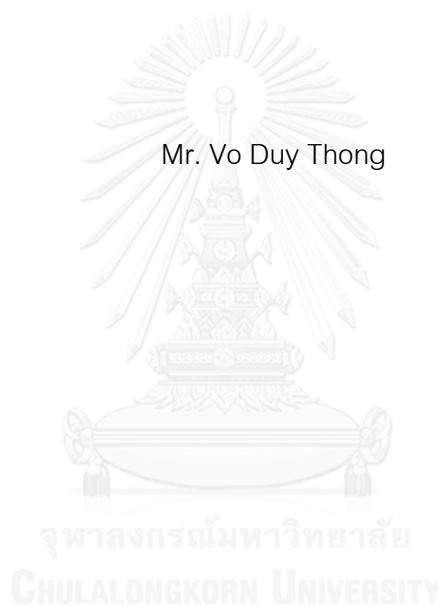INFLUENCE OF HOST AND VIRAL FACTORS IN HEPATITIS C VIRUS INFECTION:

ROLE OF TA REPEAT, IFNL3 AND IFNL4 POLYMORPHISMS IN HCV INFECTION AND

OUTCOME OF TREATMENT

Mr. Vo Duy Thong

A Dissertation Submitted in Partial Fulfillment of the Requirements

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บทบาททางพันธุกรรมของมนุษย์ และปัจจัยของไวรัสในการติดเชื้อไวรัสตับอักเสบซี: ความหลากหลายทางพันธุกรรมของ IFNL3 และ IFNL4 ในผู้ติดเชื้อไวรัสตับอักเสบซี และผลของการรักษา

นายวอ ยุย โทง

Thesis Title	INFLUENCE OF HOST AND VIRAL FACTORS IN HEPATITIS C VIRUS INFECTION: ROLE OF TA REPEAT, IFNL3 AND IFNL4 POLYMORPHISMS IN HCV INFECTION AND OUTCOME OF TREATMENT
By	Mr. Vo Duy Thong
Field of Study	Medical Science
Thesis Advisor	Professor Yong Poovorawan, M.D.
Thesis Co-Advisor	Professor Pisit Tangkijvanich, M.D. Ph.D

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

-- Dean of the Faculty of Medicine

(Associate Professor Sophon Napathorn, M.D.)

THESIS COMMITTEE

-- Chairman

(Associate Professor Wilai Anomasiri, Ph.D.)

-- Thesis Advisor

(Professor Yong Poovorawan, M.D.)

-- Thesis Co-Advisor

(Professor Pisit Tangkijvanich, M.D. Ph.D)

-- Examiner

(Professor Sittisak Honsawek, M.D. Ph.D)

-- Examiner

(Associate Professor Piyawat Komolmit, M.D. Ph.D)

-- External Examiner

(Associate Professor PTeeraporn Chinchai, Ph.D.)

วอ ยุย โทง : บทบาททางพันธุกรรมของมนุษย์ และปัจจัยของไวรัสในการติดเชื้อไวรัสตับอักเสบซี: ความหลากหลาย ทางพันธุกรรมของ IFNL3 และ IFNL4 ในผู้ติดเชื้อไวรัสตับอักเสบซี และผลของการรักษา (INFLUENCE OF HOST AND VIRAL FACTORS IN HEPATITIS C VIRUS INFECTION: ROLE OF TA REPEAT, IFNL3 AND IFNL4 POLYMORPHISMS IN HCV INFECTION AND OUTCOME OF TREATMENT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ยง ภู่วรวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. นพ. พิสิฐ ตั้งกิจวานิชย์, 155 หน้า.

ไวรัสตับอักเสบ ซี เป็นปัญหาทางสารณสุขที่สำคัญโดยมีผู้ป่วยทั่วโลกกว่า 170 ล้านราย การติดเชื้อ ไวรัสตับอักเสบ ซี เป็นผลให้เกิดตับอักเสบเรื้อรัง ตับแข็งและมะเร็งตับ โดยเป็นสาเหตุหลักของการเปลี่ยนตับทั่วโลก ไวรัสตับอักเสบ ซี สายพันธุ์ที่ 6 เป็นสายพันธุ์ที่จำเพาะในแถบจีนตอนใต้และเอเชียตะวันออกเฉียงใต้โดยพบมากถึง 30% ของการติดเชื้อทั้งหมดในกลุ่มประเทศ เหล่านี้ จากข้อมูลการศึกษาของผู้วิจัยพบว่าการตรวจแอนติเจนในแกนหลักของไวรัสตับอักเสบซีเป็นการตรวจใหม่ที่จะมีประโยชน์ ในการยืนยันการวินิจฉัยการติดเชื้อไวรัสตับอักเสบซีและสามารถใช้ในการประเมินผลการรักษาและการดำเนินโรค จากการศึกษา พบว่าปริมาณของแอนติเจนในแกนหลักของไวรัสตับอักเสบซีมีความสัมพันธ์กับปริมาณอาร์เอ็นเอของไวรัสตับอักเสบซีโดยเฉพาะ ในกลุ่มผู้ป่วยที่มีการติดเชื้อร่วมกับไวรัสเอชไอวี นอกจากนี้ระดับแอนติเจนในแกนหลักของไวรัสตับอักเสบซียังมีความสัมพันธ์กับ ลักษณะทางพันธุกรรมของผู้ป่วยที่ตำแหน่ง *ss469415590* การตรวจแอนติเจนในแกนหลักของไวรัสตับอักเสบซีเป็นการตรวจที่มี ความน่าเชื่อถือสามารถตรวจได้รวดเร็วและมีความเที่ยงตรงโดยอาจใช้เป็นทางเลือกในการตรวจแทนการตรวจอาร์เอ็นเอของไวรัส ตับอักเสบซีในบริบทที่มีข้อจำกัดในการตรวจอาร์เอ็นเอของไวรัสตับอักเสบซี

ปัจจัยทางพันธุกรรมมีผลต่อการติดเชื้อของไวรัสตับอักเสบซีโดยอาจมีผลให้ร่างกายสามารถกำจัดเชื้อออกไปหรือเกิด การติดเชื้อเรื้อรังจนนำไปสู่ภาวะตับแข็ง ความหลากหลายทางพันธุกรรมของยีนอินเตอร์ลิวคิน 28B เป็นปัจจัยหลักที่มีผลต่อการ หายของไวรัสตับอักเสบ ซีหลังการรักษาด้วยยา ความยาวของตำแหน่งไทมีนอดีนีนไดนิวคลีโอไทด์รีพีท (TA) ในยีนอินเตอร์ลิวคิน 28B มีผลต่อการสร้างอินเตอร์เฟอร์รอน ผู้วิจัยได้ทำการศึกษาความสัมพันธ์ระหว่างความยาวของตำแหน่งไทมีนอดีนีนไดนิวคลีโอ ไทด์รีพีท สายพันธุ์ไวรัส อาการทางคลินิก ลักษณะทางพันธุกรรมของอินเตอร์เฟอรอนแลมดา 3 (IFNL3) และ ลักษณะทาง พันธุกรรมของอินเตอร์เฟอรอนแลมดา 4 (IFNL4) กับผลของการรักษาไวรัสตับอักเสบ ซี ผลการศึกษาพบว่าความยาวของตำแหน่ง ไทมีนอดีนีนไดนิวคลีโอไทด์รีพีทมีความยาวตั้งแต่ 6 ถึง 16 และพบความยาวที่ 12 มากที่สุดในกลุ่มประชากรที่ทำการศึกษา การศึกษาพบว่าความยาวของตำแหน่งไทมีนอดีนีนไดนิวคลีโอไทด์รีพีทไม่เกี่ยวข้องกับการหายของไวรัสตับอักเสบซีโดยไม่ได้รับ การรักษาแต่เกี่ยวข้องกับการตอบสนองต่อการรักษาในไวรัสตับอักเสบ ซี สายพันธุ์ที่ 1, 6 และ 3 ส่วนลักษณะทางพันธุกรรมของ อินเตอร์เฟอรอนแลมดา 3 (IFNL3) และ ลักษณะทางพันธุกรรมของอินเตอร์เฟอรอนแลมดา 4 (IFNL4) เกี่ยวข้องกับการตอบสนอง ต่อการรักษาในไวรัสตับอักเสบ ซี สายพันธุ์ที่ 1 เท่านั้น

การศึกษาเพิ่มเติมในการทบทวนข้อมูลที่มีการเผยแพร่อย่างเป็นระบบในไวรัสตับอักเสบซีสายพันธุ์ที่ 6 พบว่าการ รักษาด้วยยาเพคไกเลตต์อินเตอร์เฟอรอนและยาไรบาไวรินมีประสิทธิภาพในการรักษาโดยมีอัตราการหายขาดโดยรวม 79.8% นอกไปจากนี้ผลการรักษานั้นดีกว่าการรักษาไวรัสตับอักเสบซีสายพันธุ์ที่ 1 โดยเทียบเคียงได้กับผลการรักษาไวรัสตับอักเสบซีสาย พันธุ์ที่ 2 และ 3 โดยระยะเวลาการรักษา 48 สัปดาห์ได้ผลการรักษาที่ดีกว่า 24 สัปดาห์ ผลของการรักษาขึ้นกับระดับพังผืดในตับ โดยไม่เกี่ยวข้องกับเพศของผู้ป่วยและลักษณะทางพันธุกรรมของผู้ป่วยที่ตำแหน่งอินเตอร์ลิวคิน 28B และ ลักษณะทางพันธุกรรม ของอินเตอร์เฟอรอนแลมดา 4 (IFNL4) ไม่มีผลต่อการรักษาไวรัสตับอักเสบซีสายพันธุ์ที่ 6.

สาขาวิชา วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต	_____
ปีการศึกษา 2557	ลายมือชื่อ อ.ที่ปรึกษาหลัก	_____
	ลายมือชื่อ อ.ที่ปรึกษาร่วม	_____

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VO DUY THONG: INFLUENCE OF HOST AND VIRAL FACTORS IN HEPATITIS C VIRUS INFECTION: ROLE OF TA REPEAT, IFNL3 AND IFNL4 POLYMORPHISMS IN HCV INFECTION AND OUTCOME OF TREATMENT. ADVISOR: PROF. YONG POOVORAWAN, M.D., CO-ADVISOR: PROF. PISIT TANGKIJVANICH, M.D. Ph.D, pp.

Hepatitis C virus (HCV) is a serious public health problem affecting 170 million carriers worldwide. It is a leading cause of chronic hepatitis, cirrhosis, and liver cancer and is the primary cause for liver transplantation. HCV genotype 6 (HCV-6) is uniquely prevalent in Southern China and Southeast Asia, contributing to almost 30% of all HCV infections in patients and emigrants from these countries. Our data showed that HCV core antigen (HCVcAg) levels in the serum has emerged as a potential marker for active HCV infection and may be used to evaluate response to antiviral therapy and disease progression. There was an excellent correlation between HCV RNA and HCVcAg concentrations, particularly in HCV/HIV co-infected individuals. Serum levels of HCVcAg were associated with ss469415590 polymorphism. As the HCVcAg assay is a reliable test and has the advantages of being rapid and reproducible, it could be used as an alternative to HCV RNA assays in resource-limited settings. Host genetic factors can affect the outcome of HCV infection resulting in either spontaneous clearance from acute infection without treatment or persistence leading to chronic HCV and liver cirrhosis. The interleukin-28B (*IL28B*) gene polymorphism is a strong baseline predictor of sustained virological response (SVR) in treatment. The length of thymine–adenine dinucleotide repeats, or $(TA)_n$, in the regulatory region of *IL28B* can affect interferon transcription. In order to determine predictive values in HCV infection, we explored the correlation among factors including $(TA)_n$ genotypes, clinical features, interferon-λ-3 (IFNL3) and interferon-λ-4 (IFNL4) polymorphisms, and HCV treatment outcome. We found that the variation of $(TA)_n$ ranged from 6 to 16 and the most frequent $(TA)_n$ was 12 in our population. The $(TA)_n$ genotypes was not associated with spontaneous clearance of HCV infection but was associated with treatment response in patients infected with HCV-1, HCV-3 and HCV-6. In contrast, IFNL3 and IFNL4 polymorphisms were predictive of treatment outcome only for patients infected with HCV-1. In our meta-analysis study, the PEG-IFN plus RBV combination were effective for HCV-6 patients, with a pooled SVR rate of 79.8% in our study. Moreover, treatment outcomes of HCV-6 patients were superior to HCV-1, and comparable to those of HCV-2 and HCV-3. Regardless of treatment duration and type of PEG-IFN, efficacy of treatment for 48 weeks was superior to that for 24 weeks. The level of fibrosis affected SVR in HCV-6 patients, while sex (male or female) had no significant influence on treatment outcome. Moreover, *IL28B* and IFNL4 polymorphisms were not significantly associated with treatment outcome in HCV-6 patients.

Field of Study: Medical Science	Student's Signature _____
Academic Year: 2014	Advisor's Signature _____
	Co-Advisor's Signature _____

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF ABBREVIATIONS

ALT	=	Alanine transaminase
ARFP	=	Alternate reading frame protein
BMI	=	Body mass index
CHC	=	Chronic hepatitis C virus
CI	=	Confidence Intervals
CLDN1	=	Claudin 1
CMIA	=	Chemiluminescence microparticle immuno assay
DAA	=	Direct-acting antiviral agent
E	=	Envelope
EGFR	=	Epidermal growth factor receptor
ELISA	=	Enzyme-linked immunosorbent assay
EVR	=	Early virologic response
GWAS	=	Genome-wide association studies
HBV	=	Hepatitis B virus
HCC	=	Hepatocellular carcinoma
HCV	=	Hepatitis C virus
HCVcAg	=	Hepatitis C virus core antigen
HIV	=	Human immunodeficiency virus
HSPG	=	Heparan sulphate proteoglycans
IDU	=	Injection drug user
IFN	=	Interferon
IFNL3	=	Interferon-λ3
IFNL4	=	Interferon-λ4
IL28B	=	Interleukin-28B
ISDR	=	Interferon sensitivity determining region

LIST OF ABBREVIATIONS (CONT.)

ISGs	=	Interleukin-stimulated genes
IVDU	=	Intravenous drug use
LDLR	=	Low-density-lipoprotein receptor
NANB	=	Non-A, non-B hepatitis
NCR	=	non-coding region
NR	=	not reported
NS	=	Non-structural
OR	=	Odds ratio
PCR	=	Polymerase chain reaction
PEG-IFN	=	Pegylated interferon
PI	=	Protease inhibitor
RBV	=	Ribavirin
RdRp	=	RNA dependent RNA polymerase
RNA	=	Ribonucleic acid
RVR	=	Rapid virologic response
SNP	=	Single nucleotide polymorphism
SRB1	=	Scavenger receptor class B member 1
SVR	=	Sustained virologic response
TFR1	=	Transferrin receptor 1
$(TA)_n$	=	Thymine–adenine dinucleotide repeat
UTR	=	Untranslated region

CHAPTER I

GENERAL INTRODUCTION

Hepatitis C virus (HCV) infection is an important worldwide public health problem. Acute HCV infections are usually asymptomatic and rarely diagnosed. In infected individuals, a very rapid interferon (IFN)-mediated immune response is associated with the induction of IFN-stimulated genes (ISGs) in the liver during the first 4–10 weeks of infection; this is then followed by an HCV-specific T cell response (1, 2). Most HCV cases become chronic hepatitis C (CHC), which may advance to liver fibrosis, cirrhosis, and hepatocellular carcinoma (Figure 1).
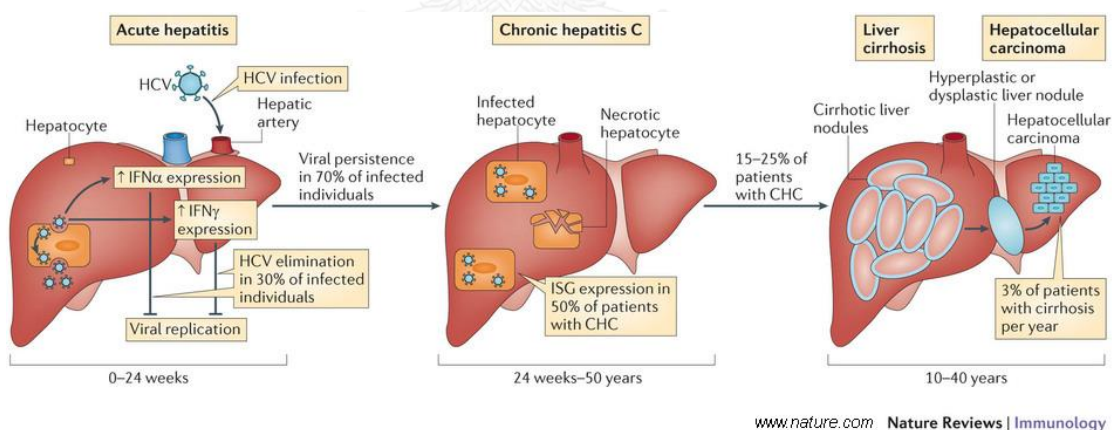
Figure 1. Natural history of CHC. Hepatitis C virus enters the liver through the hepatic artery and the portal vein, which are the two blood vessels that transport blood into the liver. Acute HCV infection lasts from 0 to 24 weeks and often remains undetected. Approximately 70% of HCV-infected individuals develop chronic hepatitis C (CHC). Most patients do not develop substantial liver fibrosis or clinically relevant liver disease. However, in 15–25% of the cases, cirrhosis develops over 10–40 years. Decompensated cirrhosis and hepatocellular carcinoma are the most important causes of mortality in end-stage CHC. IFN, interferon; ISG, IFN-stimulated gene (3).

The global prevalence of HCV infection is estimated at more than 170 million people (4-6), with approximately 9 million people infected in the United States and Western Europe (7, 8), and some studies estimate that mortality related to HCV infection (death from liver failure or hepatocellular carcinoma) will continue to increases over the next two decades (9).

The development of diagnostic tests for hepatitis A and B viruses in the 1970s, provided exclusionary criteria for the identification of another form of viral hepatitis, called non-A, non-B (NANB) hepatitis which had spread predominantly via transfusion of blood and blood products and intravenous drug abuse. Since the discovery of HCV in 1989, there have been many advances in the area of diagnostic and treatment for hepatitis C. The screening tests for HCV infection in clinical practice rely on the detection of anti-HCV antibodies using enzyme-linked immunosorbent assays (ELISA). However, these tests cannot differentiate between resolved HCV infection and an active viral replication. Therefore, the measurement of serum HCV-RNA generally serves to confirm the diagnosis of an ongoing infection (10). In addition, monitoring of HCV RNA is crucial for assessing the treatment response to anti-viral therapy (11). Currently, real-time polymerase chain reaction (PCR)-based quantitative determination is the diagnostic gold standard. However, this assay is limited in developing countries due to costs and the requirement for a real-time PCR machine.

Figure 2. Milestones in hepatitis C virus research and in chronic hepatitis C treatment. Milestones in the stepwise improvement of the treatment regimens are shown in black. Advances in HCV virology that were essential for the development of new treatments are shown in orange (3).

Various factors determine treatment response. In the case of host factors, age, sex, race, fibrosis and steatosis level all have important influences on treatment outcome (12-14), while the most important viral factors for predicting response to IFN-based therapy are genotype and viral load at baseline. In the past 10 years, the standard treatment of HCV patients has been a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) with a 24 to 48 weeks regimen depending on the viral genotype of each infected individual. Thus, HCV genotype is considered one of the most robust independent predictors for sustained virological response (SVR). The infection with different viral genotypes are important to therapeutic outcome, for genotype 2 and 3 have better response than genotype 1 (15). Currently, the few available studies suggest that treatment with the longer 48-week regimen may lead to a higher rate of SVR; on the

other hand, treatment with the 24-week regimen may also lead to a similar SVR rate in subgroups of patients, as in the case of patients with genotypes 2 and 3 (16). In 2011, newer agents known as direct-acting antivirals agents (DAA) were approved for use in conjunction with PEG-IFN and RBV for the treatment of HCV-1.

In addition, an understanding of the difference in host resistance to HCV infection and in response to treatment would be clinically important and may lead to novel therapeutic interventions. Host innate and adaptive immunity also has a profound impact on HCV infection (17). It has been suggested that chronic HCV infection may result from an impaired host immunity in particular cellular immune response against HCV (18-20). It was recognized that genetic factor(s) may be responsible for the distinct consequences of HCV infection in different hosts (21, 22). IFNs are the key cytokines produced by immune cells and play a pivotal role in natural host defense against HCV infection. IFN a by far is the most effective means for clinical treatment of HCV infection.

Genome-wide association studies have shown an association between *IL28B* SNPs, coding for interferon-λ-3 (IFNL3) on chromosome 19, and response to antiviral treatment in individuals infected with genotype HCV-1 (23). Patients with a CC allele in the rs12979860 had a much higher SVR than those who have a T allele. This *IL28B* polymorphism influenced virologic response. *IL28B* polymorphism also strongly influenced the rate of spontaneous clearance of the virus after acute hepatitis C. The frequency of the *IL28B* C allele in the general population is 90% in East

Asians, 75% in Caucasians, 70% in Hispanics, and 50% in African Americans (15). Studies have shown the rs12979860 CC genotype to be associated with natural clearance of HCV (24). More recently, a variant upstream of IFNL3 creates a novel gene, designated as IFNL4, has been discovered (25). This region harbored a dinucleotide variant (ss469415590) that is found in two alternative forms (ΔG or TT alleles). By analyzing data from HCV-infected patients from several clinical trials, it was demonstrated that the IFNL4 ΔG variant was associated with poorer clearance of HCV and response to PEG-IFN/RBV therapy than the TT allele. Compared to rs12979860, the SNP ss469415590 was more strongly associated with treatment response of HCV-1 infection in African-American individuals, although it provides comparable information in European and Asia populations (25).

HCV genome organization and viral life cycle

HCV is a member of *Hepacivirus* within the *Flaviviridae* family. It is an envelope, single stranded, positive sense RNA virus. The viral genome is encapsulated in the nucleocapsid protein and core protein. The viral genome is approximately 9.6 kb in length, and consists of a single open reading frame flanked by a 5'-untranslated region (UTR), which includes an internal ribosome entry site (IRES), and a 3'-UTR. It encodes a polyprotein precursor of about 3,000 amino acid (26). The translational process of viral genome is control by an internal ribosome entry site. The precursor is

cleaved into at least 10 different proteins after cleavage by viral and cellular protease. The structural proteins include the core and the two envelope proteins (E1 and E2), which lie at the N-terminus of the polyprotein. The non-structural proteins are NS2, NS3, NS4A, NS4B, NS5A and NS5B. Between the structural and the non-structural proteins are two proteins most likely non-structural (p7 and NS2), which are dispensable for replication, but essential for assembly (Figure 3) (27).

The 5'-UTR and 3'-UTR play an important role in both the replication and translation process. HCV has a high replication rate with an estimated 10^{12} virions per day in an infected individual (28). The viral genome is synthesized by RNA dependent RNA polymerase (RdRp) which has no proof-reading capacity. Lack of post-replicative repair mechanisms, together with the extreme rate of virus production, are at the root of HCV's pronounced genetic diversity. These characteristics are similar in most RNA virus. The envelope glycoproteins E1 and E2 interact with the viral receptors on permissive cells and mediate viral entry. E2 also contains hypervariable regions that are targeted by neutralizing antibodies. P7 acts as a viroporin or ion channel. NS2 possesses an autoprotease activity necessary for the polyprotein cleavage between NS2 and NS3. NS3 acts as serine protease in combination with NS4A that acts as a cofactor to catalyze the processing of the HCV polyprotein. NS3 also harbors RNA helicase/NTPase activity that unwinds RNA–RNA substrates and is essential for viral replication (27, 29). The functions of NS4B and NS5A are poorly characterized.

However, studies show that NS4B induces the formation of a membranous web compartment where viral replication takes place (30), and cell-culture adaptive mutations mapped to the NS5A enhance RNA replication suggesting its importance for viral replication (31). NS5A was also shown to harbor a region that may determine response to alpha interferon (IFN-**α**) therapy known as interferon sensitivity determining region (ISDR) (32). NS5B is the viral RNA-dependent RNA-polymerase responsible for HCV–RNA replication.
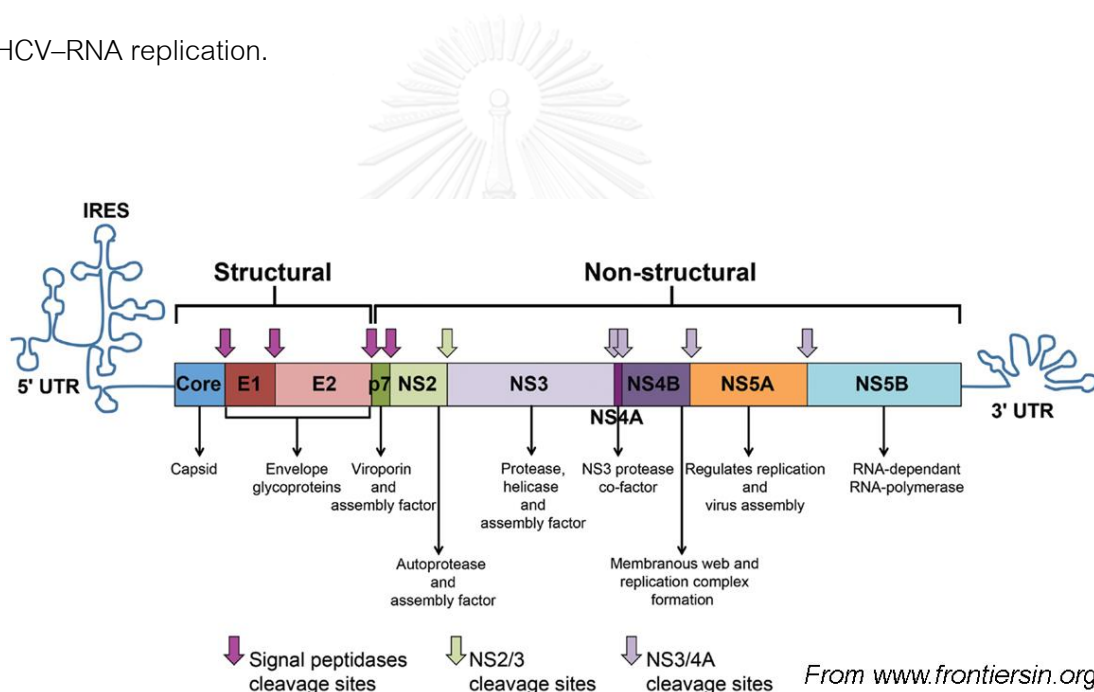
Figure 3. HCV genome and polyprotein. The HCV genome is composed of an open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). IRES-mediated translation of the ORF leads to the formation of a polyprotein that is processed into 10 viral proteins. Cleavage of the core protein from E1 involves cellular signal peptidases, which also cleave E1, E2, and p7 from the polyprotein (pink arrows). The NS2–NS3 protease auto-cleaves itself (green arrow). The NS3 protease assisted by its membrane-bound cofactor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A, and NS5B (violet arrows) (26).

Replication of HCV involves several steps. The virus replicates mainly in the hepatocytes of the liver, where it is estimated that each infected cell produces approximately fifty virions (virus particles) daily with a calculated total of one trillion virions generated. The virus may also replicate in peripheral blood mononuclear cells, potentially accounting for the high levels of immunological disorders found in chronically infected HCV patients. HCV has a wide variety of genotypes and mutates rapidly due to a high error rate on the part of the virus' RNA-dependent RNA polymerase (33). Entry into host cells occur through complex interactions between virions and cell-surface molecules CD81, LDL receptor, SR-BI, DC-SIGN, Claudin-1, and Occludin (34, 35) (Figure 4).
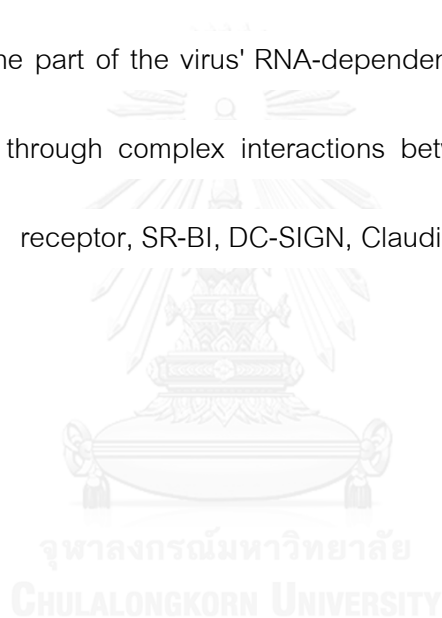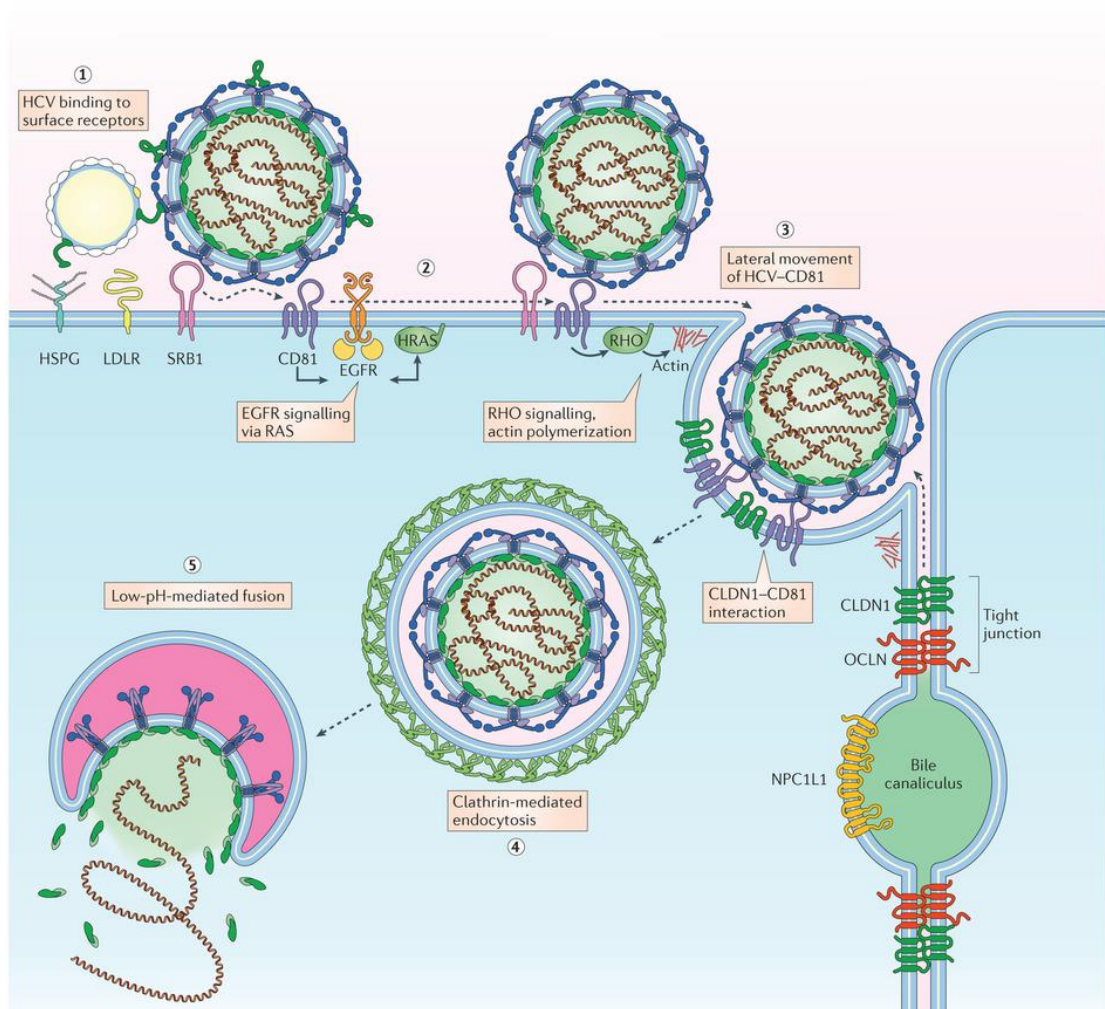
Figure 4. HCV entry. HCV lipoviral particles (LVPs) attach to the cell surface by interacting with heparan sulphate proteoglycans (HSPGs), low-density-lipoprotein receptor (LDLR) and scavenger receptor class B member 1 (SRB1). SRB1 might delipidate HCV-associated lipoproteins and induces conformational changes in the E2 glycoprotein, exposing the CD81-binding site (step 1). Transferrin receptor 1 (TFR1) has an unknown, post-CD81 role in HCV entry (not shown). Interaction of E2 with CD81 then activates signal transduction through epidermal growth factor receptor (EGFR) and HRAS, as well as through RHO GTPases (step 2). These signalling events promote lateral movement of HCV– CD81 complexes to sites of cell–cell contact (step 3), interaction of CD81 with claudin 1 (CLDN1), and HCV internalization via clathrin-mediated endocytosis (step 4). The low pH of the endosomal compartment induces HCV fusion (step 5). NPC1L1, Niemann–Pick C1-like 1; OCLN, occluding (36).

Once inside the hepatocyte, HCV takes over portions of the intracellular machinery to replicate (37). The HCV genome is translated to produce a single protein of around 3011 amino acids. The polyprotein is then proteolytically processed by viral and cellular proteases to produce three structural (virion-associated) and seven nonstructural (NS) proteins. Alternatively, a frameshift may occur in the Core region to produce an Alternate Reading Frame Protein (ARFP). The NS proteins then recruit the viral genome into an RNA replication complex, which is associated with rearranged cytoplasmic membranes. RNA replication takes places via the viral RNA-dependent RNA polymerase NS5B, which produces a negative strand RNA intermediate. The negative strand RNA then serves as a template for the production of new positive strand viral genomes. Nascent genomes can then be translated, further replicated or packaged within new virus particles. New virus particles are thought to bud into the secretory pathway and are released at the cell surface. The virus replicates on intracellular lipid membranes. The endoplasmic reticulum in particular is deformed into uniquely shaped membrane structures termed 'membranous webs'. These structures can be induced by sole expression of the viral protein NS4B. The core protein associates with lipid droplets and utilises microtubules and dyneins to alter their location to a perinuclear distribution (38). Release from the hepatocyte may involve the very low density lipoprotein secretory pathway (39).

HCV classification and geographical distribution of HCV

Until now, six primary genotypes (1 to 6 and 7a) and numerous subtypes have been classified based on HCV nucleotide sequences (40). Each of the six major genetic groups of HCV contains a series of more closely related subtypes that typically differ from each other by 20–25 % in nucleotide sequences, compared with the >30 % divergence between genotypes (41). The genetic heterogeneity in the population of HCV genomes indicates the existence of HCV quasispecies in infected individuals (42). Several methods for HCV genotyping have been applied such as RFLP, type specific primers, probes and sequencing after subgenomic amplification by RT-PCR. HCV genotyping by phylogenetic analyses of amplified nucleotide sequences of specific regions (core, NS3 and NS5) is considered the most precise method (43).

HCV genotypes have a varied geographic distribution around the world. HCV-1 is found worldwide including in developed regions such as America and Europe. HCV-2 has high prevalence in Central and West Africa as well as some western countries while HCV-3 is predominantly found in Far Eastern countries and the Indian subcontinent (44). Meanwhile, HCV genotypes 4 and 5 are endemic to specific geographical areas: HCV-4 is mainly found in Egypt and Sub-Saharan Africa, and HCV-5 has accumulated in South Africa (45). Of the primary HCV genotypes, genotype 6 is one of the most prevalent in Southern China and Southeast Asia, contributing to almost 30% of all HCV-infected patients in these areas. Among the genotypes of HCV, HCV-6 is the most geographically

limited having been discovered only in Thailand, Vietnam, Myanmar, Macau and Hong

Kong, or emigrants from those countries (24, 42, 46).
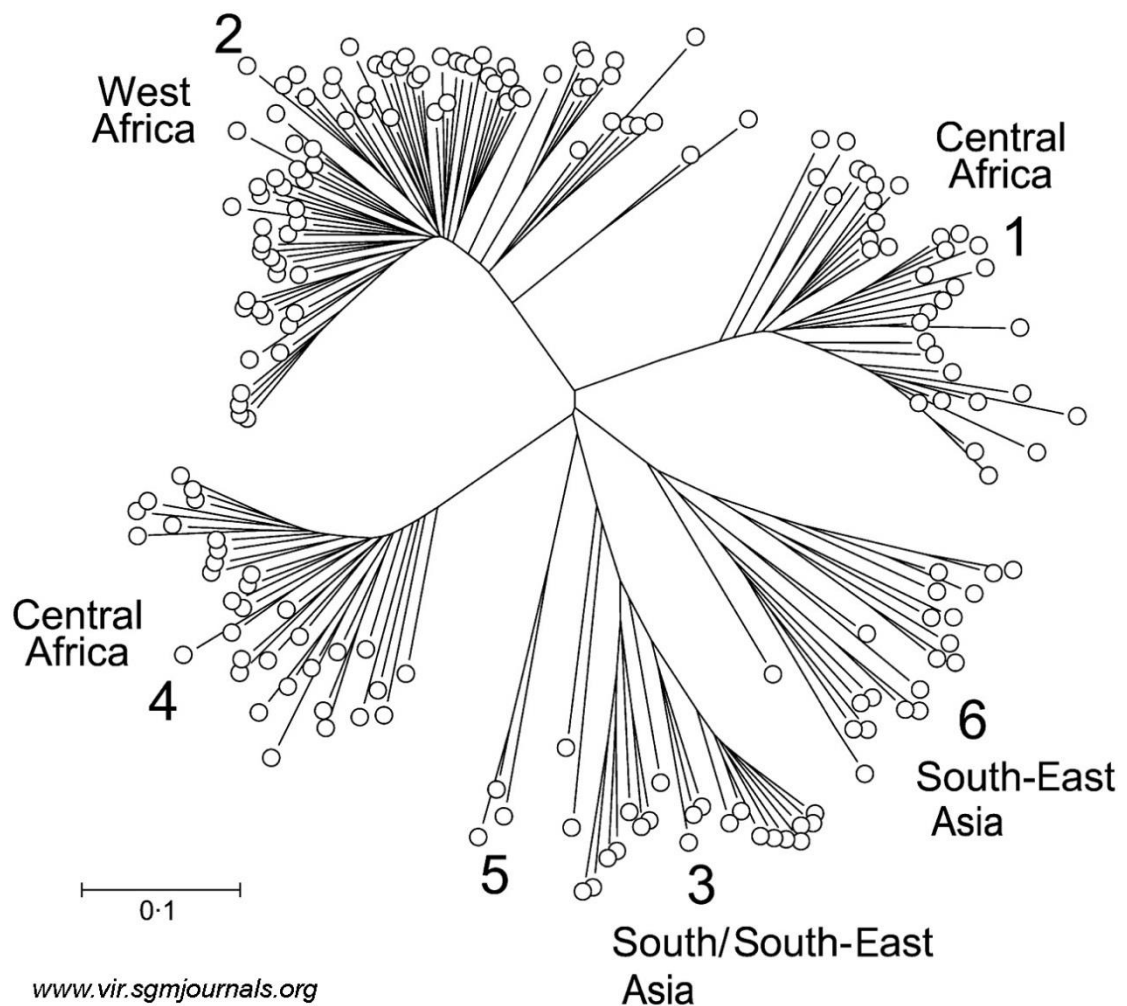
Figure 5. Evolutionary tree of all known subtypes and genotypes of HCV, including those found in high-diversity areas of genotypes 1, 2 and 4 (sub-Saharan Africa) and 3 and 6 (Southeast Asia). HCV variants still fall into six distinct clades, but with far greater numbers of genetic variants corresponding to subtypes in industrialized countries. The tree was constructed by using the neighbour-joining method as implemented in the mega package, using Jukes–Cantor-corrected distances between partial NS5B sequences (320 bp) (44).

Outline and aim of the thesis

Recently, a highly sensitive assay for measuring serum HCV core antigen (HCVcAg) concentrations using a fully automated chemiluminescent microparticle immunoassay (CMIA) has become commercially available (47). A number of studies have demonstrated good correlation between HCVcAg and HCV RNA levels, particularly in HCV mono-infected individuals (48-55). As a result, HCVcAg testing is considered to be an alternative assay to conventional HCV RNA quantification with the advantages of its rapidity, reproducibility and feasibility in resource limited settings (56). To date, data regarding the correlation between HCVcAg and HCV RNA levels in patients co-infected with human immunodeficiency virus (HIV) are still limited (57-59). In addition, the effect of different viral genotypes on HCVcAg levels and the correlation with HCV RNA levels remains to be established. However, it is unclear whether there is an association between this SNP and HCVcAg concentrations in patients with chronic HCV infection. Thus, we evaluated the use of HCVcAg measurement with respect to HIV status, HCV genotypes, IFNL4 polymorphism and clinical parameters. Detection of HCV core antigen levels in the serum served as a potential marker for active HCV infection and was used to evaluate response to antiviral therapy, and disease progression and will reduce cost of treatment.

Genome-wide association studies (GWAS) have recently identified host genetic variation critical for predicting treatment response and spontaneous clearance in

patients infected with hepatitis C virus (HCV). Successful treatment of chronic HCV infection with peginterferon and ribavirin was strongly associated with host factors - genetic polymorphisms (60). Several studies reported that *IL28B* is one of the strongest baseline predictors of SVR in HCV treatment at approximately 80%. Sugiyama *et al.* showed that the length of thymine–adenine dinucleotide repeats or $(TA)_n$ in the promoter region of *IL28B* has a wide variation and the transcriptional activity of the promoter increased gradually in a $(TA)_n$ length-dependent manner (61). These findings suggested that $(TA)_n$ could be associated with the transcriptional activity of *IL-28B* as well as applied to improve prediction of the response to interferon-based HCV treatment (61). The aim of this study was to investigate the distribution of the length of $(TA)_n$ and the correlation of $(TA)_n$ genotypes with treatment outcome, clinical characteristics and IFNL3, IFNL4 polymorphisms in HCV infection and outcome of treatment. This study revealed the SNPs (IFNL3, IFNL4 and number of TA repeat) prevalence in Thai population may be useful for public health surveillance and treatment.

The HCV genotype is a crucial predictor of anti-viral therapeutic response, and is also important in determining treatment duration (15). HCV-6 is common among patients from Southeast Asia and the surrounding regions, where HCV prevalence is also high. HCV genotype 6 has great genetic diversity and different response to antiviral therapy compared with the more commonly known genotype 1 (40). Recent advances in the development of direct-acting antiviral agents (DAAs), which inhibit viral proteins and

block the HCV life cycle, have revolutionized HCV therapy (62). DAAs, such as telaprevir and boceprevir, can achieve higher antiviral responses when combined with pegylated interferon (PEG-IFN) plus ribavirin (RBV). They also have the potential to eradicate HCV without IFN (63). Subsequently, a number of additional DAAs are being tested in ongoing clinical trials, and two are now commercially available. However, in many countries, DAAs are still not available or are too expensive for general use. As a result, in those places, a combination of PEG-IFN with RBV remains the standard therapy for chronic hepatitis C (64). However, the data on expected response rates and optimal treatment duration for HCV-6 are limited compared to what is known for HCV-1 and HCV-3. Therefore, we aimed to conduct a meta-analysis of influence of host and viral factors on virologic response in HCV-6 patients treated with PEG IFN+ RBV. We systematic reviewed the epidemiology, classification, diagnosis and treatment as it pertain to HCV-6.

Part 1: The correlation between hepatitis C core antigen and HCV RNA levels with respect to HIV status, HCV genotype and IFNL3, IFNL4 polymorphism.

Research question

1. What is the correlation between HCVcAg and HCV RNA levels in mono-infected patients and co-infected with HIV?

2. Are there any correlations between HCVcAg and HCV genotypes?

3. Are there any association between HCVcAg levels and SNPs (IFLN3 and IFNL4 polymorphisms)?

4. Are there any association of HCVcAg levels and clinical parameters?

Objective

1. To determine the association between HCVcAg and HCV RNA levels in mono-infected patients and co-infected with HIV.

2. To determine the association between HCVcAg levels and HCV genotypes.

3. To validate the association of HCVcAg levels with IFLN3 and IFNL4 polymorphisms

4. To characterize the HCVcAg levels in hepatic necroinflammation and fibrosis progression.

Part 2: The correlation of number of TA repeat and IFNL3, IFNL4 in healthy individuals, and with treatment outcome, clinical characteristics in HCV-infected patients

Research question

1. What are the prevalence of IFNL3, IFNL4 polymorphism and number of TA repeat among healthy individual, HCV-infected patient?

2. Are there any correlation between number of TA repeat and IFLN3, IFNL4 polymorphisms?

3. What is the role of number of TA repeat in HCV spontaneous clearance? Do they have any association between number of TA repeat and virological characteristics?

4. Are there any association of treatment response (SVR) and number of TA repeat?

Objective

1. To determine the prevalent SNPs among healthy individual, HCV-infected patient.

2. To determine the association between number of TA repeat and IFLN3, IFNL4 in HCV infected patients.

3. To determine the role of number of TA repeat, IFNL3 and IFNL4 polymorphisms in HCV spontaneous clearance and the outcome of treatment.

Part 3: Meta-analysis: influence of host and viral factors on virologic response in HCV-6 patients treated with PEG IFN+ RBV. A systematic review: virology, epidemiology, genetic variation and clinical implication.

Research question

1. What are the prevalent of HCV-6 and subtypes in the world and Southeast Asian countries? What is the evolution of HCV-6 in this region? And methods use for HCV-6 genotyping accuracy?

2. Are there any clinical characteristic differences between HCV-6 and others genotypes?

3. What is the optimal duration and treatment outcome in HCV-6 patients?

4. What is influence of host and viral factors on virologic response in HCV-6 patients treated with PEG IFN+ RBV in meta-analysis?

Objective

1. To determine the prevalent and evolution of HCV-6 and subtypes in the world and Southeast Asian countries, methods use for HCV-6 genotyping

2. To characterize the clinical characteristics among HCV-6 patients.

3. To validate the optimal duration and treatment outcome in HCV-6 patients.

4. To characterize the influence of host and viral factors on virologic response in HCV-6 patients treated with PEG IFN+ RBV in meta-analysis.

Conceptual framework part I


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                    │  Sample collection HCV-patients │
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Sample collection HCV-patients

Mono-infection

Co-infection with HIV

Clinical and laboratory data records

Collection of blood from HCV patients

- Hepatic inflammation
- Fibrosis progression
- HCV Genotype

- *IL28B* (rs12979869)
- IFNL4

HCV core Ag levels by CMIA

HCV-RNA by real time PCR

• Characterize the HCVcAg levels in hepatic necroinflammation and fibrosis progression
• Determine the association between HCVcAg levels and HCV genotypes.

Validate association of HCV core Ag levels with IFLN3 and IFNL4 polymorphisms

Determine the association between HCV core Ag and HCV RNA levels in HCV mono-infection and HIV/HCV co-infection.

The correlation between hepatitis C core antigen and HCV RNA levels with respect to HIV status, HCV genotype and IL28B, IFNL4 polymorphism

Conceptual framework part II

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┌─────────────────────────┐                    ┌──────────────────────────┐
│    Sample collection     │                    │ Healthy individual's     │
│  (HCV infected patients) │                    │ collection               │
│                          │                    │ from blood donors        │
└─────────────────────────┘                    └──────────────────────────┘
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| Clinical and laboratory data records | Collection of PBMCs from:
 - HCV patients treated
 - Chronic HCV patient
 - HCV spontaneous clearance | | Non HCV, HBV and HIV screening by ELISA and PCR |

| Hepatic inflammation
 Viral load (HCV RNA)
 HCV genotype
 SVR | - $IL28B$ (rs12979869)
 - IFNL4 (ss46941559 | TA repeat in the promoter region of $IL28B$ | - $IL28B$ (rs12979869)
 - IFNL4 (ss46941559 | TA repeat in the promoter region of $IL28B$ |

- Association of $(TA)_n$ repeat with HCV spontaneous clearance
- Association of $(TA)_n$ repeat with treatment outcome
- Correlation between $(TA)_n$ repeat and clinical parameters
- Correlation between $(TA)_n$ genotypes and fibrosis progression

Correlation of $(TA)_n$ repeat with $IL28B$ and IFLN4

- Allele frequency of $(TA)_n$ distribution
- Prevalence of $(TA)_n$ repeat

Conceptual framework part III

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                        │ Cochrane Library                       │
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│ ➤ Evaluation of     │              └──────────────┘    │ ● Not original data        │
│   HCV-6             │                      │            │ ● Not naïve treatment      │
│ ➤ Methods use for   │                      ▼            │ ● Patients with co-        │
│   genotyping        │              ┌────────────────┐   │   infections               │
│ ➤ Clinical          │              │ Articles and   │   │ ● Patients with other      │
│   characteristics   │              │ abstracts      │   │   liver diseases           │
│ ➤ Treatment         │              │ meeting        │   │ ● Redundant studies        │
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                            ┌──────────────────────────────────────┐
                            │ Influence of host and viral factors   │
                            │ on virologic response in HCV-6         │
                            │ patients treated with PEG IFN+ RBV     │
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CHAPTER II

HEPATITIS C VIRUS GENOTYPE 6: VIROLOGY, EPIDEMIOLOGY, GENETIC VARIATION AND CLINICAL IMPLICATION

Vo Duy Thong[1], Srunthron Akkarathamrongsin[1], Kittiyod Poovorawan[2], Pisit Tangkijvanich[3], Yong Poovorawan[1*]

[1]*Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[2]*Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[3]*Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

Abstract

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

Introduction

Hepatitis C virus (HCV) infection is an important worldwide public health problem. Most HCV cases become chronic hepatitis C (CHC), which may advance to liver fibrosis, cirrhosis, and hepatocellular carcinoma. The global prevalence of HCV infection is estimated at more than 170 million people (4-6), and some studies estimate that mortality related to HCV infection (death from liver failure or hepatocellular carcinoma) will continue to increases over the next two decades (9).

Hepatitis C virus is a member of the *Flaviviridae* family and belongs to the genus *Hepacivirus*. HCV is classified into six major genotypes (1-6) and subdivided into various subtypes named in alphabetical order from a to z. Currently, sequencing of HCV isolates has identified more than 83 subtypes from the six genotypes (40). The genomes among HCV genotypes differ from each other by ~30-35% while the genomes among the subtypes differ by 15-20%. The prevalence of HCV genotypes varies geographically: HCV-1 is found worldwide including developed regions such as North America and Europe. HCV-2 has high prevalence in Central and West Africa as well as some western countries, while HCV-3 is predominantly found in the Far Eastern countries and the Indian subcontinent (44). Meanwhile, HCV genotypes 4, 5 and 6 are endemic to specific geographical areas: HCV-4 is mainly found in Egypt and Sub-Saharan Africa, HCV-5 in

South Africa (45), and HCV-6 in South China and South-East Asian countries (24, 42, 46)

(Figure 6)
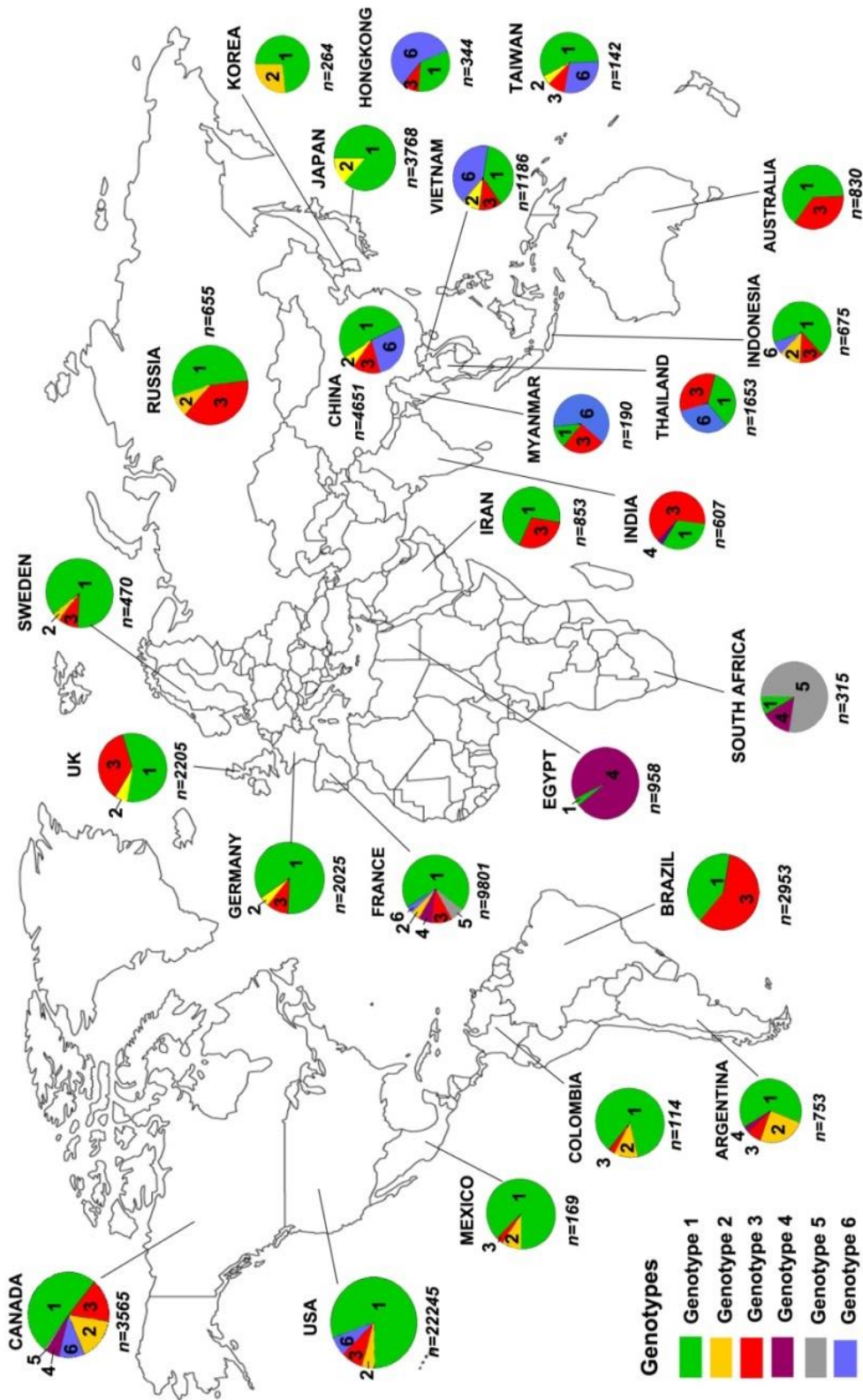
Figure 6. The prevalence of the various HCV genotypes varies considerably between countries and regions (7, 46, 65-87).

HCV-6 is highly diverse with 23 subtypes currently known (24, 42, 83). This genetic diversity may be the result of a long period of viral circulation (67). In addition, repeated viral exposure through activities such as intravenous drug use leads to more than one viral strain circulating in the host, which in turn increases the chance of viral recombination events among circulating HCV genotypes and strains. The high variation and accumulation of HCV-6 in Southeast Asia also supports the idea that this area may be the origin and worldwide distribution center of this genotype.

Various factors determine treatment response. In the case of host factors, age, sex, race, fibrosis and steatosis level all have important influences on treatment outcome (12-14), while the most important viral factors for predicting response to IFN-based therapy are genotype and viral load at baseline. In the past 10 years, the standard treatment of HCV patients has been a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) with a 24 to 48 weeks regimen depending on the viral genotype of each infected individual. Thus, HCV genotype is considered one of the most robust independent predictors for sustained virological response (SVR). Most clinical studies on PEG-IFN/RBV efficacy have been based on common genotypes such as genotype 1, 2 and 3, while scant clinical data has been generated concerning genotype 6. However, due to this genotype's extreme genetic diversity, it may be important to study it in a controlled, clinical setting in order to gauge the standard therapy's efficacy (40).

Currently, the few available studies suggest that treatment with the longer 48-week regimen may lead to a higher rate of SVR. On the other hand though, treatment with the 24-week regimen may also lead to a similar SVR rate in subgroups of patients, as in the case of patients with genotypes 2 and 3 (16). In 2011, newer agents known as direct-acting antivirals (DAA) were approved for use in conjunction with PEG-IFN and RBV for the treatment of HCV-1. However, the efficacy and safety of DAA in the treatment of the HCV-6 patients still needs to be assessed.

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

Epidemediology

Within endemic countries, HCV-6 shows variability in subtype prevalence. Vietnam has reported HCV-6 prevalence of 51-54.4% in Ho Chi Minh City and 47.1% elsewhere with the most common subtypes being 6a followed by 6e and 6l. In total, 12 subtypes have thus far been identified in Vietnam (6a, 6c, 6e, 6f, 6h, 6k, 6l, 6n, 6o, 6p, 6r and 6t) (88-90). Although there is limited information regarding HCV epidemiology in Laos and Cambodia, a few studies have reported a high proportion of HCV-6 in these countries also. Among Laos blood donors, 95.6% of the HCV RNA positive samples

were classified as HCV-6 with various subtypes being found including 6b, 6h, 6k, 6l, 6n, 6o, 6q and unclassified subtypes (91, 92). A study of Cambodian migrants in Thailand reported that HCV seroprevalence in this group is similar to their guest country (2.3%). It was also found that HCV-6 is the most dominate genotype in Cambodian migrants with 52% of the HCV-RNA carriers testing positive for this genotype with the subtype breakdown as the following; 6e (20%), 6r (20%), 6f (8%) and 6p (4%) (91). Similar to its neighboring countries, Thailand also has several endemic HCV-6 subtypes (6b, 6c, 6e, 6f, 6i, 6m and 6n) along with novel subtypes (6u and 6v), which are found in the North and Northeast (92).

However, unlike the Cambodian migrant population in Thailand, this genotype contributes a lower proportion (20.1%) of overall infection in comparison with HCV-3 and HCV-1 (85). In Myanmar, HCV-6 prevalence has gone from being the third most prevalent HCV genotype in 2004 to the first after analysis of a large number of blood samples from 2007. HCV-6 is especially prevalent in the Northern part of Myanmar with subtypes 6f, 6n and 6m predominating (93-96). Despite is ubiquitous presence in the above-mentioned countries, however, HCV-6 is only rarely reported in other proximal countries. For example, only three samples of subtype 6g (previously designated as genotype 1a) have been reported in Indonesia since 1996 (97),while only a single recent

report of subtype 6n has been found in Kuala Lumpur, Malaysia, and notably, this

patient was co-infected with HIV-1 and had a history of IVDU (98).

HCV-6 can also be found outside of the immediate South East Asian region in countries

such as South China, Hong Kong and Taiwan (Figure 7).
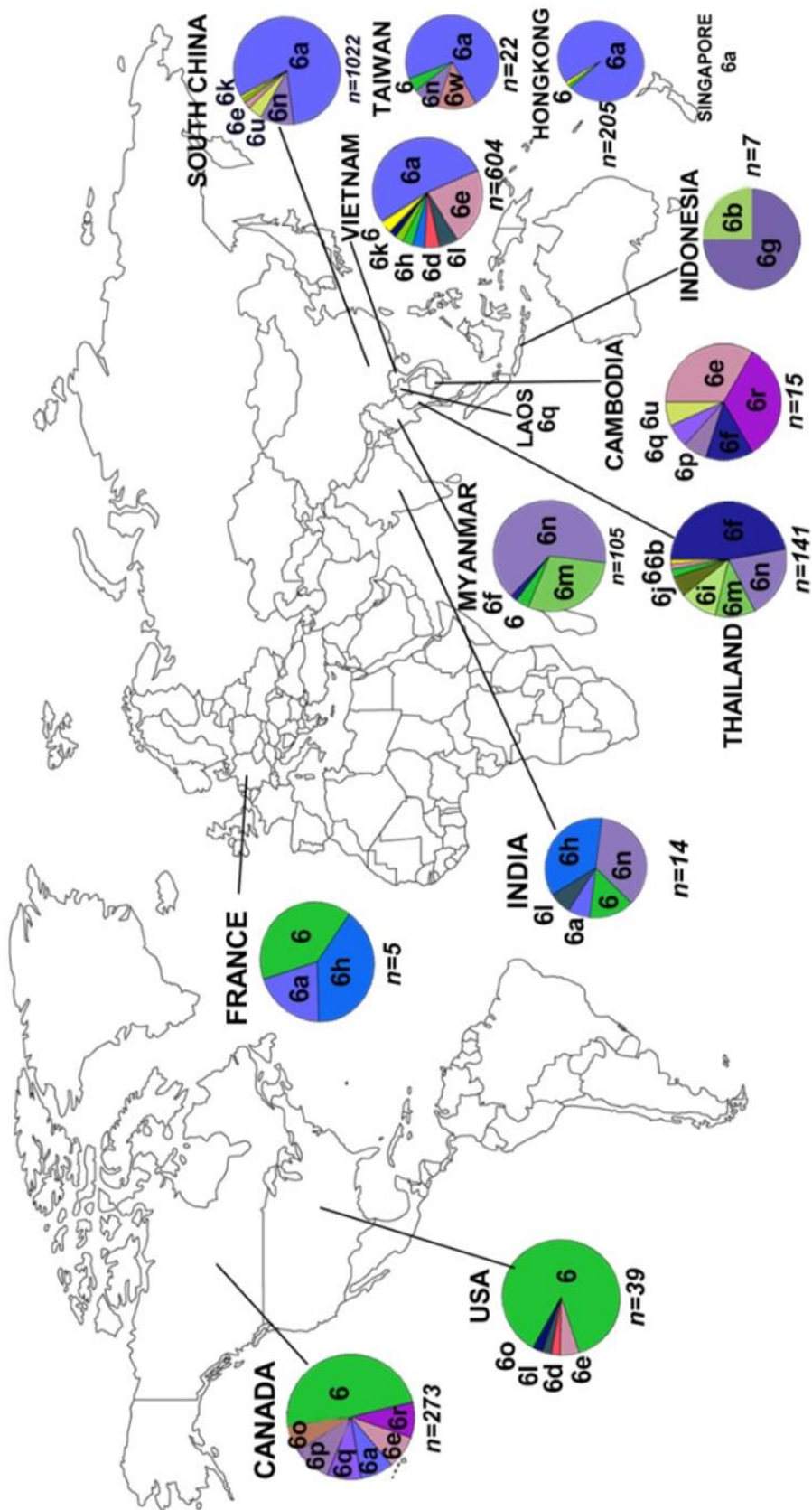
Figure 7. Distribution of the prevalence of subtype of HCV-6 in the world (81, 89, 92, 95-97, 99-104).

Intravenous drug abuse is the transmission route suspected of being most responsible for the high frequency of this genotype in certain parts of Asia and the factor driving a continuous discovery of new subtypes. In China, HCV is frequently found in the South in patients with HIV-1 co-infection and IDU history (105). For example, prevalence of HCV-6 in chronic hepatitis cases is12.9% to 14.2% while being 28.2% to 51.5% in IDUs. In addition, similar to Vietnam, 6a is the major subtype in IDUs from all study groups (70, 106, 107). A similar trend of HCV-6 infection can also be observed in Hong Kong. More than half of infected IVDU have this genotype (53.2% to 58.5%), which is much higher than the prevalence in the general population (23.6%), and subtype 6a is the most common subtype (101, 108). In Taiwan, there has been no report of HCV-6 until 2010, but since then there is a growing prevalence of HCV-6 and subtype 6a along with novel subtypes being reported in prisoners and IDUs(102).

Evolution of hepatitis C virus genotype 6

There is now considerable evidence to support the hypothesis that HCV-6 originated in Southeast Asia. First, this genotype is mainly observed in countries such as Vietnam, Cambodia, Laos, Myanmar and Thailand with the prevalence of HCV-6 in these countries ranging from approximately 20% to more than 50% of all genotypes. Second, there are a great number of known and novel subtypes circulating within these

populations (90). Third, when HCV-6 is found outside of its endemic area, such as in countries like U.S., Canada and Australia, the virus is almost exclusively isolated from Asian immigrants (45, 109). Fourth, a study in Laos showed that HCV-6 is highly divergent from other genotypes, and that it has distinct genetic differences from other strains, which suggests that there may yet be unclassified subtypes existing in this area. Thus, the accumulation of such genetic heterogeneity suggests that this genotype has circulated, adapted and evolved in this area for a long period of time (Table 1).

Table 1. Prevalence of HCV-6 in Asians.

Country	Author	Publication year	n	Genotype 6 Prevalence (%)
Myanmar	Lwin (95)	2007	1333	49
Vietnam (Hanoi, Vietnam)	Pham (88)	2009	238	47.1
Thailand				
- (Blood donors throughout country)	Kanistanon (110)	1997	NR	18
	Jutavigittum (111)	2009	326	31
- (Blood donors in northern Thailand provinces)				
	Akkarathamrongsin(112)	2010	375	30
- (Blood donors from central Thailand)				
Hong Kong (Blood donors)	Leung (113)	2006	910	27

NR, not reported

Evolutionary analysis of HCV-6 subtypes hypothesizes that these subtypes evolved from a common ancestor more than 1000 years ago, and that some subtypes

may have maintained their endemicity via local epidemics during the 20th century initiated and propagated by modern medicine, blood transfusion and IVDU(91). However, each of the subtypes seems generally restricted to different locations such as subtype 6d in Vietnam, 6f in Thailand, 6g in Indonesia and 6r in Cambodia. In addition, strains isolated from the same country tend to cluster together in a HCV-6 phylogenetic tree (Figure 8) (91, 96).
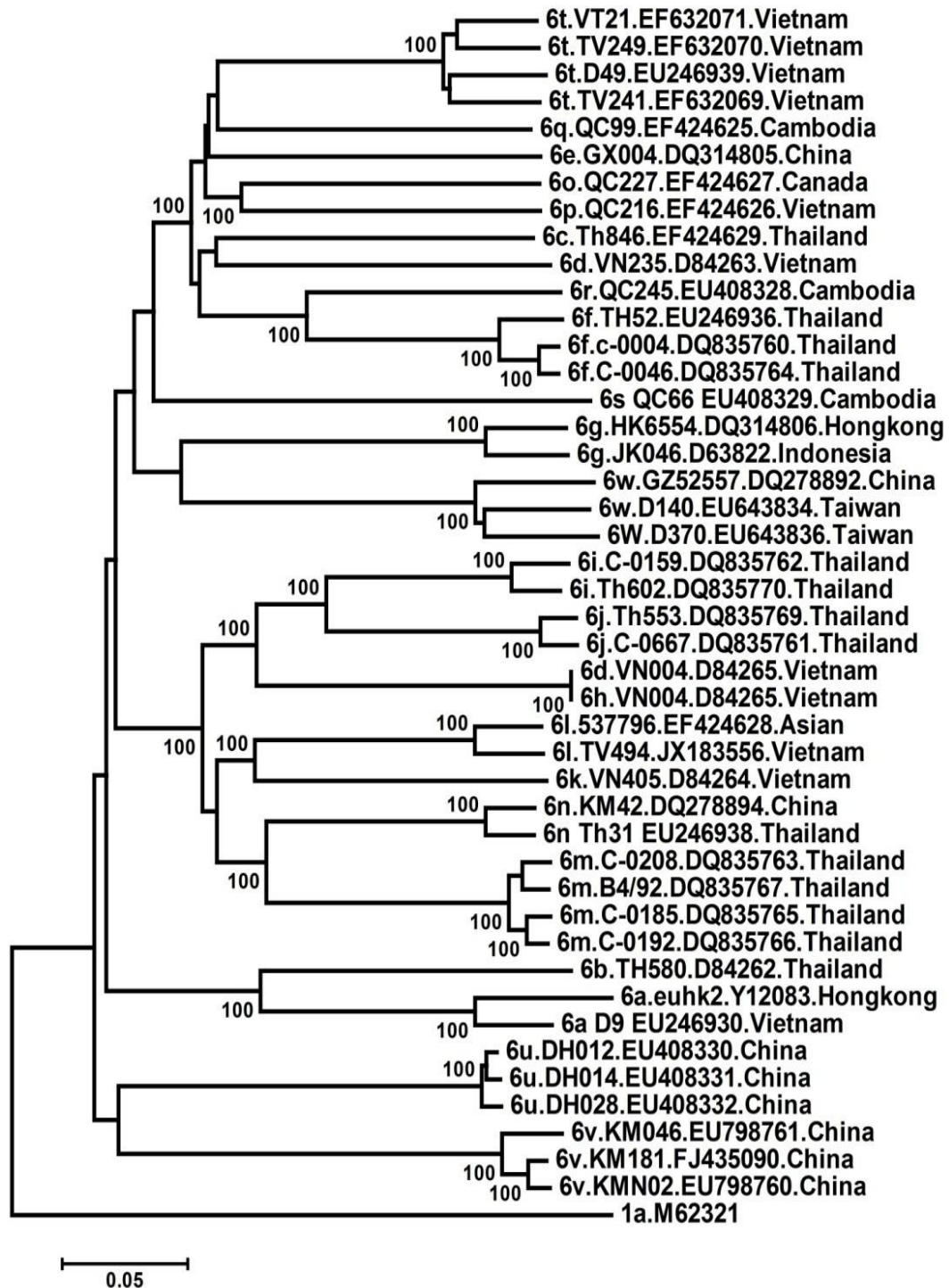
Figure 8. Phylogenetic relationships among subtypes HCV-6. Phylogenetic tree constructed from whole genome sequence of all HCV-6 subtypes (subtype 6a to 6w) which

was analyzed by K2P model and MEGA version.5. Bootstrap value of 1000 replicates was indicated at each node.

HCV-6 has spread through other East Asian countries by multiple transmission routes with one of the most effective routes being IDU. In the general population, HCV-1 is still the predominant genotype across Asia. However, HCV-6 - and especially subtype 6a - is the dominant genotype among IVDUs (102, 107, 108). An example of this is the phylogeographic and coalescent analysis of HCV-6's spread through China. Analysis shows that subtype 6a and some other Chinese strains may have originated in Vietnam and spread to neighboring Guangxi and Yunnan provinces (114, 115). Finally, since Guangdong is a major gateway to China, this province may have been the origin of subtype 6a dispersion throughout other regions of the country (107).

Similar to other RNA viruses, HCV has a high tendency for genetic heterogeneity due to a lack of proof reading activity by its viral RNA polymerase. This genetic drift results in an accumulation of viral mutations, which will then be selected for by forces of environmental pressure. Thus, only the fittest strains survive and become the major circulating viral population. Of course, however, this process of fixation requires a relatively long period of time. Meanwhile, viral recombination can result in novel strains overnight, although the drastic nature of recombination can be a danger to viral survival. So far, the evidence suggests that novel HCV strains mainly accumulate through genetic drift by collecting viral mutation instead of recombination, as there have only been two

reported cases of HCV-6-specific recombination occurring (116, 117); recombinants RF_2i/6p and RF_2b/6w from a Vietnamese blood donor and an IDU in Taiwan, respectively (102, 118).

Methods used for genotyping

Since HCV genotype is such an important consideration for predicting an effective treatment regimen, several different genotyping methods have been developed. These methods include direct nucleic acid sequencing (41), a reverse hybridization line probe assay (LiPA) (119), subtype-specific reverse transcription (RT)-PCR(120), DNA restriction fragment length polymorphism (121), heteroduplex mobility analysis (122), primer-specific and mispair extension analysis (67), melting curve analysis with fluorescence resonance energy transfer probes (123) and serologic genotyping (Figure 9) (124).

Figure 9. The 5'-UTR and Core regions targeted by INNO-LiPA (the 5'-UTR only for INNO-LiPA I or both the 5'-UTR and core regions for INNO-LiPA II), sequencing of NS5B are used the standard methods for classifying HCV genotype and subtype (40, 125).

However, not every method is equal. The Asian Pacific Association for the Study of the Liver (APASL) states that genotype discrimination based on primers from the 5'-UTR do not distinguish some of the HCV-6 subtypes prevalent in Southeast Asia, and that these subtypes should instead be classified as genotype 1 or 1b (126), available methods generally use distinct motifs found within the HCV genome for HCV genotype (40). In addition, previous studies reported the mistyping of HCV-6 as genotype 1 by the INNO-LiPA I assay (Innogenetics, Zwijnaarde, Belgium) (127, 128). However, the INNO-LiPA HCV II (Innogenetics, Zwijnaarde, Belgium) genotyping assay seems to overcome the deficiencies demonstrated by the INNO-LiPA I assay, as it has shown remarkable improvement in genotyping accuracy and differentiation between HCV-1 and HCV-6 variants by using core sequencing data as well as 5' UTR data (129, 130).

Clinical Characteristics

Acute HCV infection is infrequently diagnosed and leads to chronic infection in about 80% of cases (131). Clinical manifestations can occur, usually within 7 to 8 weeks (range, 2 to 26 weeks) after exposure to HCV, but the majority of persons have either no symptoms or only mild symptoms, and fulminant thepatic failure due to acute HCV infection is very rare. Although clinical features will be present in less than 25% of infected patients, symptoms of acute hepatitis include jaundice, malaise, nausea and right upper quadrant pain (132). While the infection becomes chronic in most cases,

chronic infection is either asymptomatic or has only mild nonspecific symptoms such as fatigue as long as cirrhosis and hepatocellular carcinoma are not present. Other clinical manifestations are possible, however, such as nausea, weakness, myalgia, arthralgia and weight loss (133). Although there have been many papers describing HCV-6's epidemiology, the clinical characteristics have not been well described in those studies. Nguyen et al. reported that patients with HCV-6 presented similar clinical manifestations as those with genotype 1 or 2/3. They also foundthat people with HCV genotype 1 and 6 had a somewhat higher baseline viral load than those with others genotypes (134). However, when comparing HCV-6 patients with patients infected with other genotypes, these differences were not statistically significant with regard to host factors (e.g. age, history of smoking, alcohol use, family history of CHC, hepatitis B, hepatocellular carcinoma and liver-related death), baseline laboratory values (e.g. ALT, total bilirubin, albumin, white blood cell count, platelet count), and liver histology (104, 135, 136). However, steatosis is a chief modulator of clinical course of HCV infection (119).

Treatment

The current standard-of-care for treatment of HCV-infected patients is a combination of PEG-IFN and RBV. Among the various viral and host factors, HCV genotype is one of the most important predictors of response to treatment and is used to guide the duration of treatment. Patients with HCV genotype 1 are typically treated for 48

weeks, whereas patients with genotype 2 and 3 are treated for 24 weeks (12, 13, 137). Limited studies have suggested that the response rate of HCV-6 may be at an intermediate level between those of genotypes 3 and 1 (134, 138-140).

Virological response kinetics during therapy has emerged as an important prognostic factor of treatment outcome in patients with chronic HCV infection (11, 64). Absence of an early virological response (EVR) at week 12 during therapy is the best negative predictor for non-response to treatment. In contrast, rapid virological response (RVR; defined as undetectable HCV RNA at week 4) is regarded as the most important predictor for SVR (defined as undetectable HCV RNA at week 24 after the end of therapy) and has emerged as an important milestone to guide the appropriate duration of therapy. For example, in patients with genotype 1, an individualized approach to therapy designed according to early viral kinetics has been adopted to optimize therapeutic outcome in patients. Recent clinical trials have used RVR to identify those patients with low baseline viral load that may benefit from shortened treatment duration of 24 weeks (141-143).

Treatment outcomes of HCV-6 vs. other genotypes

Currently, although the treatment outcome of patients with HCV-6 has so far not been exhaustively studied, a few studies exit which give hints as to what the standard course of care should be. Most previous studies have reported that genotype 6 behaves

more similar to genotypes 2 and 3 (SVR rates of 76-80%) (134, 144, 145) and thus

responds better to therapy than genotype 1 (SVR rates of 46-52%) (146). For example,

Nguyen et al. demonstrated that patients with HCV-6 had significantly better SVRs to

PEG-IFN and RBV combination therapy than patients with genotype 1 (74% vs. 49%)

(134). Furthermore, Tangkijvanich et al. reported that the SVR of HCV-6 is higher than

that of genotype 1 but lower than that of genotype 3 (147) (Figure 10).
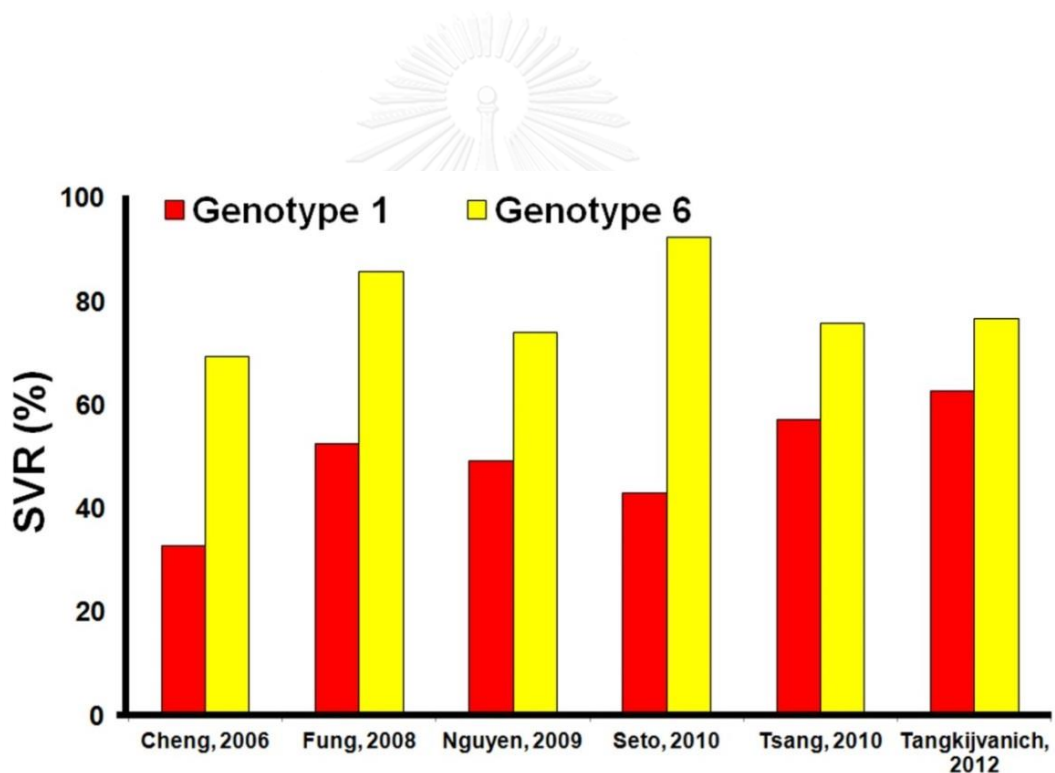
Figure 10. SVR in genotype 6 vs. genotype 1

Optimal treatment duration of HCV-6

Most prior studies of HCV-6 included patients treated for 48-52 weeks (148-150).

A small study of Asian-American patients comparing a 48-week to a shortened 24-week

regimen showed a significantly higher SVR rate in those treated by the 48-week course (74% vs. 49%) (16). However, the limitation of the study was its retrospective design and the results were not analyzed with regard to an intention-to-treat method. A retrospective study conducted in China showed that the rate of SVR in 22 patients with HCV-6 treated for 24 weeks was comparable to that of genotypes 2/3 (82% and 83%, respectively) (139). In that study, the positive predictive values of RVR and EVR for HCV-6 were comparable with those for genotypes 2/3 (87% vs. 91% and 86% vs. 87%, respectively).

A randomized controlled trial of 60 patients with HCV-6 demonstrated that there was no significant difference in SVR rates in patients treated with 48-week and 24-week regimens (79% and 70%, respectively) (146). In that study, RVR was a significant predictor of SVR in the 48-week group and tending towards significance in the 24-week group, although a sizeable number of patients did not have RVR measurement performed. Recently, Thuy et al. conducted an open-label randomized trial in Vietnam, which aimed at assessing the rate of SVR in HCV-6 chronic HCV following 48 and 24 weeks of PEG-IFN and RBV combination therapy (151). They demonstrated that RVR was achieved in the majority of HCV-6 patients and similar and high rates of SVR were noted following 48-week and 24-week therapy (71% vs. 60%, respectively; *p= 0.24*).

The feasibility of a response-guided therapy by individualizing the duration of treatment according to viral kinetics in patients with genotype 6 was first investigated by

Tangkijvanich et al. In that pilot study, more than 70% of patients with HCV-6 achieved RVR and received an abbreviated 24-week regimen. Among them, the rate of relapse was approximately 10%, and nearly 90% of them eradicated the virus. These data were consistent with observations regarding treatment of HCV genotypes 1, 2, 3 and 4 (152), which suggest that monitoring RVR might be useful to guide treatment duration for patients with HCV-6. In particular, therapy might be shortened to 24 weeks in patients with low pretreatment viral load who achieve RVR, whereas a 48-week course was appropriate for those who cleared the virus after week 4. The abbreviated regimen could offer advantages by reducing unnecessary medication exposure, which may make the treatment of HCV-6 more affordable and maximize the cost effectiveness of therapy. However, further prospective randomized trials are required to evaluate the response-guided strategy in a larger number of patients with HCV-6.

Optimal dose of RBV in treating HCV-6

Although PEG-IFN represents the backbone of treatment, combination with RBV has been shown to help prevent relapse. Current guidelines recommend a weight-adjusted dose of RBV in combination with PEG-IFN for treating patients with genotype 1, while a flat, low dose of RBV (800 mg/day) is recommended for treating patients with genotype 3 (64). However, a weight-adjusted dose of RBV might be useful to enhance the response rate in patients with genotype 3 who do not achieve RVR and in those with

RVR undergoing abbreviated therapy (153, 154). Currently, the optimal dose of RBV for treatment of patients with HCV-6 is unknown. In previous studies, daily weight-based or fixed doses of RBV had been used, rendering comparisons rather complicated. Nonetheless, recent prospective trials adopted a weight-based dosage of RBV (1000–1200 mg/day) for abbreviated treatment (24 weeks), which might result in achieving SVR equivalent to that obtained with longer treatment duration (48 weeks) (146). These data might reflect the need of a weight-based dosage of RBV in patients with HCV-6 undergoing abbreviated therapy.

Safety and side effect profile of treatment

Treatment with PEG-IFN and RBV has been shown to be safe in patients with HCV-6, but treatment discontinuation or dose reduction due to side-effects is typical. Although HCV genotypes play a role in achieving SVR, there is no significant difference in the frequency or types of side effects experienced among patients of genotypes 1, 2, 3 or 6 (134, 149, 150) taking PEG-IFN and RBV, although side effect profiles do appear to differ among patients of different ethnicities. For example, compared with Caucasians, Asian patients are more likely to decrease their RBV dose or discontinue the therapy due to anemia. In addition, Asian patients reported symptoms of depression, more commonly than Caucasian patients (155, 156). Other common side effects include flu-like symptoms (fever, fatigue, headache, malign, and loss of appetite), dyspepsia

and some cases with rash, weight loss, arthralgia and alopecia (155). However, these symptoms are often mild and tolerable and without the requirement for PEG-IFN and/or RBV dose modification.

Predictors of SVR

As in studies of patients with other HCV genotypes, pretreatment predictors of response are useful for advising patients on their likelihood of SVR. Pretreatment host and viral characteristics affect early viral kinetics. Once treatment has been initiated, outcome depends on how fast HCV RNA becomes undetectable. Multivariate analyses have identified various predictors of response in HCV-6 such as youth (<40–50 years) (145, 150), low BMI, treatment adherence and RVR (12, 145, 150). Among these predictors - and concordant with observations in other HCV genotypes - the importance of RVR (undetectable HCV RNA after 4 weeks of treatment) in the prediction of SVR has been further substantiated in HCV-6, wherein the positive predictive value to achieve SVR in patients with RVR has been 83–87% (134, 150).

Role of Interleukin IL28-B

Recent studies have reported that one of the strongest baseline predictors of SVR in HCV genotype 1 are single-nucleotide polymorphisms (SNPs) on chromosome 19 in or near the interleukin-28B gene (*IL28B,* encoding interferon lambda-3). Following

antiviral treatment, patients carrying the CC genotype of one of these predictive SNPs (rs12979860) have a twofold (95% confidence interval 1.8–2.3) greater rate of SVR than patients who carry the TT alleles (78% for the CC genotype, 38% for the TC genotype, and 26% for the TT genotype). Interestingly, the C allele frequency is much higher in white and Asian populations than in black populations (157). More recently, a variant upstream of IFNL3 creating a novel gene, designated as IFNL4, has been discovered (158). This region harbors a dinucleotide variant (ss469415590) that is found in two alternative forms (ΔG or TT alleles). The ss469415590 indel is more strongly associated with treatment response of HCV-1 infection in African-American individuals compared to rs12979860 (158).

Data regarding the association of the SNPs with antiviral response in HCV-6 infected patients are still very limited. A recent study from Hong Kong showed that rs8099917, another *IL28B* polymorphism, was associated with response to PEG-IFN/RBV therapy in HCV-6 infected patients (159). In that report, the favorable TT genotype of rs8099917, when compared to the unfavorable TG genotype, was significantly associated with an increased SVR rate (96.2% and 62.5%, respectively) and was the only clinical parameter that predicted SVR. In contrast, Akkarathamrongsin et al. found that in Thai patients the *IL28B* SNPs and the IFNL4 indel was equally associated with the treatment outcome in patients with HCV genotype 1, but not genotype 3 and 6 (unpublished data). Thus, future large-scale studies of patients

infected with HCV-6, including those from different ethnicities, should be conducted to validate the association of SVR with the *IL28B* and IFNL4 polymorphisms.

Direct-acting antiviral agents (DAAs)

In 2011, Direct-acting antivirals (telaprevir and boceprevir) were approved by the U.S. Food and Drug Administration (FDA) for treatment of HCV genotype 1. They are first generation NS3–4A protease inhibitors (PI), targeting the protease enzyme that cleaves the HCV polyprotein thus inhibiting the replication process. The addition of a DAA to PEG-IFN/RBV has reduced treatment duration and side effects, and improve efficacy and cost (160). Thus, the development of DAA represents a significant milestone in improving the efficacy of HCV treatment, especially in patients with HCV genotype 1.

Currently, clinical results of the use of DAAs for patients with HCV-6 are limited (161).For example, monotherapy with TMC 435, a second-generation NS3/4A PI with pan-genotype antiviral activity, could induce a significant mean viremia decrease of -4.35 $\log_{10}$ UI/mL after 8 days in patients with HCV-6. In addition, five patients with HCV-6 were included in the ATOMIC study and treated with sofosbuvir (GS 7977), a NS5B polymerase inhibitor, plus PEG-IFN/RBV for 24 weeks. The RVR rate at week 4 and the SVR rate 12 weeks after the end of the treatment were both 100%.

Conclusions

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

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

CHAPTER III

THE CORRELATION BETWEEN HEPATITIS C CORE ANTIGEN AND HCV RNA LEVELS WITH RESPECT TO HIV STATUS, HCV GENOTYPE AND IFNL4 POLYMORPHISM

Vo Duy Thong[a], Srunthron Akkarathamrongsin[a], Anchalee Avihingsanon[b], Apiradee Theamboonlers[a], Yong Poovoravan[a], and Pisit Tangkijvanich[c*]

[a]Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

[b]HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT), Bangkok, Thailand.

[c]Research Unit of Hepatitis and Liver Cancer, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Abstract

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

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

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

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

Introduction

Hepatitis C virus (HCV) infection is a major etiology of liver fibrosis, cirrhosis, and hepatocellular carcinoma, affecting up to 170 million people worldwide (162). HCV has been classified into seven major genotypes, all of which display different patterns of geographic distribution. In Thailand, approximately 2.2% of the general populations are chronically infected with HCV and the most common genotypes are genotype 3 (HCV-3), genotype 1 (HCV-1) and genotype 6 (HCV-6) (112). The screening tests for HCV infection in clinical practice rely on the detection of anti-HCV antibodies using enzyme-linked immunosorbent assays (ELISA). However, these tests cannot differentiate between resolved HCV infection and an active viral replication. Therefore, the measurement of serum HCV-RNA generally serves to confirm the diagnosis of an ongoing infection (10). In addition, monitoring of HCV RNA is crucial for assessing the treatment response to anti-viral therapy (11). Currently, real-time polymerase chain reaction (PCR)-based quantitative determination is the diagnostic gold standard. However, this assay is limited in developing countries due to costs and the requirement for a real-time PCR machine.

Recently, a highly sensitive assay for measuring serum HCV core antigen (HCVcAg) concentrations using a fully automated chemiluminescent microparticle immunoassay (CMIA) has become commercially available (47). A number of studies

have demonstrated good correlation between HCVcAg and HCV RNA levels, particularly in HCV mono-infected individuals (48-55). As a result, HCVcAg testing is considered to be an alternative assay to conventional HCV RNA quantification with the advantages of its rapidity, reproducibility and feasibility in resource limited settings (56). To date, data regarding the correlation between HCVcAg and HCV RNA levels in patients co-infected with human immunodeficiency virus (HIV) are still limited (57-59). In addition, the effect of different viral genotypes, particularly HCV-6 prevalent in Southeast Asia (163), on HCVcAg levels and the correlation with HCV RNA levels remains to be established.

Genome wide association studies (GWAS) have reported the association between single nucleotide polymorphisms (SNPs) adjacent to the *interleukin-28B (IL-28B)* gene (principally rs12979860) and treatment response in patients with chronic HCV infection (157, 164, 165). Recently, a transiently induced region (interferon lambda-4; IFNL4) harboring a dinucleotide variant ss469415590 (TT or ΔG) showed strong linkage disequilibrium to rs12979860 (25). So far, this novel ss469415590 SNP is the only functional variant identified in association with HCV clearance (166) and also a good predictor of treatment response to pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy (167). However, it is unclear whether there is an association between this SNP and HCVcAg concentrations in patients with chronic HCV infection. Thus, the aims of this study were to evaluate the use of HCVcAg measurement with respect to HIV status, HCV genotypes, ss469415590 polymorphism and clinical parameters.

Materials and Methods

Patients

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

Methods

HCV RNA quantification and genotypes

HCV RNA quantification was performed using the real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) (Abbott Molecular Inc. Des Plaines,

IL, USA) in accordance with the manufacturer's instructions. The lower and upper detection limits of the assay were <12 IU/ml and 100,000,000 IU/ml, respectively.

HCV genotypes were determined by nucleotide sequencing of the core and NS5B regions followed by phylogenetic analysis as described previously (112).

HCVcAg quantification

Quantification of HCVcAg was performed using a fully automated CMIA according to the manufacturer's instructions (Abbott Diagnostics, Tokyo, Japan). The assay allows the quantitative determination of HCVcAg in a linear range from 3 to 20,000 femtomoles/liter (fmol/l), with the possibility of 1: 9 automated dilutions that extended the assay linearity to 180,000 fmol/l (47).

IFNL4 genotyping

The presence of IFNL4 polymorphism ss469415590 was identified by RT- PCR assay using Taqman genotyping assay with MGB probes (Apply Biosystem, NY) as described previously (163). Briefly, genomic DNA of patients with chronic HCV infection was extracted from 100 μL of peripheral blood mononuclear cells (PBMC) using the QIAamp DNA Mini Kit (Qiagen, Germany). The reaction mixture consisted of 1 μL of DNA extract, 200 nM of each probe (ss469415590_IFNL4_VIC: 5'-ATCGCAGAAGGCC-3' and ss469415590_IFNL4_FAM: 5'-ATCGCAGCGGCCC-3'), 900 nM of each primer (ss469415590_IFNL4_F: 5'-GCCTGCTGCAGAAGCAGAGAT-3' and

ss469415590_IFNL4_R: 5'-GCTCCAGCGAGCGGTAGTG-3') (Applied Biosystems, NY), 5 μL of 2x Perfect Taq Plus Master Mix (5 PRIME, Gaithersburg, MD) adjusted to 10 μL final volume by distilled water. The PCR conditions were 2 minutes at 50°C, 10 minutes at 95 °C, 45 cycles of 15 seconds at 95 °C and 2 minutes at 60 °C. The fluorescent signal was detected at the end of each cycle. The genotype of ss469415590 was analyzed by applying the End Point Genotyping method (Light Cycler 480, Roche Diagnostics, IN). For this SNP, TT and ΔG are defined as major and minor alleles, respectively.

Liver stiffness measurement

After fasting for at least 2 hours, liver stiffness measurement was obtained from each patient using transient elastography (FibroScan, Echosens, Paris, France) according to the manufacturer's instructions. Results were recorded in kilopascals (kPa) as the median value of all measurements. The procedure was based on at least 10 validated measurements: the success rate (ratio between numbers of validated and total measurements) was over 60% and interquartile range was less than 30% (168).

Data analysis

The Mann-Whitney U test or Student's test were used to compare continuous variables, and the χ^2 test or Fisher's exact test were used to compare categorical variables. The Spearman rank correlation was used to evaluate the relationships

between variables. All data were analyzed by using SPSS Statistic Software Package for Windows version 20.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics

A total of 189 patients with chronic HCV infection were included in this study. There were 104 patients with HCV mono-infection and 85 patients with HCV/HIV co-infection. Table 2 summarizes the demographic and clinical characteristics of the patients. There were no significant differences in the baseline characteristics between each group in terms of mean age, body mass index (BMI), mean ALT level, the distribution of HCV genotypes and ss469415590 genotypes. HCV mono-infected patients had higher proportion of female gender compared with HCV/HIV co-infected patients ($p<0.001$). In contrast, HCV/HIV co-infected patients had significantly higher mean liver stiffness, $\log_{10}$ HCV RNA and $\log_{10}$HCVcAg levels compared with HCV mono-infected patients ($p=0.004$, $p=0.018$ and $p=0.035$, respectively).

Table 2. Demographics and characteristics of HCV mono- and co-infected patients.

Characteristics	Mono-infection (n=104)	Co-infection (n=85)	P value
Age, years	45.6 ± 9.7	44.1± 7.2	NS
Sex			<0.001
Males	61 (58.7)	74 (87.1)	
Females	43 (41.3)	11 (12.9)	
BMI, kg/m^2	23.9 ± 4.2	22.6 ± 6.5	NS
ALT, U/L	74.3±55.6	88.8 ± 53.1	NS
Liver stiffness, kPa	9.1 ± 5.6	14.1 ± 12.0	0.004
CD4+T cell counts, cell/µl	441.4 ± 216.6		
HCV RNA, $\log_{10}$ IU/ml	6.0 ± 1.0	6.3 ± 1.0	0.018
HCVcAg, $\log_{10}$ fmol/l	3.5 ± 0.6	3.7 ± 0.5	0.035
HCV genotypes			NS
1	38 (36.5)	29 (34.1)	
3	41 (39.4)	44 (51.8)	
6	25 (24.1)	12 (14.1)	
IFNL4 genotypes			NS
TT/TT	92 (88.5)	74 (87.1)	
TT/ΔG	7 (6.7)	10 (11.8)	
ΔG/ΔG	5 (4.8)	1 (1.1)	

BMI, Body mass index; ALT, alanine aminotransferase

Data are presented as mean ± standard deviation or number (%)

Correlation between $\log_{10}$HCVcAg and $\log_{10}$HCV RNA levels

In this study, serum HCV RNA and HCVcAg levels were detected in all cases.

Mean $\log_{10}$HCV RNA was 6.1±1.0 IU/ml (range, 3.4-7.9IU/ml) and mean $\log_{10}$HCVcAg

was 3.6±0.6fmol/l (range, 1.9-4.6fmol/l). In the overall cohort, $\log_{10}$HCVcAg levels

significantly correlated with corresponding $\log_{10}$HCV RNA levels (r=0.889, *p<0.001*) as shown in Figure 11. Regarding HIV status, there were significant correlation between both markers in mono-infected patients (r=0.845, *p<0.001*) and in co-infected patients (r=0.939, *p<0.001*). However, mean $\log_{10}$HCV RNA/HCVcAg ratio was not significantly different between mono-infected and co-infected patients (1.69±0.18 vs. 1.70±0.12, respectively, *p=0.774*).
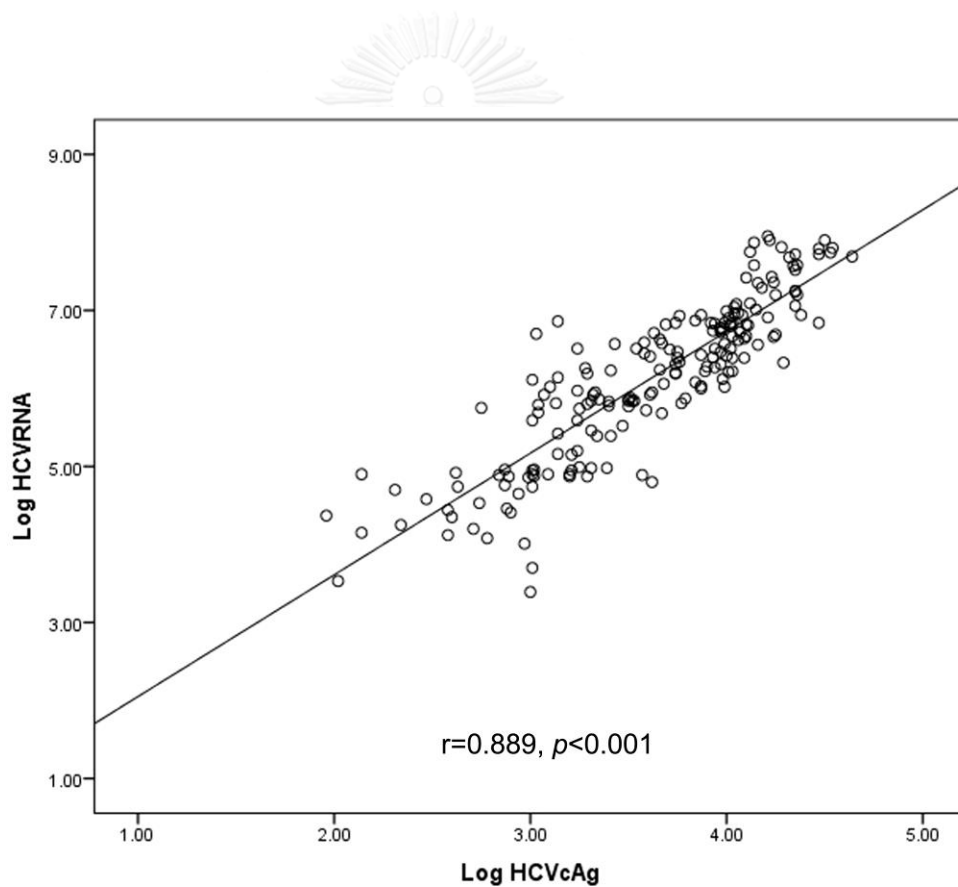
Figure 11. Correlation between $\log_{10}$HCVcAg and $\log_{10}$HCV RNA levels in all patients

Additionally, we investigated the correlation between both markers in terms of HCV viremia. Overall, high viral load samples ($\log_{10}$HCV RNA $\geq$5.0 IU/ml) showed a better correlation (r=0.815, *p<0.001*) than low viral load samples ($\log_{10}$HCV RNA < 5.0 IU/ml (r=0.555, *p<0.001*). The same trends were also observed in the mono-infected group (r=0.731, *p<0.001* and r=0.462, *p=0.015*, respectively) and the co-infected group (r=0.902*, p<0.001* and r=0.724, *p=0.003*, respectively). Regardless of HIV status, mean $\log_{10}$HCV RNA/HCVcAg ratio was significantly higher in the high viral load group compared with the low viral load group (1.72±0.13 vs. 1.63±0.23, respectively, *p=0.002*).

Correlation between $\log_{10}$HCVcAg levels and HCV genotypes

The correlation between $\log_{10}$HCVcAg and $\log_{10}$HCV-RNA levels was also evaluated with respect to HCV genotypes. Our data showed that $\log_{10}$HCVcAg level significantly correlated with $\log_{10}$HCV-RNA across HCV genotypes with the best correlation found in HCV-3 [HCV-1 (r=0.796, *p<0.001*), HCV-3 (r=0.919, *p<0.001*) and HCV-6 (r=0.895, *p<0.001*)]. When comparing the levels of $\log_{10}$HCV-RNA and $\log_{10}$HCVcAg among different HCV genotypes, we did not observe significant difference although patients with HCV-6 had slightly higher levels of both markers than the other groups (Table 3). In addition, the levels of both markers regarding HIV status were not significantly different across HCV genotypes (data not shown).

Table 3. Serum levels of $\log_{10}$HCV-RNA and $\log_{10}$HCVcAg according to HCV genotypes.

	Genotype 1 (n=67)	Genotype 3 (n=85)	Genotype 6 (n=37)	*P* value
HCV RNA, $\log_{10}$ IU/ml	6.0 ± 0.9	6.1 ± 1.0	6.4 ± 1.1	0.227
HCVcAg, $\log_{10}$fmol/l	3.5 ± 0.5	3.6 ± 0.6	3.8 ± 0.6	0.058

The relationship between HCV genotypes and clinical parameters was further examined. Patients with HCV-3 infection had significantly higher mean ALT level than patients with HCV-6 infection (90.8±53.6 vs. 60.0±50.2 U/L, respectively, *p=0.004*), but did not significantly differ from patients with HCV-1 infection (79.8±56.1 U/L, *p=0.229*). There was no significant difference in liver stiffness among patients infected with HCV-1, HCV-3 and HCV-6.

Correlation between $\log_{10}$HCVcAg levels and ss469415590 genotypes

Since a relatively small proportion of individuals displayed TT/ΔG or ΔG/ΔG genotypes of ss469415590, we grouped these genotypes together for statistical analysis. As shown in Table 4, patients harboring TT/TT genotype had significantly higher levels of $\log_{10}$HCV RNA than those with non-TT/TT genotype (6.2±1.0 vs. 5.6±1.0 IU/ml, respectively, *p=0.014*). The similar observation was found for $\log_{10}$HCVcAg levels (3.6±0.6vs. 3.3±0.5 fmol/l, respectively, *p=0.004*).

Table 4. Serum levels of $\log_{10}$HCV-RNA and $\log_{10}$HCVcAg according to ss469415590 genotypes

	TT/TT genotype (n=166)	Non-TT/TT genotype (n=23)	*P* value
HCV RNA, $\log_{10}$ IU/ml	6.2 ± 1.0	5.6 ± 1.0	0.014
HCVcAg, $\log_{10}$fmol/l	3.6 ± 0.6	3.3 ± 0.5	0.004

In the mono-infected group, there was no significant difference between patients with TT/TT and non-TT/TT genotypes in term of $\log_{10}$ HCV RNA (6.0 ± 0.9 vs. 5.4 ± 1.2 U/L, respectively, *p=0.119*) and $\log_{10}$ HCVcAg levels (3.6±0.6 vs. 3.3±0.6 fmol/l, respectively, *p=0.193*), although a trend towards increased both markers were observed among the TT/TT genotype. In the co-infected group, patients harboring TT/TT genotype had significantly higher levels of $\log_{10}$ HCV RNA and $\log_{10}$ HCVcAg than those with the non-TT/TT genotype (6.4± 1.0 vs. 5.8 ± 0.8 U/L, respectively, *p=0.034* and 3.8±0.5 vs. 3.3±0.3 fmol/l, respectively, *p=0.002*).

The relationship between ss469415590 genotypes and clinical parameters was also explored. Regarding baseline serum ALT level, there was no significant difference between patients with TT/TT and non-TT/TT genotypes (83.8±56.1 vs. 60.7±40.2 U/L, respectively, *p=0.058*), although a trend towards increased ALT levels was observed among the TT/TT genotype group. Similarly, there was no significant difference in liver stiffness between patients with TT/TT and non-TT/TT genotypes (11.9±10.1 vs. 13.9±11.2 kPa, respectively, *p=0.543*).

Correlation between HCVcAg levels and clinical parameters

In addition to associations with HCV genotypes and ss469415590 genotypes, the relationship between HCVcAg levels and clinical parameters was examined. In this study, $\log_{10}$HCVcAg levels were not correlated with serum ALT levels (r=-0.045, $p=0.542$) and liver stiffness (r=-0.122, $p=0.155$). Similarly, $\log_{10}$HCV RNA levels were not correlated with serum ALT (r=-0.031, $p=0.676$) and liver stiffness (r=-0.078, $p=0.365$). We also analyzed the correlation between HCVcAg levels and CD4+T cell counts in the co-infection group. In this study, $\log_{10}$ HCVcAg levels were not correlated with CD4+T cell counts (r=0.093, $p=0.396$). Similarly, $\log_{10}$HCV RNA levels were not correlated with CD4+T cell counts (r=0.063, $p=0.569$).

Discussion

Detection of HCVcAg levels in the serum has emerged as a potential marker for active HCV infection and may be used to evaluate response to antiviral therapy and disease progression. Several recent data have demonstrated that serum HCVcAg concentrations correlate well with HCV RNA levels in HCV mono-infected patients (48-54). In the present study, we further investigated the use of HCVcAg measurement in HCV/HIV co-infected patients. In particular, we directly compared the use of HCVcAg measurement between HCV mono-infected and HCV/HIV co-infected individuals, with

respect to HCV genotypes, host genetic variations and other disease parameters that could influence the clinical outcome of chronic HCV infection.

Previous studies estimated that the sensitivity of the ARCHITECT HCVcAg assay is approximately 3 fmol/l, which corresponds to 500-2500 IU/ml of HCV-RNA (49, 50, 54). Thus, the concordance of detectable HCV RNA and HCVcAg of all samples in this cohort likely resulted from high viremia, which is a typical feature of untreated individuals. Our data confirmed previous reports that HCVcAg levels had a good correlation with the corresponding HCV RNA levels in both mono- and co-infection groups. It should be noted that the correlation between both assays appeared to be higher among the co-infected group than the mono-infected group (r=0.939 and r=0.845, respectively). Indeed, the correlation coefficients between the two markers in co-infected patients exceeded 0.90 in most previous studies (57-59). For example, a recent cross-sectional survey conducted in China demonstrated that the correlation coefficients between HCVcAg and HCV RNA concentrations was higher in the co-infected than mono-infected group (r=0.952 and r=0.808, respectively) (59). Another study conducted in Germany showed that the correlation coefficients between these two markers in HCV/HIV co-infection was 0.97 compared to 0.75 in HCV mono-infection (58).

It should be mentioned that the correlation between HCVcAg and HCV RNA levels was also typically high among other groups of patients with impaired immune

response, including liver and kidney transplant recipients (169) and patients with end-stage kidney disease (170). In contrast, such correlation was less consistent among immunocompetent individuals. Thus, it appears that the correlation between both markers of HCV replication might be in part influenced by the immune status of infected individuals. The explanation of these interesting findings are unclear, but might be related to an interaction between HCVcAg and anti-HCV antibodies. In general, it was proposed that anti-HCV antibodies might mask the detection of HCVcAg or could lead to an enhanced clearance of circulating antigen. However, among patients with impaired immune response including HIV-infected individuals or patients receiving immunosuppressive therapy, lower antibody levels presumably do not interfere with the measurement of HCVcAg (58, 169). In contrast, patients with HBV/HCV co-infection did not show good correlation between the two markers (58), which might result from the complexity of viral interaction and fluctuation of virological dominance over time (171). Collectively, these data indicate that HCVcAg measurement is an excellent surrogate marker for monitoring HCV viral replication in HCV/HIV co-infected patients.

Of note, mean serum concentrations of HCV RNA and HCVcAg in our study were significantly higher in co-infected than mono-infected groups. These results are consistent with previous reports that HCV/HIV co-infection is associated with persistent HCV viremia and higher HCV viral load (172). The increased HCV RNA levels among co-

infected patients are thought to be in part associated with the decline in CD4+ and CD8+ T-cell responses to HCV infection. Our data also showed that co-infected patients had significantly higher liver stiffness, representing more advanced liver fibrosis compared to mono-infected patients. These findings are in agreement with previous studies that the prevalence of significant liver fibrosis is usually high among co-infected individuals (173). In the context of HCV/HIV co-infection, emerging data have suggested that multiple conditions, including increases in pro-fibrogenic cytokine expression and secretion, enhancement of oxidative stress production, and increases in hepatocyte apoptosis all contribute to accelerated fibrosis (174). In contrast to previous studies, our results did not demonstrate the correlation between HCVcAg concentration and liver inflammatory activity and liver fibrosis (175).

Unlike previous reports of HCVcAg, our study included a proportion of patients infected with HCV-6. As mentioned previously, the data on this HCV genotype are sparse due to its restricted distribution in south China and Southeast Asia (163). In this study, we observed slightly different correlations between HCVcAg and HCV RNA levels across HCV genotypes with the best correlations found in HCV-3, followed by HCV-6 and HCV-1, respectively. However, HCVcAg and HCV RNA levels did not vary according to HCV genotypes. Of note, samples with high viral load showed a better correlation of HCVcAg and HCV RNA concentrations than those with low viral load. In

addition, mean HCV RNA/HCVcAg ratio was significantly higher among samples with high viremia compared to those with low viremia. These observations were presumably related to a higher proportion of unmasked HCVcAg detectable by the HCVcAg assay in the setting of high viremia, as described previously in HCV/HIV co-infected patients and transplant recipients (58, 169).

Recent reports have shown that the **Δ**G variant of ss469415590 is associated with poorer HCV clearance and response to antiviral therapy than the TT allele (25, 167). However, the association of this polymorphism with HCVcAg levels remains to be elucidated. To our knowledge, our study is the first to assess IFNL4 genetic status in relation to HCVcAg levels in patients with chronic HCV infection. We found that patients harboring TT/TT genotype had significantly higher levels of HCV RNA and HCVcAg than those with the non-TT/TT genotype, regardless of HIV status and HCV genotypes. This finding is consistent with previous data in that the favorable CC genotype of rs12979860, which is associated with better treatment response, is also related to higher baseline viral load (157, 176). The effects on HCV RNA and HCVcAg concentrations might be in part related to biological interaction between HCV replication and IFNL4 protein. As shown in previous studies, the IFNL4 **Δ**G variant could induce weak expression of interferon-stimulated genes, which in turn provides an antiviral response in reducing HCV viral load. In contrast, the **Δ**G variant also diminishes the responsiveness

to type I and type III interferon required for effective treatment during antiviral therapy (25, 177). More information is needed to understand the mechanisms that underlie this association, as well as the clinical impact of ss469415590 polymorphisms on HCVcAg detection.

In conclusion, our data showed that there was an excellent correlation between HCV RNA and HCVcAg concentrations, particularly in HCV/HIV co-infected individuals. We also demonstrated that serum levels of HCVcAg were association with ss469415590 polymorphism. As the HCVcAg assay is a reliable test and has the advantages of being rapid and reproducible, its measurement could be used as an alternative to HCV RNA assays in resource-limited settings.

CHAPTER IV

PREVALENCE OF THYMINE-ADENINE DINUCLEOTIDE REPEAT, IL28B AND IFNL4 IN THAI POPULATION AND CORRELATION WITH SPONTANEOUS CLEARANCE AND TREATMENT OUTCOME OF HEPATITIS C INFECTION

Vo Duy Thong[1,3], Rujipat Wasitthankasem[1], Pisit Tangkijvanich[2], Sompong Vongpunsawad[1], Yong Poovorawan[1*]

[1]*Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[2]*Research Unit of Hepatitis and Liver Cancer, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[3]*Department of Internal Medicine, Division of Gastroenterology and Hepatology, Faculty of Medicine, University of Medicine and Pharmacy, Ho Chi Minh, Vietnam*

Abstract

Background: The interleukin-28B (*IL28B) gene polymorphism is* a strong baseline predictor of sustained virological response (SVR) in hepatitis C virus (HCV) treatment. The length of thymine–adenine dinucleotide repeats, or $(TA)_n$, in the regulatory region of *IL28B* can affect interferon transcription. In order to determine predictive values in HCV infection, we explored the correlation among factors including $(TA)_n$ genotypes, clinical features, interferon-λ-3 (IFNL3) and interferon-λ-4 (IFNL4) polymorphisms, and HCV treatment outcome.

Methods: Sera from 492 patients with chronic HCV infection, 101 individuals with spontaneous HCV clearance and 123 healthy blood donors (control group) were analyzed. Genotyping of the $(TA)_n$ was performed by direct sequencing. The rs12979860 (IFNL3) was identified using nested PCR and sequencing, while ss469415590 (IFNL4) was identified by real-time PCR.

Results: The distribution of $(TA)_n$ was similar between individuals with spontaneous HCV clearance and chronic HCV infection, but differed significantly from healthy controls. Individuals with both $(TA)_n$ alleles ≥12 had significantly higher SVR rate compared to individuals with at least one $(TA)_n$ <12 allele. This strong correlation was seen for patients infected with HCV-1, HCV-3, and HCV-6. The $(TA)_n$ genotypes were not associated with HCV viral load, ALT levels and liver stiffness, but were correlated with

platelet counts ($p<0.001$).   In contrast, rs12979860 (CC) and ss469415590 (TT/TT) genotypes were associated with higher SVR rated only in patients with HCV-1.

Conclusions: The $(TA)_n$ genotypes was not associated with spontaneous clearance of HCV infection but associated with treatment response in patients infected with HCV-1, HCV-3 and HCV-6. In contrast, IFNL3 and IFNL4 polymorphisms were predictive of treatment outcome only for patients infected with HCV-1.

Introduction

Hepatitis C virus (HCV) infection is a significant global public health problem affecting an estimated 160 million people (~2.35% of the population) worldwide (178). The progression to chronic HCV sometimes necessitates the need for liver transplantation and is often the leading cause of hepatocellular cancer (179). A combination of pegylated interferon (PEG-IFN) combined with ribavirin (RBV) for the duration of 24 to 48 weeks has been the standard-of-care therapy for HCV infection for the past decade. Virus genotype and host factors such as age, sex, race, fibrosis, and steatosis can determine the treatment outcome (12-14). In 2009, several genome-wide association studies reported that single nucleotide polymorphisms (SNPs) upstream of the interleukin-28B (*IL28B*) gene, which encodes interferon-**λ**-3 (IFNL3), were strongly associated with response to PEG-IFN/RBV therapy and spontaneous HCV clearance (157, 180, 181). The rs12979860 of CC genotype is associated with a two-fold greater sustained virological response (SVR) rate than the TT genotype (157). Interestingly, the gene frequency of C allele is much higher in European and Asian ancestries than in African ancestry. Recently, it was shown that the polymorphism in IFN-**λ**-4 (*IFNL4)* gene, ss469415590 of TT genotype, is more strongly associated with treatment-induced response and spontaenous HCV clearance than rs12979860 in Europeans and Asian, but especially in individuals of African ancestry (158).

Recently, an insertion/deletion polymorphism in the promoter region of *IL28B* consisting of thymine–adenine dinucleotide repeats $(TA)_n$ has been linked to *IL28B* gene expression. The length of $(TA)_n$ reportedly varies from 10 to 18 repeats with the most frequent genotype of 12/12 (61). Luciferase assay showed that the transcriptional activity of the promoter increased gradually with increasing $(TA)_n$ length. Therefore, $(TA)_n$ could be associated with the transcriptional activity of *IL-28B* and could potentially be used to improve predictions of the response to interferon-based HCV treatment. In this study, we focused on the distribution of the length of $(TA)_n$ and the correlation of $(TA)_n$ genotypes with treatment outcome, clinical features, *IL28B* and IFNL4 polymorphisms in HCV infection.
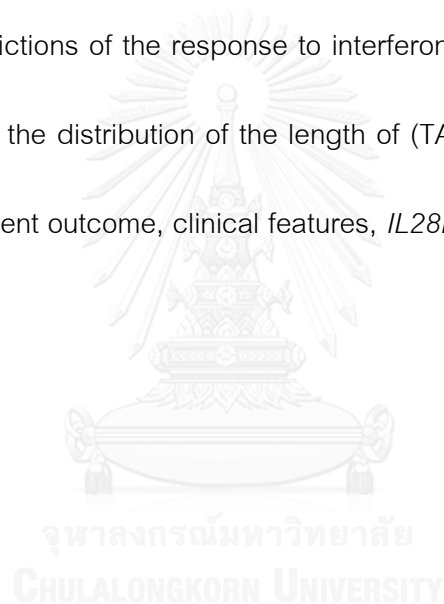
Patients and Methods

Patients

The study followed the Helsinki Declaration on medical research. The study obtained written informed consents from patients and the protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 517/57). A total of 593 HCV-infected Thai individuals comprising of 101 patients with spontaneous HCV clearance (defined as anti-HCV sero-positive and undetectable HCV RNA in patients without previous antiviral treatment) and 492 patients with chronic HCV (defined as anti-HCV sero-positive and detectable HCV RNA for more than 6 months) were followed-up at King Chulalongkorn Memorial Hospital in Bangkok, Thailand. Among the 492 patients chronically infected with HCV, 264 underwent treatment and 228 did not. Patients who received ≥ 80% of the recommended dose of PEG-IFN/RBV were considered assessable for response to treatment. SVR was defined as an absence in detectable HCV RNA in serum at 24 weeks after treatment termination. All other patients were considered non-responders. For comparison, 123 healthy Thai blood donors who tested negative for HBsAg and anti-HCV comprised the control group (Figure 12). Among these, 225 individuals from a previous study to investigate the association of rs12979860 and ss469415590 with HCV treatment response (182) were included in this study for better data analysis.
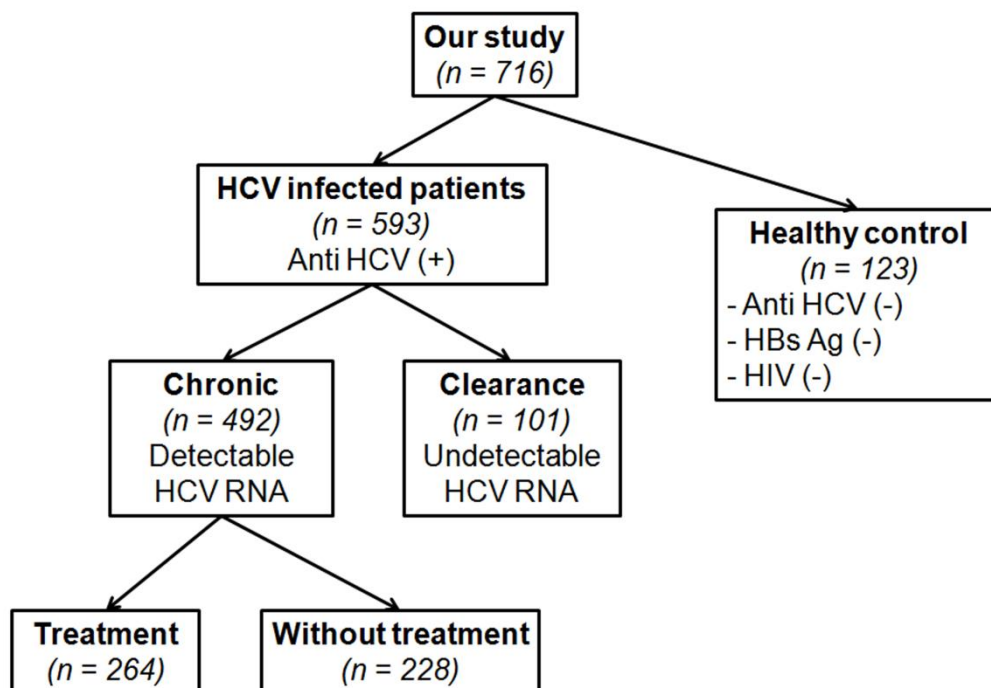
Figure 12. Diagram on the collection of cohort in this study.

Methods

Clinical, biochemical, liver assessment evaluation and SNP genotyping

During screening, we obtained patient information including demographical data (age, sex, height, weight, and body mass index) and laboratory tests at baseline including blood cell counts, aspartate transaminase (AST) and alanine transaminase (ALT). Liver fibrosis was evaluated through fibroscan stiffness. HCV treatment was also extracted from clinical database.

Two SNPs, rs12979860 (*IL28B*) and ss469415590 (IFNL4), were genotyped using nested PCR (182) and real-time PCR (158), respectively. For rs12979860, PCR products were sequenced and the major (CC) and minor (TT) alleles were determined.

In order to investigate an upstream variation of *IL28B*, ss469415590, real-time PCR using the Taqman genotyping assay with MGB probes (Applied Biosystems, Carlsbad, CA) was performed as previously described (158, 183). TT and ΔG were major and minor alleles, respectively.

HCV RNA quantification and genotypes

HCV RNA quantification was performed using the real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (COBAS TaqMan HCV assay, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

HCV genotypes were determined by nucleotide sequencing of the core and NS5B regions followed by phylogenetic analysis as previously described (112).

TA repeat genotyping

Genomic DNA of patients was extracted from peripheral blood mononuclear cells (PBMC) or plasma using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). PCR was performed using primers TA-CU-R (5'-CAATTCTTGAGCAGAGCCTCA-3') and TA-CU-F (5'- GGAAGGTATGTTCCCAAGAGG-3') and contained 5 μL DNA, 5 pmol of each primer, 10 μl of 2x Perfect *Taq* Plus MasterMix (5 PRIME, Gaithersburg, MD) in a total volume of 25 μl. The amplification cycles were: 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 54.9°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 7 min. PCR fragment was resolved by

2% agarose gel electrophoresis. PCR products were purified using GelExtract Mini Kits (5 PRIME, Gaithersburg, MD) and subjected to sequencing (First BASE Laboratories, Selangor, Malaysia) using both forward and reverse primers. The length and genotypes of TA repeat were analyzed manually based on the chromatograms (Chromas LITE, version 2.01) and compared to the reference sequence retrieved from GenBank (http://www.ncbi.nlm.nih.gov/).

Data analysis

The Mann-Whitney U test or Student's test was used to compare continuous variables, and the χ^2 test or Fisher's exact test was used to compare categorical variables. The Spearman rank correlation was used to evaluate the relationships among variables. All data were analyzed using SPSS Statistic Software Package for Windows version 20.0 (SPSS, Chicago, IL). Statistical significance was set at *p<0.05* and all tests were two-tailed. Numerical data are presented as mean +/- standard deviation (SD) or median and interquartile range (IQR).

Results

Patients with spontaneous HCV clearance, chronic HCV, and the control group had comparable mean age, body mass index (BMI), and *IL28B* genotype distribution (Table 5). Chronic HCV group had slightly different IFNL4 and $(TA)_n$ genotype

distribution compared to other groups, but these values were not statistically significant.

However, twice as many males than females had chronic HCV (*p<0.001*).

Table 5. Demographics and characteristics of healthy controls, patients with spontaneous HCV clearance, and patients with chronic HCV.

Characteristic	All (n = 716)	Control (n = 123)	Spontaneous Clearance (n = 101)	Chronic HCV (n = 492)	P value
Age, years	44.78 ± 10.1	46.76 ± 5.8	41.17 ± 10.9	44.89 ± 10.7	0.351
Sex					0.015
Males	460 (64.2%)	73 (59.3%)	53 (52.5%)	334 (67.9%)	
Females	256 (35.8%)	50 (40.7%)	48 (47.5%)	158 (32.1%)	
BMI , kg/m^2	25.33 ± 11.2	N/A	24.13 ± 4.2	25.59 ± 12.2	0.327
rs12979860 genotypes					NS
CC	624 (87.2%)	109 (88.6%)	88 (87.1%)	427 (86.8%)	
CT	76 (10.6%)	13 (10.6%)	11 (10.9%)	52 (10.6%)	
TT	16 (2.2%)	1 (0.8%)	2 (2%)	13 (2.6%)	
ss469415590 genotypes					NS
TT/TT	615 (85.9%)	110 (89.4%)	90 (89.1%)	415 (84.3%)	
TT/ΔG	86 (12.0%)	12 (9.8%)	9 (8.9%)	65 (13.2%)	
ΔG/ΔG	15 (2.1%)	1 (0.8%)	2 (2%)	12 (2.4%)	
$(TA)_n$ genotypes Hetero-/homozygous	37/679 (5.2%/94.8%)	12/111 (9.8%/90.2%)	9/98 (8.9%/91.1%)	16/476 (3.3%/96.7%)	NS

In parentheses are percentages unless otherwise noted. p-value <0.05 is considered statistically significant. N/A=Information not available. NS=not significant.

Homozygous is defined as having two identical alleles.

Heterozygous is defined as having different alleles.

Overall distribution of the allele frequencies of (TA)$_n$ in the study population

When both alleles from all 716 individuals in this study were examined (1432 alleles altogether), the observed variation of (TA)$_n$ ranged from 6 to 16 with the mode of 12 (91.7%) (Figure 13). The second and third most frequent (TA)$_n$ were 13 (4.0%) and 10 (2.1%), respectively. Other (TA)$_n$ genotypes comprised less than 1% each, and no (TA)$_n$ of 9 was observed.
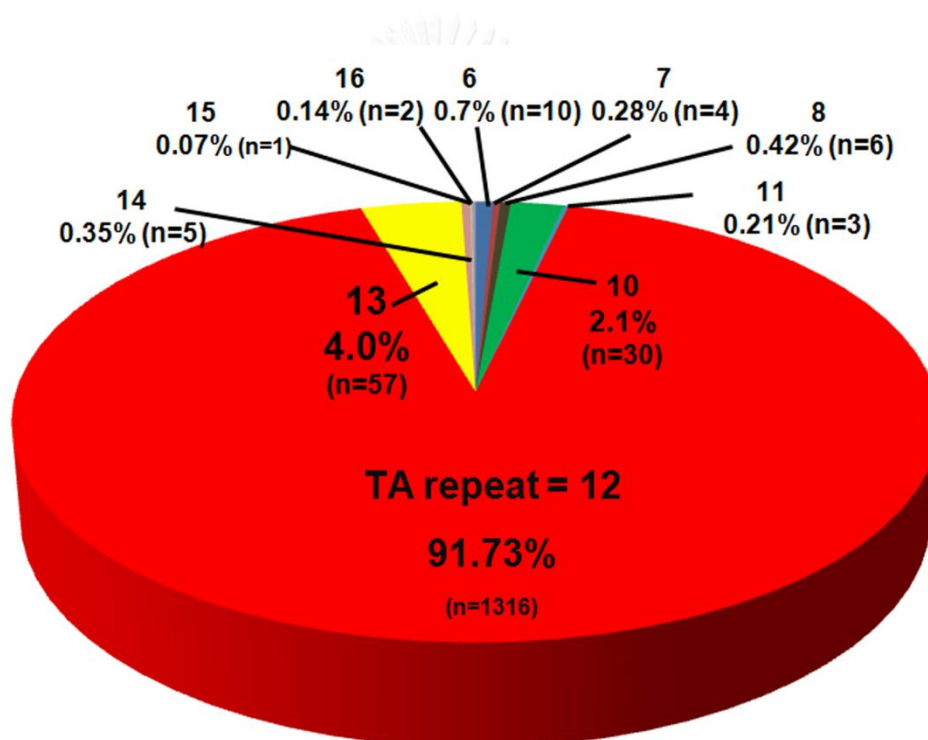
Figure 13. Allele frequencies of (TA)$_n$ in this study (n = 1432).

Genotype (TA)$_n$ of 12 was predominant in all 3 groups (Figure 14A). Although all individuals had (TA)$_n$ of 13, individuals in the spontaneous clearance and chronic HCV groups did not have (TA)$_n$ >13. As a result, these groups had significantly fewer (TA)$_n$

>12 than the control group (*p<0.001*). Furthermore, $(TA)_n$ <10 was not observed in the control group. Comparison between the spontaneous clearance and chronic HCV groups showed that there was no significant difference in the frequency of allele $(TA)_n$ >12 (*p=0.217*) or allele $(TA)_n$ <10 (*p=0.352*) (Figure 14B).
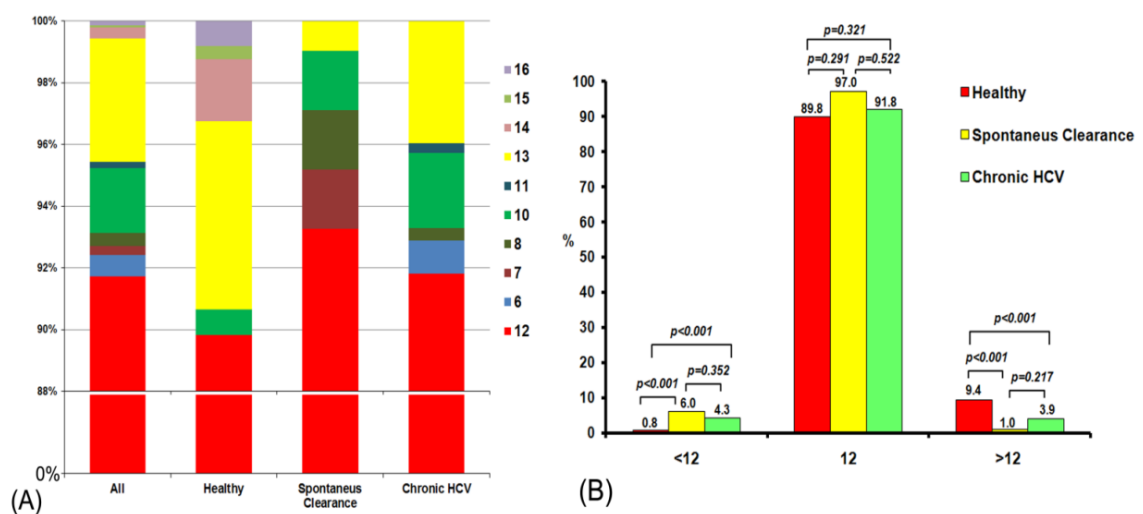
Figure 14. Allele distribution in our population. (A) Allele frequency of $(TA)_n$ distribution in the control, spontaneous clearance, and chronic HCV groups (n = 1432). (B) The frequency distribution of $(TA)_n$ genotypes with <12, 12 and >12 alleles in control, spontaneous clearance, and chronic HCV groups (n = 1432).

Prevalence of $(TA)_n$ genotypes in spontaneous clearance, chronic HCV, and control groups.

For the purpose of analysis, we defined an individual as homozygous for $(TA)_n$ genotype when that person possessed the same $(TA)_n$ for both alleles. In contrast, an individual is heterozygous for $(TA)_n$ genotype when that person possessed different

$(TA)_n$ alleles. When all 3 groups were analyzed, we found that the $(TA)_n$ genotype was 94.8% homozygous (679/716) and 5.2% heterozygous (37/716). The most prevalent $(TA)_n$ homozygous genotype was 12/12, meaning that an individual possessed $(TA)_n$ of 12 for both alleles. When analyzing each group separately, there were more individuals with $(TA)_n$ of 12/12 in the spontaneous clearance group than in the control or chronic HCV groups (97.0%, 89.8%, and 91.1%, respectively).

To further simplify the analysis for individuals with heterozygous $(TA)_n$, we defined an individual genotype "L" when both alleles were $\geq$ 12 and "S" when at least one allele is <12. Using this definition, the most prevalent heterozygous $(TA)_n$ genotype in all 3 groups was L (Figure 15). Genotype L was less frequent in the spontaneous clearance (94.4%) and chronic HCV (95.2 %) groups than the control group (99.2%) (*p<0.001*). The difference in genotype L between spontaneous clearance and chronic HCV groups was not statistically significant (*p=0.487*). In contrast, genotype S was more frequent in the spontaneous clearance (4.61%) and chronic HCV (3.85%) groups compared to the control group (0.81%) (*p<0.001*).
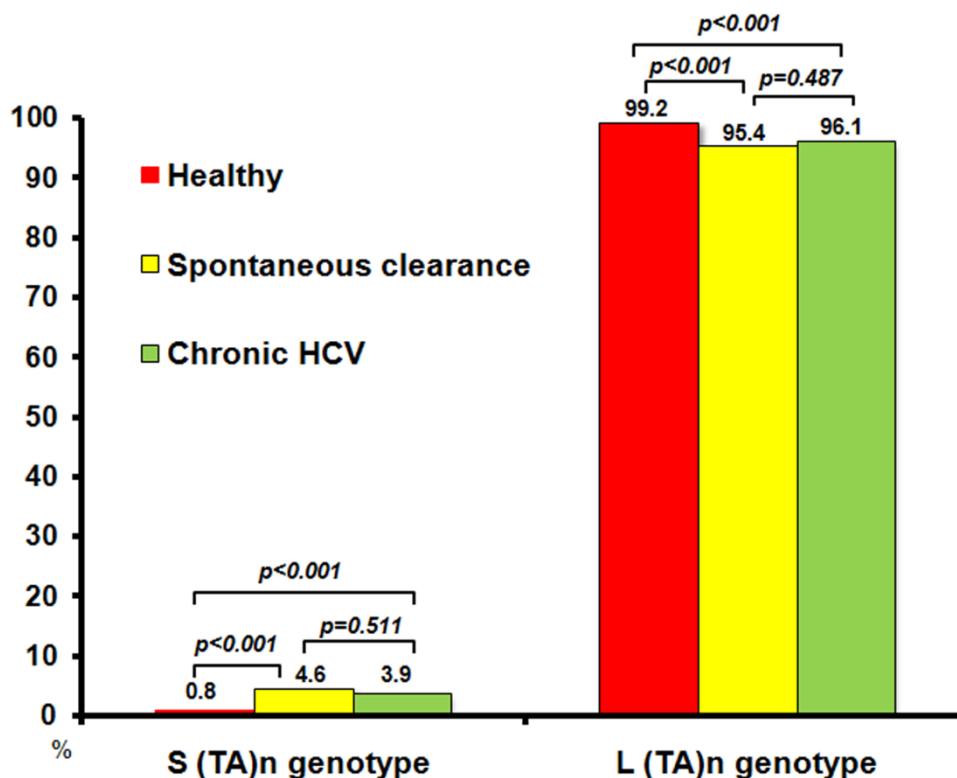
Figure 15. Prevalence of $(TA)_n$ genotypes in control, spontaneous clearance, and chronic HCV groups (n = 716). The $(TA)_n$ genotype is defined as "L" (when both alleles are $\geq$ 12) and "S" (when at least one allele is <12).

SVR rates according to $(TA)_n$, rs12979860 and ss469415590

The 264 individuals with chronic HCV underwent PEG-IFN/RBV therapy. When the $(TA)_n$ <12, SVR and non-SVR rates were similar (1.70% versus 1.33%, *p=0.427*) (Figure 16A). In contrast, individuals with $(TA)_n$ $\geq$12 were more likely to have SVR than non-SVR (79.36% versus 17.61%, *p<0.001*). Furthermore, individuals with genotype L had significantly higher SVR rate than those with genotype S (84.9% vs. 53.8%, *p<0.001*). Collectively, this observation shows striking concordance for all HCV

genotypes (HCV-1: 87.0% vs. 20.0%, *p<0.001*; HCV-3: 85.3% vs. 25.0%, *p<0.001*; and

HCV-6: 89.5% vs. 33.3%, *p<0.001*) (Figure 16B).
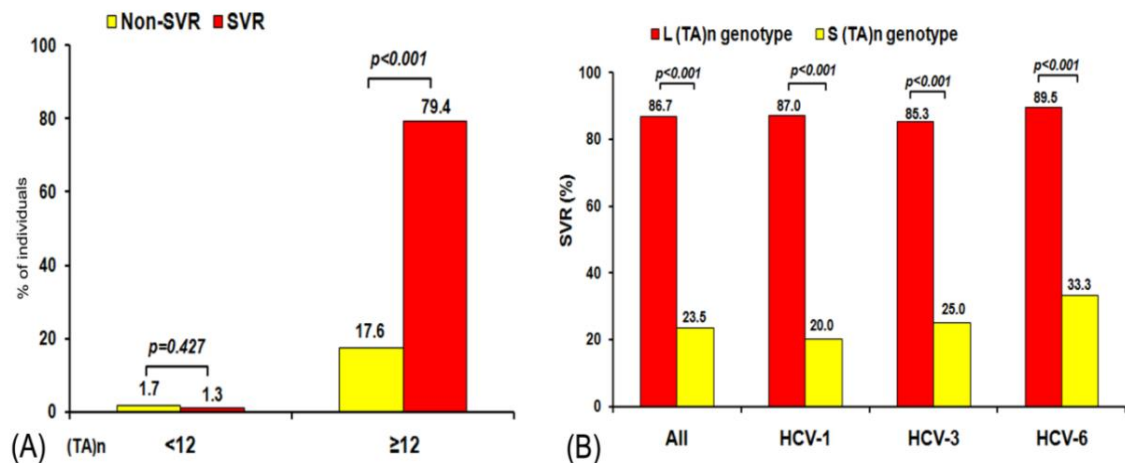
Figure 16. Association of $(TA)_n$ with SVR. (A) Association of $(TA)_n$ with SVR to PEG-IFN/RBV therapy among individuals with chronic HCV. (B) Percentage of $(TA)_n$ genotypes L and S relative to SVR in individuals with chronic HCV who underwent PEG-IFN/RBV therapy. The $(TA)_n$ genotype is defined as "L" (when both alleles are ≥ 12) and "S" (when at least one allele is <12).

Overall, significantly higher SVR rates were observed in chronic HCV with the

favorable CC genotype than non-CC genotype for rs12979860 (84.2% vs. 59.5%,

p<0.001) and TT/TT genotype than non-TT/TT genotype for ss469415590 (84.9% vs.

53.8%, *p<0.001*) (Figures 17A and 17B). The differences in SVR between favorable and

unfavorable genotypes for both rs12979860 and ss469415590 were greatest for HCV-1.

For rs12979860, 88.9% of patients with CC genotype achieved SVR compared to 45.8%

of patients with non-CC genotype (*p<0.001*). For ss469415590, 90.3% of patients with

TT/TT genotype achieved SVR compared to 41.7% of patients with non-TT/TT ($p<0.001$).

The differences in SVR for HCV-3 and HCV-6, however, were not statistically significant.

Although results from our previous study suggested association between rs12979860

IL28B and ss469415590 IFNL4 (*p<0.001*), these polymorphisms were not associated

with $(TA)_n$ (*p=0.129* with rs12979860*, p=0.108* with ss469415590 IFNL4, respectively).
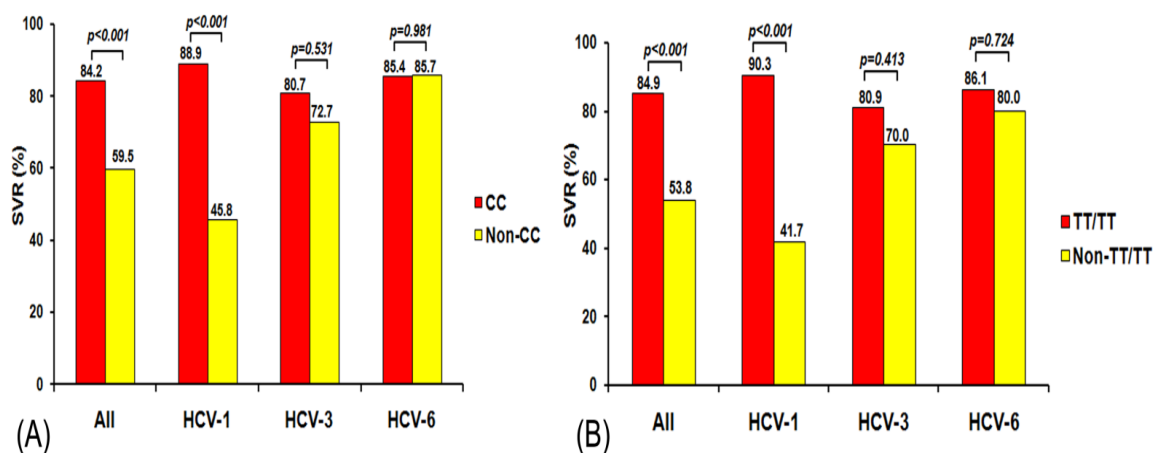
Figure 17. Association of rs12979860 genotype (A) and ss469415590 genotypes (B) with SVR to PEG-IFN/RBV therapy in patients with HCV infection.

Association of $(TA)_n$ genotypes with clinical parameters

Several clinical parameters were examined in patients with chronic HCV (Table

6). Individuals with $(TA)_n$ genotypes S and L did not demonstrate statistically significant

differences in hemoglobin (*p=0.975*) or serum ALT levels (*p=0.199*). Similarly, $(TA)_n$

genotypes did not correlate with liver stiffness (*p=0.294*) or HCV RNA (*p=0.305*).

However, $(TA)_n$ genotype S correlated with increased platelet counts ($p<0.001$).

Table 6. Correlations between $(TA)_n$ genotypes and clinical parameters.

| Characteristic | TA repeat genotypes | | P value |
	S $(TA)_n$ genotype	L $(TA)_n$ genotype	
Hemoglobin, g/dL	14.16±1.2	14.19±1.5	0.957
ALT, U/L	61.27±54.2	85.70±61.6	0.199
Platelets, × 10^9 per liter	265.45±64.2	197.12±62.1	<0.001
HCV RNA, $\log_{10}$ IU/ml	5.60±1.3	5.88±0.9	0.305
Liver stiffness, kPa	7.04±4.5	9.22±5.6	0.294

p-value <0.05 are considered statistically significant

Discussion

Host genetic factors can affect the outcome of HCV infection resulting in either spontaneous clearance from acute infection without treatment or persistence leading to chronic HCV and liver cirrhosis. Polymorphisms near the *IL28B* gene can determine the outcome of infection and the response to treatment. In the present study, we explored the overall prevalence of $(TA)_n$ genotypes among Asians of Thai descent with HCV infection resulting in natural clearance or chronic HCV. We found that the variation of $(TA)_n$ ranged from 6 to 16 and the most frequent $(TA)_n$ was 12 (91.73%) in our population. This finding was consistent with an earlier study in a Japanese cohort, which also found that 75% of individuals examined possessed $(TA)_n$ of 12 (61). The allele <10 $(TA)_n$ was significantly more frequent in the spontaneous clearance and chronic HCV groups than in the healthy controls (*p<0.001*). This difference may in part be attributed

to the genetic background represented by the fewer number of controls (n=123) compared to infected individuals (n=593). Otherwise, the allele >12 $(TA)_n$ was found in significantly higher number in healthy individuals group compared with spontaneous clearance and chronic HCV groups (*p<0.001*). Although the determination of the $(TA)_n$ genotypes was performed manually from chromatograms, two independent sequencing experiments were done for each sample to ensure data reproducibility.

Previous studies found that transcription of *IL-28B* was upregulated in the CC genotype of rs12979860, which was associated with SVR (180, 181, 184), suggesting that the expression levels of *IL-28B* could be one of the key factors to clear HCV under PEG-IFN/RBV therapy and could also affect spontaneous clearance of acute HCV infection (185), whereas the length of $(TA)_n$ in the regulatory region of *IL-28B* could affect the regulation of *IL-28B* transcription (61). The most prevalent $(TA)_n$ genotype in our population was when both alleles were ≥12, with higher frequency in healthy individuals (99.2%) compared to spontaneous clearance (94.4%) and chronic HCV (95.2 %) (*p<0.001*). The distribution of the $(TA)_n$ genotype S (when at least one allele was <12) was similar among HCV-infected individuals, but interestingly, the $(TA)_n$ genotype S was significantly more frequent in the spontaneous clearance and chronic HCV groups than in the healthy controls (*p<0.001*). Although we confirmed previous observations that favorable SNPs rs12979860 (CC) and ss469415590 (TT/TT) strongly correlated with

improved SVR with HCV-1, but not HCV-3, and HCV-6, we found that $(TA)_n \geq 12$ correlated with increase SVR for HCV-1, -3, and -6.

A recent study from Japan demonstrated the promise of $(TA)_n$ genotype in predicting spontaneous HCV clearance (186). The most frequent allele of $(TA)_n$ found in that Japanese cohort was also 12, which accounted for approximately 80% of individuals. In contrast, African-American cohort in that study demonstrated a gradient of $(TA)_n$ alleles ranging from 6 to 18, and although allele 12 was the most common, it only accounted for 30% of the individuals. More importantly, African-Americans with longer $(TA)_n$ were significantly associated with spontaneous HCV clearance, which attest to the promise of the predictive ability of $(TA)_n$ towards desirable clinical outcome in HCV infection.

Several SNPs in linkage disequilibrium upstream or within the *IL28B* gene on chromosome 19q13 are strongly associated with SVR to PEG-IFN/RBV therapy (180, 181, 184). One such polymorphism, rs12979860 (CC) genotype, is associated with greater rate of SVR than CT or TT genotypes in European-Americans, African-Americans, and Hispanics infected with HCV-1 (157). In particular, African-Americans with the CC genotype responded better to treatment than European-Americans with the TT genotype, suggesting that an individual's rs12979860 genotype is a better predictor of SVR than ethnicity. Furthermore, it is a better predictor of HCV clearance, whether natural or in response to treatment, than the baseline viral load or fibrosis. This finding

has been confirmed with Egyptians, Europeans, and Sub-Sahara Africans infected with

HCV-4 (63, 187).

Another variant in the upstream region of IFNL3, designated as IFNL4, is also

associated with treatment efficacy in HCV-infected patients (158). This region,

ss469415590, harbors a dinucleotide variant that is found in two alternative forms (ΔG

or TT alleles). The ss469415590 is more strongly associated with treatment response of

patients infected with HCV-1 than rs12979860 (158). However, 20% of patients show

discordance between *IL28B* genotype and the response, suggesting other factors

including $(TA)_n$ genotypes might be involved in HCV clearance. The lack of association

between rs12979860 (*IL28B*) and ss469415590 (IFNL4) and the $(TA)_n$ in this study may

be unique to the Thai cohort as compared to other population. Another possibility may

be that our study was under-powered and therefore could not identify such association.

The TA dinucleotide repeats, located precisely at the transcriptional start site of

IL-28B gene, could be a biomarker for improved prediction of the response to interferon-

based HCV treatment (61). We demonstrated the correlation of $(TA)_n$ genotypes with

SVR. The $(TA)_n \geq 12$ in the promoter region of *IL28B* was associated with HCV

spontaneous clearance. It is not clear whether the variation originates from genetic or

epigenetic mechanisms (188), and further studies will be needed to explore this

observation in other populations. There have been several reports that implicated *IL28B*

genotypes in inflammatory status and progression of fibrosis as measured by clinical

parameters (ALT levels, alpha-fetoprotein, histological activity, levels of fibrosis and platelet-derived growth factor) (189, 190). In our study, the baseline serum ALT level was significantly higher in patients with rs12979860 CC genotype compared to patients with non-CC genotype ($p=0.011$). Similar observations were found in patients with ss469415590 TT/TT genotype compared to those with non-TT/TT genotype ($p=0.028$). There were no significant differences in the baseline viral load between patients with rs12979860 CC and non-CC genotypes ($p=0.075$), and patients with ss469415590 TT/TT genotype compared to those with non-TT/TT ($p=0.083$). Finally, the rs12979860 and ss469415590 polymorphisms were not correlated with levels of fibrosis and platelet counts. To our knowledge, this study is the first to assess $(TA)_n$ genotypes in relation to clinical characteristics in HCV-infected patients. Although $(TA)_n$ genotypes were not associated with HCV viral load, liver inflammatory activity and liver fibrosis, they correlated with platelet counts ($p<0.001$). In clinical practice, genotyping HCV-infected patients to examine $(TA)_n$ may predict the effectiveness of PEG-IFN/RBV therapy even before treatment has begun. Since approximately 2.2% of the Thai population has chronic HCV and financial burden can restriction access to needed antiviral treatment, the ability to reliably predict efficacy of therapy will be useful in the overall disease management.

Since many polymorphisms are associated with *IL-28B* and at least $(TA)_n$ has been shown to regulate *IL-28B transcription, this cytokine* likely influence HCV

clearance under PEG-IFN/RBV therapy and could also affect spontaneous clearance of acute HCV infection (185). Administration of IL-28B has been shown to have antiviral effects (191-193), therefore lower expression of IL-28B as a result of unfavorable polymorphism might lead to a decrease in this effect.

Not only an individual's genetic background plays an important role in the course of HCV infection, viral genotypes can also determine the course of infection. The observation that *rs12979860 and ss469415590 polymorphisms were* associated equally with the treatment outcome in response to PEG-IFN/RBV therapy in patients with HCV-1 infection, but not with HCV-3 and HCV-6, suggest that viral factors may also influence SVR in patients. Although significant differences between ethnicities in response to PEG-IFN/RBV therapy were reported (194), there were no significant associations between *IL28B* genotypes and response to PEG-IFN/RBV in patients infected with HCV genotype 2 or 3 (14). In addition, some studies showed that the *IL28B* genotype did not predict response to treatment in HCV-5 and HCV-6 (159, 195).

In summary, our results demonstrated that $(TA)_n$ genotypes was strongly linked to treatment response to PEG-IFN/RBV therapy in HCV-infected patients of Asian descent regardless of the viral genotype and led to a higher rate of SVR. Thus, prescreening for $(TA)_n$ could assist clinical decision-making for the treatment of HCV infection and will be useful for making decisions on suitable regimens and treatment duration in patients in the forthcoming era of direct acting antiviral drugs.

CHAPTER V

INFLUENCE OF HOST AND VIRAL FACTORS ON PATIENTS WITH

CHRONIC HEPATITIS C GENOTYPE 6 TREATED WITH

PEGYLATED INTERFERON AND RIBAVIRIN: A SYSMEMATIC

REVIEW AND META-ANALYSIS

Vo Duy Thong[1,4], Kittiyod Poovorawan[2], Pisit Tangkijvanich[3], Rujipat Wasitthankasem[1],

Sompong Vongpunsawad[1], Yong Poovorawan[1*]

[1]*Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[2]*Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*

[3]*Research Unit of Hepatitis and Liver Cancer, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[4]*Department of Internal Medicine, Division of Gastroenterology and Hepatology, Faculty of Medicine, University of Medicine and Pharmacy, Ho Chi Minh, Vietnam*

Abstract

Background: Hepatitis C virus (HCV) genotype 6 (HCV-6) is uniquely prevalent in Southern China and Southeast Asia, contributing to almost 30% of all HCV infections in patients and emigrants from these countries. The main aims of this study were to conduct a systematic review and meta-analysis of the influence of host and viral factors on virologic response in HCV-6 patients treated with pegylated interferon and ribavirin (PEG-IFN+RBV).

Methods: All data were acquired from Medline, Embase, Pubmed and the Cochrane Library. Inclusion criteria included efficacy of PEG-IFN+RBV based on sustained virologic response (SVR), 24- or 48-week course of therapy and treatment-naïve patients. Patients with hepatitis B, D, E and HIV co-infection or another concurrent liver disease were excluded. Pooled standard difference, odds ratio (OR) and confidence intervals (CI) were calculated using a random-effects model with STATA 11.

Results: Fourteen studies were included in the meta-analysis. The pooled SVR rate was 80% (95% CI: 0.78-0.83, p<0.0001; I^2=71.2%). SVR to the PEG-IFN+RBV-treated HCV-6 group was markedly higher than that to the HCV-1 infection group (80.1% vs. 55.3%). The SVR rate at 48 weeks of treatment was significantly higher than that at 24 weeks. No significant differences in SVR were observed among HCV-infected patients with rs12979860 and ss469415590 polymorphisms of the *ILFN4* gene (80.6% in CC vs.

66.7% in non-CC, *p=0.593*; 81.1% in TT/TT vs. 60% in non-TT/TT, *p=0.288*). Based on gender and type of PEG-IFN, SVR rates were not significantly different.

Conclusions: PEG-IFN+RBV therapy is effective for HCV-6. Treatment outcomes for HCV-6 are superior to those for HCV-1 and comparable to those of HCV-2 and HCV-3. Treatment efficacy at 48 weeks is higher than that at 24 weeks. However, the level of fibrosis affects treatment outcomes, but SVR rates are not significantly different between male and female patients. *IL28B* and IFNL4 polymorphisms are not significantly associated with treatment outcome in HCV-6 patients.
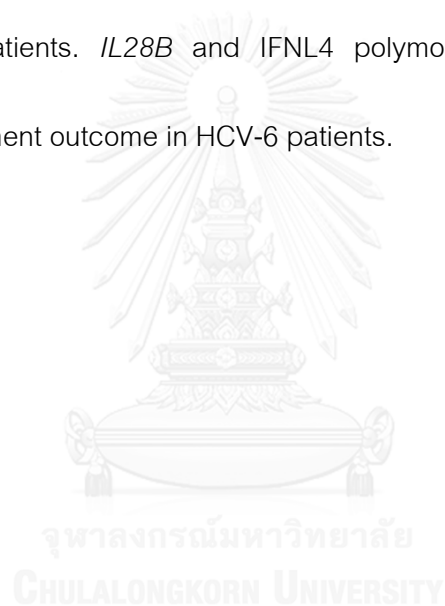
Introduction

Hepatitis C virus (HCV) is an important public health problem with more than 160 million cases of chronic infection worldwide (178). HCV infection commonly progresses to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC), and related mortality is predicted to increase over the next two decades (9). Genotype 1 (HCV-1) is found globally, especially in developed regions, such as America and Europe. HCV-3 and -6 are predominantly detected in Asian countries, which have a high prevalence of HCV (196).

Various factors can be effectively used to identify treatment responses in HCV patients. The standard treatment for HCV is a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) with a 24- to 48-week regimen, depending on the viral genotype of infected individuals(12, 13, 137). Previous studies have reported that treatment outcomes of HCV-6 are more similar to those of HCV-2 and HCV-3(134, 144, 145). And patients infected with HCV-6 respond better to therapy than those with HCV-1(196), defined as 'sustained virologic response' (SVR). Several host factors, including age, sex, race, and fibrosis level, additionally influence treatment outcomes. Single nucleotide polymorphisms on chromosome 19 within or near the *interleukin-28B* gene (*IL28B* encoding interferon lambda-3) represent one of the strongest baseline predictors of SVR in HCV treatment. Recent studies have additionally shown that the IFN-λ-4 (*IFNL4)* gene polymorphisms are in high linkage disequilibrium with those near *IL28B,*

and more strongly associated with spontaneous or treatment-induced HCV clearance than *IL28B* genotypes.

While advances in HCV therapy continue to evolve rapidly with the development of potent direct-acting antiviral agents (DAA) (197), limited data availability has prevented general recommendations for HCV-6 therapy. PEG-IFN+RBV combination therapy continues to be a predominant option in resource-limited settings due to the high costs associated with new agents. However, this treatment option remains elusive for patients in developing countries, such as Asia, where HCV-6 infection is high and funding for medications is inadequate. The current study aims to provide a systematic review and meta-analysis of the influence of host and viral factors on virologic response in HCV-6 patients treated with PEG-IFN+RBV.

Materials and methods

Data sources and search strategy

Pubmed, Embase, Medline and the Cochrane Library were used for comprehensive literature searches. To ensure maximum sensitivity of our search strategy, we kept the search string as simple as possible, using only key words without filters. The string "genotype 6" was used in all databases. Search and study selection were performed without language limitations. We reviewed the bibliographies of relevant published articles for inclusion in our study. References concerning previous meta-analyses were additionally explored for eligibility.

Inclusion and exclusion criteria

Data were included based on the following criteria: (i) treatment-naive patients, (ii) assessment of the efficacy of PEG-IFN plus RBV therapy based on SVR, defined as undetectable HCV-RNA, at least 24 weeks after the end of treatment, (iii) course of treatment (24 weeks or/and 48 weeks), and (iv) reports in English. We excluded studies comprising patients with hepatitis B, D, E or HIV co-infection and those with other concurrent liver diseases. Studies were additionally excluded if insufficient data were available for pooling.

Data extraction

Information extracted from published material included study data (author, publication year, publication status, country of origin, continent, study design, total sample size, HCV genotype, type of PEG-IFN used), demographic data on study participants (age, sex and ethnicity), and treatment outcomes (number of patients who achieved or failed to achieve SVR). Data on treatment characteristics were additionally collected, including *IL28B* and IFNL4 polymorphisms, duration of treatment (48 vs. 24 weeks), baseline HCV-RNA levels, and stage of liver fibrosis/cirrhosis.

Statistical analysis

Pooled standard differences in mean (overall SVR rate) and 95% confidence intervals (CI) were calculated for each group using a random-effects model and inverse variance method (198). Heterogeneity was tested with χ^2 -based Cochran's Q statistic with *P*-values up to 0.05, and the degree of heterogeneity quantified using the I^2 statistic representing the percentage of total variability across studies due to heterogeneity. I^2 values of 25%, 50% and 75% corresponded to low, moderate and high degrees of heterogeneity, respectively. Univariate and multivariate random-effects meta regression on study-level characteristics was performed to explain any observed heterogeneity in primary outcomes and identify associated patient-level factors (198, 199). We quantified publication bias using Egger's regression model. All analyses were performed with STATA 11 (Stata Corporation, College Station, TX) (200, 201).

Results

Study selection

A total of 207 articles relevant to HCV genotype 6 were initially identified from our searches. The modified process employed for study search and selection is summarized in Figure 18. After the titles were screened and abstracts read, 193 studies were removed based on exclusion criteria and 14 potentially eligible articles included. Among these studies (138, 146, 147, 150, 151, 182, 202-209), 11 were full texts and 3 were abstracts (202, 204, 209).
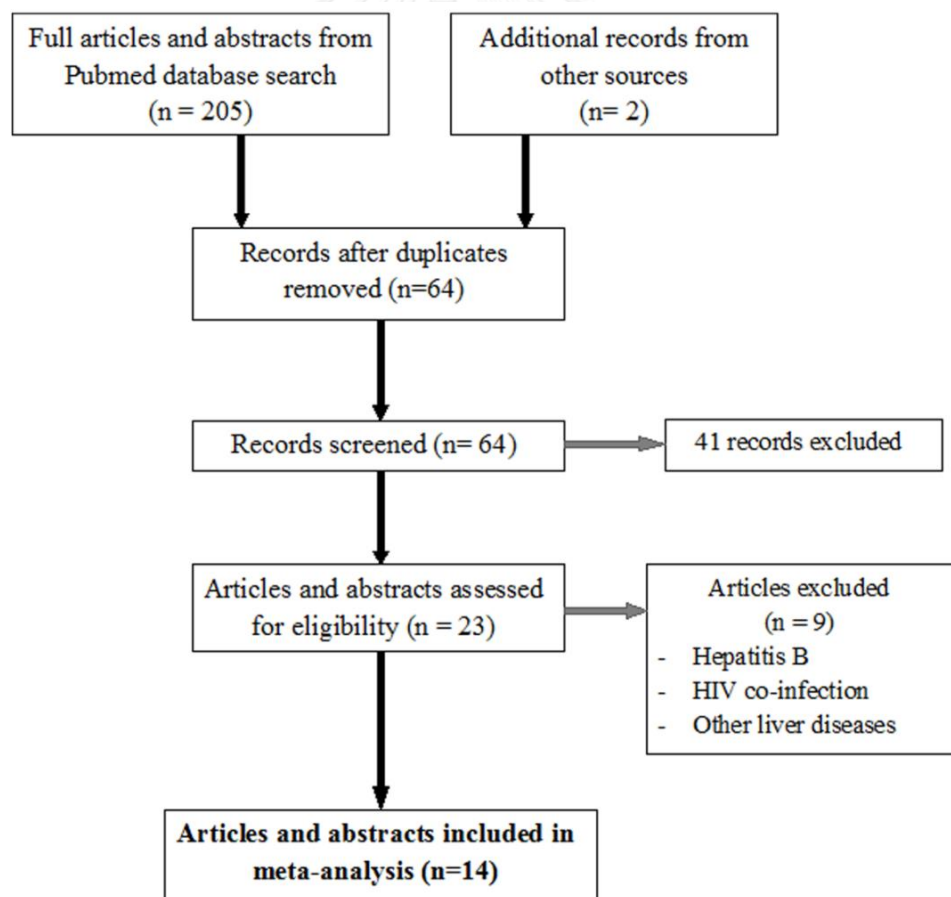
Figure 18. Overview of the study selection procedure. Flow diagram of study inclusion criteria for meta-analysis.

Clinical study and patient characteristics

Characteristics of the included studies are presented in Table 7. The pooled number of HCV-6 patients was 1176 and study sizes ranged from 12 to 70 (one study included 242 patients). Six studies evaluated patients with HCV-1 and 6, and three evaluated patients with HCV-1, 2, 3 and 6. Seven of the studies evaluated HCV-6 only. The majority of studies were nonrandomized clinical trials (11 non-RCT and 2 RCT) with predominantly male patients. Mean age ranged from 19 to 50 years. All studies underwent treatment PEG IFN with RBV for 48 weeks, but only five studies evaluated the effects of therapy for 24 weeks. Funnel plot was symmetrical and Egger's regression for publication bias was not statistically significant ($p=0.912$) (Figure 19).
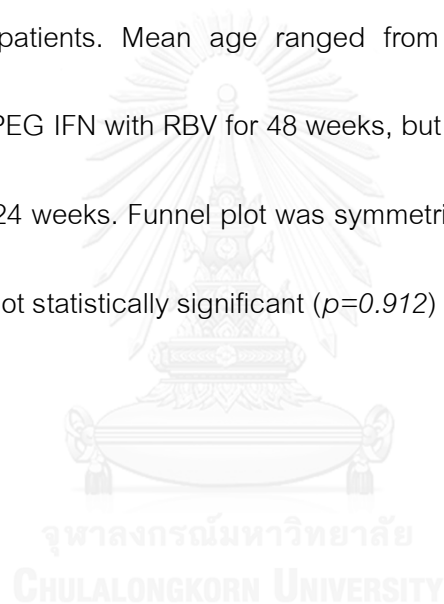
Table 7. Characteristics of studies included in the meta-analysis of HCV-6 patients treated with PEG-IFN plus RBV

Authors & Year	Study design / Publication type	Country & Population	Treatment regime (PEG-IFN+RBV)	Genotype	Treatment Duration (weeks)	Number of HCV-6 patients	Males (%)	Mean Age (years)	SVR (%)
Nguyen et al. (16) (2008)	Nonrandomized Trial	US (Asian American)	PEG-IFN α-2a	6	24	23	16 (69.6%)	49±10	9 (39%)
			PEG-IFN α-2b	6	48	12	7 (58.3%)	50±10	9 (75%)
Fung et al. (150) (2008)	Nonrandomized Trial	Hong Kong (Chinese)	PEG-IFN α-2a	1	48	21	12 (57%)	52 (30-63)	11 (52%)
			PEG-IFN α-2b	6	48	21	11 (52%)	49.6(14-64)	18 (86%)
Lam et al. (146) (2010)	Randomized Trial	US (Asian American)	PEG-IFN α-2a	6	24	27	13 (48)	49.6±12.4	19 (70%)
			PEG-IFN α-2a	6	48	33	15 (46)	52.8±8.0	26 (79%)
Nguyen et al.(104) (2010) [a]	Nonrandomized Trial	US (Asian American)	PEG-IFN α-2a	1	48	70	51 (73%)	50±9.7	25 (49%)
			PEG-IFN α-2b	6	24	27	15 (56)	49.4±12.0	17 (63%)
			PEG-IFN α-2b		48	34	19 (56%)	49.4±10.8	
Tsang et al. (138) (2010)	Nonrandomized Trial	Hong Kong (Chinese)	PEG-IFN α-2a	1	48	70	44 (63)	48 (18-64)	40 (57.1)
			PEG-IFN α-2b	6	48	70	23 (33%)	50 (28-75)	53 (75.7)
Zhou et al. (203) (2011) [a]	Nonrandomized Trial - Full	China (Chinese)	PEG-IFN α-2a	1	48	39	22 (56.4)	15 (38.5)	23 (59.0%)
			PEG-IFN α-2b	6	24	22	14 (63.6)	19 (84.6)	18 (81.8%)
Qing-Xian et al. (202) (2011)	Nonrandomized Trial - Abstract	China (Chinese)	PEG-IFN α-2b	6	48	84	NR	NR	74 (88.1%)
Tangkijvanich et al. (147) (2012) [a]	Nonrandomized Trial - Full	Thailand (Thai)	PEG-IFN α-2a	1	48	16	9 (56.3%)	46.4±12.5	10 (62.5%)
			PEG-IFN α-2a	6	48	34	23 (67.6%)	41.2±8.4	26 76.5%

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Study	Study type	Country (Ethnicity)	Treatment			Number		Age	SVR
Shao et al. (204) (2012)	Nonrandomized Trial - Abstract	China (Chinese)	PEG-IFN α-2a	6	48	28	NR	NR	26 (92.8%)
Mauss et al. (208) (2012)[a]	Nonrandomized Trial - Full	Germany (Cauasian, African, Asian, Hispanic)	PEG-IFN α-2a	6	48	27	17 (63)	47 (37-52)	16 (59%)
Thu Thuy et al. (151) (2012)	Randomized Trial - Full	Vietnam (Vietnamese)	PEG-IFN α-2a	6	24	35	22 (62.85)	46.82±7.2	21 (60%)
			PEG-IFN α-2a	6	48	70	43(61.42)	48.57±8.4	50 (71%)
Seto et al. (159) (2013)	Nonrandomized Trial - Full	Hong Kong (Chinese)	PEG-IFN α-2b	6	48	60	41 (68.3)	49 (14-71)	55 (91.7%)
Qing-Xian et al. (210) (2013)	Randomized Trial – Abstract	China (Chinese)	PEG-IFN α-2a	6	24	242 (total)	NR	NR	NR
			PEG-IFN α-2a	6	48		NR	NR	NR
Akkarathamrongsin et al. (182) (2014)[a]	Nonrandomized Trial - Full	Thailand (Thai)	PEG-IFN α-2a	1	48	69	43 (62.3%)	49.0±10.6	47 (68.1%)
			PEG-IFN α-2b	6	48	42	30 (71.4%)	42.0± 8.5	33 (78.6%)

PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; NR, Not Reported

[a]Including HCV-2, 3 and HCV-6.
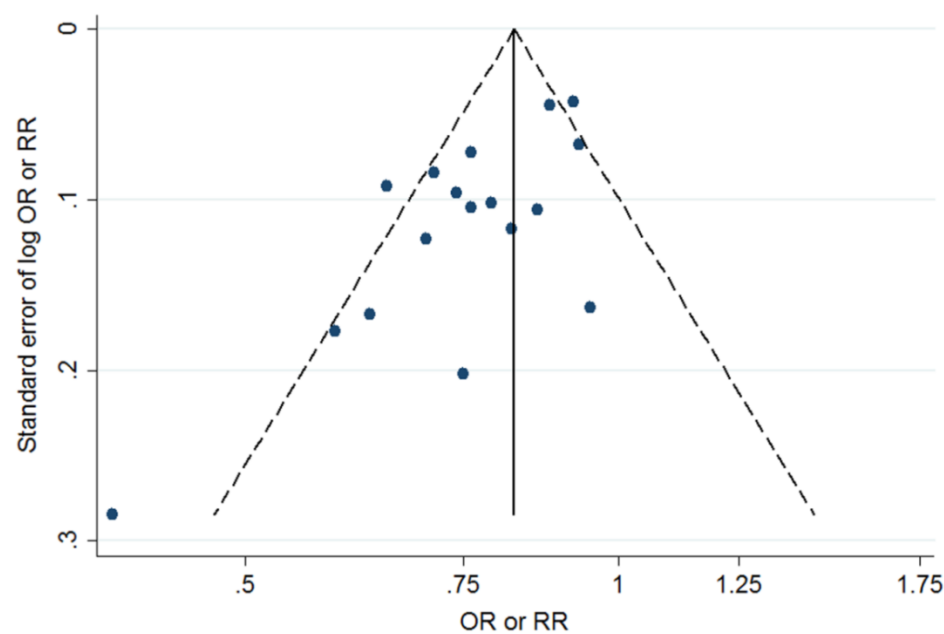
Figure 19. Funnel plot of the included studies.

Overall SVR rates

Thirteen trials reported SVR data from a total of 891 HCV-6 patients. SVR to PEG-IFN plus weight-based RBV therapy in HCV patients ranged from 39% to 92.8%. In influence analysis, the pooled SVR rate for all studies was 80% (95% CI: 0.78-0.83) (p<0.0001; I^2=71.2%) (Figure 20). Upon comparing RCT versus non-RCT, we observed a pooled SVR rate of 70% (95% CI: 0.64-0.77) in two RCTs (146, 151), compared with 83% (95% CI: 0.79-0.86) in 11 non-RCTs. This finding was statistically significant (p=0.032). Heterogeneity was observed in both subgroups (I^2=72.1% for non-RCT; I^2=67.1% for RCT).

Study name	Year	Study design	Duration	Total	Proportion (95%CI)	Weight (%)
Nguyen et al.	2008	Nonrandomized Trial	24 weeks	9/23	0.39 (0.20, 0.61)	2.31
Nguyen et al.	2008	Nonrandomized Trial	48 weeks	9/12	0.75 (0.43, 0.95)	1.44
Fung et al.	2008	Nonrandomized Trial	48 weeks	18/21	0.86 (0.64, 0.97)	3.57
Nguyen et al.	2010	Nonrandomized Trial	24 weeks	17/27	0.63 (0.42, 0.81)	2.55
Nguyen et al.	2010	Nonrandomized Trial	48 weeks	34/48	0.74 (0.59, 0.86)	5.33
Lam et al.	2010	Randomized Trial	24 weeks	19/27	0.70 (0.50, 0.81)	4.04
Lam et al.	2010	Randomized Trial	48 weeks	26/33	0.79 (0.61, 0.91)	4.31
Zhou et al.	2010	Nonrandomized Trial	24 weeks	18/22	0.82 (0.60, 0.95)	3.17
Tsang et al.	2010	Nonrandomized Trial	48 weeks	53/70	0.76 (0.64, 0.85)	8.80
Qingxian et al.	2011	Nonrandomized Trial	48 weeks	74/84	0.88 (0.79, 0.94)	17.26
Shao et al.	2012	Nonrandomized Trial	48 weeks	26/28	0.93 (0.76, 0.99)	7.34
Mauss et al.	2012	Nonrandomized Trial	48 weeks	16/27	0.59 (0.39, 0.78)	2.55
Tangkijvanich et al.	2012	Nonrandomized Trial	24 weeks	26/34	0.76 (0.59, 0.89)	4.31
Thu Thuy et al.	2012	Randomized Trial	24 weeks	21/35	0.65 (0.53, 0.76)	7.34
Thu Thuy et al.	2012	Randomized Trial	48 weeks	50/70	0.71 (0.59, 0.82)	7.34
Seto et al.	2013	Nonrandomized Trial	48 weeks	55/60	0.92 (0.82, 0.97)	17.26
Akkarathamrongsin et al.	2014	Nonrandomized Trial	48 weeks	33/42	0.95 (0.67, 1.27)	1.08
Overall (I-squared = 71.2%, P<0.0001)				504/661	0.80 (0.77, 0.83)	100

Figure 20. Overall SVR rate in HCV genotype 6 patients treated with PEG-IFN+RBV.

SVR rates in relation to host and viral factors

Male versus female patients

Among the 14 studies with 891 HCV-6 patients, genders for 537 patients were reported in eleven studies [320 (60%) males and 217 (40%) females] that included information on the influence of sex on SVR. The SVR rate was calculated as 72% in males (95% CI: 0.62–0.87) and 73% in females (CI: 0.61–0.89). Notably, no significant differences in SVR were evident between males and females with an OR of 0.81 (95% CI: 0.71-1.12, *p=0.71*). Weak heterogeneity was observed in our model (I^2=2%, *p=0.42*).

IL28B and IFNL4 polymorphisms

Two studies involving 102 patients assessed SVR in relation to *IL28B* polymorphisms while one focused on IFNL4 polymorphisms (182, 205). Among the two studies on *IL28B* polymorphisms, one with 60 HCV-6 patients [52 (90%) TT and 8 (10%) GT/GG] assessed SVR in rs8099917 TT, compared with GT/GG. One study evaluated SVR in rs12979860 CC versus CT/TT and ss469415590 TT/TT vs. non-TT/TT (Table 8). With *IL28B* rs8099917, the SVR rate in TT patients was 96.2% (50 of 52), compared to 62.5% (five of eight) in patients with the *IL28B* TG genotype (*p=0.014*, OR 15.0, 95% CI 2.0–112.1). Upon analysis of the allelic frequency of major allele T versus minor allele G, the same significant association was found (*p=0.001*, OR 10.3, 95% CI 1.7–45.6). In

contrast, for rs12979860 and ss469415590, no such differences in SVR were observed

among patients infected with HCV-6 (80.6% in CC vs. 66.7% in non-CC, *p=0.593*; 81.1%

in TT/TT vs. 60% in non-TT/TT, *p=0.288*).

Table 8. Overall SVR rates in *IL28B* (rs8099917 and rs12979860) and IFNL4 (ss469415590)

Study name	Number SVR/Total		Statistics			
	rs8099917-TT	*rs8099917–GT/GG*	*OR*	*Lower limit*	*Upper limit*	*p-value*
Seto *et al.* (2012) (159)	50/52	5/8	15.0	2.0	112.1	0.014
	rs12979860-CC	*rs12979860-nonCC*	*OR*	*Lower limit*	*Upper limit*	*p-value*
Akkarathamrongsin *et al.* (2014) (182)	29/36	4/6	2.07	0.31	13.68	0.450
	ss4694 15590-TT/TT	*ss4694 15590-nonTT/TT*	*OR*	*Lower limit*	*Upper limit*	*p-value*
Akkarathamrongsin *et al.* (2014) (182)	30/37	3/5	NR	NR	NR	NR

SVR, sustained virological response

OR, confidence interval

Mild versus advanced/severe hepatic fibrosis

A total of seven studies involving patients with mild and advanced fibrosis were

included to evaluate the influence of fibrosis on treatment response. Mild and advanced

fibrosis were defined as F0-F2 and F3-F4 based on METAVIR scoring system,

respectively. Five studies used the METAVIR scoring system whereas two others used a

different scoring system to classify patients into either mild or severe fibrosis groups. In

influence analysis, the SVR rate was 76% (95% CI: 0.67–0.84) for patients with mild

fibrosis and 67% (95% CI: 0.61–077) for those with severe fibrosis. Our data indicate that the SVR rate in patients with mild fibrosis is significantly higher, compared to patients with advanced fibrosis ($p<0.001$).

HCV-6 versus other genotypes

Six trials assessed treatment response in patients infected with HCV-6, compared with those infected with HCV-1 (104, 138, 147, 150, 182, 203). Among these, three were direct comparisons of response to antiviral therapy by HCV-6 versus HCV-3 and/or HCV-2 patients. Patients with HCV-6 displayed significantly better SVR to PEG-IFN+RBV combination therapy than those with HCV-1 (80.1% vs. 55.3%), with a relative risk of 1.33 (95% CI: 1.14–1.53, *p=0.904*) (Figure 21). Our results are consistent with the majority of previous studies reporting that HCV-6 behaves in a more similar manner to HCV-2 and HCV-3 (SVR rates of 76–80%) (134, 144, 145).

Study name	Year		RR (95% CI)	Weight (%)
Fung et al.	2008		1.64 (1.05, 2.55)	6.73
Nguyen et al.	2010		1.28 (0.86, 1.92)	13.48
Zhou et al.	2010		1.39 (1.00, 1.93)	17.51
Tsang et al.	2010		1.33 (1.04, 1.69)	35.84
Tangkijvanich et al.	2012		1.22 (0.80, 1.87)	13.23
Akkarathamrongsin et al.	2014		1.28 (0.82, 1.89)	13.23
Overall (I-squared = 0%, P = 0.967)			1.33 (1.14, 1.53)	100

RR   0   .5   1   1.5   2   2.5

Better genotype 1   Better genotype 6

Figure 21. Meta-analysis of SVR rate in HCV-6 versus HCV-1 patients.

Lower versus higher viral load

Eight studies explored the association between higher baseline HCV-RNA and treatment outcome. The definition of higher viral load varied across studies, ranging from a threshold of >200 000 IU/ml (138, 207), >400 000 IU/ml (104, 150, 208), >600 000 IU/ml (182) to >800 000 IU/ml (146, 147). The SVR estimate was 73% (95% CI: 0.67–0.81) in patients with lower viral load and 58% (95% CI: 0.45–0.69) in those with higher viral load. We observed a similar association between lower baseline viral load and higher SVR rates, relative to those with higher viral load (OR 4.01; 95% CI: 1.62-5.43, *p*<0.001). Significant heterogeneity was evident in our analysis (Q-statistic=17.94, *p*<0.05; I^2=71.52).

Treatment duration of 24 versus 48 weeks

Four studies including 261 patients [149 (57.1%) treated for 48 weeks and 112 (42.9%) treated for 24 weeks] compared treatment responses in patients treated with PEG IFN+RBV for 48 versus 24 weeks (16, 104, 146, 151). We conducted a second meta-analysis of these studies, which showed that SVR rate at 24 weeks of treatment is significantly lower than that at 48 weeks. Pooled SVR rates were 67% for 24 weeks (95% CI: 0.61-0.74) (*p=0.038*; I^2=57.6%) (Figure 22) and 84% for 48 weeks (95% CI: 0.80-0.87) (*p=0.006*; I^2=59.7%) (Figure 23). However, the risk difference was 14% (95% CI: -0.25 to -0.02).

Study name	Year	Study design	Total	Proportion (95%CI)	Weight (%)
Nguyen et al.	2008	Nonrandomized Trial	9/23	0.39 (0.20, 0.61)	9.74
Lam et al.	2010	Randomized Trial	19/27	0.70 (0.50, 0.81)	17.03
Nguyen et al.	2010	Nonrandomized Trial	17/27	0.63 (0.42, 0.81)	10.76
Zhou et al.	2010	Nonrandomized Trial	18/22	0.82 (0.60, 0.95)	13.36
Tangkijvanich et al.	2012	Nonrandomized Trial	26/34	0.76 (0.59, 0.89)	18.18
Thu Thuy et al.	2012	Randomized Trial	21/35	0.65 (0.53, 0.76)	30.94
Overall (I-squared = 56.7%, P = 0.038)			110/168	0.67 (0.61, 0.79)	100

Figure 22. Overall SVR rate in HCV genotype 6 patients treated with PEG-IFN+RBV.

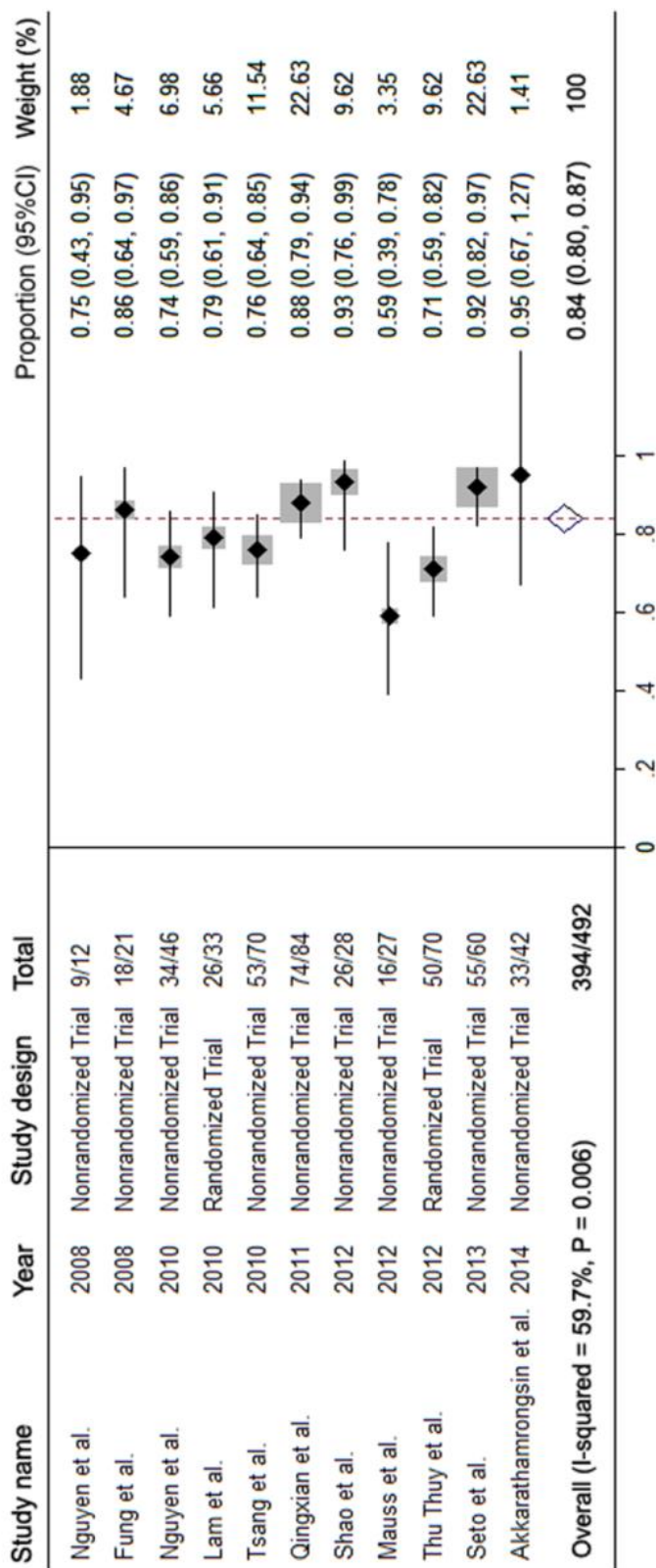| Study name	Year	Study design	Total	Proportion (95%CI)	Weight (%)
Nguyen et al.	2008	Nonrandomized Trial	9/12	0.75 (0.43, 0.95)	1.88
Fung et al.	2008	Nonrandomized Trial	18/21	0.86 (0.64, 0.97)	4.67
Nguyen et al.	2010	Nonrandomized Trial	34/46	0.74 (0.59, 0.86)	6.98
Lam et al.	2010	Randomized Trial	26/33	0.79 (0.61, 0.91)	5.66
Tsang et al.	2010	Nonrandomized Trial	53/70	0.76 (0.64, 0.85)	11.54
Qingxian et al.	2011	Nonrandomized Trial	74/84	0.88 (0.79, 0.94)	22.63
Shao et al.	2012	Nonrandomized Trial	26/28	0.93 (0.76, 0.99)	9.62
Mauss et al.	2012	Nonrandomized Trial	16/27	0.59 (0.39, 0.78)	3.35
Thu Thuy et al.	2012	Randomized Trial	50/70	0.71 (0.59, 0.82)	9.62
Seto et al.	2013	Nonrandomized Trial	55/60	0.92 (0.82, 0.97)	22.63
Akkarathamrongsin et al.	2014	Nonrandomized Trial	33/42	0.95 (0.67, 1.27)	1.41
Overall (I-squared = 59.7%, P = 0.006)			394/492	0.84 (0.80, 0.87)	100

Figure 23. Overall SVR rate in HCV-6 patients treated with PEG-IFN+RBV for 48 weeks.

Discussion

Although DAAs have been recently approved for treatment of chronic HCV infection, PEG-IFN+RBV combination therapy remains appropriate for HCV-6, since the relevant data obtained are insufficient to make general recommendations (211, 212) and not available in most countries in Southeast Asia (196). To our knowledge, this is the first systematic review and meta-analysis to evaluate the influence of host and viral factors in patients with chronic HCV-6 treated with PEG-IFN+RBV. In our analysis, which included 14 studies comprising a total of 819 HCV-6 patients treated with PEG-IFN+RBV, the pooled SVR rate was 79.8% (95% CI: 0.77-0.83). No publication bias was found. Only one earlier study performed a meta-analysis of HCV-6 patients treated with PEG-IFN+RBV (213). The pooled SVR rate in this report (75%) was slightly lower than our results. However, their study did not include subgroup analysis of host and viral factors on SVR while our investigation incorporated recent studies with a large number of patients and evaluated the influence of host and viral factors on SVR rates in HCV-6 patients. Differences in SVR rates between non-RCT and RCT were evident, with significantly higher response in non-RCTs (*p=0.032*).

The optimal duration of PEG-IFN+RBV therapy in HCV-6 patients depends on several factors and is yet to be determined. Recent studies reported no significant differences in SVR rates in patients subjected to 48-week and 24-week regimens. The abbreviated regimen has many advantages, including reducing unnecessary

medication exposure, increasing affordability of treatment and maximizing the cost-effectiveness of therapy. Interestingly, in our subgroup analysis of four studies (16, 104, 146, 151), the SVR rate at 24 weeks of treatment was significantly lower than that at 48 weeks.   Specifically, pooled SVR rates were 67% for 24 weeks (95% CI: 0.61-0.74) ($p=0.038$; I^2=57.6%) and 84% for 48 weeks (95% CI: 0.80-0.87) ($p=0.006$; I^2=59.7%). While one retrospective study demonstrated that SVR in HCV-1 and HCV-6 patients are not significantly different(214), the majority of investigations have shown that HCV-6 is more similar to HCV-2,-3 in that patients in these groups respond better to therapy, compared to those with HCV-1 infection. With regard to the treatment regimen, we did not observe significant differences in SVR rates between patients treated with PEG-IFN α-2a and PEG-IFN α-2b ($p=0.352$).

Several host factors (such as age, sex and fibrosis levels) play an important role in response to HCV therapy (12-14). In our analysis, no significant differences in SVR were found between males and females (OR of 0.81, 95% CI: 0.71-1.12, $p=0.71$). Notably, the SVR rate in patients with mild fibrosis was markedly higher than that for advanced fibrosis (76% for mild fibrosis and 67% for severe fibrosis; $p<0.001$). Baseline viral load has been established as the determinant of treatment response. Lower starting HCV-RNA levels are reported to be associated with higher SVR rates (64). Consistent with these results, our data showed that lower baseline viral load is significantly related to increased SVR, compared to the higher baseline viral load (OR 4.01, 95% CI: 1.62-

5.43, *p<0.001*). However, larger-scale studies are required to further validate our findings on the effects of gender, fibrosis levels and baseline viral load on treatment outcome.

A number of studies have reported that rs8099917 and rs12979860 polymorphisms in *IL28B* are significantly associated with treatment outcome, especially in HCV-1 patients. The two studies on *IL28B* in our subgroup analyses showed a favorable association between SVR and rs8099917 TT genotype versus GT/GG (*p=0.014*, OR 15.0, 95% CI 2.0–112.1) (205). In contrast, the effectiveness of rs12979860 and ss4694 15590 polymorphisms in predicting treatment response was shown to be attenuated in patients infected with HCV-6 (182). Our findings do not indicate a sufficiently strong association to support the utility of these polymorphisms in predicting response to therapy in HCV-6 patients.

Although all related studies have been included in our systematic review, the current analysis has several limitations. First, since the number of studies included in this meta-analysis was limited, the overall results were not robust. Furthermore, only abstracts of three studies were available and some trials included a few patients, but since all met the inclusion criteria and provided data on the outcome of interest, they were included in our meta-analysis. Second, although the efficacy of PEG-IFN+RBV varies depending on the type of treatment regimen and dose schedule, we were not able to perform sensitivity analyses on these parameters owing to limited data. Despite

the above drawbacks, this study provides solid evidence that PEG-IFN+RBV treatment significantly improves the SVR rate in HCV-6 patients and validates the effects of specific host and virus factors on treatment outcome.

In conclusion, the PEG-IFN plus RBV combination is effective for HCV-6 patients, with a pooled SVR rate of 79.8% in our study. Moreover, treatment outcomes of HCV-6 patients are superior to HCV-1, and comparable to those of HCV-2 and -3. Regardless of treatment duration and type of PEG-IFN, efficacy of treatment at 48 weeks is superior, compared to that at 24 weeks. The level of fibrosis affects SVR in HCV-6 patients, while sex (male or female) has no significant influence on treatment outcome. Moreover, *IL28B* and IFNL4 polymorphisms are not significantly associated with treatment outcome in HCV-6 patients.
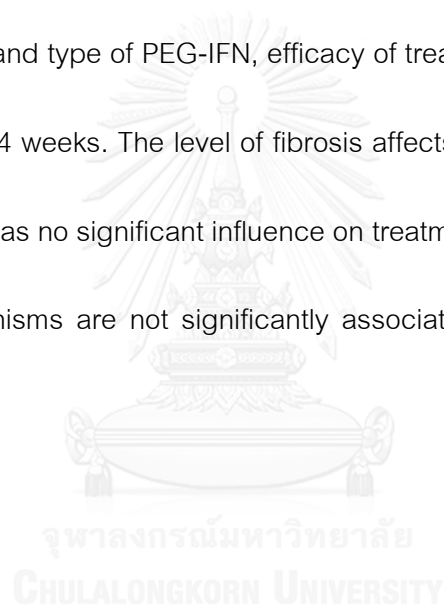
CHAPTER VI

SUMMARIZING DISCUSSION

Hepatitis C virus infection is a major global health problem, with leading to development of chronic HCV, cirrhosis and hepatocellular carcinoma in many areas of the world. Southeast Asia is considered an area with high rate of hepatitis C virus infection (215). HCV genotype 6 (HCV-6) is one of the unique prevalent in Southern China and Southeast Asia, contributing to almost 30% of all HCV-infected patients in these areas or emigrants from these countries. As we discussed in chapter II, HCV-6 has considerable genetic diversity with 23 subtypes (a to w). Evolutionary analysis of HCV-6 subtypes hypothesizes that these subtypes evolved from a common ancestor more than 1,000 years ago, and that some subtypes may have maintained their endemicity via local epidemics during the 20th century initiated and propagated by modern medicine, blood transfusion and IVDU (91). However, each of the subtypes seems generally restricted to different locations such as subtype 6d in Vietnam, 6f in Thailand, 6g in Indonesia, 6r in Cambodia (91, 96) and strains isolated from the same country tend to cluster together in a HCV-6 phylogenetic tree (Chapter II, Figure 8). Our systematic review found that previous literature suggests older tests may have

misclassified HCV-6 as genotype 1, but newer line probe assays have shown impressive improvement in genotyping accuracy and differentiation between HCV genotype 1 and HCV-6 variants. In addition, HCV-6 patients often presented with similar clinical manifestations as patients infected with other genotypes.

In clinical practice, screening for anti-HCV antibodies remains the first step for diagnosis of HCV infection in hospital, while measurement of HCV-RNA is usually used to determine the treatment duration and monitoring of the response to the antiviral therapy (216, 217). However, this measurement is limited in developing countries due to high cost and requirement for considerable technical skill and related equipment. The HCV core antigen test focused on both the complete HCV virion and RNA-free core protein structures that can be detected in the bloodstream of infected individuals. Quantification of HCVcAg has been applied as screening tests for decades and was followed in the next few years (4, 47, 218-220). Our data confirmed previous reports that HCVcAg levels had a good correlation with the corresponding HCV RNA levels in mono-HCV infected patients and co-infected with HIV (Chapter III). It should be noted that the correlation between both assays appeared to be higher among the co-infected group than the mono-infected group. Furthermore, mean serum concentrations of HCV RNA and HCVcAg in our study were significantly higher in co-infected than mono-infected groups. These results are consistent with previous reports that HCV/HIV co-infection is associated with persistent HCV viremia and higher HCV viral load (172). The increased

HCV RNA levels among co-infected patients are thought to be in part associated with the decline in CD4+ and CD8+ T-cell responses to HCV infection. In contrast to previous studies, our results did not demonstrate the correlation between HCVcAg concentration and liver inflammatory activity and liver fibrosis (175). However, HCVcAg and HCV RNA levels did not vary according to HCV genotypes. Patients harboring rs12979860 CC and ss469415590 TT/TT had significantly higher levels of HCV RNA and HCVcAg than those with the non-CC and non-TT/TT genotype, regardless of HIV status and HCV genotypes. The effects on HCV RNA and HCVcAg concentrations might be in part related to biological interaction between HCV replication and IFNL4 protein. As results, the HCVcAg assay is a reliable test and has the advantages of being rapid and reproducible, its measurement could be used as an alternative to HCV RNA assays in resource-limited settings.

Although the molecular mechanism underlying the genetic effects of *IL28B* on the anti-HCV reaction remains obscure, previous studies reported the effect of *IL28B* genotype on spontaneous HCV clearance and treatment efficacy in HCV infected patients. Moreover, significant differences between ethnicities in response to PEG-IFN/RBV therapy were reported (194). Recently studies reported that there were no significant associations between *IL28B* genotypes and response to PEG-IFN/RBV in patients infected with HCV genotype 2 or 3 (14), although the *IL28B* genotype correlates with response to PEG-IFN/RBV therapy in HCV-4 infected patients (63, 187). In addition,

the remaining 20% patients showed discordance between *IL28B* genotype and the response. Recently, an insertion/deletion polymorphism in the promoter region of *IL28B* consisting of thymine–adenine dinucleotide repeats has been linked to *IL28B* gene expression. Luciferase assay showed that the transcriptional activity of the promoter increased gradually with increasing $(TA)_n$ length. Therefore, $(TA)_n$ could be associated with the transcriptional activity of *IL-28B* and could potentially be used to improve predictions of the response to interferon-based HCV treatment.

In this study, we explored the length of the TA repeat in Thai individuals. We found that the variation of $(TA)_n$ ranged from 6 to 16 and the most frequent $(TA)_n$ was 12 (91.73%) in our population. This finding was consistent with an earlier study in a Japanese cohort, which also found that 75% of individuals examined possessed $(TA)_n$ of 12 (61). The allele <10 $(TA)_n$ was significantly more frequent in the spontaneous clearance and chronic HCV groups than in the healthy controls (*p<0.001*). This difference may in part be attributed to the genetic background represented by the fewer number of controls (n=123) compared to infected individuals (n=593). Otherwise, the allele >12 $(TA)_n$ was found in significantly higher number in healthy individuals group compared with spontaneous clearance and chronic HCV groups (Chapter III).

Moreover, $(TA)_n \geq 12$ correlated with increase SVR for HCV-1, -3, and -6. The TA dinucleotide repeats, located precisely at the transcriptional start site of *IL-28B* gene, could be a biomarker for improved prediction of the response to interferon-based HCV

treatment (61). We demonstrated the correlation of $(TA)_n$ genotypes with SVR. The $(TA)_n$ ≥12 in the promoter region of *IL28B* was associated with HCV spontaneous clearance. It is not clear whether the variation originates from genetic or epigenetic mechanisms (188), and further studies will be needed to explore this observation in other populations. Of note, this study found that rs12979860 and ss469415590 polymorphisms were associated equally with the treatment outcome in response to PEG-IFN/RBV therapy in patients with HCV-1 infection, but not with HCV-3 and HCV-6, suggesting that viral factors may also influence SVR in patients. In contrast, our results demonstrated that $(TA)_n$ genotypes was strongly linked to treatment response to PEG-IFN/RBV therapy in HCV-infected patients of Asian descent regardless of the viral genotype and led to a higher rate of SVR (Chapter IV, Figure 16). Thus, approximately 2.2% of the Thai population has chronic HCV and financial burden can restriction access to needed antiviral treatment, prescreening for $(TA)_n$ could assist clinical decision-making for the treatment of HCV infection and will be useful for making decisions on suitable regimens and treatment duration in patients in the forthcoming era of direct acting antiviral drugs.

While advances in HCV therapy continue to evolve rapidly with the development of potent direct-acting antiviral agents (DAAs) (197) and the data have been too limited to make a general recommendation in HCV-6, PEG-IFN+RBV combination therapy will continue play an important role in resource-limited settings due to the high costs associated with these new agents. Until these issues are resolved, this treatment option

may remain elusive for patients in developing countries, as Asia, where HCV-6 is high, and funding for medications remains limited. In our systematic review and meta-analysis, we demonstrated that PEG-IFN plus RBV still is effective for HCV-6 patients with the pooled SVR rate of 79.8% in our data (chapter V). In addition, treatment outcome of HCV-6 patients is superior to HCV-1, and nearly comparable to HCV-2, 3. Although, the optimal treatment duration of PEG-IFN+RBV therapy in HCV-6 patients have not yet been determined and recently studies reported that there was no significant difference in SVR rates in patients treated with 48-week and 24-week regimens, this study found that the SVR rate for 24 weeks of treatment was significantly lower than that for 48 weeks. Moreover, viral factors and host factors including age, sex, race, and fibrosis level all influence the treatment outcome. Regardless of viral load, our data showed that lower baseline viral load was significantly related to an increased SVR rate compared to the higher baseline viral load. Level of fibrosis effects to SVR in HCV-6 patients, but there is no significant difference between males and female in treatment outcome. Of note, our meta-analysis study found that *IL28B* and IFNL4 polymorphisms were not significantly related to treatment outcome in HCV-6 patients.

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VITA

Mr. Vo Duy Thong was born on December 15th, 1982 in Vietnam. In 2000, he received oversea government scholarship for Medical student. He received his Medical Doctor degree (1st Honor) and Internist in 2008 from Faculty of Medicine, Moscow State of Medicine and Dentistry, Moscow, Russia. He works at Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of Medicine and Pharmacy, Ho Chi Minh city, Vietnam. He has enrolled the International Ph.D program for Doctor of Philosophy in Medical Sciences, Faculty of Medicine, Chulalongkorn University since 2012 as an advisee of Prof Yong Poovorawan, M.D. He has published a number of international scientific articles as follow:

The publications from his thesis:

Thong VD, Wasitthankasem R, Tangkijvanich P, Vongpunsawad S, Poovorawan Y. Prevalence of Thymine-Adenine Dinucleotide Repeat, IL28B and IFNL4 in Thai Population and Correlation with Spontaneous Clearance and Treatment Outcome of Hepatitis C Infection. PLoS One. 2015 May 4; 10(5):e0125400. doi: 10.1371/journal.pone.0125400

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General publications during his study:

Akkarathamrongsin S, Thong VD, Payungporn S, Poovorawan K, Prapunwattana P, Poovorawan Y, Tangkijvanich P. IFNL3 (IL28B) and IFNL4 polymorphisms are associated with treatment response in Thai patients infected with HCV genotype 1, but not with genotypes 3 and 6. J Med Virol. 2014 Sep;86(9):1482-90.

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