.437
Log_{10} cccDNA, copies/cEq	(-0.1)±0.8	1.1±1.0	0.002*	(-0.7)±2.4	(-0.3)±1.6	0.386
Log_{10} pgRNA, copies/cEq	2.2±0.7	3.3±1.2	0.011*	1.4±1.4	1.2±1.3	0.670
Mean reduction						
Log_{10} HBV DNA, IU/ml	6.5±1.7	2.3±1.9	<0.001*	5.1±0.8	4.3±1.6	<0.001*
Log_{10} HBsAg, IU/ml	2.5±2.2	0.7±0.9	0.002*	1.7±1.1	0.4±0.5	<0.001*
Log_{10} HBcrAg, U/ml	2.6±1.1	1.0±1.1	<0.001*	1.1±0.8	0.7±0.8	0.009*
Log_{10} intrahepatic HBV DNA, copies/cEq	2.4±0.9	1.3±1.3	0.016*	0.0±2.5	0.4±2.0	0.503
Log_{10} intrahepatic cccDNA, copies/cEq	1.7±0.8	0.4±1.0	0.001*	1.1±2.2	0.7±1.7	0.347
Log_{10} intrahepatic pgRNA, copies/cEq	2.1±1.4	1.1±0.9	0.024*	0.1±1.6	0.1±1.6	0.995

Data expressed as mean ± SD or percentage; ALT, alanine aminotransferase,* *P*<0.05

4.2 Predictive roles of viral markers in HBeAg-positive CHB

4.2.1 Baseline and viral kinetics in HBeAg-positive CHB

In order to fully investigate role of viral kinetics during PEG-IFN therapy in patients with HBeAg-positive CHB, this part of the study included only those who had serum samples available at many time points (week 0, 4, 12, 24, 48 and 72). In this respect, there were only 46 patients included for the analysis.

4.2.1.1 Baseline characteristics regarding treatment-response

Among 46 patients (31 male, average age of 33.2 years), VR was achieved in 15 patients (32.6%), and HBsAg clearance was achieved in 4 patients (8.7%). Baseline characteristics of patients with respect to VR are showed in Table 18. The responders had significantly lower prevalence of BCP mutations (A1762T and/or G1764A) than non-responders. Responders trended to have lower prevalence of PC mutation (G1896A) than non-responders, though did not reach statistical significance. There was no difference between groups in terms of mean age, gender distribution, the prevalence of HBV genotype, serum ALT, HBV DNA, HBsAg and HBcrAg levels, as well as intrahepatic HBV DNA, cccDNA and pgRNA levels.

Table 18. Baseline characteristics of patients with HBeAg-positive in relation to

virological response

Characteristics	Responders	Non-responders	P
	(n = 15)	(n = 31)	
Age, years	31.5 ±6.7	34.0 ± 8.8	0.294
Gender (% Males)	8 (53.3%)	23 (74.2%)	0.157
ALT, U/L	114.3 ± 60.1	87.6 ± 62.1	0.174
Log_{10} HBV DNA, IU/ml	7.3 ± 1.0	7.2 ± 1.2	0.788
Log_{10} HBsAg, IU/ml	3.8 ± 0.6	4.0 ± 0.7	0.529
Log_{10} HBcrAg, U/ml	7.9 ± 1.1	8.2 ± 1.1	0.538
HBV genotypes			0.709
B	2(13.3%)	3 (9.7%)	
C	13(86.7%)	28 (90.3%)	
HBV mutations (n=43)			
PC mutation	0 (0%)	5 (17.2%)	0.098
BCP mutations	4 (28.6%)	19 (65.5%)	0.023*
PC and BCP mutations	4 (28.6%)	19 (65.5%)	0.023*
Log_{10} intrahepatic HBV DNA, copies/cEq	4.2±1.1	3.9±1.1	0.405
Log_{10} intrahepatic cccDNA, copies/cEq	1.7 ± 0.8	1.5 ± 1.1	0.514
Log_{10} intrahepatic pgRNA, copies/cEq	4.3±1.0	4.4±1.1	0.840

Values are presented as means (95% confidence intervals) and percentages.
PC: precore, BCP: basal core promoter, *P<0.05

4.2.1.2 Correlation of viral markers at baseline

At pre-treatment, there was a positive correlation between log_{10} serum HBsAg and log_{10} serum HBcrAg ($r = 0.616$, $P < 0.001$), and log_{10} intrahepatic cccDNA ($r = 0.424$, $P = 0.0020$). Similarly, pretreatment log_{10} serum HBV DNA was correlated with log_{10} serum HBcrAg ($r = 0.841$, $P < 0.001$). Moreover, serum HBcrAg was correlated with log_{10} intrahepatic cccDNA ($r = 0.564$, $P = 0.001$) (Figure 12A, 12B, 12C and 12D).

(A)

(B)

(C)

(D)
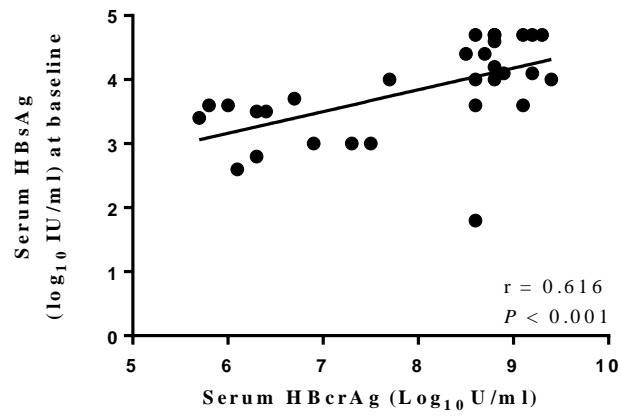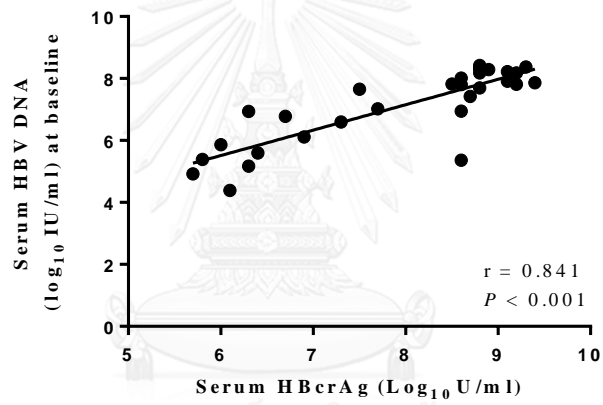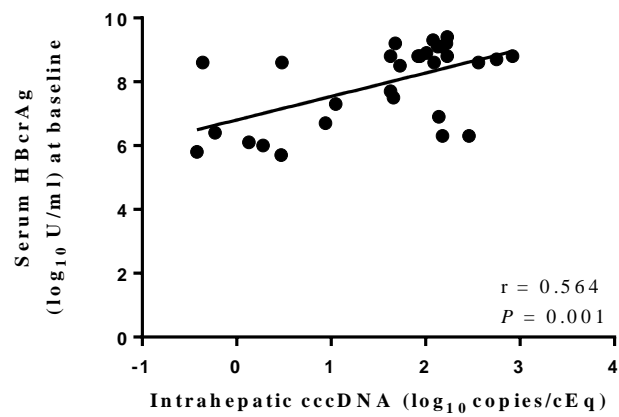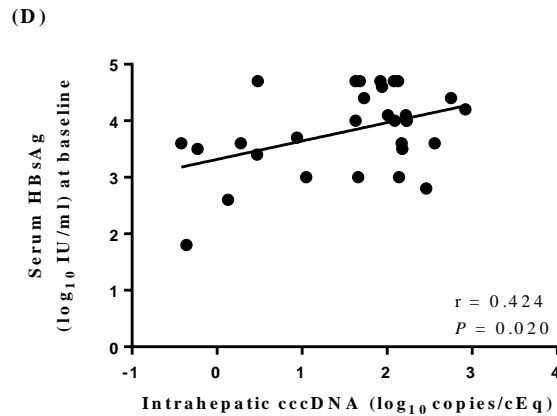
Figure 12. Correlation of viral markers at baseline in patients
with HBeAg-positive CHB

4.2.1.3 Viral markers at end of treatment

Compared with non-responders, responders had significantly lower serum levels of HBV DNA, HBsAg and HBcrAg and intrahepatic levels of HBV DNA, cccDNA and pgRNA at the end of treatment. In addition, the magnitude of reduction in serum and intrahepatic viral markers between responders and non-responders was compared. The average change in serum HBV DNA, HBsAg and HBcrAg levels, and intrahepatic HBV DNA, cccDNA and pgRNA levels in responders were significantly lower than non-responders. (Table 19)

Table 19. HBV viral markers at end of PEG-IFN treatment

End of treatment	Responders (n = 15)	Non-responders (n = 31)	*P*
Serum			
Log_{10} HBV DNA, IU/ml	0.7±1.1	4.7±2.1	<0.001*
Log_{10} HBsAg, IU/ml	1.9±1.4	3.4±1.0	0.001*
Log_{10} HBcrAg, U/ml	5.2±0.9	7.1±1.2	<0.001*
Intrahepatic			
Log_{10} HBV DNA, copies/cEq	1.9±0.9	2.6±1.0	0.043*
Log_{10} cccDNA, copies/cEq	(-0.1)±0.8	1.1±1.0	0.002*
Log_{10} pgRNA, copies/cEq	2.2±0.7	3.3±1.2	0.011*
Mean reduction			
Log_{10} HBV DNA, IU/ml	6.6±1.6	2.5±1.6	<0.001*
Log_{10} HBsAg, IU/ml	2.0±1.7	0.6±0.5	0.006*
Log_{10} HBcrAg, U/ml	2.8±1.2	1.0±1.1	<0.001*
Log_{10} intrahepatic HBV DNA, copies/cEq	2.4±0.9	1.3±1.3	0.016*
Log_{10} intrahepatic cccDNA, copies/cEq	1.7±0.8	0.4±1.0	0.001*
Log_{10} intrahepatic pgRNA, copies/cEq	2.1±1.4	1.1±0.9	0.024*

Data was presented as mean ± SD and percentage,
ALT, alanine aminotransferase, *P<0.05

4.2.1.4 Kinetics of HBsAg regarding to treatment response

Patients with VR showed a steady decrease in serum HBsAg during the study period. In contrast, non-responders showed relatively stable HBsAg levels during therapy and trended to rebound after cessation of treatment. Average levels of log_{10} HBsAg decline in the responder and non-responder groups were as follows; week 4 (0.1 ± 0.2 vs. 0.1 ± 0.3, P = 0.870), week 12 (0.4 ± 0.6 vs. 0.2 ± 0.3, P = 0.132), week 24 (1.4 ± 1.3 vs. 0.3 ± 0.4, P < 0.001), week 48 (1.8 ± 1.7 vs. 0.6 ± 0.5, P = 0.001), and week 72 (1.8 ± 2.0 vs. 0.3 ± 0.5, P < 0.001) (Figure 13).

Among the responder group, patients with HBsAg clearance (n=4) showed a marked decline in serum HBsAg compared to patients without HBsAg

clearance (n=11), particularly after week 12 until the end of follow-up. Average

levels of $\log_{10}$ HBsAg decrease in those with or without HBsAg clearance were

as follows; week 4 (0.2 ± 0.2 vs. 0.1 ± 0.2, $P = 0.191$), week 12 (0.4 ± 0.6 vs

0.4 ± 0.7, $P = 0.958$), week 24 (2.6 ± 1.4 vs. 1.0 ± 1.0, $P = 0.026$), week 48 (3.3

± 2.0 vs. 1.2 ± 1.2, $P = 0.028$) and week 72 (4.2 ± 0.3 vs. 0.9 ± 1.4, $P = 0.001$).

(Figure13)
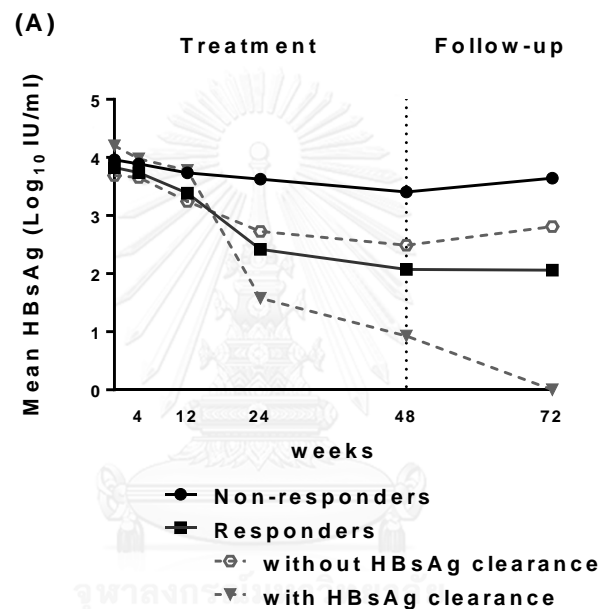
Figure 13. Kinetics of HBsAg level according to treatment response

4.2.1.5 Kinetics of HBcrAg regarding to treatment response

Responders also showed a significant decrease in serum HBcrAg levels

compared with non-responders, with the average decline of $\log_{10}$ HBcrAg as

follows: week 4 (0.4 ± 0.6 vs. 0.2 ± 0.3, $P = 0.269$), week 12 (1.2 ± 1.1 vs 0.6

± 0.8, $P = 0.068$), week 24 (2.0 ± 1.2 vs. 0.8 ± 0.9, $P < 0.001$), week 48 ($2.8 \pm$

1.2 vs. 1.0 ± 1.1, $P < 0.001$) and week 72 (3.4 ± 1.4 vs. 0.6 ± 1.5, $P < 0.001$).

Among responders, patients with or without HBsAg clearance had comparable decrease of HBcrAg level, though those achieving HBsAg clearance trended to have more HBcrAg decline at the end of follow-up. Average levels of $\log_{10}$ HBcrAg decrease in the HBsAg clearance and non-clearance groups were as follows; week 4 (0.2 ± 0.2 vs. 0.4 ± 0.6, $P = 0.192$), week 12 (1.7 ± 1.7 vs. 1.1 ± 0.9, $P = 0.535$), week 24 (2.7 ± 1.6 vs. 1.8 ± 0.9, $P = 0.346$), week 48 (3.2 ± 1.5 vs. 2.6 ± 1.1, $P = 0.545$) and week 72 (4.7 ± 1.2 vs. 3.0 ± 1.2, $P = 0.053$). (Figure 14)
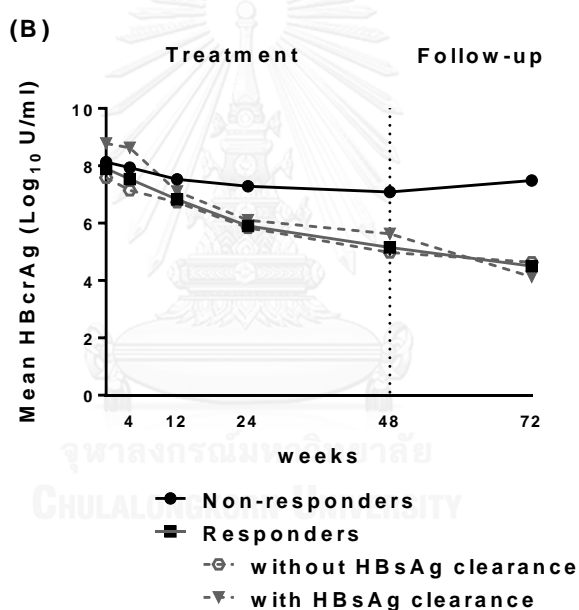
Figure 14. Kinetics of HBcrAg level according to treatment response

4.2.1.6 Predictors of VR at baseline and during therapy

To identify variables that might be associated with VR, baseline characteristics and viral kinetics during treatment were investigated. Potential baseline indicators included gender, age, serum ALT, HBV DNA, HBsAg and HBcrAg levels, HBV genotypes and mutants. On-treatment indicators at week

12 included HBsAg and HBcrAg levels. Based on multivariate analysis, the presencof WT virus at baseline and serum HBcrAg at week 12 were identified as predictors of VR (Table 20).

Table 20. Baseline characteristics of patients in relation to virological response

Factors	Category	Virological response (week 72)*			
		Univariate analysis		Multivariate analysis	
		OR (95%CI)	P	OR (95%CI)	P
Baseline Age, years	< 35 vs. ≥ 35	2.89 (0.68-12.35)	0.152		
Gender (% Males)	Male vs. Female	0.40 (0.11-1.45)	0.163		
ALT, U/L	< 80 vs. ≥ 80	2.38 (0.67-8.38)	0.179		
Log_{10} HBV DNA, IU/ml	< 7.0 vs. ≥ 7.0	1.44 (0.40-5.24)	0.576		
Log_{10} HBsAg, IU/ml	< 4.3 vs. ≥ 4.3	2.53 (0.59-10.85)	0.213		
Log_{10} HBcrAg, U/ml	< 8.0 vs. ≥ 8.0	2.14 (0.60-7.67)	0.243		
HBV genotypes	B vs. C	0.70 (0.10-4.69)	0.710		
PC/BCP mutants	Wild type vs. Mutants	4.75 (1.18-19.06)	0.028*	46.16 (2.77-777.78)	0.008*
Log_{10} cccDNA, copies/cEq	<1.5 vs. ≥ 1.5	0.31 (0.05-1.88)	0.205		
Week 12					
Log_{10} HBsAg, IU/ml	< 4.3 vs. ≥ 4.3	2.26 (0.42-12.28)	0.345		
Log_{10} HBcrAg, U/ml	< 8.0 vs. ≥ 8.0	17.00 (1.98-145.73)	0.010*	69.67 (3.26-988.15)	0.007*

To compare the predictive values of log_{10} HBsAg and log_{10} HBcrAg in predicting VR, the areas under ROC (AUROC) curve at baseline and week 12 were analyzed. At baseline, the AUROC of log_{10} HBcrAg [0.55; 95% confidence interval (CI), 0.37-0.73; $P = 0.606$) was similar to the AUROC of log_{10} HBsAg (0.57; 95%CI, 0.39-0.75; $P = 0.432$). At week 12, the AUROC of log_{10} HBcrAg (0.69; 95%CI, 0.53-0.85; $P = 0.040$) was significantly higher

than the AUROC of $\log_{10}$ HBsAg (0.56; 95%CI, 0.38-0.73; $P = 0.527$).

(Figure15)
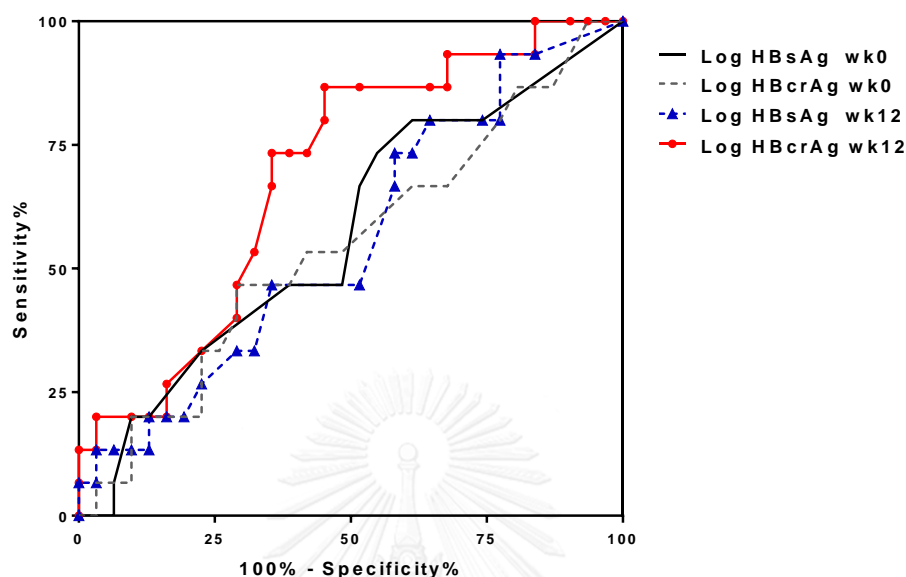
Figure 15. Predictive values of $\log_{10}$ HBsAg and $\log_{10}$ HBcrAg for VR based on the receiver operating characteristic curve

Upon the analysis of ROC, multiple levels of serum HBcrAg at week 12 were tested and compared in order to validate the optimal cut-off point in predicting VR. As shown in Table 21, $\log_{10}$ serum HBcrAg level of 8.0 U/mL was considered to be the best cut-off level, with its sensitivity of 93.3%, specificity of 54.8%, PPV of 50.0% and NPV of 94.4%. In fact, it is generally accepted that the optimal NPV to be used as a "stopping rule" should be approximately 95% or higher.

For the cut-off level of serum HBsAg, previous data have demonstrated that patients treated with PEG-IFN would have very low response rates if HBsAg level is still higher than 20,000 IU/mL ($\log_{10}$ 3.4 IU/mL) at 12-week of therapy (NPV: approximately 92-98%) (101). As a result, this level of quantitative HBsAg has been generally used as a "stopping rule" for treatment

cessation. Regarding to our study, patients with HBsAg levels $> \log_{10} 3.4$ IU/mL at weeks 12 had NPV of 80% (20% chance of response). When both serum markers at these cut-off points were applied together, the NPV in achieving VR was 88.9%, which was considered to be inferior to that of using HBcrAg alone (Table 21).

Table 21. Predictive power of HBcrAg and HBsAg levels for VR

Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Week 12				
Log_{10} HBcrAg (U/mL)				
7.0	60.0	64.5	45.0	76.9
7.5	73.3	58.1	45.8	81.8
8.0	93.3	54.8	50.0	94.4
8.5	93.3	32.3	40.0	90.9
Log_{10} HBsAg (IU/mL)				
4.3	86.7	25.8	36.1	80.0
Combination of both markers*	93.3	25.8	37.8	88.9

*Combined HBcrAg ($\log_{10}$ 8.0 U/mL) and HBsAg ($\log_{10}$ 4.3 IU/mL or 20,000 IU/mL);PPV: positive predictive value, NPV: negative predictive value

4.2.2 Characteristics of HBV mutations detected by NGS

The above-mentioned data showed that the presence of WT strains in the PC/BCP region at baseline detected by Sanger sequencing was associated with VR in patients with HBeAg-positive CHB. To confirm these results, baseline mutational patterns of HBV genome, particularly in the EnhII/BCP/PC and Pre-S/S regions were identified by NGS. As mutational patterns might be diverse among different HBV genotypes, only serum samples of patients infected with genotypes C, which represented the most common genotype in the cohort, were selected for NGS.

4.2.2.1 The concentration of PCR products and DNA library

In this part, 48 pre-treatment serum samples of patients infected with genotype C were available for further investigation. After HBV genome was amplified and processed on 2% of agarose gel, the concentration of PCR product fragment of each sample was examined and calculated for DNA library preparation (Table 22).

Table 22. Concentration of PCR products and DNA library

Sample	Concentration of each PCR product fragment (ng/µL)											DNA library (nM)
	1	2	3	4	5	6	7	8	9	10	11	
V1	13.5	12.1	13.2	4.6	15.4	10.4	6.5	14.6	11.3	12.7	6.1	4.9
V2	16.6	15.9	42.4	6.9	12.9	28.6	9.4	18	16.1	18.6	7.1	5.4
V3	20.2	18.1	18.0	12.9	19.5	17.6	7.2	17.6	18.9	20.5	8.8	14.6
V5	23.3	8.7	25.4	16.5	17.2	17.0	13.1	22.4	17.4	19.0	20.6	8.0
V4	8.4	8	11.7	32.2	11.6	9.6	34.5	9.2	7.1	7.2	5.3	0.9
V6	10.6	9.4	8.1	6.9	9.0	7.4	6.8	9.8	8.1	10.7	3.9	4.3
V7	14.7	16.2	18.2	7.5	12.6	12.5	6.2	18.6	13.1	16.8	6.5	3.8
V8	17.9	19.4	19.6	7.7	14.8	20.9	8.9	25.3	18.8	19.4	18.4	5.9
V9	16.0	19.2	22.9	9.5	13.7	18.1	10.1	16.6	16.1	16.5	11.4	7.3
V10	15.9	14.5	14.7	8.2	7.4	18.4	19.4	16.7	15.2	27.4	11.1	7.4
V11	14.3	10.4	10.9	5.6	10.3	10.7	6.8	13.3	15.0	13.9	4.1	4.7
V12	14.5	13.2	12.9	7.0	12.5	17.8	23.0	17.3	14.0	13.6	4.6	6.3
V14	13.8	11.7	11.2	7.5	10.9	10.2	7.4	16.3	7.2	12.1	7.2	2.9
V15	16.1	15.7	15.7	33.3	7.1	16.2	5.2	15.1	18.3	17.8	16.1	5.3
V17	8.1	9.9	15.6	6.4	9.8	10.3	6.8	25.2	13.4	12.6	10.9	3.1
V18	15.4	17.1	9.5	6.6	9.3	16.1	16.0	12.0	11.2	13.5	6.5	3.8
NR2	15.6	15.0	14.3	6.9	14.0	14.8	10.7	14.2	12.8	26.4	5.8	6.4
NR3	8.9	6.2	7.3	28.5	7.2	7.6	7.9	6.2	4.8	5.6	5.7	2.8
NR4	19.9	22.4	11.7	9.0	13.6	19.7	4.2	24.7	17.2	24.5	10.8	6.4
NR5	21	17.8	17.3	7.5	14.1	19.2	5.4	21.5	17.7	27.1	6.9	8.4
NR6	7.8	8.7	11.5	5.9	28.2	8.9	25.3	9.6	14.2	15.1	3.3	3.1
NR7	13.5	13.1	13.3	5.6	9.8	14.3	6.2	20.8	19.7	16.1	7.9	4.5
NR8	11.8	10.3	10.1	31.1	9.8	10.1	14.9	12.6	10.5	11.9	7.2	5.3
NR9	14.9	15.0	16.6	8.2	13.5	17.9	5.4	6.6	17.4	15.3	18.0	4.4
NR10	11.8	12.3	15.1	4.1	11.7	15.4	26.1	16.6	14.9	13.9	6.7	5.7
NR11	7.8	8.7	8.4	8.6	7.3	8.9	12.3	7.4	6.3	7.9	6.4	3.4
NR12	8.0	13.2	15.3	8.4	10.1	11.8	4.0	20.4	13.7	16.2	6.2	8.7
NR13	20.4	18.2	22.3	8.0	14.3	21.7	28.8	21.7	19.0	22.1	10.0	10.0
NR14	14.4	13.7	9.9	7.0	11.3	14.1	8.6	23.5	13.5	15.4	8.7	3.3

Sample	Concentration of each PCR product fragment (ng/ µL)											DNA library (nM)
	1	2	3	4	5	6	7	8	9	10	11	
NR15	14.6	12.4	16.3	9.5	11.4	12.8	12.6	19.5	23.8	15.8	7.4	4.1
NR16	14.5	19.1	14.3	18.0	11.1	18.2	12.1	18.4	17.2	21.2	5.2	3.5
NR18	20.8	18.3	13.4	11.5	12.2	15.0	23.4	14.7	11.7	19.3	8.0	4.8
NR19	21.0	22.9	18.1	12.4	14.5	17.1	18.0	24.9	28.5	22.8	11.2	5.9
NR20	28.4	30.0	13.8	9.9	14.3	15.6	19.5	20.5	20.1	21.0	21.8	6.4
NR21	19.6	22.5	16.0	13.6	14.4	23.4	19.8	19.6	24.9	19.1	28.8	6.7
NR22	11.8	11.8	12.0	7.3	9.9	13.6	15.7	12.4	10.7	13.7	12.3	3.8
NR23	14.0	12.5	13.6	9.0	12.5	23.2	23.4	24.7	19.9	26.6	10.3	7.2
NR24	15.6	16.1	16.9	5.7	5.6	12.4	13.6	18.0	13.8	22.7	4.8	4.9
NR26	16.5	18.6	22.4	8.2	13.2	22.8	17.5	15.1	22.7	25.1	7.0	6.2
NR28	17.8	10.9	11.6	8.7	8.8	8.9	23.4	13.6	12.1	15.1	3.6	3.1
NR29	23.6	18.6	22.1	9.8	11.5	17.2	5.3	18.7	14.8	20.1	10.5	5.1
NR31	14.6	17.8	16.4	7.9	6.3	15.9	18.5	14.8	16.5	21.3	8.6	4.4
NR32	16.4	18.3	16.3	8.8	13.4	18.7	14.8	23.0	16.7	20	10.6	6.6
NR33	10.2	6.1	6.7	6.5	4.1	7.0	7.7	7.0	7.1	6.6	11.6	2.5
NR34	15.4	11.5	17.8	7.5	14.1	14.5	10.0	15.2	13.9	16.9	11.4	5.1
NR35	15.3	18.4	11.9	6.5	8.7	16.7	19.6	12.7	8.0	14.6	12.5	2.7

4.2.2.2 Summary results of primary NGS data analysis

Only data of 47 samples with adequate quality score (more than 30) were selected for further analysis. Total yield, Q-score, Total reads and Passed filter (PF) reads were reported in Table 23.

Table 23. Primary NGS data analysis

Run 1 (23 samples) Total yield = 8.35 Gbp Quality score (>Q30) = 56.84% Total reads = 18,639,512 reads Passed filter (PF) reads = 17,038,872 reads			Run 2 (24 samples) Total yield = 8.19 Gbp Quality score (>Q30) = 73.81% Total reads = 17,306,724 reads Passed filter (PF) reads = 16,225,164 reads		
Sample	% PF reads	Passed filter (PF) reads	Sample	% PF reads	Passed filter (PF) reads
V1	2.3003	428,765	V14	2.3811	412,090
V2	1.2768	237,989	V15	3.4058	589,432
V3	4.6612	868,825	V17	6.3594	1,100,604
V5	4.5018	839,114	V18	5.1125	884,806
V6	5.5867	1,041,334	V19	3.3245	575,362
V7	2.7515	512,866	NR14	2.9583	511,985
V8	5.0156	934,883	NR15	3.2105	555,632
V9	3.8379	715,366	NR16	3.7389	647,081
V10	4.5241	843,270	NR17	3.5664	664,760
V11	4.0106	747,556	NR18	3.4872	603,520
V12	3.8861	724,350	NR19	3.1665	548,017
NR2	3.0435	567,294	NR20	3.6957	639,605
NR3	2.7589	514,245	NR21	3.7332	646,095
NR4	3.493	651,078	NR22	3.6276	627,819
NR5	2.9585	551,450	NR23	3.6101	624,790
NR6	3.1638	589,717	NR24	2.3408	405,116
NR7	3.5664	664,760	NR26	3.3245	575,362
NR8	3.629	676,428	NR28	3.2361	560,063
NR9	3.3452	623,529	NR29	3.5819	619,910
NR10	3.9448	735,291	NR31	2.8582	494,661
NR11	2.8968	539,949	NR32	3.145	544,296
NR12	4.2989	801,294	NR33	3.3887	586,473
NR13	4.4827	835,553	NR34	3.7243	644,554
			NR35	3.0501	527,872

4.2.2.3 Mutations in the EnhII/BCP/PC region

This part of the study was performed in 47 patients with HBeAg-positive CHB. The distribution and percentages of EnhII/BCP/PC mutations detected in each patient are shown in Table 24.

There were 19.1% patients (9 out of 47) with EnhII mutations; 40.4% (19/47) with T1753C/A/G mutations, 59.6% (28/47) with BCP mutations, 19.1% (9/47) with PC mutations, and 19.1 (9/47) with both BCP and PC mutations. None of participants had G1899A mutation. The prevalence of nucleotide at C1653T in EnhII was 1.6-95.1%, A1753C/A/G in BCP was 1.3-59.8%, A1762T/G1764A in BCP was 1.1-97.7% and G1896A in the PC region was 1.0-71.0%.

Table 24. Prevalence of mutations in the EnhII/BCP/PC region

ID	EnhII	BCP (nt1742-nt1849)			precore (nt1814-nt2450)		
	C1653T	T1753C/A/G	A1762T	G1764A	T1858C	G1896A	G1899A
VR1	0	0	0	0	0	0	0
VR2	0	0	0	0	0	0	0
VR3	0	0	7.8	7.6	99.3	0	0
VR5	0	0	0	0	0	0	0
VR6	95.1	0	9.3	11.6	4.4	9.1	0
VR7	0	0	0	0	0	0	0
VR8	0	0	0	0	0	0	0
VR9	0	0	0	0	0	0	0
VR10	0	0	0	0	0	0	0
VR11	79.3	0	88.2	88.1	0	0	0
VR12	49.0	43.9	47.8	79.0	6.2	0	0
VR14	0	0	99.1	99.0	0	0	0
VR15	0	0	0	0	0	0	0
VR17	0	0	0	0	0	0	0
VR18	0	5.3	19.0	19.0	0	0	0
VR19	0	1.0	20.4	34.6	0	0	0

ID	EnhII	BCP (nt1742-nt1849)			precore (nt1814-nt2450)		
	C1653T	T1753C/A/G	A1762T	G1764A	T1858C	G1896A	G1899A
NR2	49.4	0	5.8	6.0	0	0	0
NR3	8.8	38.1	38.6	38.4	0	40.4	0
NR4	0	0	21.1	21.3	0	0	0
NR5	0	49.7	97.7	97.7	99.2	0	0
NR6	1.6	4.4	96.3	96.3	0	0	0
NR7	0	1.6	1.8	2.6	1.5	4.9	0
NR8	10.5	44.9	95.9	95.3	0	49.8	0
NR9	0	0	0	0	0	0	0
NR10	0	0	40.1	40.1	0	1.0	0
NR11	0	92	92.7	92.4	0	71.0	0
NR12	0	0	0	0	0	0	0
NR13	0	1.3	2.2	2.2	0	0	0
NR14	0	0	0	0	0	0	0
NR15	0	5.7	19.0	18.8	0	0	0
NR16	0	3.6	0	0	0	0	0
NR17	0	0	14.5	36.4	0	13.8	0
NR18	0	3.3	0	0	0	0	0
NR20	0	3.6	1.1	1.9	0	0	0
NR21	0	0	0	0	0	0	0
NR22	0	2.9	3.0	3.0	0	0	0
NR23	0	1.1	0	0	0	0	0
NR24	0	0	3.6	3.5	0	9.9	0
NR26	0	59.8	95.1	95.1	0	0	0
NR28	0	0	0	0	0	0	0
NR29	0	0	0	0	0	0	0
NR31	38.4	16.8	89.7	89.7	0	0	0
NR32	0	2.0	20.9	20.9	0	0	0
NR33	0	0	24.0	24.0	0	6.5	0
NR34	0	0	0	0	0	0	0
NR35	10	0	87.6	87.6	0	0	0

Data were expressed as percentage of mutations, VR; responders,
NR; non-responders

4.2.2.4 Mutations in the Pre-S/S region

In general, there are four common mutational patterns in the Pre-S/S region that have clinical correlation. These variations comprise of Pre-S1 and Pre-S2 start codon mutations, as well as Pre-S1 and Pre-S2 deletions. The distribution and amino acid changes of these variations are shown in Table 25.

Table 25. Prevalence of mutations in Pre-S/S region

ID	Pre-S1 start codon	Pre-S2 start codon	Pre-S1 deletion	Pre-S2 deletion
V1	0	0	0	0
V2	0	0	0	0
V3	0	0	0	0
V5	0	0	0	0
V6	0	0	0	0
V7	0	0	0	0
V8	1.4 (silent)	0	7.8	0
V9	0	0	1.6	0
V10	0	0	0	0
V11	0	12.9 (M to I)	1.0	0
V12	0	0	0	0
V14	0	26.5 (M to I)	0	0
V15	0	0	0	0
V17	0	0	0	0
V18	0	0	0	0
V19	0	0	0	0
NR2	0	0	0	0
NR3	0	0	13.7	1.5
NR4	0	0	0	0
NR5	0	0	0	0
NR6	0	0	0	0
NR7	0	0	0	0
NR8	0	0	4.8	0
NR9	0	0	0	0
NR10	0	0	43.8	0
NR11	0	0	0	0

ID	Pre-S1 start codon	Pre-S2 start codon	Pre-S1 deletion	Pre-S2 deletion
NR12	0	0	0	0
NR13	0	0	0	0
NR14	0	0	0	0
NR15	0	0	0	0
NR16	0	0	11.8	0
NR17	0	0	0	0
NR18	0	0	0	0
NR19	0	0	0	0
NR20	0	0	0	0
NR21	0	0	0	0
NR22	0	5.0 (silent)	0	0
NR23	0	0	0	0
NR24	0	0	5.8	0
NR26	0	0	5.2	0
NR28	0	0	0	0
NR29	0	0	0	0
NR31	0	0	2.9	0
NR32	2.3 (silent)	16.3 (M to I)	22.5	0
NR33	0	82.3 (M to I)	0	0
NR34	0	0	0	0
NR35	0	0	2.2	0

Data were expressed as percentage of mutations; silent; silent mutation (no amino acid change), (M to I); methionine changed to isoleucine

4.2.2.5 Mutational patterns in relation to VR

The comparison of baseline characteristics between responders (n = 17) and non-responders (n = 30) is shown in Table 26. There were no difference between groups in terms of age, gender ratio, baseline ALT, serum HBV DNA, HBsAg and HBcrAg levels.

Among various mutational patterns found in the EnhII/BCP/PC region, T1753C/A/G, G1896A and A1762T/G1764A mutations were more prevalent in non-responders than responders, although no statistical significance was reached. However the combined mutations of T1753C/A/G, and

A1762T/G1764A variants were significantly higher in responders than in non-responders. On the other hand, there was no difference in the distribution of Pre-S/S variants between groups.

Table 26. Baseline characteristics and HBV mutations detected by NGS

Characteristics	All patients (n=47)	Responders (n=17)	Non-responders (n=30)	*P*
Age, years	32.4±9.4	33.1±11.3	32.0±8.4	0.707
Gender (%males)	32 (68.1%)	9 (52.9)	23 (76.7%)	0.094
ALT, U/L	92.8±66.2	75.9±41.7	101.9±74.8	0.195
Log_{10} HBV DNA, IU/ml	7.3±1.1	7.4±0.9	7.2±1.1	0.434
Log_{10} HBsAg, , IU/ml	4.0±0.7	4.0±0.8	4.0±0.7	0.959
Log_{10} HBcrAg, , IU/ml	8.2±1.0	8.4±0.8	8.1±1.0	0.398
Mutational patterns in the EnhII/BCP/PC				
C1653T	9 (19.1%)	3 (17.6)	6 (20.0)	0.844
T1753C/A/G	19 (40.4%)	4 (23.5)	15 (50.0)	0.076
A1762T/G1764A	28 (59.6%)	8 (47.1)	20 (66.7)	0.188
T1753C/A/G +A1762T/G1764A	31 (66.0%)	8 (47.1)	23 (76.7)	0.040*
T1858C	5 (10.6%)	3 (17.6)	2 (6.7)	0.404
G1896A	9 (19.1%)	1 (5.9)	8 (26.7)	0.082
G1899A	-	-	-	-
Mutational patterns in the Pre-S/S				
Pre-S1 start codon	2 (4.3%)	1 (5.9)	1 (3.3)	0.677
Pre-S2 start codon	5 (10.6%)	2 (11.8)	3 (10.0)	0.850
Pre-S1 deletion	12 (25.5%)	3 (17.6)	9 (30.0)	0.351
Pre-S2 deletion	1 (2.1%)	-	1 (3.3)	0.447

Data were expressed as mean ± SD or n (%); ALT, alanine aminotransferase, *$P<0.05$

4.2.3 Intrahepatic HBsAg and HBcAg expression by immunohistochemistry

4.2.3.1 Pretreatment and end of treatment expression

Twenty paired liver biopsies obtained from patients with HBeAg-positive CHB were selected for immunohistochemistry. All liver sections were positive for intrahepatic HBsAg with various degrees of expression ranked on a scale of 0-5. The expression of HBsAg was exclusively positive on the surface of the hepatocytes (Figure 16), but was negative in the nucleus. In contrast, the expression of intrahepatic

HBcAg was strongly positive in the nucleus of the hepatocytes (Figure 17).

The comparison between intrahepatic HBsAg and HBcAg expression between baseline and end of treatment were further analyzed. Mean degree of intrahepatic HBsAg expression at end of therapy was significantly lower than baseline expression (2.5 ± 1.3 vs. 1.1 ± 1.1, $P < 0.001$) (Figure 16). Similarly, the expression of HBcAg at the end of treatment was significantly lower than baseline (4.1 ± 1.0 vs. 3.3 ± 1.2, $P = 0.002$) (Figure 17).
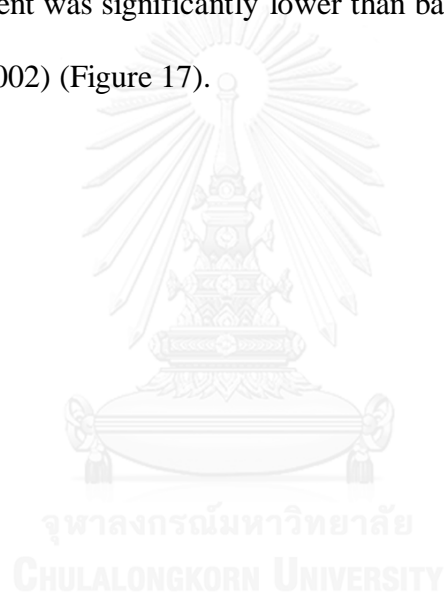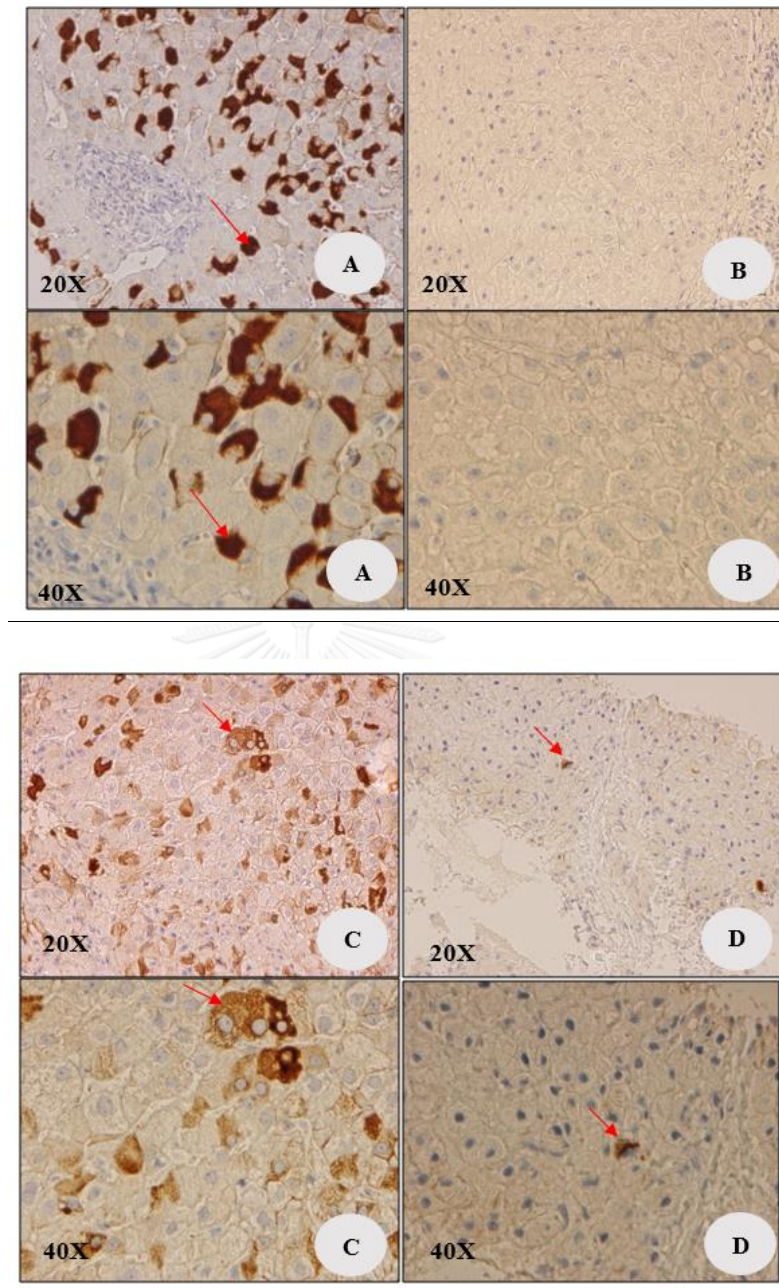
Figure 16. Expression of HBsAg in two patients with HBeAg-positive CHB. (A&C) represent baseline tissue biopsy of each participants and (B&D) represent tissue biopsy at the end of treatment of each participants. HBsAg was stained at surface of the hepatocytes (arrow heads). The degrees of expression were classified into 2-5 scores. Magnifications: 20x and 40x, respectively.
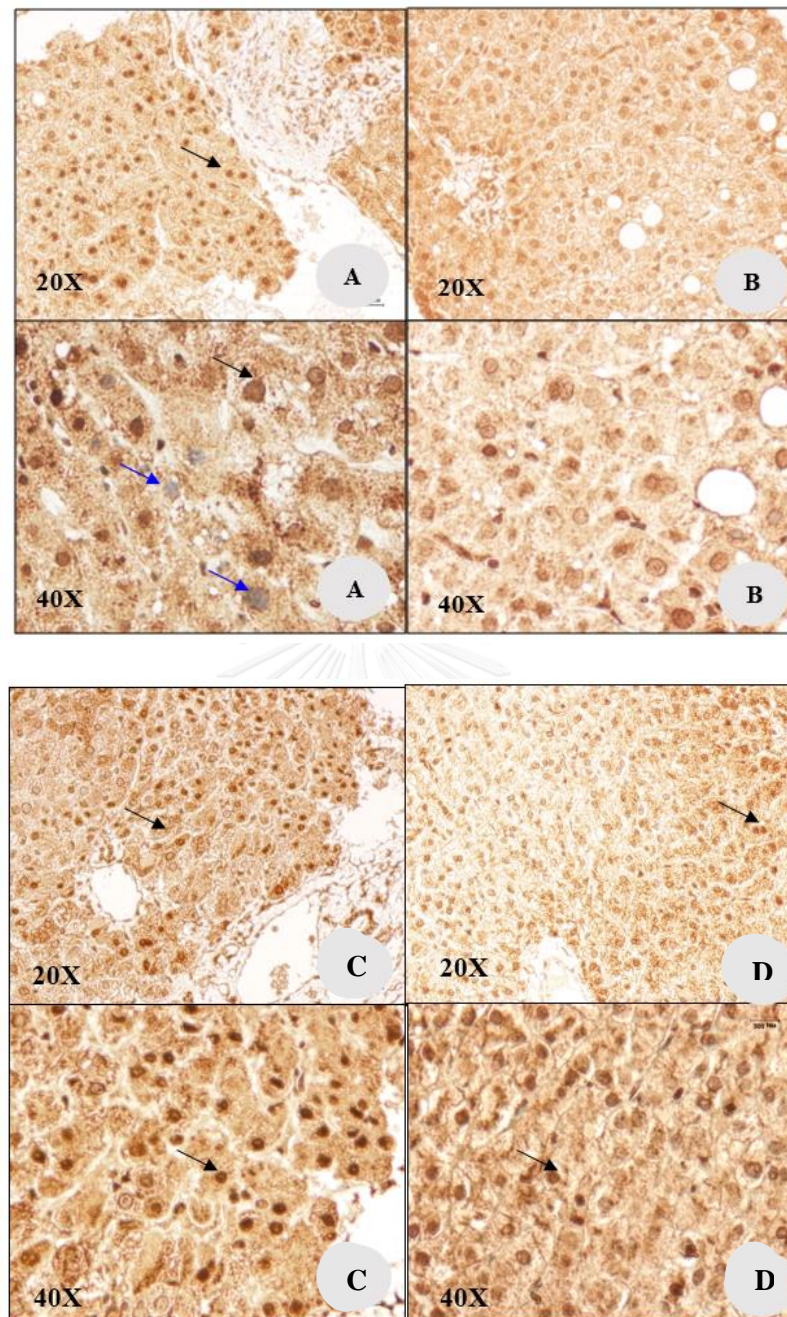
Figure 17. Expression of HBcAg in two patients with HBeAg-positive CHB. (A&C) represent baseline tissue biopsy of each participants and (B&D) represent tissue biopsy at the end of treatment of each participants. HBcAg was stained at nucleus of the hepatocytes (black arrow). Nucleus with negative HBcAg were marked (blue arrows). Magnifications: 20x and 40x, respectively.

4.2.3.2 Associations of intrahepatic expression and serum markers

The correlation between degree of intrahepatic HBsAg and HBcAg expression and serum HBsAg and HBcrAg levels in relation to PEG-IFN therapy were further examined. At end of treatment, intrahepatic HBsAg expression decline of ≥ 2 point scores was observed in 25% patients (5 out of 20) but the rest 75% (15/20) had no HBsAg decline. It was found that 80% of patients with HBsAg expression decline of ≥ 2 point could achieve VR ($P = 0.035$). In contrast, there was no correlation between the reduction of HBcAg expression and VR.

Patients with intrahepatic HBsAg decline of ≥ 2 points tented to have more reduction of serum HBsAg levels compare with those with <2 points HBsAg decline, although no statistical significance was observed (Figure 18A). Mean reduction of serum $\log_{10}$ HBsAg levels of the corresponding groups were as follows: week 4 (3.8 ± 1.0 vs. 4.0 ± 0.7, $P = 0.579$), week 12 (3.4 ± 0.9 vs 3.9 ± 0.8, $P = 0.236$), week 24 (3.1 ± 1.5 vs. 3.1 ± 1.2, $P = 0.956$), and week 48 (2.1 ± 1.5 vs. 3.0 ± 1.5, $P = 0.289$).

Patients with a decline of intrahepatic HBcAg expression of ≥ 2.0 points also exhibited a slightly greater reduction in serum HBcrAg levels than those who had no such HBcAg decline, with mean reduction of $\log_{10}$ HBcrAg of the corresponding groups as follows: week 4 (7.2 ± 1.8 vs. 8.1 ± 1.2, $P = 0.400$), week 12 (7.2 ± 2.0 vs 7.9 ± 1.4, $P = 0.391$), week 24 (6.8 ± 1.2 vs. 6.6 ± 1.0, $P = 0.763$), and week 48 (5.9 ± 1.6 vs. 6.3 ± 1.4, $P = 0.588$). (Figure 18B)

(A)

(B)
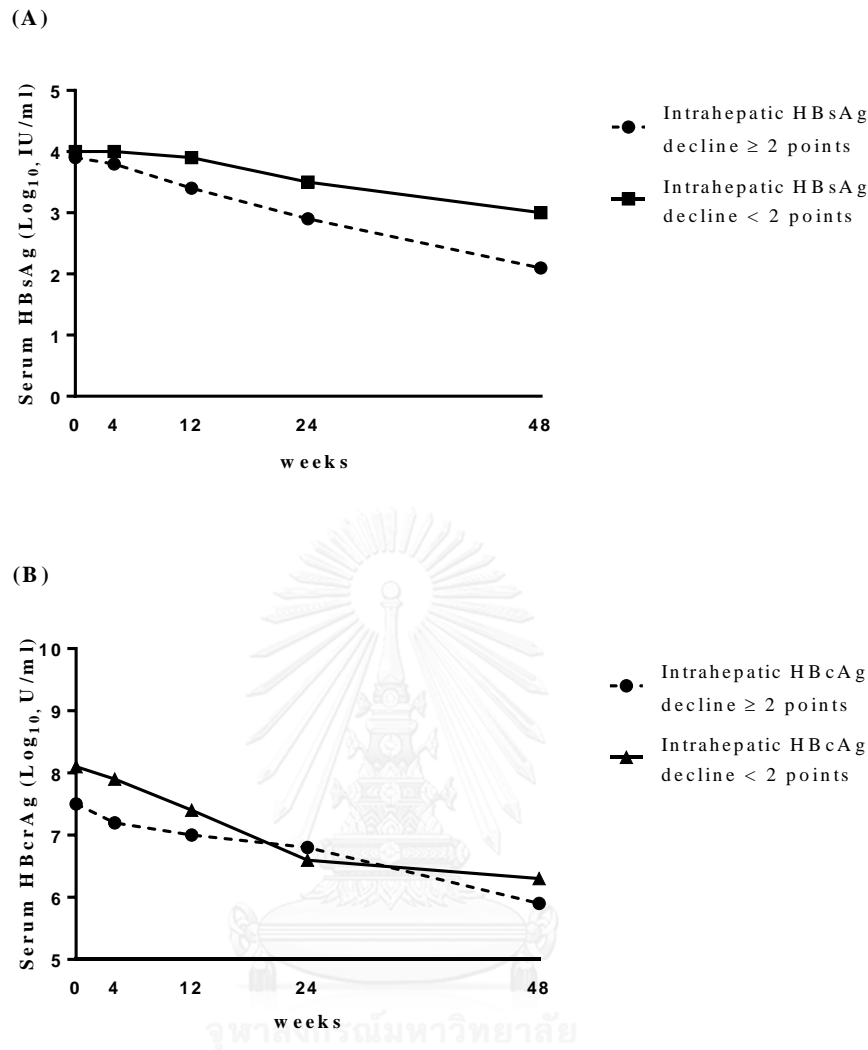
Figure 18. Serum HBsAg (A) and HBcrAg (B) declines in patients with high and low reduction scores of intrahepatic expression

4.3 Predictive roles of viral markers in HBeAg-negative CHB

4.3.1 Characteristics of patients based on treatment response

One-hundred twenty-one patients (85 male) were recruited in this part of the study. At week 72, subjects were classified into 2 groups as responders (n = 48) and non-responders (n = 73). At baseline, responders had significantly lower serum HBsAg levels and higher proportion of HBV genotype C than non-

responders. There was no significant difference between groups with respect to age and gender distribution, degree of liver fibrosis, serum HBV DNA and HBcrAg levels, as well as intrahepatic HBV DNA and cccDNA levels (Table 27).

Table 27. Baseline characteristics of the patients with HBeAg-negative in relation to VR.

Characteristics	Responders (n=48)	Non-responders (n=73)	P
Age, years	39.3±10.4	40.7±8.9	0.418
Gender (%males)	34 (70.8%)	51 (69.9%)	0.909
ALT, U/L	71.7±33.8	74.6±38.2	0.665
HBV genotypes			0.004*
B	3 (6.2%)	20 (27.4%)	
C	45 (93.8%)	53 (72.6%)	
Log_{10} HBV DNA, IU/ml	5.4±0.9	5.4±0.8	0.794
Log_{10} HBsAg, IU/ml	3.3±0.4	3.6±0.5	0.006
Log_{10} HBcrAg, , IU/ml	4.2±1.3	4.3±1.2	0.820
Log_{10} intrahepatic HBV DNA, copies/cEq	2.0±1.6	2.7±2.4	0.149
Log_{10} intrahepatic cccDNA, copies/cEq	0.4±1.3	0.3±1.4	0.932
Log_{10} intrahepatic pgRNA, copies/cEq	1.5±1.6	1.3±2.0	0.762
Liver fibrosis (kPa)	7.7±2.1	7.9±3.2	0.742

Data was expressed as mean±SD and n (percentage), *$P<0.05$

4.3.2 Baseline characteristics of patients

Similar results were found in patients with HBeAg-negative CHB, as log_{10} serum HBcrAg levels correlated with log_{10} serum HBV DNA levels ($r = 0.672$, $P < 0.001$) and intrahepatic cccDNA levels ($r = 0.313$, $P = 0.006$) (Figure 19B and 19C), but were not correlated with HBsAg levels ($r = 0.064$, $P = 0.483$) There was no correlation between serum HBsAg and HBcrAg levels ($r = 0.064$, $P = 0.483$), as well as with intrahepatic cccDNA levels ($r = 0.069$, $P = 0.549$) (Figure 19A and 19D).

(A)
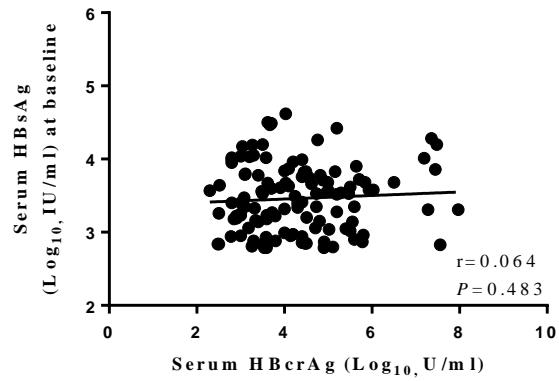
(B)
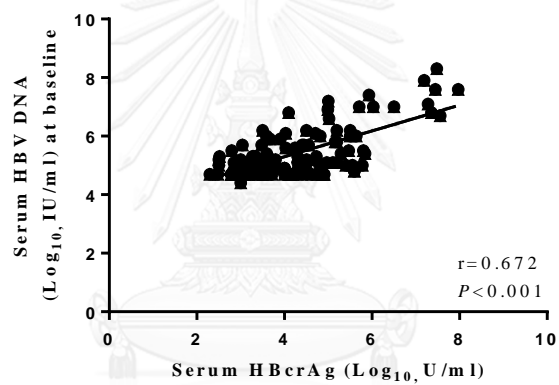
(C)
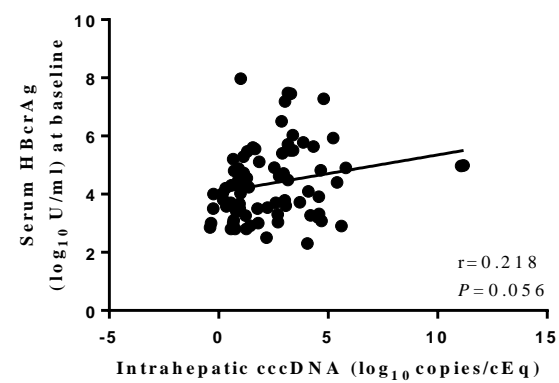
(D)
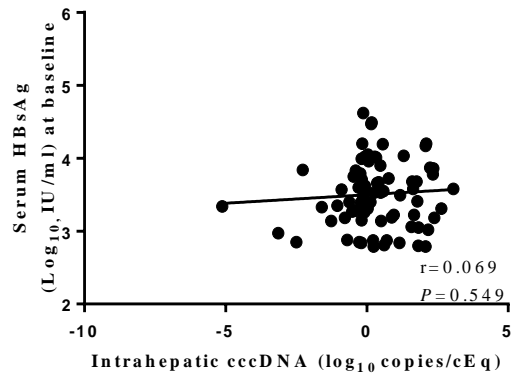
Figure 19. Correlation of viral markers at baseline in patients with
HBeAg-negative CHB

4.3.3 Viral markers at end of treatment

The mean of serum and intrahepatic markers at end of treatment in
responders versus non-responders were explored (Table 28). Responders had
significantly lower mean serum HBV DNA, HBsAg and HBcrAg levels than
non-responders. However, no significant difference of intrahepatic markers
including HBV DNA, cccDNA and pgRNA between groups were identified.
PEG-IFN treatment leaded to significant reduction of $\log_{10}$ serum HBV DNA,
HBsAg and HBcrAg levels in responders than non-responders. However, no
significant difference between groups in $\log_{10}$ intrahepatic HBV DNA,
cccDNA, and pgRNA was found.

Table 28. HBV viral markers at end of PEG-IFN treatment

End of treatment	Responders (n=48)	Non-responders (n=73)	P
Serum			
Log_{10} HBV DNA, IU/ml	0.3±0.6	1.2±1.6	<0.001*
Log_{10} HBsAg, IU/ml	1.6±1.2	3.1±0.7	<0.001*
Log_{10} HBcrAg, U/ml	3.1±1.0	3.5±0.9	0.012*
Intrahepatic			
Log_{10} HBV DNA, copies/cEq	1.9±2.4	2.3±1.9	0.437
Log_{10} cccDNA, copies/cEq	(-0.7)±2.4	(-0.3)±1.6	0.386
Log_{10} pgRNA, copies/cEq	1.4±1.4	1.2±1.3	0.670
Mean reduction			
Log_{10} HBV DNA, IU/ml	5.1±0.8	4.3±1.6	<0.001*
Log_{10} HBsAg, IU/ml	1.7±1.1	0.4±0.5	<0.001*
Log_{10} HBcrAg, U/ml	1.1±0.8	0.7±0.8	0.009*
Log_{10} intrahepatic HBV DNA, copies/cEq	0.0±2.5	0.4±2.0	0.503
Log_{10} intrahepatic cccDNA, copies/cEq	1.1±2.2	0.7±1.7	0.347
Log_{10} intrahepatic pgRNA, copies/cEq	0.1±1.6	0.1±1.6	0.995

Data were expressed as mean ± SD and n (percentage), *P<0.05

4.3.4 Kinetics of HBsAg regarding to treatment response

To identify the effect of PEG-IFN on serum levels of HBV markers in patients with HBeAg-negative CHB, the kinetics of serum HBsAg and HBcrAg were examined. Responders presented significant decline in serum HBsAg levels compared with non-responders, with the mean reduction of log_{10} HBsAg as follows: week 4 (0.2 ± 0.3 vs. 0.0 ± 0.2, P = 0.005), week 12 (0.9 ± 0.8 vs 0.2 ± 0.3, P < 0.001), week 24 (1.3 ± 1.0 vs. 0.3 ± 0.4, P < 0.001), week 48 (1.7 ± 1.1 vs. 0.4 ± 0.5, P < 0.001), and week 72 (1.3 ± 1.0 vs. 0.2 ± 0.3, P < 0.001).

Among the responder group, patients with HBsAg clearance (n = 9) showed distinct decline in serum HBsAg levels compared to those without HBsAg clearance (n = 39), particularly after week 12 through the end of follow-

up. Responders with HBsAg clearance had greater HBsAg decline at the end of treatment. Means level of $\log_{10}$ HBsAg decrease in patients with or without HBsAg clearance were as follows; week 4 (0.4 ± 0.5 vs. 0.2 ± 0.3, $P = 0.082$), week 12 (1.5 ± 1.0 vs. 0.8 ± 0.7, $P = 0.011$), week 24 (2.4 ± 0.8 vs. 1.0 ± 0.8, $P < 0.001$), week 48 (2.9 ± 0.5 vs. 1.4 ± 1.0, $P < 0.001$), and week 72 (3.0 ± 0.3 vs. 0.9 ± 0.7, $P < 0.001$). (Figure 20)
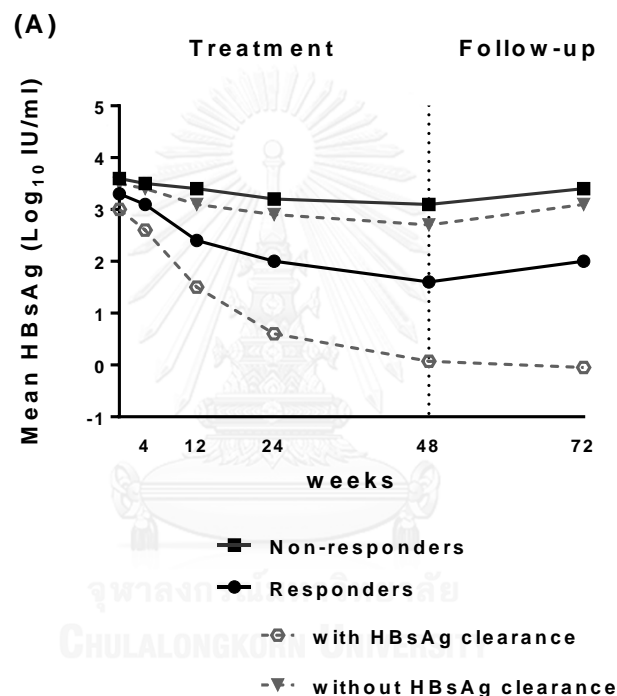
Figure 20. Kinetics of HBsAg level according to treatment response

4.3.5 Kinetics of HBcrAg regarding to treatment response

The mean decrease of $\log_{10}$ HBcrAg levels was higher in responders than non-responders; week 4 (0.4 ± 0.6 vs. 0.2 ± 0.6, $P = 0.182$), week 12 (0.6 ± 0.7 vs. 0.3 ± 0.7, $P = 0.015$), week 24 (0.7 ± 0.8 vs. 0.4 ± 0.7, $P = 0.011$), week 48 (1.1 ± 0.8 vs. 0.7 ± 0.8, $P = 0.009$), and week 72 (1.0 ± 0.9 vs. 0.4 ± 1.1, $P = 0.001$)

Among responders, patients with or without HBsAg clearance had comparable decline in HBcrAg levels. Mean levels of $\log_{10}$ HBcrAg decline in the corresponding groups were as follows; week 4 (0.4 ± 0.4 vs. 0.4 ± 0.7, P = 0.967), week 12 (0.5 ± 0.4 vs 0.6 ± 0.8, P = 0.735), week 24 (0.5 ± 0.4 vs. 0.8 ± 0.8, P = 0.324), week 48 (1.0 ± 0.6 vs. 1.2 ± 0.9, P = 0.522) and week 72 (0.6 ± 0.5 vs. 1.1 ± 1.0, P = 0.109). (Figure 21)
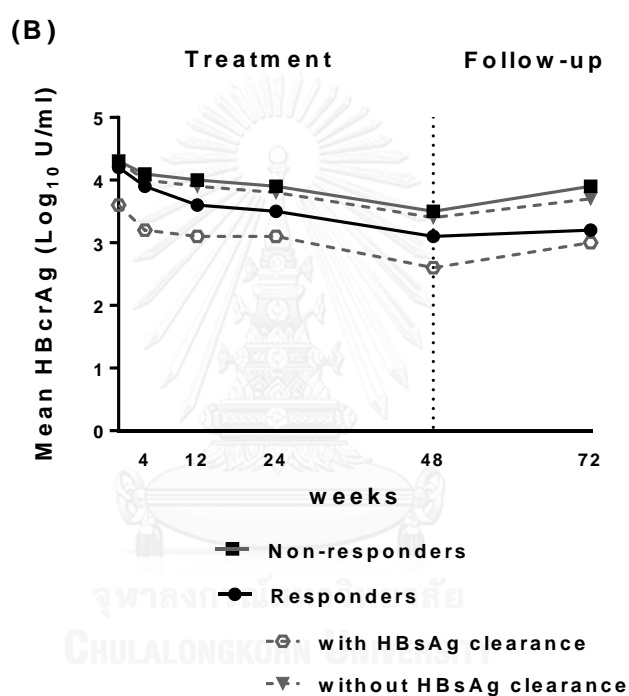
Figure 21. Kinetics of HBcrAg level according to treatment response

4.3.6 Predictors of VR during therapy

At week 12, the absence of HBsAg decline from baseline had a NPV of 81.0%, while no decline of HBcrAg levels had a NPV of 73.5%. When both criteria were used together, the NPV of achieving VR at week 12 was 100%. (Table 29)

In contrast, HBsAg decline from baseline ≥0.5 log IU/mL at week 12 provided a PPV of 77.5%, while the corresponding figure for HBcrAg decline ≥0.5 log U/mL was 52.3%. Patients in whom both declines were present had a PPV of 84.2%. (Table 30)

Table 29. The absence of HBsAg and HBcrAg decline at week 12 in predicting VR

Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Log$_{10}$ HBsAg (IU/mL)	91.7	23.3	44.0	81.0
Log$_{10}$ HBcrAg (U/mL)	85.4	34.2	46.1	78.1
Combination of both markers*	100.0	15.1	43.6	100.0

*Combined HBsAg and HBsAg levels (no decline);
PPV: positive predictive value, NPV: negative predictive value

Table 30. Decline in HBsAg and HBcrAg levels at week 12 in predicting VR

Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Log$_{10}$ HBsAg (IU/mL)				
≥0.5 log IU/mL	64.6	87.7	77.5	79.0
Log$_{10}$ HBcrAg (U/mL)				
≥0.5 log IU/mL	47.9	71.2	52.3	67.5
Combination of both markers*				
≥0.5 log IU/mL	33.3	95.9	84.2	68.6

*Combined HBsAg and HBsAg levels (decline ≥0.5 log IU/mL);
PPV: positive predictive value, NPV: negative predictive
value

CHAPTER V

DISCUSSION AND CONCLUSION

Overall, HBeAg-positive and HBeAg-negative CHB are characterized as different phases in the natural manifestation of chronic HBV infection because they have diverse immunological profiles and different viral replicative activity (9). Typically, the HBeAg-negative group trends to be older and has more progressive liver disease. This is likely related to the fact that HBeAg-negative CHB usually manifests in the later course of chronic HBV infection. The results in this study confirmed previous reports that HBeAg-positive CHB, compared with the HBeAg-negative group, had significantly higher baseline serum viral markers (HBV DNA, HBsAg and HBcrAg) and intrahepatic markers (HBV DNA, cccDNA) (9, 102).

Besides their difference in viral replication, these two groups of patients with CHB appear to have rather different response rates to PEG-IFN therapy. For example, two essential studies have reported that the HBeAg-positive and HBeAg-negative groups could achieve VR in approximately 30-40% and 20-30%, respectively (103, 104). In addition to its low response rates, therapeutic predictors of response to PEG-IFN in patients with HBeAg-positive and HBeAg-negative CHB are currently limited. Thus, identification of effective baseline and on-treatment viral markers during PEG-IFN therapy is essentially needed.

The quantification of intrahepatic cccDNA, which is the template of viral replication, could theoretically be an ideal marker in predicating treatment outcome. However, its measurement in clinical practice is restricted by the need of liver biopsy, which is considered to be an invasive procedure. Moreover, there is no commercially

available assay for cccDNA at present. In the past years, serum HBsAg level has emerged as a surrogate of cccDNA and has been used as a predictor to PEG-IFN treatment. In this study, however, a positive correlation between serum HBsAg and intrahepatic cccDNA was detected exclusively in HBeAg-positive CHB, but not in the HBeAg-negative group. This observation is consistent with previous reports indicating that there is no correlation between the levels of cccDNA and serum HBsAg in HBeAg-negative CHB (102, 105, 106). This finding might be related to the complexity of HBsAg regulation and expression in HBeAg-negative CHB, which includes its transcriptional activity and viral integration into host genome that could generate HBsAg from another source beyond cccDNA (107). The dissociation between serum HBsAg and intrahepatic replication in the HBeAg-negative group supports the concept that baseline HBsAg level might not be a reliable serum marker of cccDNA in this group of patients.

The study also examined the performance of serum HBcrAg, which represents a novel quantitative marker of HBV. The data demonstrated that quantitative HBcrAg at baseline correlated well with intrahepatic cccDNA in patients with HBeAg-positive and HBeAg-negative CHB, although a stronger correlation was found in the HBeAg-positive group. These findings are in agreement with other studies in patients with HBeAg-positive CHB treated with NA and those underwent liver transplantation (14, 15). Taken together, these data indicate that serum HBcrAg might represent a better surrogate marker of intrahepatic cccDNA compared with serum HBsAg.

Interestingly, the decline of both HBsAg and HBcrAg levels during therapy paralleled with the reduction in cccDNA levels in both HBeAg-positive and HBeAg-negative CHB. These findings suggest that dynamic changes of these viral antigens

could indirectly reflect a reduced intrahepatic cccDNA pool in response to antiviral therapy. Our data also showed that serial monitoring HBsAg and HBcrAg levels during PEG-IFN therapy may have clinical applications as responders and non-responders have distinct viral kinetics. For example, among responders in the HBeAg-positive group, the average reduction of serum HBsAg and HBcrAg after standard course of PEG-IFN therapy were approximately 2.0 and 3.0 $\log_{10}$, respectively. The corresponding data were relatively lower in the HBeAg-negative group, with an average reduction of 1.7 and 1.0 $\log_{10}$, respectively. In contrary, patients without achieving VR had limited reduction in these viral markers. As a result, early viral kinetics, particularly at week 12 during therapy, could help select individuals who might have limited chance of achieving response as NPV of the viral markers at this time-point are high.

In HBeAg-positive CHB, recent pooled data of three randomized trials have shown that the stopping rule at week 12 on the basis of HBsAg > $\log_{10}$ 3.4 IU/mL has high NPV (92–98%) in patients infected with genotypes B or C (101). However, its ability to predict non-responders in our study was limited (NPV: 80%), as an ideal stopping rule should reach NPV of $\geq$ 95% (108). On the other hand, HBcrAg level at this time-point had a NPV of almost 95%. It should also be mentioned that the NPV of combined HBcrAg and HBsAg was better than using HBsAg alone but was still lower than that of HBcrAg level. Thus, applying quantitative HBcrAg could be a better stopping rule than using HBsAg level or the combination of both markers in HBeAg-positive CHB.

Currently, there is no effective predictor for PEG-IFN therapy in patients with HBeAg-negative CHB. Our results showed that the absence of HBsAg decline from

baseline at week 12 yielded NPV of 81.0%. The corresponding figures for the absence of HBcrAg decline was approximately 74%. When both criteria were used together, the NPV of predicting VR was 100%. Thus, the combined use of HBsAg and HBcrAg levels during PEG-IFN therapy could identify patients with very low probability of response and may help individualize on-treatment decision-making in patients with HBeAg-negative CHB.

The final goal of therapy is achieving HBsAg clearance or seroconversion, which results in a better clinical outcome, such as reduced incidence of cirrhosis and HCC development (1). This end-point is, however, uncommon, accounting for approximately 5% of patients treated with PEG-IFN. Remarkably, among responders achieving HBsAg clearance, the activity of HBcrAg was still observed in serum samples. This finding is in line with recent reports demonstrating that low HBcrAg levels are detectable in some patients after spontaneous or treatment-associated HBsAg clearance (109). Thus, the prediction of HBsAg clearance after PEG-IFN therapy might be rather limited based on monitoring HBcrAg reduction alone. In contrast, our results demonstrated clearly that patients who had HBsAg clearance achieved more rapid decline in serum HBsAg levels than those without clearance.

Currently, the effect of HBV mutations on the therapeutic outcome of PEG-IFN in patients with HBeAg-positive CHB is mostly unknown. In fact, the most well-known variants that have clinical association are located within the EnhII/BCP/PC and Pre-S/S regions. The results obtained by conventional Sanger sequencing demonstrated that the presence of A1762T/G1764A at baseline was associated with significantly lower rates of PEG-IFN response. This finding is consistent with some previous studies demonstrating that patients harboring double BCP mutants responded poorer to the

therapy (110, 111). In contrast, some other reports showed that presence of viral mutants harboring mutations in the PC/BCP region could enhance the achievement of VR (76, 77). Together, these results generated conflicting data with regard to the role of viral mutants in determination of therapeutic efficiency.

The assessment of viral mutations based on PCR-based direct Sanger sequencing has limitations relating to its relatively low sensitivity and inability to detect novel mutations. To overcome these limitations, we determined the pre-existing common mutants by NGS method, which could help analyze genetic heterogeneity of HBV quasispecies and enhance ability to detect minority variants. Using the high-efficiency technology, our data showed that combined variants of T1753C/A/G and A1762T/G1764A, instead of the BCP double mutants, were associated with poor treatment outcome. Thus, the detection of these variants at baseline might be used as biomarker to identify patients who might have a high or low chance of achieving treatment response.

There are some limitations in the present reports. First, the number of patients with HBeAg-positive CHB recruited in the studying of viral kinetics was relatively small compared with the HBeAg-negative group. In addition, the assessments of intrahepatic cccDNA and the expression of viral antigens were restricted due to the requirement of liver biopsy, which is considered to be an invasive procedure. Thus, pre- and post-treatment liver tissue specimens were not available in all patients enrolled. In this respect, a larger sample size could certainly improve the statistical power and consistency of the data. Finally, although we investigated several serum and intrahepatic viral markers, but did not yet evaluate the roles of host and environmental factors that might be associated with treatment outcome.

In conclusion, our data demonstrated that baseline serum and intrahepatic viral markers differed significantly between the HBeAg-positive and HBeAg-negative groups. Baseline quantitative HBcrAg correlated well with intrahepatic cccDNA in both groups. Conversely, baseline HBsAg levels had a correlation with cccDNA only in the HBeAg-positive group but not in the HBeAg-negative group. However, kinetics of both serum HBsAg and HBcrAg levels had positive correlation with intrahepatic cccDNA levels in both groups. Thus, reduction in serum HBsAg and HBcrAg might represent the efficacy of PEG-IFN in decreasing cccDNA levels and could help predict treatment outcome. Our data also revealed that patients with HBeAg-positive CHB infected with HBV variants in the EnhII/BCP/PC region had significantly lower response rates compared with those infected with WT strains. The decline in the expression of intrahepatic HBsAg, but not intrahepatic HBcAg, was associated with treatment response. However, further studies with larger sample sizes are required to confirm these observations.

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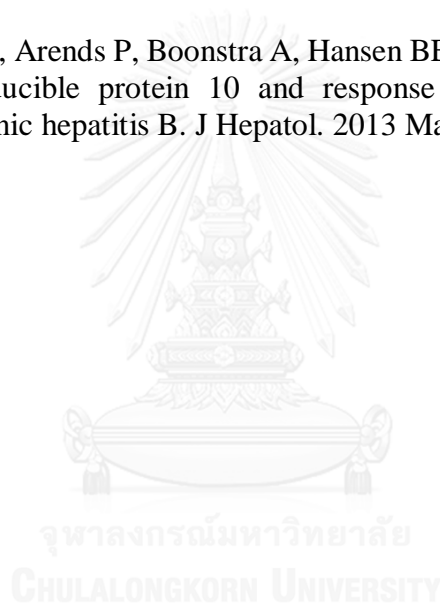
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APPENDIX
REAGENTS AND BUFFER

1. 5X Tris borate buffer (5X TBE, 1,000 ml)
1.1 Tris-base	54	g
1.2 Boric acid	27.5	g
1.3 EDTA (pH 8.0)	4.16	g
1.4 Distilled water	1,000	ml

2. 2% of agarose gel
2.1 Agarose	2	g
2.2 1X TBE buffer	100	ml

3. Lysis Buffer (50 ml)
3.1 Tris-HCl	0.105	g
3.2 EDTA	0.125	g
3.3 SDS	0.335	g
3.4 Distilled water	50	ml

4. Proteinase K [20mg/ml]
4.1 Proteinase K	100	mg
4.2 Distilled water	5	ml

5. Glycogen [20mg/ml]
5.1 glycogen	0.25	g
5.2 Dilstilled water	12.5	ml

6. 2M NaOAC (25 ml)
6.1 NaOAC	4.10	g
6.2 Distilled water	25	ml

7. 6X loading dye
7.1 Sucrose	6.0	g
7.2 Dromopheol blue	0.038	g
7.3 Distilled water	15	ml

8. LB agar (100 ml)
8.1 Agar	1.5	g
8.2 Tryptone	1.0	g
8.3 Yeast extract	0.5	g
8.4 NaCl	0.5	g
8.5 Distilled water	100	ml

*After autoclaved, add ampicillin [100 mg/ml] 100 µL

9. LB broth
9.1 Tryptone	1.0	g
9.2 Yeast extract	0.5	g

9.3 NaCl   0.5   g
9.4 Distilled water   100   ml

*After autoclaved, add ampicillin [100 mg/ml] 100 µL

10. SOC medium

10.1 Tryptone   1.0   g
10.2 Yeast extract   0.5   g
10.3 1M NaCl   1.0   ml
10.4 1M KCl   0.25   ml
10.5 Distilled water   100   ml

*After autoclaved, add 10 µL of [2M Mg^{2+}] and 10 µL of [2M glucose]

11. 0.1M Sodium citrate in 10% ethanol (50 ml)

11.1 Sodium citrate dehydrate   1.47   g
11.2 10% ethanol   50   ml

12. 8M NaOH

12.1 NaOH   3.2   g
12.2 Distilled water   10   ml

13. 0.3M Guanidine hydrochloride in 95% ethanol (50 ml)

13.1 Guanidine hydrochloride   1.43   g
13.2 95% ethanol   50   ml

14. 1% SDS

14.1 SDS   0.5   g
14.2 Distilled water   50   ml

15. Formula for DNA library preparation

Moles	$\dfrac{m\ (g)}{M.W.}$	=	$\dfrac{CV\ (ml)}{1000}$
ng/uL covert to g/ml	$\dfrac{m \times 10^{(-9)}}{660 \times length^*}$	=	$\dfrac{= C \times 10^{(-3)}}{1000}$
	$C\ [moles]$	=	$\dfrac{m \times 10^{(-3)}}{660 \times length}$
Moles convert to nmoles	$C\ [nM]$	=	$C \times 10^9$

*length (bp) = mean or median (430 bp)

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Education: 2010-2011   Master of Science degree in Medical Biochemistry, Faculty of Medicine, Chulalongkorn University

2006-2009 Bachelor of Science (BS) degree in Medical Science, Faculty of Medical Science, Naresuan University

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Published journal:

1. Chuaypen N, Boonla C, Dissayabutra T, Predanon C, Ruangvejvorachai P, Waivijit U, Tosukhowong P. Increased intrarenal expression of Sodium-Dicarboxylate Cotransporter-1 nephrolithiasis patients associates with acidic urine pH. Asian Biomedicine. 4 August 2013 (7); 571-577. DOI: 10.5372/1905-7415.0704.214.

2. Chuaypen N, Posuwan N, Payungporn S, Tanaka Y, Shinkai N, Poovorawan Y, Tangkijvanich P. Serum hepatitis B core-related antigen as a treatment predictor of pegylated interferon in patients with HBeAg-positive chronic hepatitis B. Liver international : official journal of the International Association for the Study of the Liver. 2016;36(6):827-36.

Academic award:

Outstanding academic performance award in master degree (2011)

Young investigator award (APASL at Tokyo, Japan, 2016)